Original Paper

Curcumin Attenuates Retinal Vascular Leakage by Inhibiting Calcium/Calmodulin-Dependent Protein Kinase II Activity in Streptozotocin-Induced Diabetes

Jun Li^{a,c} Peipei Wang^{a,b} Jia Ying^{a,c} Zhen Chen^{a,c} Songping Yu^{a,c}

^aDepartment of Ophthalmology, and ^bDepartment of Stomatology, Lishui Hospital affiliated with Zhejiang University, Lishui, ^cDepartment of Ophthalmology Lishui Central Hospital Fifth Affiliated Hospital of Wenzhou Medical University, Lishui, PR China

Key Words

Curcumin • Retinal vascular leakage • CaMKII • Diabetic retinopathy

Abstract

Background: Curcumin possesses many pharmacological properties including anti-inflammatory effects. Although prior studies indicate that curcumin has beneficial effects for diabetic retinopathy, the mechanism of action is not known. To address this issue, we investigated the effect of curcumin against diabetes-induced retinal vascular damage and its mechanism of action by using cultured retinal Müller cells stimulated with high glucose. **Methods:** We studied the effects of curcumin *in vivo* in the retinas of rats rendered diabetic by streptozotocin and *in vitro* in Müller cells stimulated with high glucose. We administered curcumin, or KN93, an inhibitor of calcium/calmodulin dependent protein kinase II (CaMKII), or saline vehicle to experimental animals on a daily basis for 12 weeks. Primary cultures of rat Müller cells were incubated with normal glucose or high glucose, with or without curcumin, KN93, or pyrrolidine dithiocarbamate (PDTTC), an inhibitor of the transcription protein nuclear factor κ B (NF- κ B). We examined mRNA and protein levels of vascular endothelial growth factor (VEGF), inducible nitric oxide synthase (iNOS) and intercellular adhesion molecule-1 (ICAM-1) by real-time RT-PCR and Western blotting, respectively. Retinal levels of CaMKII and NF- κ B were examined by Western blotting. Vascular leakage was evaluated using Evans blue. **Results:** Curcumin and KN93 significantly inhibited the activation of CaMKII/NF- κ B signaling induced by diabetes or elevated glucose, and subsequently decreased the expression of VEGF, iNOS and ICAM-1. These changes were associated with a decrease of diabetes-induced retinal vascular leakage. **Conclusion:** Curcumin protects the diabetic rat retina against early retinal vascular damage, by inhibition of CaMKII activity. Curcumin is currently used to treat a number of clinical conditions, and may prove beneficial for the management of diabetic retinopathy.

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Songping Yu,

Department of Ophthalmology, Lishui Hospital affiliated with Zhejiang University,
Kuocang Road 289, Lishui 323000, (PR China)
Tel. / Fax +86 0578 2285316, E-Mail yusongpin_zhj_c@163.com.

Introduction

Diabetic retinopathy is a microvascular complication of diabetes and a leading cause of adult blindness [1]. It is a chronic inflammatory disorder where pro-inflammatory proteins, including intercellular adhesion molecule-1 (ICAM-1), inducible nitric oxide synthase (iNOS) and vascular endothelial growth factor (VEGF), are increased in the early stages of the disease process [2]. These increases are correlated with increased leukocyte adhesion to retinal vessels and blood-retinal barrier (BRB) breakdown [3-5], which are believed to be important contributors to the development of clinically significant diabetic retinopathy [6, 7]. Therefore, anti-inflammatory agents have important potential for the treatment of diabetic retinopathy.

Calcium/calmodulin-dependent protein kinase II (CaMKII), a ubiquitous multifunctional serine-threonine protein kinase, has been implicated in regulating transcriptional activity of nuclear factor- κ B (NF- κ B), resulting in pro-inflammatory responses and the release of inflammatory cytokines [8-10]. Activated CaMKII has been shown to play an important role in mediating the development of abnormal vascular dysfunction in diabetes, including diabetic retinopathy [11-14]. For example, autocamtide-2-related inhibitory peptide, a potent inhibitor of CaMKII, prevents vascular leakage in diabetic mice [14]. Thus, it has been proposed that CaMKII is a key player in the diabetes-induced retinal vasculature damage through NF- κ B mediated inflammatory responses [9, 14].

Curcumin (diferuloylmethane), a polyphenol, is the major active compound in *Curcuma longa*. In traditional medicine, curcumin has found applications in a number of diseases, particularly as an anti-inflammatory agent [15, 16]. In relation to diabetes, curcumin ameliorates many complications including diabetic cardiomyopathy [17], diabetic encephalopathy [18], diabetic nephropathy [19], and diabetic retinopathy [20, 21]. As the cellular mechanism(s) by which curcumin achieves these effects are unclear, we investigated whether curcumin modifies CaMKII-dependent retinal vascular leakage in a rat model of diabetes induced by streptozotocin (STZ), and whether it inhibits CaMKII in rat retinal Müller cells. We report here that curcumin inhibits the CaMKII/NF- κ B signaling pathway, both *in vivo* and *in vitro*, and thereby decreases the expression of inflammatory mediators such as VEGF, iNOS and ICAM-1. We also report that comparable results were obtained with KN93, a pharmacological inhibitor of CaMKII inhibitor. These results indicate a prominent role for CaMKII-mediated signaling in the vascular damage of diabetic retinopathy and that curcumin may have effective therapeutic potential for these changes.

Materials and Methods

Ethics Statement

All studies involving 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. The protocol was approved by the Committee on the Ethics of Animal Experiments of Zhejiang University.

Rat model of diabetic retinopathy and drug treatment

Male Sprague-Dawley rats (8 weeks of age) weighing 180–200 g (Shanghai Laboratory Animal Center, Chinese Academy of Sciences) were used in this study. Rats were housed in ventilated microisolator cages with free access to water and food. The rats were randomly assigned to receive either 60 mg/kg STZ intraperitoneally or citrate buffer alone. Rats were categorized as diabetic when blood glucose levels exceeded 16.7 mmol/L at 48 h after STZ treatment [22]. Two weeks after the induction of diabetes, rats were divided randomly into three subgroups: STZ-diabetic rats (n=12), STZ-treated diabetic rats administered curcumin (n=12), or STZ-diabetic rats administered KN93 (n=12) for a 12-week period. Curcumin was suspended in saline containing 0.5% carboxymethylcellulose at a concentration of 20 mg/ml and administered via oral gavage at a total dose of 100 mg/kg/day [17]. KN93 was administered by intraperitoneal injection at 1 mg/kg/day [13]. Control STZ-treated diabetic rats and non-diabetic controls (n=12) were gavage administered saline containing 0.5% carboxymethylcellulose on a daily basis. Body

weights and blood glucose levels were measured every 2 weeks. At the completion of the administration protocol, animals were deeply anesthetized with pentobarbital and subsequently sacrificed. The eyes were then enucleated for investigation.

Measurement of retinal vascular permeability

Retinal vascular permeability was assessed with Evans blue using a published protocol [23, 24]. Briefly, after anesthesia by intraperitoneal injection of 1% pentobarbital sodium (10 mg/kg body weight), the right jugular vein and right iliac artery were cannulated with 0.28- and 0.58-mm internal diameter polyethylene tubing, respectively, and filled with heparinized saline (400 units heparin/ml saline). Evans blue was injected through the jugular vein over a 10 s period at a dose of 45 mg/kg. Two minutes after the rats turned visibly blue, 0.2 ml of blood was drawn from the right iliac artery to obtain the initial Evans blue plasma concentration. Subsequently, 0.1 ml of blood was drawn at 15-min intervals for up to 2 h after injection to obtain the time-averaged Evans blue plasma concentration. After the dye had circulated for 2 h, the chest cavity was opened and rats were perfused via the left ventricle at 37°C with 1% paraformaldehyde in 0.05M citric acid (pH 3.5). The perfusion lasted 2 min at a physiological pressure of 120 mm Hg, and then both eyes were enucleated and bisected at the equator. The retinas were then carefully dissected away under an operating microscope. After measurement of the retinal wet weight, retinas were thoroughly dried in a Speed-Vac for up to 5 h. The Evans blue dye was extracted by incubating each retina in 0.3 ml of formamide for 18 h at 70°C. The extract was ultracentrifuged at 70,000 rpm for 45 min at 4°C to precipitate any proteins with absorbance at 620 nm. Aliquots of 60 µl of supernatant were used to measure the absorbance at 620 nm. The concentration of dye in the extracts was calculated from a standard curve of Evans blue in formamide. BRB breakdown was calculated using the following equation, with the results expressed in µl plasma×g retinal wet wt⁻¹·h⁻¹.

$$\frac{\text{Evans blue } (\mu\text{g})/\text{Retina wet weight (g)}}{\text{Time averaged Evans blue concentration } (\mu\text{g})/\text{Plasma } (\mu\text{l}) \times \text{Circulation time (h)}}$$

For qualitative observation of retinal capillary leakage, the rats were anesthetized with pentobarbital and then administered Evans blue (100 mg/kg) via the tail vein. Animals were kept on a temperature controlled heating pad for 2 h, after which they were sacrificed by 1% pentobarbital overdose (>45mg/KG). The eyes were immediately enucleated and fixed with 2% paraformaldehyde in PBS for 2 h. Retinas were then dissected, and flat mounts were mounted on glass slides and visualized under a confocal microscope (Eclipse E800; Nikon, Tokyo, Japan).

Cell culture

For primary culture studies, rat retinal Müller cells were obtained and identified as described previously [25]. Briefly, Sprague–Dawley rats at postnatal (PN) day 5 to PN7 were sacrificed and the enucleated eyes were washed under sterile conditions, and the anterior portions were discarded. The retinas were isolated, chopped into 1×1 mm fragments, treated with 0.1% trypsin at 37°C for 20 min, and then passed through mesh to remove any large retinal pieces. The strained isolates were centrifuged at 800 rpm for 5 min, and the supernatant fluid was removed. The precipitated cells were resuspended and seeded onto plastic culture flasks containing Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mmol/L glutamine, 0.1% penicillin/streptomycin and 10% fetal calf serum. The cultures were maintained in 5% CO₂ at 37°C. The medium was routinely replaced every 3–4 d. Müller cells were identified by their expression of glutamine synthetase (GS) and vimentin, as judged by immunocytochemical staining. Nuclei were stained with DAPI (4',6-diamidino-2-phenylindole). All experiments were conducted using 80%–85% confluent cells. Before each experiment, the plated cells were incubated with serum-free DMEM medium for 1 h. After this, the medium was replaced with serum-free DMEM and the cells were treated with normal D-glucose (5.5 mmol/L) or high glucose (HG; 30 mmol/l glucose) in the presence or absence of 10 µmol/L KN93, 100 µmol/L PDTC (pyrrolidine dithiocarbamate, a NF-κB inhibitor), or curcumin at the indicated concentrations (all from Sigma, St. Louis, MO).

Evaluation of Cell Viability

Cell viability was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide (MTT) assay. Briefly, Müller cells were seeded at a density of 10×⁴ cells per well in 96-well plates and

cultured until sub-confluence. Next, cells were treated with curcumin for 24 h before incubation with MTT (5 mg/mL) at 37°C in 5% CO₂ atmosphere for 4 h. The culture medium was then removed, and the formazan formed in the reaction was dissolved in 150 µL DMSO (dimethyl sulphoxide). The optical density of the solution was measured at 490 nm using a multifunctional microplate reader (SpectraMax M5, Molecular Devices, CA, USA). Cell viability in each well was presented as a percentage of the control (vehicle-treated group).

Real-time reverse transcription-polymerase chain reactions (RT-PCR)

Total RNA was extracted from rat retinal tissue, harvested as described above, or from Müller cells using TRIzol reagent (Invitrogen Life Technologies, Gaithersburg, MD) and stored at -80°C. The DyNAmo Flash SYBR Green qPCR Kit (Finnzymes Oy, Espoo, Finland) was used according to the manufacturer's instructions. The primer sequences (sense/antisense) used were:

VEGF, 5'-AAT GAT GAA GCC CTG GAG TG -3' / 5'-AAT GCT TTC TCC GCT CTG AA-3'; ICAM-1, 5'- CCC CAC CTA CAT ACA TTC CTA C-3' / 5'-ACA TTT TCT CCC AGG CAT TC-3'; iNOS, 5'-TCC CCC ACA TTC TCT TTC C-3' / 5'-GCA GCT AAA TAT TAG AGC AGC G-3'; β-actin 5'-CGA CAA CGG CTC CGG CAT GT-3' / 5'-GGG GCC ACA CGC AGC TCA TT-3').

The specificity of the amplification product was determined by performing a melting curve analysis. Standard curves were generated the expression of each gene by using serial dilutions of known quantities of the corresponding cDNA gene template. Relative quantification of the signals was performed by normalizing the signals of different genes with the β-actin signal.

Western blotting

The protein concentration in the supernatant was measured using the Bio-Rad DC protein assay. Protein (50 µg) obtained from each retinal and Müller cell sample were subjected to SDS-PAGE in a Bio-Rad miniature slab gel apparatus and electrophoretically transferred onto a nitrocellulose membrane. The membrane was blocked in 5% nonfat dried milk solution and incubated overnight with partially purified mouse anti-VEGF monoclonal antibody (mAb; 1:500; Chemicon, Temecula, CA), rabbit anti- ICAM-1 mAb (1:500) (Cell Signaling Technology), rabbit antiphospho- iNOS mAb (1:500) (Cell Signaling Technology), rabbit mAb against rat CaMKII and p-CaMKII (Thr-286) (Abcam, Cambridge, MA, USA), or human anti-NF-κB antibody (Enzo Life Sciences Inc., NY). Detection of β-actin expression with a mAb (1:1,000; Sigma Chemical) was used as an internal control to confirm equivalent total protein loading. All measures are expressed relative to the signal intensities measured in the control lanes, which were assigned a value of 1.0. Western blots were repeated 3 to 5 times and qualitatively similar results were obtained.

Immunofluorescence Staining for Detection of NF-κB p65 in retinal Müller cells

NF-κB p65 translocation into the nucleus, as an index of NF-κB activation, was measured using immunofluorescence staining. Cells were seeded onto chamber slides and grown into confluence. After exposures, slides were washed twice with ice-cold PBS, fixed with 3.7% paraformaldehyde, and permeabilized with 0.1% Triton X-100. Slides were blocked with 10% goat serum (Vector Laboratories, Burlingame, CA) for 1 h and incubated overnight at 4°C with anti-NF-κB p65 antibody (1:1000, Cell Signaling Technology Inc., Beverly, MA). The following day, slides were incubated at room temperature with anti-rabbit IgG antibody labeled with Alexa Fluor 555 (Molecular Probes, Eugene, Oregon, USA). Sections were mounted with Vectashield mounting medium with DAPI (Vector Laboratories, Burlingame, CA) and visualized and photographed under a confocal laser scanning microscopy (Zeiss LSM510; Carl Zeiss, Thornwood, NY). The number of cells with p65 nuclear translocation were counted in six random fields in a masked fashion. Counts were expressed as a percentage of the number of translocated cells in comparison to that of total cells. Experiments were performed in triplicate and were repeated at least three times.

Statistical Analysis

Data are expressed as mean ± SEM of at least three independent experiments. Differences between mean values of multiple groups were analyzed by one-way analysis of variance with Dunnett's test for post hoc comparisons. A P-value less than 0.05 was considered statistically significant. All computations were performed with the SPSS16.0 (Chicago, IL) software.

Results

Curcumin does not affect body weight and blood glucose levels in diabetic rats

Figure 1 presents basic measures of the STZ model. In comparison to non-diabetic controls, blood glucose levels were chronically elevated at the first time that this measurement was obtained (2 weeks following STZ), and this level was maintained throughout the experiment (Fig. 1A). Daily administration of curcumin or KN93 to STZ-treated rats had no effect on blood glucose ($P>0.05$). Body weight gradually increased in control rats, but remained stable in rats treated with STZ (Fig. 1B). Daily administration of curcumin or KN93 to STZ-treated rats had no effect on body weight ($P>0.05$). These data indicate that these basic features of the STZ rat model are not affected by long-term curcumin administration.

Curcumin and KN93 inhibit retinal vascular leakage induced by diabetes

Evans blue was used in retinal flat mounts to evaluate the effect of curcumin on retinal blood vessel leakage. In control retinas, Evans blue fluorescence was located within blood vessels (Fig. 2A). In STZ-treated rats, focal leakage of the dye from capillaries and larger vessels was noted (Fig. 2B, arrows), in agreement with other reports [26]. This leakage was not seen in STZ-treated rats which were administered curcumin (Fig. 2C) or KN93 (Fig. 2D). Evans blue in the retina was measured to assess BRB permeability (Fig. 2E). Evans blue levels were elevated in the retinas of STZ-treated diabetic rats (2.89 ± 0.47 μg Evans blue/g wet wt retina) as compared to control animals (0.82 ± 0.11 μg Evans blue/g wet wt retina). In agreement with the reduced vascular leakage indicating by whole mount imaging, this elevation was significantly reduced in STZ-treated rats administered curcumin (1.24 ± 0.21 μg) or KN93 (1.37 ± 0.35 μg).

Curcumin and KN93 reduce VEGF, iNOS and ICAM-1 expression in the diabetic retina

Vascular leakage and adhesion of leukocytes to retinal vessels is mediated by pro-inflammatory cytokines [27]. Therefore, the effect of curcumin on the expression levels of VEGF, iNOS and ICAM-1 was measured. In comparison to non-diabetic controls, mRNA (Fig. 3A) and protein (Fig. 3B, C) measures were significantly elevated in the retina of STZ-treated diabetic rats. These increases were significantly reduced by administration of curcumin or KN93.

Curcumin protects retinal Müller cells against high glucose-induced cytotoxicity

Cultured Müller cells were used to examine the impact of high glucose on cell viability. As shown in Figure 4, the viability of cells grown in 30 mM glucose (HG) for 24 h was reduced

Fig. 1. Blood glucose and body weight characteristics of experimental animals. (A) In STZ-treated rats, blood glucose was elevated by a factor of 4-5 over that seen in non-diabetic controls (Con). This elevation was comparable for STZ-treated diabetic rats administered vehicle (DM) or curcumin (DM+Cur) or KN93 (DM+KN93). (B) Body weight of STZ-treated rats increased progressively over the 12 week follow-up period. This increase was comparable for DM and DM+Cur and DM+KN93 animals. Data points indicate the average $\pm$ SEM of 12 animals.

Fig. 2. Evans blue fluorescence. (A) Representative image of retinal fluorescence in an untreated control animal (Con), in which Evans blue fluorescence is limited to the blood vessels. (B) In STZ-treated diabetic retinas (DM), dye leakage is observed (white arrows). This leakage was not seen in STZ-treated diabetic rats administered (C) curcumin (DM+Cur) or (D) KN93 (DM+KN93). (E) Quantitative analysis of the Evans Blue leakage. Bars indicate average ($\pm$ SEM) of at least 6 rats. Scale bars indicate 100 μ m.

Fig. 3. Retinal cytokine levels. (A) mRNA levels measured by RT-PCR for VEGF, iNOS and ICAM-1 in non-diabetic control rats (Con), STZ-treated diabetic rats administered vehicle (DM), curcumin (DM+Cur) or KN93 (DM+KN93). Results are expressed relative to average values measured in control retinas. (B) Representative western blots for VEGF, iNOS and ICAM-1 protein expression in the four experimental groups. Equal protein loading was confirmed with the β -actin antibody. (C) Quantification of signal intensity by VEGF, iNOS and ICAM-1 densitometry of retinal tissue. Error bars indicate average + SEM of at least 6 retinas per group. * p <0.01 versus Con; # p <0.01 versus DM.

to 58.28% $\pm$ 4.9% of cells grown in 5.5 mM glucose (NG). Pretreatment of Müller cells with curcumin significantly improved cell viability in a dose-dependent fashion within the concentration range of 5-15 μ M/L. This effect was not observed at a concentration of 30 μ M curcumin, indicating that this dose was higher than these cells could tolerate.

Curcumin and KN93 suppress phosphorylation of CaMKII and NF- κ B in diabetic retina

Phosphorylation of the p65 subunit of NF- κ B plays an important role in regulating the expression of many genes, including those that encode pro-inflammatory cytokines

Fig. 4. Effects of curcumin on retinal Müller cell viability. Retinal Müller cells were exposed for 24 h to normal glucose (NG, 5.5 mM) or high glucose (HG, 30 mM) along with the indicated concentrations of curcumin (5, 10, 15 or 30 μ M). Cell viability was determined by MTT assay. Results are presented as percentage of normal glucose without curcumin. Error bars indicate average + SEM of at least 8 animals per group. * $p < 0.01$; # $p < 0.05$.

Fig. 5. Western blot analysis of retinal levels of p-p65, p65, p-CaMKII, and CaMKII. (A) Representative Western blots obtained for control rats (Con), and STZ-treated diabetic rats administered vehicle (DM), curcumin (DM+Cur) or KN93 (DM+KN93). β -actin served as the loading control. (B) Western blot quantification. Bars indicate average + SEM from at least three independent experiments. * $p < 0.05$ versus Con; # $p < 0.05$ versus DM.

and adhesion molecules [28]. In addition, phosphorylation of CaMKII is a critical factor in the development of retinal vascular damage in diabetic mice [14]. To evaluate the role of curcumin in the regulation of the phosphorylation of CaMKII and NF- κ B p65, we examined retinas by Western blot. As shown in Figure 5, levels of phosphorylated CaMKII (Thr²⁸⁶) and NF- κ B p65 (Ser⁵³⁶) were significantly elevated in retinas of STZ-treated diabetic rats as compared to controls. This elevation was normalized in STZ-treated diabetic rats that were administered curcumin (100 mg/kg/day) or KN93 (1 mg/kg/day).

Curcumin suppresses the activation of NF- κ B through a CaMKII-dependent pathway

To further explore the mechanism of curcumin on the action of NF- κ B p65 in cultured retinal Müller cells, we measured NF- κ B p65 translocation from the cytoplasm to nuclei, an index of NF- κ B activation [29]. As shown in Figure 6A-a, NF- κ B was distributed in the cytoplasm of the cells maintained in normal glucose (5.5 mM). This distribution was not changed when curcumin (Fig. 6A-b) or KN93 (Fig. 6A-c) was added to the medium. In comparison, high glucose medium (30 mM) resulted in a high degree of nuclear translocation of NF- κ B p65, as shown by the high degree of co-localization of the NF- κ B and nuclear labels in Fig. 6A-d. The translocation induced by high glucose was significantly inhibited by 15 μ M curcumin (Fig. 6A-e) and also by KN93 (Fig. 6A-f), with an apparently greater effect at the dose used. Western blot analysis of cultured Müller cells showed that high glucose resulted

Fig. 6. Immunohistochemical analysis of NF- κ B p65 distribution. (A) Representative sections stained for NF- κ B p65 antibody (red) and for DAPI (blue). Immunostaining of NF- κ B p65 in rat Müller cells from untreated (a), 15 μ M curcumin (b) and 10 μ M KN93 (c) under normal glucose (NG, 5.5 mM) exhibit the cytoplasmic localization of p65. Cells stimulated with high glucose (HG, 30 mM) display a significant increase in the translocation of p65 into the nucleus (d). p65 immunostaining was predominantly cytoplasmic in cells stimulated with HG and treated with 15 μ M curcumin (e) or 10 μ M KN93 (f) (B) Western blot analysis showing the expression of p65 and p-p65 protein in cells raised in NG or HG and also treated with Curcumin (+Cur), KN93 (+KN93) or PDTC (+PDTC). β -actin served as the loading control. (C) Western blot quantification. Error bars indicate average +SEM from at least three independent experiments. * $p < 0.01$ versus NG; # $p < 0.01$ versus HG.

in increased levels of phosphorylated p65 NF- κ B, and that this increase was normalized by curcumin and KN93, without affecting total p65NF- κ B (Fig. 6B,C).

Inhibition of CaMKII, or NF- κ B attenuated HG-induced upregulation of cytokines

In comparison to Müller cells cultured in normal glucose, those cultured in high glucose show elevated levels of mRNAs for the pro-inflammatory cytokines VEGF, iNOS and ICAM-1 (Fig. 7A). This increase was reduced to levels found in normal glucose conditions by curcumin (15 μ M), KN93 (10 μ M), or the NF- κ B inhibitor PDTC (100 μ M). Similar changes were seen when protein levels of these molecules were examined by Western blot (Fig. 7B,C).

Discussion

In the current study, we provide evidence that administration of curcumin, a natural phenolic compound with vasoprotective properties, to a rat model of diabetes can effectively suppress the action of transcription factor NF- κ B through inhibition of CaMKII. We also demonstrate that this action downregulates the inflammatory cytokines VEGF, iNOS and

ICAM-1. These changes are accompanied by a reduced vascular permeability of blood vessels in the diabetic retina. Although it is possible that curcumin could impact a number of pathways, comparable results were obtained with KN93, a specific CaMKII inhibitor, supporting our conclusion that the CaMKII pathway is the primary pathway through which curcumin exerts its beneficial effects.

Diabetic retinopathy is the most common complication of diabetes and is a leading cause of blindness among working-age adults. Retinal vascular lesions are a major cause of vision loss in diabetic retinopathy; occurring at early stages and reflecting a breakdown of the BRB [30]. There is currently no effective treatment with which to prevent the development of the retinal vascular lesions that are induced by diabetes. The identification of an effective treatment for this complication of diabetic retinopathy would be an important advance.

In this report, we focused on curcumin, a pharmacological agent which is commonly used in Asian traditional medicine due to its anti-inflammation and anti-oxidization features [16, 31]. To evaluate the impact of curcumin *in vivo*, we applied it to the well-characterized STZ rat model of diabetes. In these studies, we found that curcumin alleviates the BRB leakage observed in STZ-treated rats. The inhibition of vascular lesions by curcumin was independent of a change in blood glucose, as this measure (and body weight) was comparable in STZ rats that were administered curcumin or the vehicle control (Fig. 1). Instead, our results indicate that curcumin reduces pro-inflammatory cytokines, which are known to play a critical role in the development of diabetic retinopathy [2, 32]. Specifically, curcumin administered on a daily basis to the STZ rat model of diabetic retinopathy reduced retinal levels of VEGF, iNOS and ICAM-1. This result was also obtained in our study of cultured Müller cells, in which these cytokines were elevated by high glucose media. These elevations were normalized by 24 h administration of curcumin. Kim et al. [14, 33] recently demonstrated that resveratrol, another polyphenol with curcumin-like physiological properties, regulates CaMKII activity. Consistent with a key role for CaMKII, we found that levels of phospho-CaMKII were increased in the retina of diabetic rats and also in Müller cells cultured in high glucose. Our results are in agreement with those of other laboratories who suggest activated CaMKII is an important mediator in diabetes-mediated

Fig. 7. Cytokine levels in cultured Muller cells. (A) RT-PCR measures of mRNA for VEGF, iNOS, and ICAM obtained from cells raised in normal glucose (NG), or high glucose (HG), and administered Curcumin (+Cur), KN93 (+KN93) or PDTC (+PDTC). Results are expressed relative to untreated NG controls. (B, C) Western blot analysis of protein levels of VEGF, iNOS, and ICAM in the experimental conditions defined in (A). β -actin served as the loading control. Error bars indicate average $\pm$ SEM from at least three independent experiments. * $p < 0.01$ versus NG; # $p < 0.05$ versus HG.

vascular dysfunction [14]. Other groups have established that curcumin treatment may prevent retinopathy due to its anti-oxidant and anti-inflammatory properties [21]. In muscle, and other reactive oxygen species rich environments, cellular levels of Ca^{2+} channels along with downstream effectors such as CaMKII are elevated [34], while CaMKII knockdown attenuates H_2O_2 -induced oxidative stress [35]. These suggest that CaMKII has functions beyond simply connecting “upstream” oxidant stress and Ca^{2+} signals to “downstream” cellular responses. Accordingly, we hypothesize that curcumin regulates CaMKII to impact the oxidative stress-induced expression of multiple inflammatory cytokines in the retina. This conclusion is supported by our *in vitro* results with cultured retinal Müller cells and *in vivo* measures of vascular leakage.

CaMKII is activated by sustained increases of intracellular cytosolic free calcium ($[\text{Ca}^{2+}]_i$) and by oscillations in $[\text{Ca}^{2+}]_i$, serving to translate these $[\text{Ca}^{2+}]_i$ signals into cellular responses in an autophosphorylation-dependent manner [36]. While noting that posttranslational modifications other than phosphorylation (i.e. oxidation, O-GlcNAcylation, nitrosylation) can trigger CaMKII activity, phosphorylation of CaMKII is known to play important roles in a variety of cellular functions including neurons and vascular system [14, 35, 37]. CaMKII is encoded by 4 genes [38]. Three isoforms of CaMKII are found in skeletal muscle (CaMKII β , CaMKII γ , and CaMKII δ) while CaMKII α is the predominant isoform in the retina and the central nervous system [39]. Learning, memory, and cognition are thought to involve long-term potentiation of synaptic strength, which in turn involves binding of CaMKII to the N-methyl-D-aspartate (NMDA)-type glutamate receptor [40]. In developing CaMKII inhibition as a clinical treatment for human diabetes, it will be critically important to identify dose ranges and/or molecules that spare learning, memory and cognition while being effective against diabetic retinopathy.

CaMKII activation by phosphorylation also contributes to a neuroprotective response against glutamate or NMDA-induced retinal neurotoxicity, both *in vitro* and *in vivo* [41, 42]. Our previous work and other studies indicate that curcumin and resveratrol attenuate diabetes-induced apoptosis in retinal neurons by reducing glutamate levels and downregulating CaMKII, and suggest CaMKII to be a potential target for the treatment of vision loss due to diabetes induced neuropathy [22, 33]. CaMKII α autophosphorylation at Thr²⁸⁶ is involved in physiological and pathological events in neurons, and also in diabetic retinopathy [22, 33]. In particular, CaMKII has been implicated in regulating NF- κ B activation, and to be involved in inflammatory diseases [9, 14]. Our results in diabetic rats (Fig. 5) and in Müller cell culture (Fig. 6) suggest that high glucose induces NF- κ B activation through a CaMKII-dependent mechanism. Curcumin normalizes these responses, including NF- κ B p65 phosphorylation and p65 nuclear translocation, indicating that the anti-inflammatory actions of curcumin are achieved through transcriptional suppression of NF- κ B through a CaMKII-dependent pathway.

CaMKII responds to increases in $[\text{Ca}^{2+}]_i$ resulting from the stimulation of NMDA receptors. The elevation of VEGF expression and BRB breakdown in STZ-induced diabetic rats is blocked by NMDA receptor channel blockers and antagonists [43]. Our previous work has shown that curcumin can reduce retinal glutamate to near-normal levels in diabetic rats [22]. These observations indicate that high glucose induces elevations in glutamate that in turn activate CaMKII and NF- κ B, and thereby regulate cytokine expression and subsequent retinal vascular leakage in diabetes. This model is supported by our observations that high glucose-induced expression of cytokines (iNOS, ICAM-1, and VEGF) was blocked by PDTC, a NF- κ B inhibitor, and also by KN93, a CaMKII inhibitor. This data therefore identifies CaMKII as a potential treatment target for patients with diabetic retinopathy. In view of the global healthcare challenge presented by diabetic retinopathy, and the current use of curcumin for other human disorders, it would be of interest to conduct a clinical trial to determine the value of curcumin in the management of patients with diabetes to ameliorate the development and progression of diabetic retinopathy, and potentially other complications of this condition.

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Disclosure Statement

No competing financial interests exist.

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