Baicalein Reduces Inflammatory Process in a Rodent Model of Diabetic Retinopathy

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PURPOSE. This study was designed to elucidate the role of inflammatory process in diabetic retinopathy and to investigate the effect of baicalein treatment on diabetic rat.

METHODS. Retinal microglial cells were identified with CD11b antibody, and retinal Müller cells were identified with glial fibrillary acidic protein (GFAP). The gene expression of interleukin (IL)-18, tumor necrosis factor (TNF)-α, and IL-1β was examined by quantitative real-time PCR. The expression of GFAP and vascular endothelial growth factor (VEGF) was examined by quantitative real-time PCR, immunohistochemistry, and Western blot analysis. Vascular permeability was measured in vivo by bovine serum albumin conjugated with FITC. Baicalein and Western blot analysis. Vascular permeability was measured in vivo by bovine serum albumin conjugated with FITC. Baicalein was given by oral administration (150 mg/kg/d) with an animal feeding needle beginning 5 days after streptozotocin (STZ) injection.

RESULTS. By 24 weeks after onset of diabetes, microglial cells were activated and proliferated, and Müller cells upregulated their GFAP and VEGF expression. Pro-inflammatory factors, including IL-18, TNF-α, and IL-1β, were significantly upregulated. Obvious vascular leakage and abnormality were demonstrated, and ganglion cell loss was significant. Baicalein treatment ameliorated diabetes-induced microglial activation and pro-inflammatory expression, reduced the GFAP and VEGF expression from Müller cells, and significantly reduced vascular abnormality and ganglion cell loss within the retina.

CONCLUSIONS. Inflammatory process, characterized by microglial activation and Müller cells dysfunction, was implicated in STZ-induced diabetic retinopathy. Baicalein treatment ameliorated inflammatory process, and therefore inhibited vascular abnormality and neuron loss in diabetic retinas. (Invest Ophthalmol Vis Sci. 2009;50:2319–2327) DOI:10.1167/iovs.08-2642

Diabetic retinopathy (DR) is the leading cause of adult blindness and the most common complication of diabetes. It affects more than 90% of people with diabetes, ultimately leading to retinal edema, neovascularization, and vision loss in some patients.1,2 Vascular changes, including breakdown of the blood-retinal barrier, thickening of the capillary basement membrane, and reduction in the number of pericytes and increment in the number of acellular capillaries, have been widely documented in DR. Capillary cells are not the only retinal cells that undergo apoptotic death in diabetes. A greater-than-normal frequency of nonvascular cells, mostly ganglion cells, have also been reported to become TUNEL-positive in retinas of humans and animals with diabetes.3

In previous studies, emerging evidence supported the notion that inflammation in the retina, characterized by the activation of microglia and astroglia, is involved in the pathogenesis of DR. DR is a chronic, low-grade inflammatory disease.1–6 Diabetic conditions lead to an elevation of pro-inflammatory cytokine expression within the retina, which activates microglial cells.7 In response to an activating stimulus, quiescent microglia undergo a series of stereotyped morphologic, phenotypic, and functional changes.8 Activated microglia thereby stimulate a cycle of inflammation that recruits leukocytes, causes vascular breakdown, and directly induces glial dysfunction and neuronal cell death through the release of cytotoxic substances.7

The root of Scutellaria baicalensis (Chinese name, Huang-qin) has been used as a folk medicine in China and Japan for the treatment of chronic hepatitis, allergy, thrombotic stroke, and inflammatory disease for centuries.9 Huangqin is known to contain numerous flavone derivatives,10 and their pharmacological properties have been extensively investigated. Among them, baicalein (5,6,7-trihydroxy-2-phenyl-4H-1-benzopyran-4-one) has attracted considerable attention. As the major active component of S. baicalensis, baicalein has also been reported to exhibit potent anti-inflammatory effect. Baicalein was found to inhibit acetic acid–induced increase in vascular permeability in mice and to reduce acute paw edema in the rats induced by compound 48/80.11 It also suppressed development of secondary lesion in adjuvant-induced arthritis in rats.12 Microglial suppressive effect of baicalein is also well documented. In this regard, it has been reported to abolish the LPS-induced expression of inducible nitric oxide synthase (iNOS), tumor necrosis factor (TNF)–α, and interleukin (IL)-1β in murine BV-2 cell and rat primary microglial cells12 and to downregulate the microglial markers CD68, CD80, and CD86 expression in G93A/SOD1 mouse (an animal model of amyotrophic lateral sclerosis).13

Given that inflammatory process plays an important role in the pathogenesis of DR, we speculate that baicalein, with its anti-inflammatory properties, may exert the capacity to block high-glucose–induced microglial and astroglial activation, and consequently protect neurons and vascular damage in DR.

MATERIALS AND METHODS

Animals

After a 16-hour fast, SD female rats weighing 190 to 200 g received a single 60 mg/kg intraperitoneal injection of streptozotocin (STZ; Sigma-Aldrich, St. Louis, MO) in 10 mM sodium citrate buffer (pH 4.5).
Control animals were fasted and received the buffer alone. Animals with blood glucose levels higher than 16.7 mM 5 days after receiving STZ were considered to be diabetic. Body weight, mean systolic blood pressure (by tail cuff plethysmography in conscious rats; equipment from IITC Life Science, Woodland Hills, CA), and blood glucose levels were checked every 4 weeks. All experiments were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. All animals were husbanded in accordance with the guidelines of the Association for the Assessment and Accreditation of Laboratory Animal Care.

For the in vivo baicalein (Sigma-Aldrich) treatment, rats were treated by oral administration with an animal feeding needle beginning 5 days after STZ injection. We chose oral administration of baicalein because administration in humans is by the oral route. Baicalein was administered at concentrations of 130, 260, and 520 mg/kg. Referring to these previous studies, we chose to give treatment at concentrations of 75, 150, and 300 mg/kg/d. We chose 150 mg/kg/d as the dosage in the present study, there is no significant difference between 75, 150, and 300 mg/kg/d. We chose 150 mg/kg/d as the dosage to present our results in this article.

**Tissue Preparation**

Animals were euthanatized with an overdose of pentobarbital, and their eyes were immediately enucleated and fixed in 4% (w/v) paraformaldehyde (PFA) in PBS for 1 hour. The anterior segments were removed and the posterior segments were further fixed in the same fixative for an additional period of 5 hours. The tissue samples were transferred to 20% sucrose buffer overnight at 4 °C for cryoprotection and then embedded in OCT compound. Frozen sections were cut 8 μm thick through the optic nerve head and ora serrata with a cryostat, and the sections were kept in a −80°C freezer until use.

Flatmounted retinas were prepared by bisecting the eyes at the ciliary body into anterior and posterior sections. The lens and vitreous were removed, and the retina was subsequently separated from the underlying retinal pigment epithelium and choroid. Flatmounted retinas were kept at 4°C until use.

**Vessel Leakage Study**

For leakage study, rats were deeply anesthetized using a ketamine–xylazine mixture, and were perfused through the left ventricle using a FITC-dextran stock solution, 10 mg/mL (Sigma-Aldrich), at a 0.05 mL/g body weight concentration. Animals were killed 1 minute after perfusion. The eyes were enucleated and fixed in 4% PFA overnight. The corneas and lens were then removed, and the peripheral retinas were dissected and flatmounted on microscope slides for examination under confocal laser scanning microscope (510 META; Carl Zeiss, Oberkochen, Germany).

**Immunohistochemical Study**

For immunohistochemical study, the frozen sections were fixed with chilled fresh acetone for 10 minutes. Sections were incubated with mouse anti-VEGF monoclonal (1:50, SC-507; Santa Cruz Biotechnology, Santa Cruz, CA) or goat anti-VEGF polyclonal (1:50, SC-9965; Santa Cruz Biotechnology) antibodies. The secondary anti-rabbit or anti-mouse antibodies were labeled with HRP. Control sections were treated in the same way, but with omission of primary antibodies. The sections were developed with DAB. Flatmounted retinas were examined by confocal microscope (Carl Zeiss) and counted by a masked observer. Positively stained microglial cells in the inner and middle retinal layer were counted in a standard area of retina using a graticule at 40X objective magnifications. At least 10 nonadjacent areas per eye were counted for each stain.

For double staining, sections were first blocked in 10% rabbit serum for 30 minutes at room temperature, then they were incubated in primary antibody to CD11b (Serotec Ltd.) at 4°C overnight. After washing in PBS for 15 minutes, TRITC- or FITC-conjugated secondary anti-mouse IgG (1:100; Jackson Laboratories, West Grove, PA) was added for 45 minute at 37°C then the sections were washed in PBS for 15 minutes and further added primary goat anti-TNF-α antibody (1:100; SC-1351; Santa Cruz Biotechnology) or primary rabbit anti–Factor VIII-related Ag (1:100, 18-0018; Invitrogen Corp., Grand Island, NY) for 3 hours at room temperature. After washing, sections were incubated in corresponding secondary antibodies (1:100; Jackson Laboratories) for 45 minute at 37°C. The sections were observed with a confocal laser scanning microscope (Carl Zeiss) with 568 nm filter for TRITC and 488 nm filter for FITC.

**Western Blot Assays**

After the animals were killed as described previously, the eyes were enucleated and bisected, and the retinas were peeled from the eyecups and immediately homogenized with 0.5 mL ice-cold lysis buffer. The insoluble material was removed by centrifugation at 12,000 g for 20 minutes. Final protein concentrations were determined using a protein assay kit (BCA; Pierce Biotechnology, Rockford, IL) according to the manufacturer’s specifications. Western blot analysis was then performed. Antibodies specific for GFAP and VEGF were used for immunodetection. Blots were visualized using an enhanced chemiluminescent technique (SC-2048; Santa Cruz Biotechnology). As a control for equal loading of proteins, a β-actin antibody (1:2000, A2228; Sigma-Aldrich) was used. For quantitative evaluation of the Western blot studies, the films were scanned and the optical densities were quantified with analysis software (Quantity One 1-D; Bio-Rad, Hercules, CA). The Western blot experiments were repeated four times from separate samples.

**Quantitative Real-Time PCR**

After the animals were killed as described previously, the eyes were enucleated and bisected, and the retinas were peeled from the eyecups and immediately homogenized in an RNA isolation agent (TRIzol; Invitrogen Corp.). Real-time PCR was performed in 96-well plates using standard protocols with a fluorescent detection dye (SYBR Green; Invitrogen Corp.) in a real-time detection system (Cycler; Bio-Rad). All PCR reactions were at a final volume of 30 μL comprised of fluorescent dye/PCR mix, 600 μM forward and reverse primers, and 1 ng of cDNA. We used the following PCR cycle parameters: polymerase activation for 15 minutes at 95°C, 40 cycles of 95°C for 15 seconds, 60°C for 30 seconds, and 72°C for 1 minute. Fluorescence was measured at 72°C. The quantity of mRNA was calculated by normalizing the Ct (threshold cycle) of the target gene to the Ct of the β-actin housekeeping gene in the same sample, according to the following formula: The average β-actin Ct (each multiplex PCR was performed in triplicate) was subtracted from the average target gene Ct; the result represents the ΔCt. This ΔCt is specific and can be compared with the ΔCt of a calibration sample. The subtraction of control ΔCt from the ΔCt of the target gene is referred as ΔΔCt. The relative quantification of expression of a target gene (in comparison with control) was determined by using 2−ΔΔCt.
The Role of Baicalein in Diabetic Retinopathy

**RESULTS**

**Animals**

The body weight, blood pressure, and blood glucose levels for diabetic rats with or without baicalein treatment and the age-matched control rats are shown in Table 1. By 4 weeks after onset of diabetes, diabetic rats had a 2% gain in weight during this period. The blood pressure levels and blood glucose levels for diabetic rats with or without baicalein treatment and the age-matched control rats are shown in Table 1. By 4 weeks after onset of diabetes, whereas the diabetic rats had no weight gain, and the baicalein-treated diabetic rats had a 2% gain in weight during this period. The blood pressure levels had no significant difference among control rats and diabetic rats with or without baicalein treatment at all ages examined. The blood glucose levels differed significantly between control rats and diabetic rats with or without baicalein treatment at all ages studied.

**Amelioration of Diabetes-Induced Retinal Morphologic Alterations by Baicalein**

In the age-matched control retinas, the photoreceptor inner and outer segments as well as the photoreceptor nuclei were well aligned (Fig. 1A). By 24 weeks after onset of diabetes, the outer and inner segments of photoreceptor cells showed moderate disorganization, and large number of pyknotic nuclei appeared in the inner nuclear layer (INL) (Fig. 1B). After treatment with baicalein, the disorganized inner and outer segment as well as the pyknotic nuclei in the INL disappeared (Fig. 1C). By 24 weeks after onset of diabetes, the thickness of retina of diabetic rats was less than that in age-matched control and the baicalein-treated rats.

Additional measurements were made of the number of cells in the ganglion cell layer (GCL) of diabetic and control rats. Retinas of control and baicalein-treated rats had a GCL in which the cells were densely packed; except for occasional blood vessels, there was little space intervening between cells. In contrast, diabetic retinas without baicalein treatment demonstrated a loss of cells in the GCL, with some areas devoid of any cells.

To determine whether there is a significant difference in the number of cells in the GCL of diabetic versus control rats, the cells were counted in sections by a masked observer. The cells in the ganglion cell layer were counted in a standard length of retina (1.2 mm) centered on the optic nerve head using a graticule at 40× objective magnification. As shown in Figure 1D, control retinas typically had from 27 to 35 cells in cross-sections of this layer. By 24 weeks after onset of diabetes, there were significantly fewer (40%) cells in the GCL in diabetic retina compared with that in control retina (18.00 ± 2.92 vs. 30.00 ± 3.39). After treatment with baicalein, the number of cells in the GCL in diabetic retina was 48% more than that in untreated retinas (26.8 ± 1.93 vs. 18.00 ± 2.92), and there did not appear to be a significant difference when compared with age-matched control retinas (26.8 ± 1.93 vs. 30.00 ± 3.39).

**Prevention of Diabetes-Induced Vascular Changes by Baicalein**

Increases in retinal vascular permeability in animal models of diabetes have been reported by multiple investigators. In the present study, retinal vascular permeability was measured by extravasation of BSA-FITC. Diabetic rats without baicalein treatment (Fig. 2B) had significant lesions in the retinal capillary network as well as large vessels compared with that in age-matched controls (Fig. 2A) and baicalein-treated (Fig. 2C) rats. The diabetic retina demonstrated focal dilatation of retinal capillaries, large-vessel tortuosity or beading, and small-like bulges in the vessel wall. Obvious leakage of the fluorescein from capillaries and larger vessels was also noted in the flat-mounted retinas of diabetic rats without baicalein treatment.

**Reduction of Microglial Reactivity in the Retina of Diabetic Mouse by Baicalein**

Microglias were visualized in flatmounted retinas from control and diabetic rats with and without baicalein treatment. Two subpopulations of CD11b-labeled microglial cells were identified in age-matched control retina. One subpopulation had...
long, slender processes, localized at the perivascular region in the neural fiber layer and GCL (Fig. 3A). The other, also with long and slender processes, was mostly found in the retinal parenchyma, did not run along the retinal vessels, and was more localized in the inner plexiform layer (Fig. 3D).

In the diabetic retinas, the microglial cells in the inner retinal layer were characteristically ameboid, with few stout and dark-stained processes localized at the vascular walls (Fig. 3B). The microglial cells in the middle retinal layer were characterized with much shorter and wider processes (Fig. 3E). The number of microglial cells both in the inner and middle retinal layer was significantly higher than those in the control retinas (Fig. 3G). The morphologic and number changes show that microglial cells in the diabetic retinas were activated and proliferated. In diabetic retinas with baicalein treatment, the microglial cells in the inner retinal layer were ramified with dark-stained processes (Fig. 3C), and the cell number was significantly fewer than that in the untreated diabetic retinas, but it was still higher than that in the control retinas (Fig. 3G).

The microglial cells with baicalein treatment in the middle retinal layer showed more slender processes (Fig. 3F), and the cell number was significantly fewer than that in the untreated diabetic retinas, but there was no significant difference when compared with the control retinas (Fig. 3G). The morphologic and number changes show that baicalein treatment ameliorated high glucose-induced microglial activation and proliferation.

To determine whether activated microglial cells in the inner retinal layer were responsible for the cell loss in the GCL, we double-labeled TNF-α/CD11b on diabetic retinas. Our results demonstrated that TNF-α expression was most evident in the inner retinal layer (Fig. 3H). CD11b labeled microglial cells was most evident in the inner and middle retinal layers (Fig. 3I). Colocalization of TNF-α and CD11b showed that TNF-α was mainly expressed by microglial cells in the inner retinal layer; microglial cells in the middle retinal layer did not express TNF-α (Fig. 3J).
To further demonstrate the relationship between activated microglial cells and vascular abnormality, we double-labeled CD11b and anti-Factor VIII related Ag, which labeled vascular endothelial cells, on diabetic retinas. Our results demonstrated that CD11b labeled microglial cells were most evident in the inner and middle retinal layers (Fig. 3K). The anti–Factor VIII-related Ag expression was evident in the inner and middle retinal layers, which corresponded to the vascular distribution in the retina (Fig. 3L). Colocalization of CD11b and anti–Factor VIII-related Ag showed that some of the activated microglial cells surrounded the abnormal vascular in the inner and even in the middle retinal layer (Fig. 3M).

**Reduction of GFAP and VEGF Immunoreactivity in the Retina of Diabetic Mouse by Baicalein**

The expression of GFAP and VEGF was determined by quantitative real-time PCR, immunohistochemistry, and Western blot analysis. In the age-matched control retinas, the GFAP-positive staining was mainly located in the neural fiber layer, where the stellate astrocyte is located; the Müller cells were not labeled (Fig. 4A). By 24 weeks after onset of diabetes, a marked increase in the number of GFAP-positive processes of Müller cells was observed throughout the whole neural retinal layer (Fig. 4B). After treatment with baicalein, the GFAP-positive labeling was much weaker than that in untreated retinas (Fig. 4C). A representative gel image of GFAP protein expression is shown in Figure 4D. The GFAP mRNA expression was detected by real-time PCR analysis using forward primer (GFAP): TTGGAAGGATGGTTGT GGATTC. The results show that the age-matched control retina consistently expressed modest quantities of GFAP. By 24 weeks after onset of diabetes, the GFAP expression was significantly upregulated. After treatment with baicalein, the GFAP expression was significantly downregulated when compared with that in untreated retinas, and

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**FIGURE 3.** Microglial cells in the age-matched control (A, D) and diabetic retinas with (B, E) or without (C, F) baicalein treatment. In the age-matched control retina, the microglial cells in the inner retinal layer had long, thin processes, located at the peri-vascular region (A); in the middle retinal layer, most cells were vessel-independent (D). In the diabetic retinas (24 weeks after onset of diabetes), the microglial cells in the inner retinal layer were characteristically ameboid with few stout processes (B); the cells in the middle retinal layer were characterized with short wide processes (E). In diabetic retinas with baicalein treatment (treated by oral administration with 150 mg/kg/d beginning 5 days after STZ injection), the microglial morphology in the inner retinal layer (C) and middle retinal layer (F) has fine, extended processes which were marked different from that in the diabetic retinas. (G) The quantitative studies showed that the number of microglial cells both in the inner retinal layer and middle retinal layer were significantly increased in diabetic retinas, and baicalein treatment significantly reduced high glucose–induced microglial increment. (H–J) Double-immunofluorescent labeling of TNF-α and CD11b on diabetic retina. TNF-α is green and CD11b in red. Overlay of labeling for both TNF-α and CD11b in orange. (H) TNF-α expression was most evident in the inner retinal layer (arrows). (I) CD11b labeled microglial cells were demonstrated both in the inner retinal layer and middle retinal layer (arrows). (J) Co-localization of TNF-α and CD11b showed that TNF-α was mainly expressed by microglial cells in the inner retinal layer (arrows); microglial cells in the middle retinal layer did not express TNF-α. (K–M) Double immunofluorescent labeling of CD11b and Factor VIII related Ag on diabetic retina. CD11b in green and Factor VIII related Ag in red. (K) CD11b labeled microglial cells was most evident in the inner and middle retinal layer (arrows). (L) The Factor VIII related Ag expression was evident in the inner and middle retinal layer (arrows). (M) Co-localization of CD11b and Factor VIII related Ag showed that some of the activated microglial cells surrounded the abnormal vascular in the inner and even in the middle retinal layer (arrows).

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**To further demonstrate the relationship between activated microglial cells and vascular abnormality, we double-labeled CD11b and anti-Factor VIII related Ag, which labeled vascular endothelial cells, on diabetic retinas. Our results demonstrated that CD11b labeled microglial cells were most evident in the inner and middle retinal layers (Fig. 3K). The anti–Factor VIII-related Ag expression was evident in the inner and middle retinal layers, which corresponded to the vascular distribution in the retina (Fig. 3L). Colocalization of CD11b and anti–Factor VIII-related Ag showed that some of the activated microglial cells surrounded the abnormal vascular in the inner and even in the middle retinal layer (Fig. 3M).**

**Reduction of GFAP and VEGF Immunoreactivity in the Retina of Diabetic Mouse by Baicalein**

The expression of GFAP and VEGF was determined by quantitative real-time PCR, immunohistochemistry, and Western blot analysis. In the age-matched control retinas, the GFAP-positive staining was mainly located in the neural fiber layer, where the stellate astrocyte is located; the Müller cells were not labeled (Fig. 4A). By 24 weeks after onset of diabetes, a marked increase in the number of GFAP-positive processes of Müller cells was observed throughout the whole neural retinal layer (Fig. 4B). After treatment with baicalein, the GFAP-positive labeling was much weaker than that in untreated retinas (Fig. 4C). A representative gel image of GFAP protein expression is shown in Figure 4D. The GFAP mRNA expression was detected by real-time PCR analysis using forward primer (GFAP): CCGTTCTCTGGAAGACACTGAAC and reverse primer (GFAP): TTGGAAGGATGGTTGT GGATTC. The results show that the age-matched control retina consistently expressed modest quantities of GFAP. By 24 weeks after onset of diabetes, the GFAP expression was significantly upregulated. After treatment with baicalein, the GFAP expression was significantly downregulated when compared with that in untreated retinas, and
there did not appear to be a significant difference when compared with age-matched control retinas (Fig. 4E and F).

In the age-matched control retina, no clear VEGF-positive staining was observed (Fig. 5A). By 24 weeks after onset of diabetes, clear vertical thread-like positive-VEGF staining was observed (Fig. 5B). Colocalization study demonstrated that VEGF was expressed in Müller cells (data not shown). After treatment with baicalein, the VEGF-positive labeling was much weaker than that in untreated retinas (Fig. 5C). A representative gel image of VEGF protein expression is shown in Figure 5D. The VEGF mRNA expression was detected by real-time PCR analysis using forward primer (VEGF): CTGTACCTCCA CCATGCCAAGT and reverse primer (VEGF): CTGCGCTGGTATA GCCGTCCTCAT. The results show that the age-matched control retina expressed very low levels of VEGF protein. By 24 weeks after onset of diabetes, the gene expression of IL-18, TNF-α, and IL-1β was significantly upregulated. After treatment with baicalein, the gene expression of IL-18, TNF-α, and IL-1β was significantly downregulated when compared with those in untreated retinas, but there did not appear to be a significant difference when compared with age-matched control retinas (Fig. 6).

**DISCUSSION**

Our studies demonstrated that inflammatory process, characterized by microglial activation and Müller cell dysfunction, is implicated in STZ-induced DR. Anti-inflammatory drug baicalein treatment ameliorated inflammatory process, and therefore inhibited vascular abnormality and neuron loss in diabetic retinas.

Microglia belongs to the mononuclear phagocyte system, and forms the resident macrophages in the brain and retina. Resting microglial cells promote neuronal cell viability and maintain homeostasis in the normal CNS. However, reactive microglial cells were implicated in the progression of many retinal diseases, including retinal degeneration, glaucoma, and DR. Consistent with previous studies, our work demonstrates that microglial cells in the inner and middle retinal layers were markedly activated in diabetic retinas. Furthermore, colocalization studies demonstrate that some of the activated microglial cells surround the abnormal vascular in the inner and even in the middle retinal layer. Krady et al. have hypothesized that activated microglia stimulate a cycle of inflammation that recruits leukocytes, causing vascular break-

**Reduction of IL-18, TNF-α, and IL-1β Expression in the Retina of Diabetic Mouse by Baicalein**

Gene expression of IL-18, TNF-α, and IL-1β were examined by quantitative real-time PCR using the following primers: for IL-18, CAACCGCAGTAATACGGAGCAT (forward primer) and TCTGGGATTCGTTGGCTGTT (reverse primer); for TNF-α, GGTTGATCGGTCTCCAACA (forward primer) and TGGGCTACGGGCTTGACTCA (reverse primer); for IL-1β, CAAAATGCTCTGTTGCTGTTGCT (forward primer) and TGTACAAGGCTCATGGAGAATACCA (reverse primer); for βactin, AGGCCAACCGTGAAAGATG (forward primer) and ACCAGGAGCATACGGGACAA (reverse primer). Our results showed that age-matched control retinas expressed very low levels of IL-18, TNF-α, and IL-1β. By 24 weeks after onset of diabetes, the gene expression of IL-18, TNF-α, and IL-1β was significantly upregulated. After treatment with baicalein, the gene expression of IL-18, TNF-α, and IL-1β was significantly downregulated when compared with those in untreated retinas, but there did not appear to be a significant difference when compared with age-matched control retinas (Fig. 6).

**FIGURE 4.** Expression of glial fibrillary acidic protein (GFAP) in the age-matched control and diabetic retinas with or without baicalein treatment. (A) Retina from the age-matched control rat. (B) Retina from the diabetic rat (24 weeks after onset of diabetes) exhibited strong GFAP-positive staining of Müller cells. (C) With baicalein treatment (treated by oral administration with 150 mg/kg/d beginning 5 days after STZ injection) for 24 weeks, the GFAP-positive labeling was significantly decreased. Western blot analysis (D, E) and real-time PCR analysis (F) demonstrated that GFAP expression was significantly upregulated in the diabetic retinas, and baicalein treatment markedly reduced high glucose-induced GFAP expression.
Mueller cell dysfunction was involved in STZ-induced diabetic expression was demonstrated in Mueller cells, indicating that high levels of GFAP. In the present study, increased GFAP therapeutic interventions. Given that functions, the microglial cells represent targets for contributors to the enhanced death of neurons in these diseases. proinflammatory and/or cytotoxic factors might be major con-
death. The activated microglial cells and their secretions of leading to increased leukocyte infiltration and neuronal cell can propagate the inflammatory response within the retina, Mueller cells. When under stress, activated Mueller cells express normal mammalian retina, GFAP is marginally detectable in extrinsic proapoptotic signaling pathways. TNF-Our study confirmed that IL-18, TNF-α, and IL-1β are toxic factors secreted from activated microglial cells. Elevated levels of IL-18, TNF-α, and IL-1β measured in serum and vitreous are increased in subjects with DR compared with healthy control subjects. Our study confirmed that IL-18, TNF-α, and IL-1β are markedly upregulated in diabetic retinas, and TNF-α is mainly expressed by activated microglial cells in the inner retinal layer. Binding of IL-18 to its receptor complex can lead to activation of JNK and MAPK p38, and this can activate both intrinsic and extrinsic proapoptotic signaling pathways. TNF-α has been shown to induce the death of neurons via a direct apoptotic effect after receptor binding and by indirectly interfering with intracellular substances used by growth factors such as IGF-1 and the insulin receptor. The most likely sources of these cytokines are retinal microglial cells; however, other retinal cell types (e.g., pericytes) release proinflammatory cytokines in response to diabetic stress. On their release, these cytokines can propagate the inflammatory response within the retina, leading to increased leukocyte infiltration and neuronal cell death. The activated microglial cells and their secretions of proinflammatory and/or cytotoxic factors might be major contributors to the enhanced death of neurons in these diseases. Given that functions, the microglial cells represent targets for therapeutic interventions.

GFAP is an established indicator of retinal stress. In the normal mammalian retina, GFAP is marginally detectable in Mueller cells. When under stress, activated Mueller cells express high levels of GFAP. In the present study, increased GFAP expression was demonstrated in Mueller cells, indicating that Mueller cell dysfunction was involved in STZ-induced diabetic retinopathy, which is consistent with previous studies. Muller cell dysfunction leads to glutamate transport abnormality, which is toxic to neuronal cells. Neuronal dysfunction or cell loss in diabetic retinas might partly be due to Muller cell dysfunction.

Vision loss and blindness from diabetic retinopathy are usually the result of vascular leakage or ischemia. Vascular leakage involves hemorrhage or the formation of hard exudates. Ischemia from vascular damage and disruption in local perfusion result in angiogenesis and neovascularization. The new blood vessels formed are fragile and prone to hemorrhage, which can impair vision, ultimately causing blindness. VEGF is a major regulator of blood vessel formation and function. It controls several processes in endothelial cells, such as proliferation, survival, and migration. Retinal VEGF expression is correlated with diabetic blood-retinal barrier breakdown and ischemia related neovascularization in animals and humans. In the present study, VEGF expression in Muller cells was significantly upregulated in diabetic retina, indicating that VEGF overexpression plays a crucial role in retinal vascular abnormality in STZ-induced diabetes.

Baicalein has been shown to exert anti-inflammatory effects. In the present study, baicalein treatment ameliorated high glucose-induced microglial activation and their secretion of inflammatory and/or cytotoxic factor expression; baicalein treatment also inhibited high glucose-induced Muller cell dysfunction and VEGF overexpression. Limitations of the in vivo studies prevented the present study from providing a direct answer to the question of whether the inhibitory effect on activated microglial and Muller cells contributed to the rescue of retina, or the inhibitory effect on activated microglial and Muller cells were the result of the rescued retina. Nevertheless, several groups have confirmed baicalein’s microglial inhibitory
In vitro studies performed by Chen et al.\textsuperscript{12} and Li et al.\textsuperscript{41} demonstrated that baicalein treatment abolished the LPS-induced microglial morphologic activation and secretion of iNOS, TNF-\(\alpha\), and IL-1\(\beta\), and attenuated LPS-induced neuron loss in neuron-glia cultures. In vivo studies from Ignacio et al.\textsuperscript{13} demonstrated that baicalein treatment downregulated the microglial markers CD68, CD80, and CD86 expression, all of which were upregulated in untreated mutant animals. Furthermore, studies by Suk et al.\textsuperscript{42} suggest that baicalein could be a key template for the development of therapeutic agents to selectively modulate inflammatory responses and cellular apoptosis in central nervous system. Although we cannot exclude the possibility that baicalein may execute protective activity through other mechanisms, from the evidence we mentioned above and our own studies, we suggest that baicalein treatment attenuated the inflammatory process in diabetic retinas, and subsequently inhibited high glucose–induced vascular abnormality and neuronal cell loss in diabetic retinas.

Aside from its antimicroglial and anti-Müller effect, oral baicalein dramatically reduced sorbitol levels in RBC without affecting blood glucose levels. Its mechanism is presumably through inhibition of aldose reductase.\textsuperscript{43} Antioxidant effect of baicalein is also well documented. It has been reported to Scavenge reactive oxygen species, including superoxide, hydrogen peroxide, and hydroxyl radicals.\textsuperscript{44} Antioxidants and aldose reductase inhibitors have been found to have potent anti-inflammatory actions in diabetic retinopathy.\textsuperscript{45,46} From previous studies, baicalein or baicalin treatment has few side effects, which facilitates its application for human DR.

In conclusion, our study demonstrated that inflammatory process plays an important role in vascular abnormality and neuron cell loss in DR. Drugs designed to counter the inflammatory process should, therefore, have numerous benefits in disease such as DR.

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