European Review for Medical and Pharmacological Sciences 2020; 24: 7092-7100

Curcumin exerts protective effect on PC12 cells against lidocaine-induced cytotoxicity by suppressing the formation of NLRP3 inflammasome

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Abstract. – OBJECTIVE: To explore the protective effect of curcumin on lidocaine-insulted PC12 cells.

MATERIALS AND METHODS: We first treated PC12 cells with different doses of lidocaine, and then treated the cells with curcumin or Nod-like receptor pyrin domain3 (NLRP3) inhibitor (MCC950). Subsequently, the cell viability, apoptosis, reactive oxygen species (ROS) production and NLRP3 inflammasome were detected by cell counting kit-8 (CCK8), Annexin V/PI staining, FCM and Western blot analysis, respectively, and the level of IL-1β in PC12 cells was determined by an enzyme-linked immunosorbent assay (ELISA) kit.

RESULTS: Lidocaine inhibited the viability of PC12 cells, and it induced cell apoptosis, promoted ROS release and activated NLRP3 inflammasome in PC12 cells, but its effects were reversed by the treatment of curcumin. Moreover, NLRP3 over-expression also induced cytotoxicity in PC12 cells, which was also rescued by the treatment of curcumin.

CONCLUSIONS: Our study indicates that curcumin exerts protective effect against lidocaine-induced cytotoxicity on PC12 cells by suppressing the activity of NLRP3 inflammasome, which provides new ideas on screening natural product for neurological damage therapy.

Key Words:
Curcumin, Cytotoxicity, PC12 cells, NLRP3, Inflammation.

Introduction

Lidocaine (2-diethylamino-2',6'-acetoxylidide) is a well-known local anesthetic drug, and it is also used for arrhythmia treatment[1,2]. As an arrhythmia drug, it is not often used now, because some people worry about its long-term side effects and a few people are allergic to lidocaine. It is reported that local anesthetics can induce neurotoxicity in clinical application[3], such as transient neurological syndrome and cauda equina syndrome[3]. If the local anesthetics entering into the blood vessels by mistake or absorbed into the blood in unit time are excessive, or the patients have poor nutrition regulation and incomplete liver and kidney functions, the concentration of local anesthetics in the blood will be too high, which will result in toxic reactions, mainly manifested as central nervous system toxicity and cardiovascular dysfunction[4]. Lidocaine-induced cytotoxicity includes cell apoptosis, increased reactive oxygen species (ROS) production, and a series of inflammatory reactions[4]. However, the detailed mechanism of lidocaine-induced neurotoxicity still remains unclear, which results in a number of unfavorable therapeutic effects of some clinical drugs and settings.

Curcumin is an orange crystal powder, mainly used for coloring food, and it is also a naturally occurring autophagy modulator[5,6]. Medical research has revealed that curcumin exerts the

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functions of decreasing blood lipid[7], anti-tumor[8,9], anti-inflammatory[10], cholagogic and anti-oxidation[11]. In addition, researchers[12] have reported that curcumin exerts protective effects against neurological injury. Hagl et al[13] have found that curcumin could relieve the mitochondrial injury in PC12 cells and brain tissues of NMRI mice. Zhang et al[14] have reported that curcumin relieves cerebral ischemia injury in rats and PC12 cells *via* the suppression of overactivated autophagy. In addition, Xu et al[15] have uncovered that curcumin analogues relieve Aβ-induced oxidative injury in PC12 cells through the Nrf2 signaling pathway. However, the protective effect of curcumin on lidocaine-induced PC12 cells and the underlying mechanisms have not been reported.

In this study, we first studied the effect of lidocaine treatment on cell proliferation, apoptosis, ROS level, and NLRP3 inflammasome in PC12 cells. Then, we analyzed the effect of curcumin intervention on lidocaine-induced cytotoxicity in PC12 cells. Additionally, we also determined whether curcumin played the neuroprotective role by regulating the NLRP3 inflammasome, and studied the molecular mechanism involved.

Materials and methods

Cell Culture

PC12 cells obtained from the cell bank of the Chinese Academy of Sciences (Shanghai, China) were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (HyClone, South Logan, UT, USA) containing 10% horse serum (Thermo Fisher Scientific, Inc., Waltham, MA, USA), 5% fetal bovine serum (FBS; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and 1% penicillin streptomycin mixture (Solarbio Science & Technology Co., Ltd, Beijing, China) in humidified 5% CO_2 at 37°C.

Cell Transfection

For NLRP3 over-expression, PC12 cells were transfected with pCDNA3.1(+)-NLRP3 (GenePharma Co., Ltd., Shanghai, China) by Lipofectamine™ 2000 (Thermo Fisher Scientific, Inc., Waltham, MA, USA). After transfection for 24 h, the mRNA level of NLRP3 was determined by qRT-PCR.

CCK-8 Viability

PC12 cells with or without transfection were pretreated with or without curcumin (0, 2.5, 5, 10, 20 and 40 μM; Solarbio Science & Technology Co., Ltd, Beijing, China) or MCC950 (5 μM, Solarbio Science & Technology Co., Ltd, Beijing, China) for 6 h, and then treated with lidocaine (0, 0.5, 1 and 2 mM) for 12, 24 and 48 h. Subsequently, cell proliferation was detected by a Cell Counting Kit-8 (Sigma-Aldrich, St. Louis, MO, USA). The absorbance was measured at 450 nm by an enzyme analytical apparatus (Beijing PLANON New Technology Co., Ltd, Beijing, China).

Cell Apoptosis

PC12 cells with or without transfection were pretreated with or without curcumin or MCC950 for 6 h, and then treated with lidocaine for 24 h. subsequently, the cells were harvested, washed with phosphate-buffered saline (PBS) (Solarbio Science & Technology Co., Ltd, Beijing, China), and stained with a Annexin V-FITC apoptosis detection kit (Beyotime Biotech Co., Ltd., Shanghai, China). Finally, the apoptosis rate of PC12 cells and data analysis were performed by a flow cytometer (BD Biosciences, San Jose, CA, USA).

ROS Production Detection

The level of ROS was evaluated by DCFH-DA fluorescent dye. PC12 cells were treated with DCFH-DA at 5% CO_2 and 37°C, and then cultured in serum-free media, and washed three times to get rid of redundant DCFH-DA. Flow cytometry (BD Biosciences, San Jose, CA, USA) was used for fluorescence detection. The emission of 525 nm was measured by 480 nm excitation. The ROS positive cells showed strong green fluorescence, corresponding to FL1 detection channel of flow cytometry.

qRT-PCR

Cells were transferred to a 1 ml TRIzol homogenate tube, and RNA was extracted from the cells with TRIzol reagent (Thermo Fisher Scientific, Inc., Waltham, MA, USA) and cDNA was synthesized by Transcriptor HiFi cDNA Synth (Roche, Applied Science, Penzberg, Upper Bavaria, Germany) under the manufacturer's instruction. The following PCR primers were used: NLRP3 forward sequence, 5'-ACCTCAACAGACGCTACACCC-3' and reverse, 5'-GCTGCTCCCTGGAACACC-3' (196 bps) and GAPDH forward sequence, 5'-GGAGTCTACTGGCGTCTTCAC-3' and reverse, 5'-ATGAGCCCTTCCACGATGC-3' (237 bps). The PCR re-

actions were performed using the SYBR Green PCR (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The NLRP3 expression was calculated by the $2^{-\Delta\Delta Ct}$ method.

Western Blot

Total protein lysates were separated by 10% sodium-dodecyl-sulfate-polyacrylamide gel-electrophoresis (SDS-PAGE) and transferred to polyvinyl difluoride (PVDF) membranes (Solarbio Science & Technology Co., Ltd, Beijing, China). Tris-Buffered Saline (TBS) containing 0.1% Tween-20 (TBS-T, Amresco, Life Science, Lehi, UT, USA, BYL40713) and 5% skimmed milk were sealed at 37°C for 30 minutes, and then the membranes were washed in TBS-T four times, and incubated with primary antibody at 4°C overnight. The primary antibodies were purchased from Abcam (Cambridge, MA, USA), Cell Signaling Technology (CST Biological Reagents Co., Ltd. Danvers, MA, USA) and NOVUS Biologicals (Shanghai, China), and used at the following dilutions: Anti-NLRP3 (1:1000; Ab210491), anti-IL-1β (1:1000; Ab205924), anti-GAPDH (1:1000; CST#5174), anti-Pro-IL-1β (1:1000; NOVUS Biologicals, Shanghai, China, NBP1-19775), anti-Caspase1 (1:1000; Ab1872) and anti-Pro-Caspase1 (1:1000; Ab179515). After extensive washing, the membranes were incubated with horseradish peroxidase (HRP)-linked goat polyclonal anti-rabbit immunoglobulin G (IgG) secondary antibody (A0208; Beyotime Biotechnology Co., Ltd., Shanghai, China) at 1:2000 for 1 h at 25°C. GAPDH served as the loading control.

Enzyme-Linked Immuno Sorbent Assay (ELISA)

The level of IL-1β in PC12 cell supernatant was detected by a Rat IL-1β/IL-1β Quantikine ELISA Kit (Elabscience, Berlin, Germany) according to the manufacturers' protocols.

Statistical Analysis

Data were expressed as the mean ± standard deviation. The statistics were analyzed by GraphPad Prism 7.0. Comparisons between different experimental groups were carried out using ANOVA, followed by Tukey's post-multiple test. Student's *t*-test was performed to analyze the difference between two groups. $p< 0.05$ indicates a significant difference.

Results

Lidocaine Inhibited Cell Activity, Induced Cell Apoptosis and ROS Release, and Activated NLRP3 Inflammasome In PC12 Cells

We first evaluated the effect of lidocaine on PC12 cells. In Figure 1A, lidocaine significantly suppressed cell proliferation as compared with the non-treated PC12 cells ($p<0.01$). Cell apoptosis was examined by flow cytometry (FCM), and it was turned out that lidocaine treatment for 24 h significantly promoted the apoptosis of PC12 cells compared with the non-treated PC12 cells ($p<0.001$, Figure 1B-C). The ROS levels in PC12 cells treated with different doses of lidocaine were increased significantly as compared with that in the non-treated PC12 cells ($p<0.001$, Figure 1D-E). Moreover, the expression of NLRP3, active caspase-1, pro-caspase-1, and IL-1β was evaluated, and the expression of which was increased dose-dependently with the lidocaine treatment (Figure 1F-G). The results indicated that lidocaine could activate NLRP3 inflammasome in PC12 cells.

Curcumin Reversed the Effect of Lidocaine on Cell Viability and NLRP3 Inflammasome in PC12 Cells

We subsequently treated PC cells with lidocaine (1 mM), and then treated them with curcumin (0, 5, 10, and 20 μM) or MCC950 (5 μM) (Figure 2A). MCC950 was known as an inhibitor of NLRP3. The status of NLRP3 inflammasome was analyzed by Western blot and ELISA. It was shown that in Figure 2B, the expression of NLRP3, active caspase-1, pro-caspase-1, IL-1β and pro-IL-1β in PC12 cells were significantly up-regulated by lidocaine destruction, and the expression of which was then decreased by curcumin or MCC950 treatment. These results reveal that curcumin could increase cell viability and inhibit the activity of NLRP3 inflammasome in lidocaine-insulted PC12 cells.

Curcumin Suppressed the Cell Apoptosis and ROS Production Induced By Lidocaine in Pc12 Cells

We further identified the effect of curcumin on cell apoptosis and ROS production in lidocaine-treated PC12 cells. As shown in Figure 3A-B, lidocaine notably promoted the apoptosis and ROS production of PC12 cells compared

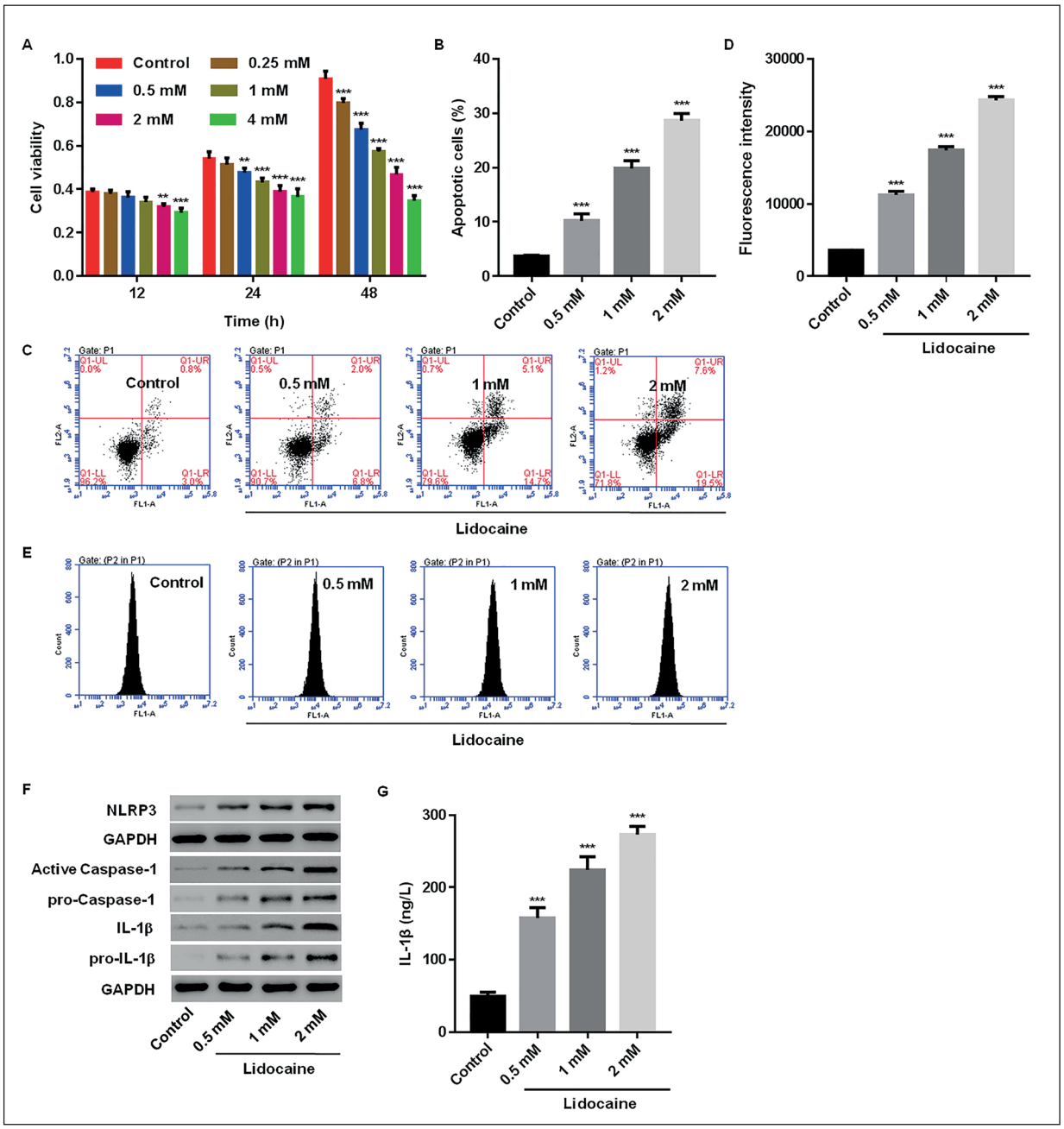

Figure 1. Lidocaine inhibits PC12 cell activity, induces apoptosis and increases ROS, and activates NLRP3 inflammasome. **A,** PC12 cells were treated with Lidocaine (0, 0.25, 0.5, 1, 2 and 4 mM) for 12, 24 and 48 h, cell viability was examined by cell counting kit-8 (CCK8). **B-E,** Cell apoptosis and ROS level of PC12 cells were evaluated by FCM assay. **F,** The expressions of NLRP3, active caspase-1, pro-caspase-1, pro-IL-1β, and IL-1β were tested by Western blot. **G,** The level of IL-1β in PC12 cells was examined by ELISA assay. Data are shown as mean ± SD. **p<0.01 *vs.* control; ***p<0.001 *vs.* control.

with the non-treated PC12 cells (p<0.001), while pretreatment of curcumin (5, 10, and 20 μM) or MCC950 (5 μM) significantly reversed the effect of lidocaine on cell apoptosis and ROS production of PC12 cells (p<0.05, Figure 3A-B).

Curcumin Protected PC12 Cells Against Apoptosis, ROS Production and NLRP3 Inflammasome Activation Induced By NLRP3Overexpression

To further explore whether curcumin had protective effect through regulating NLRP3 in-

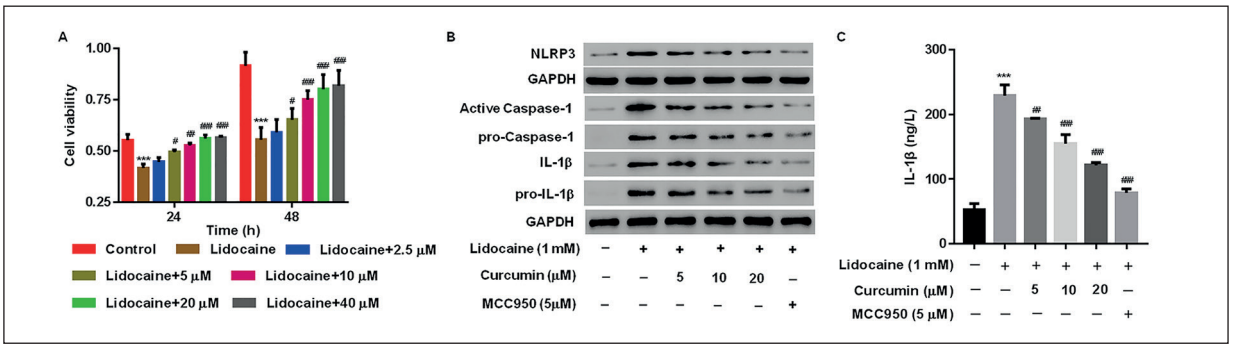

Figure 2. Curcumin reverses the effect of Lidocaine on cell viability and NLRP3 inflammasome in PC12 cells. **A,** PC12 cells were treated with lidocaine (1 mM), and then with curcumin (0, 2.5, 5, 10, 20, and 40 mM) for 24 and 48 h, cell viability was examined by CCK-8. **B,** PC12 cells were treated with Lidocaine (1 mM), and then with curcumin (0, 5, 10, and 20 μM) or MCC950 (5 μM) for 24 and 48 h, the expressions of NLRP3, active caspase-1, pro-caspase-1, IL-1β, and pro-IL-1β were tested by Western blot. **C,** The content of IL-1β in PC12 cells was examined by ELISA analysis. Data are shown as mean ± SD. ***p<0.001 *vs.* control; $^{\#}p$<0.05, $^{\#\#}p$<0.01, $^{\#\#\#}p$<0.001 *vs.* Lidocaine.

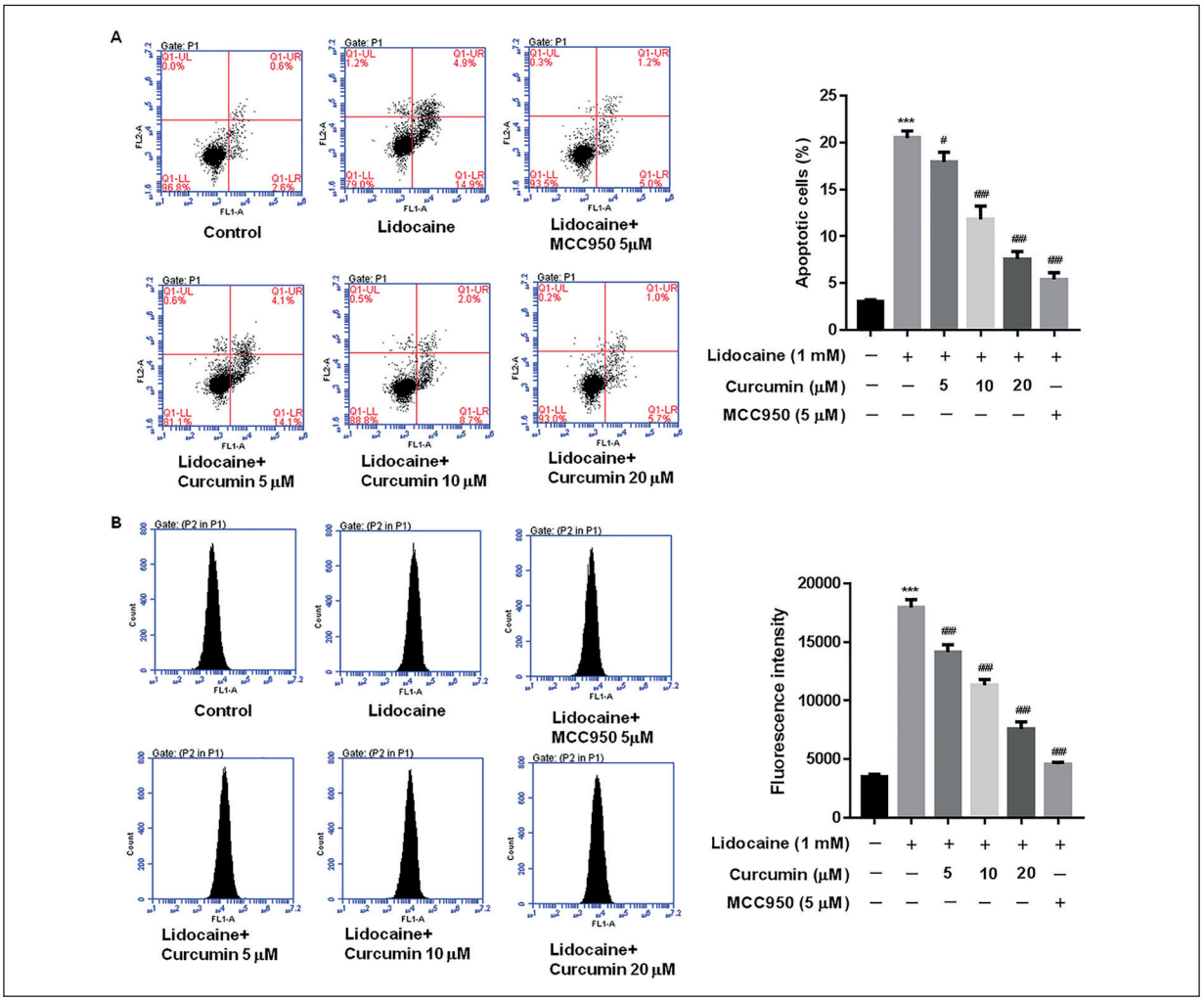

Figure 3. Curcumin inhibits the cell apoptosis and ROS production induced by lidocaine in PC12 cells. **A-B,** PC12 cells were treated with lidocaine (1 mM), and then treated with curcumin (0, 5, 10, and 20 μM) or MCC950 (5 μM) for 24 h, cell apoptosis and ROS production were examined by FCM analysis. Data are shown as mean ± SD. ***p<0.001 *vs.* control; $^{\#}p$<0.05, $^{\#\#}p$<0.01, $^{\#\#\#}p$<0.001 *vs.* Lidocaine.

flammasome, PC12 cells were transfected with oeNLRP3 and then treated with curcumin, and cell apoptosis, ROS production and NLRP3 inflammasome-related protein expression were subsequently evaluated. As shown in Figure 4A, NLRP3 was over-expressed in PC12 cells. Moreover, NLRP3 overexpression significantly induced cell apoptosis ($p<0.001$, Figure 4B-C), enhanced ROS production ($p<0.001$, Figure 4D-E) and stimulated the activity of NLRP3 inflammasome ($p<0.001$, Figure 4F-G). However, curcumin could remarkably suppress the effect of NLRP3 overexpression on PC12 cells ($p<0.001$, Figure 4B-G).

Discussion

Curcumin with multiple therapeutic effects attracts increasing attention of scientists in recent decades[16,17]. Curcumin can capture free radicals by phenolic hydroxyl, protect against liver injury and oxidative damage caused by radiation drugs, inhibit tumor by regulating cell cycle, inducing apoptosis and regulating gene expression, inhibit inflammation by inhibiting the inflammatory factors, and have antiviral and antibacterial effects[17]. The development of curcumin enjoys a great application value. Curcumin is one of the most sold natural food pigments in the world. Emerging evidence showed that curcumin could protect nervous system against mechanical or chemical damage[18]. Lidocaine is an anesthetic drug, which also has cytotoxic effect on PC12 cells. Hong et al[19] have reported that lidocaine induces cell apoptosis through endoplasmic reticulum (ER) stress in SD rats and in PC12 cells, and Wang et al[3] have also reported that lidocaine has cytotoxicity on PC12 cells. In the present work, we reported the effect of curcumin intervention on cytotoxicity induced by lidocaine in PC12 cells. We firstly determined the injury effect from different doses of lidocaine on PC12 cell proliferation, apoptosis and ROS production. The results indicated that lidocaine exerted inhibitory effect on cell proliferation and stimulated cell apoptosis and ROS release in a dose-dependent manner. As known, an excessive level of ROS can induce the opening of mitochondrial outer membrane pore, release of calcium, cytochrome c, and apoptosis induced by AIF, finally resulting in cell apoptosis[20]. Furthermore, we found that lidocaine increased the expression of NLRP3, active caspase-1, procaspase-1, and IL-1β, and these are main factors of NLRP3 inflammasome[21]. Inflammasome is a kind of cytoplasmic protein complex, which mainly mediates the host's immune response to microbial infection and cell damage[22]. The aggregation of inflammasome causes the cleavage of Pro caspase-1 and the formation of activated caspase-1[23]. Caspase-1 promotes the transformation of pro-IL-1β, and pro-IL-1βtransforms into mature IL-1β. In many immune responses, mature IL-1β is an effective pro-inflammatory medium, which can recruit innate immune cells to the infected site and regulate acquired immune cells[24]. While, mature IL-1β can promote the production of interferon γ (IFN -γ), and enhance the killing activity of natural killer cells and T cells. Activated caspase-1 can induce cell death in the pro-inflammatory form. The components of inflammasome include pattern recognition receptor (PRR), apoptosis related spot like protein and caspase-1[25]. These results reveal that lidocaine induces cell apoptosis by promoting ROS production and NLRP3 inflammasome activation.

We further examined whether curcumin treatment could exert protective effect against the cytotoxicity induced by lidocaine in PC12 cells. MCC950, often used as aNLRP3 inhibitor[26], was employed as the positive control. The results showed that curcumin reversed the effect of lidocaine on cell viability and NLRP3 inflammasome in PC12 cells, and curcumin inhibited the cell apoptosis and ROS production induced by lidocaine in PC12 cells. Previous studies have reported that NLRP3 can intensify the activity of NLRP3 inflammasome[27]. The present investigation identified the protective effect of curcumin for regulating NLRP3 inflammasome, and for NLRP3 overexpression in PC12 cells. Our results showed that NLRP3 induced cell apoptosis, enhanced ROS production and stimulated the activity of NLRP3 inflammasome. Of note, curcumin could remarkably suppress the effect in NLRP3 overexpression on PC12 cells. The data demonstrated that curcumin reduced cytotoxicity through restraining the NLRP3 inflammasome formation. However, in addition to neuronal toxicity, the role of curcumin in others still needs further exploration.

Curcumin has been reported to regulate autophagy via Beclin-1-dependent or mTOR signaling pathways[28,29]. Moreover, it can induce autophagy to protect vascular endothelial cell from apoptosis under oxidative stress[30]. Consistent with this, our results also suggest that the protective effect of curcumin on PC12 cells is associated with

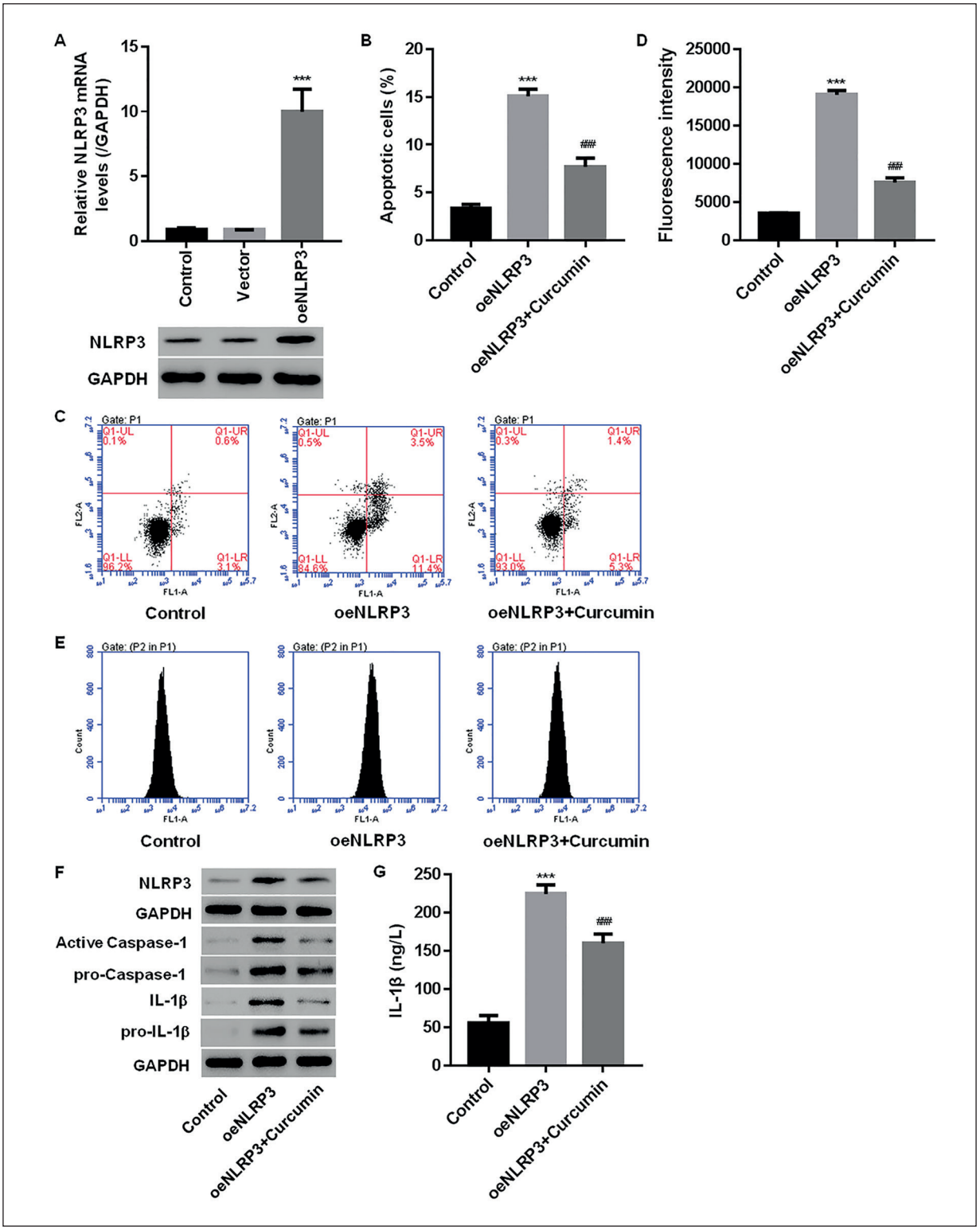

Figure 4. Curcumin rescues the effect of NLRP3 overexpression on cell apoptosis, ROS production and NLRP3 inflammasome. **A,** PC12 cells were transfected with vector and oeNLRP3, and the transfection efficiency was identified by Western blot. **B-E,** PC12 cells were transfected with oeNLRP3 and then were treated with Curcumin, and cell apoptosis and ROS production were tested by FCM analysis. **F,** The expression of NLRP3, active caspase-1, pro-caspase-1, and IL-1β and pro-IL-1β was tested by Western blot. **G,** The level of IL-1β in PC12 cells was measured by ELISA analysis. Data are shown as the mean ± SD. ***p<0.001 *vs.* control; $^{###}p$<0.001 *vs.* oeNLRP3 (NLRP3 overexpression).

reduction in oxidative stress damage. Therefore, curcumin supplementation could be beneficial for the neuron against the cytotoxicity of lidocaine, suggesting that NLRP3 activation and oxidative stress damage underlined the mechanism of curcumin inhibition of side effects of local anesthetic application.

Conclusions

In summary, the present work indicates that curcumin exerts neuroprotective action against lidocaine-induced cytotoxicity including cell apoptosis, ROS production and NLRP3 inflammasome formation in PC12 cells, which might provide novel ideas for understanding lidocaine--induced nerve injury and exploring new agents for nerve injury therapy.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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