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Requirement of the phosphatidylinositol 3-kinase/Akt signaling pathway for the effect of nicotine on interleukin-1beta-induced chondrocyte apoptosis in a rat model of osteoarthritis

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ABSTRACT

Chondrocyte apoptosis is mainly responsible for the progressive degeneration of cartilage in osteoarthritis (OA). Interleukin-1beta (IL-1β) was widely used as a modulating and chondrocyte apoptosis-inducing agent. Nicotine is able to confer resistance to apoptosis and promote cell survival in some cell lines, but its regulatory mechanism is ambiguous. We aimed to investigate the effect of nicotine on IL-1β-induced chondrocyte apoptosis and the mechanism underlying how nicotine antagonizes IL-1β-induced apoptosis of rat chondrocytes. Chondrocytes isolated from newborn rat joints were exposed to IL-1β. The cell viability was analyzed by the MTT (3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide) assay, and the apoptotic cells were counted with DAPI staining. The levels of Akt, phosphorylated-Akt (p-Akt) and downstream protein targets of Akt were detected by western blotting. The results showed that nicotine neutralized the effect of IL-1β on chondrocytes by activating PI3K/Akt signaling pathways, including the PI3K/Akt/Bcl-2 pathway, to block IL-1β-induced cell apoptosis and the PI3K/Akt/p70S6K (p70S6 kinase)/S6 pathway for promoting protein synthesis, modulating its downstream effectors such as TIMP-1 and MMP-13. Activation of the PI3K/Akt pathway is, in part, required for the effect of nicotine on IL-1β-induced chondrocyte apoptosis in a rat model of osteoarthritis.

1. Introduction

Osteoarthritis (OA) is a common, predominantly age-related joint disorder characterized by chondrocyte loss, degradation of the extracellular matrix (ECM), subchondral bone remodeling and synovial inflammation [1,2]. Proinflammatory cytokines secreted by chondrocytes, such as interleukin-1beta (IL-1β) and tumor necrosis factor-α (TNF-α), contribute to the progression of OA [3–5]. For example, the number of type I IL-1 receptor (IL-1R) is significantly increased in OA chondrocytes and synovial cells, giving these cells a higher sensitivity to stimulation by IL-1β [4,5]. IL-1 has especially been shown to trigger chondrocyte apoptosis [3]. Thus, IL-1β is commonly used as a modulating and chondrocyte apoptosis-inducing agent [6–8]. It has been found that chondrocyte death by apoptosis is associated with the initiation and severity of articular cartilage degradation [9], and chondrocyte apoptosis increases in human OA cartilage [10]. Chondrocyte apoptosis is mainly responsible for the progressive cartilage degeneration in OA and gradually becomes one of the potential therapeutic targets for OA [9–12]. Therefore, it is necessary to clarify the molecular pathways involved in chondrocyte apoptosis in OA.

It is well known that elements of the cholinergic system, including acetyltransferase, acetylcholinesterase, and acetylcholine receptors (AChRs), are expressed in a large array of non-neural cells including chondrocytes, osteocytes, endothelial cells, monocytes, and epithelial cells [13–16]. Nicotine, an agonist of the nicotinic acetylcholine receptors (nAChR), could confer resistance to apoptosis and promote the proliferation of various types of cells via different cellular signal transduction pathways [17].

Akt is a serine/threonine protein kinase that can be phosphorylated and activated by extracellular factors in a phosphatidylinositol 3-kinase (PI3K)-dependent fashion. As a potent inhibitory signal for apoptosis, Akt plays an important role in regulating

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chondrocyte apoptosis and survival and potentially preventing OA [12,18,19]. 17β-Estradiol promotes cell proliferation in chondrocytes from a rat OA model via the PI3K/Akt pathway [12]. The increased expression of the Akt inhibitor TRB3 in OA chondrocytes inhibits insulin-like growth factor 1-mediated cell survival and proteoglycan synthesis [19]. Recent studies found that nicotine induced cancer cell proliferation by activating PI3K/Akt signaling, which is mediated by the alpha7-nACh receptor [20,21]. However, whether nicotine modulates chondrocyte proliferation and the link between the regulatory mechanism of nicotine, apoptosis and PI3K/Akt signaling in chondrocytes remain unclear.

Our results showed that nicotine neutralized the effect of IL-1β on chondrocytes by activating the PI3K/Akt signaling pathway, including the PI3K/Akt/Bcl-2 pathway for blocking cell apoptosis and the PI3K/Akt/p70S6K/S6 pathway for promoting protein synthesis. These data suggest that the PI3K/Akt signaling pathway plays a critical role in the nicotine-mediated inhibition of IL-1β-induced rat chondrocyte apoptosis.

2. Materials and methods

2.1. Reagents and antibodies

LY294002 and antibodies against Akt, p-Akt-Ser473, GAPDH, p-mTor, p-S6, p-p70S6k, Bcl-2, Bcl-xl, TIMP-1 and MMP-13 were purchased from Cell Signaling Technology Inc. (Beverly, MA, USA) and Santa Cruz Biotechnology (Santa Cruz, CA, USA). Recombinant rat IL-1β was purchased from PeproTech (Rocky Hill, NJ, USA). Collagenase II and DMEM/F12 were obtained from Gibco (Carlsbad, CA, USA). Nicotine, trypsin and the rest of the reagents were purchased from Sigma–Aldrich (Missouri, USA).

2.2. Isolation and culture of rat chondrocytes

Neonatal male Sprague–Dawley rats (within 24 h after birth) were killed after approval of the Ethical Committee of the Medical School, University of Xiamen, and the articular cartilages were removed under sterile conditions. Rat articular chondrocytes were cultured as previously described [12,22].

2.3. Stimulation with IL-1β and treatment with nicotine

With the cells at subconfluence, the medium was changed to DMEM/F12 supplemented with 0.5% FBS, antibiotics and recombinant IL-1β (10 ng/ml) for 2 h. Then, cells were treated with the indicated concentrations of nicotine or Ly294002 and harvested at different treatment durations as required to be subjected to different experimental procedures.

2.4. Cell viability analysis

Cells were plated in 96-well plates (3×10^3 cells/well) and starved with non-serum medium for 6 h. The medium was then replaced with fresh culture medium and the cells were exposed to IL-1β for 2 h alone or sequentially treated with different concentrations of nicotine for 24 h further, followed by a 3-(4,-dimethylthiazol-2-y)-2,5-diphenyl-tetrazolium bromide (MTT) assay [23]. The product was then dissolved in dimethylformamide and quantified spectrophotometrically at a wavelength of 490 nm with a reference wavelength of 630 nm. The OD values correspond to the number of viable cells.

2.5. Immunofluorescence assay

Cells were treated with or without nicotine. Then, the harvested cells were stained with 50 μg/ml 4,6-diamidino-2-phenylindole (DAPI) and observed using fluorescence microscope as previously described [12].

2.6. Protein extraction and western blotting analysis

Cells collected by centrifugation were lysed as previously described [7]. Protein extracts were subjected to SDS–PAGE (8–10%) and transferred to a nitrocellulose membrane for western blotting analysis [12]. The signal was detected using a chemiluminescent detection system according to the manufacturer's instructions (Pierce, USA).

2.7. Statistical analysis

All experiments were done at least three times to ascertain the reproducibility of the results. The Student's *t* test and one-way ANOVA were used to calculate statistical significance.

3. Results

3.1. Nicotine modulates cell viability and apoptosis through activating PI3K/Akt signaling in IL-1β-stimulated rat chondrocytes

We investigated the induction effects of IL-1β on chondrocyte apoptosis. When exposed to IL-1β (10 ng/ml) for 2 h, the cell viability was reduced compared to the control group, as determined by an MTT assay (Fig. 1A, $P < 0.05$). Meanwhile, the percentage of apoptotic cells was determined by DAPI staining, a common method used to observe the apoptotic nuclear morphology (such as nuclear condensation and fragmentation). The apoptotic cells displayed condensed chromatin that is brightly and uniformly stained by DAPI. The proportion of cells exhibiting nuclear chromatin condensation increased in IL-1β-stimulated rat chondrocyte (Fig. 1B, $P < 0.05$). Thus, IL-1β could induce chondrocyte apoptosis. Subsequently, the effect of nicotine on IL-1β-stimulated rat chondrocytes was evaluated. As shown in Fig. 1C, IL-1β-stimulated chondrocytes were treated with different doses of nicotine (10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-4} and 10^{-2} mol/L) for 24 h, after which the cell viability increased significantly ($P < 0.05$). Of the six nicotine doses used (10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-4} and 10^{-2} mol/L), 10^{-7} mol/L nicotine showed the most significant protection effect. Nicotine (10^{-7} mol/L) significantly suppressed IL-1β-induced apoptosis (Fig. 1D, $P < 0.05$).

To determine whether PI3K/Akt signaling was involved in the protective effect of nicotine on IL-1β-stimulated rat chondrocytes, protein levels of p-Akt and Akt were assessed in the presence or absence of nicotine. The western blotting analysis showed that different does of nicotine dramatically up-regulated p-Akt expression without alteration of the total Akt level in IL-1β-stimulated rat chondrocytes, and the p-Akt level appeared to be saliently enhanced by nicotine at a dose of 10^{-7} mol/L (Fig. 1E, $P < 0.05$). The nicotine-mediated (10^{-7} mol/L) increase in the p-Akt level in IL-1β-stimulated chondrocytes was time dependent (5, 30, 60, 120 and 360 min of nicotine treatment) (Fig. 1F, $P < 0.05$). Taken together, these findings show that nicotine can confer protection against IL-1β-induced apoptosis in rat chondrocytes, triggering the activation of PI3K/Akt signaling in IL-1β-stimulated rat chondrocytes.

3.2. Nicotine reduces IL-1β-induced apoptosis of rat chondrocytes via the PI3K/Akt/Bcl-2 pathway

To provide further evidence for a critical role of PI3K/Akt signaling in the anti-apoptotic effect of nicotine in IL-1β-stimulated rat chondrocytes, the PI3K-specific inhibitor LY294002 was used. Similar to our previous results, treatment with nicotine (10^{-7} mol/L) resulted in a significant decrease in cell apoptosis determined by

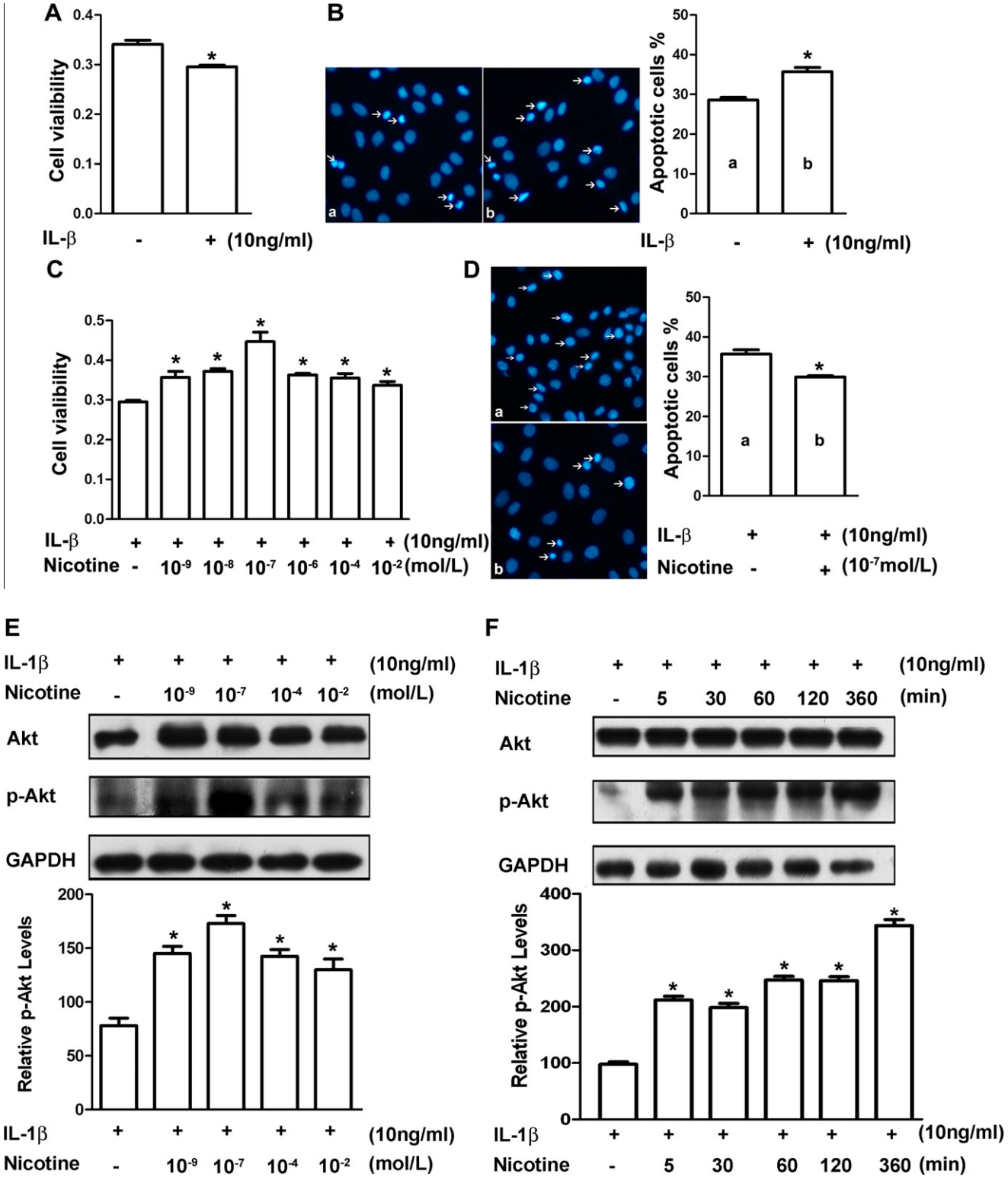

Fig. 1. Nicotine modulates cell viability and apoptosis through activating PI3K/Akt signaling in IL-1β-stimulated rat chondrocytes. (A and C) Cells were treated with IL-1β (10 ng/L) for 2 h alone or followed by treatment with different does of nicotine (10^{-9}, 10^{-8}, 10^{-7}, 10^{-6},10^{-4} and 10^{-2} mol/L) for 24 h, and the cell viability was measured by the MTT assay according to the Section 2. (B and D) Cells were treated with IL-1β (10 ng/L) for 2 h alone or followed by treatment with nicotine (10^{-7} mol/L) for 24 h and stained with DAPI as described in the Materials and Methods section. The apoptotic cells were observed and counted using fluorescence microscopy. (E) Cells were pretreated with IL-1β (10 ng/L) for 2 h prior to treatment with different doses of nicotine (10^{-9}, 10^{-8}, 10^{-7}, 10^{-6},10^{-4} and 10^{-2} mol/L) for 6 h, and the protein expression of Akt and p-Akt was detected by western blotting using rat anti-Akt, p-Akt and GAPDH antibodies. (F) Cells were pretreated with IL-1β (10 ng/L) for 2 h prior to treatment with nicotine (10^{-7} mol/L) for the indicated time, and the protein expression of Akt and p-Akt was detected by western blotting using rat anti-Akt, p-Akt and GAPDH antibodies. The blots were normalized to an endogenous protein (GAPDH). The values represent the mean ± S.E.M. of three to five independent experiments, each yielding similar results ($^{*}p < 0.05$ compared to the control group).

DAPI staining (Fig. 2A, $P < 0.05$), a remarkably increased cell viability (Fig. 2B, $P < 0.05$), as well as an increase in the p-Akt level (Fig. 2C, $P < 0.05$). The addition of LY294002 (25 mmol/L) partly blocked the protective effect of nicotine on cell apoptosis (Fig. 2A, $P < 0.01$; Fig. 2B, $P < 0.05$, versus those treated with nicotine) and inhibited the nicotine-induced increase of p-Akt levels (Fig. 2C, $P < 0.05$ versus those treated with nicotine), indicating the involvement of PI3K/Akt signaling in nicotine-mediated protection against IL-1β-induced chondrocyte apoptosis. It has been reported that the activation of PI3K/Akt regulates cell apoptosis through the regulation of pro- and anti-apoptotic proteins, including the Bcl-2 family in mitochondria, such as Bcl-2 and Bcl-xl. Thus, the expression level of both Bcl-2 and Bcl-xl was evaluated in rat chondroytes. Treatment with nicotine elevated the expression level of Bcl-xl and Bcl-2 protein in IL-1β-stimulated rat chondrocytes (Fig. 2C, $P < 0.05$). LY294002 attenuated nicotine-mediated up-reg-

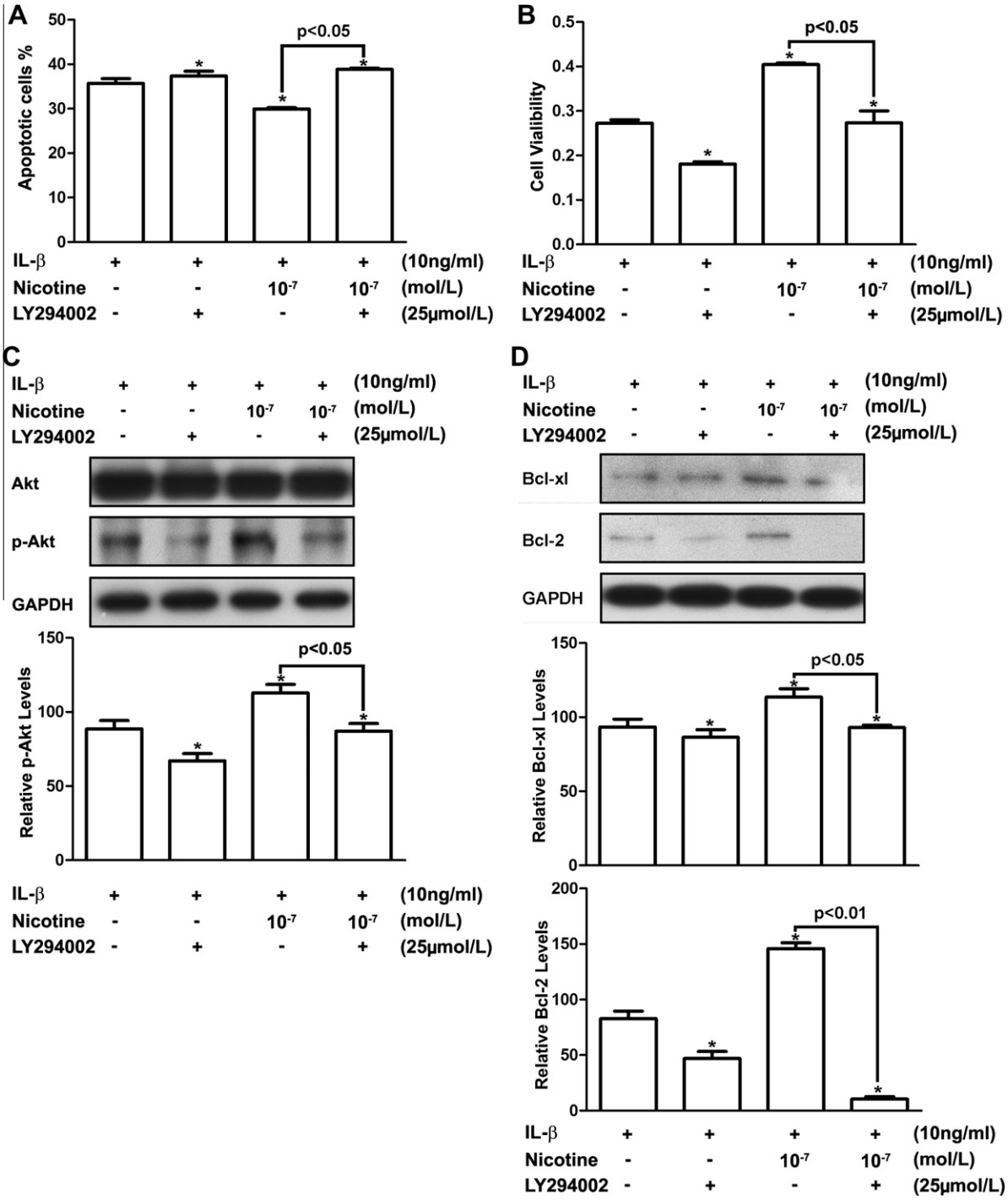

Fig. 2. Nicotine inhibits cell apoptosis via the PI3K/Akt/Bcl-2 pathway in IL-1β-stimulated rat chondrocytes. IL-1β-stimulated chondrocytes were pretreated with or without LY294002 (25 μmol/L) for 1 h, followed by nicotine (10^{-7} mol/L) for 6 h. (A) Cells were stained with DAPI as described in the Materials and Methods section. The apoptotic cells were observed and counted using fluorescence microscopy. (B) The cell viability was measured by the MTT assay according to the Materials and Methods section. (C) The protein expression of Akt and p-Akt was detected by western blotting using rat anti-Akt, p-Akt and GAPDH antibodies. (D) The protein expression of Bcl-2 and Bcl-xl was detected by western blotting using rat anti-Bcl-2, Bcl-xl and GAPDH antibodies. The blots were normalized to an endogenous protein (GAPDH). The results represent the mean ± S.E.M. of three to five independent experiments, each yielding similar results (*$p < 0.05$ compared to the control group).

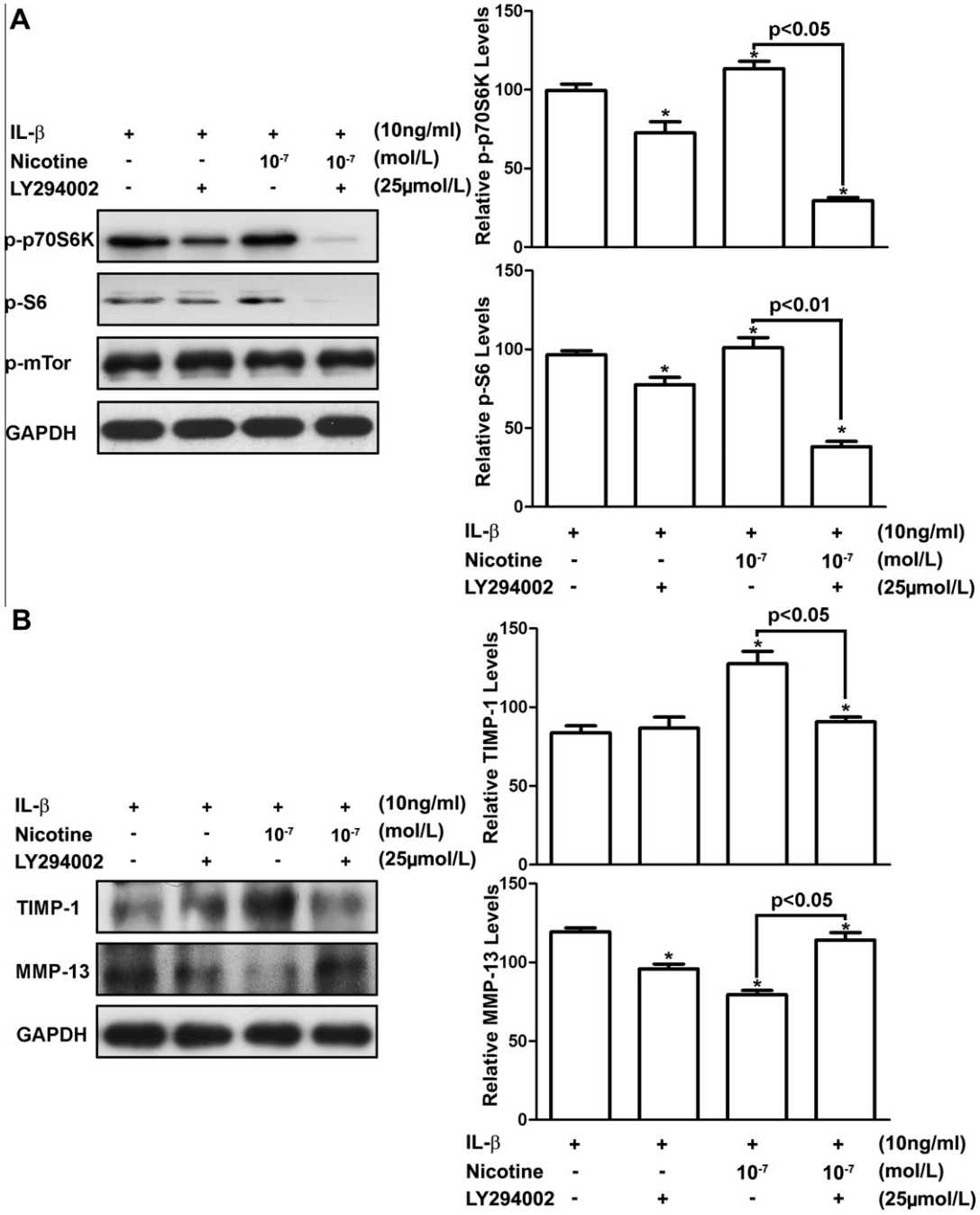

Fig. 3. Nicotine enhances protein synthesis via the PI3K/Akt/p70S6K/S6 pathway in IL-1β-stimulated rat chondrocytes. IL-1β-stimulated chondrocytes were pretreated with or without LY294002 (25 μmol/L) for 1 h, followed by nicotine (10^{-7} mol/L) for 6 h. A, The protein expression of p-mTor, p-p70S6 K and p-S6 was detected by western blotting using rat anti-p-mTor, p-p70S6 K, p-S6 Bcl-2, and GAPDH antibodies. B, The protein expression levels of MMP-13 and TIMP-1 were detected by western blotting using rat anti-MMP-13, anti-TIMP-1 and GAPDH antibodies. The blots were normalized to an endogenous protein (GAPDH). The results represent the mean ± S.E.M. of three to five independent experiments, each yielding similar results (*$p < 0.05$ compared to the control group).

ulation of Bcl-2 and Bcl-xl protein expression (Fig. 2C, $P < 0.01$ and $P < 0.05$, versus those treated with nicotine). The data indicated that nicotine might protect rat chondrocytes against IL-1β-induced apoptosis via PI3K/Akt/Bcl-2 pathway activation in IL-1β-stimulated rat chondrocytes.

3.3. Nicotine enhances protein synthesis via the PI3K/Akt/p70S6K/S6 pathway in IL-1β-stimulated rat chondrocytes

In addition to regulating the expression of anti-apoptotic genes, PI3K/Akt signaling has also been reported to modulate protein synthesis through phosphorylation of its substrates such as mammalian target of rapamycin (mTor), p70 S6 kinase (p70S6K) and S6. The phosphorylation level of mTor, p70S6K and S6 in IL-1β-stimulated chondrocytes was evaluated. The level of p-p70S6K and p-S6 significantly increased after treatment with nicotine (10^{-7} mol/L) for 6 h (Fig. 3A, $P < 0.05$). However, nicotine did not affect p-mTor expression (Fig. 3A). The enhanced effect of nicotine on p-p70S6K and p-S6 was dramatically inhibited by pretreatment with Ly294002 (Fig. 3A, $P < 0.05$ and $P < 0.01$, versus those treated with nicotine), suggesting that nicotine promotes protein synthesis via the PI3K/Akt signaling pathway. Because matrix metallopeptidase

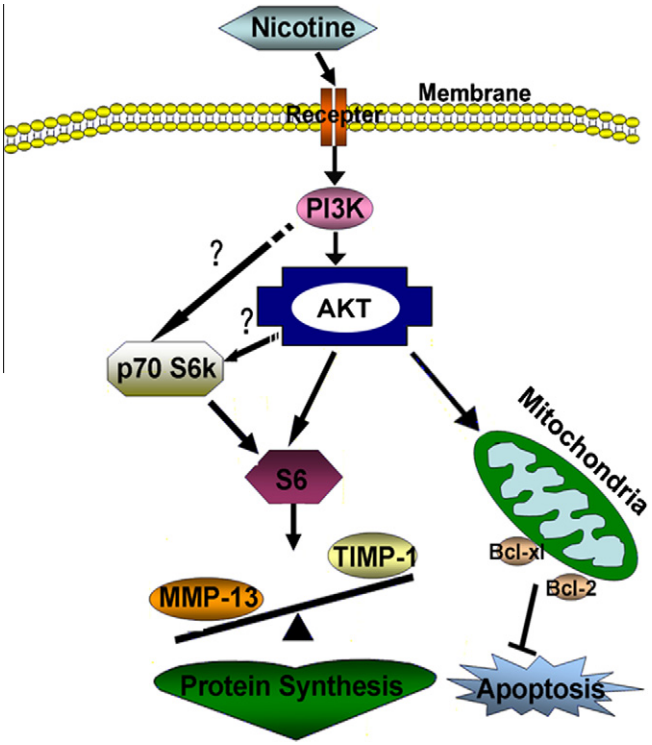

Fig. 4. Proposed model for nicotine modulation of cell apoptosis in IL-1β-stimulated rat chondrocytes. Akt activated by nicotine promotes protein synthesis through the phosphorylation of p70S6K and S6, which might adjust the balance between MMP-13 and TIMP-1, and modulates its substrates in the mitochondria, such as Bcl-2 and Bcl-xl, to inhibit IL-1β-induced apoptosis of rat chondrocytes.

13 (MMP-13) and tissue inhibitor of metalloproteinase 1 (TIMP-1) are directly responsible for damaging the cartilaginous matrix [24,25], we next detected the effect of nicotine on the expression of MMP-13 and TIMP-1 in IL-1β-stimulated chondrocytes. Interestingly, nicotine increased the expression of TIMP-1 and restrained the expression of MMP-13, and such changes were blocked by LY294002, suggesting that nicotine might also regulate the expression of MMP-13 and TIMP-1 via the PI3K/Akt signaling pathway (Fig. 3B, $P < 0.05$ and $P < 0.01$, versus those treated with nicotine). Thus, nicotine enhances protein synthesis via the PI3K/Akt/p70S6K/S6 pathway in IL-1β-stimulated rat chondrocytes.

4. Discussion

There is increasing evidence that smokers have a lower incidence of some inflammatory diseases and the protective effect involves the activation of a cholinergic anti-inflammatory pathway that requires the alpha7nAChR on immune cells [26]. Human growth plate chondrocytes expressed alpha7nAChR, indicating that chondrocytes might be one of the targets of nicotine [16]. In this study, we investigated the effects of nicotine on IL-1β-induced chondrocyte apoptosis and found that nicotine promotes cell viability and resists cell apoptosis in IL-1β-stimulated rat chondrocytes, one type of model OA chondrocytes (Fig. 1). Recently, Ying et al. also found that nicotine significantly promotes cell proliferation and collagen synthesis of chondrocytes derived from normal human cartilage, and slightly less for chondrocytes from OA patients at the same concentration of nicotine [27]. Therefore, nicotine appears to be a protector against cell apoptosis in IL-1β-stimulated rat chondrocytes.

Previous reports have supported the view that PI3K/Akt signaling is the positive regulator for sustaining chondrocyte survival and promoting matrix synthesis [18,19,28–30]. For example, the expression of constitutively active Akt results in significant increases in proteoglycan synthesis and cell survival in human articular chondrocytes [18]. Conversely, inhibition of PI3K/Akt signaling blocks proteoglycan synthesis in chondrocytes and reduces chondrocyte survival [19,28–30]. Here, we showed that nicotine could modulate cell survival and apoptosis of IL-1β-stimulated chondrocytes through triggering PI3K/Akt signaling (Fig. 1). Accordingly, the activation of PI3K/Akt signaling appears to involve, at least in part, the regulatory effect of nicotine on IL-1β-stimulated chondrocytes.

Increased chondrocyte apoptosis is considered a key pathological feature of OA [9–11]. Recent findings suggested that OA chondrocytes exhibit a loss of mitochondrial function, which precedes the classic signs of apoptosis [31,32]. The pro-apoptotic Bcl-2 family protein, Bad, translocates from the cytosol to mitochondria in response to death signaling. Bcl-2, Bcl-xl and Bad constitute a complex to sustain the balance between pro- and anti-apoptotic members. Phosphorylation of Bad by Akt triggers its dissociation with the Bcl-2/Bcl-xl complex and leads to the loss of its pro-apoptotic function [33]. Nicotine could protect cells against apoptosis by activating the PI3K/Akt/Bcl-2 pathway in some lung cancer cell lines. For example, short-term nicotine exposure moderately activated mitogenic signaling pathways (such as protein kinase C and Akt) and enhanced the expression of Bcl-2, accompanied with increased resistance to cisplatin-induced apoptosis [34]. Nicotine also has been shown to up-regulate Akt-mediated anti-apoptotic X-linked inhibitor of apoptosis protein and phosphorylated pro-apoptotic Bcl-2-antagonist of cell death [35]. Our data indicate that nicotine increases the protein level of Bcl-2 and Bcl-xl by activating PI3K/Akt signaling. It is very likely that nicotine induces phosphorylation of Bad by Akt and results in its disassociation from the Bcl-2/Bcl-xl complex, thus attenuating the pro-apoptotic function of Bad, eventually leading to apoptosis suppression in IL-1β-stimulated chondrocytes (Fig. 2).

PI3K/Akt/mTor activated p70S6K in a phosphorylation cascade, which subsequently phosphorylates S6 on Ser235/Ser236, one of the 40S ribosomal subunits that promote protein synthesis [36]. However, our data showed that activation of PI3K/Akt signaling by nicotine did not affect mTor phosphorylation, and the pretreatment of LY294002 only blocked nicotine-induced activation of p70S6K (Thr389) and S6 (Ser235/236) but not p-mTor. This suggests that mTor is not involved in the nicotine-mediated activation of p70S6K and S6. S6 possesses the RxRxxS/T motif, which can be phosphorylated by AGC kinase family members such as Akt, RSK and p70S6K, indicating that the PI3K/Akt-mediated phosphorylation of S6 could be accomplished in an mTor-independent manner in IL-1β-stimulated chondrocytes, possibly through p70S6K and Akt [37–40].

Increasing evidence suggests a role for PI3K/Akt in the regulation of protein synthesis by MMP-13 and TIMP-1 signaling. For example, PI3K, Akt and p70S6K are required for TGF-β1-mediated regulation of steady-state levels of MMP-13 mRNA in hepatic stellate cells [41]. Chloramphenicol-induced PI3K/Akt phosphorylation might function as a novel mitochondrial stress signal that results in an increase of MMP-13 expression in various cancer cells [42]. TGF-beta induces TIMP-3 gene expression in human chondrocytes partially through the PI3K/Akt pathway [43]. It is also found that nicotine could enhance the expression of cartilage specific type II collagen in chondrocytes isolated from normal human and OA patients [27], implying a regulatory effect of nicotine on the ECM. Thus, it is credible that nicotine activates PI3K/Akt signaling, which sequentially phosphorylates p70S6K and S6 and modulates protein synthesis such as MMP-13 and TIMP-1 to enhance the synthesis of ECM in IL-1β-stimulated chondrocytes. In addition, the effect of LY294002 on the expression of TIMP-1 and MMP-13 further sup-

ports the requirement of the PI3K/Akt pathway in the nicotine-mediated induction of these genes. This suggests that nicotine promoted the synthesis of cartilage ECM components via activation of PI3 K/Akt signaling in IL-1β-stimulated chondrocytes.

Based on our data and previous studies by others, we propose a model illustrating the regulatory mechanism of nicotine in IL-1β-induced chondrocyte apoptosis through PI3K/Akt signaling (Fig. 4). In this model, nicotine-mediated activation of PI3K/Akt signaling reduces IL-1β-induced apoptosis by regulating Bcl-2/Bcl-xl expression. In addition, it also triggers the phosphorylation of S6 through p70S6K or Akt, which regulates protein synthesis including the increase of TIMP-1 and the repression of MMP-13 for balancing ECM degradation and synthesis, contributing to the maintenance of cell survival.

Acknowledgments

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