

Interleukin-10 Protects Schwann Cells against Advanced Glycation End Products-Induced Apoptosis via NF- κ B Suppression

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ABSTRACT

Demyelination resulting from Schwann cell injury is a main pathological feature of diabetic neuropathy, and a key contributor to this process may be inflammation due to advanced glycation end products (AGEs). Therefore, protection by anti-inflammation agents is anticipated. In this study, we showed that interleukin-10 (IL-10), an anti-inflammatory cytokine, inhibits apoptosis of Schwann cells induced by AGEs *in vitro*. We isolated and cultured Schwann cells from rat sciatic nerves. As detected by flow cytometry, apoptosis of Schwann cells markedly increased following incubation with AGEs for 48 h. However, pretreatment with IL-10 inhibited AGE-induced apoptosis. The effect of IL-10 on NF- κ B, which is a very important regulator of inflammation, was also evaluated, and results showed high levels of phospho-NF- κ B and nuclear localization of NF- κ B in cells incubated with AGEs but low levels of phospho-NF- κ B and cytoplasmic localization in the cells incubated with IL-10, indicating the activation of NF- κ B by AGEs and inhibition of NF- κ B by IL-10. Moreover, incubating Schwann cells with an NF- κ B inhibitor (caffeic acid phenethyl ester) for 30 min before adding AGEs mimicked IL-10, lowering the amount of reactive oxygen species and activity of caspase-3 and also decreasing apoptosis in Schwann cells. These results indicate that IL-10 may protect Schwann cells against AGE-induced apoptosis by attenuating oxidative stress via the inhibition of activation of NF- κ B.

Introduction

Peripheral neuropathy is one of the most common complications of diabetes, and as many as 50 % of diabetic patients suffer from diabetic neuropathy [1]. The pathology of peripheral neuropathy includes damage to neurons, Schwann cells, and blood vessels within the nerves.

Schwann cells are susceptible to hyperglycemic toxicity because they uptake glucose through an insulin-independent glucose transporter [2, 3]. Goncalves et al. reported the involvement of Schwann cells in diabetic neuropathy [4]. Dysfunction and apoptosis of Schwann cells can cause demyelination, which is involved in the development of peripheral neuropathy in diabetic patients. In diabetic rat models, a great number of osmiophilic inclusion bodies, which are often related to the destruction of myelin sheath, were found in Schwann cells [5]. Pathological findings in spontaneous diabetes animals also showed that their nerve fiber abnormalities were mainly restricted to the myelin sheath and Schwann cells [6]. There was obvious splitting and ballooning of the myelin sheath, accompanied by reactive, degenerative, and proliferative Schwann cells. This evidence suggests that injury to Schwann cells has significance in the pathogenesis of diabetic neuropathy [6]. Apoptosis of Schwann cells has been detected in diabetic models [7–10], which could be a cause of demyelination in the peripheral nervous system [11].

Oxidative stress in and apoptosis of Schwann cells may result from high glucose levels *in vitro* [12–14]. However, advanced glycation end products (AGEs) are the major threat for hyperglycemia *in vivo*. These are produced from non-enzymatically catalyzed sugar reactions with amino groups of proteins to form reversible Schiff bases. The early glycation products then undergo further complex reactions, such as rearrangement, dehydration, and condensation, to become irreversibly cross-linked, heterogeneous fluorescent derivatives known as AGEs [15]. The formation and accumulation of AGEs are positively correlated with the progression of diabetes. In particular, the pathological role of AGEs has been reported in various diabetic complications [15–19]. AGEs, by binding to their receptor (RAGE), can lead to dysfunction and death in various retinal cells [20] and decrease pericyte adherence [21, 22]. It has also been shown *in vitro* that incubation of Schwann cells with AGEs induces cell death [23]. Binding to RAGE activates various signaling pathways, leading to increased oxidative stress and synthesis of local growth factors, cytokines, and adhesion molecules [24].

Data obtained from diabetic humans and animals showed that AGE levels increased not only in the serum but also in the peripheral nerves [25]. AGEs were found in the peripheral nerves of diabetic rats, and the expression of RAGE was found in endothelial and Schwann cells, which may be responsible for impaired nerve function and pathological nerve alterations [26]. Glycolaldehyde (a precursor of AGEs) at physiological concentrations can decrease the viability of rat Schwann cells and thereby contribute to the pathogenesis and development of diabetic neuropathy [27].

Upon the binding of AGEs with RAGE, oxidative stress levels in cells increase and various cytokines are secreted through the activation of nuclear factor κ B (NF- κ B), which cause an inflammatory response that results in injury [28, 29]. Targeting the AGE–RAGE system may be a novel strategy for diabetic complication therapy. Inhibiting AGE formation [30], RAGE deletion [31], and down-

stream effect attenuation of the AGE–RAGE system [32, 33] are common strategies. Inhibiting the inflammatory response is very important for blocking the downstream effect of AGE–RAGE [34]. Interleukin-10 (IL-10) is one of the most important anti-inflammatory cytokines. It can inhibit neuroinflammation-induced apoptosis [35]. Moreover, the inflammation mediated by RAGE is related to low levels of IL-10. In a sepsis model, RAGE was found to be suppressed by IL-10, and exogenous IL-10 administered to animals enhanced the survival of these animals, indicating protection against RAGE signaling by IL-10 [36]. Therefore, IL-10 may be a useful drug for diabetic neuropathy therapy. In this study, the effect of IL-10 on the apoptosis of Schwann cells induced by AGEs and its related mechanisms were investigated.

Materials and Methods

Isolation of Schwann cells from rat sciatic nerves

The animal study was approved by the Animal Ethics Committee of the China-Japan Friendship Hospital. 6-week-old male Wistar rats (200–250 g) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). The rats were maintained on a 12 h:12 h light:dark cycle with unlimited access to rodent chow and water.

Rats were euthanized, and their sciatic nerves were surgically obtained. The sciatic nerves were cut into small pieces and digested in 0.2 % type I collagenase solution for 15 min at 37 °C. The mixture was then diluted with 10 ml M199 medium and passed through a 100-mesh sieve. The filtrate was collected and centrifuged to harvest Schwann cells. The Schwann cells were washed twice with M199 medium, and cultured in M199 medium containing 10 % fetal bovine serum (FBS) and 50 ng/ml nerve growth factor (Staidson Beijing Biopharmaceuticals Co, Ltd, China). Cells between passages 3–6 were used in this study.

Immunohistochemistry staining

Rat sciatic nerve tissue was fixed in 4 % paraformaldehyde for 24 h, and processed by paraffin embedding. Samples of 5- μ m thickness were prepared on slides and immunostained using mouse anti-rat myelin basic protein (MBP) (Abcam, UK) and horseradish peroxidase-conjugated secondary antibodies. The sections were rinsed in PBS and developed in diaminobenzidine solution. The results were observed and photographed using a microscope (Olympus IX71, Japan).

Identification of Schwann cells by flow cytometry

A single cell suspension of Schwann cells was prepared and fixed with 4 % paraformaldehyde. After being washed with 0.1 % BSA/PBS thrice, the cells were stained with mouse monoclonal antibody to rat MBP for 1 h at room temperature. As control, irrelevant, isotype-matched antibody was used. Cells were further stained with Alexa 488-conjugated donkey anti-mouse IgG antibody for 30 min. Cells were washed thrice and then resuspended in 200 μ l of PBS/BSA. Cell fluorescence intensity was measured using flow cytometry (Beckman Coulter, Germany).

Cell viability assay

Schwann cells were seeded in a 96-well plate at a cell density of 2×10^3 cells/well. After being initially cultured overnight, the cells were then cultured in medium containing different concentrations of AGEs (0, 100, 200, 300, and 400 $\mu\text{g/ml}$) for 7 days. The medium was changed every 2 days. The survival of Schwann cells was evaluated at 0, 1, 3, 5, and 7 days by MTT assay. In brief, at each time point, 0.5% MTT (3-(4,5-dimethylthiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) was added to 3 wells from each dose group (50 μl /well). After 4-h incubation at 37 °C, the culture medium was discarded, the water-insoluble formazan dye in the cell was solubilized with DMSO (150 μl /well), and optical density was measured by a microplate reader (Multiscan, Thermo, USA) at 492 nm. The experiment was independently repeated thrice.

Flow cytometry for apoptosis

Schwann cells were inoculated on 6-well plates and cultured in medium containing AGEs (300 $\mu\text{g/ml}$) alone, AGEs and IL-10 (50 ng/ml, PeproTech, UK; added 30 min before AGEs treatment) or AGEs and NF- κB inhibitor (caffeic acid phenethyl ester, 10 μM ; added 30 min before AGEs treatment). Normally cultured cells were used as a control. The cells were collected after 72 h, incubated with annexin V-FITC and PI (Life Technologies, USA) at room temperature for 15 min, then analyzed by FACScan (Beckman Counter Epics XL, Miami, FL, USA).

Western blotting

Schwann cells were lysed in RIPA buffer containing protease inhibitors (Roche Diagnostics, Indianapolis, USA) and phosphatase inhibitors (Sigma, Shanghai, China). Extracts were separated using SDS-polyacrylamide gels, and transferred to 0.2 μm nitrocellulose membranes (Immobilon Millipore, Bedford, MA, USA). After blocking, the membranes were incubated with primary antibodies: anti-NF- κB p65 (1:1000, Cell Signaling Technology, Beverly, MA), and anti-phospho-NF- κB p65 (1:1000, Cell Signaling Technology, Beverly, MA). Anti- β -actin (1:10000, Sigma, St Louis, MO, USA) was used as a loading control. Further incubation with horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG followed, then samples were visualized using an enhanced chemiluminescence solution (Millipore, Billerica, USA).

Immunofluorescent staining for the intracellular location of NF- κB p65

Schwann cells were plated onto 24-well plates and cultured in medium containing 0.5% FBS for 24 h. Then the cells were treated with AGEs with or without pre-treated with IL-10 for 30 min. Cells cultured under normal condition were used as control. The cells were rinsed with PBS, fixed with 4% paraformaldehyde, then permeabilized with 0.1% Triton X-100/0.1% citrate for 10 min at room temperature. Cells were then stained with mouse anti-NF- κB p65 as a primary antibody and Alexa 488-conjugated donkey anti-mouse IgG as a secondary antibody. The intracellular location of NF- κB p65 was observed using inverted fluorescence microscopy (Olympus, Japan).

Reactive oxygen species (ROS) assay

Schwann cells were treated with AGEs only, AGEs + IL-10, or AGEs + NF- κB inhibitor (caffeic acid phenethyl ester, 10 μM , added 30 min before AGE treatment). 3 wells were included in each group.

The cells were cultured for 48 h. The reactive oxygen species (ROS) level was measured using an ROS assay kit (Genmed, Shanghai, China), following the manufacturer's protocol. A plate fluorescence reader (Molecular Devices Corp, Sunnyvale, CA, USA) was used for quantification.

Detection of caspase-3 activity

To investigate whether NF- κB -mediating AGEs induced apoptosis, Schwann cells were treated with AGEs alone, AGEs + IL-10, or AGEs + NF- κB inhibitor (caffeic acid phenethyl ester, 10 μM , added 30 min before AGE treatment). 4 wells were included in each group. The cells were cultured for 48 h. Caspase-3 activity was measured using a caspase-3 assay kit (Genmed, Shanghai, China), following the manufacturer's protocol. A plate fluorescence reader (Molecular Devices Corp, Sunnyvale, CA, USA) was used for quantification.

Statistical analysis

SPSS 13.0 software was used to conduct statistical analyses. Data were presented as mean \pm SEM. One-way ANOVA followed by Tukey's multiple comparison test were used to analyzing the difference between multiple groups. $P < 0.05$ was deemed statistically significant.

Results

Identification of Schwann cells

The myelin sheath of the sciatic nerve is formed mainly by Schwann cells. MBP is a well-known marker of Schwann cells that is frequently expressed in sciatic nerve tissue (**► Fig. 1a**). After digesting sciatic nerve tissue with collagenase, Schwann cells were obtained that exhibited fibroblast-like morphology (**► Fig. 1b**). The purity of cultured Schwann cells was as high as 99% by flow cytometry for MBP (**► Fig. 1c**).

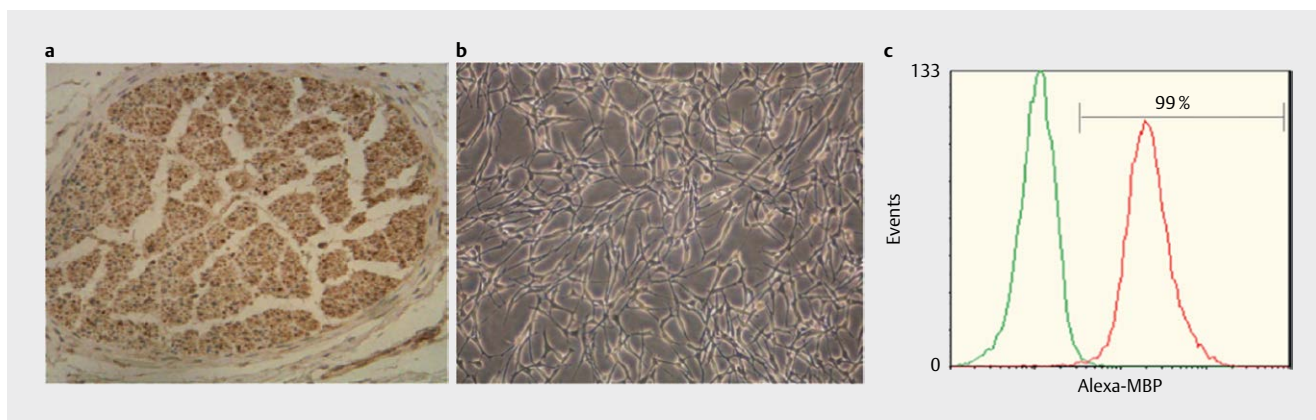
Effects of AGEs and IL-10 on Schwann cell survival

Schwann cells were treated with different concentrations of AGEs for up to 7 days to examine the cytotoxic effect of AGEs. The viability of Schwann cells was significantly reduced in a dose-dependent and time-dependent manner (**► Fig. 2a**). These results suggest that AGEs decrease the survival of Schwann cells, which might be through the induction of apoptosis in Schwann cells.

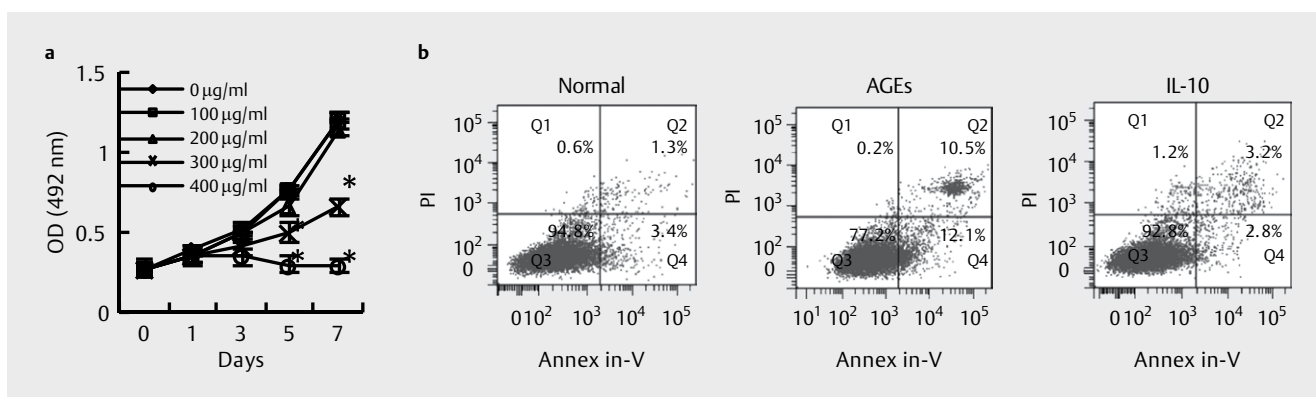
To investigate whether AGEs induce apoptosis of Schwann cells, and the effect of IL-10, Schwann cells were treated with AGEs or a combination of AGEs and IL-10. Apoptosis was then evaluated. As shown in **► Fig. 2b**, exposure to AGEs significantly increased the early and late apoptotic cell populations. The presence of IL-10 reduced the percentage of apoptotic cells. This result indicated that IL-10 could protect Schwann cells against AGEs.

The effect of IL-10 on the activation of NF- κB

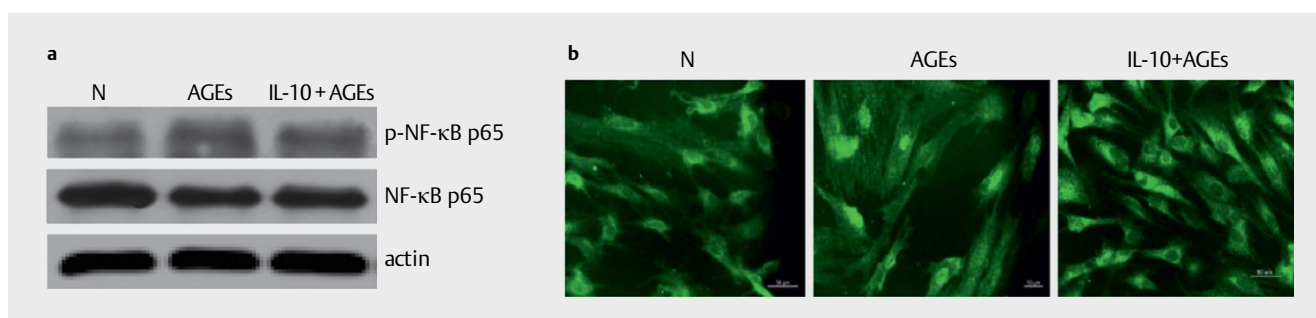
It has been reported that exposure to AGEs could play a significant role in activating NF- κB . Therefore, the effect of IL-10 on NF- κB was analyzed. Schwann cells were treated with AGEs or a combination of AGEs with IL-10. The cell lysates were subjected to Western blotting for the evaluation of NF- κB p65 phosphorylation. It was found that p65 phosphorylation was increased by AGEs, but this was reversed by the presence of IL-10 (**► Fig. 3a**). In normal culture con-



► **Fig. 1** Identification of Schwann cells derived from rat sciatic nerve tissue. **a** Immunohistochemistry staining for myelin basic protein on rat sciatic nerve tissue. **b** The morphology of cultured Schwann cells. **c** Flow cytometry for myelin basic protein on cultured Schwann cells.



► **Fig. 2** The effect of IL-10 on AGE-induced apoptosis of Schwann cells. **a** The effect of AGEs on the survival of Schwann cells by an MTT assay. Schwann cells were cultured with different AGE doses, and the survival of Schwann cells was measured by MTT assay. Data are presented as mean \pm SEM, $n = 3$, * $P < 0.01$ vs. 0 $\mu\text{g/ml}$ group. **b** The effect of IL-10 on AGE-induced apoptosis of Schwann cells. Some Schwann cells were pre-treated with IL-10 (50 ng/ml) for 30 min, and then all cells were treated with AGEs (300 $\mu\text{g/ml}$). The cells were cultured for 72 h followed by Annexin V/PI staining.

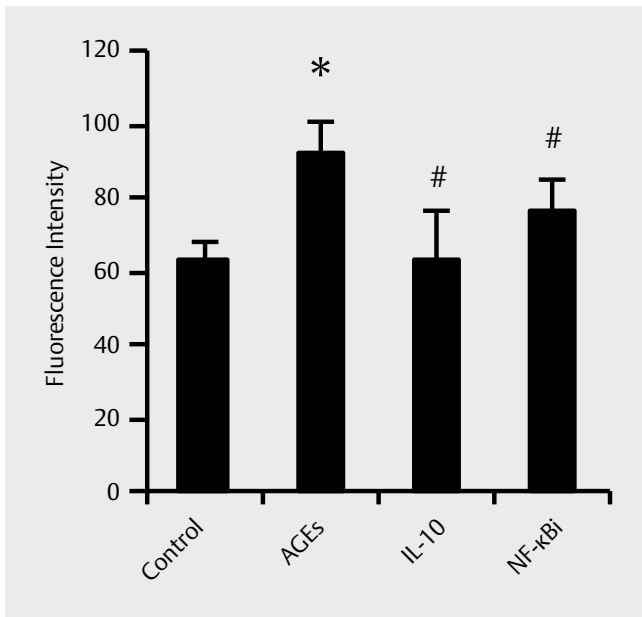


► **Fig. 3** The effect of IL-10 on the activation of NF- κ B. **a** Western blotting for p65 phosphorylation. Schwann cells were treated with AGEs or AGEs + IL-10 for 24 h. The cell lysates were subjected to Western blotting. **b** Intracellular localization of NF- κ B by immunofluorescence staining.

ditions, p65 was mainly located in the cytoplasm, but translocated into the nucleus following exposure to AGEs. If cells were pre-incubated with IL-10 before being treated with AGEs, then the distribution of p65 was similar to that seen in normally cultured cells (► **Fig. 3b**). These results indicate that AGEs activate NF- κ B, and IL-10 inhibits NF- κ B activation.

Inhibiting NF- κ B decreased AGE-induced ROS and apoptosis

To confirm that the protective action of IL-10 was mediated by NF- κ B suppression, we tested whether NF- κ B inhibitors could mimic the protection afforded by IL-10. As shown in ► **Fig. 4**, AGEs signif-



► **Fig. 4** The effect of IL-10 and NF-κB on ROS level. Schwann cells were treated with AGEs, AGEs + IL-10 and AGEs + NF-κB inhibitor (caffeic acid phenethyl ester, 10 μM) for 48 h. The ROS levels in Schwann cells were measured with a DCFH-DA probe-based assay kit. Data are presented as mean ± SEM, n = 3, * *P* < 0.01 vs. Control group; # *P* < 0.01 vs. AGEs group.

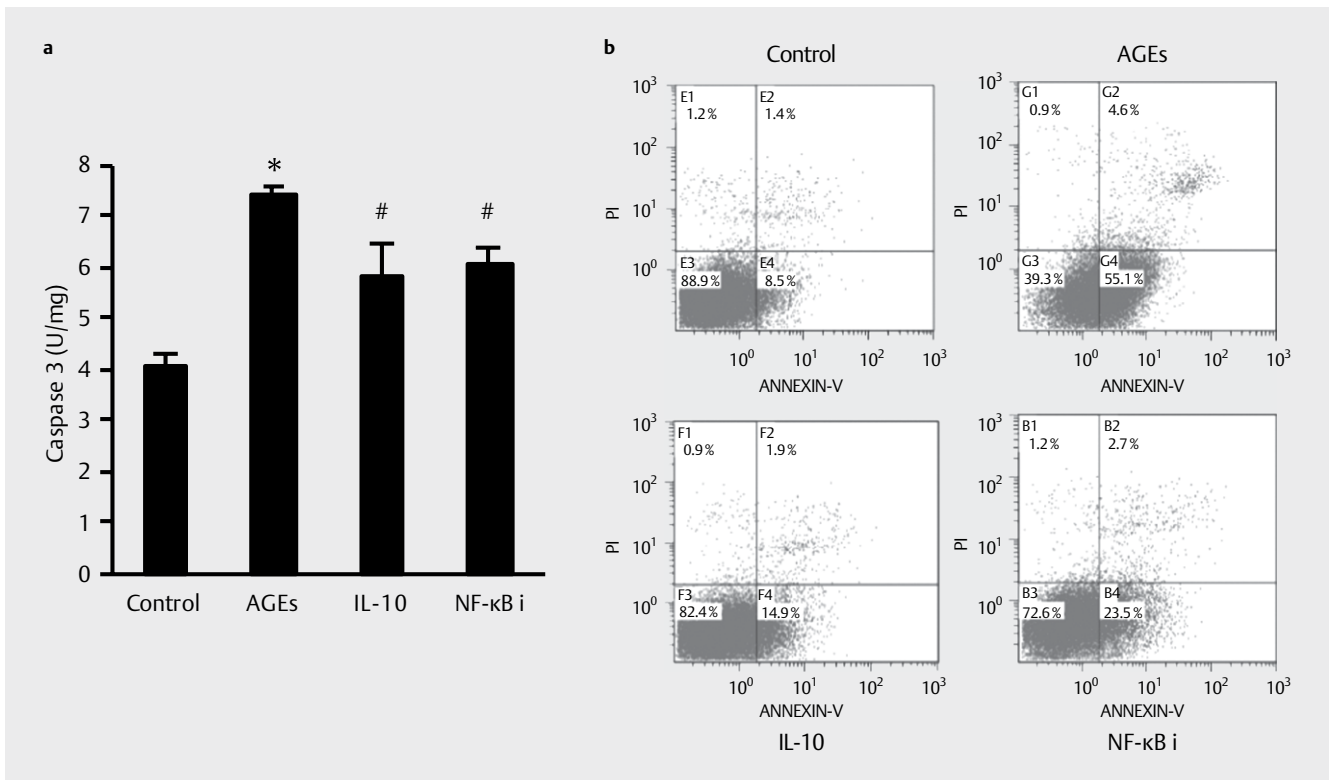
icantly increased the ROS level in Schwann cells, but NF-κB inhibitors could mimic IL-10 leading to decreased ROS levels

Exposure to AGEs also increased caspase-3 activity, which was lowered by IL-10 and NF-κB inhibitors to a similar level (► **Fig. 5a**). Moreover, the early and late apoptotic cell populations were increased by AGEs but decreased by NF-κB inhibitors and IL-10 as well (► **Fig. 5b**). These results indicate that inhibiting NF-κB could protect Schwann cells against AGE-induced apoptosis and that inhibiting NF-κB activity with IL-10 will, therefore, result in the protection of Schwann cells.

Discussion and Conclusions

AGE-induced inflammation and oxidative stress are key contributors to diabetic complications, including diabetic neuropathy. Attenuating these processes may have potential therapeutic effects for diabetic neuropathy. In this study, we showed that an anti-inflammation cytokine, IL-10, could protect Schwann cells against AGE-induced apoptosis, which has important implications for the prevention of and therapy for diabetic peripheral neuropathy.

Chronic high levels of blood glucose lead to the accumulation of AGEs, which interact with the receptor RAGE, and this can ultimately lead to cellular injury mediated by an inflammation reaction and oxidative stress [37]. In this study, Schwann cells were isolated from rat sciatic nerve tissue, and a large number of primary Schwann cells of high purity were obtained. Schwann cells were



► **Fig. 5** The effect of NF-κB on AGE-induced apoptosis and caspase-3 activity. Schwann cells were treated with AGEs, AGEs + NF-κB inhibitor (caffeic acid phenethyl ester, 10 μM) for 48 h. The activity of caspase-3 was measured using a fluorescence-based kit **a** Apoptosis was detected by flow cytometry **b** Data are presented as mean ± SEM, n = 3, * *P* < 0.01 vs. Control group; # *P* < 0.01 vs. AGEs group.

treated with AGEs to mimic the diabetic condition. We found that the survival of Schwann cells was decreased by AGEs in a dose-dependent manner. AGEs induced obvious apoptosis in Schwann cells, which may contribute to diabetic demyelination [23]. However, pre-treatment with IL-10 significantly reduced AGE-induced apoptosis, which suggested IL-10 was highly protective for Schwann cells.

IL-10 is an important immuno-regulatory cytokine with multiple functions. It has an anti-inflammatory action and influences the activity of some types of immune cells. IL-10 has also been involved in proliferation, survival, and anti-apoptotic activities [38]. IL-10 is reportedly involved in both central and peripheral neuropathy, as well as diabetic neuropathy. In a peripheral neuropathic pain model in type 1 diabetic animals, continuous delivery of IL-10 by vectors in the nerve fibers mediated transduction in the dorsal root ganglion, and alleviated the nociceptive and stress responses. IL-10 also inhibits the development of painful neuropathy by decreasing stress in the dorsal root ganglion [39].

In recent years, the involvement of IL-10 in diabetes has been demonstrated by experimental and clinical research. Plasma IL-10 levels were found to be significantly lower in patients with prediabetes or diabetes compared with controls, whereas TNF- α levels were significantly higher in patients with type 2 diabetes [40]. It was also reported that IL-10 serum concentration decreases in patients with diabetic distal sensorimotor polyneuropathy [41]. An investigation in India showed that IFN γ and IL-10 genes are significantly associated with peripheral neuropathy in South Indian type 2 diabetic patients. It was found that the "high-producer" IL-10-1082 G/G genotype and the "low-producer" IFN γ + 874 A/A genotype may be responsible for the down-regulation of immune responses [42]. This evidence suggests that IL-10 is a protective factor for diabetes.

Some diabetic drugs exhibit neuroprotection by up-regulating IL-10. In experimental diabetic peripheral neuropathy models, sulforaphane or liraglutide normalized motor coordination and the latency withdrawal time of the tail-flick test, increased the latency withdrawal time of the cold allodynia test, and ameliorated histopathological changes. The mechanism of both drugs was partly related to the up-regulation of IL-10 [43, 44]. The glucagon-like peptide-1 (GLP-1) receptor agonist exenatide exhibits neuroprotection through the autocrine release of IL-10 [45].

IL-10 binds to its receptor and regulates downstream signaling, such as JAK1/STAT3 [46, 47], PI3K/Akt/NF- κ B [48, 49], and MAPK [38]. NF- κ B plays a very important role, along with IL-10, in diabetic complications, for NF- κ B is also associated with AGE-RAGE signaling. Activation of RAGE in diabetic animal models contributes to the onset and progression of diabetic neuropathy. Mice with RAGE knockout showed tolerance to STZ and attenuated neuropathy. Diabetic RAGE knockout mice have also shown decreased expression of NF- κ B in their peripheral nerves, particularly in Schwann cells [50]. NF- κ B is one of the most important transcription factors activated by RAGE. It has been reported that there was a positive feed-forward loop of NF- κ B activation and increased RAGE expression [51]. Sustained activation of NF- κ B by the AGE-RAGE system was implicated in cell stress and dysfunction in diabetes cases [51]. NF- κ B appears to be a key node in the network of the development of diabetic neuropathy. Some pathways were reported to have involvement in diabetic complications via the activation of NF- κ B [52].

In this study, we showed that the phosphorylation of NF- κ B was increased by AGEs in Schwann cells, but inhibited by IL-10. Signal-dependent nuclear translocation of NF- κ B is required for the activation of downstream target genes that regulate immune and inflammatory responses. We found AGEs induced obvious nuclear translocation of NF- κ B, but AGE-induced nuclear translocation was significantly inhibited by IL-10. Furthermore, NF- κ B inhibitors could also decrease apoptosis induced by AGEs and attenuated caspase-3 activity in Schwann cells. All of our data support the hypothesis that IL-10 reduces the apoptosis of Schwann cells induced by AGEs via NF- κ B suppression.

Mounting evidence has shown that besides of the benefit of endogenous IL-10, exogenous IL-10 also had obvious neuroprotection and neuro-recovery properties in animal models; even more efficient than some anti-inflammatory and anti-oxidative agents [53]. Considering these data together with the findings from this study, IL-10 may be a potentially useful drug for the treatment of diabetic neuropathy.

Contribution Statement

X.S., B.W., M.X., L.Y., S.J. and L.J. researched and collected data. L.H. performed statistical analysis. C.H. designed the study and edited the manuscript, Z.W. performed literature research and prepared the manuscript. L.J. and P.L. had the study concept and revised the manuscript.

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Conflicts of Interest

The authors declare that they have no conflict of interest.

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