Sinomenine inhibits activation of rat retinal microglia induced by advanced glycation end products

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Abstract

Diabetic retinopathy involves an inflammatory response in the retina characterized by an increase in inflammatory cytokines and activation of microglia. The degree of microglia activation may influence the extent of retina injury following an inflammatory stimulus. Cytokines, released by activated microglia, regulate the influx of inflammatory cells to the damaged area. Thus, therapeutic strategy to reduce cytokine expression in microglia would be neuroprotective. Sinomenine, an alkaloid isolated from the stem and root of *Sinomenium acutum*, has long been recognized as an anti-inflammatory drug for rheumatoid arthritis and also inhibits macrophage activation. In this study, we activated retinal microglia in culture with advanced glycation end products (AGEs) treatment and attempted to determine whether sinomenine could reduce the production of cytokines from the activated microglia at both gene and protein levels. Changes in inflammatory cytokines, TNF alpha, IL-1 beta and IL-6, were measured by semi-quantitative RT-PCR and enzyme-linked immunosorbent assay (ELISA) both in the presence and absence of AGEs. The effect of sinomenine on levels of reactive oxygen species (ROS) and the nuclear translocation of NF-kB p65 were studied with a laser confocal scanning microscope. AGEs treatment induced a significant release of TNF alpha, IL-1beta, and IL-6 from retinal microglia. Sinomenine could inhibit release of these cytokines. Sinomenine attenuated ROS production in a dose-dependent fashion and reduced the nuclear translocation of NF-kB p65 in AGEs-activated retinal microglia in culture.
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1. Introduction

Microglia in the brain and retina are highly dynamic and are capable of assuming different morphologies and functions in response to the changes in their local cellular environment [1]. Microglia, which are normally quiescent
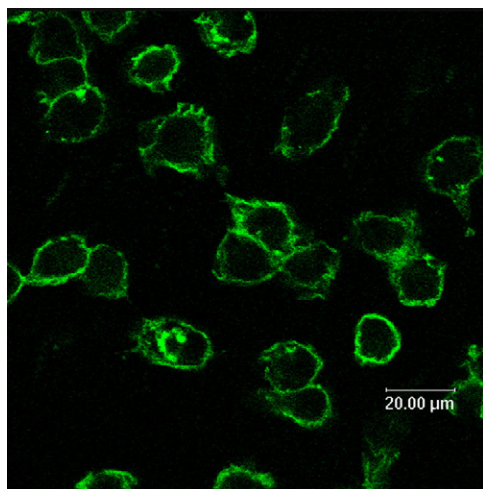

Fig. 1. Homogeneity of retinal microglia. Immunocytochemical labeling of the cells showed that majority were positive for CD11b and the labeling was primarily in the plasma membrane.

in retina, may be activated by diabetes [2,3]. Our unpublished data showed that AGEs, one of the factors that are thought to contribute to diabetic retinopathy (DR), induced retinal microglial activation in an in-vitro study. This suggested that AGEs might act directly on microglia to initiate and promote the advancement of DR. Activated microglia have been shown to release cytokines in in-vivo and in-vitro studies [4,5]. Up-regulation of cytokine expression by microglia and macrophages in the retina might cause an influx of inflammatory cells into retinal vasculature and tissues, inducing endothelial cells and neuronal impairment [6].

We hypothesize that hyperactivity of microglia in the retina may contribute to the inflammation and neuronal degeneration observed in DR. Initial development of diabetic neuropathy may be related to altered microglial behavior. Thus modification of microglial activation may also be an essential target for pharmacological studies aimed at amelioration of DR [7].

Sinomenine (7, 8-Didehydro-4-hydroxy-3, 7-dimethoxy-17-methylmorphinan-6-one) is an alkaloid isolated from the stem and root of *Sinomenium acutum* which chemical structure contains a hydrophenanthrene base [8]. *S. acutum* has long been used in traditional Chinese medicine to treat various rheumatoid arthritis [9,10]. Minocycline, which showed beneficial effects in therapy of rheumatoid arthritis, was recently demonstrated to be neuroprotective against light-induced loss of photoreceptor, possibly via inhibition of microglial activation [11]. We have also observed that minocycline inhibited LPS-induced microglial activation [12,13]. Given the effect of sinomenine on arthritis, we undertook the present

study to determine the efficacy of sinomenine inhibition on AGEs-induced retinal microglial activation.

2. Materials and methods

2.1. Microglia culture

Microglia were isolated from retinas of newborn Sprague Dawley (SD) rats by methods previously described [12–14]. Briefly, retinas were dissected from newborn (within 72 h) SD rat pups with retinal blood vessels carefully removed under a microscope. Tissues were collected, and digested with 0.125% trypsin for 20 min before mechanical dissociation. The trypsin was inactivated with DMEM/F12 (1:1) (Invitrogen, CA) plus 10% fetal bovine serum (FBS) (Hyclone, UT). The cells were collected by centrifugation, re-suspended in culture medium and plated onto T75 cell culture dishes at a density of 1×10^6 cells/mm^2. After 2 weeks, microglia were harvested by shaking the flasks at 100 rpm for 1 h. The cell suspension was centrifuged and the detached cells were re-plated in DMEM/F12 (1:1) plus 10% FBS at designated densities for various experiments described below. The morphology of microglia in culture was examined by phase contrast and fluorescent microscopy. The culture of retinal microglia appeared to be homogeneous under the phase contrast microscope. Immunocytochemical labeling with CD11b, a microglial marker, showed that the majority of cells in the cultures were positively labeled for CD11b (Fig. 1) and the labeling was localized in the cell membrane.

2.2. Measurement of TNF alpha, IL-1beta, IL-6 secretion

Microglia collected from culture flasks were seeded at a density of 3×10^5 cells/well onto 24-well tissue culture plates. One day after seeding, each culture well was fed with medium containing 0 (control) or 0.01, 0.1, 1 mM sinomenine (Xi'an High-tech Co, Xi'an, China), 1 h prior to the addition of 0 (control) or 500 µg/ml AGEs for 24 h. Fifty microliter aliquots of medium were taken directly from the well for the measurement of TNF alpha, IL-1 beta, and IL-6 by ELISA (R&D Systems, MN).

Table 1
Effects of different concentrations of sinomenine (SIN) on the nuclear translocation of NF-kB p65 in cultured microglia induced by AGEs

Treatment group	SIN concentration (mM)	NF-κB p65 (FN/FC)
Control	–	1.042 ± 0.34
AGEs (500 µg/ml)	–	2.024 ± 0.36 [a]
AGEs (500 µg/ml)	0.1	0.909 ± 0.26 [b]
AGEs (500 µg/ml)	1	1.103 ± 0.428 [b]

Retina microglia were pre-treated with different concentrations of sinomenine and then treated with 500 µg/ml AGEs for 24 h. NF-kB translocation from the cytosol to the nucleus was used as an index of activation. FN-green fluorescence in the nucleus, FC-green fluorescence in the cytosol. Results were means ± S.E.M. of 3 individual experiments.

[a] Compared with the control.

[b] Compared with AGEs-treated cultures.

2.3. Semi-Quantitative reverse transcription-polymerase chain reaction (RT-PCR)

RT-PCR was performed as previously described [15,16]. Briefly, RT was performed using ThermoScript™ RT reagents (Invitrogen, CA). The sequence of primers for rat IL-1 beta, TNF alpha, IL-6 and beta-actin, used in this study, are listed in Table 1. PCR amplification was performed using 28 cycles (TNF alpha and IL-6) or 25 cycles (IL-1 beta) of 94 °C for 30 s, 60 °C for 45 s, and 72 °C for 60 s. For beta-actin, amplification was performed using 25 cycles at 94 °C for 30 s, 55 °C for 45 s, and 72 °C for 60 s. PCR was stopped by a final extension for 10 min at 72 °C. Equal volumes of reaction mixture from each sample were loaded onto 2% agarose gels. The fluorescent images were digitally captured for analysis of intensity with Quantity One 1-D Analysis software (Bio-Rad, CA). Levels of TNF alpha, IL-1 beta, IL-6 mRNA were normalized relative to beta-actin in the same sample.

2.4. Semi-quantitative measurement of intracellular ROS

Microglia collected from culture flasks were seeded at a density of 1×10^6 cells/ml onto glass bottom culture dishes. One day after seeding, each culture well was fed with medium containing 0 (control) or 0.1, 1 mM sinomenine, 1 h prior to the addition of 0 (control) or 500 µg/ml AGEs for 24 h. This was followed by addition of a ROS probe 2-, 7-dichlorodihydro-fluorescein diacetate (H_2DCF-DA, 5 µM/l) to the cells. The oxidation of the nonfluorescent H_2DCF-DA by intracellular ROS results in the formation of the fluorescent compound 2-, 7-dichlorofluorescein (DCF) [17,18]. DCF mean fluorescence intensity (MFI) was monitored with a laser confocal scanning microscope (Model, TCS NT, Leica, Germany). The obtained data were analyzed using Zeiss LSM software and numerical presentation was conducted with Excel.

2.5. Localization of NF-kB

Microglia collected from culture flasks were seeded at a density of 1×10^6 cells/ml onto glass bottom culture dishes. One day after seeding, each culture well was fed with medium containing 0 (control) or 0.1, 1 mM sinomenine, 1 h prior to the addition of 0 (control) or 500 µg/ml AGEs for 24 h. The cells were fixed with freshly prepared 4% paraformaldehyde for 15 min and permeabilized with 0.5% Triton X-100 for 15 min. After 1 h incubation with 10% normal goat serum/PBS, cells were incubated with rabbit anti-NF-kB p65 antibody (Santa Cruz, CA) diluted at 1:50 in PBS for 2 h, washed and then incubated with FITC-conjugated IgG, diluted at 1:100 in PBS, for 1 h. To identify the nuclei, the FITC-labeled samples were counterstained with 5 µg/ml propidium iodide (PI) for 15 min.
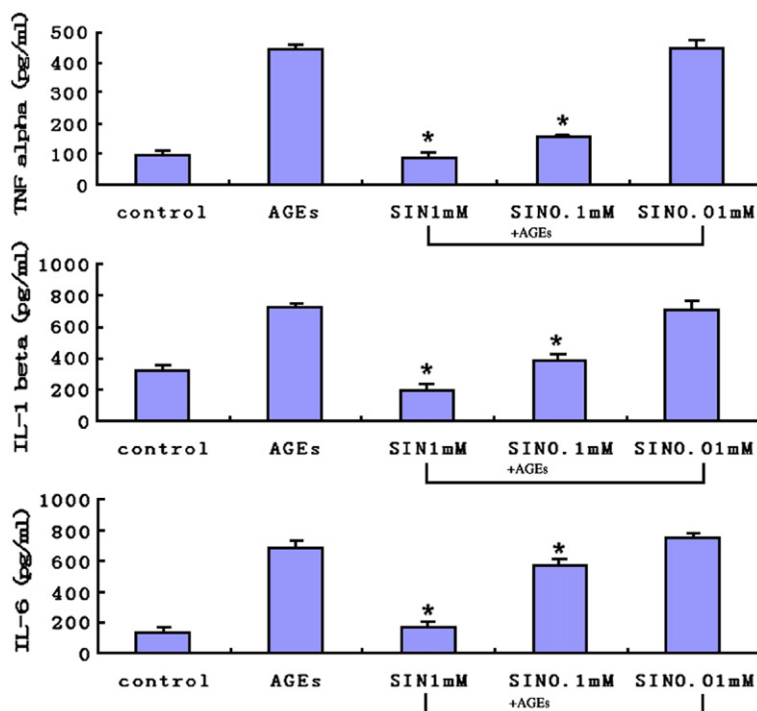

Fig. 2. Effects of AGEs and SIN on TNF alpha, IL-1 beta, and IL-6 levels in retinal microglial culture. Retinal microglia were pre-treated with 0.01, 0.1, 1 mM sinomenine for 1 h followed by co-treatment with 500 µg/ml AGEs for 24 h. Culture supernatants were collected and assayed for TNF alpha, IL-1 beta and IL-6. Results were means ± S.E.M. of 3 individual experiments. *$p < 0.05$ compared with the Control, **$p < 0.05$ compared with AGEs-treated cultures.
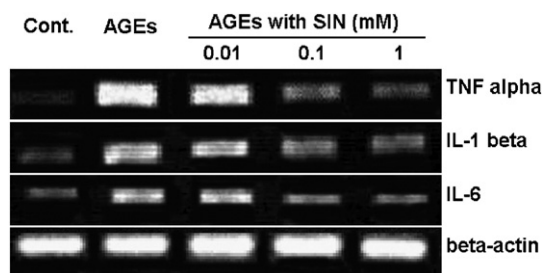
Fig. 3. Effects of AGEs and SIN on the mRNA of TNF alpha, IL-1 beta, and IL-6 in retinal microglial culture. Comparison of the mRNA levels of TNF alpha, IL-1 beta, and IL-6 in microglia cultures with or without 500 µg/ml AGEs-treatment in the presence or absence of 0.01, 0.1, 1 mM sinomenine pre-treatment than co-treatment. Beta-actin was used as an internal reference.

To acquire dual-color images, the double-stained cells were observed with laser confocal scanning microscopy (Leica). The samples labeled with both FITC and PI were excited at 488 nm, and the fluorescent emissions captured through 510 to 550 nm (530 nm in center) and 590 to 620 nm (605 nm in center) band pass with spectral grating, respectively. A change in color of the nucleus from red to yellow (due to co-localization of green FITC fluorescence and red PI fluorescence) was indicative of NF-kB translocation in the cells. NF-kB translocation from the cytosol (FC) to the nucleus (FN) was used as an index of activation of NF-kB (FN/FC). Images were recorded and analyzed via digital photography with the accompanied software.

2.6. Western blot

An enriched nuclear fraction was prepared by homogenizing cultured microglia in isotonic HIM buffer (10 mM HEPES, 60 mM sucrose, 240 mM mannitol, pH 7.4). Centrifugation at 600 ×g for 10 min at 4 °C produced the nuclear pellet and the supernatant. Protein concentrations were determined using the Bradford colorimetric assay. Thirty micrograms of each protein lysate were loaded in each lane in sample buffer (2% SDS, 10% glycerol, 0.001% bromophenol blue, 1% DTT, and 0.05 M Tris–HCl, pH 6.8), separated on 10% SDS-PAGE (Invitrogen), and transferred to a PVDF membrane (Millipore, Temecula, CA). The blots were blocked with 5% nonfat milk in PBS and incubated with specific rabbit polyclonal antibody against NF-kB p65, followed by peroxidase-conjugated goat anti-rabbit IgG_{2a} (Millipore) and the enhanced chemoluminescence detection system (Amersham Biosciences, Arlington Heights, IL).

2.7. Statistics

All experiments were performed at least in triplicate. Graphical representation of data was from combined results rather than representative data from one single experiment. They were presented as the arithmetic mean percentages of control ± standard errors of the mean (S.E.M.). Experimental data were analyzed with one-way analysis of variance (ANOVA) for significant differences ($p < 0.05$).

3. Results

3.1. Effects of sinomenine on IL-1 beta, TNF alpha, and IL-6 release

Microglia were treated with different concentrations of sinomenine for 24 h and cell viability was detected by [3H] thymidine conversion. Sinomenine at concentrations from 0.01 to 1 mM did not induce any detectable cell death in microglia cultures during the 24 h incubation (data not shown). The effect of sinomenine on the release of cytokines into the culture medium was measured in microglia pretreated with 0.01, 0.1, 1 mM sinomenine for 1 h and then co-treated with 500 µg/ml AGEs for 24 h (Fig. 2). In the presence of AGEs, 0.01 mM sinomenine had no inhibitory effect on AGEs-induced release of TNF alpha, IL-1 beta and IL-6. However, the inhibitory effect of 0.1 mM, 1 mM sinomenine on AGEs-induced release of TNF alpha, IL-1 beta and IL-6 was obvious and significant in a dose-dependent fashion.

3.2. Effect of sinomenine on TNF alpha, IL-1 beta, and IL-6 mRNA expression

TNF alpha, IL-1 beta, and IL-6 mRNA levels was measured by RT-PCR. AGEs treatment induced an increase in mRNA levels of TNF alpha, IL-1 beta and IL-6 in microglia (Fig. 3). Incubation of microglia with 0.01, 0.1, 1 mM sinomenine alone for 24 h did not induce any detectable changes in TNF alpha, IL-1beta, and IL-6 mRNA levels (data not shown); but the same concentration of sinomenine reduced the AGEs-induced TNF alpha, IL-1 beta, and IL-6 gene expression (Fig. 2). Only low levels of these cytokine mRNAs were detected in cultured microglia without AGEs stimulation. The expression of TNF alpha, IL-1 beta and IL-6 mRNA was significantly higher after AGEs exposure. Incubation of microglia with 0.01, 0.1, 1 mM sinomenine reduced the AGEs-induced TNF alpha, IL-1 beta and IL-6 gene expression in a dose-dependent fashion.
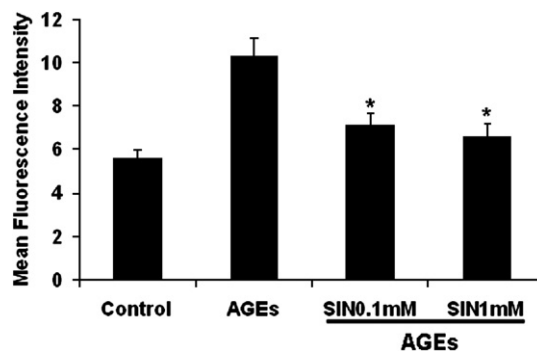

Fig. 4. Effects of sinomenine on DCF fluorescence in AGEs-induced retinal microglial activation. H_2DCF-DA was used to determine intracellular ROS production. The fluorescence intensity reflected the amount of ROS formation. Results were means ± S.E.M. of 3 individual experiments. *$p < 0.05$ compared with the control, **$p < 0.05$ compared with AGEs-treated cultures.

3.3. Effect of sinomenine on intracellular ROS

To examine whether AGEs might induce ROS production in cultured retinal microglia, we measured intracellular ROS level using the redox-sensitive fluorescent dye H_2DCF-DA. Due to the lack of significant effect of 0.01 mM sinomenine on AGEs-induce cytokine release, only 0.1 and 1 mM sinomenine were used in this experiment. Incubation of microglia with 0.1, 1 mM sinomenine alone for 24 h did not affect the levels of the fluorescence intensity of DCF (data not shown). The fluorescence intensity of DCF was significantly increased after adding 500 µg/ml AGEs for 24 h (Fig. 4). The inhibitory effect of 0.1, 1 mM sinomenine on AGEs-induced release of ROS was significant and dose-dependent.

3.4. Effect of sinomenine on NF-kB p65 nuclear translocation

For immunofluorescence localization of NF-kB by laser confocal scanning microscopy, fixed cells were reacted with a rabbit polyclonal antibody against the p65 subunit of NF-kB,
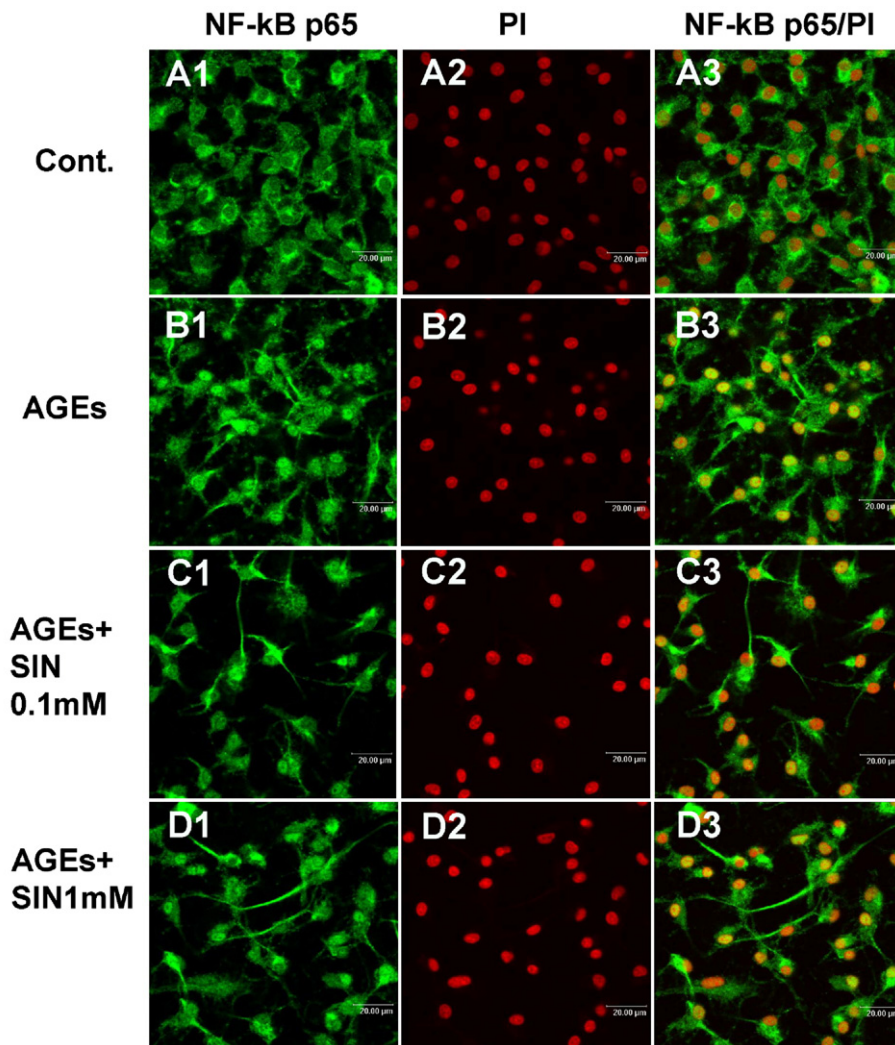

Fig. 5. Effect of sinomenine on p65 NF-kB translocation during activation of retinal microglia induced by AGEs. A. Cells were pretreated with different concentrations of sinomenine and then stimulated for 24 h with AGEs in the presence of it. Representative images shown are as follows: (A) untreated microglia showing red nuclei indicating no nuclear NF-kB translocation. A1: FITC labeled p65 NF-kB; A2: PI staring of nuclei; A3: Translocation of p 65 NF-kB. (B) Microglia activated by 24 h treatment of 500 µg/ml AGEs, where the nuclei appeared yellow indicating p65 NF-kB translocation. B1: FITC labeled p65 NF-kB; B2: PI staining of nuclei; B3: Translocation of p65 NF-kB. (C) microglia pre-treated with 0.1 mM sinomenine and then stimulated with 500 µg/ml AGEs for 24 h, showing a mixture of red and yellow colors in the nuclei indicative of partial p65 NF-kB translocation. C1: FITC labeled p65 NF-kB; C2: PI staring of nuclei; C3: Translocation of p 65 NF-kB (D) microglia pre-treated with 1 mM sinomenine and then stimulated with 500 µg/ml AGEs for 24 h, showing a mixture of red and yellow colors in the nuclei indicative of partial p65 NF-kB translocation. D1: FITC labeled NF-kB; D2: Nuclear of microglia labeled by PI; D3: Translocation of NF-kB by confocal microscopy observing minimal inhibition of NF-kB nuclear translocation. Bar = 20 µm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
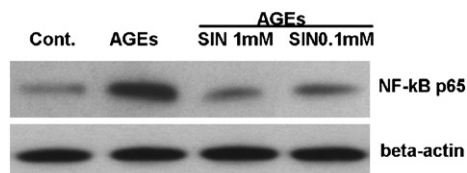
Fig. 6. Western blot: NF-kB p65 nuclear translocation. Retinal microglia were pre-treated with 0.1, 1 mM sinomenine for 1 h followed by co-treatment with 500 µg/ml AGEs for 24 h. Nuclear extracts were prepared, fractionated on SDS-PAGE, and electrotransferred to a PVDF membrane. The analysis was performed using p65 antibodies. First row, Control; second panel, 500 µg/ml AGEs; third row, SIN 1 mM+AGEs; fourth Row, SIN 0.1 mM+AGEs.

followed by FITC-conjugated goat anti-rabbit IgG antibody. The nuclei were stained with PI. NF-kB translocation from the cytosol to the nucleus was used as an index of activation of NF-kB. A change in color of the nucleus from red to yellow (due to co-localization of green FITC fluorescence and red PI fluorescence) was indicative of NF-kB translocation in the cells (Fig. 5). In the unstimulated group, NF-kB p65 protein remained localized in the cytoplasm. There was little staining of p65 in the nuclei indicating a lack of translocation of NF-kB to the nucleus (Fig. 4A). In the microglia activated by AGEs, an increase in NF-kB translocation was observed as most nuclei appeared to be yellow (Fig. 5B). In the presence of sinomenine (0.1, 1 mM), AGEs-induced NF-kB translocation was notably reduced in cultured retinal microglia in a dose-dependent fashion (Fig. 5C and D).

3.5. Western blot

As shown by Western blot analysis in Fig. 6, AGEs induced NF-kB p65 nuclear translocation. SIN 0.1 and 1 mM abolished AGEs-induced nuclear translocation of p65 significantly. Beta-actin in the same sample is an internal control.

4. Discussion

S. acutum has long been used in traditional Chinese medicine to treat various rheumatic diseases [9,10]. Previous reports have demonstrated that the pharmacological profile of sinomenine includes immunosuppression [19,20], anti-inflammation [12,13] and protection against hepatitis induced by lipopolysaccharide [21]. In addition, using intramuscular injection and multiple dosing, a combination of sinomenine and cyclosporin A showed immunomodulatory effects in a cardiac transplant model [22]. In the present work, we found that sinomenine significantly reduced AGEs-induced TNF alpha, IL-1 beta, IL-6, and also mRNA and protein levels, resulting in decreased microglial activation (Figs. 2 and 3). Sinomenine concentrations chosen for this study did not cause

detectable cell damage in our microglial culture system during the 24 h treatment.

We found that sinomenine treatment could suppress AGEs-induced activation of microglia, by decrease of ROS activity and as indicated by a reduction of DCF fluorescence (Fig. 4). Considering that enhanced formation of ROS oxidatively damage cellular macromolecules and/or alter signal transduction, it is possible that neuroprotection by sinomenine is achieved, at least partially, by a reduction in ROS-induced damage. In this study, we found that sinomenine inhibited NF-kB translocation from the cytosol to the nucleus in AGEs-induced microglial activation (Figs. 5 and 6). Sinomenine has been shown to inhibit induction of IL-2, and IL-8 protein by increasing degradation of TNF alpha and iNOS beta mRNA [23]. This is consistent with our results that sinomenine inhibits cytokine gene expression and protein release possibly by reducing NF-kB translocation.

Sinomenine has emerged as a potent inhibitor of microglial activation. Its potential as a drug is favored by its easy absorption from the gastrointestinal tract with a half-life of 4 h. It has been considered to be relatively safe for humans in clinical trials for rheumatoid arthritis. Moreover, it has an ability to penetrate the blood–brain barriers [9,10]. Therefore, sinomenine may be a good candidate for treating retinal microvascular and neuro-degenerative diseases, such as diabetic retinopathy. However, some studies suggest that microglia probably have dual functions. The observations from experimental work in animals support a role of microglia as neuroprotective cells after acute neuronal injury. These studies point towards an important role of neuronal–microglial crosstalk in the facilitation of neuroprotection. Conceptually, injured neurons are thought to generate rescue signals that trigger microglial activation and, in turn, activated microglia produce trophic or other factors that help damaged neurons recover from injury. Thus, whether sinomenine will inhibit, prevent or induce trophic factors from activated microglia is one topic for future study. Further understanding of the mechanisms of action of sinomenine will provide insights into potential therapeutic interventions.

In conclusion, sinomenine plays a role in modulating AGEs-induced expression and release of TNF alpha, IL-1 beta, and IL-6 in microglia in culture via ROS production and NF-kB translocation.

Acknowledgements

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