miR-124 Regulates Amadori-Glycated Albumin-Induced Retinal Microglial Activation and Inflammation by Targeting Rac1

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Purpose. To characterize whether the activation of Rac1 is involved in the inflammatory effects produced by Amadori-glycated albumin (AGA) in retinal microglia and to further explore the pathologic pathways of AGA-induced retinal microglial activation and inflammation via a microRNA-dependent mechanism.

Methods. Primary rat retinal microglia were separated and cultured. The levels of TNF-α mRNA and soluble TNF-α produced by the retinal microglia in response to AGA were measured with quantitative RT-PCR (qRT-PCR) and ELISA. In addition, the GTPase activity of Rac1 was measured using a Rac activation assay kit. Luciferase reporter assays were used to validate the regulation of a putative target of microRNA-124 (miR-124).

Results. Amadori-glycated albumin significantly stimulated the expression of TNF-α mRNA and protein in cultured retinal microglial cells in a dose- and time-dependent manner. MicroRNA-124 expression was consistently suppressed by AGA, and the inhibitory effect was controlled by histone deacetylases (HDACs). Amadori-glycated albumin induced an increase in Rac1 activation in a dose- and time-dependent manner. Furthermore, our data indicated that Rac1 activation–mediated reactive oxygen species production stimulates p65 NF-κB phosphorylation and induces TNF-α release from retinal microglial cells. Finally, we demonstrated that miR-124 directly controls Rac1 expression.

Conclusions. The current study indicated that AGA-induced retinal microglial activation and inflammation occur via a miR-124-dependent mechanism.

Keywords: diabetic retinopathy, Amadori-glycated albumin, miR-124, microglia, Rac1

Diabetes mellitus (DM) is a global health problem that has dramatically increased in recent years, with no evidence of the trend abating. The prevalence of DM is expected to increase to 642 million individuals worldwide by 2040, with Asia accounting for 60% of the world's diabetic population.[1,2] With the increasing prevalence of DM, the number of diabetic-related complications will also increase. Diabetic retinopathy (DR) is the most common microvascular complication of DM. Diabetic retinopathy leads to retinal ischemia, retinal permeability, retinal neovascularization, and diabetic macular edema; however, the pathogenesis of DR is not well understood.[3] Longer duration of diabetes, poor metabolic control, hypertension, high blood cholesterol, nephropathy, age, sex, smoking, and genetic disposition are risk factors for the development of DR, but the development of this diabetic complication has not yet been fully explained.[4]

Increasing evidence has revealed new pathways, such as those associated with inflammation, that may be involved in the pathogenesis of DR.[5–10] Inflammation has been particularly associated with the early stages of DR, and it results in increased nuclear factor–κB (NF-κB) activation, as well as increased production of cytokines, chemokines, and adhesion molecules.[11] It is generally acknowledged that microglia serve as the resident immunocompetent and phagocytic cells in the central nervous system and potentially modulate inflammatory processes. Because microglia are the resident immune cells in the retina, it is likely that microglial activation plays a role in DR. Previous studies have demonstrated that microglial activation represents a major histopathological change in DR.[6] Currently, it is known that activated microglia not only act as scavengers but also release immunomodulatory molecules that can directly or indirectly cause damage to neural cells.[6] Various mechanisms, including hyperglycemia, ischemia, hypoxia, increased cellular oxidative stress, and the production of advanced glycation end products (AGEs), have been hypothesized to contribute to the inflammatory component of DR.[12,13] We previously showed that stimulation with AGEs significantly increased the expression of monocyte chemotactic protein-1 (MCP-1) in retinal neurons in vitro, which in turn increased microglial activation and TNF-α expression via the p38, ERK, and NF-κB pathways.[7,9] Then, activated microglia accelerate retinal ganglion cell (RGC) death by secreting cytotoxic substances, such as TNF-α, as well as effectively phagocytosing damaged cells and debris.[7,9]

It is well established that early glycation leads to the formation of Schiff's bases and Amadori products and further produces AGEs.[14] To date, most in vitro and in vivo studies have shown that AGEs activate multiple signaling pathways, which induce oxidative stress, inflammation, and cytokine release, leading to a series of pathophysiological changes.[15] However, only a small portion of Amadori products undergo complex rearrangements that result in the formation of AGEs, and most

glycated proteins in plasma exist as Amadori-glycated proteins rather than as AGEs. Although Amadori products are the major glycated modifications, thus far, only a few studies have focused on the role of Amadori-glycated proteins in DR. In recent years, human and animal studies have demonstrated that Amadori-glycated albumin (AGA) is the prominent form of circulating glycated protein. Furthermore, AGA is considered a key inducer of proinflammatory response.[16] A previous study demonstrated that AGA was a proinflammatory trigger in a rodent DR model after streptozotocin (STZ) injection, and stimulation with AGA significantly increased the expression of TNF-α in retinal microglia in vitro due to reactive oxygen species (ROS) formation and subsequent activation of mitogen-activated protein kinase (MAPK).[16] It is generally acknowledged that hyperglycemia results in increased cellular oxidative stress and that oxidative stress contributes to the pathogenesis of DR.[17,18] Ras-related C3 botulinum toxin substrate 1 (Rac1), which is a member of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase family, has been shown to control multiple cellular processes, including ROS generation.[19,20] However, to the best of our knowledge, whether AGA can induce retinal microglia to release TNF-α following the activation of Rac1, contributing to the pathologic changes of DR, has not yet been elucidated.

MicroRNAs (miRNAs) are a well-established class of small (22 nucleotides in length) endogenous noncoding RNAs that are capable of regulating the posttranscriptional expression of protein-coding mRNAs. Mechanistically, miRNAs function by binding to the 3′ untranslated regions (UTRs) of target mRNAs, causing translation to be blocked and/or mRNA degradation to proceed.[21–23] An increasing body of evidence indicates that some miRNAs play a role in the pathogenesis of diabetes and DR.[24,25] We previously showed that baicalein inhibits AGA-induced MCP-1 expression in RGCs via a microRNA-124–dependent mechanism[23]; however, whether AGA can induce activation of retinal microglia via a microRNA-dependent mechanism to contribute to the pathologic changes associated with DR has not been elucidated.

Therefore, the aim of the present study was to characterize whether the activation of Rac1 is involved in the inflammatory effect of AGA in retinal microglia. More importantly, we aimed to further explore the pathologic pathways associated with AGA-induced retinal microglial activation and inflammation via a microRNA-dependent mechanism.

Materials and Methods

Amadori-Glycated Albumin Preparation

The AGA was purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA), purified according to Ibrahim's published protocol.[16] The AGA was purified by affinity chromatography, concentrated, delipidated, and desalted into PBS. The AGA and nonglycated albumin (NGA) preparations were tested for fluorescent AGEs, determined by fluorescence assays (360–600 nm) on excitation at 370 nm or 350 nm. The fluorescent AGEs were not detectable. In addition, the major nonfluorescent AGE Nε(carboxymethyl)lysine (CML) concentrations were determined by using a CML-ELISA kit (Cusabio Biotech, Wuhan, Hubei, China). The AGA contained 130 ng CML/mg albumin protein, and the NGA contained 50 ng CML/mg albumin protein. So the CML was present in only minute amounts.

Cell Culture

Primary retinal microglia were cultured from 3-day-old Sprague-Dawley (SD) rats. All experiments were conducted according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The method of cell culture has been described in detail previously.[7,9] In brief, retinas were collected and digested with 0.125% trypsin for 20 minutes at 37°C. The trypsin was subsequently inactivated with Dulbecco's Modified Eagle's Medium (DMEM)/Ham's Nutrient Mixture F-12 (F-12; Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS; Invitrogen). Subsequently, the tissue was passed through 200-μm filters. Then, the filtered cells were resuspended in DMEM/F-12 culture medium containing 10% FBS, 100 U/mL penicillin, and 100 mg/mL streptomycin. Next, the cells were seeded into 75-cm^2 tissue culture flasks (Corning, Oneonta, NY, USA) at a density of 1×10^6 cells/cm^2. The cells were kept in a humidified atmosphere of 5% CO_2 and 95% air. The culture medium was changed at 24 hours and twice weekly thereafter. After 2 weeks, the microglia were harvested by shaking the flasks at 200 rpm for 1 hour, then the cells were in serum-free media containing 10 mM glucose for 24 hours, and then the cells were grown for 12 to 48 hours in the media containing physiological (5.5 mM) glucose concentrations for various experiments.

Immortalized cells (BV-2) were purchased from the Institute of Basic Medical Sciences of the China Science Academy (Beijing, China). The cells were maintained in DMEM (4.5 g/L glucose; 2% FBS; 50 mg/mL gentamycin) and incubated at 37°C with 5% CO_2 for 24 hours. Then, the cells were cultured for 24 hours in the media containing 1% FBS and physiological (5.5 mM) glucose concentrations for various experiments.

Primary microglial cells were used to examine the role of miR-124 in microglial activation in vitro study. Only for the luciferase study, BV-2 cells were used to examine the regulation of miR-124 on the Rac1.

MTT Cell Viability Assay

The method of MTT cell viability assay has been described in detail previously.[7,9,22,23] 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.[7,9,22,23] In brief, Dicer-specific siRNAs (Cell Signaling Technology, Beverly, MA, USA) or Rac1 siRNAs (Dharmacon, Lafayette, CO, USA) were complexed with Lipofectamine 2000 (Invitrogen) in six-well plates according to the manufacturer's instructions. Two microliters Lipofectamine 2000 was diluted in 50 μL DMEM/F-12 (Sigma-Aldrich Corp.) and combined with 0.01 to 0.20 μg siRNA after 15-minute incubation at room temperature. The transfection was continued for 24 hours at room temperature. The knockdown of Dicer and Rac1 in microglial cells was determined by Western blot analysis.

Transfection

The method for cell transfection has been previously described in detail.[21–23] Briefly, miR-124 mimics, anti–miR-124 molecules, miR-124 mimics negative control, and anti–miR-124 negative controls were obtained from GenePharma (Shanghai, China). The 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.

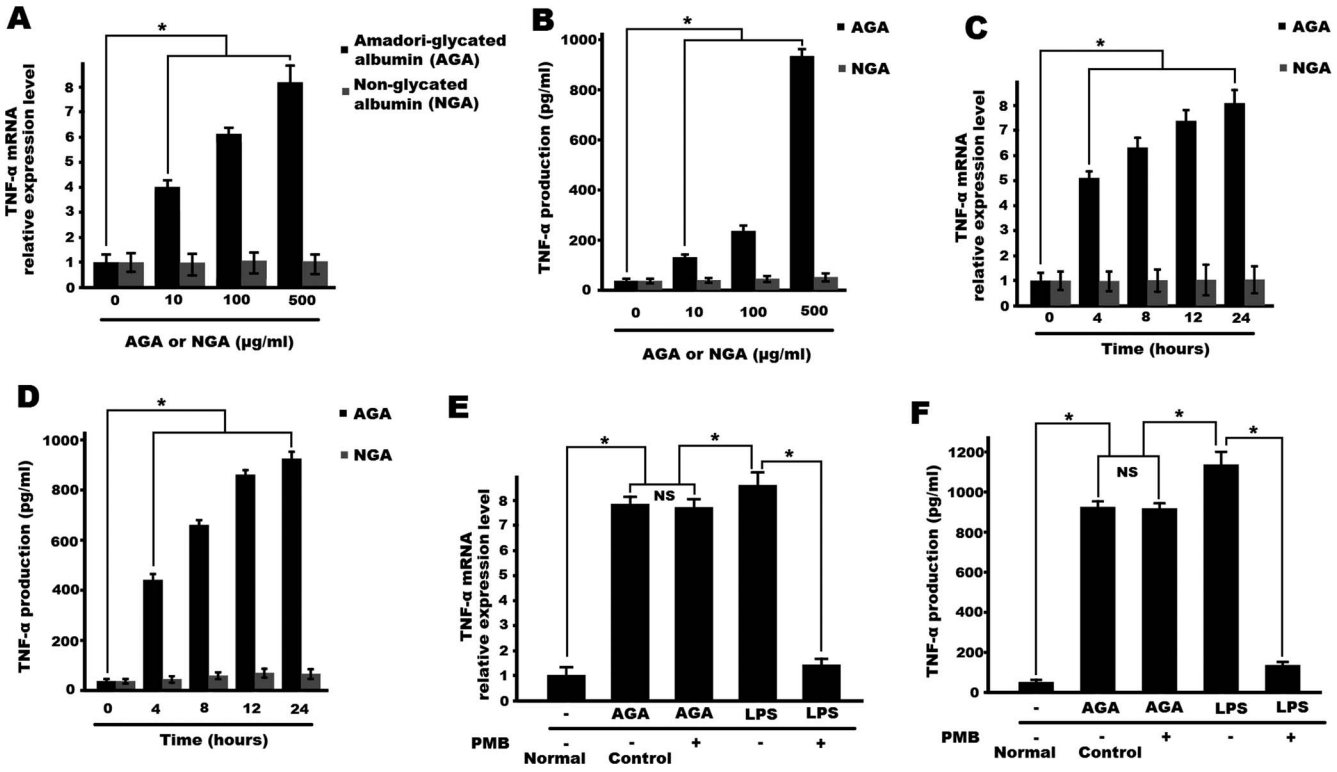

FIGURE 1. Amadori-glycated albumin induces TNF-α release by retinal microglial cells. (**A**, **B**) Amadori-glycated albumin stimulation increased the expression of TNF-α mRNA and protein in cultured retinal microglial cells. Microglial cells were incubated with the indicated concentration of AGA for 24 hours. Amadori-glycated albumin treatment increased the expression of TNF-α in a dose-dependent manner. Data shown are the mean ± SE of five experiments. (**C**, **D**) A total of 500 μg/mL AGA treatment increased the expression of TNF-α in a time-dependent manner. Data shown are the mean ± SE of five experiments. (**E**, **F**) Polymyxin B (PBM) (1 μg/mL) resulted in 90% inhibition of TNF-α mRNA and protein in microglia activated by purified LPS (1 μg/mL). However, pretreated with polymyxin B (1 μg/mL) did not reduce the TNF-α mRNA and protein induced by AGA. Data shown are the mean ± SE of five experiments. Results are statistically significant (*$P < 0.01$). *Error bars* denote SEM.

Quantitative RT-PCR

The quantitative RT-PCR (qRT-PCR) method has been described in detail previously.[9,21–23] Quantitative RT-PCR of miRNAs was performed using small nuclear RNA U6 as the normalization control. Quantitative RT-PCR of mRNAs of TNF-α was performed using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as the normalization control. The primers were as follows: miR-124 sense, 5′-GGACTTTCTTCATTCACACCG-3′; miR-124 antisense, 5′-GACCACTGAGGTTAGAGCCA-3′; TNF-α, sense 5′-CCCTCACACTCAGATCATCTTCT-3′; TNF-α, antisense 5′-GCTACGACGTGGGCTACAG-3′; GAPDH sense, 5′-AGGTCGGTGTGAACGGATTTG-3′; GAPDH antisense, 5′-TGTAGACCATGTAGTTGAGGTCA-3′.

Enzyme-Linked Immunosorbent Assay

The method for ELISA has been previously described in detail.[7,9,23] The concentrations of TNF-α were tested with ELISA kits (Pierce, Rockford, IL, USA). Briefly, samples were incubated in 96-well plates coated with TNF-α antiserum for 2 hours. The samples were treated with enzyme working reagent for 30 minutes and with TMB One-Step substrate reagent (DAKO, Carpinteria, CA, USA) for 30 minutes in the dark, and the reaction plates were read within 30 minutes in an ELISA plate reader (Molecular Devices, Eugene, OR, USA) at 450 nm, with 620 nm serving as the reference. The detection limit was 1 pg/mL.

Rac GTPase Activation Assay

The GTPase activity of Rac1 was measured using a Rac activation assay kit (Upstate, Lake Placid, NY, USA) according to the published protocol of Yi et al.[26] The microglial cells were rinsed with cold PBS and lysed in Mg2+ lysis buffer (MLB). After 10 minutes at 48°C under agitation, cells were scraped and lysates were cleared by centrifugation in a precooled rotor. Then, the pull-down proteins (500 μg per sample) were incubated with 10 μg PAK-1 PBD agarose and the reaction mixture was gently rocked at 4°C for 1 hour to bind Rac-GTP. The beads were washed three times with MLB, resuspended in 25 μL 2X Laemmli reducing sample buffer, and boiled for 5 minutes. Then, the bound proteins were loaded on SDS-PAGE gels. The bound active GTP-Rac was analyzed by Western blotting by using anti-Rac antibody.

Western Blot Analysis

The method used for Western blot analysis has previously been described in detail.[7,9,21–23] The primary antibodies were anti-Dicer, anti-p65 NF-κB, anti-phosphorylated (Thr536) p65 NF-κB, and anti-actin, which were from Cell Signaling Technology. Anti-Rac1 antibody was purchased from Abcam (Cambridge, MA, USA).

Dichlorofluorescein (DCF) Assay for ROS Formation

Dichlorofluorescein is the oxidation product of the reagent 2′,7′-dichlorofluorescin diacetate (H_2DCFDA; Molecular

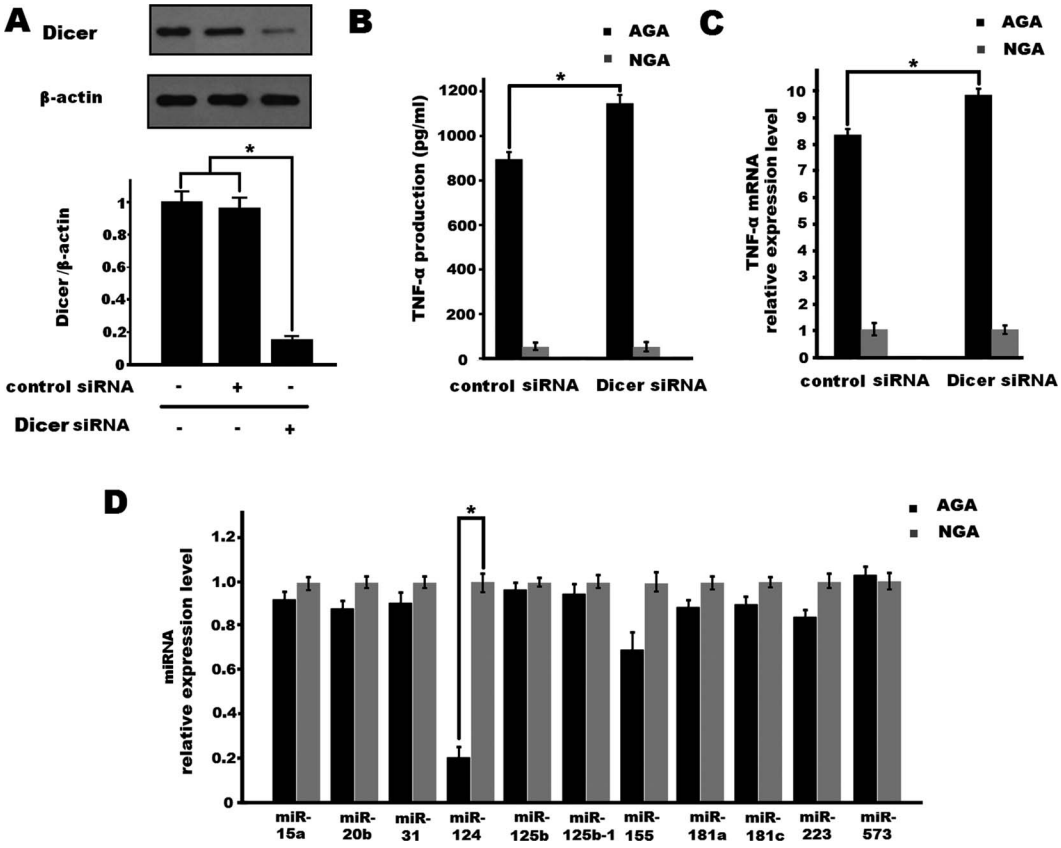

FIGURE 2. Amadori-glycated albumin induces TNF-α release by retinal microglial cells via a microRNA-dependent mechanism. (**A**) Direct knockdown of Dicer downregulated the expression of Dicer by Western blot analysis. Data shown are the mean ± SE of three experiments. (**B**, **C**) Retinal microglial cells were transfected with control siRNA or Dicer siRNA for 24 hours, and stimulated with 500 μg/mL AGA or 500 μg/mL NGA. Tumor necrosis factor-α production was measured by ELISA and qRT-PCR. The percentages were calculated using NGA alone as 100%. The activated ratio of AGA-induced TNF-α protein and mRNA was increased from 45.52-fold to 58.36-fold and from 8.38-fold to 9.81-fold by knockdown of Dicer, respectively. Data shown are the mean ± SE of five experiments. (**D**) MicroRNA-124 decreased by nearly 5-fold in cultured microglial cells stimulated with 500 μg/mL AGA for 24 hours by qRT-PCR. Data shown are the mean ± SE of three experiments. Results are statistically significant (*$P < 0.01$). *Error bars* denote SEM.

Probes, Eugene, OR, USA), is used as a marker of cellular oxidation according to Ibrahim's published protocol.[16]

Luciferase Assay

The method of luciferase assay has been described in detail previously.[21–23] In brief, the 3′-UTRs of Rac1 containing the predicted miR-124 binding or mutant sites were amplified by PCR by using the following primers: Rac1 sense 5′-AAACTC GAGATGTCTCAGCCCCTCGTTCT-3′; Rac1 antisense, 5′-AAAGCGGCCGCCTCCACAATTCTGCAACTGTCA-3′; mutant Rac1 sense 5′-AAAGAATTCATGCAGGCCATCAAGTGT-3′; mutant Rac1 antisense, 5′-AAAGGATCCTTACAACAGCAGG CATTTTC-3′. Fragments were subcloned into the Not I and Xho I sites in the 3′-UTR of Renilla luciferase of the psiCHECK-2 reporter vector. The psiCHECK-2/Rac1 3′-UTR or psiCHECK-2/Rac1 3′-UTR mutant reporter plasmids (200 ng) were cotransfected with the miR-124 mimics or miR-124 control mimics into BV-2 cells. After 24 hours, the cells were lysed, and reporter activity was assessed using the dual-luciferase reporter assay system (Promega, Madison, WI, USA) in accordance with the manufacturer's protocols.

Statistical Analysis

All experiments were performed at least three times. Quantitative data are presented as the mean ± SE and were analyzed by 1-way ANOVA or Student's *t*-test. A *P* value less than 0.05 was considered statistically significant.

RESULTS

Amadori-Glycated Albumin Induces TNF-α Release From Retinal Microglial Cells

An MTT cell viability assay was used to generate a dose-response curve, which showed that a 500-μg/mL concentration of AGA did not affect retinal microglial cell viability (Supplementary Fig. S1). To determine whether AGA could induce the expression of TNF-α in cultured retinal microglial cells, we analyzed TNF-α mRNA and protein expression levels by qRT-PCR and ELISA, respectively. As shown in Figures 1A and 1B, AGA significantly stimulated TNF-α production in a dose-dependent manner, whereas incubation with nonglycated albumin (NGA) had no effect. In addition, AGA promoted the expression of TNF-α mRNA and protein in a time-dependent manner, and these levels remained elevated over the 24-hour experimental period (Figs. 1C, 1D). To investigate whether the AGA-induced expression of TNF-α in cultured retinal microglial cells was mediated by AGA alone and not by an endotoxin or any other contaminant, we preincubated media with purified lipopolysaccharide (LPS; 1 μg/mL) or LPS-binding polymyxin B (1 μg/mL). Incubation with polymyxin B resulted in a 90% inhibition of TNF-α mRNA and protein production in

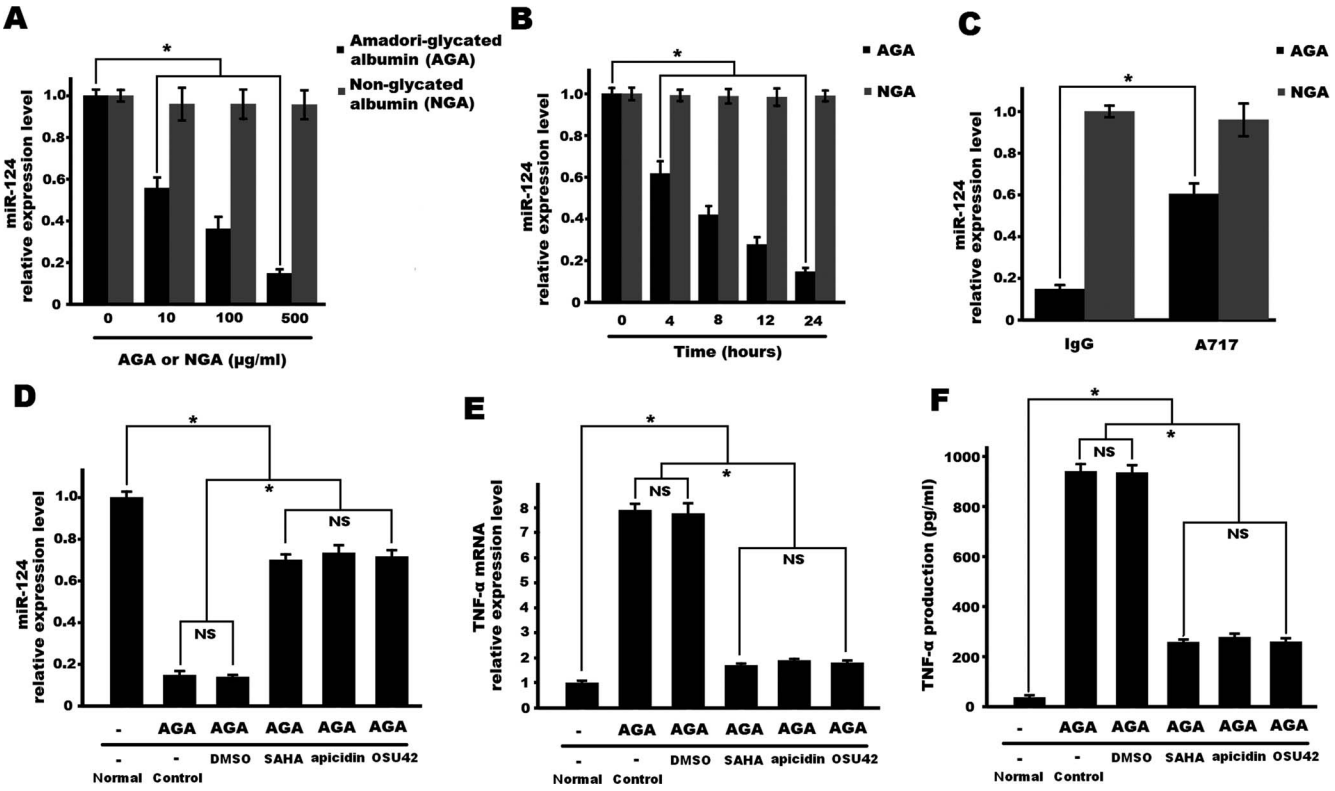

FIGURE 3. MicroRNA-124 expression is inhibited by AGA and the suppressed effect is controlled by histone deacetylases. (**A**, **B**) Amadori-glycated albumin stimulation inhibited the expression of miR-124 in retinal microglial cells. Retinal microglial cells were incubated with the indicated concentration of AGA for 24 hours or 500 μg/mL AGA for the indicated time. Amadori-glycated albumin treatment decreased the expression of miR-124 in a dose- and time-dependent manner. Data shown are the mean ± SE of five experiments. (**C**) A total of 500 μg/mL of NGA or AGA was neutralized by A717 or IgG. The inhibition of miR-124 expression in retinal microglial cells treated with AGA was attenuated by A717. Data shown are the mean ± SE of five experiments. (**D**) Suberoylanilide hydroxamic acid (10 μmol/L), apicidin (3 μmol/L), or OSU42 (2.5 μmol/L) significantly increased the expression of miR-124 in retinal microglial cells treated with AGA. Data shown are the mean ± SE of five experiments. In addition, suberoylanilide hydroxamic acid (10 μmol/L), apicidin (3 μmol/L), or OSU42 (2.5 μmol/L) significantly decreased the expression of TNF-α mRNA (**E**) and protein (**F**) in cultured retinal microglial cells treated with AGA. Data shown are the mean ± SE of five experiments. Results are statistically significant (*$P < 0.01$). *Error bars* denote SEM.

microglia that were activated with purified LPS (Figs. 1E, 1F). However, pretreatment with polymyxin B (1 μg/mL) did not reduce AGA-induced TNF-α mRNA and protein expression (Figs. 1E, 1F); therefore, AGA-induced TNF-α production in cultured retinal microglial cells occurs independently of endotoxin contamination.

Amadori-Glycated Albumin Induces TNF-α Release by Retinal Microglial Cells via a MicroRNA-Dependent Mechanism

To further analyze whether miRNAs, which cause translation inhibition and/or mRNA degradation by binding to the 3′-UTRs of target mRNAs, are involved in the inflammatory actions of AGA, Dicer was knocked down. It is well established that Dicer, a ribonuclease III endonuclease, plays pivotal roles in miRNA maturation.[27] We achieved the downregulation of Dicer protein by using Dicer-specific siRNAs as monitored by Western blot analysis, with a maximum knockdown of approximately 85% (Fig. 2A). When Dicer was knocked down, the AGA-induced expression of TNF-α protein and mRNA significantly increased (Figs. 2B, 2C). These findings prompted us to identify what miRNAs were involved in AGA-induced TNF-α production in cultured retinal microglial cells. A number of miRNAs have been found to regulate inflammation and immunity.[23,28–30] To identify the potential miRNAs involved in the inflammatory actions of AGA, the expression levels of these miRNAs were measured by qRT-PCR. As shown in Figure 2D, the expression of miR-124 decreased by nearly 5-fold in the presence of AGA, whereas the levels of the other assessed miRNAs were not affected by AGA treatment.

MicroRNA-124 Expression Is Consistently Suppressed by AGA and the Inhibitory Effect Is Controlled by Histone Deacetylases

To further investigate whether AGA affects the expression of miR-124 in retinal microglial cells, we analyzed miR-124 expression by qRT-PCR. As shown in Figure 3A, AGA significantly downregulated miR-124 in a dose-dependent manner, whereas incubation with nonglycated albumin had no effect. In addition, AGA inhibited the expression of miR-124 in a time-dependent manner (Fig. 3B). Furthermore, A717, an antibody against AGA, neutralized the biological activity of AGA.[16] The data showed that inhibition of miR-124 expression in retinal microglial cells following treatment with AGA was attenuated by treatment with A717 (Fig. 3C). Because miRNA expression can be modulated by acetylation,[23,31,32] to elucidate the stimulus responsible for the decreased expression of miR-124 in retinal microglial cells treated with AGA, we tested whether small molecule histone deacetylase inhibitors (HDACi) can rescue miR-124 expression in microglia stimulated with AGA. Suberoylanilide hydroxamic acid (10 μmol/L), apicidin (3 μmol/L), or OSU42 (2.5 μmol/L) significantly increased the expression of miR-124 in retinal microglial cells treated with AGA and concomitantly decreased the expression of TNF-α mRNA and protein in cultured retinal microglial cells (Figs. 3D–F).

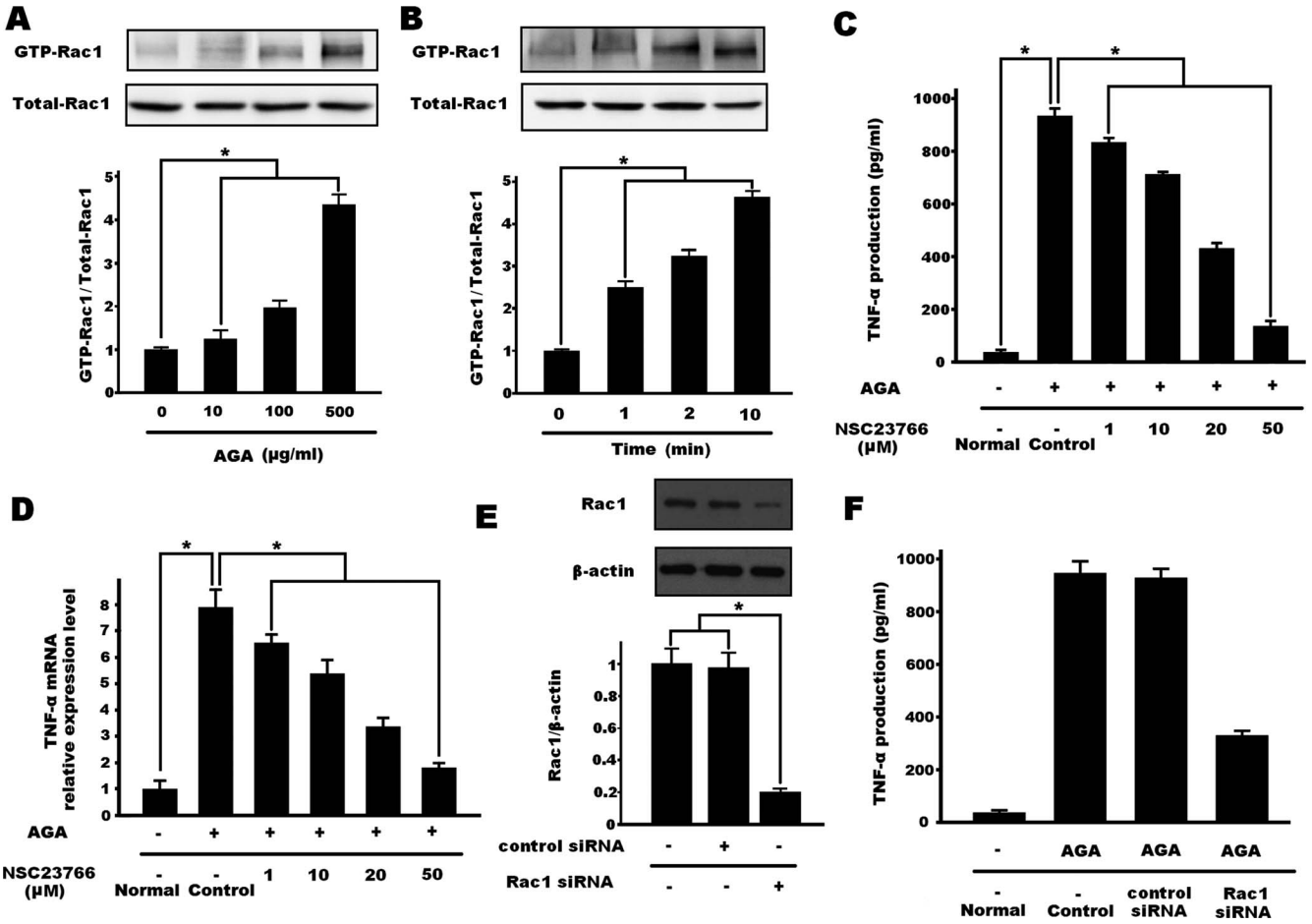

FIGURE 4. Amadori-glycated albumin enhances Rac1 activity in retinal microglial cells. (**A**, **B**) Amadori-glycated albumin treatment induced an increase in Rac1 activity in a dose- and time-dependent manner using the Rac1 activation assay. Retinal microglial cells were incubated with the indicated concentration of AGA for 10 minutes or 500 μg/mL AGA for the indicated time. Data shown are the mean ± SE of three experiments. (**C**, **D**) Retinal microglial cells were preincubated with the indicated concentrations of a Rac1-GTPase inhibitor, NSC23766 for 24 hours before incubation with 500 μg/mL AGA for 24 hours. Tumor necrosis factor-α production was measured by ELISA and qRT-PCR. The activated effects of AGA on TNF-α protein and mRNA were significantly attenuated by treatment with NSC23766. Data shown are the mean ± SE of five experiments. (**E**) Direct knockdown of Rac1 downregulated the expression of Rac1. Rac1 protein was measured by Western blot analysis. Data shown are the mean ± SE of three experiments. (**F**) Retinal microglial cells were transfected with control siRNA or Rac siRNA for 24 hours, and stimulated with 500 μg/mL AGA. Tumor necrosis factor-α protein was measured by ELISA. The expression of TNF-α protein induced by AGA was significantly decreased, when cultured retinal microglial cells were transfected with Rac1-specific siRNAs. Data shown are the mean ± SE of five experiments. Results are statistically significant (*$P < 0.01$). *Error bars* denote SEM.

Amadori-Glycated Albumin Induces TNF-α Release From Retinal Microglial Cells via Rac1 Activation

To test whether AGA affects the activation of Rac1 in retinal microglial cells, we analyzed Rac1 activity in retinal microglial cells treated with AGA. Rac1 activation assay results showed that AGA induced an increase in Rac1 activation in a dose- and time-dependent manner (Figs. 4A, 4B). To confirm that Rac1 is associated with the expression of TNF-α in cultured retinal microglial cells, the effects of AGA on TNF-α protein and mRNA expression were assessed in the presence of NSC23766, a Rac1 inhibitor. The effects of AGA were significantly attenuated by treatment with NSC23766, which led to a decrease in Rac1 activity (Figs. 4C, 4D). In addition, we achieved downregulation of Rac1 protein using Rac1-specific siRNAs as monitored via Western blot analysis, with a maximum knockdown of approximately 70% (Fig. 4E). To further confirm the relationship between Rac1 and the effects of AGA on TNF-α production, Rac1 was knocked down. The data showed that AGA-induced expression of TNF-α protein significantly decreased after cultured retinal microglial cells were transfected with Rac1-specific siRNAs (Fig. 4F).

Intracellular ROS Levels and AGA-Induced TNF-α Production Correlate With Rac1 Activity in Retinal Microglial Cells

A previous study demonstrated that ROS production is an early event that is induced in response to glycated protein stimulus,[16] and Rac1 can control ROS generation.[19,20] To identify whether ROS generation is causally related to Rac1 activation-mediated TNF-α release, we first explored the contribution of ROS formation to AGA-induced TNF-α release. Amadori-glycated albumin significantly stimulated ROS formation in a dose- and time-dependent manner in retinal microglial cells (Figs. 5A, 5B). Amadori-glycated albumin–mediated TNF-α release was prevented by pretreatment with cannabidiol (CBD) (2 μmol/L), which is an antioxidant (Fig. 5C). Furthermore, AGA-mediated ROS formation was inhibited by pretreatment with NSC23766 and Rac1-specific siRNAs

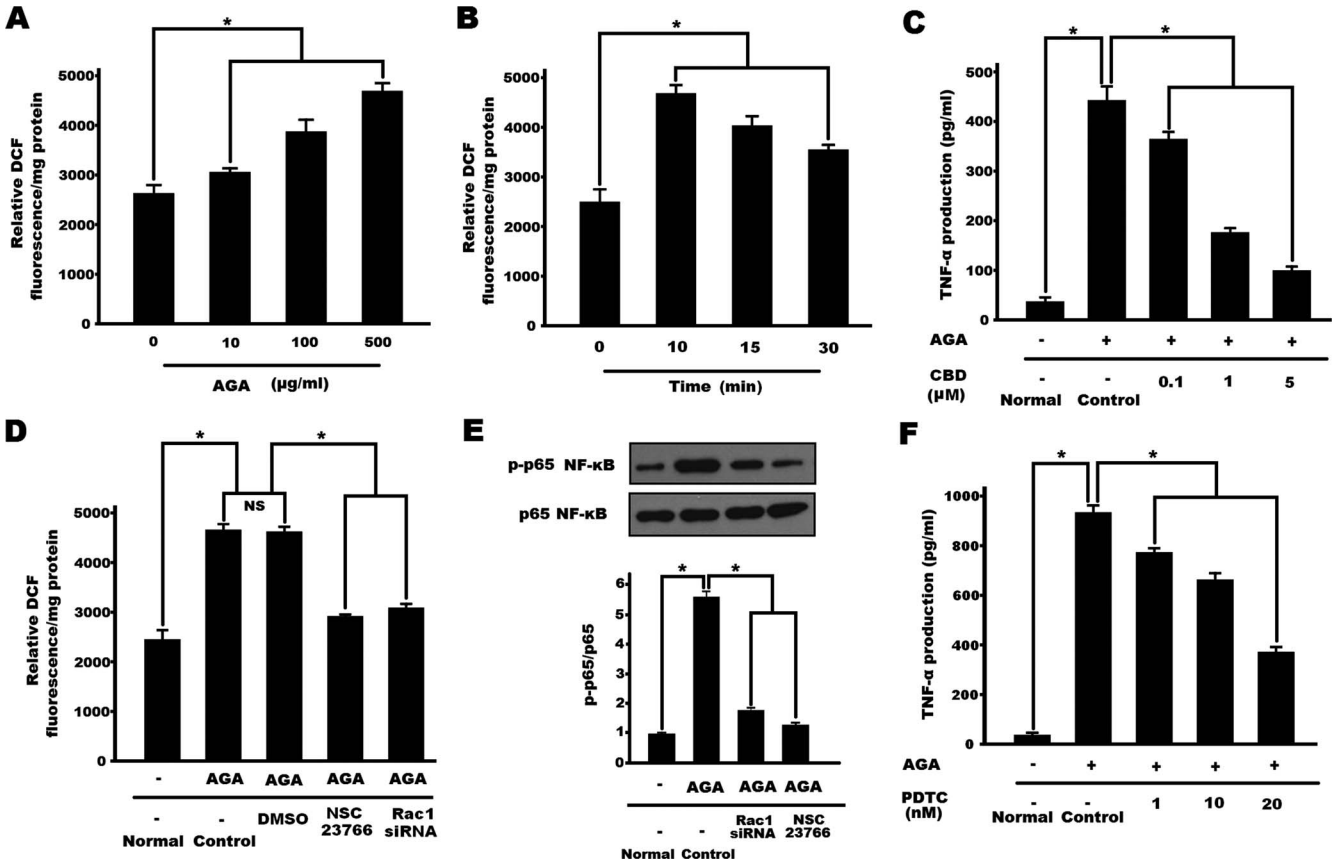

FIGURE 5. Rac1 activation is an early event involved in AGA-induced TNF-α release. (**A**, **B**) Amadori-glycated albumin significantly stimulated ROS formation in a dose- and time-dependent manner in retinal microglial cells. Retinal microglial cells loaded with H_2DCFDA were treated with AGA in the indicated concentration of AGA for 30 minutes or 500 μg/mL AGA for the indicated time, and the fluorescence of DCF was measured. Reactive oxygen species formation was expressed as changes in DCF fluorescence/mg protein. Data shown are the mean ± SE of five experiments. (**C**) Amadori-glycated albumin–mediated TNF-α release was prevented by pretreatment with CBD (2 μmol/L). Retinal microglial cells were preincubated with 2 μmol/L CBD for 1 hour before incubation with 500 μg/mL AGA for 4 hours. Data shown are the mean ± SE of five experiments. (**D**) Amadori-glycated albumin–mediated ROS formation was inhibited by pretreatment with NSC23766 and Rac1-specific siRNAs. Retinal microglial cells loaded with H_2DCFDA were pretreated with NSC23766 or Rac1-specific siRNAs for 24 hours and treated with 500 μg/mL AGA, and the fluorescence of DCF was measured at 30 minutes. Data shown are the mean ± SE of five experiments. (**E**) Amadori-glycated albumin stimulated p65 NF-κB phosphorylation, but this effect was prevented by pretreatment with NSC23766 and Rac1-specific siRNAs in retinal microglial cells, as shown by Western blot analysis. Retinal microglial cells were preincubated with NSC23766 or Rac1-specific siRNAs for 24 hours before incubation with 500 μg/mL AGA for 24 hours. Data shown are the mean ± SE of three experiments. (**F**) Treatment with PDTC significantly prevented soluble TNF-α release in a dose-dependent manner, as shown by ELISA. Retinal microglial cells were preincubated with the indicated concentration of PDTC for 1 hour before incubation with 500 μg/mL AGA for 24 hours. Data shown are the mean ± SE of five experiments. Results are statistically significant (*$P < 0.01$). *Error bars* denote SEM.

(Fig. 5D). We also analyzed levels of p65 NF-κB phosphorylation. As shown in Figure 5E, AGA stimulated p65 NF-κB phosphorylation in retinal microglial cells; however, this effect was prevented by pretreatment with NSC23766 and Rac1-specific siRNAs. Additionally, treatment with PDTC (an NF-κB inhibitor) significantly inhibited the release of soluble TNF-α (Fig. 5F) in a dose-dependent manner. These findings indicated that Rac1 activation–mediated oxidative stress is a common signaling event that occurs upstream of the NF-κB pathway, which mediates TNF-α release in AGA-treated microglia.

MicroRNA-124 Downregulates Rac1 Expression by Directly Targeting Its 3′-UTR in Retinal Microglial Cells

MicroRNA-124 was downregulated in retinal microglial cells following treatment with AGA, suggesting that it has a potential role in the biological properties of microglia. MicroRNA-124 expression was significantly increased in retinal microglial cells transfected with miR-124 mimics compared with cells transfected with miR-124 control mimics according to qRT-PCR (Fig. 6A). Furthermore, miR-124 expression was significantly decreased in retinal microglial cells transfected with anti–miR-124 compared with microglia transfected with anti–miR-124 control according to qRT-PCR (Fig. 6B). Overexpression of miR-124 inhibited the expression of Rac1 in cultured retinal microglial cells, as shown by Western blot analysis (Fig. 6C). In agreement with this finding, the downregulated expression of miR-124 increased the expression of Rac1 in cultured retinal microglial cells (Fig. 6D). We used miRanda (available in the public domain at www.microrna.org) to search for 3′-UTR sequences of mRNAs encoding Rac1, and we found that Rac1 mRNA contained a seed sequence for miR-124, which suggests that miR-124 binds directly to its 3′-UTR (Fig. 6E). To test this proposal, we performed a luciferase reporter assay to verify that miR-124 directly targets Rac1 in the microglia (Fig. 6E).

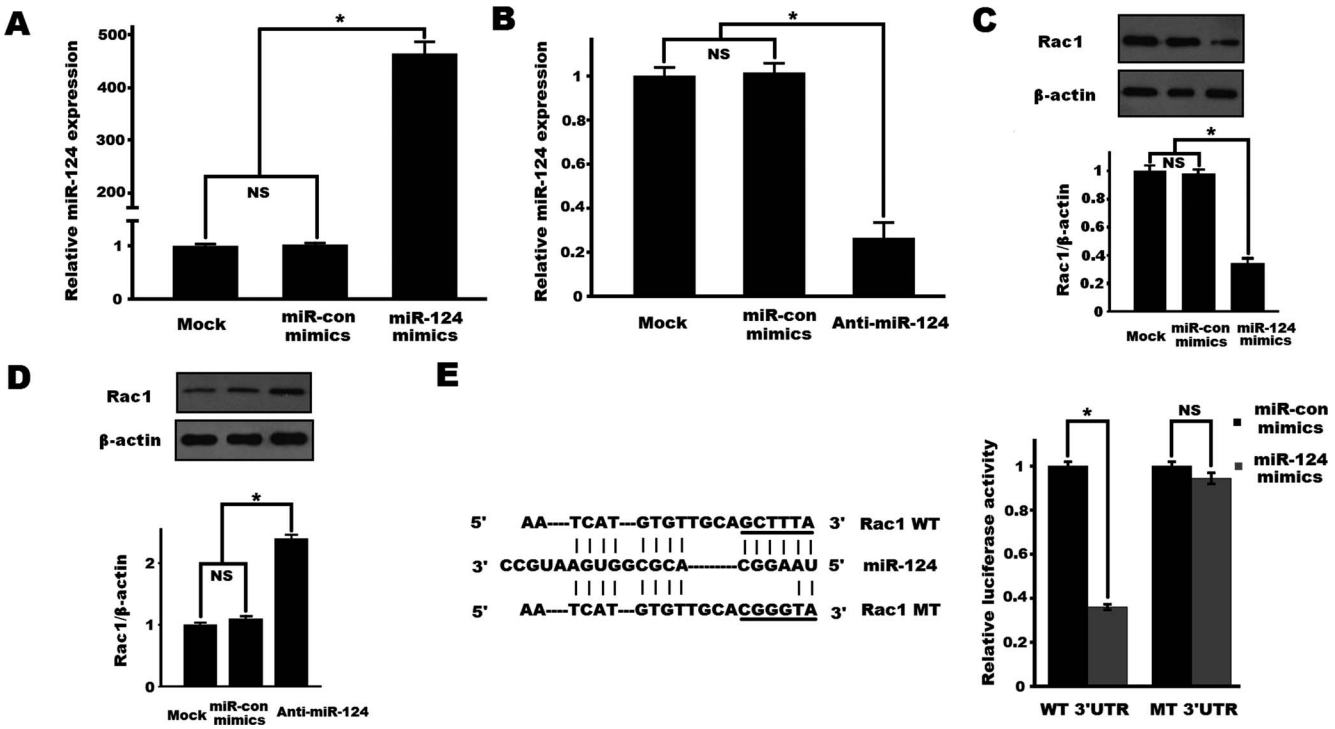

FIGURE 6. MicroRNA-124 directly controls Rac1 expression in retinal microglial cells. (**A**) MicroRNA-124 expression was significantly high in cultured retinal microglial cells transfected with miR-124 mimics compared with the microglia transfected with miR-124 mimics control (472-fold, $^*P < 0.01$) by qRT-PCR. Data shown are the mean ± SE of five experiments. (**B**) MiR-124 expression was significantly deceased in retinal microglial cells transfected with anti–miR-124 compared with the microglia transfected with anti–miR-124 control (4.2-fold, $^*P < 0.01$) by qRT-PCR. Data shown are the mean ± SE of five experiments. (**C**, **D**) The expressions of Rac1 in cultured retinal microglial cells transfected with miR-124/miR-control or anti–miR-124/anti–miR-control are measured by Western blot. The overexpression of miR-124 prevented the expression of Rac1. In line with this finding, the downregulated expression of miR-124 increased the expression of Rac1 in the microglia. Data shown are the mean ± SE of three experiments. (**E**) The region of the Rac1 mRNA 3′-UTR predicted to be targeted by miR-124. Dual-luciferase report assays were performed on BV-2 immortalized cells. The luciferase activity of wild-type (WT) reporter transfected with miR-124 mimics was significantly decreased compared with miR-124 mimics control ($^*P < 0.01$). However, the luciferase reporter activity was not inhibited by miR-124 mimics when the seeding sites were mutated ($P > 0.05$). Data shown are the mean ± SE of five experiments. Results are statistically significant ($^*P < 0.01$). *Error bars* denote SEM.

Amadori-Glycated Albumin Induces TNF-α Release From Retinal Microglial Cells via an miR-124–Dependent Mechanism

The findings that miR-124 downregulated Rac1 expression by directly targeting its 3′-UTR prompted us to explore whether miR-124 is involved in TNF-α production and release from cultured retinal microglial cells by directly controlling Rac1. As shown in Figure 7A, the overexpression of miR-124 inhibited AGA-induced release of soluble TNF-α in retinal microglial cells based on ELISA. In contrast, the downregulation of miR-124 expression increased the release of soluble TNF-α. To further confirm that Rac1 is a functional target of miR-124, we next examined Rac1 expression in retinal microglial cells transfected with a Rac1 expression vector containing the entire coding sequence of Rac1 but with a mutated 3′-UTR. As shown in Figure 7B, the ectopic expression of the Rac1 3′-UTR mutant significantly abrogated the effects associated with the downregulation of Rac1 expression induced by miR-124. Similar to these findings, the release of soluble TNF-α and the production of TNF-α mRNA were both inhibited by the overexpression of miR-124 (Figs. 7C, 7D). In contrast, the ectopic expression of the Rac1 3′-UTR mutant significantly abrogated the effects of the downregulation of TNF-α production induced by miR-124 (Figs. 7C, 7D). Finally, Figure 8 illustrates the pathologic pathways associated with AGA-induced retinal microglial activation and inflammation via an miR-124–dependent mechanism and the various drugs that can be used to block these processes.

DISCUSSION

Our data clearly showed that AGA stimulation in retinal microglial cells both inhibited the expression of miR-124 by controlling histone deacetylases and activated Rac1. Furthermore, Rac1 activation mediated ROS production, stimulated p65 NF-κB phosphorylation, and induced TNF-α release from retinal microglial cells. Finally, we demonstrated that miR-124 directly controlled Rac1 expression by binding directly to the 3′-UTR of Rac1. In addition, the transcriptional repression of miR-124 by histone deacetylases resulted in the dysregulation of Rac1, which in turn drove retinal microglial activation and inflammation. These findings clearly showed that AGA-induced retinal microglial activation and inflammation occur via an miR-124–dependent mechanism.

Amadori-glycated albumin is one of the major forms of Amadori-glycated proteins generated in the environment of hyperglycemia and is considered as a key inducer of proinflammatory response.[33,34] Diabetes mellitus results in persistently elevated levels of blood glucose, or hyperglycemia. Hyperglycemia, increased cellular oxidative stress, and AGA have been hypothesized to play important roles in the pathogenesis of DR. In relation to DR, elevated AGA has been detected in the retinal capillaries of diabetic patients.[35] In addition, it has been reported that AGA concentrations in the

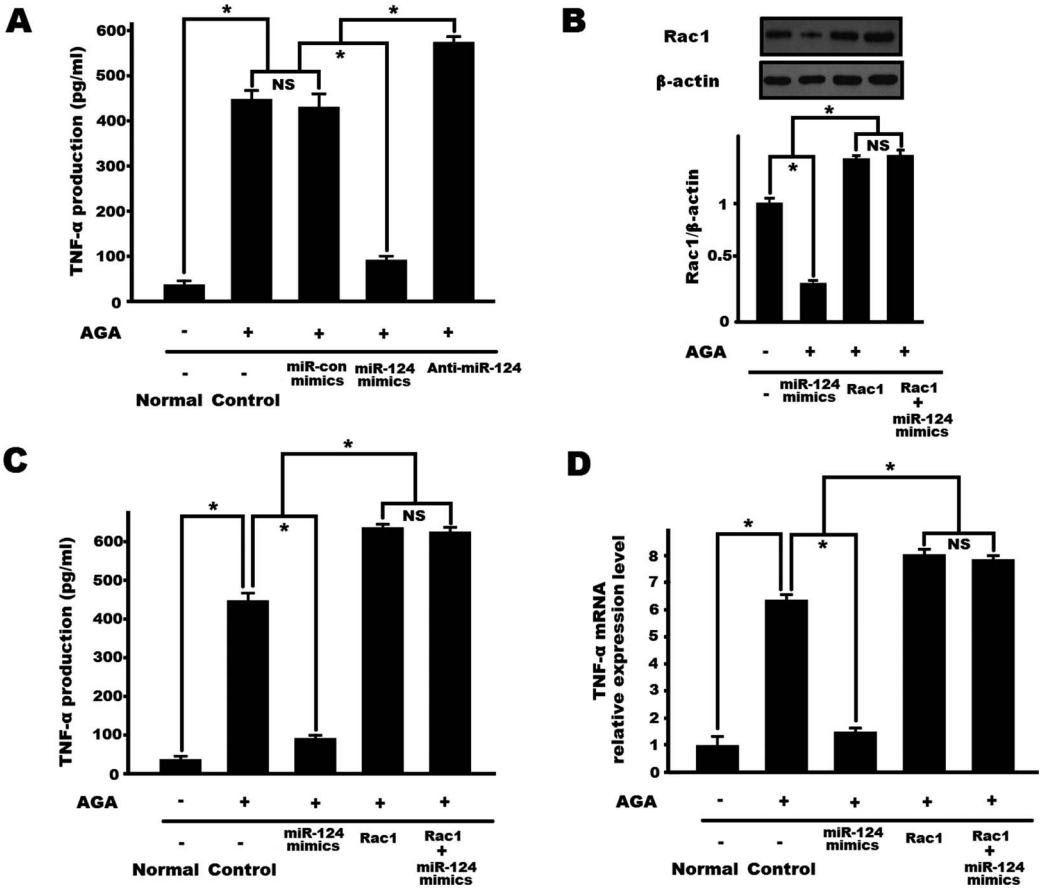

Figure 7. Amadori-glycated albumin induces TNF-α release from retinal microglial cells via a miR-124–dependent mechanism. (**A**) The soluble TNF-α release in cultured retinal microglial cells transfected with miR-124/miR-control or anti–miR-124/anti–miR-control are measured by ELISA. The overexpression of miR-124 inhibited soluble TNF-α release induced by AGA in retinal microglial cells; however, the downregulated expression of miR-124 increased soluble TNF-α release. Data shown are the mean ± SE of five experiments. (**B**) The overexpression of miR-124 prevented the expression of Rac1. In contrast, the ectopic expression of mutated 3′-UTR Rac1 significantly abrogated the effect of downregulated Rac1 expression induced by miR-124. Rac1 protein expression levels were confirmed by Western blotting. Data shown are the mean ± SE of three experiments. (**C**, **D**) The soluble TNF-α release and TNF-α mRNA were inhibited by overexpression of miR-124. In contrast, the ectopic expression of mutated 3′-UTR Rac1 significantly abrogated the effect of downregulated TNF-α production induced by miR-124. The soluble TNF-α release and TNF-α mRNA were measured by ELISA and qRT-PCR, respectively. Data shown are the mean ± SE of five experiments. Results are statistically significant (*$P < 0.01$). *Error bars* denote SEM.

retinas of diabetic animals are increased compared with nondiabetic animals.[36] Furthermore, retinal basement membrane thickening in diabetic db/db mice was ameliorated when db/db mice were treated with the A717 antibody, which specifically neutralizes AGA.[37] Moreover, inhibiting the formation of glycated albumin ameliorated vitreous changes in angiogenesis and metabolic factors associated with the development of DR.[38] Glycated albumin accumulates in the diabetic retina,[35,36] the treatment of diabetic mice with AGA antibodies ameliorates retinal retinopathy,[37,38] and AGA increases oxidative stress and activates NF-κB and ERK in macrophage RAW cells[33]; therefore, AGA rather than AGE may have important effects on the initiation and progression of DR. Consistent with previous studies,[16] the current study provides evidence that AGA significantly stimulates the expression of TNF-α mRNA and protein in a dose- and time-dependent manner in cultured retinal microglial cells.

The Rho GTPase family belongs to the Ras superfamily of low molecular weight (~21 kDa) guanine nucleotide-binding proteins.[19,20] Rac1 belongs to the Rho family of GTPases and is a NADPH oxidase, and it increases the production of ROS.[19,20] Previous studies have shown that the NF-κB pathway can be regulated by Rac1 and that this regulation is mediated by ROS.[39] Consistent with previous studies, the present work also demonstrated that AGA induces TNF-α release from retinal microglial cells via Rac1 activation, which mediates ROS production and stimulates p65 NF-κB phosphorylation.

Increasing evidence has indicated that miRNAs play important roles in the pathogenesis of diabetes and DR.[24,25] Therefore, we studied the effects of AGA on TNF-α release from retinal microglial cells after knocking down Dicer to verify that the inflammatory actions of AGA were mediated by miRNA. Under this condition, AGA-induced TNF-α protein and mRNA expression significantly increased. Collectively, the above evidence indicates that AGA induces TNF-α release from retinal microglial cells via a microRNA-dependent mechanism.

MicroRNAs are capable of regulating the posttranscriptional expression of protein-coding mRNAs by binding to the 3′-UTRs of target mRNAs, targeting them for degradation and/or inhibiting their translation and thereby downregulating the expression of the corresponding proteins.[21–23] Micro RNAs have been found to regulate many biological processes, including the inflammatory response.[23,28–30] Interestingly, some studies have shown that miR-124 directly controls Rac1 expression by binding to the 3′-UTR of Rac1 mRNA.[40,41] MicroRNA-124 acts as a tumor suppressor in MG-63 and U2OS

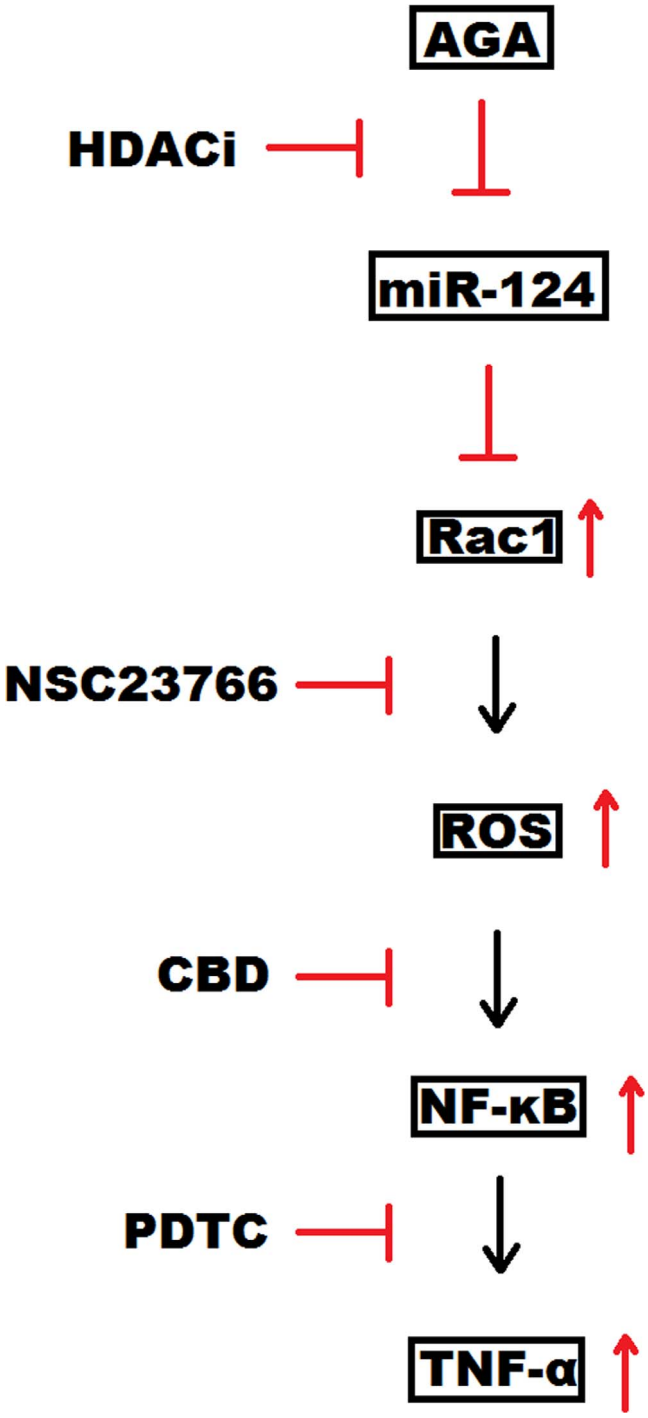

FIGURE 8. Amadori-glycated albumin induces retinal microglial activation and inflammation via a miR-124–dependent mechanism.

cells by suppressing Rac1 protein expression; attenuating cell proliferation, migration, and invasion; and inducing apoptosis.[40] Furthermore, miR-124 also inhibits cell proliferation, invasion, and metastasis by directly controlling Rac1 in pancreatic cancer.[41] Therefore, to investigate the possible mechanism through which miRNAs are involved in the inflammatory action of AGA, the expression of miRNAs that have been found to either regulate Rac1 expression or undergo inflammatory response was examined by qRT-PCR. We found that miR-124 expression decreased by nearly 5-fold in the presence of AGA, whereas the levels of other miRNAs were not affected. Moreover, in the current study, we identified Rac1 as a direct and functional target of miR-124 in microglial cells. In addition, we demonstrated that AGA induces retinal microglial activation and inflammation via a miR-124-dependent mechanism.

Histone deacetylases (HDACs) regulate transcription in an epigenetic manner by affecting chromatin structure and transcription factor activity. Previous studies have shown that HDACs 2, 4, and 5 are upregulated in the kidneys of STZ-induced diabetic rats, and AGEs stimuli significantly increased HDAC4 expression in a concentration-dependent manner in podocytes.[42] Furthermore, we have previously indicated that miR-124 is induced by baicalein-mediated HDAC4 and HDAC5 expression in cultured rat RGCs.[23] These findings prompted us to explore whether HDAC inhibitors (HDACi) can rescue miR-124 expression in retinal microglial cells stimulated with AGA. We found that different HDACi significantly increased the expression of miR-124 in retinal microglial cells treated with AGA and decreased the expression of TNF-α mRNA and protein in cultured retinal microglial cells. The evidence indirectly indicates that miR-124 is epigenetically silenced and HDACs are involved in AGA-induced miR-124 downregulation in retinal microglial cells.

However, research conducted by Wu et al.[43] showed increased levels of miR-124 in the retinas of a rodent DR model after STZ injection. These findings are inconsistent with our current results. This may be because they used whole retina tissues instead of cultured retinal microglial cells that were treated with AGA, which was our experimental design.

In summary, the current study provides new insights into the pathogenic processes associated with the early features of DR and indicates that AGA-induced retinal microglial activation and inflammation occur via an miR-124–dependent mechanism.

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