Baicalein Inhibits Amadori-Glycated Albumin-Induced MCP-1 Expression in Retinal Ganglion Cells via a MicroRNA-124–Dependent Mechanism

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目的。本研究的目的是为了表征阿莫拉-甘化白蛋白（AGA）对培养的视网膜节细胞（RGCs）的炎症效应，并进一步探讨白藜芦醇抑制AGA诱导的MCP-1表达的潜在机制。

方法。实验主要涉及视网膜节细胞的分离和培养。通过定量反转录PCR（qRT-PCR）和酶联免疫吸附试验（ELISA）分别检测视网膜节细胞中MCP-1的mRNA和蛋白表达。同时，通过实时荧光报告子检测miR-124的表达。

结果。AGA刺激视网膜节细胞显著增加了MCP-1的mRNA和蛋白表达。白藜芦醇处理后，MCP-1的表达显著降低，并且miR-124的表达也得到了显著的上调。

结论。白藜芦醇通过miR-124介导的机制抑制AGA诱导的MCP-1表达，这为糖尿病视网膜病变的治疗提供了新的靶点。

糖尿病视网膜病变是一种全球性健康问题，特别是在亚太地区，其患病率较高。糖尿病视网膜病变是由于葡萄糖氧化和糖化终产物（AGEs）的形成而引起的，AGEs的存在会激活视网膜神经元中的核因子κB（NF-κB）通路，促进炎症反应。研究中，我们通过定量PCR和Western blot技术检测了视网膜节细胞中MCP-1的表达水平，结果显示白藜芦醇能够显著抑制AGA诱导的MCP-1表达。

此外，我们还通过实时荧光报告子检测了miR-124的表达，发现白藜芦醇处理后miR-124的表达显著上调，这表明miR-124可能通过靶向MCP-1的3'UTR来抑制其表达。

综上所述，本研究首次揭示了白藜芦醇通过miR-124介导的机制抑制AGA诱导的MCP-1表达，为糖尿病视网膜病变的治疗提供了新的靶点。
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 baikalensis 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 (5’-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 used. 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-L-lysine (20 ng/mL) and maintained in 0.5 mL/well. Neurobasal medium (Invitrogen) supplemented with 10% FBS and 1% penicillin-streptomycin at 37°C with 5% CO2. Cells were kept in a humidified atmosphere of 5% CO2 and 95% air. On the second day after seeding, cytosine 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.

**MITT Cell Viability Assay**

The method of MITT cell viability assay has been described in detail previously. Briefly, seven-day-old primary cultured retina neurons (1 × 10^6 cells/well) in 96-well plates (Corning, Inc., Corning, NY, USA) were used for the 3-(4,5-dimethyl-2-y)-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 microtitters 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′-GGACTTTTCTTCACTTCCAGCG-3′; miR-124 antisense, 5′-GACCATGGGTTAGAGCGCA-3′; MCP-1 sense, 5′-GCAACGACACCTCTCTCT-3′; MCP-1 antisense, 5′-TTCCCTATGGGCTAGCAC-3′; HDAC1 sense, 5′-GGGAGGAGGAGGCCGACACT-3′; HDAC1 antisense, 5′-GTCAGGCTCTATTGGGTGG-3′; HDAC2 sense, 5′-TGCAGGAAACTCTCCTAAAGCA-3′; HDAC2 antisense, 5′-GGAGAGGACAGGAGGAGATG-3′; HDAC3 sense, 5′-CACATCTCATCTCGATTTCTC-3′; HDAC3 antisense, 5′-
GGCATGGCTCTCTGAAACCTTA-3′; HDAC4 sense, 5′-GAGTACTGCGGACGCTGCTGAAGT-3′; HDAC4 antisense, 5′-CAGCAGTGCTGCTGCTGGCGAGGAGGAG-3′; HDAC5 sense, 5′-TTCCTAATCCTGCTGCTGAAGT-3′; HDAC5 antisense, 5′-TCCATTGTGGCTGCTGCTGAAGT-3′; HDAC6 sense, 5′-TGGGGCTGCGGCTGCTGCTGAAGT-3′; HDAC6 antisense, 5′-GGGGCTGCGGCTGCTGCTGAAGT-3′; HDAC7 sense, 5′-ACCAACCTGCGGCTGCTGAAGT-3′; HDAC7 antisense, 5′-GATGCCAACGAGAAGGAG-3′; HDAC8 sense, 5′-CAGCAGGCCGAGAAGGAG-3′; HDAC8 antisense, 5′-CTCGAGTCCCCAGACACCCTGTTTTA-3′; HDAC9 sense, 5′-CAGCAGGCCGAGAAGGAG-3′; HDAC9 antisense, 5′-TGTAGACCATGTAGTTGAGGTCA-3′.

Enzyme-Linked Immunosorbent Assay

The method for enzyme-linked immunosorbent assay has been previously described in detail.7,9 The concentrations of MCP-1 were tested with ELISA kits (Pierce, Rockford, IL, USA). Briefly, samples were incubated in 96-well plates coated with MCP-1 antiserum for 2 hours. The samples were treated with enzyme reporter assay system (Promega, Madison, WI, USA) in accordance with the manufacturer’s protocols.

Western Blot Analysis

The method used for Western blot analysis has previously been described in detail. The primary antibodies were anti-Dicer, anti–MCP-1, and antiactin (Cell Signaling Technology).

Luciferase Assay

The method of luciferase assay has been described in detail previously.20,21 Briefly, the 3′-UTRs of MCP-1 containing the predicted miR-124 binding or mutant sites were amplified by PCR, using the following primers: MCP-1 sense 5′-CTCGAGTCCCCAGACACCCTGTTTTA-3′; MCP-1 antisense, 5′-CAGCAGGCCGAGAAGGAG-3′; mutant MCP-1 sense 5′-ACATATACGCTGTAATGTTAATTC-3′; mutant MCP-1 antisense, 5′-CTCGAGTCCCCAGACACCCTGTTTTA-3′. Fragments were subcloned into the NotI and Xbol sites in the 3′-UTR of Renilla luciferase of the psiCHECK-2 reporter vector. The psiCHECK-2/MCP-1 3′-UTR or psiCHECK-2/MCP-1 3′-UTR mutant reporter plasmid (200 ng) was cotransfected with the miR-124 mimics or miR-124 control mimics into HEK293 cells. After 24 hours, cells were lysed, and serum-free media was removed. Luciferase activity was measured using dual-luciferase reporter assay system (Promega, Madison, WI, USA) in accordance with the manufacturer’s protocols.

Results

Baicalein Inhibits AGA-Induced MCP-1 Expression in RGCs

MCP-1 sense 5′-GAGTACTGCGGACGCTGCTGAAGT-3′; HDAC4 sense, 5′-GAGTACTGCGGACGCTGCTGAAGT-3′; HDAC4 antisense, 5′-CAGCAGTGCTGCTGCTGGCGAGGAGGAG-3′; HDAC5 sense, 5′-TTCCTAATCCTGCTGCTGAAGT-3′; HDAC5 antisense, 5′-TCCATTGTGGCTGCTGCTGAAGT-3′; HDAC6 sense, 5′-TGGGGCTGCGGCTGCTGCTGAAGT-3′; HDAC6 antisense, 5′-GGGGCTGCGGCTGCTGCTGAAGT-3′; HDAC7 sense, 5′-ACCAACCTGCGGCTGCTGAAGT-3′; HDAC7 antisense, 5′-GATGCCAACGAGAAGGAG-3′; HDAC8 sense, 5′-CAGCAGGCCGAGAAGGAG-3′; HDAC8 antisense, 5′-CTCGAGTCCCCAGACACCCTGTTTTA-3′; HDAC9 sense, 5′-CAGCAGGCCGAGAAGGAG-3′; HDAC9 antisense, 5′-TGTAGACCATGTAGTTGAGGTCA-3′.

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Statistical Analysis

All experiments were performed at least three times. Quantitative data are presented as the mean ± SE and were analyzed by one-way analysis of variance (ANOVA) or Student’s t-test. A P value < 0.05 was considered statistically significant.
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).
Baicalin Attenuated Expression of MCP-1 Induced by AGA in Cultured Rat RGCs by an MiR-124–Dependent Mechanism

To further assess whether baicalin 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 baicalin 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. In addition, it is important to elucidate the functional role of individual 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. However, baicalein attenuated the expression levels of HDAC4 and HDAC5 induced by AGA in cultured rat RGCs.

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

Figure 3. MicroR-124 directly controls MCP-1 expression in cultured rat RGCs. (A) MicroR-124 expression was significantly increased in cultured rat RGCs transfected with miR-124 mimics compared with RGCs transfected with miR-124 mimic control (67-fold increase; *P < 0.01; by qRT-PCR). (B) MicroR-124 expression was significantly decreased in cultured rat RGCs transfected with anti-miR-124 compared with cells transfected with anti-miR-124 control (4.9-fold, *P < 0.01; by qRT-PCR). (C, D) Expressions of MCP-1 in cultured rat RGCs cotransfected with miR-124/miR-control or anti-miR-124/anti-miR-control in the presence of AGA were assayed by Western blotting. Overexpression of miR-124 attenuated the expression of MCP-1 induced by AGA in cultured rat RGCs. In agreement with this finding, the down-regulated expression of miR-124 increased expression of MCP-1 induced by AGA in cultured rat RGCs. (E) The region of the MCP-1 mRNA 3′-UTR was predicted to be the target of miR-124. Dual-luciferase report assays were performed using HEK293 cells. Luciferase activity of wild-type (WT) reporter transfected with miR-124 mimics was significantly decreased compared with that of miR-124 mimic controls (*P < 0.01). 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.
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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.5-9 and microglial activation has been shown to be a major histopathologic change in DR.29 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.30-31 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,7,9 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.32 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.10 Previous studies have demonstrated that baicalein plays a neuroprotective role by its antioxidative and anti-inflammatory properties.33,34 In the present study, baicalein treatment prevented expression of MCP-1 induced by AGA in cultured rat RGCs via a miR-124-dependent mechanism (Fig. 4).

An increasing body of evidence indicates that miRNAs play a role in the pathogenesis of diabetes and DR.22-25 Therefore, we studied the inhibitory effects of baicalein on MCP-1 production when Dicer was knocked down, to verify that the inhibited effect of baicalein on MCP-1 release was mediated by miRNAs. The present study showed the inhibitory effects of baicalein on MCP-1 production were significantly attenuated if Dicer was knocked down. All of the evidence indicates that baicalein attenuated the expression of MCP-1 induced by AGA in cultured rat RGCs via a miRNAs-dependent mechanism.

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.20,21 A number of miRNAs have been found to regulate the expression of MCP-1.25,26 For example, miR-126 was proposed to bound directly to the 3' UTRs of MCP-1 mRNA and miR-193b regulated MCP-1 production indirectly through a network of transcription factors.25 In addition, some studies have elucidated that miR-124 directly controls MCP-1 expression by binding to the 3' UTRs of MCP-1 mRNA.35,36 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-1,25,26 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,35,36 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 miR-124-dependent mechanism (Fig. 4).

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.37 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.37 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.38 Because miRNA expression can be modulated by acetylation,27,28 we first tested whether HDACs inhibitors (HDACi) could rescue miR-
expression of miR-124 in cultured rat RGCs treated with AGA. 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.39,40 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.

Acknowledgments

The authors thank Xiao Lin, Chair, Department of Ophthalmology, Beijing Shijitan Hospital, Capital Medical University, Beijing, People’s Republic of China.

Supported by National Natural Science Foundation of China Grant 81400405, Beijing Natural Science Foundation Grant 7154210, China Railway Corporation Research and Development of Science and Technology Project Grant J2014C011-J, and Project of Beijing Integrated Traditional and Western Medicine of Beijing Municipal Administration of Traditional Chinese Medicine. The authors alone are responsible for the content and writing of the paper.

Disclosure: N. Dong, None; B. Xu, None; H. Shi, None; X. Tang, None
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