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1,25-$(OH)_2D_3$ protects Schwann cells against advanced glycation end products-induced apoptosis through PKA-NF-κB pathway

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ABSTRACT

Aims: To explore the effect and mechanism of 1, 25-$(OH)_2D_3$ on Schwann cell apoptosis induced by advanced glycation end products.

Main methods: Schwann cells, isolated from rodent sciatic nerve were incubated with AGE-modified bovine serum albumin(AGE) to mimic diabetic conditions and 1,25-$(OH)_2D_3$ was used as protector. Cell apoptosis was detected by PI/Annexin-V staining, caspase 3 activity assay and western blotting for caspase 3 and PARP. The activation of protein kinase A (PKA) and nuclear factor kappa-B (NF-κB) was evaluated by western blot. Immunofluorescent staining was used for intercellular location of NF-κB. Cytokine secretion was evaluated by enzyme-linked immunosorbent assay.

Key findings: Schwann cell apoptosis accelerated after incubating with AGE. However, if combining 1,25-$(OH)_2D_3$ with AGE, apoptosis decreased significantly. 1,25-$(OH)_2D_3$ enhanced PKA activity, but inhibited AGE-induced nuclear translocation of NF-κB. Furthermore, PKA activator (8-bromoadenoside cyclic adenoside monophosphate, 8-Br-cAMP) or NF-κB inhibitor (caffeic acid phenethyl ester, CAPE) could reduce the apoptosis, decreased cleaved caspase 3 and cleaved PARP, suggesting the involvement of PKA and NF-κB pathways in the protection of 1,25-$(OH)_2D_3$ on Schwann cells. Moreover, 8-Br-cAMP and CAPE could inhibit AGE-induced secretion of interleukin(IL)-1β, prostaglandin E2(PEG2) and cyclooxygenase 2(COX2). Interestingly, 8-Br-cAMP decreased phospho-NF-κB and inhibited nucleus translocation of NF-κB. It hinted at the regulation of PKA to NF-κB. Finally, a pre-treatment of H-89 (an inhibitor of PKA) could block the protection of 1,25-$(OH)_2D_3$ on cell apoptosis. In conclusion, 1,25-$(OH)_2D_3$ could protect Schwann cell against AGE-induced apoptosis through PKA/NF-κB pathway.

Significance: These findings provide experimental rationales for using vitamin D for diabetic neuropathy.

1. Introduction

As one of the most common chronic complications of diabetes mellitus, diabetic peripheral neuropathy (DPN) is also a major cause of worsening quality of life in individuals with diabetes. Hyperglycemia results in an accumulation of advanced glycation end products (AGEs) and becomes an important causative factor of diabetic complications. However, the molecular mechanism of DPN is so complex that multiple factors and many types of cells are involved. Both injuries of peripheral nerve and microvessels supplying peripheral nerve might contribute to DPN and result in demyelination and axonal degeneration followed by nerve dysfunction and ultimately somatic and visceral denervation [1].

Myelin sheath is generated by Schwann cells in the peripheral nervous system. Thus demyelination in the peripheral nervous system is caused mostly by insults of Schwann cells [2,3]. In addition, Schwann cell are sensitive to hyperglycemic toxicity because of its insulin-independent glucose transporter [4,5]. Pathological abnormalities of nerve fiber are generally restricted to myelin sheath and Schwann cell in rat models of diabetes [6]. Apoptosis of Schwann cell has been detected in models of diabetes [7–11]. It indicated that protecting

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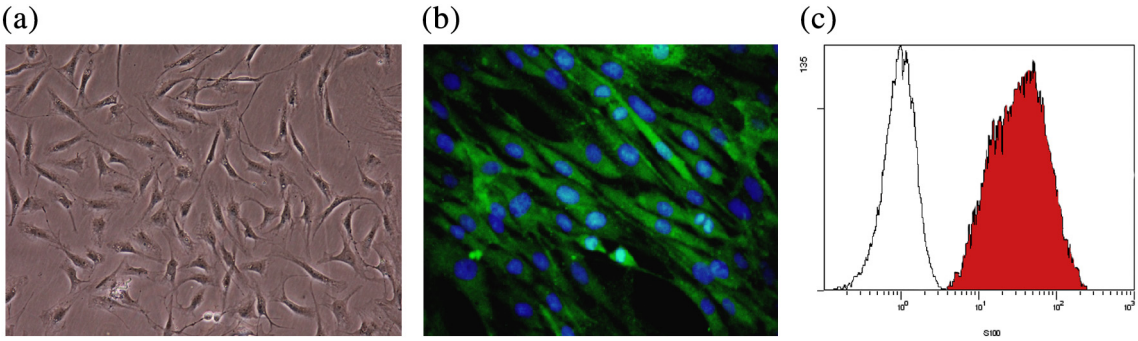

Fig. 1. Identification of Schwann cells derived from rat sciatic nerve. (a): Morphology of cultured cells, 100×; (b): Immunofluorescent staining for S-100, 400×; (c): Flow cytometry for S-100.

Schwann cell might be a potential strategy for preventing and treating DPN.

In recent year, AGEs are demonstrated as a key factor leading to diabetic complications. AGEs were produced from sugars reacting non-enzymatically with the amino groups of proteins to form reversible Schiff bases. Then the early glycation products undergo further complex reactions such as rearrangement, dehydration and condensation to become irreversibly cross-linked, heterogeneous fluorescent derivatives termed AGE [12]. The formation and accumulation of AGE are positively correlated with the progress of diabetes. Especially, the pathological role of AGE has been reported in various diabetic complications [12–16]. AGE via binding to their receptor (RAGE) could lead to dysfunction and death of various types of cells [17–19]. It is also shown that in vitro incubation of Schwann cells with AGE induces cell death [20].

The data obtained from patients and animals with diabetes showed that the levels of AGEs were increased not only in the serum but also in the peripheral nerves [21]. AGEs were found in the peripheral nerve of rat with diabetes, and the expression of RAGE was found in endothelial and Schwann cells, which may contribute to the impairment of nerve function [22].

Some recent studies have demonstrated that a low serum level of vitamin D was associated with diabetes complications [23,24] and vitamin D deficiency might be correlated with DPN [25,26]. Many international studies have reaffirmed that vitamin D deficiency was an independent risk factor for DPN [27–30]. It is expected that supplementation of vitamin D could prevent or delay the onset of DPN. In another study, treatment of vitamin D improved the emotional distress of patients with painful diabetic neuropathy. Yet there was no significant effect on other painful symptoms [31]. Vitamin D was correlated positively with IL-13 and nerve growth factor [22] and negatively with IL-17 [32]. These effects of vitamin D indicated anti-inflammation and nerve protection. Chabas JF et al. examined the efficacy of vitamin D on neuropathy in a rat model of transected peripheral nerve. Vitamin D2 could increase axon diameter and potentiate nerve regeneration [33]. Both vitamins D2 and D3 improved myelination and recovery after nerve injury. However, vitamin D3 was more efficient than vitamin D2. Vitamin D3 not only increased the number of preserved or newly formed axons but also improved neurite myelination in both distal and proximal ends [34].

Despite an efficacy of vitamin D for DPN, the mechanism of vitamin D on peripheral nerve system required further investigation, especially under disease condition. Under diabetic conditions, AGE binding to RAGE activates a variety of signaling pathways leading to increased oxidative stress and synthesis of local growth factors, cytokines and adhesion molecules [35]. Activation of nuclear factor κB (NF-κB) is a key step for inflammatory response and following injury [36,37]. Moreover, in our previous studies, we found that PKA mediated protection in diabetic nephropathy [38]. Activating PKA was also reported to contribute a beneficial effect of cilostazol on DPN [39].

Considering improved myelination of vitamin D, it was speculated that vitamin D might have a beneficial effect on the survival or function of Schwann cells and PKA or NF-κB pathway may be involved. Therefore, the current study was intended for elucidating the effect and mechanism of $1,25(OH)_2D_3$ on the AGE-induced apoptosis of Schwann cells.

2. Materials and methods

2.1. Isolation and culture of Schwann cells

Schwann cells were isolated from rodent sciatic nerve as described previously with modification [40]. The experimental protocol was approved by our institutional Animal Ethics Committee. Six-week-old Wistar rats (200–250 g) (Beijing Vital River Laboratory Animal Technology, Beijing, China) were euthanized and sciatic nerves dissected. Sciatic nerves were cut into approximately 1 mm pieces and subsequently digested for 15 min at 37 °C in 0.2% type I collagenase solution. The mixture was agitated vigorously and then diluted with 10 mL M199 medium. After passing through a 100-mesh sieve, filtrate was centrifuged, rinsed with M199 medium and cultured in M199 medium supplied with 10% fetal bovine serum and 50 ng/mL nerve growth factor (Staidson Beijing Biopharmaceuticals, Beijing, China). Only cells between the third and sixth passages were used in this study.

2.2. Identification of Schwann cells

Schwann cells were identified by immunofluorescent staining for S-100. After fixing with 4% paraformaldehyde for 30 min at room temperature, sample was rinsed twice with phosphate buffered saline (PBS) and blocked with 1% bovine serum albumin (BSA)/PBS. Then labeling was performed with mouse-anti-rat S-100 IgG (Abcam, Shanghai, China) and Alexa 488-conjugated donkey anti-mouse IgG (Invitrogen, Carlsbad, CA, USA). The nuclei were stained with 4′,6-diamidino-2-phenylindole. The results were observed and photographed under an inverted fluorescent microscopy (Olympus, Tokyo, Japan). The purification of Schwann cells was evaluated by flow cytometry for S-100.

2.3. Detection of apoptosis by flow cytometry

AGE modified BSA (described as AGE in this study) (Abcam, Shanghai, China, Cat No. 51995) was used to induce apoptosis of Schwann cell. Single-cell suspension was prepared for each group. The cells were washed twice by centrifuge and then incubated for 15 min with annexin V-Alex488 and PI (Invitrogen). Apoptosis was analyzed immediately by BD FACSCalibur(Becton, Dickinson and Company, USA).

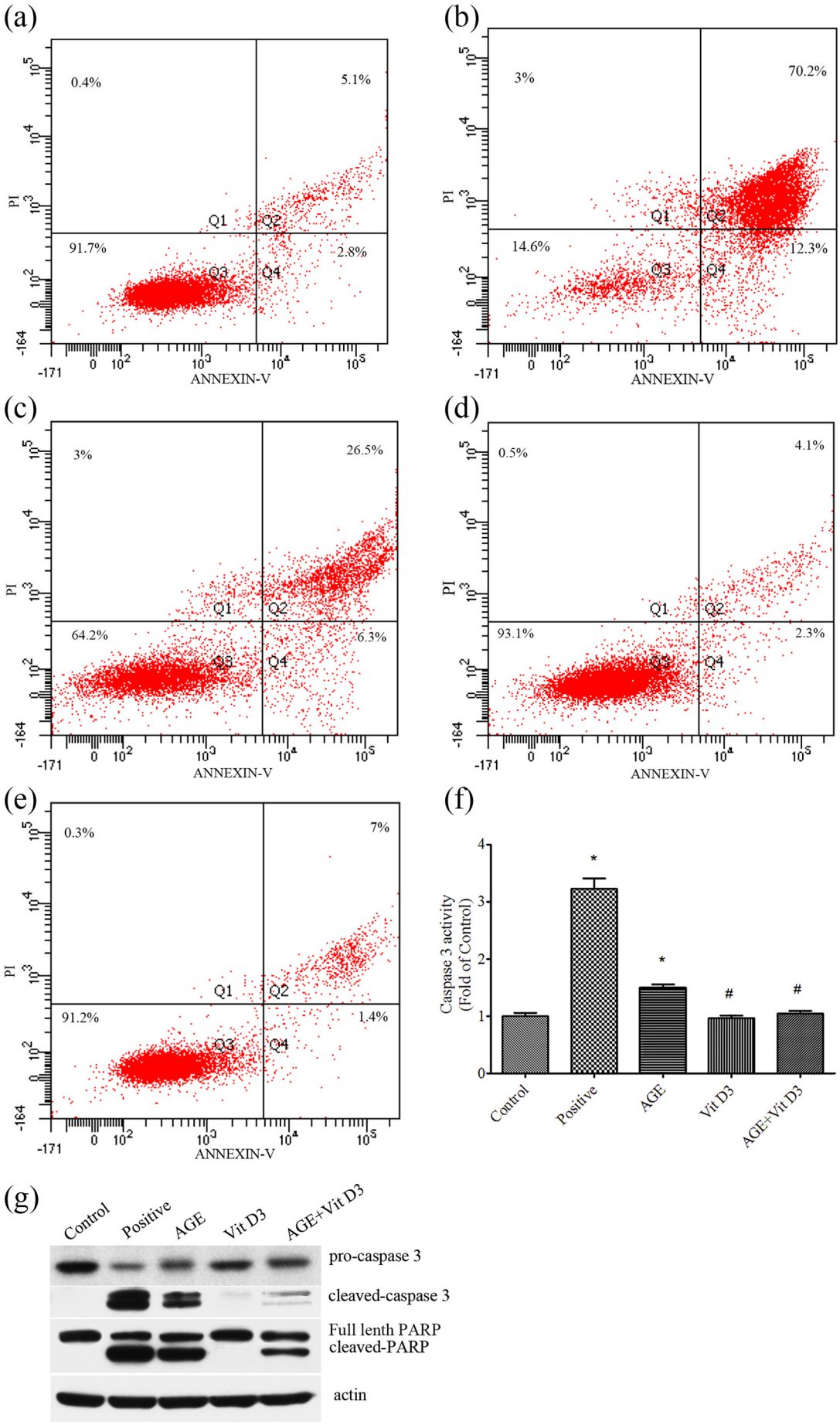

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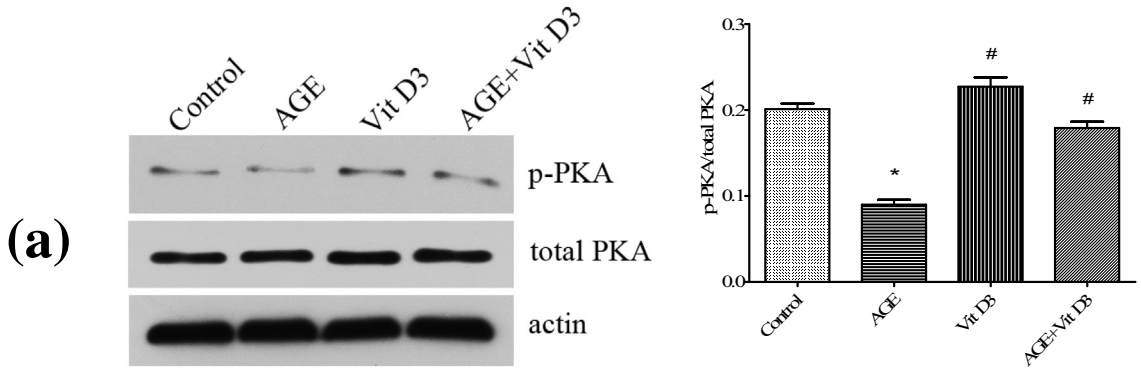
Fig. 2. Effect of 1,25-$(OH)_2D_3$ on AGE-induced apoptosis of Schwann cell. Apoptosis labeled by PI/Annexin-V and detected by flow cytometry. (a) through (e): Schwann cell cultured with BSA (200 μg/mL), Apoptosis inducer, AGE (200 μg/mL), 1,25-$(OH)_2D_3$ (1×10^{-8} M) and AGE + 1,25-$(OH)_2D_3$, respectively. (f): Activity of Caspase 3. N = 3, $*P < 0.01$ vs. Control group; $\#*P < 0.01$ vs. AGE group. (g): The caspase 3 and PARP expressions were detected by western blot.



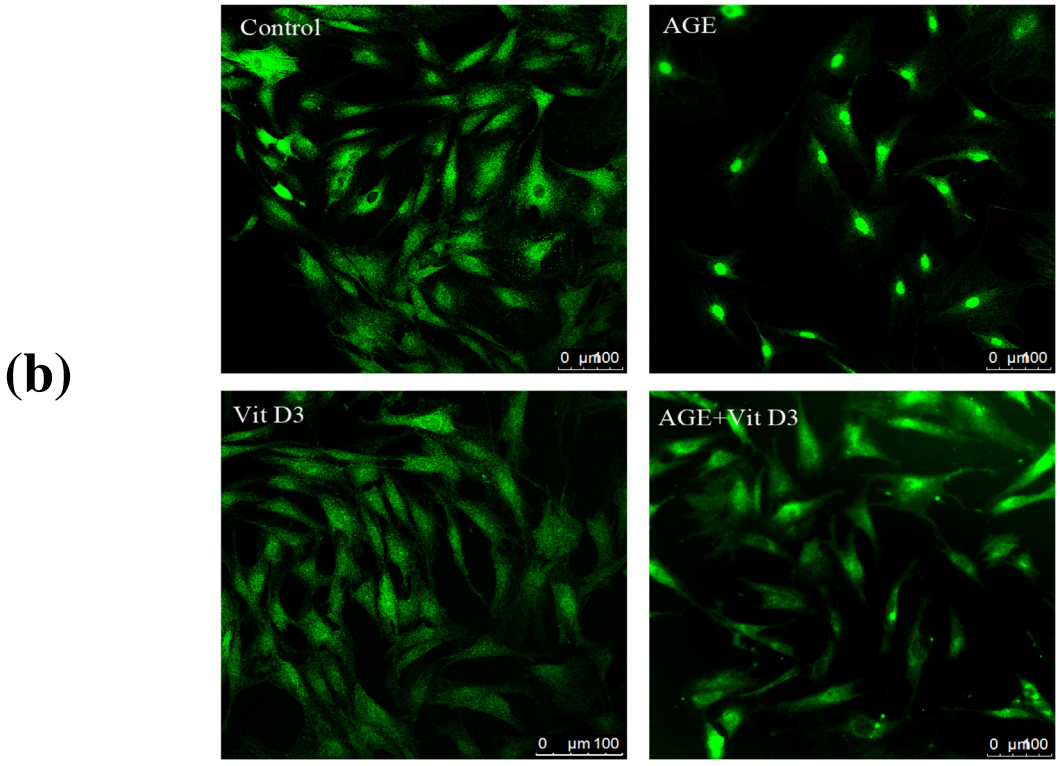


Fig. 3. Effects of 1,25-$(OH)_2D_3$ on the activities of PKA and NF-κB. (a): Effect of 1,25-$(OH)_2D_3$ on the activity of PKA by western blotting(left panel). Band intensities were quantified by densitometric analysis (right panel). N = 3, $*P < 0.01$ vs control group and $\# P < 0.01$ vs AGE group. (b): Effect of 1,25-$(OH)_2D_3$ on nuclear translocation of NF-κB.

2.4. Assay of caspase 3 activity

To confirm apoptosis, caspase 3 activity in Schwann cells treated with different reagents was measured. Three wells were set for each group. Activity of caspase-3 was measured by a fluorimetric caspase 3 assay kit (Genmed, Shanghai, China) according to the manufacturer's instructions. The results were detected by plate fluorescent reader (Molecular Devices Corp, Sunnyvale, CA, USA).

2.5. Western blot

Total protein of cells from each group was extracted with RIPA buffer containing protease inhibitors (Roche Diagnostics, Indianapolis, IN, USA) and phosphatase inhibitors (Sigma, Shanghai, China). In addition, 100 μg protein was loaded onto a sodium dodecylsulfate-polyacrylamide gel and transferred to 0.2 μm PVDF membrane (Immobilon Millipore, Billerica, MA, USA). After blocking with 5% BSA solution, membranes were incubated with primary antibodies (Cell Signaling Technology, Beverly, MA, USA). Anti-β-actin (Sigma) was used as a loading control. After incubating with horseradish peroxidase-conjugated secondary antibody (Sigma), proteins were visualized by enhanced chemiluminescence solution (Millipore, Billerica, MA, USA).

2.6. Intracellular location of NF-κB by immunofluorescent staining

Schwann cells were plated into coverglass-bottom dishes and

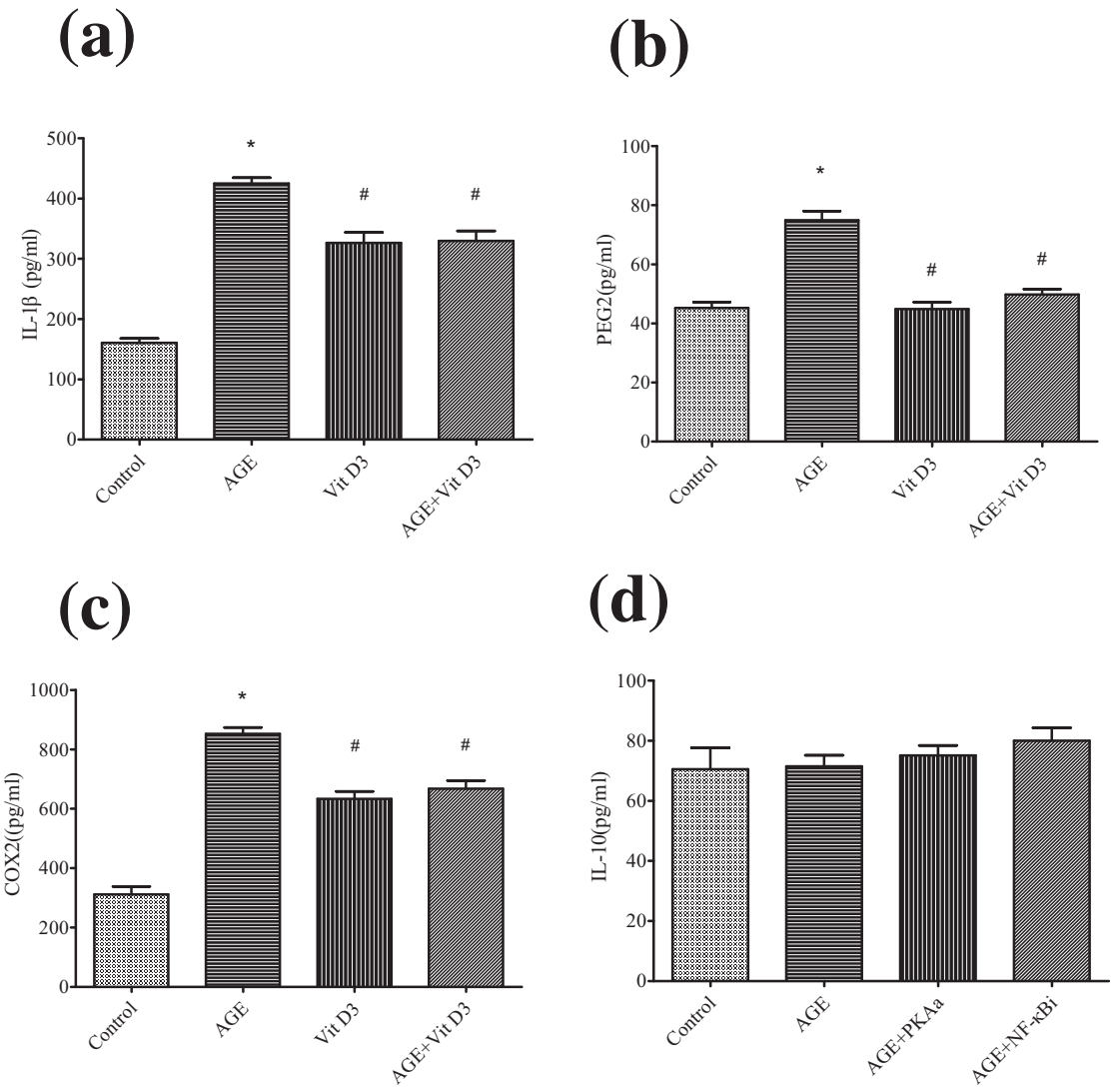


Fig. 4. 1,25-$(OH)_2D_3$ decreased AGE-induced cytokine secretion. N = 3, *$P < 0.01$ vs control group and # $P < 0.01$ vs AGE group.

cultured for 24 h in medium contain 0.5% fetal bovine serum. Then the cells were treated with AGE (200 μg/mL), 1,25$(OH)_2D_3$(10^{-8} M), AGE plus 1,25$(OH)_2D_3$, or AGE plus 1,25$(OH)_2D_3$ and PKA activator (a 30-min pretreatment of 8-Br-cAMP prior to adding AGE and 1,25$(OH)_2D_3$). All groups were treated for 3 h. After rinsing twice with PBS, cells were fixed with 4% paraformaldehyde at room temperature and permeabilized for 10 min with 0.1% Triton X-100. Then staining was performed with mouse anti-NF-κB p65 as a primary antibody and Alex488-conjugated donkey anti-mouse IgG as a secondary antibody. Intracellular distribution of NF-κB p65 was observed under a fluorescent microscope (Olympus, Tokyo, Japan).

2.7. Enzyme-linked immunosorbent assay (ELISA)

For measuring cytokine secretion, Schwann cells were inoculated into 24-well plate, and treated with AGE (200 μg/mL), 1,25$(OH)_2D_3$(10^{-8} M), or AGE plus 1,25$(OH)_2D_3$. Cell treated with BSA(200 μg/mL) was used as control. After incubation for 24 h, the supernatant from each group was collected, and content of interleukin (IL)-1β, prostaglandin E2(PEG2), cyclooxygenase 2(COX2) and IL-10 were measured with ELISA kit (USCN, Wuhan, China).

2.8. Statistical analyses

The data were expressed as means ± SEM and represented three independent experiments. One-way ANOVA after Tukey's multiple comparison test was used for statistical analyses (Prism software, GraphPad Inc., La Jolla, CA, USA). A value of $P < 0.05$ was deemed as statistically significant.

3. Results

3.1. Identification of Schwann cells

Cultured Schwann cells exhibited a monolayer with fibroblast-like morphology (Fig. 1a). All cells expressed S-100 by immunofluorescent staining (Fig. 1b). Purity of cultured cells was as high as 99% by flow cytometry for S-100 (Fig. 1c).

3.2. Effects of 1, 25$(OH)_2D_3$ on AGE-induced apoptosis of Schwann cell

For detecting the protection of 1, 25$(OH)_2D_3$ on Schwann cells, AGE were used for simulating diabetic condition. As shown in Fig. 2, Annexin-V positive cells were scarce under normal culture with BSA

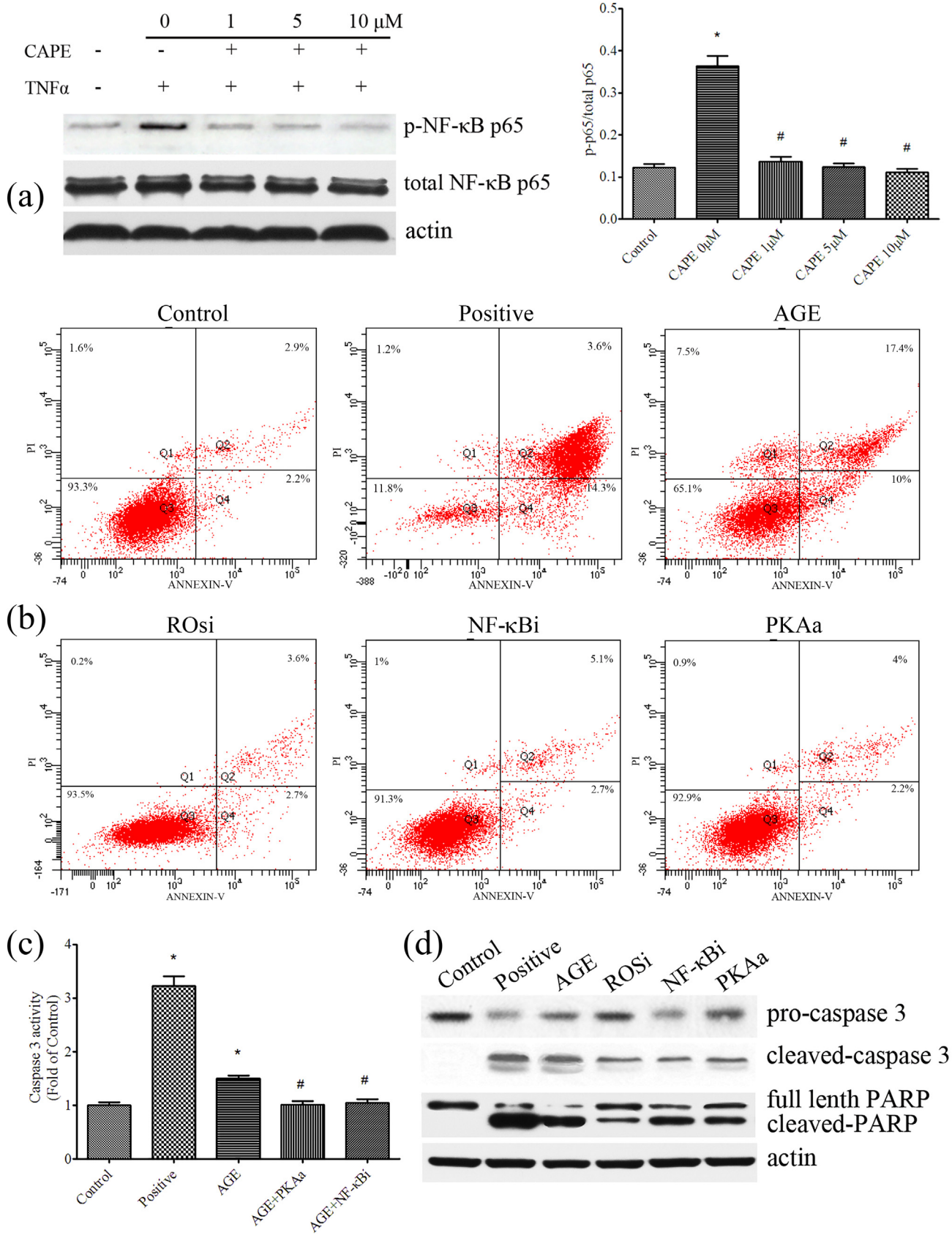


Fig. 5. Effects of PKA and NF-κB on AGE-induced apoptosis of Schwann cell. (a): The efficacy of CAPE on NF-κB inhibition by western blotting (left panel). Band intensities were quantified by densitometric analysis (right panel). N = 3, $^{*}P < 0.01$ vs control group and # $P < 0.01$ vs AGE group. (b): The effect of 8-Br-cAMP and CAPE on AGE-induced apoptosis of Schwann cell. (c): The effect of 8-Br-cAMP and CAPE on caspase 3 activity. N = 3, $^{*}P < 0.01$ vs control group and # $P < 0.05$ vs AGE group. (d): The caspase 3 and PARP expressions were detected by western blot.

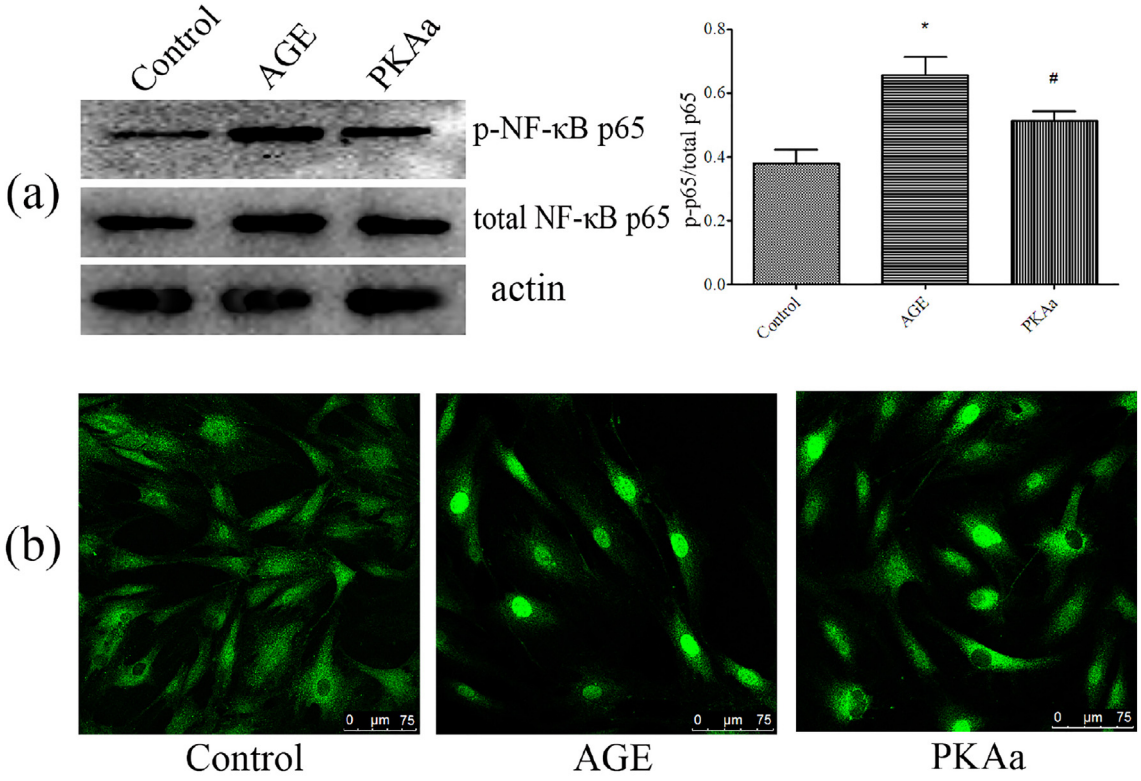


Fig. 6. PKA regulates NF-κB activation. (a): The phosphorylation of NF-κB was detected by western blot (left panel). Band intensities were quantified by densitometric analysis (right panel). N = 3, *$P < 0.01$ vs control group and # $P < 0.05$ vs AGE group. (b): Nuclear translocation of NF-κB was detected by immunofluorescent staining.

(Fig. 2a). However, exposure to apoptosis inducer (positive control, Fig. 2b) or AGE (Fig. 2c) induced dramatic apoptosis of Schwann cells. Incubating with 1, 25$(OH)_2D_3$ alone did not influence the survival of Schwann cells (Fig. 2d). If co-incubating with both AGE and 1, 25$(OH)_2D_3$, apoptosis decreased significantly (Fig. 2e) than incubating with AGE alone. The activity of caspase 3 showed similar change as apoptosis (Fig. 2f). In addition, the levels of cleaved-caspase 3 and cleaved-PARP were significantly increased with AGE in Schwann cells, whereas caspase 3 and PARP(full lenth) were consistently decreased. However, the cleavage of caspase 3 and PARP induced by AGE was inhibited by co-incubating with 1, 25$(OH)_2D_3$ (Fig. 2g). It hinted at the protective effect of 1, 25$(OH)_2D_3$ for Schwann cells.

3.3. Effect of 1,25$(OH)_2D_3$ on the activation of PKA and NF-κB

To explore the protective mechanism of 1,25$(OH)_2D_3$ on Schwann cells, the influence of 1,25$(OH)_2D_3$ on activation of PKA and NF-κB was detected. As shown in Fig. 3a, incubation of Schwann cells with AGE decreased baseline PKA activity by approxinately 70%. Co-incubation with 1,25$(OH)_2D_3$ reverted this reduction, leading to PKA activity of approximately 90% compared to control values.

As an important hallmark of NF-κB activation, translocation into nucleus is necessary for regulating downstream genes. By immunofluorescent staining (Fig. 3b), NF-κB p65 protein was predominantly located in cytoplasm under normal culture conditions. Yet p65 translocated into the nucleus after exposure to AGE. If co-incubated with 1,25$(OH)_2D_3$, translocation of NF-κB p65 declined and cellular distribution was similar to normal cultivation. These results indicated that AGE activated NF-κB and 1,25$(OH)_2D_3$ inhibited NF-κB activation.

3.4. 1,25-$(OH)_2D_3$ decreased AGE-induced cytokine secretion

Because NF-κB is a key regulator of inflammation, inhibition of NF-κB by 1,25-$(OH)_2D_3$ may change cytokine levels in Schwann cells. As shown in Fig. 4, AGE increased IL-1β (Fig. 4a), PGE2 (Fig. 4b) and COX2 (Fig. 4c) release by Schwann cells, which was inhibited by 1,25-$(OH)_2D_3$. However, both AGE and 1,25-$(OH)_2D_3$ has no significant influence on IL-10 secretion (Fig. 4d).

3.5. Activation of PKA or inhibition of NF-κB decreased AGE-induced apoptosis

For confirming the involvement of PKA and NF-κB in AGE-mediated apoptosis, we examined whether PKA activator or NF-κB inhibitor might also protect Schwann cell against AGE. First, we tested the efficacy of caffeic acid phenethyl ester (CAPE), a NF-κB inhibitor by western blotting for phospho-NF-κB level. As shown in Fig. 5a, tumor necrosis factor alpha (TNF-α) (activator of NF-κB) increased phospho-NF-κB level dramatically. That action was blocked by CAPE in a dose-dependent manner. 10 μM CAPE was selected for the following experiments.

Cell apoptosis could be induced by AGE via oxidative stress. As shown in Fig. 5b, exposure to AGE significantly increased Annexin-V positive apoptotic cell populations. N-acetl-L-cysteine (an inhibitor of reactive oxygen species) could reduce the apoptotic cell percentage. 8-Br-cAMP (PKA activator) and CAPE could reduce apoptosis. Moreover, AGE-enhanced caspase 3 activity was also lowered by 8-Br-cAMP or CAPE. The levels of caspase 3 activity, cleaved-caspase 3 and cleaved-PARP were consistent with annexin-V positive cell percentage (Fig. 5d). These results indicated that PKA and NF-κB pathways were involved in AGE-induced insults of Schwann cells. Therefore, the effects of 1, 25$(OH)_2D_3$ on PKA and NF-κB contributes to its protection.

3.6. PKA regulating activation of NF-κB

To analyze whether there was interaction between PKA and NF-κB,

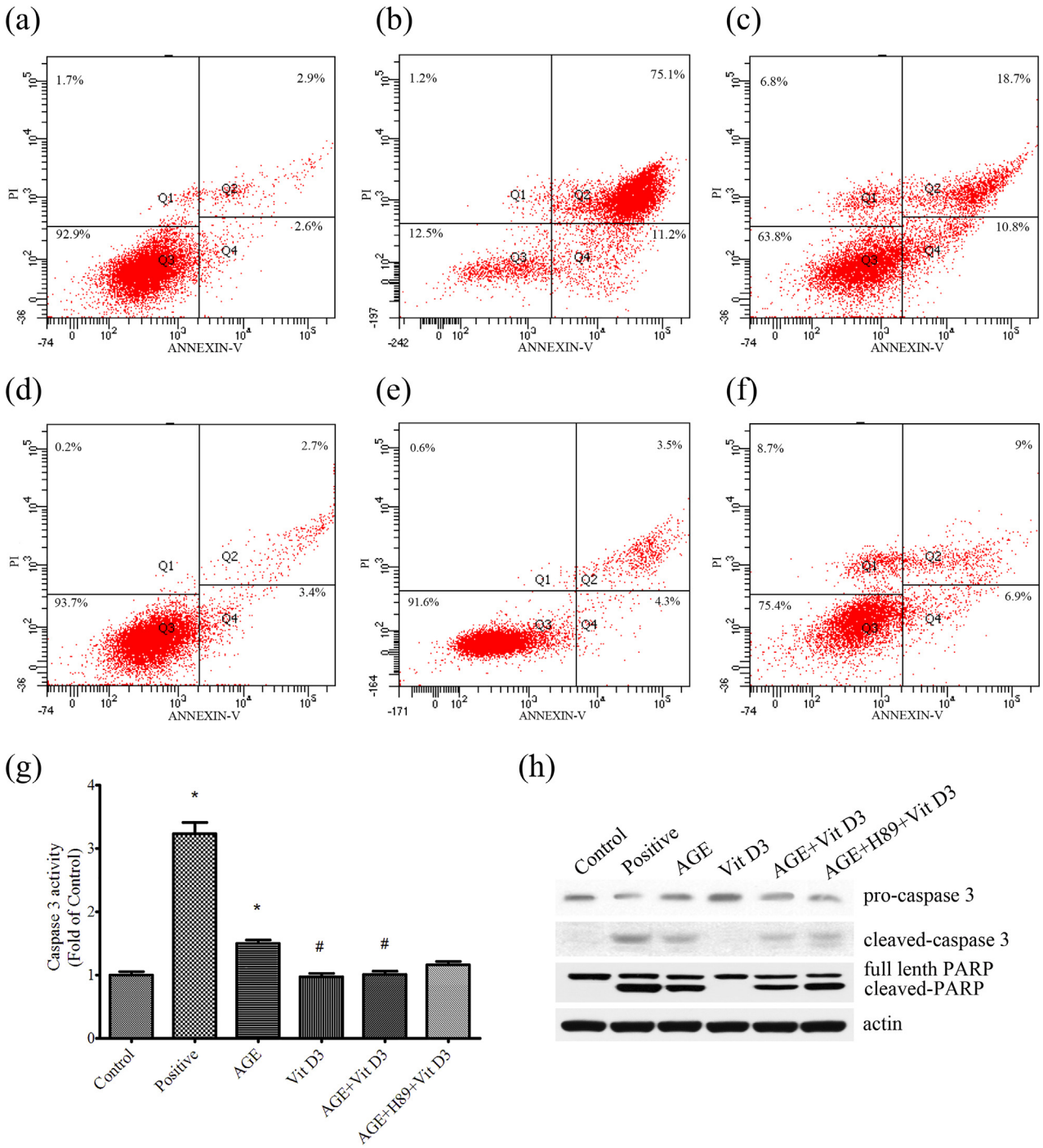


Fig. 7. Inhibition of PKA attenuated the protection of 1,25-$(OH)_2D_3$ on Schwann cells. Schwann cells were treated with BSA(a), apoptosis inducer(b), AGE(c), 1,25-$(OH)_2D_3$(d), AGE plus 1,25-$(OH)_2D_3$(e) or pretreated for 30 min with H89 prior to adding AGE plus 1,25-$(OH)_2D_3$(f), Then apoptosis was detected by PI/annexin-V staining. Caspase 3 activity was measured by fluorimetric based assay(g). N = 3, $*P < 0.01$ vs control group and $\# P < 0.05$ vs AGE group. Cleaved-caspase3 and cleaved-PARP were detected by western blotting(h).

Schwann cell was treated with 8-Br-cAMP to activate PKA and then the activity of NF-κB was evaluated. The results showed that the phospho-NF-κB was up-regulated by AGE but down-regulated by 8-Br-cAMP (Fig. 6a). Nuclear translocation of NF-κB induced by AGE could also be inhibited by 8-Br-cAMP (Fig. 6b). These data indicated that NF-κB is a downstream target of PKA.

3.7. PKA inhibition blocking the protection of 1,25(OH)$_2$D$_3$ on Schwann cell

The contribution of PKA to the protection of 1,25$(OH)_2D_3$ on Schwann cell was confirmed by using a PKA inhibitor. Fig. 7 shows pre-incubation with H89 for 30 min prior to treatment with AGE + 1,25-$(OH)_2D_3$. The protection of 1,25-$(OH)_2D_3$ on Schwann cells against apoptosis was blocked significantly, shown as increased annexin-V positive cell population, caspase 3 activity, and cleaved-caspase 3 and cleaved-PARP compared with the AGE + Vit D3 group. It indicated that

protection of Schwann cells by 1,25-$(OH)_2D_3$ was mediated via the PKA pathway.

4. Discussion

Vitamin D deficiency has been widely reported in patients with diabetic neuropathy [23,26,28,29]. However, the efficacy of vitamin D for diabetic neuropathy is not clear. Herein it was shown that 1,25-$(OH)_2D_3$ could protect Schwann cells against AGE-induced apoptosis via PKA/NF-κB pathway.

Schwann cells consist of myelin sheath in the peripheral nerve system. Such a role is vital for nerve conduction and neuron survival. During the development of diabetic neuropathy, Schwann cells became injured by a high level of blood glucose, accumulation of AGE and other pathological factors. Pathological change and apoptosis of Schwann cells was found in animals with diabetes [6–10], which indicated an important role of Schwann cells in diabetic peripheral disorders.

Regarding the effect of AGE on Schwann cells and the protection of 1,25-$(OH)_2D_3$, we isolated high-purity Schwann cells from rat sciatic nerve. Cell apoptosis could be induced by AGEs. We found that 1,25-$(OH)_2D_3$ had obvious protection on Schwann cells by greatly decreasing apoptosis. The study of rat Schwann cell line RSC96 had similar findings. It was shown that calcitriol reduced oxidative stress in RSC96 cells induced by high glucose and methylglyoxal through restoration of cystathionine beta synthase/hydrogen sulfide expression [41]. These results suggested potential application of vitamin D for patients with DPN. In some clinical trials, vitamin D supplementation might relieve symptoms and signs [42,43]. Vitamin D supplementation plus training might achieve significant improvement of sensory-motor neuropathy in women with diabetes [43].

Vitamin D can regulate cell proliferation, differentiation and apoptosis in many tissues through binding to vitamin D receptor and regulating multiple pathways. In human breast and prostate cancer cell lines, MDA-MB-231 and PC3, stable knockdown of vitamin D receptor, induced cell apoptosis and inhibited cell proliferation in vitro [44]. Calcitriol, a hormonal form of vitamin D, inhibited both caspase-3-dependent and caspase-independent apoptosis in keratinocytes [45]. Vitamin D might also protect endothelial cells from ionizing radiation-induced reactive oxygen species production and apoptosis [46]. In a model of diabetes, vitamin D was reported improving renal functions and albuminuria by inhibiting podocyte apoptosis [47].

Vitamin D regulates cell proliferation and apoptosis through various signaling pathways. Diverse mechanisms exist in different cell types, even in different conditions of the same cell type. Vitamin D facilitated proliferation of endothelial cells by activating extracellular signal-regulated kinases and protected quiescent cells by inhibiting p38 [46]. It was also capable of suppressing the apoptosis of cultured mouse podocytes [48] and human oral keratinocytes by blocking NF-κB pathways [49].

NF-κB pathway is a very important signaling method for complications related to diabetes. In the current study, we found 1,25$(OH)_2D_3$ suppressed the NF-κB pathway and inflammation of its downstream effects. In addition, as accumulating data suggested that PKA signaling exhibits protection against complications related to diabetes, we also detected the effect of 1,25$(OH)_2D_3$ on PKA activity. Results showed that 1,25$(OH)_2D_3$ not only inhibited NF-κB pathway but also the activating PKA pathway. Moreover, PKA activator or NF-κB inhibitor could both mimic the protective action of 1,25$(OH)_2D_3$ separately. Both pathways contributed to the protection of vitamin D on Schwann cells. NF-κB pathway is activated by AGE-RAGE signaling and contributes to the development of diabetic neuropathy. The RAGE knockout mice with diabetes had decreased expression of NF-κB in peripheral nerves, particularly in Schwann cell [50]. Sustained activation of NF-κB by the AGE-RAGE system is implicated as cell stress and dysfunction in diabetes [51]. Some pathways are involved in diabetic complications via regulating NF-κB [52]. Signal-dependent nuclear translocation of NF-κB is required for regulating downstream target genes. AGE induced obvious nuclear translocation of NF-κB. However, PKA activator inhibited nuclear translocation of NF-κB which indicates that NF-κB is a downstream target of PKA.

5. Conclusion

Mounting evidence has confirmed the protective role of PKA for microvascular complications related to diabetes [38,53,54]. 1, 25$(OH)_2D_3$ decreases Schwann cell apoptosis through an activation of PKA followed by NF-κB suppression. These data hint at a potential application of vitamin D for clinical DPN.

Acknowledgments

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Conflicts of interest

The authors declare there are no conflicts of interest.

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