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Involvement of mitochondrial apoptotic pathway and MAPKs/NF-κB inflammatory pathway in the neuroprotective effect of atractylenolide III in corticosterone-induced PC12 cells

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[ABSTRACT] Atractylenolide III (ATL-III), a sesquiterpene compound isolated from *Rhizoma Atractylodis Macrocephalae*, has revealed a number of pharmacological properties including anti-inflammatory, anti-cancer activity, and neuroprotective effect. This study aimed to evaluate the cytoprotective efficiency and potential mechanisms of ATL-III on corticosterone injured rat phaeochromocytoma (PC12) cells. Our results demonstrate that ATL-III increases cell viability and reduces the release of lactate dehydrogenase (LDH). The results suggest that ATL-III protects PC12 cells from corticosterone-induced injury by inhibiting the intracellular Ca^{2+} overloading, inhibiting the mitochondrial apoptotic pathway and modulating the MAPK/NF-κB inflammatory pathways. These findings provide a novel insight into the molecular mechanism by which ATL-III protected the PC12 cells against corticosterone-induced injury for the first time. Our results provide the evidence that ATL-III may serve as a therapeutic agent in the treatment of depression.

[KEY WORDS] Depression; Atractylenolide III; Corticosterone; Apoptosis; MAPKs; NF-κB

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Introduction

Atractylenolide III (ATL-III) is a sesquiterpene compound isolated from *Rhizoma Atractylodis Macrocephalae*. It has been reported that ATL-III has multiple biological activities, including antiinflammatory activity [1-2], anti-cancer activity, and neuroprotective effect [3]. Related report indicated that ATL-III might possess anti-depression effect. Recent studies demonstrated that ATL-III can significantly ameliorate learning and memory impairment induced by chronic homocysteine administration in rats [4] and prevent the neuronal apoptosis caused by glutamate [3, 5]. Our previous study demonstrated that atractylenolqides might be the main antidepressant active ingredients of Xiaoyaosan, a well-known formula for relieving depression [6]. Pharmacology network analysis indicated that ATL-III involve 11 targets in the energy metabolism-immune-signal transmutation relevant biological processes which is releated with depression [7]. Based on the aforementioned findings, we deduced that ATL-III might have an antidepressant effect and neuroprotective effect .

Corticosterone, a kind of glucocorticoids (GCs), is the final product of hypothalamic pituitary-adrenal (HPA) axis, which plays a crucial role in the pathogenesis and progression of depression [8]. High corticosterone level have been reported to induce depression-like behaviors and hippocampal neurons damage in mammals [9]. The phenomenon can be reversed by treatment with antidepressants [10]. Drugs that can protect neurone from corticosterone-induced lesion may possess antidepression effect [11-13]. The PC12 cells is reported to possess typical features of brain neurons [14]. Typically, the cells are abundant in GCs receptors. High concentration of corticosterone was usually cultured with PC12 cells to simulate the state of neurologic damage in depression [15]. Thus, the model has

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been gradually accepted as a tool for establishing an depression model in depression studies or in anti-depression pharmacological research. Wang *et al*. suggested that venlafaxine protected PC12 cells from corticosterone-induced cytotoxicity by regulating the activity of the PI3K/Akt/FoxO3a pathway [16]. Li *et al*. showed that saikosaponin D protected corticosterone-injured PC12 cells by regulating a glucocorticoid receptor (GR)-dependent pathway and mitochondrial GR translocation [17]. Zhou *et al*. demonstrated that curcumin can protect PC12 cells from corticosterone-induced cell death via inhibiting the phosphorylation of extracellular signal-regulated kinase (ERK) [18]. Therefore, the model of corticosterone-induced PC12 cells was used as a tool for the study of antidepressant effect and releated mechanism of ATL-III in the present study.

Increasing evidence has implicated that the activation of mitochondrial apoptotic pathway and mitogen-activated protein kinases (MAPKs) pathway played crucial roles in the development and progression of corticosterone-induced damage [15-18]. Besides, further study showed that ATL-III could inhibited mitochondrial apoptotic pathway in glutamate-induced PC12 cells [3], and inhibited the activation of MAPKs pathway in LPS-treated macrophages [1]. Thus, we deduced that mitochondrial apoptotic pathway and MAPKs-depedent pathway might been involved in antidepressant effect and neuroprotective effect of ATL-III.

The present study was undertaken to examine the effects of ATL-III on corticosterone-induced neurons damage and the underlying mechanisms. In the current study, we investigated the *in vitro* protective effect of ATL-III on corticosterone injured PC12 cells and futher explored potential mechanisms. Our results suggest that ATL-III protects PC12 cells from corticosterone-induced neurotoxicity by inhibiting the intracellular Ca^{2+} overloading, inhibiting the mitochondrial apoptotic pathway and modulating the MAPK/NF-κB inflammatory pathways.

Materials and Methods

Chemicals and drugs

Atractylenolide III, a compound with purity over 98%, was obtained from Chengdu Pufei De Biotech Co., Ltd. (Chengdu, China). Corticosterone with purity over 98.5% was obtained from TCI (Shanghai, China). Fetal bovine serum (FBS), penicillin and streptomycin were purchased from Gibco (Grand Island, USA). RPMI-1640 was purchased from HyClone (GE, USA). Methyl thiazolyl tetrazolium (MTT) was obtained from Sigma Aldrich (St. Louis, USA). Lactate dehydrogenase (LDH) and Hoechst 33324/PI assay kit was obtained from Nanjing Jianchen Bioengineering Institute (Nanjing, China). Annexin V-fluorescein isothiocyanate (FITC) was obtained from BD pharmingen (BD Biosciences Pharmingen, USA). Fluo-4/AMA and JC-1 assay kit were purchased from Biyuntian Bioengineering Institute (Shanghai, China). TNF-α ELISA kit was obtained from Nanjing Jianchen Bioengineering Institute (Nanjing, China). Primary antibodies for β-actin, cyt C, Bax, Bcl-2, caspase-3, p65, IκBα, p-JNK, p-p38, and p-ERK1/2 were purchased from Cell Signaling (CST, USA).

Cell culturing and treatment

Rat pheochromocytoma cells (PC12) were gained from Peking Union Medical College, Beijing. The cells were cultured in RPMI medium supplemented with 10% (*V*/*V*) heat-inactivated FBS, 100 μg·mL^{-1} streptomycin and 100 U·mL^{-1} penicillin, at 37 °C and 5% CO_2 under a humidified atmosphere.

To find appropriate damaging concentration of corticosterone, PC12 cells were exposed to different concentrations of corticosterone (50, 100, 200, 400, and 800 μmol·L^{-1}) for 48 h. Then the MTT assay was carried out to detect the cell viability. The cell viability reduced to approximately 50% when treated with 400 μmol·L^{-1} corticosterone for 48 h. Therefore, the PC12 cells co-incubated with 400 μmol·L^{-1} corticosterone for 48 h were employed in subsequent experiments.

To study the protective effect of ALT-III on corticosterone-induced PC12 cells, the treatment groups included: untreated, corticosterone (400 μmol·L^{-1}), and corticosterone (400 μmol·L^{-1}) plus ALT-III groups. Experiments were preformed for 24 h after the cells were plated. ALT-III were applied for 2 h prior to the treatment with corticosterone.

Cell viability assay

To confirm the neuroprotective effect and active dose of ALT-III, MTT colorimetric assay was performed to quantify the cell viability. In brief, PC12 cells (2×10^4 cells/well) were seeded on 96-well plates (coated with 0.01% PLL). After 24 h incubation, the medium was replaced with RPMI medium and the PC12 cells were treated with 400 μmol·L^{-1} corticosterone or 400 μmol·L^{-1} corticosterone plus various concentrations of ALT-III (1, 10 and 20 μmol·L^{-1}), for another 24 h. Then the fresh medium containing 0.5 mg·mL^{-1} MTT was added and incubated at 37 °C for another 4 h. The supernatant was then aspirated, and 100 μL of DMSO were added to dissolve the formazan. The absorbance was measured at 570 nm using a microplate reader (BioTek, USA).

Lactate dehydrogenase (LDH) release assay

LDH activity was determined with a LDH diagnostic kit. In brief, PC12 cells (1×10^5 cells/well) were seeded on 24-well plates (coated with 0.01% PLL). After cell treatment as described in section 2.2, the medium was collected and the cell pellets were lysed with 0.5 mL of lysis buffer containing 1% Trition X-100 for 30 min at 37 °C. The suspension was centrifuged at 12 000 r·min^{-1} and supernatants were collected to determine the amount of released intracellular LDH. The absorbance was measured at 450 nm using a microplate reader (BioTek, USA). The release of LDH was calculated according to the equation: LDH released = [LDH in the medium/(LDH in the medium + LDH in the cell)] × 100 [19].

Apoptosis detection by flow cytometry

The stages of apoptosis was analyzed using an Annexin-V-FITC/PI apoptosis kit. In brief, PC12 cells were cul-

tured on 6-well plates at a density of 4×10^5 cells/well. After cell treatment as described above, the cells were washed twice with PBS and then resuspended in 200 μL of binding buffer. The cells were incubated with FITC-labelled Annexin V (5 μL) and PI (10 μL) in the darkness at room temperature for 20 min. Cell apoptosis rate was determined on a FACS Culibur flow cytometer (BD Bioscience, San Jose, CA, USA) [20].

Hoechst 33342 and PI double staining

To further investigate the anti-apoptosis effects of ALT-III, Hoechst 33342 and PI double fluorescent staining were carried out. Briefly, PC12 cells were seeded on 6-well culture plates. After the treatment as described above, the cells were co-incubated with Hoechst 33342 (5 $mg \cdot mL^{-1}$) for 10 min in darkness. Followed twice washes with PBS, the cells were incubated with PI (1 $\mu g \cdot mL^{-1}$) for an another 10 min, and observed by inverted fluorescence microscopy (IX71, Olympus, Japan).

Measurement of mitochondrial membrane potentials (MMPs)

Changes in the mitochondrial transmembrane potential in the corticosterone-treated PC12 cells were determined by flow cytometry with JC-1. Briefly, PC12 cells were cultured on 6-well plates at a density of 4×10^5 cells/well. After cell treatment as described above, they were suspended in medium at a density of 1×10^6 cells/mL and then incubated with JC-1 (2 $mmol \cdot L^{-1}$) in darkness for 30 min. Followed twice washes with PBS, the cells were analysed by FACS Calibur flow cytometry (BD Bioscience, San Jose, CA, USA). The intensity of red fluorescence was served as a indicator of mitochondrial membrane potential of the cells [14].

Measurement of intracellular Ca^{2+} concentration

The intracellular Ca^{2+} concentration was determined with Fluo-4/AM. Briefly, PC12 cells (4×10^5 cells/well) were seeded on 24-well plates and treated as described above. After the treatment, the cells were harvested and centrifuged to remove the supernatant. The cells were further incubated with Fluo-4/AM (5 $\mu mol \cdot L^{-1}$) at 37 °C for 30 min and then washed twice with D-hanks. The fluorescence intensity was measured by FACS Calibur flow cytometry (BD Bioscience, San Jose, CA, USA) [14].

Measurement of TNF-α secretion

PC12 cells (4×10^5 cells/well) were cultured on 24-well plates and treated as described above. After the treatment, culture medium were collected. The levels of inflammatory mediators TNF-α in culture medium were measured by ELISA kits, according to the manufacturer's instructions. The absorbance was measured at 450 nm using a microplate reader (BioTek, USA), and values were calculated according to standard curves [21].

Western blot analysis

PC12 cells (2×10^6 cells/dish) were cultured on 100 mm dishes and cultured for 24 h. After cell treatment as described above, the PC12 cells were collected and washed twice with PBS. The nuclear and cytosolic fractions were fractionated by the Nuclear and Cytoplasmic Protein Extraction kit (Beyotime, Shanghai, China). Total proteins were extracted using the RIPA lysis buffer with 1% PMSF as described previously, and then centrifuged. The concentrations of protein were measured by the Bradford protein assay. Samples cantaining 50 μg protein were separated on 12% SDS-PAGE gels. Then the proteins were transferred onto PVDF membranes, followed by blockage with non-fat milk for 2 h at room temperature. Then the PVDF membranes were incubated with the primary antibodies overnight at 4 °C, followed by incubation with the fluorescent secondary antibodies for another 2 h at room temperature. The membranes were analyzed on a fluorescent scanner (Odclyssey CLX, Gene company limited, USA).

Statistical analysis

Statistical analysis was performed by SPSS 16.0 software, all data were expressed as mean ± SD. Multiple group comparisons were performed using one-way ANOVA. The value of $P < 0.05$ was regarded significant difference. All experiments were performed for three times.

Results

Atractylenolide III protects PC12 from corticosterone-induced cytotoxicity

The neurocytotoxicity of ALT-III on PC12 cells was evaluated. The cell viability were not significantly changed when treated with ALT-III within 1–20 $\mu mol \cdot L^{-1}$. However, 40 $\mu mol \cdot L^{-1}$ of ALT-III showed significant difference as compared with the control group (Data not shown). Consequently, 1–20 $\mu mol \cdot L^{-1}$ of ALT-III was selected in the subsequent study. As illustrated in Fig. 1A, treatment with corticosterone (400 $\mu mol \cdot L^{-1}$) for 48 h caused cytotoxicity in PC12 cells, and the survival rate was 49.70% ± 5.61% of non-treated control ($P < 0.01$). However, the relative cell survival rate of PC12 cells increased significantly with ALT-III (1–20 $\mu mol \cdot L^{-1}$) in a concentration-dependent manner. 20 $\mu mol \cdot L^{-1}$ of ALT-III increased the survival rate to 79.81% ± 7.75%, showing the best neuroprotective effect.

A similar neuroprotective effect was observed in the lactate dehydrogenase release analysis. After treatment of PC12 cells with corticosterone for 48 h, the percentage of LDH leakage in corticosterone group was 164.36% ± 3.32% while 100% ± 10.61% in control group (Fig. 1B), showing that the LDH leakage was significantly increased in the corticosterone group ($P < 0.01$). However, pretreatment with ALT-III of 1, 10 and 20 $\mu mol \cdot L^{-1}$ induced a significant decrease in LDH leakage (134.26% ± 11.86%, 124.33% ± 2.96%, 109.51% ± 5.50%, respectively) compared with the corticosterone group ($P < 0.01$).

To further study the protective effect and the possible mechanisms of ALT-III against corticosterone, 20 $\mu mol \cdot L^{-1}$ ALT-III were selected for apoptosis rate, mitochondrial membrane potentials (MMPs), intracellular calcium, and Western blot analysis.

Atractylenolide III inhibits the corticosterone-induced apoptosis in PC12 cells

Annexin V-FITC and PI double staining was performed

to explore anti-apoptosis effect of ALT-III on corticosterone-induced PC12 cells. The cells of Annexin V-positive, PI-negative were defined as early apoptotic cells, and cells with both Annexin V and PI positive were defined as late apoptotic cells. As illustrated in Fig. 1C, the treatment of corticosterone significantly increased the early apoptotic percentage. The percentage of early apoptosis in the control group was 3.56% ± 0.87%. Corticosterone treatment lead to a significant increase of early apoptosis percentage to 26.43% ± 4.13% ($P < 0.01$). On the contrary, pretreatment of PC12 cells with ALT-III at 20 μmol·L^{-1} significantly induced the percentage of early apoptosis to 16.52% ± 2.81% ($P < 0.01$). However, no significant difference was observed in late apoptosis cells. The result demonstrated that ALT-III could inhibit the corticosterone-induced apoptosis in PC12 cells.

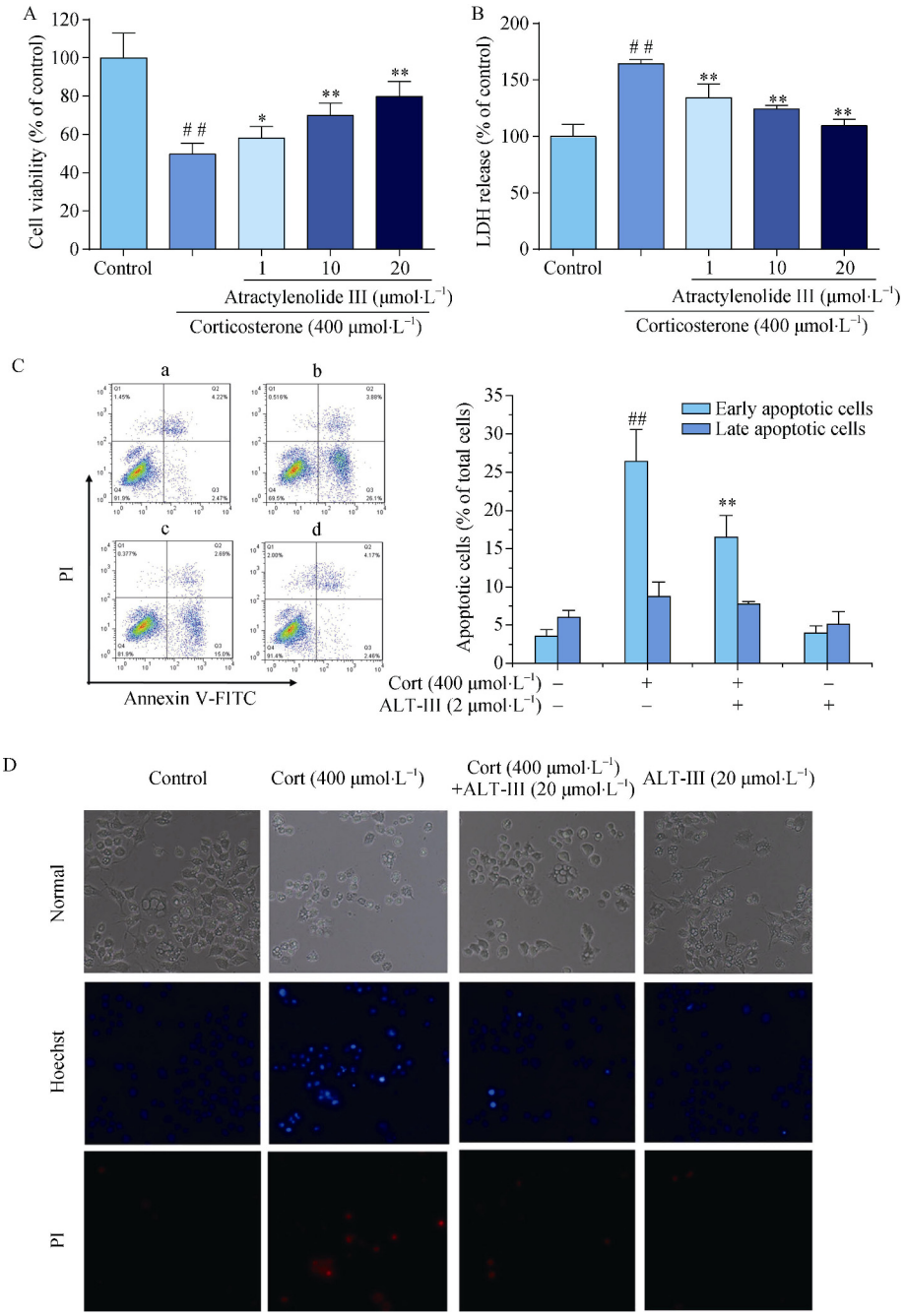

Fig. 1 Protective effect of ALT-III on corticosterone-induced cytotoxicity in PC12 cells. (A) Cell survival determined by the MTT assay ($n = 6$). (B) Effect of ALT-III on corticosterone-induced LDH release in PC12 cells ($n = 6$). (C) Apoptotic rate analyzed by flow cytometry ($n = 3$). a (Control), b (Cort), c (Cort + ALT-III), d (ALT-III). (D) Effect of ALT-III on the cell survival in corticosterone-induced PC12 cells by Hoechst 33342 and PI double staining. Results are presented as means ± SD. $^{\#\#}P < 0.01$ *vs* control group, $^{**}P < 0.01$ *vs* corticosterone-treated group (Cort)

Hoechst 33342 and PI double fluorescent staining were further carried out to investigate the anti-apoptosis effects of ALT-III. The cells of Hoechst 33342 and PI-negative were defined as normal cells, cells of Hoechst 33342-positive, PI-negative were defined as apoptotic cells, and cells of both Hoechst 33342 and PI positive were defined as necrotic cells. As presented in the microphotographs (Fig. 1D), the number of apoptotic cells and necrotic cells significantly increased after treatment with 400 μmol·L^{-1} corticosterone for 48 h. In contrast, the Hoechst 33342 and PI-positive cells was significantly decreased when the cells were dealt with ALT-III. And treatment with individual ALT-III did not lead to nuclear condensation.

The apoptotic pathway involves activation of the proapoptotic protein Bax, followed by the release of cyt C, and activation of caspase-3. To monitor the expression levels of apoptosis-regulated proteins, western blotting was performed. Our results revealed that corticosterone administration decreased the level of Bcl-2, but increased the levels of Bax, cytosolic cytochrome c, and caspase-3 in PC12 cells. Treatment with ALT-III attenuated the corticosterone-induced changes in the levels of apoptosis-related proteins (Fig. 2B–2D). These results showed that the protective effect of ALT-III on corticosterone-induced apoptosis in PC12 cells was regulated through the Bcl-2/Bax signal pathway.

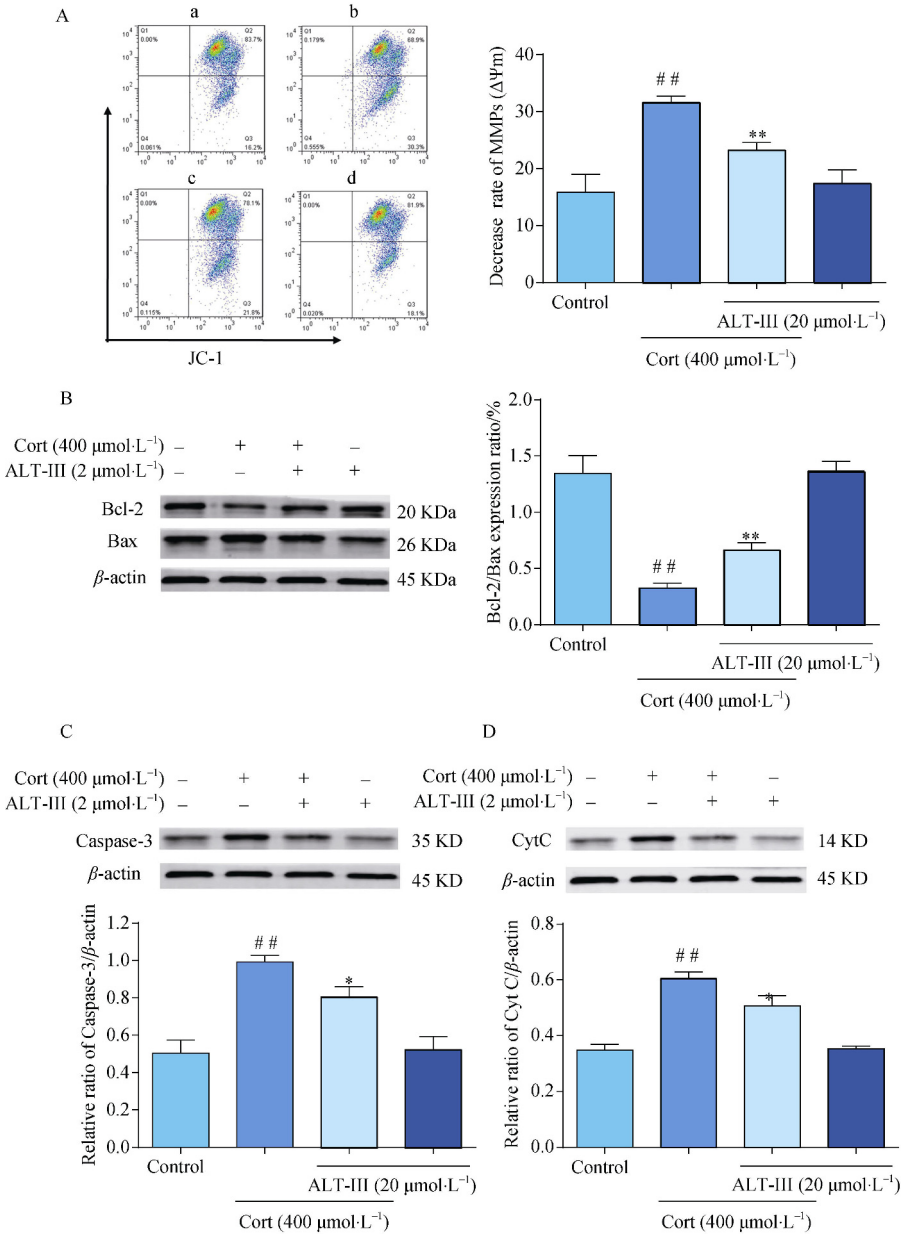

Fig. 2 Effect of ALT-III on the activation of mitochondrial apoptotic pathway in PC12 cells induced by corticosterone. (A) Effect of ALT-III on the corticosterone-induced MMP depolarization in PC12 cells. a (Control), b (Cort), c (Cort + ALT-III), d (ALT-III). (B) The ratio of Bcl-2/Bax. (C) Caspase-3 proteins. (D) Cyt C proteins. Results are presented as means ± SD (n = 3). $^{\#\#}P < 0.01$ *vs* control group, $^{**}P < 0.01$ *vs* corticosterone-treated group (Cort)

Atractylenolide III inhibits the depolarisations MMPs in corticosterone-treated PC12 cells

The effect of ALT-III on ΔΨm injured by corticosterone was assayed by JC-1 staining. Fig. 2A shows that the exposure of PC12 cells to corticosterone resulted in a significant increase in the MMP declining rate compared to that of the control group ($P < 0.01$), indicating that corticosterone caused the depolarisation of ΔΨm. However, pretreatment of PC12 cells with ALT-III at 20 μmol·L^{-1} markedly attenuated the MMP declining rate. Statistical analyses revealed that the MMP decline rate of the corticosterone-treated group increased to 31.60% ± 1.12%, while decreased to 23.19% ± 1.41% in the presence of ALT-III ($P < 0.01$). These results showed that the anti-apoptotic effect of ALT-III was probably due to the recovery of mitochondrial function.

Atractylenolide III attenuates the corticosterone-induced [Ca^{2+}]i overloading in PC12 cells

Fluo-4/AM fluorescence labeling assay was employed to measured the intracellular Ca^{2+} concentration. As illustrated in Fig. 3, after the treatment of PC12 cells with 400 μmol·L^{-1} corticosterone, the ratio of fluorescence intensity of [Ca^{2+}]i in PC12 cells was 271.62% ± 16.66%, and control group was 100% ± 7.53%, demonstrating that significant increase of [Ca^{2+}]i in PC12 cells. By contrast, pretreatment with ALT-III (20 μmol·L^{-1}) significantly decreased the fluorescence intensities of intracellular Ca^{2+} as compared with corticosterone-treated group ($P < 0.01$). The [Ca^{2+}]i concentration of ALT-III group was 194.49% ± 7.04%. The results revealed ALT-III can significantly attenuate the intracellular Ca^{2+} overloading in PC12 cells caused by corticosterone.

ALT-III inhibits the corticosterone-induced phosphorylation of MAPKs in PC12 cells

Mitogen-activated protein kinases (MAPKs), a family of serine/threonine protein kinases, plays a pivotal role in the central nerves system. The c-Jun NH2-terminal protein kinase (JNK), the extracellular signal-regulated kinases (Erks), and the p38 are well-characterized MAPK subfamilies in mammalian cells. In the present study, the expression levels of p-JNK, p-ERK1/2, and p-p38 were monitored to study the effect of ALT-III on MAPKs signaling pathways. Phosphorylations of JNK, ERK1/2, and p38 were significantly increased when treated with corticosterone (Fig. 4, $P < 0.01$). ALT-III significantly suppressed the phosphorylation of JNK, ERK1/2, and p38 in corticosterone-induced PC12 cells ($P < 0.05$, $P < 0.01$, and $P < 0.01$, respectively). The dramatic changes demonstrate that ATL-III protected PC12 cells from corticosterone-induced cytotoxicity by modulating the MAPKs signaling pathways.

ALT-III regulates NF-κB signal pathway and inhibits the release of proinlammatory cytokine in corticosterone-treated PC12 cells

Activation of NF-κB plays a vital role in inflammation and the immune response. The NF-κB pathway is activated by the release of p65 from IκB. To further confirm whether ALT-III modulate PC12 cells survival by affecting corticosterone-induced NF-κB activation, we examined the expression of nuclear NF-κB and its inhibitor IκBα by Western blot. As illustrated in Fig. 5A and Fig. 5B, there was a significant ($P < 0.01$) increase in the NF-κB (p65) accumulation in the nuclear fractions of PC12 cells from the corticosterone-induced when compared with the control PC12 cells. However, pretreatment with ATL-III significantly reduced the level of p65 in nucleus ($P < 0.01$). IκBα degradation is an critical step for NF-κB activation. Our data also showed that the levels of IκBα were significantly decreased following corticosterone treatment ($P < 0.01$), and ALT-III reversed IκBα protein levels ($P < 0.01$). These results demonstrated that the NF-κB signalling pathway plays a crucial role of in the neuroprotective mechanism of ALT-III.

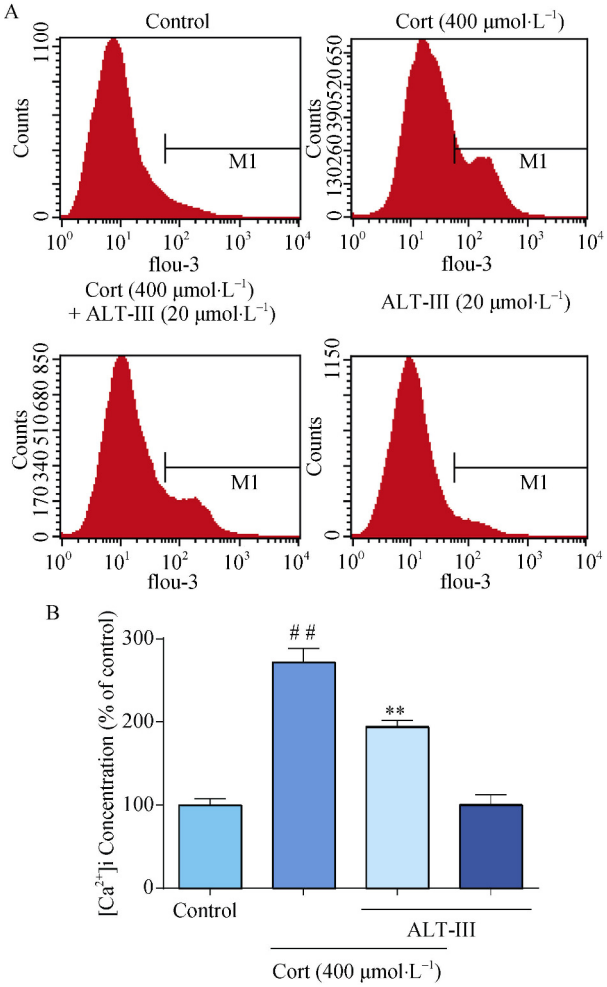

Fig. 3 Effect of ALT-III on the corticosterone-induced PC12 cell intracellular Ca^{2+} influx. (A) After the treatment, the cells were stained with Fluo-4/AM and analyzed with flow cytometry. (B) Statistical analysis of the [Ca^{2+}]i concentration after the treatment of CORT or ALT-III. Results are presented as means ± SD ($n = 3$). $^{\#\#}P < 0.01$ *vs* control group, $^{**}P < 0.01$ *vs* corticosterone-treated group (Cort)

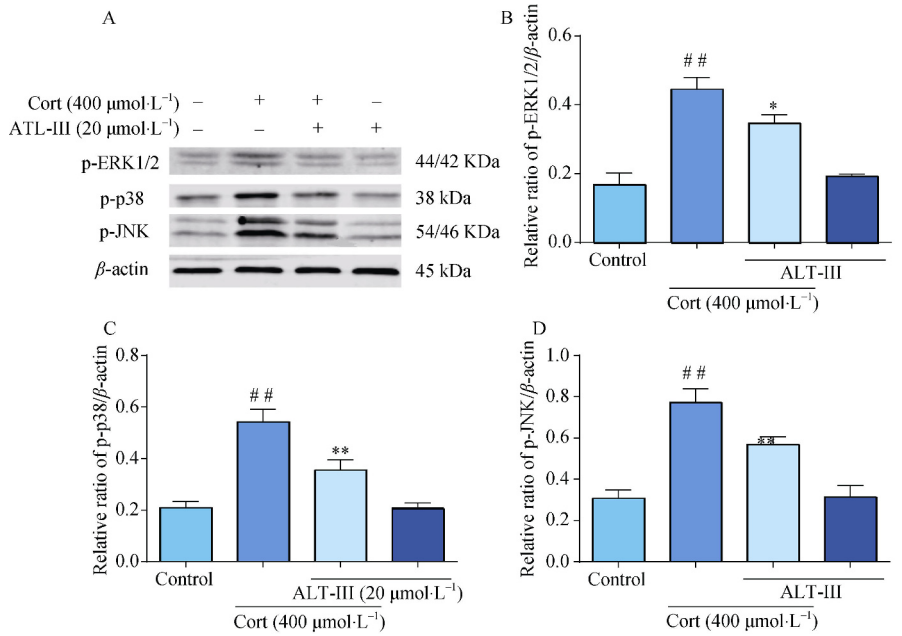

Fig. 4 Effect of ALT-III on the corticosterone-induced phosphorylation of MAPKs in PC12 cells. (A) Representative immunoblots for p-ERK1/2, p-p38 and p-JNK. *β*-actin was used as internal control. (B, C, D) Quantitative analysis of these proteins. Results are presented as means ± SD (*n* = 3). $^{\#\#}P < 0.01$ *vs* control group, $^{**}P < 0.01$ *vs* corticosterone-treated group (Cort)

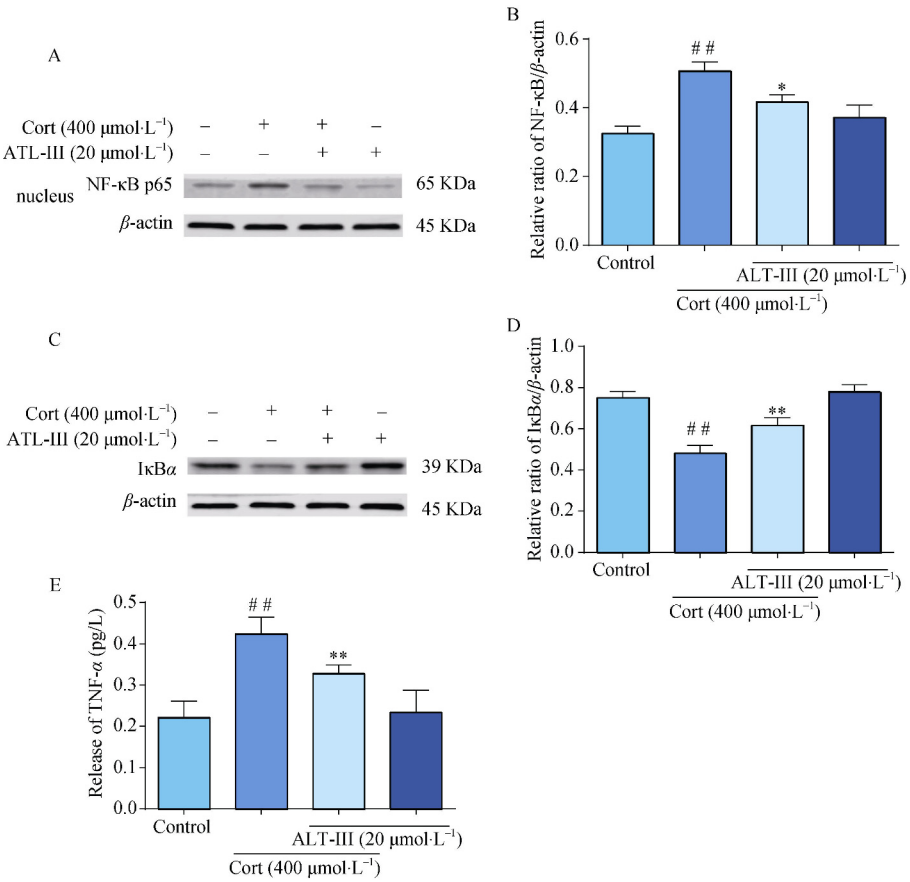

Fig. 5 Effect of ALT-III on the corticosterone-induced activation of NF-*κ*B signalling and TNF-*α* secretion in PC12 cells. (A, C) Representative immunoblots for p65 and IκB*α* in PC12 cells. *β*-actin was used as internal control. (B, D) Quantitative analysis of these proteins. (E) The release of TNF-*α* in corticosterone-treated PC12 cells. Results are presented as means ± SD (*n* = 3). $^{\#\#}P < 0.01$ *vs* control group, $^{**}P < 0.01$ *vs* corticosterone-treated group (Cort)

Inflammatory cytokines play pivotal roles in the pathogenesis of depression. In order to investigate the effect of ALT-III treatment on the inflammatory response in corticosterone-treated PC12 cell, we examined the expression levels of TNF-α in culture medium. As shown in Fig. 5E, after 24 h corticosterone exposure, the levels of TNF-α in the culture medium were significantly elevated ($P < 0.01$) as compared with the control group. When pretreated with ALT-III, TNF-α secretion were remarkably inhibited ($P < 0.01$), indicating that ALT-III could decrease inflammatory response after corticosterone damage.

Discussion

ATL-III, an active sesquiterpene lactone in *Rhizoma Atractylodis Macrocephalae,* have been reported exhibiting neuroprotection in cultured neurons [22-23] and animal stroke model [24]. Liu *et al.* demonstrated that glutamate-induced damage in cultured neurons could be attenuated by ATL-III treatment, which was due to suppression of apoptosis in cultured mice cerebral cortical neurons [3]. Furthermore, it is reported that ATL-III could exert neuroprotective activity on PC12 cells ischemia/reperfusion- like insults [5]. Our current study for the first time demonstrated the neuroprotective effect of ATL-III against corticosterone-induced cytotoxicity in PC12 cells.

The results of MTT analysis demonstrated that ATL-III did not induce neurotoxicity or proliferation effect in PC12 cells within 1–20 μmol·L^{-1}, whereas 40 μmol·L^{-1} ATL-III caused obvious cell damage on cell survival rate. LDH is a cytosolic enzyme exist in mammalian cells. The injury of cell membrane induced a change in the membrane permeability and subsequent a leakage of LDH [25]. Therefore, LDH leakage is usually regarded as a indicator of cell damage. In the current study, we found that corticosterone (400 μmol·L^{-1}) resulted in a significant decrease in cell survival rate and an increase in release of LDH in PC12 cells as compared with that in control. By contrast, ATL-III resulted in a significant increase in the cell survival rate and inhibited the LDH leakage as compared to the CORT group. These results indicated that ATL-III protect the cells from corticosterone-induced damage.

Several evidences showed that the cellular mechanisms of corticosterone- induced lesion in PC12 cells involves the production of reactive oxyen species (ROS) [26], the introduction of mitochondrial dysfunction [17], the deregulation of Ca^{2+} homeostasis [27], the fragmentation of DNA and the activation of several signaling pathways [16, 18]. In the current study, we investigate a protective effect of ATL-III in corticosterone-induced cytotoxicity in PC12 cells.

Calcium plays a crucial role in the development of neurons, and intracellular Ca^{2+} overloading may lead to neuron damage [28]. Several evidence showed that corticosterone-induced cytotoxicity in PC12 cells was closely related to the accumulation of Ca^{2+} [26-27]. The overloading of intracellular Ca^{2+} can result in mitochondrial dysfunction by activating the mPTPs, which induce a cascade of reactions that lead to cell death. In addition, the ΔΨm is considered to be regulated by the abnormal opening of mPTPs [10]. In the current study, we suggested that corticosterone (400 μmol·L^{-1}) resulted in [Ca^{2+}]i overloading, which subsequently caused the depolarisation of ΔΨm in PC12 cells as in previous reports as well. However, these phenomena were attenuated in the presence of ATL-III. These results suggested that ATL-III may exert a neuroprotective effect via the blockade of intracellular Ca^{2+} overloading and mitochondrial dysfunction.

Increasing evidence indicated that apoptosis induced by corticosterone plays a crucial role in hippocampal neuronal lesion *in vivo* and *in vitro* [10, 29]. The mitochondria-dependent apoptosis pathway is one of the main pathways of apoptosis [30-31], which is modulated by members of the Bcl-2 family of proteins [32]. Bcl-2 is an anti-apoptotic protein on the outer mitochondrial membrane, which can protect cells from apoptosis under external stimulation. It exerts a survival function through the inhibition of mitochondrial cytochrome c release [33]. Bax, a pro-apoptotic protein in Bcl-2 family, is generally located in cytoplasm. Bax can influence membrane permeability when the cell is stimulated, resulting in the release of cytochrome c from the mitochondria into the cytosol, which subsequently activates the caspase related apoptosis cascade and cause apoptosis. The ratio of Bcl-2/Bax represents the balance between the pro- and anti-apoptotic proteins of the Bcl-2 family, which is considered as a significant marker of apoptosis [34]. In the current study, using flow cytometry, we observed the increase of apoptotic cells following corticosterone treatment. Moreover, corticosterone administration led to an decrease in ratio of Bcl-2/Bax, an increase in the level of caspase-3, and an increase in the level of cytochrome c. However, on pretreatment with ATL-III in presence of 400 μmol·L^{-1} corticosterone, the apoptotic cells were decreased and the level of apoptotic proteins were reversed, supporting that the neuroprotective activity of ATL-III on corticosterone-induced PC12 cells was involved in the apoptotic pathway. These results are agreed with literature, i.e., the anti-apoptotic effect of ATL-III aganist glutamate-induced neurotoxicity in neurons as in previous reports as well [3].

Mitogen-activated protein kinases (MAPKs) are a family of serine/threonine protein kinases involved in the transduction of neurotrophic signalling from the cell surface to the nucleus [35]. It comprise three distinct signaling pathways, namely, the JNK, the Erks, and the p38 MAPK [36]. Several evidence has shown that modifications of MAPKs signalling pathways could contribute to the pharmacological action of antidepressant therapies [37-38]. Li *et al.* demonstrated that corticosterone could lead to a rapid activation of p38 and JNK in PC12 cells through a PKC-dependent pathway [39]. A recent

study by qPCR also demonstrated that glucocorticoids can affect cell viability and neurite outgrowth result from the over expression of MAPK pathway genes [40]. Our results demonstrated that the phosphorylation levels of p38, ERK1/2, and JNK were significantly increased by corticosterone treatment, and ATL-III at 20 μmol·L^{-1} markedly inhibited the activation of phosphorylated JNK, p38, and ERK1/2.

Depression is reported to be associated with various inflammatory reactions [41]. The NF-*κ*B signaling cascade has been showed to play a crucial role in the generation and regulation of pro-inflammatory cytokines. In normal circumstances, NF-*κ*B forms an inactive complex with I*κ*B*α*. However, I*κ*B*α* undergoes phosphorylation and degradation when exposed to stimuli, causing free NF-*κ*B to translocate to the nucleus and then bind to target genes to promote the expression of proinflammatory cytokines and mediator genes [42-43]. The activation of the NF-*κ*B pathway can also be regulated by MAPKs in response to stress [44]. The activation of the p44/p42 MAPK pathway is reported to regulate NF-*κ*B activity [45]. Craig *et al*. showed that the p38 pathway affected NF-*κ*B activity via the physical association of MKK6 and IKK*β* [46]. Our results demonstrated that corticosterone caused the overexpression of p65, decreased expression of its endogenous inhibitor I*κ*B*α* and increase the release of TNF-*α*, while these changes were reversed by ATL-III treatment. These results are essentially consistent with the finding that the ATL-III inhibited the activation of NF-*κ*B and MAPKs signaling pathways in macrophages as in previous reports as well [1]. Furthermore, the activation of NF-*κ*B is related to the down-regulation of Bcl-2 [47]. It is demonstrated that the Bcl-2 gene may be one of the downstream targets of the NF-*κ*B transcription complex during early B lineage apoptosis [48]. The present study demonstrates that down-regulation of Bcl-2 is medicated by NF-*κ*B activation in corticosterone-induced PC12 cells, while these changes can be reversed by ATL-III treatment.

In conclusion, as summarized in Fig. 6, our results suggest a potential cytoprotective mechanism of ATL-III. The current study provides novel evidence demonstrating that ATL-III protect PC12 cells *in vitro*, likely through the blockage of [Ca^{2+}]i overloading, consequent mitochondrial dysfunction, imbalance of the Bax/Bcl-2 ratio and caspase-3 activation. Furthermore, the protective effects of ATL-III might have been exerted by suppressing the phosphorylation of JNK, p38, and ERK1/2, resulting in blocking of the activation of NF-*κ*B pathway and thereby suppressing the release of proinlammatory cytokine TNF-*α*, which finally induced the blockage of the inflammation cascade. Our study demonstrated that ATL-III exerted a protective effect on corticosterone-induced neurotoxicity, designating ATL-III as a potential agent for therapeutic interventions in depression. However, in this study, we only determined the antidepression effect of ATL-III *in vitro* model. It is worth investigating more precisely in the future the antidepression effect of ATL-III in animal models.

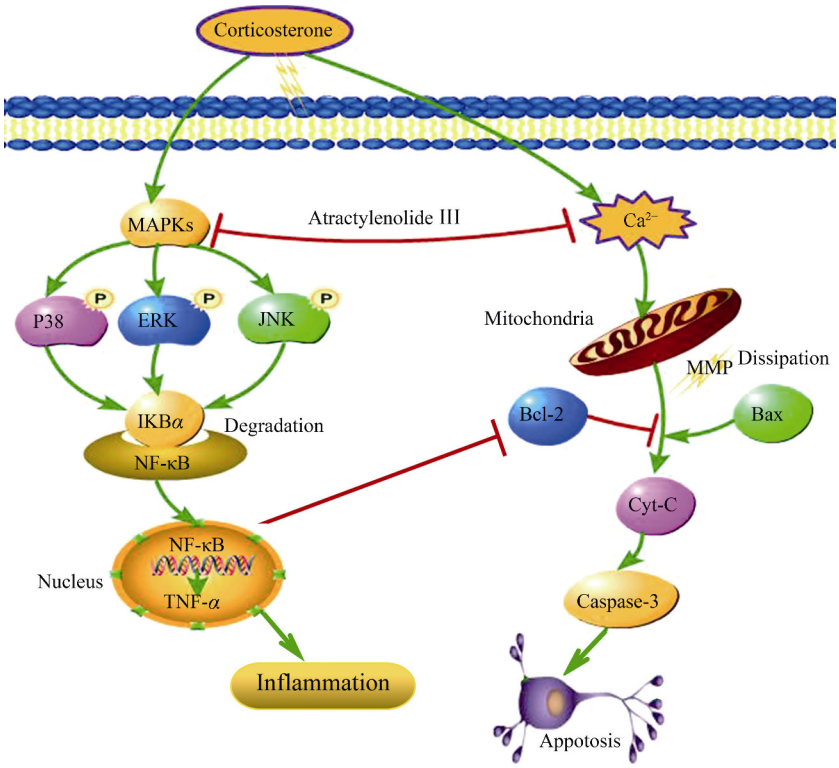

Fig. 6 Putative mechanisms underlying the protective effects of ATL-III on corticosterone-induced toxicity in PC12 cells

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