

Brazilin attenuates methylglyoxal-induced endothelial injury by enhancing eIF5A hypusination and inhibiting the AMPK/mTOR-mediated autophagy/apoptosis axis *in vitro* and *in vivo*

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Abstract. Methylglyoxal (MGO), a glycolytic metabolic byproduct and major precursor of advanced glycation end products (AGEs), accumulates and is critically involved in diabetes, particularly in diabetic vascular complications. In endothelial cells (ECs), MGO has been implicated in oxidative stress and inflammatory responses, and it can further induce autophagy and apoptosis. Brazilin (BZ), a natural compound, confers cytoprotection by allosterically activating deoxyhypusine hydroxylase (DOHH), thereby enhancing

eukaryotic initiation factor 5A (eIF5A) hypusination, a distinctive post-translational modification. Although BZ can mitigate vascular inflammation and regulate autophagy and apoptosis, its effects on MGO-induced autophagy and apoptosis and the underlying mechanism remain elusive. In the present study, it was demonstrated that BZ pretreatment conferred cytoprotection by targeting DOHH and the downstream eIF5A signaling cascade, thereby suppressing activation of the AMP-activated protein kinase/mammalian target of rapamycin (AMPK/mTOR) signaling pathway. Moreover, 3-methyladenine (3-MA) and Compound C strengthened the inhibitory effect of BZ on MGO-induced autophagy and apoptosis. By contrast, cyclopirox, a specific inhibitor of DOHH, abolished the BZ-mediated suppression of MGO-induced cellular responses. The *in vivo* experiments further confirmed that BZ suppresses MGO-triggered autophagy and apoptosis. Using *db/db* mice to mimic the diabetic microenvironment, it was further found that DOHH inhibition reversed the BZ-mediated suppression of autophagy and apoptosis. Collectively, the findings of the present study reveal a DOHH/eIF5A-AMPK/mTOR axis through which BZ antagonizes MGO-induced autophagy and apoptosis, shedding new light on the pharmacological mechanism of BZ and underscoring its therapeutic potential for diabetic vascular complications.

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Abbreviations: AGEs, advanced glycation end products; AMPK, AMP-activated protein kinase; BZ, brazilin; CCK-8, Cell Counting Kit-8; CQ, chloroquine; DOHH, deoxyhypusine hydroxylase; ECs, endothelial cells; eIF5A, eukaryotic translation initiation factor 5A; FITC, fluorescein isothiocyanate; HUVECs, human umbilical vein endothelial cells; MGO, methylglyoxal; mTOR, mammalian target of rapamycin; PI, propidium iodide; siRNA, small interfering RNA; si-DOHH, siRNA targeting DOHH; si-NC, negative control siRNA; TSA, tyramide signal amplification; WT, wild-type; 3-MA, 3-methyladenine

Key words: MGO, BZ, autophagy, apoptosis, DOHH/eIF5A

Introduction

Diabetes encompasses metabolic disorders marked by hyperglycemia (1,2). Chronic hyperglycemia induces mitochondrial metabolic disorders in pancreatic β cells, thereby markedly reducing intracellular insulin synthesis and secretion (3-5). This represents a key contributor to the gradual impairment of β -cell function as diabetes advances. Hyperglycemia and insulin resistance/hyperinsulinemia constitute core metabolic abnormalities that drive cardiovascular metabolic syndrome

and predispose individuals to diabetic vascular complications, ranging from retinopathy and neuropathy to atherosclerosis, coronary artery disease, hypertension and peripheral artery disease (6-8). Endothelial cells (ECs) form the innermost layer of blood vessels, and their injury and dysfunction serve as critical early events in diabetic vascular disease, promoting disease onset and progression (9-12).

Methylglyoxal (MGO), generated through glycolytic metabolism, commonly exists in cells as a metabolic byproduct with highly reactive dicarbonyl properties (13-15). MGO reacts with arginine and lysine residues within proteins, resulting in the formation of stable advanced glycation end products (AGEs), which in turn trigger inflammatory and oxidative processes, thereby damaging the integrity and homeostasis of vascular ECs and potentially contributing to cardiovascular diseases (16-18). The authors' previous research revealed that MGO exposure elicited apoptotic and pyroptotic responses in human umbilical vein endothelial cells (HUVECs) by triggering mitochondrial dysfunction, oxidative stress and inflammation (19-21). Plasma MGO concentrations are aberrantly increased in patients with diabetes, while its accumulation across various tissues and organs contributes importantly to the development of diabetic vascular complications (16,22,23). In addition, prolonged exposure of ECs to pathogenic stimuli and circulating factors can lead to blood flow dysregulation and barrier dysfunction, ultimately triggering autophagy and apoptosis through disruption of intracellular signaling pathways (24-27). Notably, MGO has been shown to elicit autophagy as well as apoptosis, although the underlying molecular mechanisms remain incompletely understood (28-35).

Brazilin (BZ) is an isoflavonoid compound and a representative active ingredient of *Caesalpinia sappan* (Sappanwood) (36). It exhibits vasodilatory, anti-inflammatory and hypoglycemic activities and has been extensively used for diabetes and cardiovascular disease management (37,38). Deoxyhypusine hydroxylase (DOHH) has recently been recognized as a critical cellular target of BZ (39). Via the γ -carboxyl groups within the active-site residues Glu57 and Glu208, DOHH mediates the unique hypusine post-translational modification of eukaryotic translation initiation factor 5A (eIF5A), thereby exerting cytoprotective effects (39). Furthermore, BZ appears to modulate autophagy and apoptosis through activation of the AMP-activated protein kinase/mammalian target of rapamycin (AMPK/mTOR) (40,41). However, the effects of BZ against MGO-mediated autophagy and apoptosis in ECs, the functional localization of BZ and MGO, and their interaction mechanisms in the protection against diabetic cardiovascular complications remain to be further investigated. Therefore, a focused exploration of the regulatory mechanisms of the relevant signaling pathways, particularly DOHH/eIF5A and AMPK/mTOR, may provide new strategies and targets for managing diabetic vascular diseases.

In the present study, it was examined whether BZ protects against MGO-induced autophagy and apoptosis in HUVECs *in vitro* and further assessed its ability to alleviate MGO-evoked vascular injury and related autophagic and apoptotic responses *in vivo*. The mechanistic basis by which BZ attenuates autophagy and apoptosis via the DOHH/eIF5A-regulated AMPK/mTOR pathway was further explored.

Materials and methods

Chemicals and reagents. MGO was obtained from MilliporeSigma. BZ, Compound C and ciclopirox were supplied by MedChemExpress. 3-Methyladenine was purchased from APeXBIO. The apoptosis detection kits for Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) were supplied by BD Biosciences. MGO ELISA kits (cat. no. E03M0040) were obtained from Blue Gene Biotech. LC3 (cat. no. RX201696M), p62 (cat. no. RX202212M) and cleaved caspase-3 (cat. no. RX2D240566) ELISA kits were obtained from Ruixin Biotechnology Co., Ltd. A tricolor pre-dyed marker and Omni-ECL™ ultrasensitive chemiluminescence detection kits were purchased from Epizyme. Unless otherwise specified, the remaining chemicals and reagents were obtained from Beyotime Institute of Biotechnology. Information on the antibodies used in the present study is listed in Table I.

Cell culture. Primary HUVECs used in the present study were obtained from ATCC (cat. no. PCS-100-010) as passage 1 stock cells, and cells at passages 4-10 were used for all experiments. Mycoplasma contamination was assessed using a PCR-based detection kit (HUABIO, cat. no. K0103). The PCR products were separated by electrophoresis on a 1% agarose gel and visualized after staining with GelRed nucleic acid stain. No mycoplasma-specific amplification band was detected, confirming that the cells were mycoplasma-negative. HUVECs were cultured at 37°C under humidified conditions with 5% CO₂ in EC medium supplemented with 5% fetal bovine serum (FBS), 1% EC growth supplement, and 1% penicillin/streptomycin. All related reagents were purchased from ScienCell Research Laboratories: FBS (cat. no. 0025), EC growth supplement (cat. no. 1052) and penicillin/streptomycin (cat. no. 0503).

Assessment of cell viability. Changes in cell viability after treatment were detected in a Cell Counting Kit-8 (CCK-8) assay. A total of $\sim 5 \times 10^3$ cells were seeded into each well of 96-well plates and cultured for 24 h. The cells were then incubated with different concentrations of BZ (0, 1, 2, 5, 10 and 20 μ M) for 2 h, followed by exposure to MGO (0, 10, 20, 50, 100 and 200 μ M) for 24 h. Subsequently, 100 μ l of diluted CCK-8 solution was added to each well, and the cells were incubated in the dark for 1 h. The optical density at 450 nm was measured using a microplate reader.

Flow cytometry. After treatment, the cells were collected and washed three times with 1X binding buffer (10 mM HEPES, pH 7.4, 140 mM NaCl, and 2.5 mM CaCl₂). Each sample was then resuspended in 100 μ l of 1X binding buffer, followed by staining with 5 μ l Annexin V-FITC and 5 μ l PI for 15-20 min at room temperature in the dark. The apoptotic rate was detected by flow cytometry, and data analysis was performed using Cell Quest software (version 5.2; BD Biosciences).

Immunofluorescence. Following incubation, cells were fixed in 4% paraformaldehyde (PFA) for 30 min at room temperature and then permeabilized with 0.1% Triton X-100 for 10 min. After blocking with 5% BSA (cat. no. 36101ES; Shanghai Yeasen Biotechnology Co., Ltd.) for 1 h at room temperature,

Table I. Information (including dilution, MW, cat. no. and supplier) of antibodies used for western blot analysis.

Antibody	MW (kDa)	Cat. no.	Supplier	Dilution
LC3	14,16	12741	Cell Signaling Technology, Inc.	1:1,000
mTOR	289	2983	Cell Signaling Technology, Inc.	1:1,000
p-mTOR	289	5536	Cell Signaling Technology, Inc.	1:1,000
AMPK	62	5831	Cell Signaling Technology, Inc.	1:1,000
p-AMPK	62	50081	Cell Signaling Technology, Inc.	1:1,000
Beclin1	52	11306-1-AP	Proteintech Group, Inc.	1:2,000
p62	62	18420-1-AP	Proteintech Group, Inc.	1:5,000
Bax	21	50599-2-Ig	Proteintech Group, Inc.	1:2,000
Bcl-2	26	26593-1-AP	Proteintech Group, Inc.	1:2,000
caspase-3	32	19677-1-AP	Proteintech Group, Inc.	1:2,000
Cleaved caspase-3	17	68773-1-Ig	Proteintech Group, Inc.	1:5,000
DOHH	33	68151-1-Ig	Proteintech Group, Inc.	1:5,000
eIF5A	18	11309-1-AP	Proteintech Group, Inc.	1:5,000
GAPDH	36	60004-1-Ig	Proteintech Group, Inc.	1:50,000
Anti-Hypusine	17	ATMA1034Mo	AntibodySystem	1:1,000
HRP-conjugated goat anti-rabbit and anti-mouse IgG (H+L)		A0208/A0216	Beyotime Institute of Biotechnology	1:1,000

the cells were sequentially incubated with the primary antibody overnight at 4°C, followed by incubation with the appropriate fluorophore-conjugated secondary antibody for 1 h at room temperature in the dark. Nuclei were subsequently counterstained with DAPI (1 µg/ml) for 10 min at room temperature in the dark. After anti-fade mounting medium was added, fluorescence images were captured using an IX73 inverted fluorescence microscope (Olympus Corporation) (42).

Transmission electron microscopy (TEM). Samples were initially fixed with 2.5% glutaraldehyde at 4°C overnight and subsequently post-fixed with 1% osmium tetroxide for 2 h at room temperature in the dark. After gradient ethanol dehydration, the samples were subjected to gradient infiltration and embedding using a dehydration agent and Epon-812 embedding agent. Ultrathin sections (60-90 nm thick) were prepared from the samples and mounted on copper grids. Following staining with uranyl acetate and lead citrate, the grids were dried overnight at room temperature, and images were acquired using a TEM (JEM-1400FLASH; JEOL, Ltd.) (43).

Western blotting. A BCA protein assay kit (Beyotime Institute of Biotechnology) was used to quantify protein concentration. Protein lysates were loaded at equal amounts of 30 µg per lane. SDS-PAGE was performed using 6%, 7.5%, 10%, 12.5%, or 15% separating gels selected based on target protein size. Proteins were subsequently transferred to PVDF membranes and blocked with 5% non-fat milk for 1 h at room temperature. The membranes were incubated with primary antibodies overnight at 4°C and then with secondary antibodies for 1 h at room temperature. Protein bands were visualized using ECL developer solution (Pierce; Thermo Fisher Scientific, Inc.). Densitometric analysis was conducted with ImageJ software (version 1.51j8; National Institutes of Health).

Small interfering RNA (siRNA) transfection. siRNA targeting DOHH (si-DOHH) and negative control siRNA (si-NC; cat. no. siN0000001-1) were synthesized by Guangzhou RiboBio Co., Ltd. The si-DOHH sequences were as follows: Sense, 5'-GCAGAGCUUUGGCGUCUAAtt-3'; antisense, 5'-UUA GACGCCAAAGCUCUGCgg-3'. HUVECs were seeded in 6-well plates and transfected with si-DOHH or si-NC at a final concentration of 50 nM using the riboFECT™ CP Transfection Kit (cat. no. C10511-05; Guangzhou RiboBio Co., Ltd.) according to the manufacturer's instructions when the cells reached ~60-70% confluence. At 48 h post-transfection, the cells were harvested for western blot analysis.

Animals. A total of 52 mice aged 6-8 weeks were used in the present study, including 24 C57BL/6 mice (18-25 g), 21 *db/db* mice (30-45 g), and 7 wild-type (WT) control mice (18-25 g). All mice were supplied by Huafukang Biological Technology Co., Ltd. and allowed to acclimate for 1 week before experimentation. Of these, data from 3 C57BL/6 mice were excluded from the final analysis due to incomplete data collection; therefore, data from 49 mice were included in the final analysis. The mice were maintained under standard laboratory conditions, including a 12/12-h light/dark cycle, 22°C temperature, and 60±10% humidity, with free access to food and water. Animals were randomly assigned to groups, and investigators were blinded to group allocation during outcome evaluation and data analysis. Sample sizes were determined based on previous studies to ensure adequate statistical power. Humane endpoints were predefined before the study. Mice were euthanized before the scheduled endpoint if they exhibited severe body weight loss exceeding 20% of baseline body weight, persistent anorexia or dehydration, severe lethargy, inability to access food or water, labored breathing, unrelieved pain or distress, or a moribund condition. No obvious signs of

distress or morbidity were observed in any animal throughout the study. At the end of the 7-week experimental period, mice were anesthetized via intraperitoneal injection of 1% pentobarbital sodium at 50 mg/kg body weight, followed by blood collection via cardiac puncture, euthanasia by cervical dislocation, and harvesting of vascular tissues. The study was conducted in compliance with the ARRIVE guidelines (<https://arriveguidelines.org>). All animal procedures were approved by the Animal Ethics Committee of Southwest Medical University (approval no. 2020895; Luzhou, China).

MGO, BZ and DOHH inhibitor administration. Mice were intraperitoneally administered MGO 5 days per week for 7 consecutive weeks. The dose was set at 50 mg/kg body weight for the first 2 weeks, increased to 60 mg/kg for the next three weeks, and further raised to 75 mg/kg during the final 2 weeks (44). During the final five weeks, the mice received BZ by oral gavage at 30 mg/kg/day, with the dosage calculated from published effective regimens and IP/PO bioavailability conversion (45). DOHH inhibitor (25 mg/kg/day) were administered by oral gavage once daily, five times per week, for 5 consecutive weeks (46). Both the control and WT were treated with physiological saline.

Measurement of MGO levels and autophagy/apoptosis-related factors. MGO concentrations and serum levels of autophagy/apoptosis-related markers, including LC3, p62 and cleaved caspase-3, were quantified with commercial ELISA kits according to the prescribed protocol.

H&E staining. Tissue samples underwent fixation in 4% PFA, dehydration, embedding, processing and deparaffinization, followed by pretreatment, hematoxylin staining, bluing, and eosin staining. After dehydration through a graded ethanol series and clearing in xylene, the sections were mounted with neutral resin and scanned in bright-field mode using a whole-slide scanner (Pannoramic SCAN II; 3DHISTECH Ltd.).

TUNEL staining. Paraffin-embedded mouse artery tissue samples were washed twice with xylene, soaked once with gradient ethanol (95 and 75%), rinsed twice in PBS, and subsequently treated with proteinase K solution for 20 min at room temperature. After two PBS washes, TUNEL reaction solution was applied to the slides, which were then incubated for 1 h at 37°C. The slides were rinsed three times with PBS and incubated with Converter-POD for 30 min at 37°C. Following another three PBS washes, DAB substrate was added and allowed to react for 10 min at room temperature. After a final PBS wash series, the slides were counterstained with methyl green, cover-slipped with a mounting medium, and scanned in bright-field mode using a whole-slide scanner (Pannoramic SCAN II; 3DHISTECH Ltd.). A total of five randomly selected, non-overlapping fields of view encompassing the luminal endothelial layer were evaluated at x400 magnification for the quantification of TUNEL-positive cells within the endothelial layer.

Immunohistochemical detection. Aortic paraffin sections were deparaffinized using deparaffinization solution and absolute

ethanol, and endogenous peroxidase activity was blocked with 3% hydrogen peroxide. After blocking with serum or BSA (cat. no. 36101ES; Shanghai Yeasen Biotechnology Co., Ltd.), the sections were sequentially incubated with primary and secondary antibodies, developed with DAB, counterstained with hematoxylin, and dehydrated through ethanol. The slides were finally sealed and scanned under bright-field mode using a whole-slide scanner (Pannoramic SCAN II; 3DHISTECH Ltd.).

Triple immunofluorescence. Paraffin-embedded sections were dewaxed, rehydrated, and subjected to antigen retrieval. After the staining areas were outlined, endogenous peroxidase activity was inhibited with hydrogen peroxide, and serum blocking was performed to reduce nonspecific binding. The sections were incubated with primary and secondary antibodies, followed by tyramide signal amplification (TSA) fluorescence reactions using iF488-Tyramide (cat. no. G1231), iF555-Tyramide (cat. no. G1233) and iF647-Tyramide (cat. no. G1232), all purchased from Wuhan Servicebio Technology Co., Ltd. Between consecutive staining cycles, the sections underwent microwave treatment and another round of serum blocking before the subsequent staining cycle. Finally, nuclei were counterstained with DAPI (1 µg/ml), autofluorescence was quenched, and the sections were mounted for imaging.

Statistical analysis. Data analysis was performed using ImageJ and GraphPad Prism 9.0 software (Dotmatics). Results are expressed as the mean ± standard deviation (SD). Before statistical testing, normal distribution and homogeneity of variance were evaluated. Differences between two groups were analyzed using an unpaired two-tailed Student's t-test, whereas comparisons among multiple groups were conducted by one-way ANOVA followed by Tukey's HSD post hoc test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Inhibitory effect of BZ on MGO-induced autophagy is associated with the DOHH/eIF5A pathway. To establish a model of MGO-induced endothelial injury, the effects of MGO (Fig. 1A) and BZ (Fig. 1B) on HUVEC viability were first evaluated using a CCK-8 assay. Following 24 h of exposure to MGO at concentrations of 10, 20, 50, 100, or 200 µM, HUVEC viability decreased significantly, in agreement with previous findings (Fig. 1C) (19–21). Furthermore, after 2 h of treatment with BZ at concentrations of 1, 2, and 5 µM, no marked side effects were observed. However, when the concentration increased to 10 µM, cell viability began to decrease (Fig. 1D). Additionally, 2 h of pretreatment with BZ at 1, 2, or 5 µM dose-dependently restored HUVEC viability suppressed by MGO (Fig. 1E).

To determine whether BZ modulates MGO-induced autophagy, HUVECs were exposed to BZ at 1, 2, or 5 µM for 2 h before treatment with 100 µM MGO for 24 h. Changes in the level of intracellular autophagy were detected by TEM. Compared with controls, exposure to MGO markedly increased autophagic vesicle and autolysosome formation, whereas BZ supplementation attenuated these alterations in a dose-dependent manner. In particular, the number of autophagosomes/autophagolysosomes was minimized at a

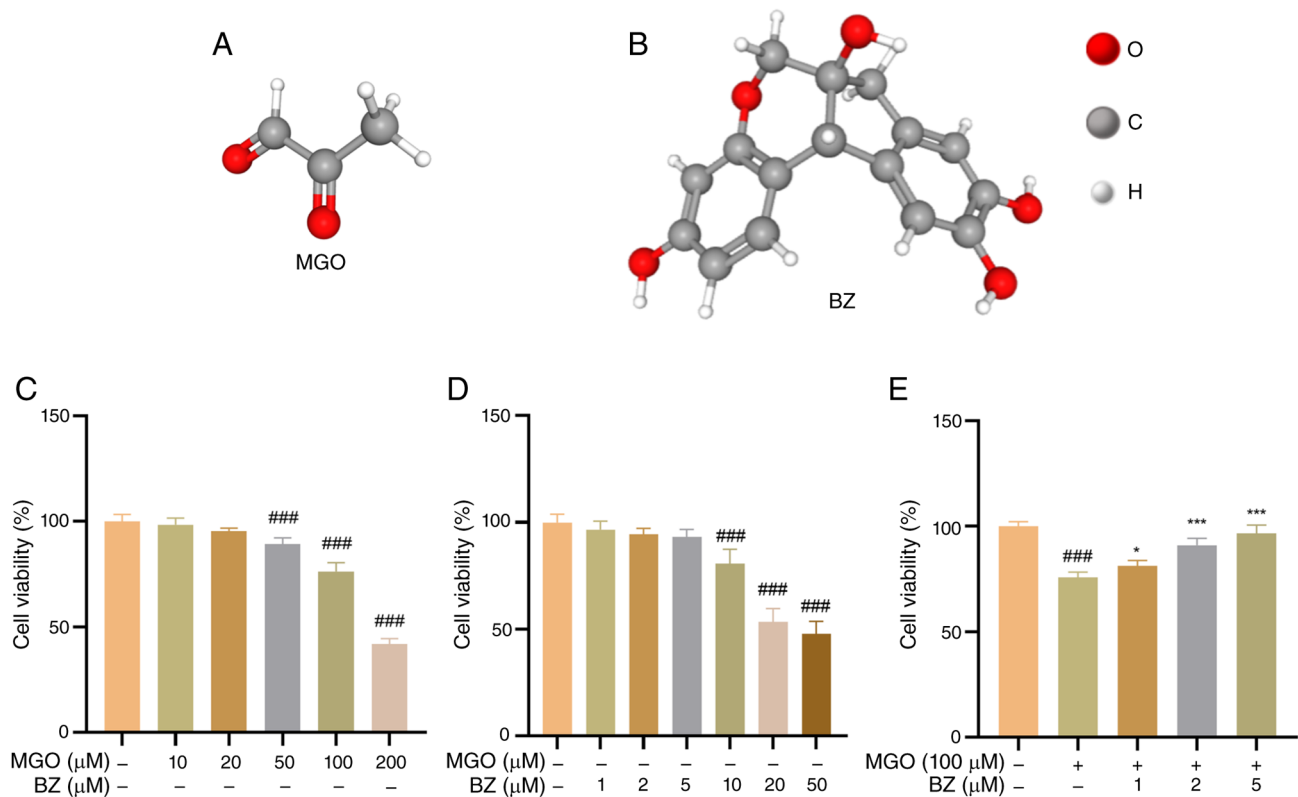


Figure 1. Stereostructures of MGO and BZ and the results of the CCK-8 method for screening the appropriate concentrations of MGO and BZ. (A and B) MGO and BZ stereoscopic molecular structures. (C and D) The effects of varying MGO and BZ concentrations on cell viability were assessed by CCK-8 assay. (E) Human umbilical vein endothelial cells were exposed to BZ at 1, 2, and 5 μ M for 2 h before treatment with 100 μ M MGO for 24 h, and cell viability was subsequently assessed. ###P<0.001 vs. Ctrl; *P<0.05 and ***P<0.001 vs. MGO. MGO, methylglyoxal; BZ, brazilin; CCK-8, Cell Counting Kit-8.

BZ concentration of 5 μ M (Fig. 2A). Similarly, LC3 immunofluorescence experiments revealed that the fluorescence intensity was the strongest after the addition of MGO, and the autophagic flux reached a maximum. However, BZ progressively attenuated cellular autophagy, with the most pronounced reduction observed at 5 μ M (Fig. 2B). Moreover, immunoblotting experiments were used to further verify the changes in autophagy-related protein levels. Relative to the control group, MGO significantly elevated the LC3-II/LC3-I ratio and Beclin1 levels while reducing p62 expression. By contrast, BZ treatment dose-dependently reversed these changes by lowering LC3-II/LC3-I and Beclin1 levels and restoring p62 expression (Fig. 2C and D). As illustrated in the schematic diagram in Fig. 2E, BZ has been reported to mediate its protective effects via DOHH, a target protein, and the downstream eIF5A signaling cascade (39).

The level of DOHH, a BZ target protein, was subsequently further examined by western blotting. Interestingly, treatment with either BZ or MGO did not significantly alter DOHH levels relative to the control group. Hypusinated eIF5A expression was assessed and the potential mechanism by which BZ exerts its effects through the DOHH/eIF5A axis was explored. Compared with the blank group, MGO stimulation did not markedly affect hypusinated eIF5A levels, whereas BZ pretreatment elevated hypusinated eIF5A, with a significant increase observed at 5 μ M BZ (Fig. 2F and G). Therefore, these findings suggest that BZ may activate the DOHH/eIF5A signaling cascade to enhance

eIF5A hypusination, thereby regulating MGO-induced cellular responses.

BZ reverses MGO-induced apoptosis in HUVECs. These findings confirmed that BZ attenuated MGO-induced autophagy in ECs. Therefore, it was further examined whether BZ modulates MGO-triggered apoptosis. As aforementioned, HUVECs were preincubated with BZ at 1, 2, or 5 μ M for 2 h and subsequently exposed to 100 μ M MGO for 24 h. Annexin V-FITC/PI double staining was performed to quantify apoptosis by flow cytometry. MGO exposure significantly increased the apoptotic rate compared with controls, while BZ pretreatment progressively reduced apoptosis with increasing concentrations (Fig. 3A and B). Molecular analyses further corroborated these findings, revealing dose-dependent reductions in Bax and the cleaved caspase-3/caspase-3 ratio, alongside a concomitant increase in Bcl-2 expression (Fig. 3C and D). These findings collectively demonstrate that BZ not only mitigates MGO-induced autophagy but also effectively blocks the subsequent apoptotic cascade.

Combined flow cytometry and western blot data revealed that BZ was able to suppress autophagic and apoptotic responses elicited by MGO in HUVECs. As the AMPK/mTOR pathway is pivotal for autophagy regulation (47,48), it was hypothesized that BZ confers protection by modulating this pathway. Accordingly, it was first examined how MGO and BZ affected core pathway proteins at the phosphorylation level. Relative to the control group, MGO treatment elevated phosphorylated

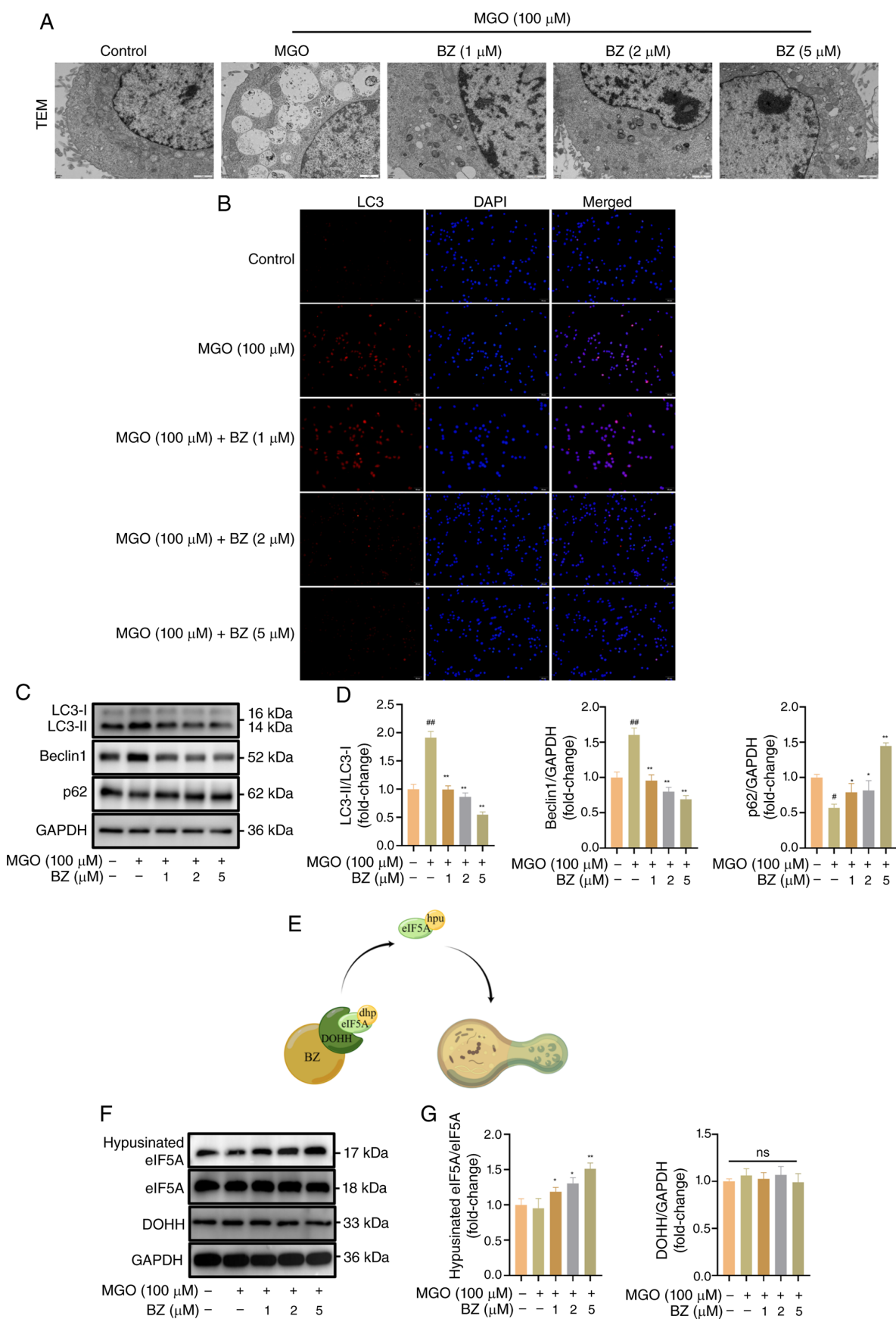


Figure 2. BZ attenuates MGO-induced autophagy in endothelial cells. Human umbilical vein endothelial cells were exposed to BZ at 1, 2, and 5 μ M for 2 h before treatment with 100 μ M MGO for 24 h. (A) Changes in the level of autophagy were detected by transmission electron microscopy. Scale bar, 1 μ m. (B) Changes in autophagy were detected by LC3 immunofluorescence, with red fluorescence representing LC3 and blue fluorescence representing DAPI. (C and D) The LC3-II/LC3-I ratio and the protein expression of Beclin1 and p62 were evaluated by western blot analysis. (E) The mechanism of action of BZ on DOHH. (F and G) Hypusinated eIF5A, eIF5A and DOHH protein expression was measured by western blot analysis. [#]P<0.05 and ^{##}P<0.01 vs. Ctrl; *P<0.05 and **P<0.01 vs. MGO. MGO, methylglyoxal; BZ, brazilin; DOHH, deoxyhypusine hydroxylase; eIF5A, eukaryotic translation initiation factor 5A.

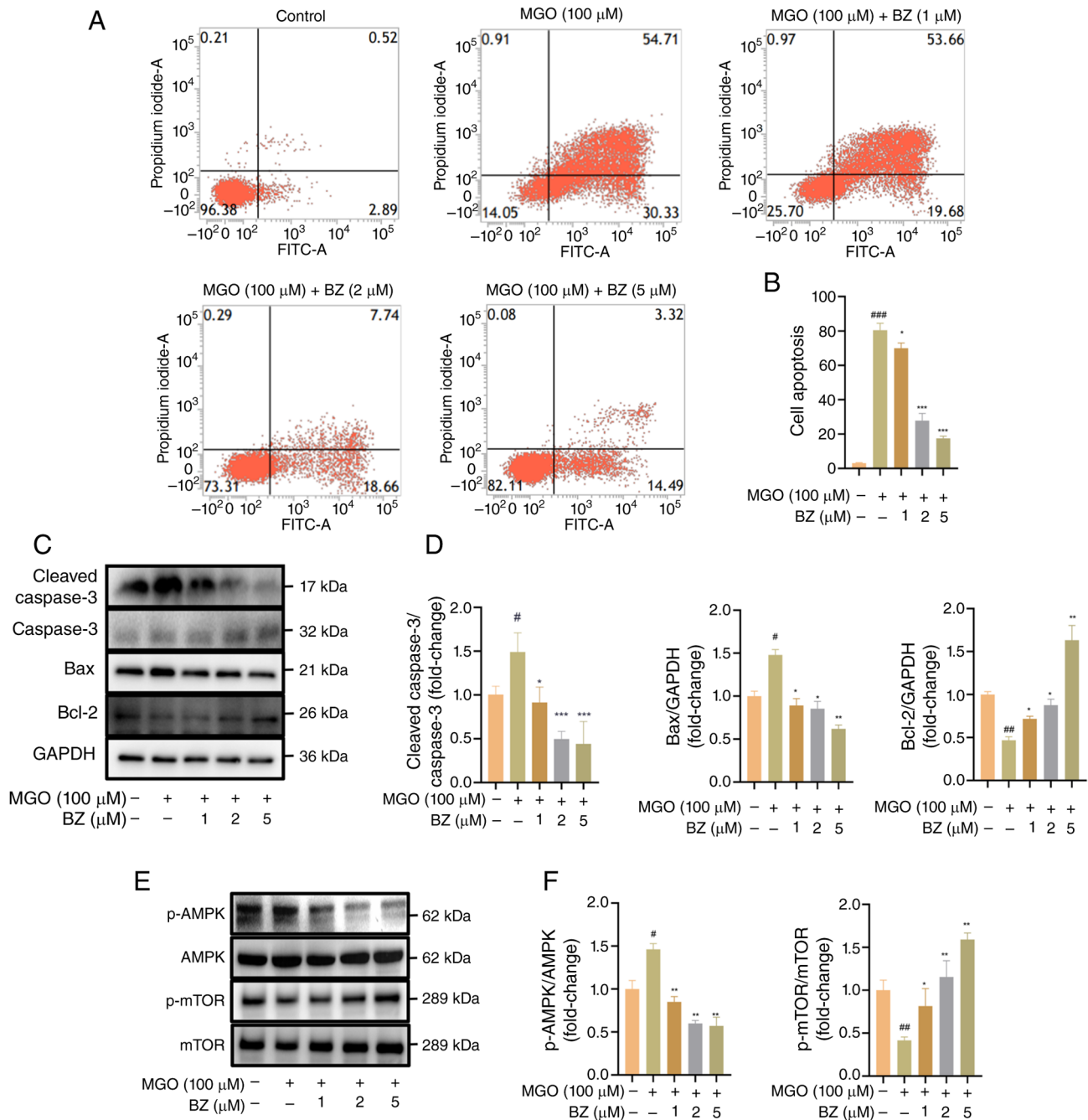


Figure 3. MGO-induced apoptosis is reversed by BZ. (A and B) After trypsinization, cells were stained with Annexin V-FITC and PI for 20 min, followed by flow cytometric quantification of apoptosis. (C-F) Bax, cleaved caspase-3/caspase-3, Bcl-2, p-mTOR, mTOR, p-AMPK and AMPK protein expression was assessed by western blot analysis. # P <0.05 ** P <0.01 and *** P <0.001 vs. Ctrl; * P <0.05 ** P <0.01 and *** P <0.001 vs. MGO. MGO, methylglyoxal; BZ, brazilin; p-, phosphorylated.

(p-)AMPK levels while reducing p-mTOR levels. Moreover, BZ pretreatment dose-dependently decreased p-AMPK levels while increasing p-mTOR levels (Fig. 3E and F). These findings suggest that MGO induces apoptosis and AMPK/mTOR signaling, which is modulated by BZ.

Effects of 3-MA on MGO-induced autophagy and apoptosis in HUVECs. To further determine whether MGO-induced cellular autophagy and apoptosis are associated with the inhibitory effect of BZ on autophagy, HUVECs were pretreated with BZ (5 μ M) + 3-MA (5 mM, an autophagy inhibitor) for 2 h and co-incubated them for 24 h with MGO (100 μ M) (49).

TEM was used to observe the changes in autophagosomes. BZ clearly reduced MGO-induced autophagy, while combined BZ and 3-MA pretreatment further suppressed this response, with reduced autophagosome and autolysosome formation (Fig. 4A). Further LC3 immunofluorescence analysis was conducted to determine whether 3-MA suppressed MGO-evoked autophagy in ECs and enhanced the protective effects of BZ. As observed in aforementioned results, the red fluorescence was strongest in the MGO-treated group. After the addition of BZ, autophagic flux was reduced. In addition, cotreatment with 3-MA and BZ further potentiated BZ-mediated suppression of MGO-induced autophagy in HUVECs (Fig. 4B).

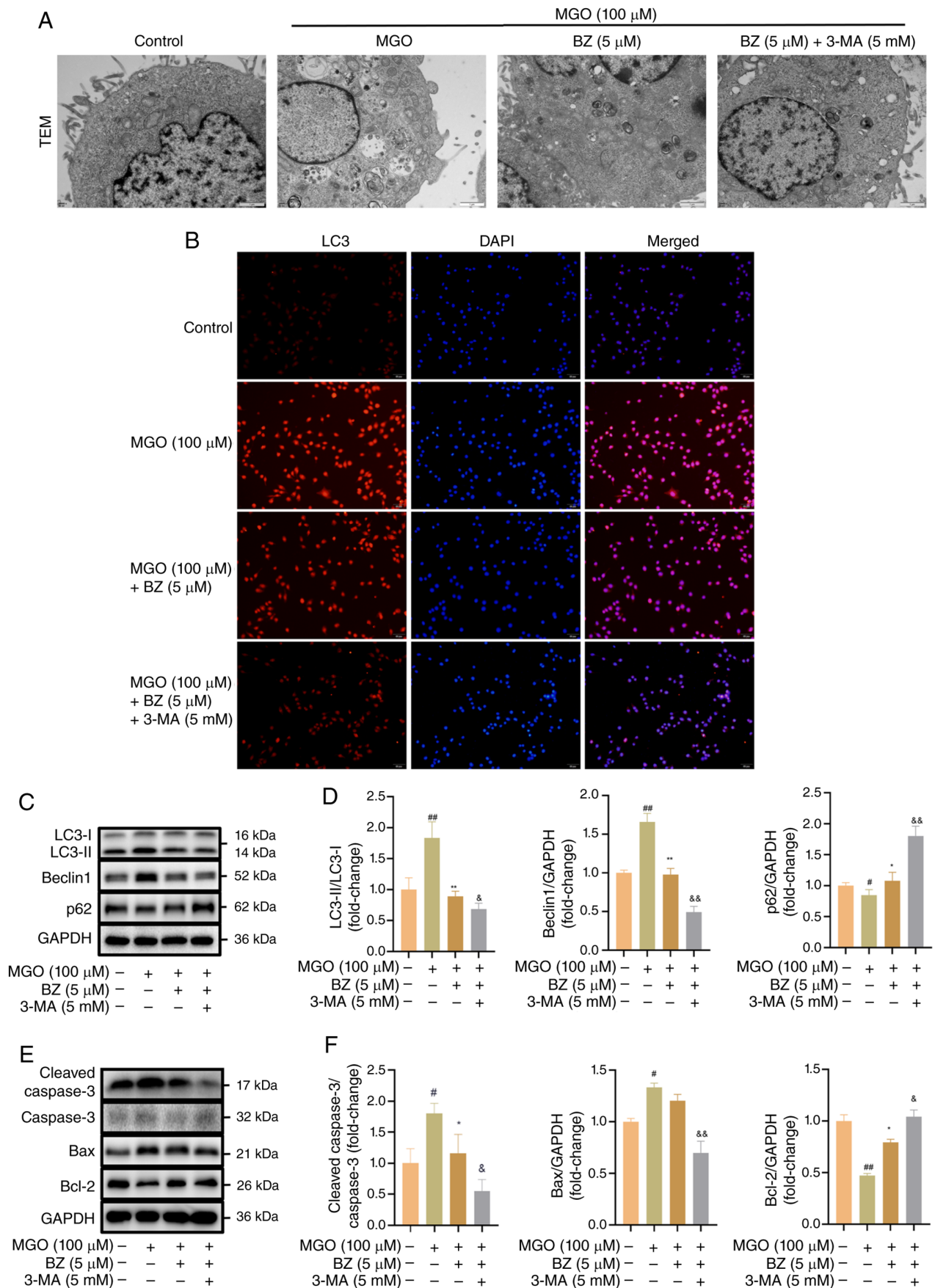


Figure 4. Inhibitory effect of BZ on MGO-induced autophagy and apoptosis in HUVECs was enhanced by 3-MA. HUVECs were preincubated with BZ (5 μ M) in the presence or absence of 3-MA (5 mM) for 2 h, followed by exposure to MGO (100 μ M) for 24 h. (A) Changes in autophagy levels were detected by transmission electron microscopy. Scale bar, 1 μ m. (B) Autophagy was detected by LC3 immunofluorescence. (C-F) Protein expression of LC3-II/LC3-I, Beclin1, p62, Bcl-2, Bax and cleaved caspase-3/caspase-3 was evaluated by western blotting. [#]P<0.05 and ^{##}P<0.01 vs. Ctrl; ^{*}P<0.05 and ^{**}P<0.01 vs. MGO; [&]P<0.05 and ^{&&}P<0.01 vs. MGO + BZ. MGO, methylglyoxal; BZ, brazilin; HUVECs, human umbilical vein endothelial cells.

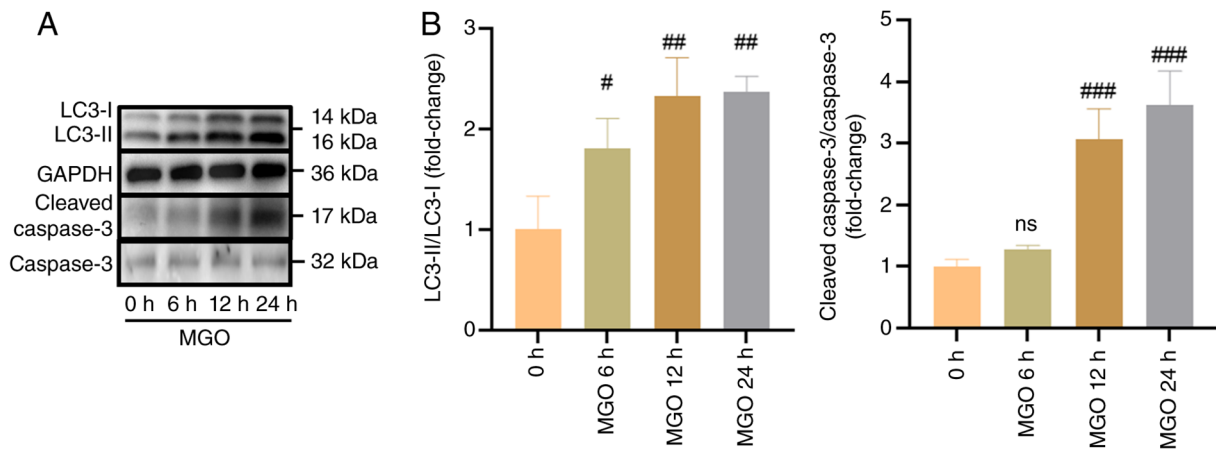


Figure 5. Dynamic time-course changes in autophagy and apoptosis markers in HUVECs. (A and B) Western blot analysis of the LC3-II/LC3-I and cleaved caspase-3/caspase-3 ratio in HUVECs treated with MGO for 0, 6, 12 and 24 h. * $P<0.05$, ** $P<0.01$ and *** $P<0.001$ vs. Ctrl. HUVECs, human umbilical vein endothelial cells; MGO, methylglyoxal; ns, not significant.

Autophagy- and apoptosis-related protein expression was examined using western blot analysis. Relative to the control group, MGO significantly enhanced cellular autophagic and apoptotic responses. However, pretreatment with BZ reversed these effects. Combined treatment with BZ and 3-MA further enhanced this reversal, significantly downregulating the LC3-II/LC3-I ratio, the expression of Beclin1 and Bax, as well as the cleaved caspase-3/caspase-3 ratio, while upregulating the expression levels of Bcl-2 and p62 (Fig. 4C-F). In summary, BZ can decrease the levels of autophagy and apoptosis and further reduce MGO-induced autophagy and apoptosis when combined with the autophagy inhibitor 3-MA. These data suggest that 3-MA and BZ have synergistic effects on the protection of vascular ECs.

To further demonstrate that MGO-induced autophagy occurs before apoptosis, the time-course changes in autophagy and apoptosis markers in HUVECs were examined at 0, 6, 12 and 24 h after MGO treatment. As shown in Fig. 5A and B, the autophagy marker LC3-II was significantly upregulated as early as 6 h after MGO stimulation, whereas the cleaved caspase-3 began to increase significantly at 12 h and remained elevated at 24 h. In addition, a chloroquine (CQ) blockade assay was performed to evaluate autophagic flux. As revealed in Fig. 6A and B, treatment with either CQ or MGO alone significantly increased LC3-II levels compared with the control group. Notably, combined treatment with MGO and CQ further increased LC3-II levels compared with MGO treatment alone. The results suggest that MGO enhances autophagic flux, rather than merely causing passive accumulation of autophagosomes due to impaired lysosomal degradation.

An AMPK inhibitor enhances the BZ-mediated suppression of MGO-induced autophagy and apoptosis. Current data indicate that MGO stimulation significantly increases the levels of p-AMPK while decreasing the levels of mTOR and that BZ can reverse these effects. Therefore, it was hypothesized that BZ may regulate MGO-triggered autophagic and apoptotic responses through AMPK/mTOR signaling. Before exposure to 100 μ M MGO for 24 h, HUVECs were preincubated for 2 h with BZ (5 μ M) in the presence or absence of

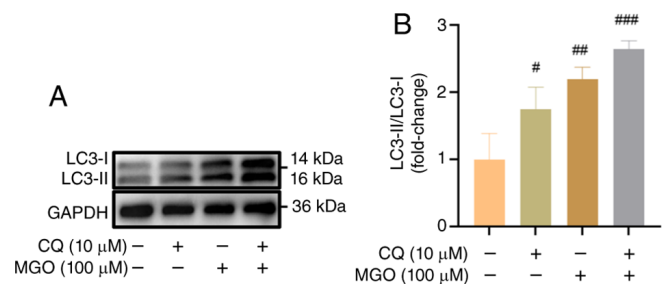


Figure 6. Effect of MGO on autophagic flux in endothelial cells. (A and B) Western blot analysis of LC3-II protein levels in human umbilical vein endothelial cells treated with MGO (100 μ M) and/or CQ (10 μ M) for 24 h. * $P<0.05$, ** $P<0.01$ and *** $P<0.001$ vs. Ctrl. MGO, methylglyoxal; CQ, chloroquine.

Compound C (50 μ M, an AMPK inhibitor) (50). TEM revealed that BZ reduced MGO-induced autophagy. Furthermore, after cotreatment with BZ and Compound C, the numbers of autophagosomes and autolysosomes further decreased (Fig. 7A). Additionally, flow cytometric analysis was further performed to assess apoptosis following AMPK blockade. The data showed that BZ attenuated MGO-induced HUVEC apoptosis. Notably, cotreatment with BZ and Compound C also attenuated HUVEC apoptosis, which blocked AMPK signaling (Fig. 7B and C). Western blot analysis revealed that Compound C, an AMPK inhibitor, further enhanced the BZ-mediated reversal, as reflected by reduced p-AMPK levels and increased p-mTOR levels (Fig. 7D and E). Overall, the inhibitory effects of BZ on MGO-induced autophagy and apoptosis appear to be further enhanced by Compound C.

BZ activates the DOHH/eIF5A pathway to regulate MGO-induced autophagy and apoptosis in ECs. The aforementioned results reveal that BZ does not alter DOHH levels in MGO-treated HUVECs but significantly increases hypusinated eIF5A levels, suggesting that BZ may inhibit MGO-triggered autophagy and apoptosis by enhancing DOHH-mediated eIF5A hypusination. To verify the aforementioned hypothesis, HUVECs were pretreated for 2 h with BZ (5 μ M) alone or

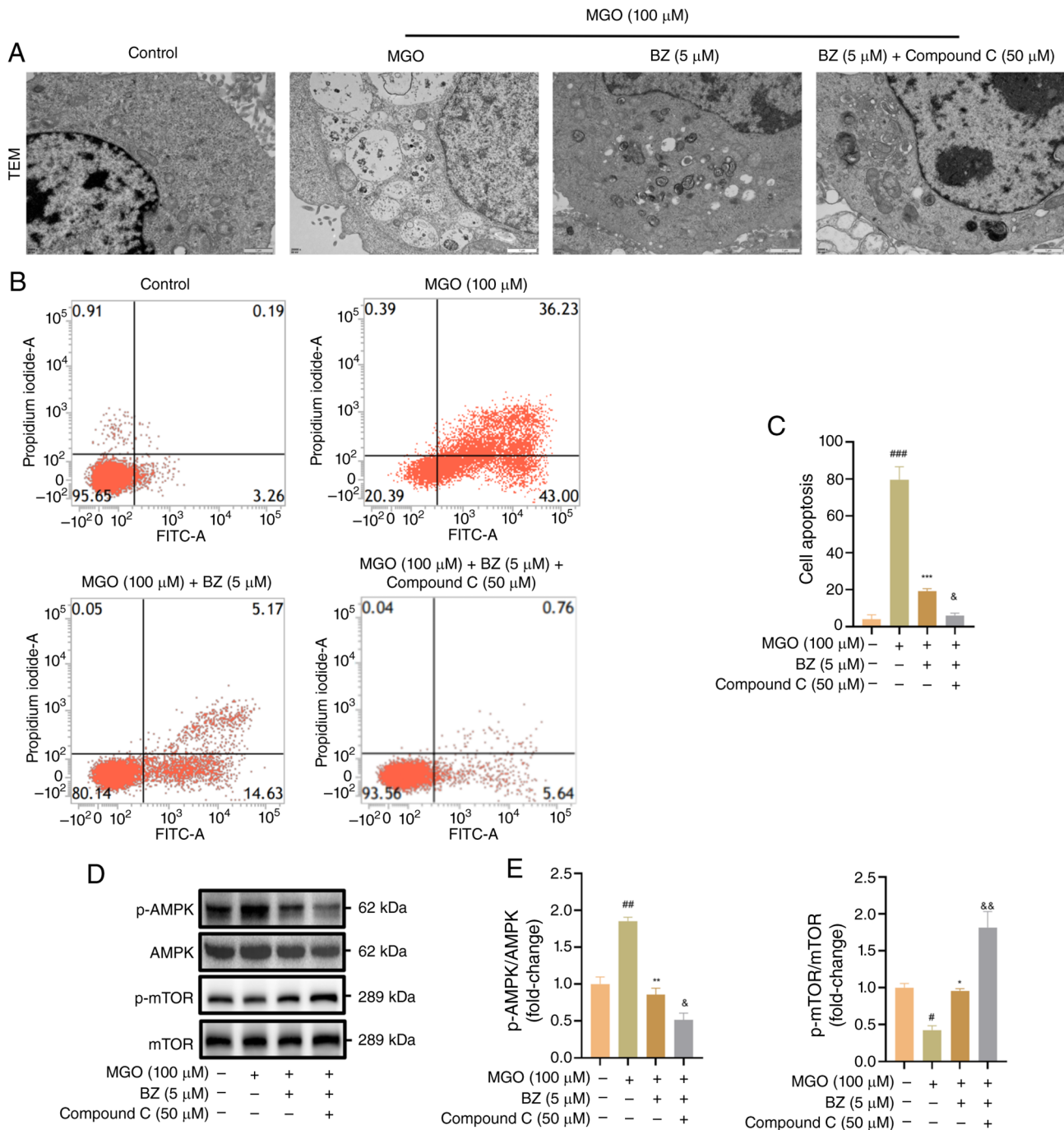


Figure 7. Effect of Compound C on BZ-mediated inhibition of MGO-induced autophagy and apoptosis in HUVECs. HUVECs were preincubated with BZ (5 μ M) in the presence or absence of Compound C (50 μ M) for 2 h, followed by exposure to MGO (100 μ M) for 24 h. (A) Changes in the level of autophagy were detected by transmission electron microscopy. Scale bar, 1 μ m. (B and C) Apoptotic changes were assessed by flow cytometry. (D and E) Protein expression of p-AMPK, AMPK, p-mTOR and mTOR was evaluated by western blotting. # P <0.05, ## P <0.01 and ### P <0.001 vs. Ctrl; * P <0.05, ** P <0.01 and *** P <0.001 vs. MGO; & P <0.05 and && P <0.01 vs. MGO + BZ. BZ, brazilin; MGO, methylglyoxal; HUVECs, human umbilical vein endothelial cells; p-, phosphorylated.

with BZ (5 μ M) combined with ciclopirox (20 μ M), a DOHH signaling pathway inhibitor, and were subsequently stimulated with MGO (100 μ M) (51,52). TEM revealed that BZ inhibited the MGO-induced increases in autophagic vesicles and autolysosomes, whereas combined treatment with BZ, ciclopirox and MGO markedly elevated autophagic activity (Fig. 8A). These results were further confirmed by LC3 immunofluorescence, which revealed that BZ reduced the extent of MGO-induced autophagy and decreased autophagic flux. However, cells receiving combined treatment with BZ, ciclopirox and MGO

exhibited a marked increase in autophagic fluorescence intensity (Fig. 8B).

More importantly, as demonstrated in Fig. 8C and D, pretreatment with BZ reversed MGO-induced apoptosis, but the apoptotic rate increased after ciclopirox treatment. The aforementioned results suggest that the DOHH inhibitor ciclopirox could resist the protective effect of BZ and promote apoptosis. Subsequently, western blotting was performed to examine the BZ-associated downstream DOHH/eIF5A signaling cascade. DOHH levels were not

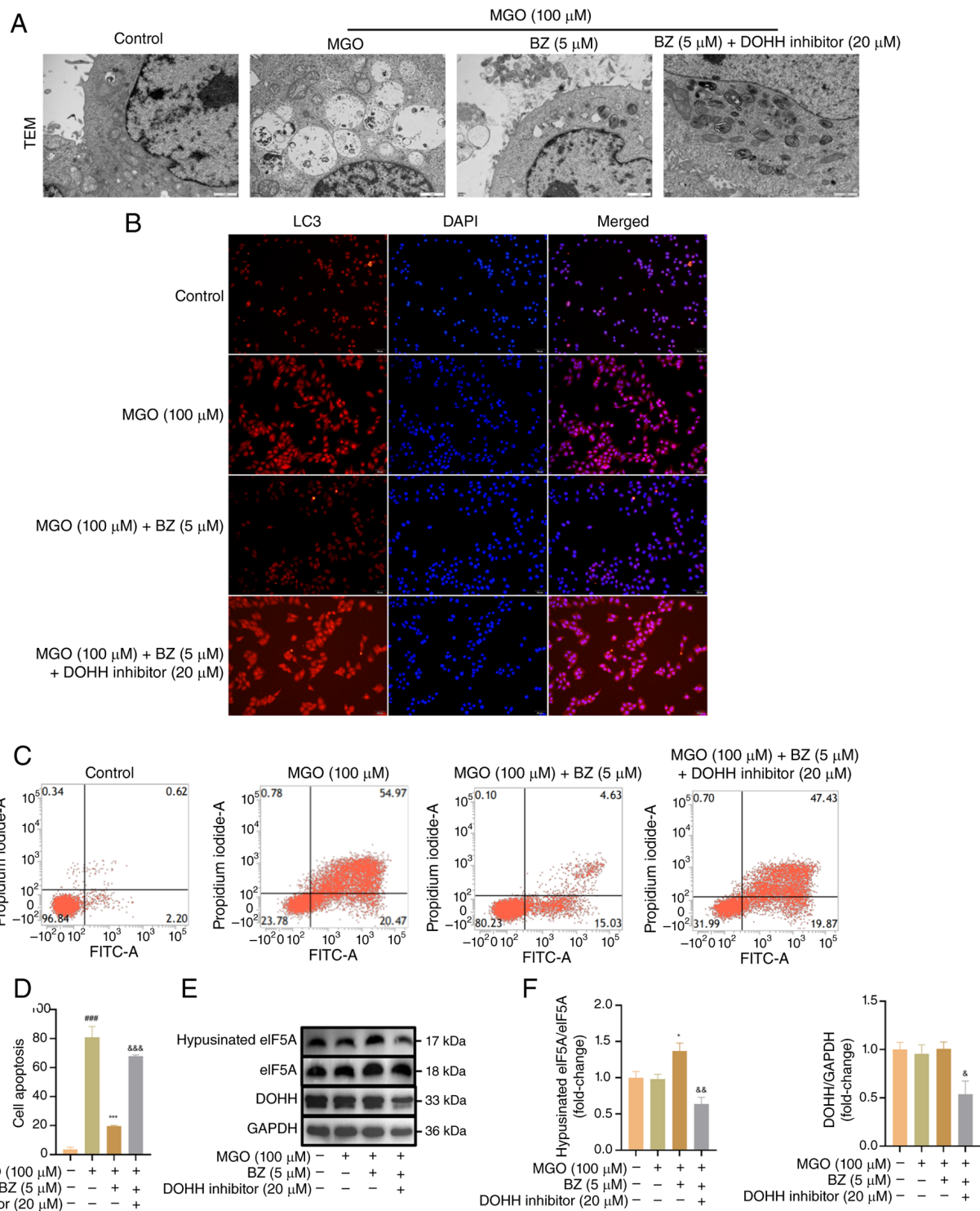


Figure 8. BZ-mediated inhibition of MGO-induced autophagy and apoptosis in HUVECs is reversed by ciclopirox. HUVECs were preincubated with BZ (5 μ M) or BZ (5 μ M) + the DOHH signaling pathway inhibitor ciclopirox (20 μ M) for 2 h and then combined with 100 μ M MGO for 24 h. (A) Changes in the level of autophagy were detected by transmission electron microscopy. Scale bar, 1 μ m. (B) Autophagy was detected by LC3 immunofluorescence. (C and D) Apoptotic changes were assessed by flow cytometry. (E and F) Hypusinated eIF5A, eIF5A and DOHH protein expression was evaluated by western blotting. *** P <0.001 vs. Ctrl; * P <0.05 and *** P <0.001 vs. MGO; & P <0.05, && P <0.01 and &&& P <0.001 vs. MGO + BZ. BZ, brazilin; MGO, methylglyoxal; HUVECs, human umbilical vein endothelial cells; DOHH, deoxyhypusine hydroxylase; eIF5A, eukaryotic translation initiation factor 5A.

significantly altered by BZ pretreatment or MGO exposure relative to the blank group; however, pretreatment with the DOHH inhibitor ciclopirox significantly reduced DOHH expression. Hypusinated eIF5A levels were not markedly altered by MGO treatment relative to the blank control group, whereas BZ pretreatment increased them; however, this

response was significantly reversed by ciclopirox pretreatment (Fig. 8E and F). These findings indicate that ciclopirox promotes autophagic and apoptotic responses elicited by MGO in HUVECs through suppression of the DOHH/eIF5A signaling cascade and abolishment of the cytoprotective effects of BZ.

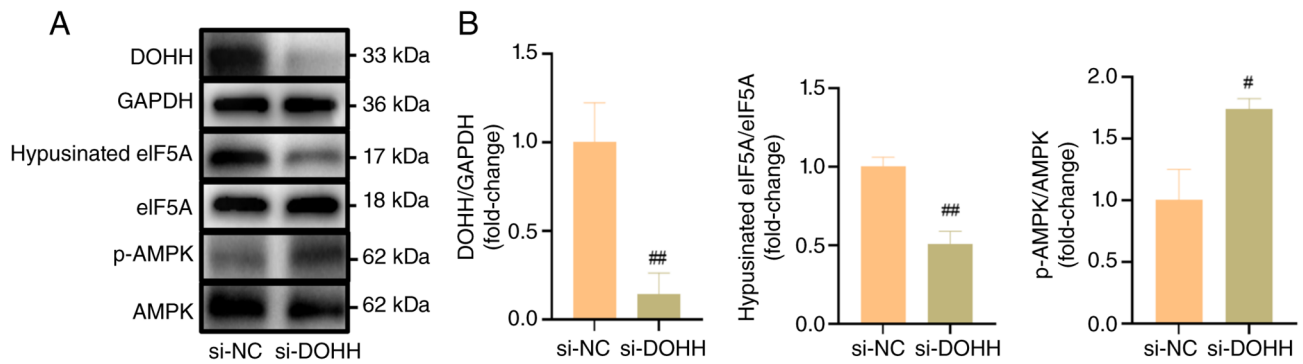


Figure 9. DOHH knockdown by si-DOHH reduces hypusinated eIF5A levels and activates AMPK signaling. (A and B) Western blot analysis of DOHH, hypusinated eIF5A, eIF5A, p-AMPK and AMPK protein levels in cells transfected with si-NC or si-DOHH. [#]P<0.05 and ^{##}P<0.01 vs. siNC. DOHH, deoxyhypusine hydroxylase; si-, small interfering; eIF5A, eukaryotic translation initiation factor 5A; p-, phosphorylated; NC, negative control.

To exclude the potential off-target effects of ciclopirox, si-DOHH transfection was further performed in the present study to verify the specificity of DOHH inhibition and to investigate its relationship with AMPK activation. As revealed in Fig. 9A and B, si-DOHH transfection significantly reduced the expression levels of DOHH and hypusinated eIF5A, while significantly increasing p-AMPK levels. These results suggest that inhibition of the DOHH/eIF5A pathway may be closely associated with AMPK activation.

Physiological and pathological changes in mice. To assess whether BZ modulates MGO-evoked vascular autophagy and apoptosis *in vivo*, C57BL/6J mice were administered MGO and BZ. Body weight, food intake and serum MGO concentration were measured after intraperitoneal injection of MGO and intragastric administration of BZ. The experimental design, including the sampling strategy and treatment regimen for the mouse study, is presented in Fig. 10A. The results showed a modest increase in body weight across all mouse groups after treatment, with no statistically significant difference from pretreatment body weights, which may be attributable to normal physiological growth during the 7-week feeding period (Fig. 10B). Interestingly, the food intake of the mice did not significantly change after administration compared with that of the pre-administration mice (Fig. 10C). These data indicate that treatment with BZ and MGO does not significantly affect the physiological functions of mice. Next, serum MGO levels were quantified, revealing a significant elevation in the MGO group and a significant reduction following BZ treatment (Fig. 10D). Dynamic alterations in autophagy- and apoptosis-related factors, including LC3, p62 and cleaved caspase-3, were examined. As shown in Fig. 10E-G, MGO significantly elevated LC3 and cleaved caspase-3 levels while reducing p62 expression. However, these effects were significantly suppressed upon the addition of BZ.

Moreover, H&E and TUNEL staining were used to evaluate whether BZ affects MGO-induced vascular injury. H&E-stained aortic sections from the control group showed an intact, smooth, and regularly organized intima. Conversely, MGO treatment led to substantial thickening, irregularity, a darker color, and a more disordered shape, indicating pronounced vascular damage. Following BZ treatment, the

dilatation and injury of the aortic intima were significantly ameliorated (Fig. 10H and J). Additionally, TUNEL staining was performed to further observe changes in EC apoptosis. MGO treatment significantly increased the apoptotic rate in mouse aortic vessels, but this effect was significantly reversed after BZ treatment (Fig. 10I and K).

BZ decreases the MGO-induced autophagy and apoptosis of blood vessels. Additionally, to determine whether BZ inhibits autophagy and apoptosis *in vivo*, immunohistochemical experiments were conducted. Relative to the control group, the MGO group showed elevated vascular autophagy levels, increased expression of LC3, Beclin1 and Bax, and reduced positivity rates for p62 and Bcl-2. However, these effects were reversed upon BZ treatment (Fig. 11A-J). Harvested mouse vascular tissue was subsequently analyzed by western blotting to further validate these findings. It was found that MGO elevated Beclin1 and Bax levels, whereas BZ counteracted these changes and attenuated autophagic and apoptotic responses (Fig. 11K-M). These data align with the cellular findings and indicate that BZ markedly suppresses vascular cell autophagic and apoptotic responses triggered by MGO, thereby supporting its vasoprotective effects.

Effects of MGO and BZ on changes in DOHH and hypusinated eIF5A *in vivo*. The aforementioned *in vitro* findings revealed that BZ suppresses autophagic and apoptotic responses elicited by MGO in ECs through the DOHH/eIF5A pathway. Immunofluorescence localization was used to examine changes in DOHH and hypusinated eIF5A in vascular ECs. In the control group, ECs (red fluorescence), DOHH (green fluorescence) and hypusinated eIF5A (yellow fluorescence) were observed. Furthermore, the results from MGO treatment alone aligned with the *in vitro* findings, showing that MGO did not alter DOHH or hypusinated eIF5A positivity in ECs. Although DOHH positivity remained unchanged after MGO and BZ treatment, hypusinated eIF5A positivity was significantly increased (Fig. 12A). Consistently, western blot analysis of mouse vascular tissues showed that MGO did not affect DOHH or hypusinated eIF5A protein levels relative to the control group, whereas MGO and BZ cotreatment significantly elevated hypusinated eIF5A levels. These results further confirmed that BZ enhances the post-translational

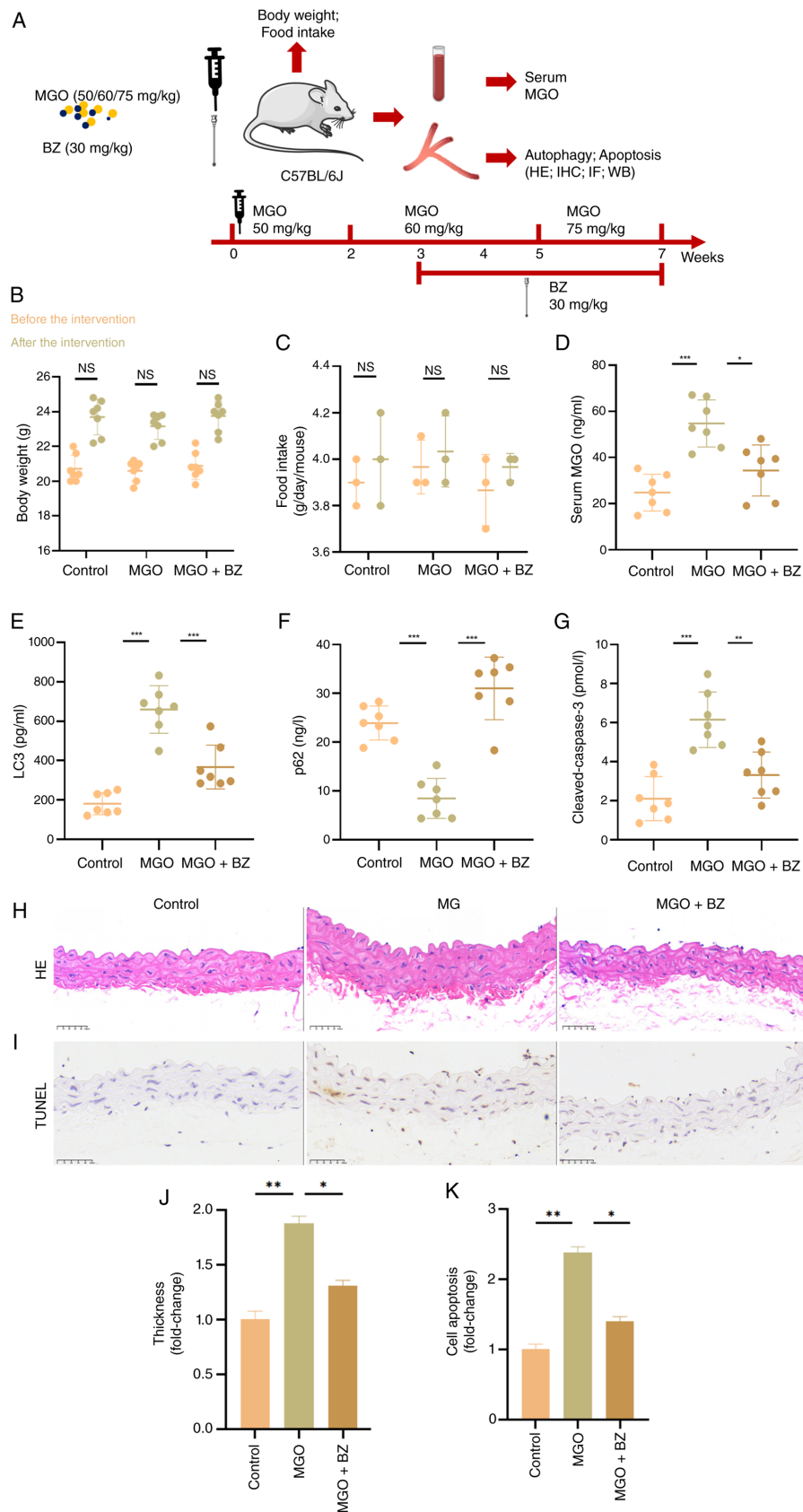


Figure 10. Changes in the physiological features of mice and the inhibition of MGO-induced aortic vascular injury and apoptosis by BZ. C57BL/6J mice were intraperitoneally injected with normal saline or MGO solution (50, 60, or 75 mg/kg/d), and BZ or normal saline was administered by gavage from the third week. (A) Experimental design, sampling strategy and treatment schedule in mice. (B) The weight changes of each mouse were measured before and after the experiment and recorded statistically. (C) Changes in food intake before and after the experiment were measured and statistically recorded. (D) Changes in serum MGO concentration were determined by extracting serum from mice euthanized after drug administration. (E-G) Expression changes in autophagy/apoptosis-related factors, including LC3, p62 and cleaved caspase-3, were evaluated. (H and I) H&E staining was performed to assess vascular endothelial morphology. (I and K) TUNEL staining was performed to evaluate apoptosis in aortic vascular tissues. The number of mice in each group was ≥ 6 ; scale bar, 50 μm . * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$. MGO, methylglyoxal; BZ, brazilin; NS, not significant.

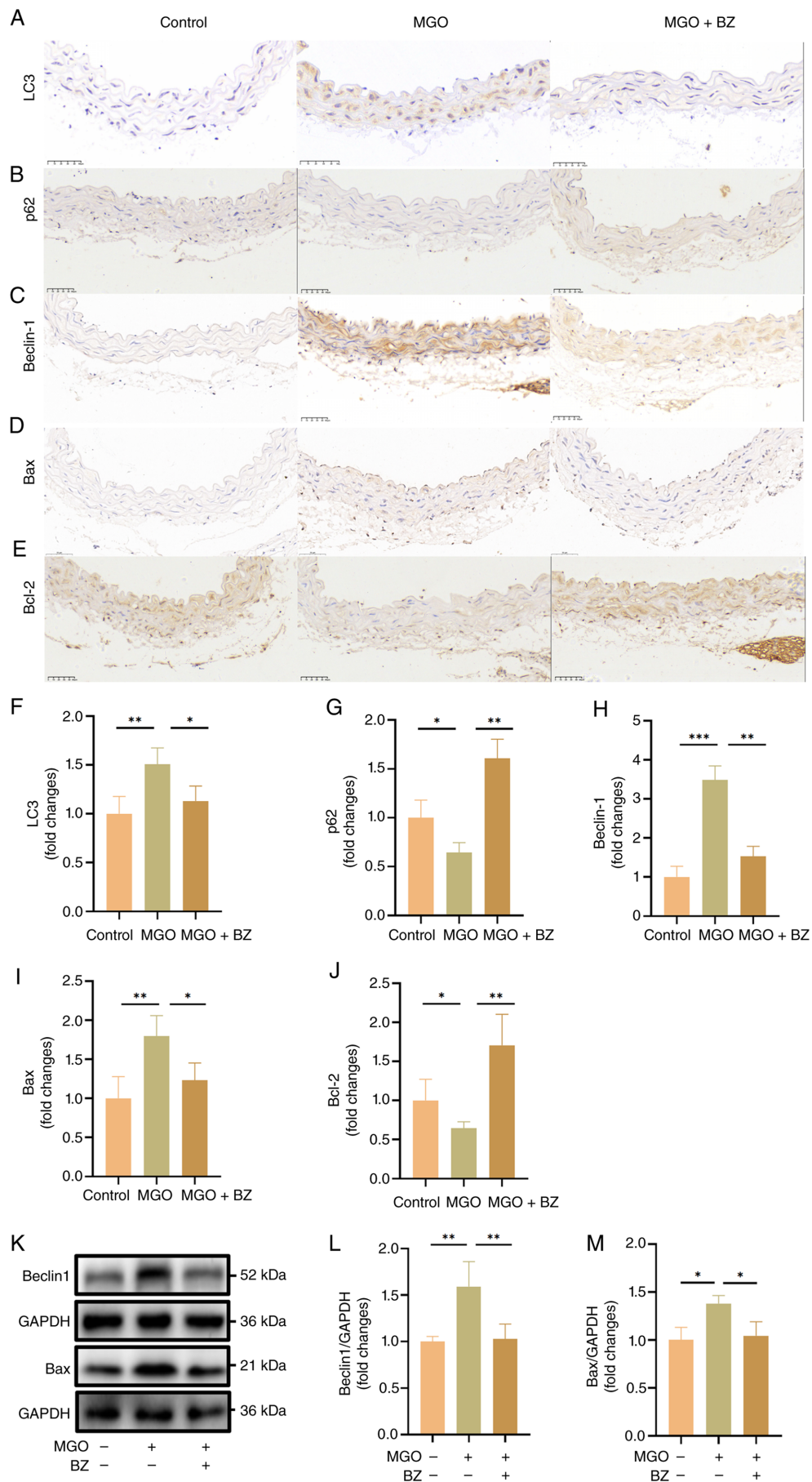


Figure 11. BZ decreases the MGO-induced autophagy and apoptosis of blood vessels. (A-J) Immunohistochemical staining was performed for LC3, p62, Beclin1, Bax and Bcl-2. Scale bar, 50 μ m. (K-M) Beclin1 and Bax protein expression was measured by western blot analysis. * P <0.05, ** P <0.01 and *** P <0.001. BZ, brazilin; MGO, methylglyoxal.

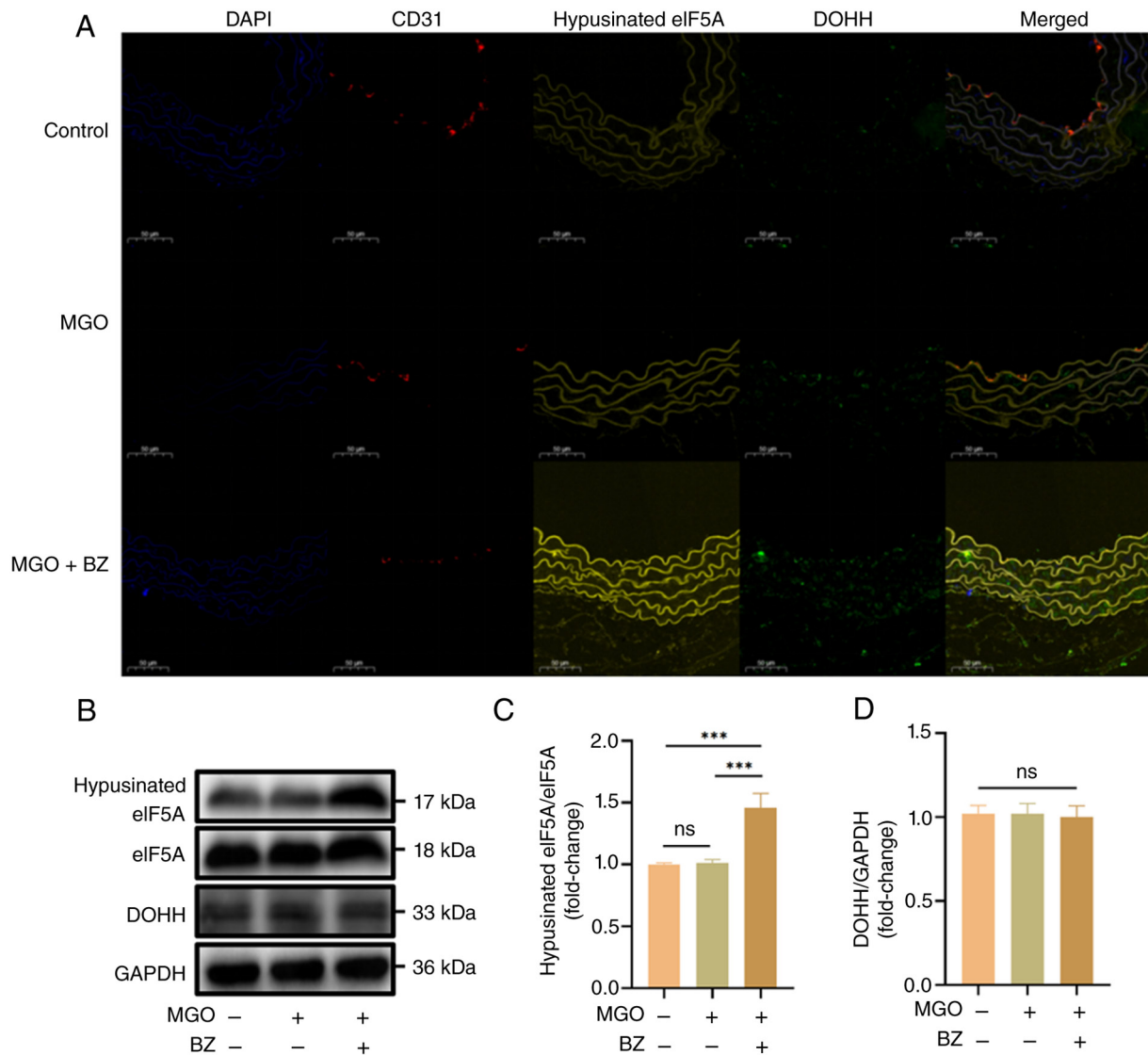


Figure 12. Effects of MGO and BZ on changes in DOHH and hypusinated eIF5A *in vivo*. (A) Changes in DOHH and hypusinated eIF5A in the vascular endothelial cells of mice were detected by immunofluorescence staining for three markers. Scale bar, 50 μ m. (B-D) Hypusinated eIF5A, eIF5A and DOHH protein expression was measured by western blot analysis. ***P<0.001. MGO, methylglyoxal; BZ, brazilin; DOHH, deoxyhypusine hydroxylase; eIF5A, eukaryotic translation initiation factor 5A; ns, not significant.

modification of the eIF5A protein by hypusine via allosteric activation of DOHH, thereby exerting its protective effects (Fig. 12B-D).

Effects of BZ and a DOHH inhibitor on autophagy and apoptosis in *db/db* mice. To more accurately mimic the pathophysiological characteristics of diabetes, *db/db* mice were used instead of MGO injection to investigate autophagy and apoptosis in vascular tissues as well as the involvement of the DOHH/eIF5A signaling pathway. Relative to the control group, DOHH and hypusinated eIF5A levels in vascular tissues from *db/db* mice showed no significant differences, whereas Beclin1 and Bax levels were elevated. This pattern was highly consistent with that observed in the MGO administration model, indicating that both diabetes and exogenous MGO stimulation increase the levels of autophagy and apoptosis in vascular tissues. After oral BZ administration to *db/db* mice, hypusinated eIF5A protein levels were significantly

elevated, whereas DOHH expression remained unchanged, and autophagic and apoptotic responses were reduced. By contrast, DOHH inhibitor treatment significantly reduced DOHH and hypusinated eIF5A levels, elevated Beclin1 and Bax levels, and enhanced autophagic and apoptotic responses (Fig. 13A-F). These findings indicate that autophagic and apoptotic responses triggered by MGO are aligned with diabetes-related vascular pathological changes, while BZ may act as a promising therapeutic candidate for diabetic vascular injury.

Discussion

MGO is a highly reactive glucose-derived metabolic byproduct that forms stable AGEs through the non-enzymatic chemical modification of proteins (13,17,53). MGO compromises vascular EC integrity and homeostasis by enhancing extracellular matrix collagen cross-linking, remodeling vascular

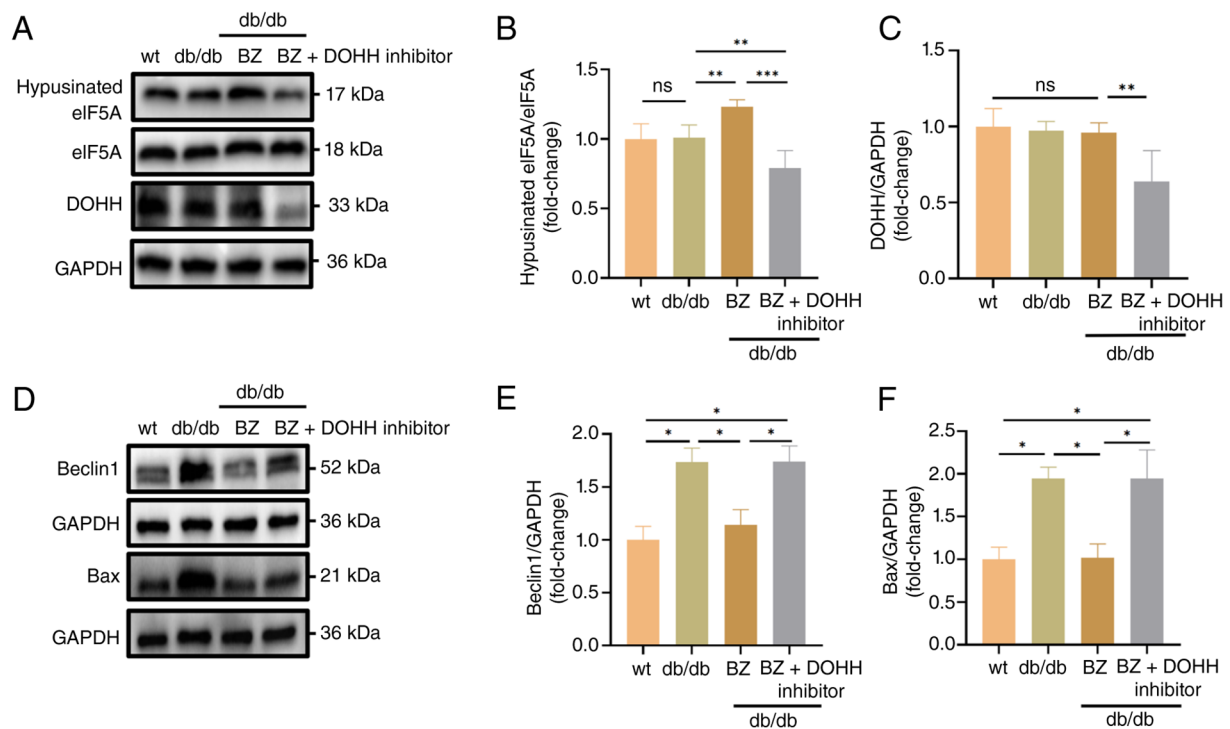


Figure 13. Effects of BZ and DOHH inhibition on autophagy and apoptosis in *db/db* mice. (A-F) Hypusinated eIF5A, eIF5A, Beclin1 and Bax protein expression was measured by western blot analysis. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$. BZ, brazilin; DOHH, deoxyhypusine hydroxylase; eIF5A, eukaryotic translation initiation factor 5A; wt, wild-type; ns, not significant.

architecture, and activating inflammatory or oxidative pathways (13,17,53). MGO accumulation is a critical contributor to diabetic progression and associated vascular complications (54-56). In previous studies by the authors, MGO was shown to induce apoptosis and pyroptosis in ECs through oxidative stress, inflammatory responses and mitochondrial damage, leading to vascular endothelial injury in mice (19-21). In addition, MGO enhances autophagy in vascular ECs via AGE receptor- and peroxynitrite-dependent mechanisms (29). BZ appears to regulate autophagy and apoptosis and exerts cytoprotective effects (39,57). However, the precise role of BZ in MGO-triggered HUVEC apoptosis and the associated signaling pathways remain unclear. Marked reductions in HUVEC viability, together with enhanced autophagic and apoptotic responses, were observed following MGO treatment, whereas these effects were dose-dependently counteracted by BZ. Activation of the DOHH/eIF5A and AMPK/mTOR signaling pathways may contribute to these effects. Further *in vivo* validation using an MGO-induced vascular injury model and *db/db* mice confirmed that BZ suppresses MGO-evoked autophagy and apoptosis. These findings align with previous studies (28,37,38,53,58), indicating that BZ exerts protective effects against diabetes-associated vascular injury and further clarifying the mechanisms underlying its action.

To further investigate the mechanism associated with autophagic and apoptotic responses triggered by MGO and clarify their temporal sequence, HUVECs were pretreated with 3-MA, an autophagy inhibitor. The results showed marked autophagy inhibition, evidenced by decreased LC3-II/LC3-I and Beclin1 levels and increased p62 expression, along with reduced Bax and cleaved caspase-3/caspase-3 and enhanced Bcl-2 expression. These findings suggest that

inhibition of autophagy attenuates MGO-induced apoptosis, indicating that autophagy may contribute to the subsequent activation of apoptotic signaling. Consistently, time-course analysis showed that LC3-II was significantly upregulated as early as 6 h after MGO stimulation, whereas cleaved caspase-3/caspase-3 began to increase significantly at 12 h and remained elevated at 24 h. Taken together, these observations support the notion that MGO-induced autophagy occurs before apoptosis and may participate in promoting apoptotic progression in HUVECs. Furthermore, the CQ blockade assay provided additional evidence that MGO-induced LC3-II accumulation is not merely attributable to impaired lysosomal degradation but rather reflects enhanced autophagosome formation and increased autophagic flux in HUVECs. The AMPK/mTOR pathway is a central signaling axis that regulates cellular energy metabolism, nutrient sensing, and growth and proliferation and is closely associated with autophagy and apoptosis in ECs (59,60). The use of BZ in the treatment of vascular inflammation and vascular regulation has been previously reported (38,61,62). The present findings indicate that BZ pretreatment dose-dependently mitigated the MGO-evoked elevation of p-AMPK expression while reversing the reduction in p-mTOR expression caused by MGO. By inhibiting AMPK/mTOR signaling, BZ significantly reduced MGO-induced EC autophagy and apoptosis *in vitro* and *in vivo*, as evidenced by reduced LC3-II/LC3-I, Beclin1, Bax and cleaved caspase-3 levels, together with increased p62 and Bcl-2 expression. These changes were accompanied by reduced autophagosome and autolysosome accumulation. Notably, Compound C, an AMPK inhibitor, further potentiated the BZ-mediated anti-autophagic and antiapoptotic effects, suppressing HUVEC autophagic and apoptotic responses

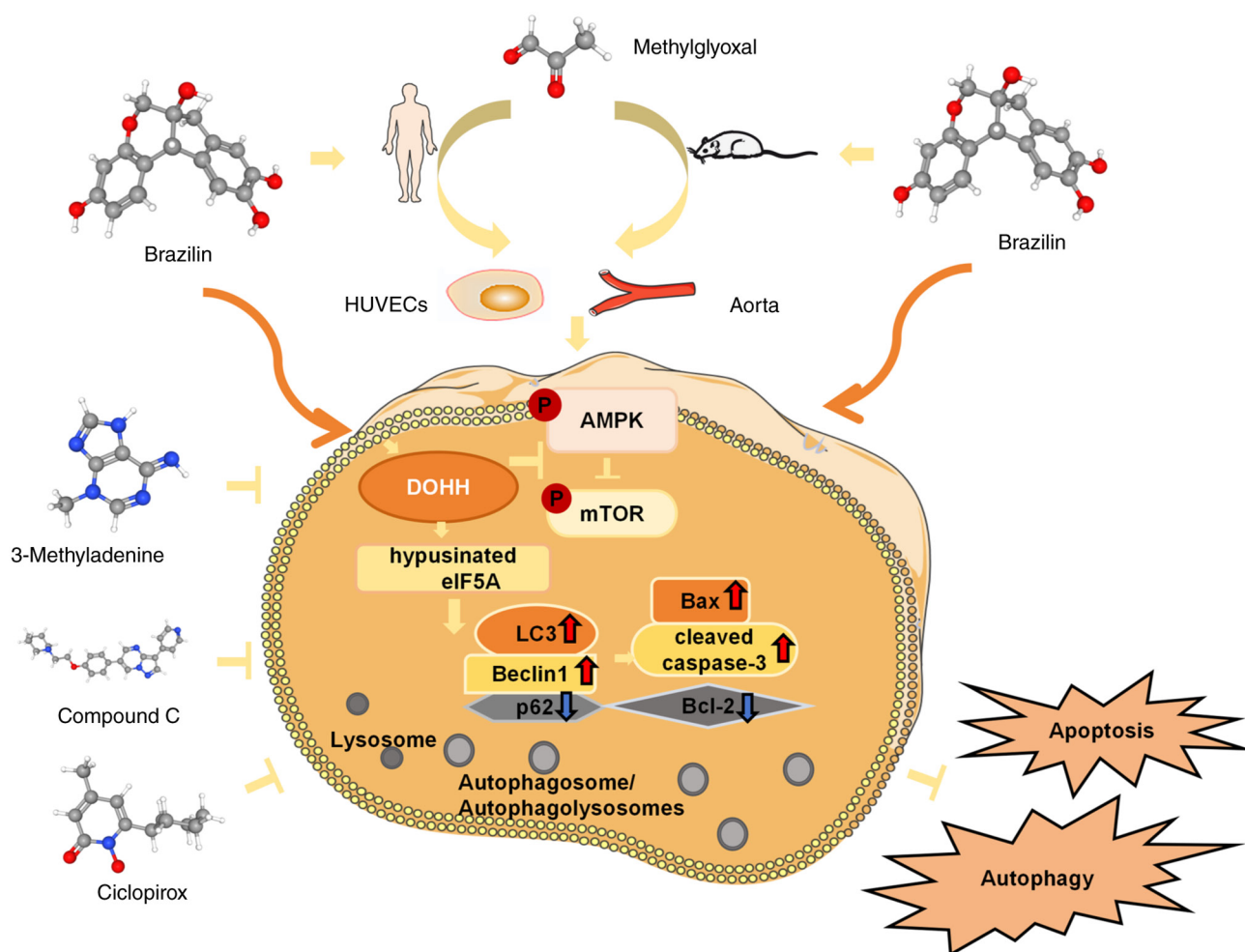


Figure 14. Summary diagram. BZ confers protection against MGO-triggered cellular autophagic and apoptotic responses and vascular endothelial injury by enhancing eIF5A hypusination, thereby regulating the downstream DOHH/eIF5A signaling cascade and the AMPK/mTOR signaling pathway. BZ, brazilin; MGO, methylglyoxal; DOHH, deoxyhypusine hydroxylase; eIF5A, eukaryotic translation initiation factor 5A; HUVECs, human umbilical vein endothelial cells.

elicited by MGO and further reducing p-AMPK levels while elevating p-mTOR levels. Taken together, the present findings indicate that MGO-induced autophagy may precede the onset of apoptosis and be modulated by BZ via AMPK/mTOR signaling, in agreement with previous studies (59,60). Moreover, the present study suggests that BZ, as a natural extract, holds considerable potential for ameliorating autophagy- and apoptosis-related diabetic vascular injury.

Previously, through protein microarray screening, Guo *et al* (39) developed a biotin-labeled BZ molecular probe and identified DOHH as a direct target of BZ. Furthermore, BZ exerts its cytoprotective effects by directly interacting with the Cys232 residue of the DOHH protein (39). DOHH shows a strong preference and strict specificity for binding to eIF5A containing deoxyhypusine and catalyzes its hydroxylation (63). In the present study, BZ pretreatment was found to leave DOHH abundance largely unchanged while markedly increasing intracellular hypusinated eIF5A levels. By contrast, treatment with a DOHH inhibitor significantly reduced both DOHH and hypusinated eIF5A levels and effectively reversed the BZ-induced increase in hypusinated eIF5A. In addition, si-DOHH transfection was used in the present study to specifically knock down DOHH, thereby further verifying the

specificity of ciclopirox action. The decreases in DOHH expression and hypusinated eIF5A levels observed after si-DOHH intervention were consistent with the results of ciclopirox treatment. Collectively, these results further support that BZ may act on DOHH to catalyze eIF5A, thereby accelerating the final step of post-translational hypusine synthesis on eIF5A and modulating cellular autophagy and apoptosis. However, the present study has several limitations. After si-DOHH treatment, both DOHH expression and hypusinated eIF5A levels were decreased, accompanied by an increase in p-AMPK levels, suggesting that AMPK activation may be associated with DOHH inhibition and reduced hypusinated eIF5A levels. Nevertheless, changes in mTOR pathway-related molecules and the precise molecular mechanisms linking DOHH/eIF5A to the AMPK/mTOR pathway, including whether direct protein-protein interactions and specific modification sites are involved, remain to be elucidated through further targeted mechanistic studies.

Marked elevations in plasma MGO concentrations have been reported in patients with diabetes (64,65), and MGO has also been implicated in the initiation and progression of diabetes-associated vascular diseases (14,35,66,67). Accordingly, in the present study, MGO was first selected

as the primary injurious factor to induce autophagy, apoptosis, and endothelial dysfunction in ECs *in vitro*, thereby mimicking diabetes-related vascular pathology. A *db/db* mouse model was subsequently used to establish a diabetic condition and to validate the *in vitro* findings *in vivo*. The findings of the present study indicate that MGO-induced vascular injury closely resembles diabetes-associated vascular disease at the molecular mechanistic level, indicating that MGO can serve as a reliable experimental surrogate for diabetic vascular damage. These findings suggest that BZ may represent a promising therapeutic candidate for diabetic vascular injury.

In summary, the current findings newly demonstrate the protective effect of BZ against MGO-evoked autophagic and apoptotic responses *in vitro* and *in vivo*. Mechanistically, BZ mitigates MGO-triggered vascular endothelial damage by enhancing the unique post-translational hypusination of eIF5A and in turn regulating the DOHH/eIF5A and AMPK/mTOR signaling cascades (Fig. 14). These findings further clarify the molecular basis of BZ action and offer new theoretical support and research perspectives for its potential therapeutic application in diabetes-associated vascular diseases.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

YL and JC designed the present study and drafted the manuscript. HL, JX and JY performed the experiments and acquired the data. LW and JW analyzed the data and conducted the initial interpretation of the results. YZ and ML contributed to the study design, critically reviewed the manuscript, and revised it for important intellectual content. YL and ML confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

The present study complied with the ARRIVE guidelines (<https://arriveguidelines.org>). All animal procedures were approved by the Animal Ethics Committee of Southwest Medical University (approval no. 2020895; Luzhou, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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