Diabetes mellitus has been a global health problem, with Asia accounting for 60% of the world’s diabetic population, and it is a proinflammatory state characterized by elevated levels of C-reactive protein (CRP), inflammatory cytokines, chemokines, adhesion molecules, monocyte activity, and adipose tissue dysregulation. With increasing prevalence of diabetes in the community, diabetic retinopathy-related visual impairment has become a serious public health issue; however, the pathogenesis of diabetic retinopathy (DR) is not well understood. Longer duration of diabetes, poor metabolic control, hypertension, high concentrations of serum cholesterol, nephropathy, age, sex, smoking, and genetic disposition are risk factors for the development of DR, but no one has been able to fully explain the development of this diabetic complication; increasing evidence indicates that inflammation plays a pivotal role in the pathogenesis of DR.

Inflammation results in increases in nuclear factor-κB (NF-κB) activation, cytokines, chemokines, and adhesion molecules. Previous studies have demonstrated that elevated levels of monocyte chemotactic protein-1 (MCP-1) can be detected in vitreous fluid, tear fluid, and aqueous humor of patients with DR. It is generally acknowledged that microglia are resident immunocompetent and phagocytic cells in the central nervous system. Currently, it is known that microglial activation represents a major histopathologic change in DR. Activated microglia act not only as scavengers but also release immunomodulatory molecules that can directly or indirectly cause damage to neural cells. However, microglia collaborate with activities of neurons and vascular cells rather than function in isolation. We previously showed for the first time that advanced glycation end products and produces AGE. Previous studies have shown that AGE activates multiple signaling pathways, which induce oxidative stress and inflammation, cytokine release, and an increase in lipid metabolism, leading to a series of pathophysiologic changes. In recent years, human and animal studies have elucidated the fact that amadori-glycated albumin (AGA) is
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the prominent form of circulating glycated proteins. Furthermore, AGA, one of the major forms of AGE, is considered a key inducer of proinflammatory response. However, whether AGA can induce RGCs release of retinal neuronal MCP-1, contributing to the pathologic changes of DR has not been elucidated, to the best of our knowledge.

Baicalein is one of the effective ingredients extracted from dried roots of the family Lamiaceae plant *Scutellaria baicalensis* Georgi and has been reported to exhibit antioxidative, antiviral, and anti-inflammatory properties. In a previous study, baicalein treatment ameliorated inflammatory process and inhibited vascular abnormality and neuron loss in a rodent DR model; however, the mechanism is not yet clearly understood. MicroRNAs (miRNAs) are a class of small, noncoding RNAs that are capable of regulating the post-transcriptional expression of protein-encoding mRNAs. Mechanistically, miRNAs function by binding to the 3′ untranslated regions (3′-UTRs) of target mRNAs, causing translation to be blocked and/or mRNA degradation. An increasing body of evidence indicates that some miRNAs play a role in regulating insulin secretion, beta cell differentiation, glucose metabolism, and inflammatory pathways, therefore, playing key roles in the pathogenesis of diabetes and DR.

Therefore, the aim of the present study was to characterize the inflammatory effect of AGA in cultured rat RGCs; more importantly, we selected baicalein treatment to further explore the mechanism of the potential anti-inflammatory effects of baicalein via a microRNA-dependent mechanism.

**MATERIALS AND METHODS**

**AGA and Baicalein Preparation**

AGA was purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA), purified according to Ibrahim’s published protocol.

Baicalein was purchased from Sigma-Aldrich Corp. Baicalein is relatively insoluble in aqueous medium. The stock solution (50 mg/mL) was prepared in dimethyl sulfoxide (DMSO; Sigma-Aldrich Corp.) and stored at −20°C until needed. The final baicalein concentrations used for the different experiments were prepared by diluting the stock solution with Dulbecco’s modified Eagle’s medium (DMEM; Gibco-BRL, Grand Island, NY, USA). The final DMSO concentrations in the medium were less than 0.1% (in control and treated samples), which did not affect cell viability.

**Cell Culture**

Primary retinal neural cells were cultured from 3-day-old Sprague-Dawley rats. All experiments were conducted according to the statement from the Association for Research in Vision and Ophthalmology (ARVO) for the Use of Animals in Ophthalmic and Vision Research. The method of cell culture has been described in detail previously. Briefly, retinas were collected and digested with 0.125% trypsin for 20 minutes at 37°C. The trypsin was subsequently inactivated with DMEM/F-12 medium containing 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA). Subsequently, the tissue was passed through 200-µm filters. Then, the filtered cells were resuspended and seeded in 6-well or 24-well culture plates precoated with poly-lysine (20 ng/mL) and maintained in 0.5 mL/well. Neurobasal medium (Invitrogen) supplemented with 50 units/mL penicillin and 50 µg/mL streptomycin. Cells were kept in a humidified atmosphere of 5% CO₂ and 95% air. On the second day after seeding, cytokine β-arabinofuranoside (10 µM; Sigma-Aldrich Corp.) was added to the cultures to suppress the proliferation of glial cells. Culture medium was changed at 24 hours and twice weekly thereafter.

HEK293 cells (American Type Culture Collection, Rockville, MD, USA) were maintained in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin at 37°C with 5% CO₂. HEK293 cells were used only for the luciferase study to examine the regulation of miR-124 on MCP-1.

**MTT Cell Viability Assay**

The method of MTT cell viability assay has been described in detail previously. Briefly, seven-day-old primary cultured retina neurons (1 × 10⁶ cells/well) in 96-well plates (Corning, Inc., Corning, NY, USA) were used for the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test. Amadori-glycated albumin was added at different concentrations (0, 100, 250, 500, 750, 1000, 1250, or 1500 µg/mL), and cells were cultured for 24 hours. Next, 15 µL MTT solution was added to each well, and plates were incubated for 4 hours at 37°C. The reaction was terminated by adding an extraction solution (100 µL/well) that consisted of 20% (w/v) sodium dodecyl sulfate, N,N-dimethylformamide to lyse the cells and dissolve the crystals. Plates were incubated overnight at 37°C. The optical density was measured at 570 nm in a dual-beam microtiter plate reader (Molecular Devices, Sunnyvale, CA, USA) with 630 nm as the reference.

**RNA Interference**

The method of small interfering RNA (siRNA) preparation and transfection has been described in detail previously. Briefly, Dicer-specific siRNAs were purchased from Cell Signaling Technology (Beverly, MA, USA) and complexed with Lipofectamine 2000 (Invitrogen) in 6-well plates according to the manufacturer’s instructions. Two microtillers of Lipofectamine 2000 were diluted in 50 µL DMEM/F12 (Sigma-Aldrich Corp.) and combined with 0.01 to 0.20 µg siRNA after a 15-minute incubation at room temperature. Transfection was continued for 24 hours at room temperature. The knockdown of Dicer in RGCs was determined by Western blot analysis.

**Transfection**

The method for cell transfection has been previously described in detail. Briefly, miR-124 mimics, anti-miR-124 molecules, miR-124 mimics negative control, and anti-miR-124 negative control were obtained from GenePharma (Shanghai, China). Cells were transiently transfected with 100 nM miR-124 mimics or anti-miR-124 molecules or miR-124 mimics negative control or anti-miR-124 negative control using GenePORTER transfection reagent (GTS, Inc., San Diego, CA, USA) according to the manufacturer’s instructions. After 6 hours, the supernatant was removed, and fresh medium was added.

**Quantitative Reverse Transcription-PCR (qRT-PCR)**

The qRT-PCR method has been described in detail previously. The primers were as follows: miR-124 sense, 5′-GGAGGCATTTCTTCATTCCAGACC-3′; miR-124 antisense, 5′-GACCATGAGGGTTAGAGCCA-3′; MCP-1 sense, 5′-CCAGAAAACGCACACTCTC-3′; MCP-1 antisense, 5′-TTCCCTATTTGGGTCAGCAC-3′; HDAC1 sense, 5′-GGGACAGATGGGCGAGACT-3′; HDAC1 antisense, 5′-GCGGCACGACCTATGCTG-3′; HDAC2 sense, 5′-GGGACACAGACGAGAGATG-3′; HDAC2 antisense, 5′-CACATCGTCTACTGTTTCT-3′; HDAC3 sense, 5′-CACATCGTCTACTGTTTCT-3′; HDAC3 antisense, 5′-
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RESULTS

Baicalein Attenuated Expression of MCP-1 Induced by AGA in Cultured Rat RGCs

MTT cell viability assay provided dose–response results, which showed that 750 μg/mL AGA didn’t affect cell viability (Supplementary Fig. S1A). To investigate whether AGA induce the expression of MCP-1 in cultured rat RGCs, we analyzed the expression levels of MCP-1 protein by ELISA. Data showed that AGA significantly stimulated MCP-1 production in a dose-dependent manner, whereas incubation with nonglycated albumin had no effect (Fig. 1A). In addition, AGA can promote expression of the MCP-1 protein in a time-dependent manner and remain elevated over the 24-hour experimental period (Fig. 1B). To further analyze whether AGA-induced MCP-1 expression in retinal ganglion cells are influenced by baicalein, we pretreated cultured rat RGCs with different concentrations of baicalein for 1 hour before incubation with 750 μg/mL AGA for 24 hours, or 750 μg/mL AGA for 24 hours and we also added baicalein for the indicated times. As shown in Figures 1C, 1D, baicalein prevented the expression of MCP-1 induced by AGA in cultured rat RGCs in a dose-dependent manner and a time-dependent manner. Moreover, these findings prompted us to ask whether miRNAs, which could cause translation to be blocked and/or mRNA degradation by binding to the 3’ UTRs of target mRNAs, are involved in the baicalein anti-inflammatory action. Dicer, which is the class of ribonuclease III endonucleases, plays pivotal roles in miRNAs maturation. 24 To verify that the inhibited effect of baicalein on MCP-1 release was mediated by miRNAs, Dicer was knocked down, the inhibitory effects of baicalein on MCP-1 production were significantly attenuated (Fig. 1E). Dicer siRNA and control siRNA did not affect cell viability by MTT (Supplementary Fig. S1B).

Baicalein Induced miR-124 in Cultured Rat RGCs

A number of miRNAs have been found to regulate the expression of MCP-1. 25,26 To investigate the possible mechanism through which miRNAs are involved in the baicalein anti-inflammatory action, the expression of these miRNAs was examined by qRT-PCR. As shown in Figure 2A, expression of miR-124 was nearly 4-fold increased in the presence of baicalein, whereas the levels of other miRNAs were not affected by baicalein treatment. Moreover, to identify whether AGA affected the expression of miR-124 in cultured rat RGCs, we analyzed the expression levels of miR-124 by qRT-PCR. Data showed that AGA significantly downregulated miR-124 in a dose-dependent manner, whereas incubation with nonglycated albumin had no effect (Fig. 2B). In addition, AGA could inhibit the expression of miR-124 in a time-dependent manner (Fig. 2C). To further analyze whether AGA-downregulated miR-124 in retinal ganglion cells are influenced by baicalein, we pretreated cultured rat RGCs with different concentrations of baicalein for 1 hour before incubation with 750 μg/mL AGA for 24 hours, or 750 μg/mL AGA for 24 hours and we also added baicalein for the indicated times. As shown in Fig. 2D and Fig. 2E, baicalein restored the expression of miR-124 prevented by AGA in cultured rat RGCs in a dose-dependent and time-dependent manner.

MiR-124 Directly Controls MCP-1 Expression in Cultured Rat RGCs

MicroRNA-124 was downregulated in cultured rat RGCs treated with AGA, suggesting a potential role in the biological
properties of RGCs. MicroRNA-124 expression was significantly higher in cultured rat RGCs transfected with miR-124 mimics than in cells transfected with miR-124 control mimics, as shown by qRT-PCR (67-fold; \( P < 0.01 \)) (Fig. 3A). In addition, miR-124 expression was significantly decreased in cultured rat RGCs transfected with anti-miR-124 compared with the cells transfected with anti-miR-124 control, as shown by qRT-PCR (4.9-fold, \( P < 0.01 \)) (Fig. 3B). As shown in Figure 3C, the overexpression of miR-124 inhibited the expression of MCP-1 induced by AGA in cultured rat RGCs. In line with this finding, the down-regulated expression of miR-124 increased the expression of MCP-1 induced by AGA in cultured rat RGCs (Fig. 3D). We used miRanda to search for the 3'-UTR sequences of the mRNAs encoding MCP-1, and we found that MCP-1 mRNA contained a seed sequence for miR-124, which suggests that miR-124 binds directly to its 3'-UTR (Fig. 3E). To test this proposal, we performed a luciferase reporter assay to verify that miR-124 directly targets MCP-1 (Fig. 3E).
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Figure 2. MicroR-124 is induced by baicalein in cultured rat RGCs. (A) MicroR-124 was nearly 4-fold increased in cultured rat RGCs stimulated with 750 μg/mL AGA in the presence of 10 μg/mL baicalein for 24 hours, according to qRT-PCR assay. (B, C) Amadori-glycated albumin stimulation inhibited expression of miR-124 in cultured rat RGCs. Retinal ganglion cells were incubated with the indicated concentrations of AGA for 24 hours or with 750 μg/mL AGA for the indicated times. Amadori-glycated albumin treatment decreased expression of miR-124 in a dose- and time-dependent manner. (D, E) Retinal ganglion cells were preincubated with the indicated concentrations of baicalein for 1 hour before incubation with 750 μg/mL AGA for 24 hours or with 750 μg/mL AGA for 24 hours, and we also added 10 μg/mL baicalein for the indicated times. Baicalein increased the AGA-downregulated miR-124 in a dose- and time-dependent manner. Expression levels of miR-124 were determined by qRT-PCR assay. Results were statistically significant (*P < 0.01). Error bars denote SEM.

Baicalein Attenuated Expression of MCP-1 Induced by AGA in Cultured Rat RGCs by an MiR-124-Dependent Mechanism

To further assess whether baicalein attenuated the expression of MCP-1 induced by AGA in cultured rat RGCs via a miR-124-dependent mechanism, RGCs were exposed to AGA in the presence of anti-miR-124 and assessed for MCP-1 protein and mRNA. As shown in Figures 4A and 4B, the down-regulated expression of miR-124 in cultured rat RGCs transfected with anti-miR-124 attenuated the effect that baicalein inhibited the expression of MCP-1 induced by AGA.
miR-124 Was Induced by Baicalein by Controlling Histone Deacetylases in Cultured Rat RGCs

Because miRNA expression can be modulated by acetylation,27,28 to elucidate the stimulus responsible for decreased expression of miR-124 in cultured rat RGCs treated with AGA, we first tested whether small molecule histone deacetylase inhibitors (HDACi) can rescue miR-124 expression in cultured rat RGCs stimulated with AGA. As shown in Figures 5A and 5B, suberoylanilide hydroxamic acid (10 μmol/L), apicidin (3 μmol/L), or OSU42 (2.5 μmol/L) significantly increased the expression of miR-124 in cultured rat RGCs treated with AGA and concomitantly decreased MCP-1 expression. However, luciferase reporter activity was not inhibited by miR-124 mimics when the seeding sites were mutated (P > 0.05). Results were statistically significant (P < 0.01). Error bars denote SEM.

DISCUSSION

Our data clearly showed that AGA stimulation inhibited expression of miR-124 and increased expression of MCP-1 in cultured rat RGCs. In addition, miR-124 directly controlled HDACs in cultured rat RGCs treated with AGA. Quantitative RT-PCR analyses showed that the expression levels of HDAC4 and HDAC5 were significantly increased in cultured rat RGCs treated with AGA. The levels of HDAC1, -2, -3, -6, -7, -8, -9, -10, and -11 had no significant changes. However, baikalein attenuated the expression levels of HDAC4 and HDAC5 induced by AGA in cultured rat RGCs.
MCP-1 expression by binding directly to 3'-UTR of MCP-1. The application of baicalein in RGCs attenuated AGA-induced MCP-1 expression and upregulated expression of miR-124 by controlling HDAC4 and HDAC5.

Increasing evidence indicates that inflammation plays a pivotal role in the pathogenesis of DR, and microglial activation has been shown to be a major histopathologic change in DR. However, in recent years, accumulating in vivo and in vitro evidence has shown that microglial activity is prominent after neuronal damage, suggesting that neurons play an important role in activating microglia. Our previous study has showed that the pathologic increase in the expression of MCP-1 originates from diseased RGCs, which activate and recruit retinal microglia, so inhibiting the development of RGCs dysfunction and attenuating a pathologic increase in the expression of MCP-1 maybe play an important role in controlling the occurrence and development of DR AGA is one of the major forms of AGEs generated in the environment of hyperglycemia, and it is considered as a key inducer of proinflammatory response. The current study provides evidence that AGA significantly stimulated MCP-1 production in a dose- and time-dependent manner in cultured rat RGCs. The dried roots of S. baicalensis Georgi are known in traditional Chinese medicine as huang qin, and baicalein, a flavonoid originally isolated from huang qin, has shown a wide range of antioxidative, antiviral, and anti-inflammatory properties. Previous studies have demonstrated that baicalein plays a neuroprotective role by its antioxidative and anti-inflammatory properties.

MicroRNAs are capable of regulating the posttranscriptional expression of protein-encoding mRNAs by binding to the 3'-UTRs of target mRNAs, causing translation to be blocked and/or mRNA degradation. A number of miRNAs have been found to regulate the expression of MCP-1. For example, miR-126 was proposed to bind directly to the 3' UTRs of MCP-1 mRNA and miR-193b regulated MCP-1 production indirectly through a network of transcription factors. In addition, some studies have elucidated that miR-124 directly controls MCP-1 expression by binding to the 3' UTRs of MCP-1 mRNA. Therefore, to investigate the possible mechanism through which miRNAs are involved in the baicalein anti-inflammatory action, the expression of these miRNAs, which have been found to regulate the expression of MCP-1, was examined by qRT-PCR. In the present study, expression of miR-124 increased by nearly 4-fold in the presence of baicalein, while the levels of other miRNAs were not affected by baicalein treatment. In addition, consistent with the previous studies, miR-124 directly controls MCP-1 expression in cultured rat RGCs. Furthermore, we demonstrated baicalein attenuated the expression of MCP-1 induced by AGA in cultured rat RGCs via a miRNAs-dependent mechanism.

Histone deacetylases regulate transcription in an epigenetic manner by affecting chromatin structure and transcription factor activity. To date, 18 mammalian HDAC proteins have been identified, and they are divided into 4 classes based on their structure and function. Class I (HDACs 1, 2, 3, and 8), II (HDACs 4, 5, 6, 7, 9, and 10), and IV (HDAC 11) enzymes depend on zinc for catalytic activity and contain a highly conserved deacetylase domain, whereas the class III sirtuins (SIRT1–7) act through a distinct NAD+-dependent mechanism. Studies have shown that the expression levels of HDAC2, HDAC4, and HDAC5 were significantly increased in the kidneys of streptozotocin-induced diabetic rats and AGEs stimuli significantly increased HDAC4 expression in a concentration dependent manner in podocytes. Because miRNA expression can be modulated by acetylation, we first tested whether HDACs inhibitors (HDACi) could rescue miR-
The present study showed different HDACi significantly increased the expression of miR-124 in cultured rat RGCs treated with AGA and concomitantly decreased MCP-1 expression. All evidence indicates that HDACs are involved in miR-124 downregulation induced by AGA in cultured rat RGCs. More interestingly, numerous dietary agents consist of many bioactive ingredients which actively regulate various molecular targets involved in epigenetic changes. Finally, we present evidence that miR-124 is induced by baicalein via controlling HDAC4 and HDAC5 in cultured rat RGCs.

In summary, the current study provides new insights in understanding the pathogenesis of early features of DR, indicating that AGA stimulation increased the expression of MCP-1 in cultured rat RGCs via an miR-124-dependent mechanism. Furthermore, this study suggests that miR-124 is recovered by baicalein via controlling HDAC4 and HDAC5 in cultured rat RGCs. Thus, our data support the idea that baicalein inhibit AGA-induced MCP-1 expression in retinal ganglion cells via a microRNA-124-dependent mechanism.

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**Figure 5.** MicroR-124 is induced by baicalein by controlling histone deacetylases in cultured rat RGCs. (A) Suberoylanilide hydroxamic acid (10 μmol/L), apicidin (3 μmol/L), or OSU42 (2.5 μmol/L) significantly increased expression of miR-124 in cultured rat RGCs treated with AGA. (B) Suberoylanilide hydroxamic acid (10 μmol/L), apicidin (3 μmol/L), or OSU42 (2.5 μmol/L) significantly decreased expression of MCP-1 mRNA in cultured rat RGCs treated with AGA. (C) Relative mRNA levels of HDAC1–11 in cultured rat RGCs by qRT-PCR analyses. Results are statistically significant (P < 0.01). Error bars denote SEM.
References


