Mangiferin inhibits cell migration and angiogenesis via PI3K/AKT/mTOR signaling in high glucose- and hypoxia-induced RRCECs

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Abstract. Mangiferin is a prominent active component that can be derived from several traditional herbs, including Mangifera indica L., Anemarrhena asphodeloides Bge., and Belamcanda chinensis (L.) DC., which displays antidiabetic properties. Diabetic retinopathy (DR), a serious complication caused by diabetes, is the leading cause of blindness. The present study aimed to evaluate the beneficial effects of mangiferin on high glucose (HG)/hypoxia-induced rat retinal capillary endothelial cell (RRCEC) angiogenesis, as well as the underlying mechanisms. To establish an in vitro model of DR, RRCECs were exposed to 30 mM glucose and hypoxia. Following treatment with different doses of mangiferin (0.05, 0.1 or 0.2 µM), RRCEC viability, migration and angiogenesis were assessed by performing Cell Counting Kit 8, immunofluorescence, wound healing, Transwell and tube formation assays. Western blotting was conducted to evaluate protein expression levels. Furthermore, LY294002 and IGF-1, an inhibitor and activator of the PI3K/AKT/mTOR signaling pathway, respectively, were used to verify the potential mechanisms underlying mangiferin. The results demonstrated that mangiferin notably inhibited HG/hypoxia-induced RRCEC migration and angiogenesis. HG/hypoxia-induced upregulation of hypoxia-inducible factor-1α, vascular endothelial growth factor, matrix metalloproteinase (MMP)2 and MMP9 expression levels and the phosphorylation of PI3K, AKT and mTOR in RRCECs was significantly reversed following treatment with mangiferin. Additionally, further activation of the PI3K/AKT signaling pathway by IGF-1 inhibited the beneficial effects of mangiferin on RRCECs, whereas deactivation of the PI3K/AKT signaling pathway by LY294002 displayed the opposite results. Collectively, the results of the present study suggested that mangiferin suppressed RRCEC angiogenesis via modulating the PI3K/AKT/mTOR signaling pathway, which could serve as an effective treatment strategy for DR.

Introduction

Diabetic retinopathy (DR) is a common complication of diabetes (1). With the increasing prevalence of diabetes, it is estimated that the number of patients with DR will reach 191 million worldwide by 2030 (2). In diabetes, hyperglycemia-mediated injury of blood vessels and capillaries causes retina ischemia and hypoxia, and vitreous hemorrhage, which leads to proliferative diabetic retinopathy, and ultimately blindness (1). At present, the local therapeutic interventions for the treatment of DR include intravitreal injection of anti-vascular endothelial growth factor (VEGF) drugs, glucocorticoids and laser photocoagulation (3). However, due to the complicated pathogenesis of DR, the current therapeutic interventions cannot completely prevent complications, including vitreous hemorrhage, traction retinal detachment and neovascular glaucoma (4). The primary pathological features of DR are retinal vascular occlusive circulation disorder, as well as retinal endothelial cell proliferation, migration and angiogenesis (5,6). Therefore, inhibiting retinal neovascularization is important for the treatment of DR.

Mangiferin (1,3,6,7-tetrahydroxyxanthone-2-β-D-glucoside) is a xanthone glucoside. Mangiferin can be extracted from plants belonging to the Liliaceae, Anacardiaceae and Gentianaceae families (7,8). Mangiferin exerts a wide range of pharmacological effects, including antitumor, anti-infectious, hypoglycemic, antioxidation, and immune regulatory effects (9). A previous study demonstrated that mangiferin attenuates dexamethasone-induced osteoblastic MC3T3-E1 cell apoptosis and oxidative stress via activating the bone morphogenetic protein 2/Smad-1 signaling pathway (10). In atherosclerosis, mangiferin promoted macrophage cholesterol efflux via enhancing ATP binding cassette subfamily (ABC)A1 and ABCG1 expression (8). Moreover, it has been reported that the PI3K/AKT and Rac1/Wiskott-Aldrich syndrome protein family member 2 (WAVE2) signaling pathways are blocked...
by mangiferin in cancer (11). In particular, mangiferin exerted beneficial effects on biochemical and hematological parameters in rats with streptozotocin-induced diabetes (12). However, whether mangiferin protects against high glucose (HG)- and hypoxia-induced injury in rat retinal capillary endothelial cells (RRCECs) is not completely understood. Thus, the present study aimed to investigate the effects of mangiferin on RRCEC viability, migration and angiogenesis following exposure to HG and hypoxia.

Materials and methods

Drug. Mangiferin (C_{19}H_{18}O_{11}; molecular weight, 422.34; purity, ≥98%; cat. no. 4773-96-0) was purchased from Sigma-Aldrich (Merck KGaA).

Cell culture. RRCECs (Procell Life Science & Technology Co., Ltd.) were cultured in HG DMEM (cat. no. PM150220; Procell Life Science & Technology Co., Ltd.) supplemented with 10% FBS (cat. no. FB15015; Clark Bioscience) and 1% penicillin/streptomycin (cat. no. sv30010; HyClone; Cytiva) with 5% CO₂ at 37°C.

Cell treatment. To select the optimum concentration (A₀), RRCECs were treated with different concentrations of mangiferin (0.01, 0.1, or 10 mM) for 24, 48 and 72 h, as previously described (10,13). Subsequently, RRCECs were pretreated with D-(+)-glucose (30 mM; cat. no. G7528; Sigma-Aldrich; Merck KGaA)/hypoxia (1% O₂, 94% N₂ and 5% CO₂) and then were treated with 0.1 mM (A₀) mangiferin for 24 or 48 h. RRCECs were also treated with D-(+)-glucose/hypoxia and followed by different doses of mangiferin [0.05 mM (1/2 A₀), 0.1 mM (A₀) and 0.2 mM (2A₀)] for 24 h at 37°C. Finally, RRCECs were treated with a PI3K/akt signaling pathway inhibitor (LY294002; Sigma-Aldrich; Merck KGaA 40 µmol/l) or a PI3K/akt signaling pathway activator (IGF-1; Sigma-Aldrich; Merck KGaA 100 ng/ml) in the presence of D-(+)-glucose/hypoxia and mangiferin (A₀) for 24 h at 37°C. The control cells were treated the same amount of DMSO for 24 h at 37°C. Cells were divided into the following nine groups: i) Control; ii) HG/hypoxia; iii) HG/hypoxia + 1/2 A₀ mangiferin; iv) HG/hypoxia + A₀ mangiferin; v) HG/hypoxia + 2A₀ mangiferin; vi) HG/hypoxia + 0.1 mM (A₀) mangiferin + LY294002; vii) HG/hypoxia + 0.1 mM (A₀) mangiferin + IGF-1; viii) HG/hypoxia + LY294002; ix) HG/hypoxia + IGF-1.

Western blotting. RRCECs were plated (1x10⁶ cells) into 100-mm culture plates. The following day, cells were treated with mangiferin as aforementioned. Following washing three times with PBS, total protein was extracted from cells using RIPA buffer (cat. no. P0013C; Beyotime Institute of Biotechnology) supplemented with 1% phenylmethylsulfonyl fluoride (Beyotime Institute of Biotechnology). Protein concentrations were determined using a BCA assay kit (CoWin Biosciences). Proteins (40 µg) were separated alongside molecular weight standards (Bio-Rad Laboratories, Inc.) via 10% SDS-PAGE. Proteins were transferred onto PVDF membranes (Amersham; Cytiva). Following blocking with 5% non-fat milk at room temperature for 1 h, the membranes were incubated overnight at 37°C with anti-matrix metallopeptidase (MMP)2 (1:1,000; cat. no. AF5330; Affinity Biosciences), anti-MMP9 (1:500; cat. no. ab119906; Abcam), anti-VEGF (1:1,000; cat. no. ab46154; Abcam), anti-hypoxia-inducible factor-1α (HIF-1α; 1:500; cat. no. ab1; Abcam), anti-PI3K (1:1,000; cat. no. ab32089; Abcam), anti-akt (1:10,000; cat. no. ab179462; Abcam), anti-mTOR (1:1,000; cat. no. ab32028; Abcam), anti-phosphorylated (p)-PI3K (1:500; cat. no. ab182651; Abcam), anti-p-akt (1:5,000; cat. no. ab81283; Abcam), anti-p-mTOR (1:1,000; cat. no. ab109268; Abcam) and anti-β-actin (1:1,000; cat. no. AC026; ABclonal Biotech Co., Ltd.). Subsequently, the membranes were incubated with a HRP-conjugated Goat Anti-Rabbit IgG secondary antibody (1:2,000; cat. no. ab6721; Abcam) or a HRP-conjugated Goat Anti-Mouse IgG secondary antibody (1:2,000; cat. no. ab6728; Abcam) at room temperature for 3 h. Protein bands were visualized using an ECL system (cat. no. KF001; Affinity Biosciences). Protein expression levels were semi-quantified using Image-Pro Plus software (version 6.0; Media Cybernetics, Inc.) with β-actin as the loading control.

Cell viability assay. RRCEC viability was assessed by performing Cell Counting Kit-8 (CCK-8) assays (cat. no. BS350B; Biosharp Life Sciences) according to the manufacturer's protocol. CCK-8 solution was added to each well and incubated at 37°C for 1.5 h. Cell viability was measured at a wavelength of 450 nm using a microplate reader.

Immunofluorescence. RRCECs were seeded (5x10⁴ cells/well) into 96-well plates and cultured at 37°C with 5% CO₂. At 90% confluence, the culture medium was removed and cells were washed with PBS. The cells were fixed with 4% paraformaldehyde at room temperature for 30 min and washed 3 times with 0.02 M PBS at room temperature for 3 min. Following blocking with 10% serum blocking solution (cat. no. 22012-8612; Zhijiang Tianhang Biotechnology Co., Ltd.) at 4°C overnight. Subsequently, cells were incubated with rhodamine-labeled goat anti-rabbit IgG antibody (1:200; cat. no. GB13030-2; Wuhan Servicebio Technology Co., Ltd.) at 4°C. Finally, cell nuclei were labeled with DAPI (cat. no. ZLI-9557; OriGene Technologies, Inc.) at room temperature for 10 min and the cells were incubated with anti-fluorescence decay blocking solution at room temperature (cat. no. AR1109; Wuhan Boster Biological Technology, Ltd.). Stained cells were observed using a fluorescence microscope (magnification, x400; Olympus Corporation).

Tube formation assay. First, 60 µl dissolved Matrigel adhesive matrix was added into each well of a pre-cooled 96-well plate and allowed to solidify at 37°C for 1 h. RRCECs (5x10⁴ cells/ml) were added into each well, cultured under hypoxic conditions (1% O₂, 94% N₂ and 5% CO₂) for 6 h and then transferred to normoxic conditions (95% air and 5% CO₂) for 12 h. Subsequently, cells were treated with mangiferin (0.05, 0.1 or 0.2 mM) at 37°C with 5% CO₂ for 24 h. Tube formation was observed under an inverted phase-contrast light microscope in five randomly selected fields of view.

Wound healing assay. RRCEC migration was assessed by performing a wound healing assay. Briefly, cells were grown...
in DMEM medium containing 10% FBS and then seeded (5x10^5 cells) into a 6-well plate. At 100% confluence, a wound was created in each cell monolayer using a sterile micropipette tip, followed by washing with PBS to remove cell debris. Subsequently, cells were cultured in serum-free DMEM under hypoxic conditions for 6 h, and then transferred to normoxic conditions for 12 h. Finally, cells were treated with different concentrations of mangiferin (0.05, 0.1 or 0.2 mM) for 24 h at 37°C with 5% CO_2. An inverted phase-contrast light microscope (Olympus Corporation) was used to obtain images of the wound at different time points. The width of the wound was measured at three different positions at 0 and 24 h. The relative wound recovery distance was calculated as 0-24 h.

Transwell assay. To evaluate cell migration, RRCECs were resuspended (1x10^6 cells/ml) in serum-free DMEM. Transwell chambers were placed into a 24-well plate. Subsequently, cell suspension (20 µl/well) was added into the upper chamber. Cells were cultured under hypoxic conditions for 6 h and then normoxic conditions for 12 h. Subsequently, 600 µl DMEM supplemented with 10% FBS (cat. no. FB15015; Clark Bioscience) was added into the lower chamber. Cells in the upper chamber were treated with mangiferin (0.05, 0.1 or 0.2 mM) for 24 h and cultured at 37°C with 5% CO_2. Following fixation with 4% paraformaldehyde for 20 min at room temperature, cells were stained with 0.1% crystal violet for 15 min at room temperature. Migratory cells were visualized using an inverted phase-contrast light microscope (Olympus Corporation).

Statistical analysis. Statistical analysis was performed on the means of triplicates that resulted from three independent replications of each experiment. Data are presented as the mean ± SD. Statistical analyses were performed using SPSS software (version 20.0; IBM Corp.). Comparisons among groups were analyzed using one-way ANOVA followed by Tukey’s post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

**Effect of mangiferin on HG/hypoxia-induced RRCEC viability.** Cell viability was assessed by performing CCK-8 assays. The results demonstrated that mangiferin (0.01-10 mM) decreased RRCEC viability in a dose-dependent manner (Fig. 1). Furthermore, treatment of RRCECs with mangiferin for 24, 48 and 72 h increased cell viability in a time-dependent manner (Fig. 1). Following treatment with A0 mangiferin for 48 h, no significant alteration in cell viability was observed compared with the control group (Fig. 1A). In addition, RRCECs were treated with A0 mangiferin and induced with HG/hypoxia for 24 and 48 h. Co-treatment of RRCECs with HG and A0 mangiferin for 24 and 48 h did not significantly alter cell viability compared with the control + A0 group (Fig. 1B). Therefore, the results indicated that A0 mangiferin did not display cytotoxicity.

**Mangiferin attenuates HG/hypoxia-induced RRCEC migration and angiogenesis.** Subsequently, the effects of mangiferin on HG/hypoxia-induced viability, migration and angiogenesis were investigated. Compared with the control group, HG/hypoxia exposure significantly increased RRCEC viability, whereas mangiferin did not display significant inhibitory or promotive effects on RRCEC viability (Fig. 2A). Furthermore, the immunofluorescence results demonstrated that compared with the control group, the number of Ki67+ cells was clearly increased following treatment with HG/hypoxia, an effect that was not notably reversed by mangiferin (Fig. 2B). In addition, HG/hypoxia exposure significantly increased RRCEC migration compared with the control group (Fig. 2C-F). Treatment with 1/2 A0, A0 or 2A0 mangiferin markedly attenuated HG/hypoxia-mediated effects on cell migration (Fig. 2C-F). Compared with the control group, RRCEC angiogenesis was markedly enhanced following treatment with HG/hypoxia, which was notably attenuated by treatment with 1/2 A0, A0 or 2A0 mangiferin (Fig. 2G). Furthermore, following exposure to HG/hypoxia, the expression levels of HIF-1α, VEGF, MMP2 and MMP9 in RRCECs were significantly upregulated compared with the control group. However, treatment with

![Figure 1. Effects of mangiferin on HG/hypoxia-induced RRCEC viability. RRCECs were treated with different doses of mangiferin (0.01, 0.1, 1 and 10 mM) for 24, 48 or 72 h. (A and B) Cell viability was assessed by performing Cell Counting Kit-8 assays. ***P<0.001 vs. control. HG, high glucose; RRCEC, rat retinal capillary endothelial cell; A0, 0.1 mM mangiferin.](image-url)
A0 or 2A0 mangiferin significantly reversed HG/hypoxia exposure-induced upregulation of HIF-1α, VEGF, MMP2, and MMP9 expression levels (Fig. 2H and I).

Mangiferin suppresses the activity of the PI3K/AKT and mTOR signaling pathways in HG/hypoxia-treated RRCECs. The PI3K/AKT and mTOR signaling pathways serve important
roles in various intracellular events, including cell migration, invasion and angiogenesis (14,15). Therefore, the present study explored whether these signaling pathways were implicated in mangiferin-mediated cell migration and angiogenesis. The western blotting results demonstrated that the protein expression levels of PI3K, p-PI3K, p-AKT, mTOR and p-mTOR were significantly upregulated in HG/hypoxia-exposed cells compared with control cells (Fig. 3). However, the levels of PI3K, AKT and mTOR phosphorylation were significantly decreased following treatment with 0.05 mM mangiferin in HG/hypoxia-exposed cells (Fig. 3). Similar results were observed for the p-AKT/AKT, p-PI3K/PI3K and p-mTOR/mTOR ratios (Fig. 3D, G and J).

Mangiferin inhibits HG/hypoxia-induced RCEC migration and angiogenesis via the PI3K/AKT and mTOR signaling pathways. The present study investigated whether the PI3K/AKT and mTOR signaling pathways were involved in cell migration and angiogenesis following exposure to HG/hypoxia. The western blotting results demonstrated that the PI3K/AKT signaling pathway inhibitor (LY294002) and the PI3K/AKT signaling pathway activator (IGF-1) significantly downregulated and upregulated the expression levels of p-PI3K, p-AKT and p-mTOR in HG/hypoxia-exposed cells (Fig. 3). Similar results were observed for the p-AKT/AKT, p-PI3K/PI3K and p-mTOR/mTOR ratios (Fig. 3D, G and J).

Additionally, mangiferin-induced inhibition of RCEC migration was significantly enhanced and inhibited following treatment with LY294002 and IGF-1, respectively (Fig. 4L-O). Furthermore, the tube formation assay results demonstrated that 0.05 mM mangiferin markedly reduced the number of branching points in HG/hypoxia-treated cells, an effect that was notably enhanced and inhibited following treatment with LY294002 and IGF-1, respectively (Fig. 4P). Overall, the results of the present study suggested that mangiferin inhibited RCEC migration and angiogenesis partially via deactivation of the PI3K/AKT and mTOR signaling pathways.

Discussion

Long-term exposure to hyperglycemia and hypoxia is highly associated with the development of DR pathological states, and is considered the primary cause of blood-retinal barrier and microvascular injury (16). Mangiferin, a natural bioactive ingredient, is commonly found in mango trees, and has been reported to display antitumor, antidiabetic, lipometabolism regulating, anti-inflammatory and antibacterial properties (17). Previous studies have reported that mangiferin exerts pharmacological effects on hypoglycemia and diabetes. In streptozotocin-induced type 2 diabetic rats, mangiferin ameliorates insulin sensitivity and dyslipidemia, depletes serum TNF-α and elevates adiponectin (18). Mangiferin suppresses increased plasma insulin and fasted plasma non-esterified fatty acid concentrations at the baseline during an oral glucose tolerance test in a rat model of fructose-induced metabolic...
Figure 4. Mangiferin inhibits HG/hypoxia-induced cell migration and angiogenesis via the PI3K/AKT/mTOR signaling pathways in RRCECs. Protein expression levels were (A) determined via western blotting and semi-quantified for (B) PI3K, (C) p-PI3K, (D) p-PI3K/PI3K, (E) AKT, (F) p-AKT, (G) p-AKT/ AKT, (H) mTOR, (I) p-mTOR and (J) p-mTOR/mTOR. (K) RRCEC viability was evaluated by performing Cell Counting Kit-8 assays. RRCEC migration was evaluated by performing (L) wound healing and (M) Transwell assay. Representative images of the (N) wound healing (x50) and (O) Transwell assays (magnification, x100). (P) Tube formation and branching points were determined magnification, x100). **P<0.01 and ***P<0.001 vs. control; #P<0.05, ##P<0.01 and ###P<0.001 vs. HG; &P<0.05, &&P<0.01 and &&&P<0.001 vs. HG+a0. HG, high glucose; RRCEC, rat retinal capillary endothelial cell; p, phosphorylated; c, control; a0, 0.1 mM mangiferin.
syndrome (19). Furthermore, at 3 weeks post‑oral administration, mangiferin decreases blood glucose levels in KK‑Ay mice, but displays no effect on blood glucose levels in healthy mice (20). Moreover, mangiferin clearly improves reactive oxygen species (ROS) generation, inflammation and endoplasmic reticulum stress in gestational diabetes mellitus model mice (21). Mangiferin increases bovine aortic cell migration, but displays no effect on cell proliferation in vitro (22). The results of the present study demonstrated that mangiferin displayed no significant effect on cell viability in HG/hypoxia‑induced RRCECs. Additionally, treatment with mangiferin markedly attenuated HG/hypoxia‑induced RRCEC migration and angiogenesis, which suggested that mangiferin may be useful for the treatment of DR. Mechanistically, mangiferin inhibits glial cell proliferation and promotes apoptosis via down‑regulating MMP9 expression (23). Furthermore, mangiferin reduces MMP7 and MMP9 expression, and inhibits breast cancer cell proliferation and epithelial‑mesenchymal transition (EMT) in vitro and in vivo via activation of the β‑catenin signaling pathway (24). Increasing evidence has demonstrated that mangiferin attenuates inflammation via inhibiting the Toll‑like receptor 4/p65 signaling pathway, and suppresses the EMT process via blocking the Smad2/3 signaling pathway and downregulating MMP9 expression in pulmonary fibrosis model mice (25). Similarly, the results of the present study demonstrated that mangiferin significantly decreased MMP2 and MMP9 expression levels in HG/hypoxia‑exposed RRCECs.

HIF‑α/VEGF expression serves a major role in mediating active retinal angiogenesis and alleviating the progression of DR. In DR, HIF‑1α regulates the transcription of VEGF, which ultimately leads to intracellular accumulation of VEGF (26). VEGF is an important stimulating factor involved in retinal and choroidal neovascularization (27,28). Clinical data indicates that the concentration of VEGF is enhanced in vitreous fluid of patients with DR (29). It has been reported that anti‑VEGF agents may be used to treat angiogenesis in eye diseases, including neovascular glaucoma, persistent vitreous hemorrhage and DR (30). In the present study, mangiferin treatment significantly downregulated HG/hypoxia‑induced HIF‑α and VEGF expression levels.

In addition, several signaling pathways, including the PI3K/AKT, STAT3 and mTOR signaling pathways, are associated with HIF‑1α/VEGF signaling in DR (31‑33). It has been reported that the PI3K/AKT/mTOR signaling pathway promotes DR via inducing retinal cell viability, apoptosis, migration and angiogenesis. The use of a combination of PI3K/AKT/mTOR drugs is safe and effective for the treatment of ocular neovascularization (34). In retinal endothelial RF/6A and microglia BV‑2 cells, erianin significantly suppresses HG‑induced increases in retinal vessels and microglia activation via deactivating the VEGF/PI3K/AKT signaling pathway (35). Furthermore, microRNA‑183 overexpression activates the PI3K/AKT signaling pathway, increases VEGF expression and downregulates B‑cell translocation gene 1 expression in DR (36). In addition, Nogo‑B induced VEGF secretion and activation of the PI3K/AKT axis in HG‑exposes human retinal microvascular endothelial cells (37). A previous study reported that Parkinsonism associated deglycase overexpression significantly reduces ROS production, p‑AKT and p‑mTOR expression, and cell apoptosis in HG‑induced retinal capillary pericytes (38). Similarly, the present study demonstrated that A0 mangiferin significantly inhibited the PI3K/AKT/mTOR signaling pathway in HG/hypoxia‑induced RRCECs. Activation of the PI3K/AKT signaling pathway by IGF‑1 clearly reversed mangiferin‑mediated effects on RRCEC migration and angiogenesis. Moreover, A20 mangiferin did not significantly reduce the phosphorylation levels of PI3K, AKT and mTOR in HG/hypoxia‑induced RRCECs, which suggested that high concentration mangiferin was not conducive to the inhibition of protein phosphorylation. It was hypothesized that high concentrations of mangiferin might activate or inhibit additional upstream signaling pathways to impair the phosphorylation levels of PI3K, AKT and mTOR; however, further investigations are required to confirm the effects of high concentration mangiferin on the upstream components of the PI3K/AKT signaling pathway.

In summary, to the best of our knowledge, the present study demonstrated for the first time that mangiferin displayed beneficial effects against DR by suppressing HG/hypoxia‑induced RRCEC migration and angiogenesis via inhibition of the PI3K/AKT/mTOR signaling pathway, suggesting the potential use of mangiferin for the treatment of DR.

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

Authors’ contributions
JS, CT, YL and HL conceptualized and designed the study. JS, CT, YL, JH and HZ performed the experiments. JS, CT and YL analyzed the data. JS, CT, YL and HL drafted the manuscript. JS and HL confirmed the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate
Not applicable.

Patient consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.
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