

Lipoic acid alleviates LPS-evoked PC12 cell damage by targeting p53 and inactivating the NF- κ B pathway

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Lipoic acid (LA) exerts several beneficial effects including anti-inflammatory and antioxidant activity. This research aims to explore the function and mechanisms of LA on lipopolysaccharide (LPS)-induced PC12 cells. PC12 cells stimulated by LPS were used to mimic an *in vitro* inflammatory model of Parkinson's disease. Cell toxicity was determined by a cell counting kit-8 assay after various treatments. The concentrations of tumor necrosis factor (TNF- α), interleukin (IL)-1 β and IL-6 were analyzed by an ELISA kit. The effects of LA on cell apoptosis and cell cycle were measured by flow cytometry. The levels of α -syn, Nurr1 and tyrosine hydroxylase (TH) were tested by immunocytochemistry and ELISA kits. Western blotting assays were used to measure the expression of NF- κ B pathway-related proteins. In PC12 cells, 100 μ mol/mL LA effectively attenuated the upregulation of TNF- α , IL-1 β and IL-6 triggered by LPS; inhibited the increase of cell apoptosis; and relieved the cell cycle arrest induced. Additionally, the increase in α -syn and the decrease in Nurr1 and TH triggered by LPS were reversed by 100 μ mol/mL LA. We also found that the elevated expression of p53 in LPS-induced PC12 cells was suppressed by LA. Significantly, knockdown of p53 enhanced the ameliorative effect of LA on LPS-triggered PC12 cell damage. The increase in levels of p-p65 NF- κ B and p-I κ B α triggered by LPS were suppressed by LA and si-p53 combination treatment. The results indicate that LA can attenuate LPS-triggered inflammation and apoptosis in PC12 cells by targeting the p53/NF- κ B pathway. These findings provide a theoretical basis for the future treatment of inflammation in Parkinson's disease.

Key words: lipoic acid, LPS, NF- κ B, PC12 cells

INTRODUCTION

Parkinson's disease (PD) is a multifactorial degenerative disease that can cause tremor, stiffness of gait and decreased movement, thereby affecting normal life (Lotankar et al., 2017). The major pathology of PD is the loss of dopaminergic neurons in the substantia nigra (Hughes et al., 1992; Bergman and Deuschl, 2002). Several reports have proposed that the inflammatory response participates in the progression of dopaminergic neuron degeneration (McGeer and McGeer, 2008; Frank-Cannon et al., 2009). Tumor necrosis fac-

tor (TNF- α), interleukin (IL)-1 β and IL-6, three critical harmful inflammatory mediators (Nagatsu and Sawada, 2005; Sawada et al., 2006), were found to exacerbate dopaminergic neuron damage (Stoll et al., 2000; More et al., 2013), resulting in chronic inflammation. Such persistent inflammation may further worsen PD. Thus, it is necessary to explore effective molecules that suppress the occurrence of inflammation in PD.

PC12 cells exhibit physiological characteristics with similar properties to dopaminergic neurons (Yagnik and Benzeroual, 2013; Zhong et al., 2015), therefore, we used PC12 cells treated with lipopolysaccharide (LPS) to simulate an *in vitro* model of PD.

Lipoic acid (LA), also called thioctic acid, has become a frequent component in multivitamin formulations, anti-aging supplements, and even pet food (Shay et al., 2009). Additionally, LA has been identified as a potential biological antioxidant, an antidote, and a diabetes drug (Rochette et al., 2015). LA was also shown to prevent age-related cardiovascular disease and cognitive and neuromuscular deficits, as well as being described as a regulator of diverse inflammatory pathways (Scott et al., 1994; Suh et al., 2004). Together these studies highlight the benefits of LA for humans. Moreover, it has been proposed that LA protects dopaminergic neurons in an LPS-evoked PD model (Li et al., 2015). However, the specific molecular mechanism of LA in PD has not been elucidated.

p53, as a stress response gene, has been implicated in various cell death pathways. Some reports have demonstrated that p53 dysregulation is involved in neuronal damage in neurodegenerative disease (Culmsee and Mattson, 2005). Significantly, p53 depletion was presented to be good under circumstance of neuronal damage (Keramiris et al., 2003). A previous study revealed that altering the p53 signaling pathway was essential for acupuncture-induced neuronal protection and dopaminergic neuron function in a mouse model of PD (Park et al., 2015). Moreover, p53 has been shown to be highly expressed in PD brains. Thus, inhibition of p53 expression is a valid potential method for improving PD.

Herein, we constructed an *in vitro* PD model by using LPS-stimulated PC12 cells and our data indicated that LA effectively reduced the LPS-induced inflammation damage of PC12 cells by targeting p53. These findings provide a theoretical basis for investigating the role of LA in PD.

METHODS

Cell culture and treatment

PC12 cells were obtained from the Shanghai Cell Bank of the Chinese Academy of Science (Shanghai, China) and were cultured in DMEM medium followed by 10% fetal bovine serum (FBS), including a 1% penicillin-streptomycin mixture. The cells were placed in a humidified incubator with 5% CO₂ and 95% air at 37°C.

An *in vitro* model of PD was established by stimulation of PC12 cells with 500 ng/mL LPS for approximately 24 h. Different concentrations of alpha-lipoic acid (LA; 50, 100, 200 µmol/mL) were applied to treat PC12 cells to detect the effect of LA in PC12 cells. Small interfering RNA p53 (si-p53) was purchased from Sangon (Shanghai, China) and used to knockdown p53 expression. The si-p53 fragment was loaded into cells

with Lipofectamine 2000 for transfection (Invitrogen, Carlsbad, CA, USA).

Cell viability detection

Exponentially growing PC12 cells were transplanted into 96-well plates for culture and detection. Cell viability was determined using a cell counting kit-8 (CCK-8, Beyotime, China) every 24 h, a mixture of 10 µL CCK-8 reagent and 100 µL of DMEM (excluding FBS) was added into each well, and the cells were cultured for 1.5 h under the above conditions. Finally, the optical density (OD) was observed at 450 nm wavelength.

Enzyme-linked immunosorbent assay (ELISA)

The levels of IL-1β, IL-6, TNF-α, α-syn, Nurr1 and tyrosine hydroxylase (TH) were tested using corresponding ELISA kits (Jianglaibio, China). All operations were carried out based on the manufacturer's instructions.

Cell apoptosis detection

The apoptosis rate was assessed with a flow cytometry assay. First, the cells were seeded into six-well plates and cultured overnight. After different stimulations, the cells were gathered, rinsed with PBS and resuspended in binding buffer. Thereafter, the cells were put into a mixture with 5 µL Annexin V-FITC and 5 µL propidium iodide (PI) and cultured at approximately 25°C for 15 min away from the light. Finally, the rate of apoptosis was determined by flow cytometry within 1 h.

Cell cycle detection

First, the cells were seeded into six-well plates and cultured overnight. After different stimulations, the cells were gathered and rinsed with PBS. Then, the cells were cultured for 30 min at 37°C with RNase A solution treatment. Thereafter, 400 µL PI was loaded and the cells were cultured for 30 min at approximately 25°C. The percentage of cells in the G1, S and G2 phase was analyzed by flow cytometry.

Immunocytochemistry

Coverslips were placed on the bottom of six-well plates and cells were seeded. Afterwards, the culture

solution was removed and the plates were rinsed with PBS. Then, the cells were fixed with 4% paraformaldehyde for 12 min, permeabilized with ice-cold methanol for 2 min and incubated with anti- α -syn, anti-Nurr1 and anti-TH at 4°C for 24 h. After rinsing, the cells were incubated with secondary antibody.

RT-qPCR

First, total RNA was reverse transcribed into cDNA. RT-qPCR solution included 2 μ L cDNA, 5 μ L mixture, 0.5 μ L forward/reverse primer and 2 μ L nanopure water. The cycles of the amplification reaction were as follows: denaturation at 95°C for 10 s, extension at 60°C for 1 min, the reaction completed for 40 cycles. The experiment was performed in replicates. GAPDH was selected as the internal control.

Western blotting

The treated cells were rinsed with PBS and lysed with RIPA solution including 1mM PMSF. The concentration of protein was detected with the BCA method. Next, 20 μ g of sample was added into loading buffer and separated by 10% SDS-PAGE. After transfer, the PVDF membrane was sealed for 2 h at approximately 25°C by using 5% skim milk. Thereafter, the membranes were probed with the primary antibodies against P65 NF- κ B, p-P65 NF- κ B, I κ B α and p-I κ B α or β -actin over night at 4°C. The next day, the membranes were incubated with specific secondary antibody for 2 h at room temperature. Finally, the proteins were developed by enhanced chemiluminescence and quantified by ImageJ software.

Statistical analyses

All data were processed with GraphPad Prism 8.0 and SPSS 22.0 software. The differences among multiple groups were evaluated by one-way ANOVA. $P < 0.05$ was considered statistically significant.

RESULTS

LA effectively reduced toxicity, inflammation, apoptosis and cell-cycle arrest triggered by LPS in PC12 cells

Different concentrations of LPS were applied to stimulate PC12 cells to mimic the inflammatory environment. Cell viability, tested by CCK-8 assay, was re-

duced by LPS (100, 200, 500, 1000 ng/mL) treatment. 500 ng/mL LPS was used for subsequent experiments due to its 50% reduction in cell viability (Fig. 1A, $F_{4,10}=47.559$, $P < 0.05$). Then, different concentrations of LA were utilized to treat PC12 cells after LPS treatment. The data showed that 100 μ mol/mL LA had a higher effect on PC12 cell viability than 50 and 200 μ mol/mL LA under the LPS condition (Fig. 1B, $F_{4,10}=24.376$, $P < 0.05$). The results from the ELISA kit revealed that the increase in IL-1 β (Fig. 1C, $F_{4,10}=35.578$, $P < 0.05$), IL-6 (Fig. 1D, $F_{4,10}=37.42$, $P < 0.05$) and TNF- α (Fig. 1E, $F_{4,10}=68.114$, $P < 0.01$) levels in LPS-induced PC12 cells was reduced by LA (50, 100, 200 μ mol/mL) treatment and the efficiency of 100 and 200 μ mol/mL LA was higher than that of 50 μ mol/mL LA, but there was no significant difference between 100 and 200 μ mol/mL LA treatment.

A flow cytometry assay was applied to detect the effect of LA on PC12 cell apoptosis and cell cycle under LPS stimulation. The increase in PC12 cell apoptosis rate triggered by LPS was suppressed by LA (50, 100, 200 μ mol/mL) stimulation. Compared with 50 μ mol/mL LA treatment, 100 and 200 μ mol/mL LA had the same high efficiency in relieving cell apoptosis (Fig. 2A, $F_{4,10}=105.822$, $P < 0.01$). Moreover, LPS-induced PC12 cell cycle arrest was relieved by LA (50, 100, 200 μ mol/mL) treatment, and 100 and 200 μ mol/mL LA were more efficient than 50 μ mol/mL of LA (Fig. 2B, $F_{4,10}=110.448$, $P < 0.01$). Thus, 100 μ mol/mL of LA significantly alleviated PC12 cell damage induced by LPS.

The expression of α -syn, Nurr1 and TH in LPS-induced PC12 cells was modified by LA

Data from the immunocytochemistry and ELISA assays showed that the expression of α -syn increased, while Nurr1 and TH expression decreased, in LPS-stimulated PC12 cells. However, LA succeeded in suppressing the phenomenon. We observed that the increase in α -syn (Fig. 3A, $F_{4,10}=19.956$, $P < 0.05$; Fig. 3D, $F_{4,10}=12.517$, $P < 0.05$) and decrease in Nurr1 (Fig. 3B, $F_{4,10}=26.999$, $P < 0.01$; Fig. 3E, $F_{4,10}=15.096$, $P < 0.05$) and TH (Fig. 3C, $F_{4,10}=20.386$, $P < 0.05$; Fig. 3F, $F_{4,10}=19.281$, $P < 0.05$) in LPS-stimulated PC12 cells was inhibited by LA (50, 100, 200 μ mol/mL) treatment. Moreover, 100 μ mol/mL LA had the highest efficiency in the regulation of α -syn, Nurr1 and TH expression compared with 50 and 200 μ mol/mL LA.

p53 was confirmed as a target of LA and jointly regulated PC12 cell damage with LA

Based on the importance of the p53 gene in PD and predictions generated by the Comparative Toxicog-

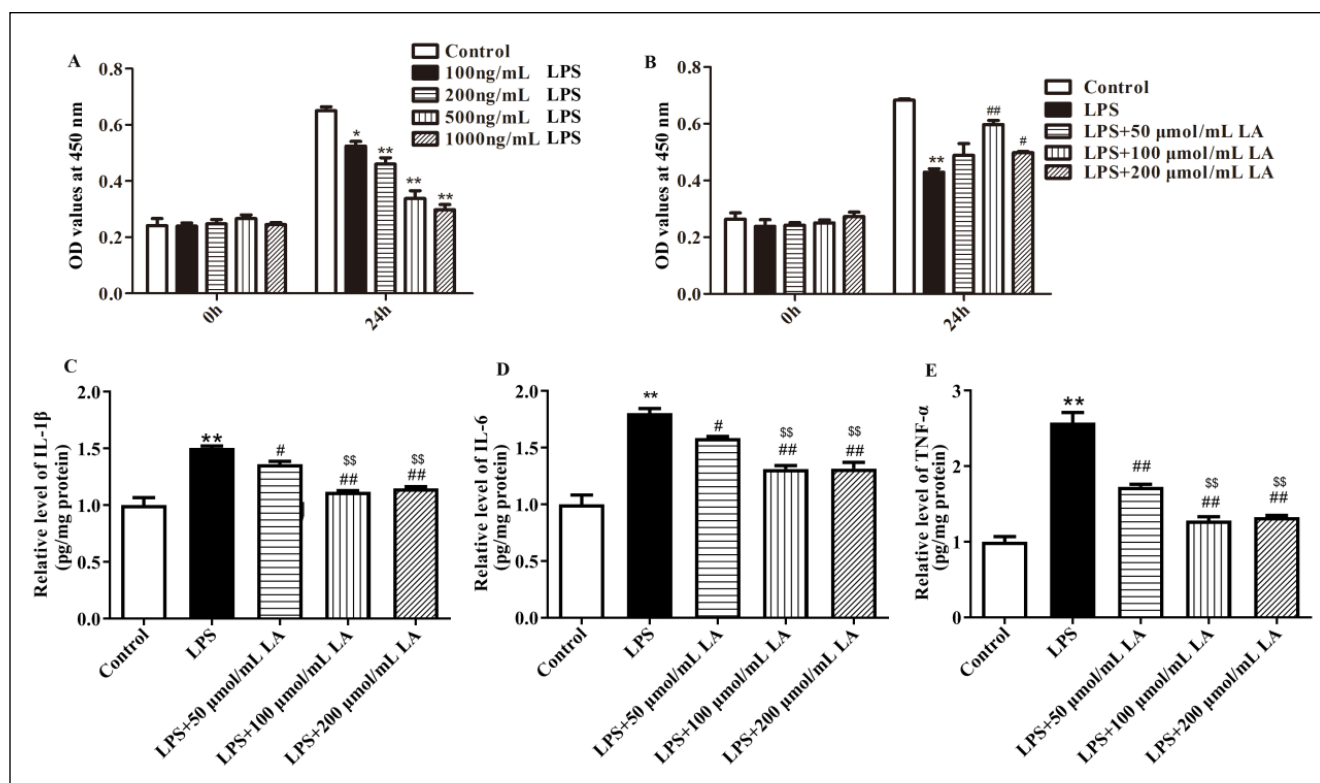


Fig. 1. The cytotoxicity and inflammation in LPS-evoked PC12 cells was suppressed by LA treatment. (A) CCK-8 assays were performed to analyze the viability of PC12 cells after different concentrations of LPS treatment. (B) Different concentrations of LA alleviated the LPS-induced toxicity of PC12 cells. (C-E) The increased levels of IL-1 β , IL-6 and TNF- α in LPS-induced PC12 cells were suppressed by different concentrations of LA. * P <0.05, ** P <0.01 vs. control; # P <0.05, ## P <0.01 vs. LPS; \$\$ P <0.01 vs. LPS+50 μ mol/mL LA.

enomics Database (CTD) website, we preliminarily speculated that p53 may be a potential target of LA. Importantly, we observed that p53, highly expressed in LPS-induced PC12 cells, was suppressed by LA (Fig. 4A, $F_{2,6}=8.386$, P <0.05) and that si-p53 could significantly reduce p53 expression (Fig. 4B, $F_{2,6}=36.692$, P <0.01). We also discovered that knockdown of p53 reduced the increased levels of IL-1 β , IL-6 and TNF- α triggered by LPS in PC12 cells. Moreover, the depletion of p53 enhanced the therapeutic effect of LA accompanied by lower levels of IL-1 β (Fig. 4C, $F_{4,10}=20.406$, P <0.05), IL-6 (Fig. 4D, $F_{4,10}=11.747$, P <0.05) and TNF- α (Fig. 4E, $F_{4,10}=26.192$, P <0.05). Silencing of p53 also reduced the increased apoptotic rate of PC12 cells (Fig. 5A, $F_{4,10}=185.406$, P <0.01) induced by LPS and inhibited the cell cycle arrest (Fig. 5B, $F_{4,10}=23.965$, P <0.01). However, the above results were also achieved after LA and si-p53 combination treatment (Fig. 5A-B). The increase in α -syn (Fig. 6A, $F_{4,10}=13.537$, P <0.05; 6D, $F_{4,10}=18.028$, P <0.05) and decrease in Nurr1 (Fig. 6B, $F_{4,10}=18.084$, P <0.05; 6E, $F_{4,10}=10.555$, P <0.05) and TH (Fig. 6C, $F_{4,10}=12.289$, P <0.05; 6F, $F_{4,10}=14.670$, P <0.05) in LPS-triggered PC12 cells were inhibited after p53 depletion. Moreover, these changes were more significant when LA and si-p53 were applied

together. Together, these data illustrate that protective effects were achieved with LA in PC12 cells by targeting p53.

The NF- κ B pathway was found to be involved in the protective effect of LA/p53 in PC12 cells

The NF- κ B signaling pathway has been shown to be activated in PD, thereby increasing the levels of TNF- α , IL-1 β and IL-6 (Han et al., 2016). Moreover, LA is also widely known as an inhibitor of NF- κ B (Packer et al., 1995). Therefore, we used the western blotting to investigate the effect of LA/p53 on the NF- κ B signaling pathway. The results revealed that the levels of p-P65 NF- κ B and p-I κ B α were reduced after either LA or si-p53 treatment. In addition, compared with LA or si-p53 treatment alone, co-treatment with LA and si-p53 reduced the expression of p-P65 NF- κ B (Fig. 7A, $F_{3,8}=32.681$, P <0.05) and p-I κ B α (Fig. 7A, $F_{3,8}=24.078$, P <0.05) even more significantly. Therefore, the mitigative effect of LA on LPS-induced PC12 cell damage was achieved by targeting p53 and inactivating the NF- κ B signaling pathway.

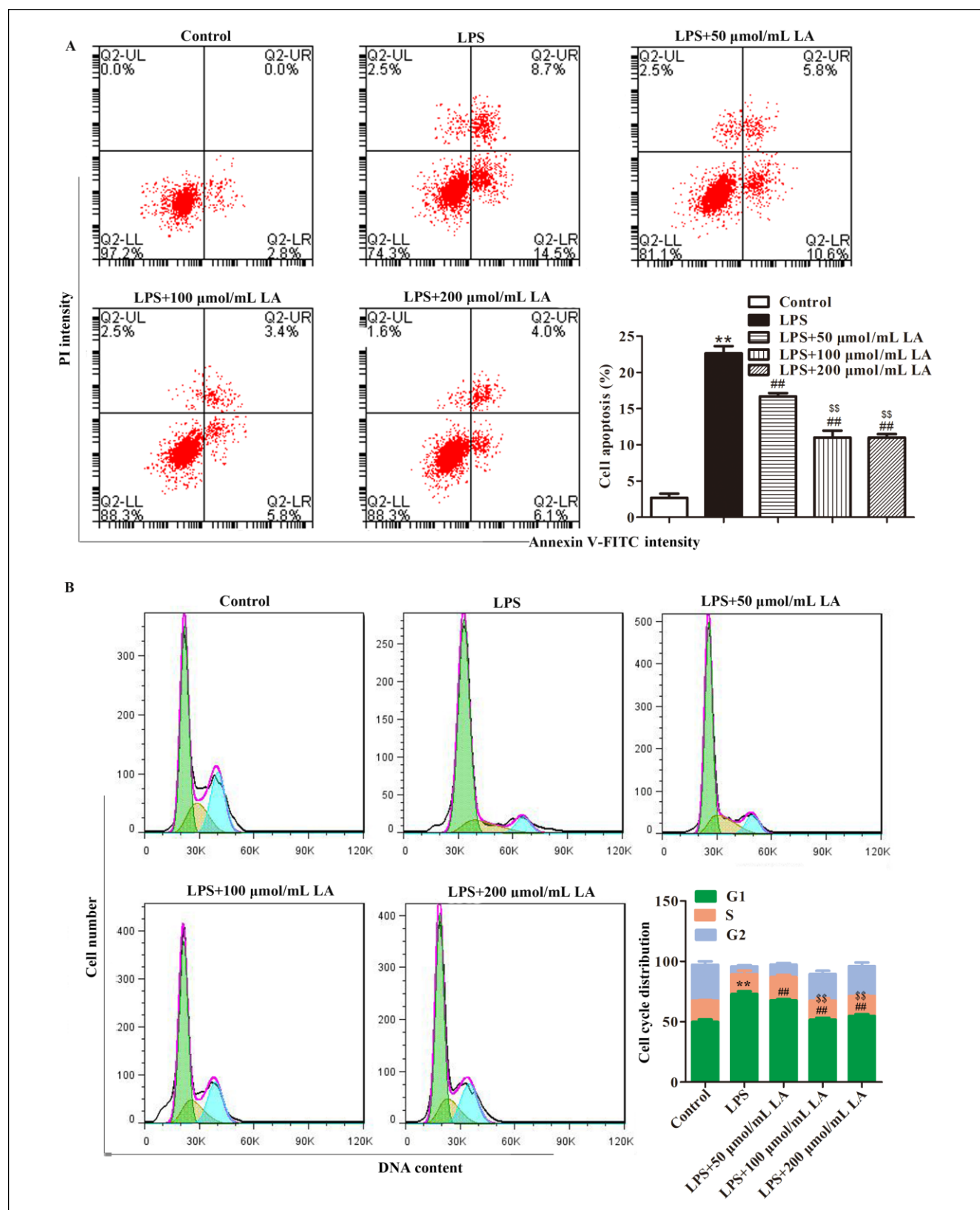


Fig. 2. The increased apoptosis rate and G1 phase arrest of PC12 cells triggered by LPS were inhibited by LA stimulation. (A) LPS induced an increase in PC12 cell apoptosis, which was suppressed by different concentrations of LA treatment. (B) LPS induced PC12 cell arrest in G1 phase, while treatment with different concentrations of LA inhibited PC12 cell cycle arrest. ** $P < 0.01$ vs. control; ## $P < 0.01$ vs. LPS; \$\$ $P < 0.01$ vs. LPS+50 µmol/mL LA.

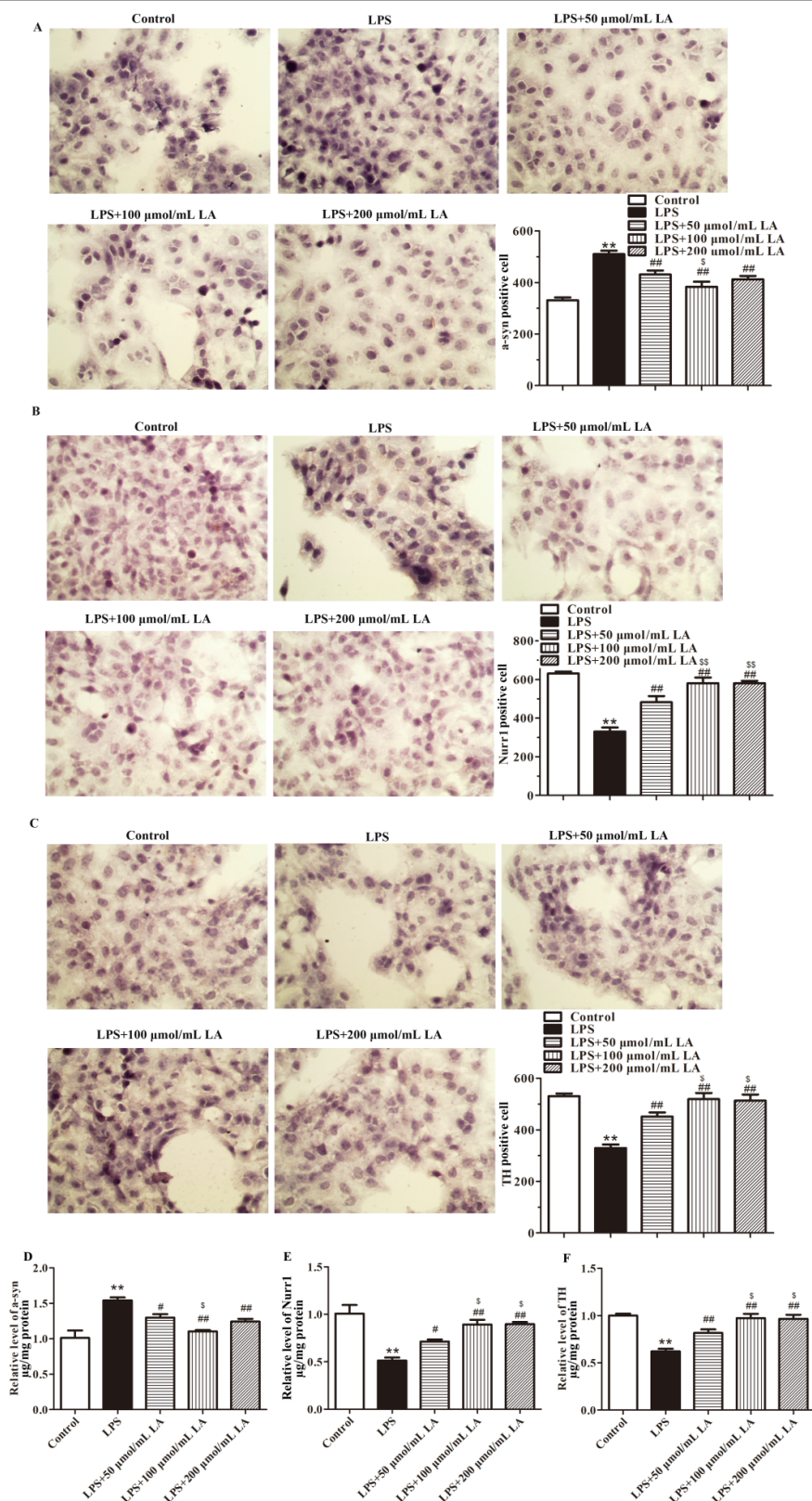


Fig. 3. The increased α -syn and decreased Nurr1 and TH in LPS-induced PC12 cells were suppressed after LA treatment. (A and D) LA treatment suppressed the increase of α -syn caused by LPS in PC12 cells. In PC12 cells, the decrease of Nurr1 (B and E) and TH (C and F) caused by LPS were suppressed by LA stimulation. ** $P < 0.01$ vs. control; # $P < 0.05$, ## $P < 0.01$ vs. LPS; $sP < 0.05$, $ssP < 0.01$ vs. LPS+50 $\mu\text{mol/mL}$ LA.

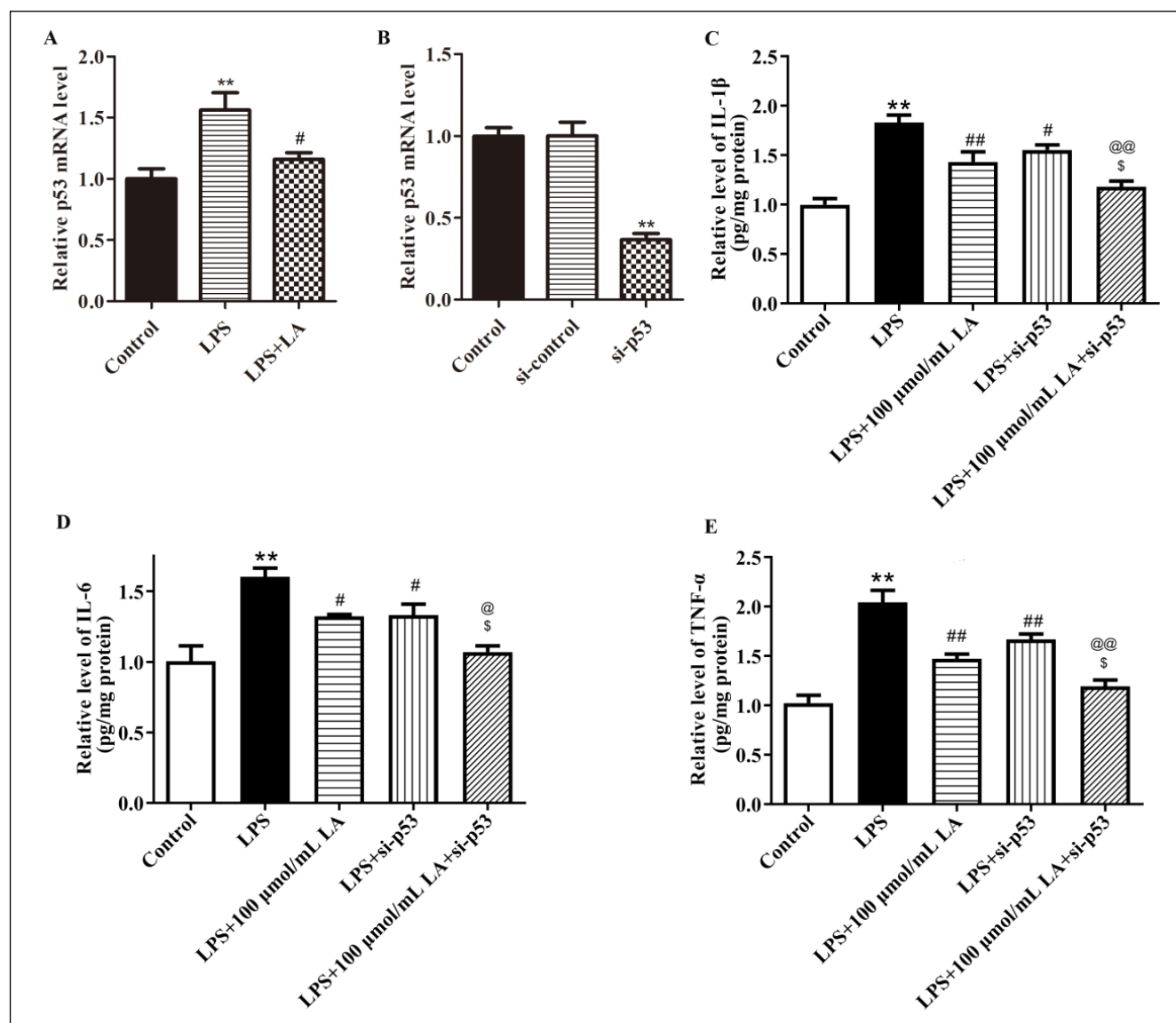


Fig. 4. p53, highly expressed in LPS-induced PC12 cells, was suppressed by LA treatment. (A) RT-qPCR was used to detect p53 expression in PC12 cells after LPS or LPS+LA treatment. (B) si-p53 clearly reduced p53 mRNA expression. (C-E) Knockdown of p53 strengthened the inhibitory effects of LA on IL-1 β , IL-6 and TNF- α expression. ** P <0.01 vs. control; # P <0.05, ## P <0.01 vs. LPS; \$ P <0.05 vs. LPS+100 μ mol/mL LA; @ P <0.05, @@ P <0.01 vs. LPS+si-p53.

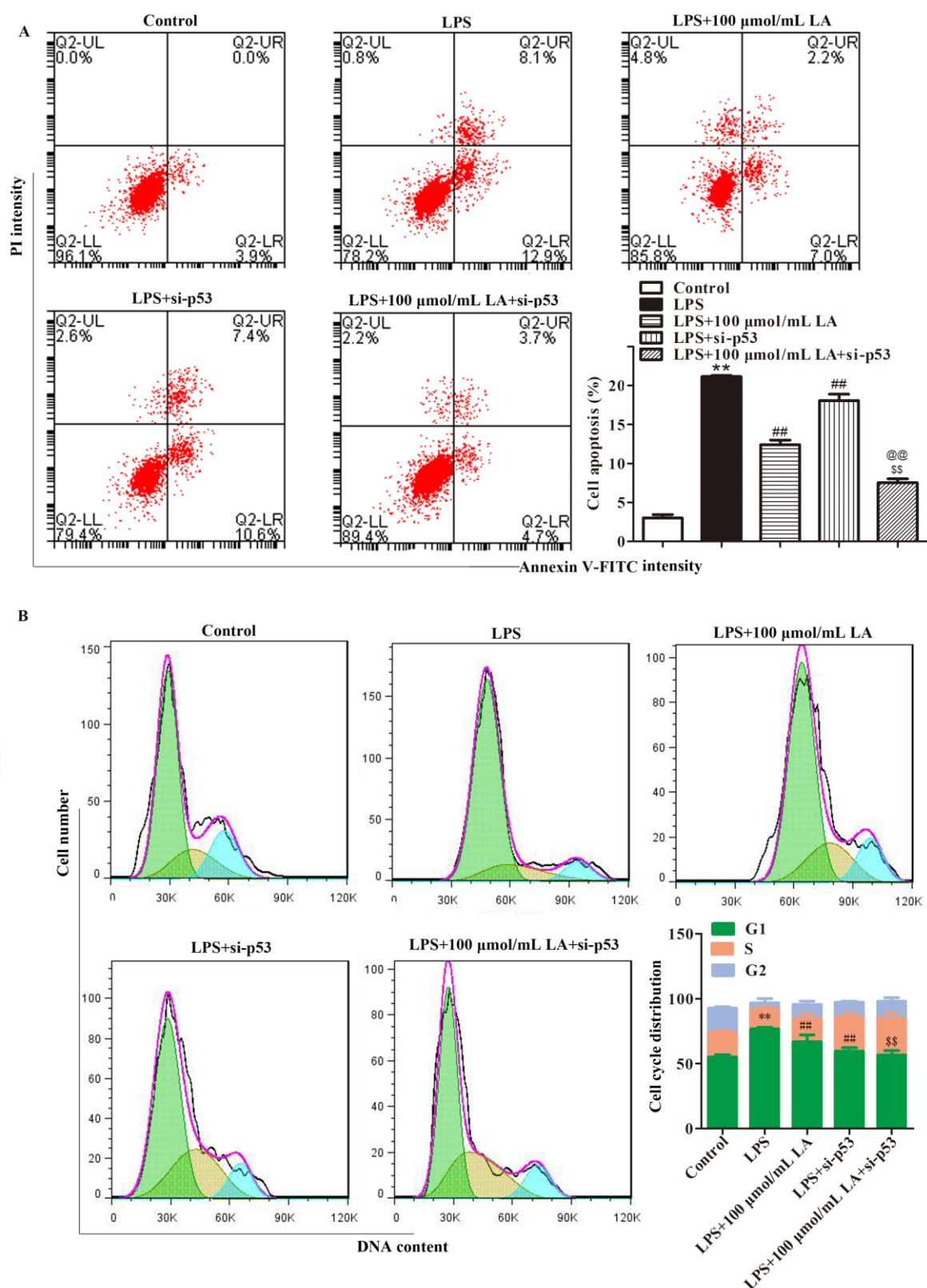


Fig. 5. LA attenuated the apoptosis and eliminated cell cycle arrest in LPS-induced PC12 cells by targeting p53. (A) Flow cytometry was used to detect the effect of LA and p53 on PC12 cells apoptosis under the LPS condition. (B) The function of LA and p53 on PC12 cell cycle was measured by flow cytometry under LPS stimulation. ** $P < 0.01$ vs. control; ## $P < 0.01$ vs. LPS; \$\$ $P < 0.01$ vs. LPS+100 μmol/mL LA; @ $P < 0.01$ vs. LPS+si-p53.

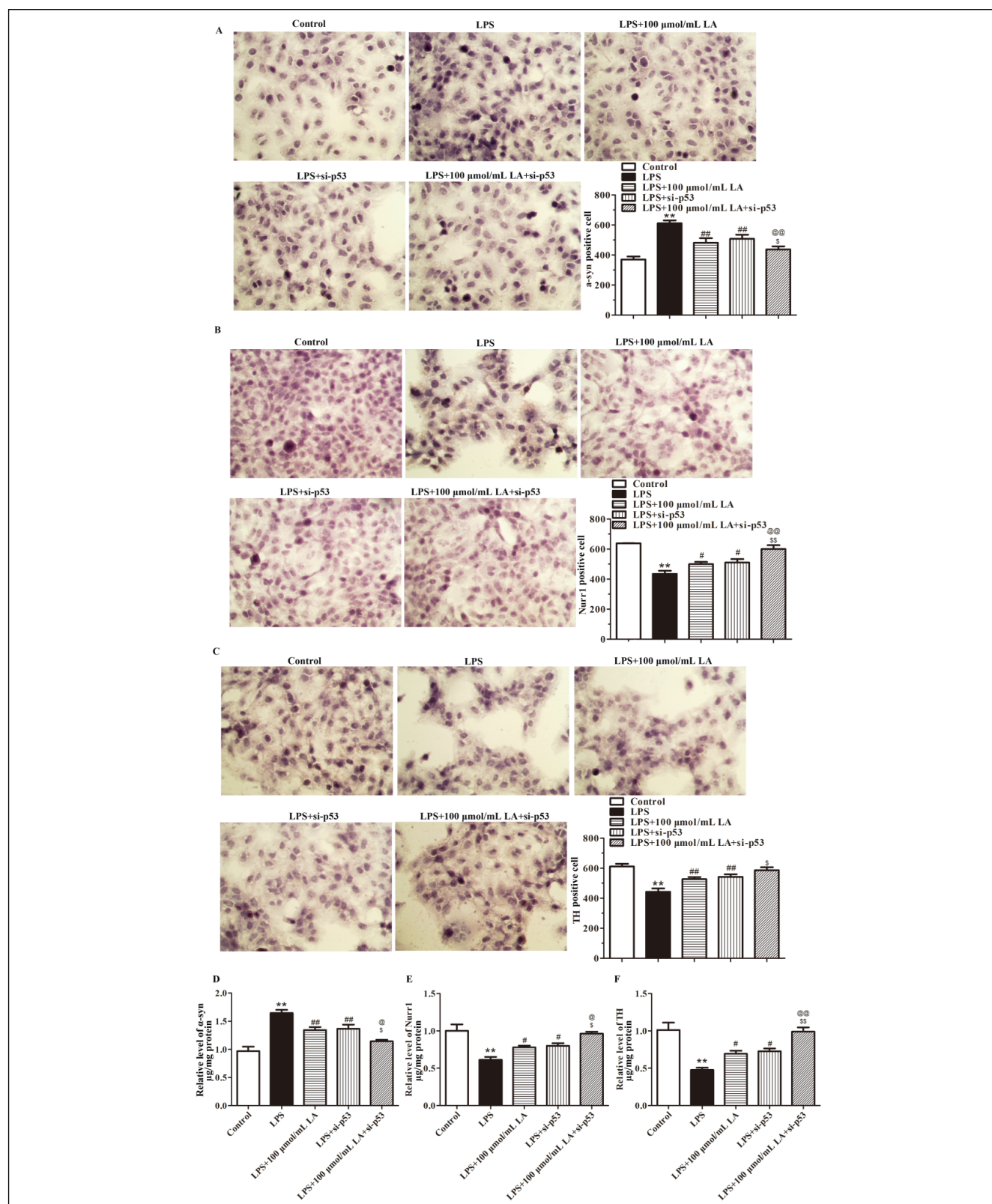


Fig. 6. The effects of LA on α-syn, Nurr1 and TH levels were strengthened by p53 depletion. (A and D) LA suppressed the increasing trend of α-syn in LPS-induced PC12 cells, and depletion of p53 enhanced the inhibitory effect of LA on α-syn level. (B, C, E and F) The decreased trends of Nurr1 and TH in LPS-induced PC12 cells were inhibited by LA treatment, which were strengthened by p53 knockdown. ** $P < 0.01$ vs. control; * $P < 0.05$, ## $P < 0.01$ vs. LPS; # $P < 0.05$, @@ $P < 0.01$ vs. LPS+100 μmol/mL LA; @ $P < 0.05$, @@@ $P < 0.01$ vs. LPS+si-p53.

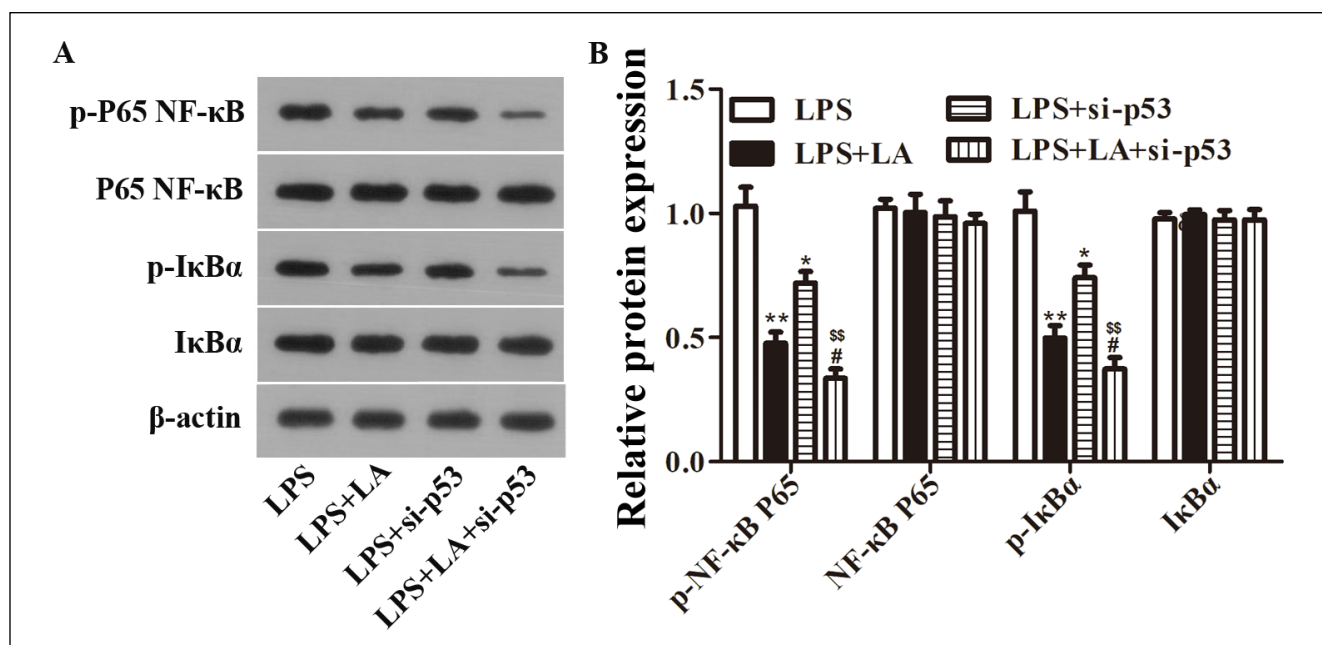


Fig. 7. The NF-κB pathway was involved in the effects of LA on LPS-induced PC12 cells. (A) Western blotting was carried out to test P65 NF-κB, p-P65 NF-κB, IκBα and p-IκBα expression. (B) Graph of relative protein expression in A. * $P < 0.05$, ** $P < 0.01$ vs. LPS; # $P < 0.05$ vs. LPS+LA; ## $P < 0.01$ vs. LPS+si-p53.

DISCUSSION

Neuroinflammation is one of the important pathogeneses of neurodegenerative diseases including PD (Hill-Burns et al., 2011). The levels of the pro-inflammatory mediators TNF- α , IL-1 and IL-6 in the brain of PD patients were found to be elevated (Reale et al., 2009). Therefore, inflammation might be a basic process leading to the death of neurons in PD, and anti-inflammatory treatment may provide a specific neuroprotective therapy for PD. Herein, PC12 cells exposed to LPS were used to construct an *in vitro* module of PD and it was found that LA can alleviate LPS damage to PC12 cells.

LA has been reported to prevent hydrogen peroxide-triggered neuronal damage, protect neurons from neurotoxicity and decrease oxidative damage (Li et al., 2015). Moreover, LA has the ability to suppress LPS-triggered inflammatory processes (Suh et al., 2015). Additionally, a study by Li and colleagues (2015) revealed that LA treatment improved motor dysfunction and suppressed the expression of pro-inflammatory molecules in M1 microglia. However, our study not only revealed an effect of LA on inflammatory factors and α -syn but also effects on apoptosis, cell cycle, Nurr1 and TH. Moreover, we also found that LA suppressed damage in PC12 cells exposed to LPS by targeting p53.

P53 has an important role in the death of neurons in many neurodegenerative diseases. The evidence

comes primarily from the fact that p53 levels are found to be greatly increased in affected neurons in both *in vivo* and *in vitro* experimental models (Dai et al., 2016). Moreover, it has been proposed that p53 overexpression regulates apoptosis, resulting in neuronal loss in the substantia nigra pars compacta, leading to PD (Fauvel et al., 2001). In our study, we observed that p53 was highly expressed in LPS-triggered PC12 cells, while LA stimulation clearly reduced p53 expression. Moreover, knockdown of p53 suppressed the release of TNF- α , IL-1 β and IL-6, inhibited cell apoptosis and eliminated cell cycle arrest in LPS-induced PC12 cells, which suggested that p53 played a promoting role in LPS-induced PC12 cell injury.

Previous studies have reported that α -syn aggregates have been found in the central nervous system in PD (Kim et al., 2019). It is well documented that Nurr1 plays an important role in the midbrain dopaminergic neuronal development, differentiation and survival. It has also been proposed that the decreases in Nurr1 are related to PD, and TH expression has been found to decrease in the substantia nigra pars compacta region with the progression of PD (Kordower et al., 2013; Dong et al., 2016). In our study, we observed that the level of α -syn was increased in LPS-induced PC12 cells, while Nurr1 and TH were reduced. However, after treatment with LA or si-p53, these phenomena were reversed, indicating that LA or si-p53 alleviated PC12 cell damage by regulating the levels of α -syn, Nurr1 and TH.

The NF- κ B signaling pathway was found to be activated in PD, thereby increasing the levels of TNF- α , IL-1 β and IL-6 (Han et al., 2016). Moreover, LA is also widely known as an inhibitor of NF- κ B (Packer et al., 1995). Interestingly, we found that the levels of p-P65 NF- κ B and p-I κ B α were significantly reduced after LA treatment when PC12 cells were exposed to LPS. Moreover, knockdown of p53 strengthened the inhibitory effect of LA on p-P65 NF- κ B and p-I κ B α expression. Therefore, our data suggests that the LA exerts an effect on PC12 cells exposed to LPS partly through targeting p53 and mediating the NF- κ B pathway.

CONCLUSIONS

Taken together, our results revealed that LA can alleviate the inflammatory response, apoptosis and cell cycle arrest of PC12 cells induced by LPS by targeting p53 and inactivating NF- κ B pathway. These findings provide a theoretical basis for further investigation of the mechanism of LA in neuroinflammation.

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