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Curcumolide reduces diabetic retinal vascular leukostasis and leakage partly via inhibition of the p38MAPK/NF-κ B signaling

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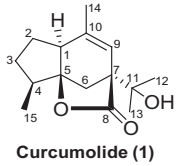
ABSTRACT

Retinal inflammation in a hyperglycemic condition is believed to play a crucial role in the development of diabetic retinopathy, and targeting inflammatory mediators is a promising strategy for the control of diabetic retinopathy. Curcumolide, a novel sesquiterpenoid with a unique 5/6/5 tricyclic skeleton, was isolated from Curcuma wenyujin. In this study, we demonstrate that treatment with curcumolide alleviated retinal inflammatory activities both in vitro and in vivo in a STZ-induced diabetic rat model and in TNF-α-stimulated HUVECs. Curcumolide alleviated retinal vascular permeability and leukostasis and attenuated the overexpression of TNF-α and ICAM-1 in diabetic retinas. Moreover, curcumolide also inhibited inducible p38 MAPK and NF-κB activation and the subsequent induction of proinflammatory mediators. These data suggest potential treatment strategies against diabetic retinopathy, particularly in the early stages of the disease.

Diabetic retinopathy (DR) is a microvascular complication of diabetes and a leading cause of vision loss in the working-age population. During diabetes, chronic hyperglycemia causes structural and functional alteration in the retina, including the breakdown of the blood retinal barrier (BRB); increased vascular permeability; apoptotic cell death of retinal neurons, endothelial cells, and pericytes; and the formation of microaneurysms and acellular capillaries.[1–5] These changes eventually result in the clinical appearance of vascular leakage from the retinal capillaries, leading to diabetic macular edema and vision loss.[6] Therapeutic options for DR have been limited. Until recently, laser photocoagulation of the peripheral retinal tissue and intravitreal injection of steroids or vascular endothelial growth factor (VEGF) inhibitors can stabilize the condition or improve vision,[7,1,8–12] but these approaches are limited or have various complications. Thus, additional treatment options are needed.[13,14]

Numerous reports have demonstrated that the pathogenesis of diabetic retinopathy is mediated by inflammatory processes in the ocular microenvironment of diabetic animals or patients.[15–18] These inflammatory changes include increased vascular permeability, leukocyte accumulation and infiltration (Leukostasis), and increased activity of inflammatory markers VEGF, tumor necrosis factor-α (TNF-α), intercellular adhesion molecule - 1 (ICAM-1), interleukin (IL) -1β, and nuclear factor kappa B (NF-κB). Thus, the inhibition of pro-inflammatory mediator production is a promising strategy to control DR.[19] Retinal inflammation is triggered by chronic hyperglycemia via receptor signals that stimulate the transcription factor NF-κB, mitogen-activated protein kinases (MAPKs), and protein kinase C (PKC). NF-κB plays an important role in the development of diabetic retinopathy via its ability to induce an inflammatory condition. NF-κB activation mediates the expression of cytokines, such as TNF-α, and adhesion molecules, such as ICAM-1. The inhibition of the NF-κB pathway prevents increases in the levels of ICAM-1 and TNF-α and reduces leukocyte adhesion and BRB leakage in diabetic retinas.[20] The MAPKs are extracellular-signal regulated kinases (ERK), the p38 mitogen-activated protein kinase (p38 MAPK) and the c-Jun NH_2-terminal kinase (JNK). The activation of MAPKs has been observed in the retinas of diabetic rats and is associated with BRB breakdown.[21–23]

Curcumolide (Cc), a novel sesquiterpenoid with a unique 5/6/5 tricyclic skeleton, was isolated from *Curcuma wenyujin*,[24] a plant that has been used in traditional Chinese medicine for the treatment of jaundice, thoracic-abdominal pain, arthralgia, and dysmenorrhea.[25] Recent pharmacological studies of this plant have demonstrated various activities, including anti-inflammatory,[26] anticancer,[27–29] antioxidation, and antidepressant properties.



Curcumolide (1)

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Previously, we used a bioassay to demonstrate that Cc suppressed lipopolysaccharide (LPS)-induced NO and reactive oxygen species (ROS) production, nuclear translocation, the DNA binding activity of NF-κ B, as well as the expression of TNF-α, IL-6, and IL-1β in RAW 264.7 macrophages.[24] Based on these findings, in this study, we explore whether Cc has therapeutic effects on DR in diabetes animal models and whether such effects are dependent on inhibition of the p38 MAPK/NF-κ B signaling pathway.

All procedures with animals were conducted in accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. Diabetes was induced in 8-week-old, pathogen-free male Wistar rats with an intraperitoneal injection of freshly prepared streptozotocin (55 mg/kg in 50 mmol/L citrate buffer, pH 4.5). 36 h later, animals with blood glucose levels higher than16.7 mmol/dL were considered diabetic. The experiments were performed 4 weeks after diabetes induction.

Data are presented as the mean ± SEM of at least three independent experiments. Statistical significance was evaluated by one-way ANOVA followed by Student's *t* test for paired populations. $P < 0.05$ showed statistical significance.

To evaluate the effect of Cc on retinal vascular leakage, we first performed a retinal vascular permeability assay using FITC-labelled dextran as previously described.[30] As shown in Fig. 1A–D, the rats were perfused with FITC - dextran for 1 h, and the retinas were isolated and flat-mounted. The diabetic rat showed increased leakage of FITC-dextran fluorescence compared to the control. The degree of FITC-dextran leakage was markedly decreased by the intravitreal injection of Cc (50 μg/eye) or triamcinolone acetonide (50 μg/eye, TA). We used the Evans blue albumin leakage assay to quantitate the amount of retinal leakage in the four groups. Consistent with FITC-labelled dextran examination, diabetes increased the permeability in diabetic rats (82.94 ± 11.20 μg Evans blue/g dry retina) compared to the control (32.92 ± 5.72 μg Evans blue/g dry retina). Treatment with Cc significantly reduced the vascular leakage in diabetic rats (43.53 ± 7.53 μg Evans blue/g dry retina) compared with models, but the effect was less than that of TA (36.33 ± 3.83 μg Evans blue/g dry retina) (Fig. 1E).

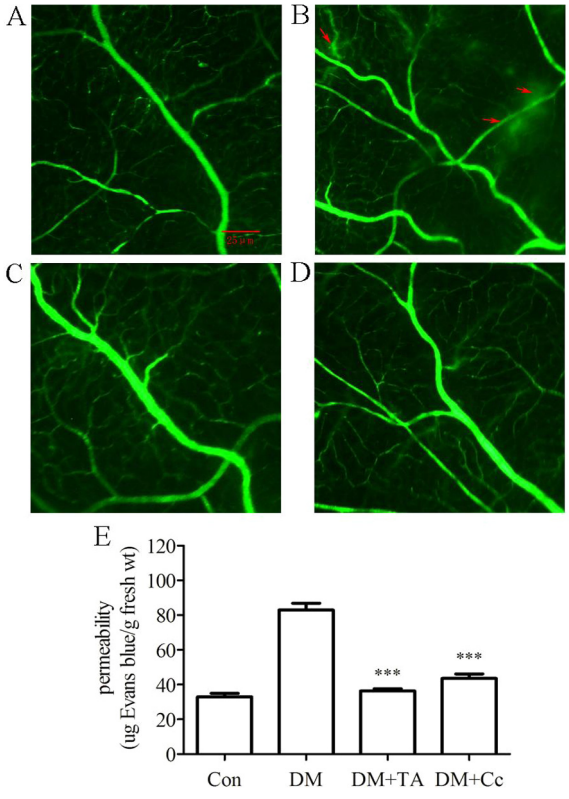

Fig. 1. Treatment with Cc inhibited the retinal vascular leakage induced by diabetes in a retinal vascular permeability assay using FITC-labelled dextran. The rats were perfused with FITC-dextran for 1 h and the retinas were isolated and flat-mounted and retinal vascular leakage was visualized by fluorescence microscopy. Representative images of retinal flat mounts from nondiabetic rats (control, A), untreated STZ-induced diabetic rats (models, B), diabetic rats treated with intravitreal injection of Triamcinolone Acetonide for 3 days (C), and diabetic rats treated with intravitreal injection of Cc for 3 days (D). Arrows indicate retinal vascular leakage. Scale bars: 25 μm. (E) Quantitative measure of retinal permeability by quantification with Evans blue. The results are presented as dye per wet dry retina and represent the mean ± SEM of three independent experiments (n = 5–6). $^{***}P < 0.001$ compared with the model group (DM).

The retinal toxicity of Cc was evaluated by histologic examination with hematoxylin and eosin stain and terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay after the intraocular injection of 75 μg/eye *Cc*, was one point five times of the therapeutic dose. The retina showed normal thickness and all retinal layers were clear with no inflammatory cells in the vitreous, retina, or choroid tissues at 3 days after injection. Additionally, there were no increases in the number of TUNEL-positive cells for all retinal layers (Supporting information Fig. S1). Thus, a dose of Cc less than 75 μg did not produce retinal toxicity in rat.

Leukostasis, occurs early in DR, can damage the retinal endothelium and promote vascular leakage, and plays a major pathogenic role in DR. We examined Cc effects on leukostasis in the retinal microvasculature in diabetic rats by perfusion-labeling with FITC-coupled Con A. Leukocyte counts were evaluated in the whole retina. The total number of adherent leukocytes was significantly higher in diabetic rats (52 ± 10 cells/retina, Fig. 2B) compared to the control (16 ± 7 cells/retina, Fig. 2A). Treatment with Cc significantly decreased the leukocyte count compared with the models (25 ± 12 cells/retina, Fig. 2D), but not to the same decreased level as the TA-treated samples (19 ± 14 cells/retina, Fig. 2C).

Diabetes-induced leukostasis is associated with an increase in the expression of ICAM-1 by retinal endothelial cells and the suppression of ICAM-1 in mice decreases both leukostasis and vascular permeability. TNF-α is an inflammatory cytokine that is upregulated in diabetic retinas, and the suppression of TNF-α can reduce diabetes-induced leukostasis and BRB breakdown. To explain the effects of Cc on permeability and leukostasis at the molecular level, we investigated the protein levels of TNF-α and ICAM-1 in retinas by western blot. The results showed that diabetes caused obvious overexpression of TNF-α and ICAM-1 and treatment with Cc greatly decreased these levels in diabetic retinas compared with models, although to a lesser extent than treatment with TA (Fig. 3). These results indicated that intravitreal injection of *Cc* attenuated overexpression of TNF-α and ICAM-1 in diabetic retinas.

Cc attenuates retinal vascular leakage and leukostasis and suppresses the expression of TNF-α and ICAM-1 in diabetic retinas, all activities that are known to be regulated by the transcription factor NF-κB. NF-κB is a key transcriptional factor involved in regulating the expression of inflammatory proteins and thus playing an important role in the development of diabetic retinopathy. The NF-κB family has five members: RelA/p65, RelB, c-Rel, NF-κB 1/p50, and NF-κB 2/p52; the NF-κB p50/p65 heterodimer is a typical member of the Rel family of transcription factors that regulate diverse cellular functions.[31]

In the inactive state, NF-κB dimers in the cytoplasm are complexed with the inhibitor IκB. Upon activation by external stimuli, the inflammatory signal converges on and activates a IκB kinase (IKK) complex composed of two catalytic subunits, IKKα and IKKβ, and a regulatory subunit, IKKγ/NEMO.[32] The activated IKK complex phosphorylates two conserved N-terminal serine residues of IκBα,