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Essential role of ERK activation in neurite outgrowth induced by α-lipoic acid

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ABSTRACT

Background: Neurite outgrowth is an important aspect of neuronal plasticity and regeneration after neuronal injury. Alpha-lipoic acid (LA) has been used as a therapeutic approach for a variety of neural disorders. We recently reported that LA prevents local anesthetics-induced neurite loss. In this study, we hypothesized that LA administration promotes neurite outgrowth. *Methods:* To test our hypothesis, we treated mouse neuroblastoma N2a cells and primary neurons with LA. Neurite outgrowth was evaluated by examination of morphological changes and by immunocytochemistry for β-tubulin-3. ROS production was examined, as were the phosphorylation levels of ERK and Akt. In separate experiments, we determined the effects of the inhibition of ERK or PI3K/Akt as well as ROS production on LA-induced neurite outgrowth. *Results:* LA promoted significantly neurite outgrowth in a time- and concentration-dependent manner. LA stimulation significantly increased the phosphorylation levels of both Akt and ERK and transiently induced ROS production. PI3K/Akt inhibition did not affect LA-induced neurite outgrowth. However, the inhibition of ERK activation completely abolished LA-induced neurite outgrowth. Importantly, the prevention of ROS production by antioxidants attenuated LA-stimulated ERK activation and completely abolished LA-promoted neurite outgrowth. *Conclusion:* Our data suggest that LA stimulates neurite outgrowth through the activation of ERK signaling, an effect mediated through a ROS-dependent mechanism.

1. Introduction

Neurite outgrowth is an important aspect of neuronal plasticity and regeneration in neuropathological conditions and neural injury [1–3]. Neurite outgrowth is also essential for neuronal pathfinding and the establishment of synaptic connections during development [1].

Alpha-lipoic acid (LA) is a naturally occurring dithiol compound synthesized enzymatically in mitochondrion from octanoic acid. LA has been described as a potent biological antioxidant and is used to treat various neural disorders, including pain disorders [4], Alzheimer's [5] and Parkinson's diseases [6], diabetic polyneuropathy [7] and chemotherapy neuropathy [8]. In addition, LA has been shown to improve spatial memory and temporal memory in ApoE4 transgenic mice [9] and to reduce the potentiation of noise-induced hearing loss [10]. The mechanisms by which LA exerts its neuroprotective effects involve antiapoptotic properties mediated by the inhibiting of proapoptotic signaling [6] and the activation of antiapoptotic signaling [5,11]. LA has also been shown to prevent mitochondrial damage [8]. We have previously reported that LA prevents local anesthetics-triggered neurite loss and neuron death [11]. Whether LA stimulates neurite outgrowth, however, is unknown.

We have recently demonstrated that LA treatment activates PI3K/Akt signaling in neurons [11]. We also found that LA activates ERK signaling (data not shown). Activation of the ERK and PI3K/Akt signaling pathways has been reported to regulate neuronal differentiation [12–14] and to protect neurons from drug-induced injury [5,11]. In addition, the activation of ERK is required for NGF-stimulated neurite outgrowth in PC12 cells [15–17]. Collectively, these data suggest that both ERK and PI3K/Akt signaling are involved in neurite outgrowth.

In the present study, we examined the effect of LA treatment on neurite outgrowth in mouse neuroblastoma N2a (N2a) cells and rat primary hippocampal neurons. To the best of our knowledge, we are the first to report that LA promotes neurite outgrowth and that this

Abbreviations: ROS, reactive oxygen species; NGF, nerve growth factor; LA, α-lipoic acid; DCFH-DA, 2',7'-dichlorofluorescein diacetate; NAC, *N*-acetylcysteine; WM, wortmannin; API, triciribine; DPI, diphenyleneiodonium chloride; MEM, minimum essential medium; FCS, fetal calf serum; NGS, normal goat serum; hCG, human chorionic gonadotropin; DHA, docosahexaenoic acid

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process is mediated by the activation of ERK but not by PI3K/Akt signaling. More importantly, LA administration induces a transient and moderate production of ROS in N2a cells. The inhibition of ROS production attenuates the LA-induced activation of ERK and completely abolishes LA-induced neurite outgrowth.

2. Materials and methods

2.1. Chemicals

LA, 2',7'-dichlorofluorescein diacetate (DCFH-DA), *N*-acetylcysteine (NAC), diphenyleneiodonium chloride (DPI), PD98059 (PD), SB202190, SP600125 and wortmannin (WM) were purchased from Sigma-Aldrich (St. Louis, MO). Hoechst 33342 reagent, minimum essential medium (MEM), neurobasal medium, fetal calf serum (FCS) and B27 supplement were purchased from Invitrogen Life Technology (Carlsbad, CA). Primary antibodies for total Akt and total ERK, phospho-Akt (p-Akt) and phospho-ERK (p-ERK) were obtained from Cell Signaling Technology (Beverly, MA). A primary antibody against β-tubulin-3 was purchased from R&D Systems, Inc. (Minneapolis, MN). Normal goat serum (NGS) and antibodies against IgG conjugated with horseradish peroxidase (HRP) or Cy3 were purchased from Vector Labs (Burlingame, CA). Complete protease inhibitor cocktail was purchased from Roche (Mannheim, Germany). MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] reagent was purchased from Bio Besic, Inc. (Markham, Ontario, Canada). The BCA protein assay kit and SuperSignal West Pico chemiluminescent substrate were obtained from Pierce (Rockford, IL). Triciribine (API) was obtained from Kangcheng BioTtech (Shanghai, China).

2.2. Cell culture

N2a cells were plated in 60-mm dishes at a density of 3×10^5 cells/dish and grown in MEM supplemented with 10% FCS. Twenty-four hours later, cell medium was changed to MEM containing 5% FCS and subjected to treatment.

Primary hippocampal neurons were isolated from neonatal Sprague–Dawley rats and cultured in Neurobasal medium containing B27 supplement using methods described previously [11,16]. After 12 h, neurons were subjected to LA stimulation (500 μM). $n=3$/group.

For the experiments using inhibitors for MEK/ERK (PD98059, 40 μM), p38 MAPK (SB202190, 10 μM), JNK (SP600125, 10 μM), PI3K (WM, 500 nM) and Akt (API, 1 mM), N2a cells were pretreated with these inhibitors 30 min prior to LA (500 μM) administration. For the experiments involving antioxidants, N2a cells were pretreated with NAC (20 mM) or DPI (10 μM) 30 min prior to LA (500 μM) administration.

2.3. Measurement of neurite outgrowth

For experimental analyses of the dose– and time–response of neurite outgrowth in N2a cells, cells were treated with LA at various concentrations (0, 100, 300 or 500 μM) for different time periods (24, 36, 48 and 60 h). Neurite outgrowth was then observed under a phase-contrast light microscope (Axiovert 200, Zeiss Ltd., Germany) at a magnification of 200×. A neurite was defined as a process with length greater than twofold the cell body diameter and possessing a terminal growth cone, as described previously [18]. The percentage of cells with neurites was calculated by counting 300–400 cells per well in randomly chosen fields. $n=3$–4/group.

Measurements of neurite outgrowth in primary hippocampal neurons were made under a phase-contrast light microscope 24 and 36 h following LA stimulation. For experiments using inhibitors of ROS production (NAC, 20 mM) or MEK (PD98059, 40 μM), primary neurons were pretreated with these inhibitors 30 min prior to LA (500 μM) administration, respectively. Neurite length was measured in 5–10 random fields in each well at a magnification of 200×. Images were analyzed using image pro-plus software. $n=3$/group.

2.4. Fluorescent immunocytochemistry

After 60 h (for N2a cells) or 36 h (for primary hippocampal neurons) treatment with LA, cells were fixed in 50% methanol and 50% acetone for 15 min. After blocking with 7.5% NGS for 1 h at room temperature, the primary β-tubulin-3 antibody (1:500) was applied to the cells overnight at 4 °C. Subsequently, a Cy3-conjugated secondary antibody (1:100) was added to the cells for 2 h at room temperature. Hoechst 33342 was used to counter-stain the nuclei. Images were observed under a fluorescent microscope (Zeiss Ltd., Germany). $n=3$/group.

2.5. Determination of intracellular ROS levels

ROS levels in N2a cells were determined using a DCFH assay in the following treatment groups: (1) N2a cells treated with at different concentrations of LA (0, 100, 300 and 500 μM) for 30 min ($n=4$/group) and (2) N2a cells treated with LA (500 μM) for 30 min in the presence or absence of antioxidants (NAC 20 mM or DPI 10 μM; $n=4$–6/group).

DCFH-DA is a cell-permeable compound. When it enters cells, the acetate group is cleaved by cellular esterases, and non-fluoresent DCFH is trapped inside. Following oxidation by ROS, DCFH yields the fluorescent product DCF. Therefore, DCFH-DA is an ROS-sensitive probe that can detect oxidative activity in living cells. In the present study, cells were incubated with DCFH-DA (10 μM) for 30 min in the dark. Data were collected by a fluorometer equipped with a 24-well plate reader (Synergy HT, BIO-TEK, USA) at an excitation/emission wavelength of 485/530 nm.

2.6. Cell proliferation

Cell proliferation was examined using a MTT assay. N2a cells were treated with LA at various concentrations (500, 1000 or 1500 μM). MTT assays were performed 24, 40 and 60 h following LA treatment, respectively, according to manufacturer's instructions. At the end of LA treatment, cells were incubated with MTT (0.5 mg/ml) for 4 h. The crystals that formed were solubilized in 100 μl of DMSO, and the color was read photometrically at 570 nm on a Synergy HT plate reader (Synergy HT, Bio-Tek, USA; $n=5$/group).

2.7. Western blot

Phosphorylation levels of Akt, ERK, p38 MAPK and JNK were examined by Western blot in N2a cells that received the following treatments: (1) N2a cells treated with LA (500 μM) for 0.5, 1, 3, 6, 12, 24 and 36 h ($n=3$/group) and (2) N2a cells treated with LA (500 μM) for 30 min in the presence or absence of an antioxidant (NAC), MEK inhibitor (PD98059), p38 MAPK inhibitor (SB202190), JNK inhibitor (SP600125), PI3K inhibitor (WM) or Akt inhibitor (API). $n=3$–7/group.

Western blots were performed as previously described [11]. Briefly, equal amounts of protein extracts were separated on a 10% SDS-PAGE gel and transferred onto an immobilon-p membrane (Milipore Corp). Following the blocking step, membranes were incubated with primary antibodies at 4 °C overnight, followed by incubation with a secondary antibody. The same membranes were also probed with anti-α-tubulin for loading control. Proteins were detected with an ECL kit, and signals were quantified using scanning densitometry.

2.8. Statistical analyses

Results are expressed as means ± standard deviation ($x\pm SD$). Comparison data between groups was performed using one-way analysis of variance (ANOVA). Tukey's procedure for multiple range tests was performed. $P<0.05$ was considered statistically significant.

3. Results

3.1. LA induces neurite outgrowth in N2a cells and primary neurons

We examined the effects of different doses of LA and periods of treatment on neurite outgrowth in N2a cells. Fig. 1A shows that LA-stimulated neurite outgrowth in N2a cells in a time- and dose-dependent manner. Following treatment with LA for 24 h, the number of neurite-bearing cells was significantly increased in the cells treated with 500 μM of LA but did not increase in the cells treated with100 μM and 300 μM of LA, when compared with untreated control cells. However, when compared with time-matched, untreated cells, the number of neurite-bearing cells increased 0.86-, 1.80- and 4.41-fold following stimulation with LA for 60 h at concentrations of 100, 300 and 500 μM, respectively ($P<0.01$ or 0.05). Based on these data, we used an LA concentration of 500 μM in the subsequent experiments.

We also examined the effect of LA on neurite outgrowth in primary hippocampal neurons. Fig. 1B shows that neurite length was

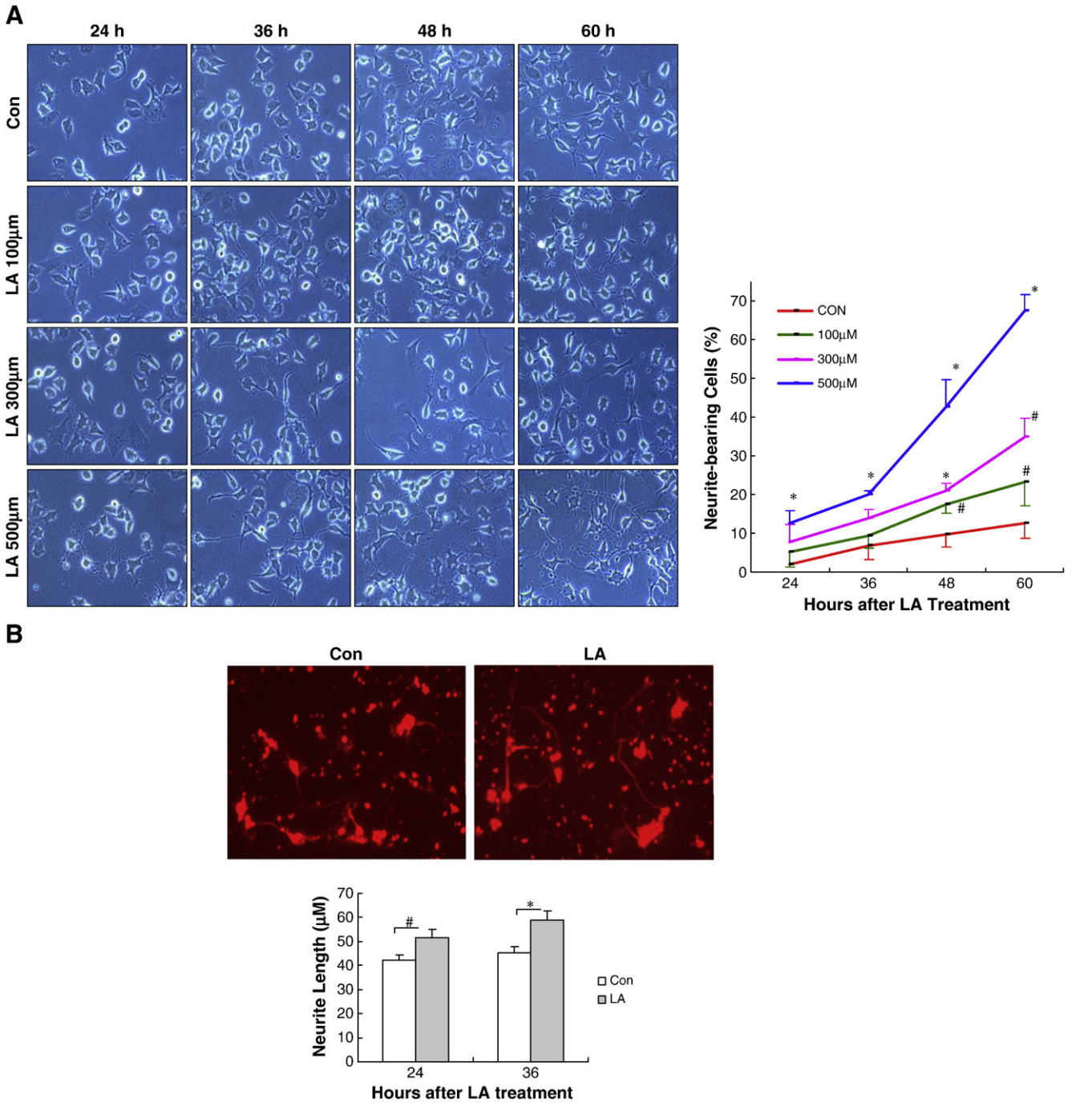

Fig. 1. LA promotes neurite outgrowth. (A) LA induces neurite outgrowth of N2a cells in a dose- and time-dependent manner. N2a cells were stimulated with LA at various concentrations (100, 300 and 500 μM) for different periods (24, 36, 48 and 60 h), respectively. Cell morphology was observed under a light microscope (200×) and the neurite-bearing cells were counted. $^{*}P<0.01$ and $^{\#}P<0.05$ vs. time-matched controls, $n=3$/group. (B) LA promotes neurite growth in primary hippocampal neurons. Neurite length was measured in neurons following incubation with LA (500 μM) for 24 and 36 h. Upper panel represents neurite morphology of neurons treated with LA for 36 h or untreated neurons, as visualized by immunostaining for β-tubulin-3. $^{*}P<0.01$ and $^{\#}P<0.05$, $n=3$/group.

significantly increased following LA treatment for 24 h ($P<0.05$) and 36 h ($P<0.01$), respectively, when compared with time-matched untreated cells. The upper panel shows the representative morphology of untreated neurons or those treated with LA for 36 h.

3.2. LA increases phosphorylation of Akt and ERK in N2a cells

To determine whether PI3K/Akt and/or ERK signaling is involved in LA-mediated neurite outgrowth in neurons, we examined the phosphorylation levels of Akt and ERK in N2a cells with or without LA treatment. As shown in Fig. 2, LA treatment significantly increased both Akt and ERK phosphorylation in a time-dependent manner. The levels of phospho-Akt were significantly increased by 206.4%, 64.1%, 92.1%, 162.3%, 187.8%, and 210.4% following LA treatment for 0.5, 1, 3, 6, 24, and 36 h, respectively, when compared with untreated cells ($P<0.01$). The levels of phospho-ERK were significantly increased by 229.4%, 162.5%, 86.3%, 75.1%, 154.0%, and 189.2% following LA treatment for 0.5, 1, 3, 6, 24, and 36 h, respectively, when compared with untreated control cells ($P<0.01$ or 0.05).

3.3. PI3K/Akt inhibition does not affect LA-induced neurite outgrowth in N2a cells

To determine whether the activation of PI3K/Akt signaling is involved in LA-induced neurite growth, we treated N2a cells with WM, a PI3K inhibitor, 30 min prior to LA administration. Fig. 3A shows that WM treatment prevented LA-induced Akt phosphorylation. However, WM administration did not affect LA-induced neurite outgrowth (Fig. 3B). We next pretreated N2a cells with API, a selective Akt inhibitor, 30 min prior to LA administration. As shown in Fig. 3C, API administration significantly reduced LA-induced phospho-Akt by 69.1%. However, similar to WM treatment, API administration did not affect the LA-induced increase in the number of neurite-bearing cells (Fig. 3D). These data suggest that activation of PI3K/Akt signaling is not involved in LA-induced neurite outgrowth.

Interestingly, the administration of either WM or API to LA-treated N2a cells significantly increased the levels of phospho-ERK (Fig. 3E, F). These data suggests that there is an interaction between PI3K/Akt signaling and the ERK pathway.

3.4. LA-induced neurite outgrowth requires activation of ERK

We next examined the role of ERK pathway activation on LA-induced neurite outgrowth. We treated N2a cells with an ERK inhibitor, PD98059 (PD), 30 min prior to LA administration. As shown in Fig. 4A, PD administration significantly reduced LA-induced increases in the levels of phospho-ERK by 76.4% when compared with cells treated with LA alone. PD administration also reduced the levels of phospho-ERK by 68.2% in LA-untreated cells. Fig. 4B shows that the inhibition of ERK activation by PD administration completely abolished LA-induced neurite outgrowth. These data suggest that LA-induced neurite outgrowth is mediated by the activation of ERK signaling.

We also examined whether the other two members of the MAPK family, p38 MAPK and JNK, are involved in LA-induced neurite outgrowth. As shown in Fig. 4C, p38 MAPK and JNK phosphorylation levels in LA-treated N2a cells were increased by 97.5% and 73.8%, respectively, when compared with untreated controls ($P<0.01$ or 0.05). Treatment with SB202190 and SP600125 significantly suppressed the LA-induced activation of p38 MAPK and JNK, respectively (Fig. 4D, F). The inhibition of JNK abolished LA-induced neurite outgrowth (Fig. 4E), whereas the inhibition of p38 MAPK did not (Fig. 4G).

3.5. LA-induced neurite outgrowth requires ROS production in N2a cells

We examined how LA administration activates ERK signaling. It has been shown that transiently produced ROS could serve as a signaling molecule. Moderate production of ROS has been reported to be essential for neuronal outgrowth and differentiation [19,20]. We therefore examined whether LA administration induces ROS production in N2a cells. We treated N2a cells with 100, 300 or 500 μM of LA for 30 min and measured ROS production. As shown in Fig. 5A, treatment with these concentrations of LA increased ROS production by 18.2%, 39.2%, and 44.0%, respectively, when compared with untreated cells ($P<0.01$). The upper panel of Fig. 5C shows

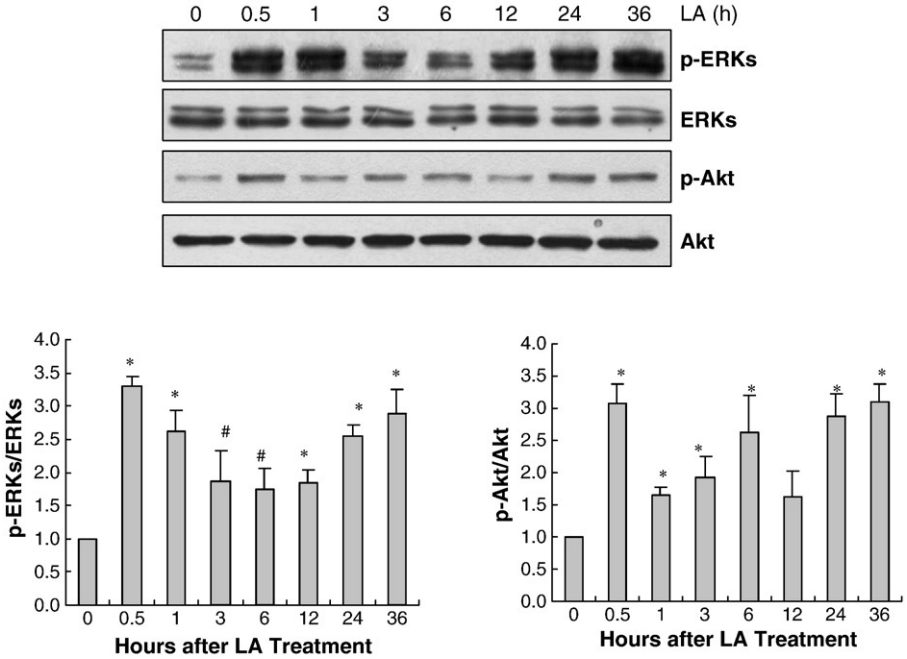

Fig. 2. LA increases the levels of phospho-ERK and phospho-Akt in N2a cells. N2a cells were challenged with LA (500 μM) for 0.5, 1, 3, 6, 12, 24 and 36 h. Protein extracts were prepared for Western blot analysis. $^{*}P<0.01$ and $^{\#}P<0.05$ vs. 0 h group, $n=3$/group.

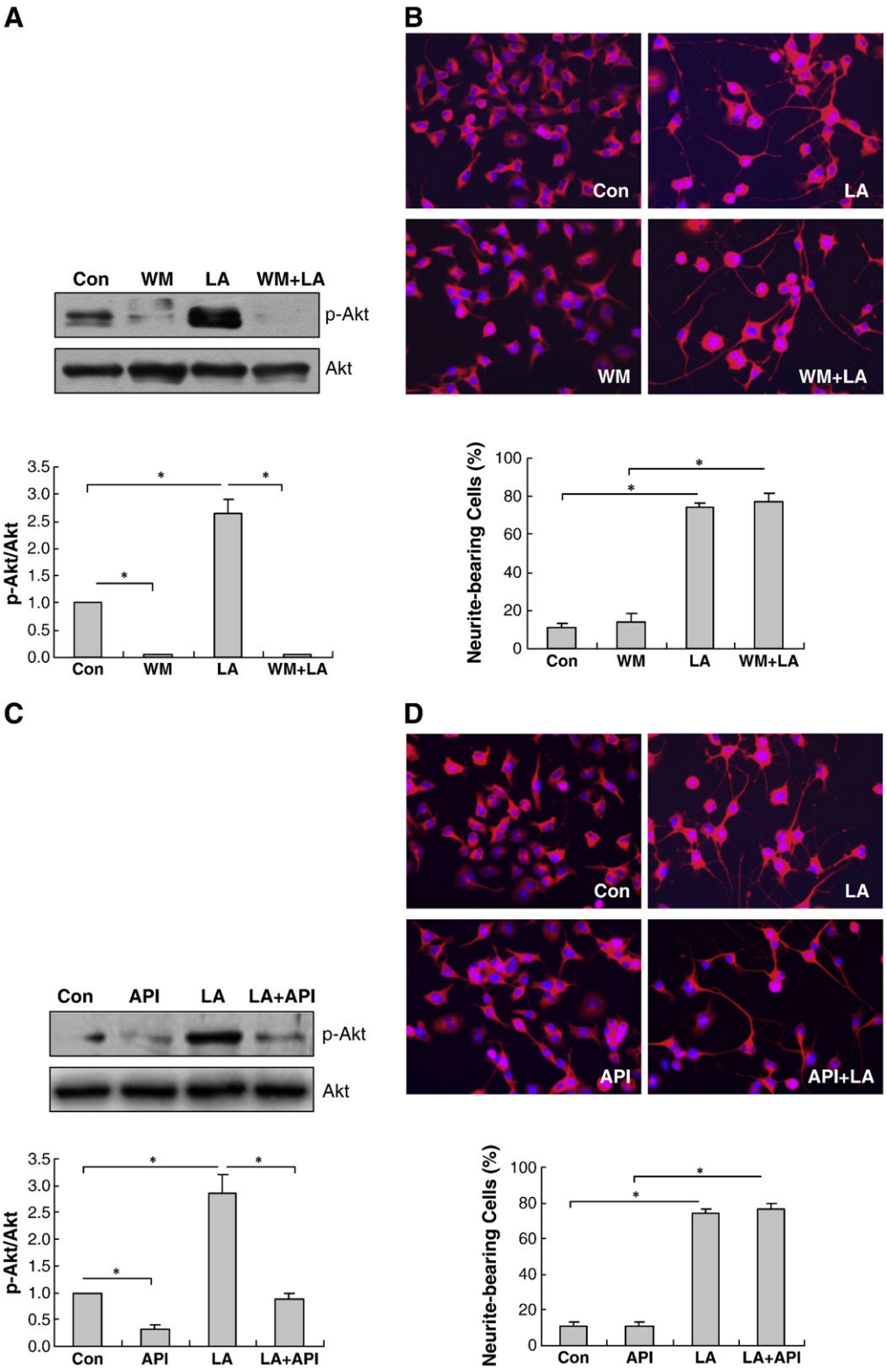

Fig. 3. Inhibition of PI3K/Akt does not affect LA-induced neurite outgrowth. (A) WM inhibits LA-induced Akt activation. N2a cells were pretreated with WM 30 min prior to LA exposure (500 μM). Thirty minutes following LA stimulation, cellular extracts were prepared and subjected to Western blot analysis. $^{*}P<0.01$, $n=3$/group. (B) WM has no effect on LA-induced neurite outgrowth. N2a cells were pretreated with WM 30 min prior to LA exposure (500 μM). Sixty hours following LA treatment, morphology was examined and neurite-bearing cells were counted under a light microscope (200×). $^{*}P<0.01$, $n=3$/group. Upper panel shows neurites visualized by immunostaining with β-tubulin-3 ($n=3$/group). (C) API inhibits LA-induced Akt activation. N2a cells were pretreated with API 30 min prior to LA exposure (500 μM). Thirty minutes following LA stimulation, cellular extracts were prepared and subjected to Western blot analysis. $^{*}P<0.01$, $n=3$–6/group. (D) API has no effect on LA-induced neurite outgrowth. N2a cells were pretreated with API 30 min prior to LA exposure (500 μM). Sixty hours following LA treatment, morphology was examined and neurite-bearing cells were counted under a light microscope (200×). $^{*}P<0.01$, $n=3$/group. Upper panel shows the neurites visualized by immunostaining with β-tubulin-3 ($n=3$/group). (E, F) Inhibition of PI3K/Akt signaling activates ERK. N2a cells were pretreated with WM or API 30 min prior to LA exposure (500 μM). Thirty minutes following LA stimulation, cellular extracts were prepared and subjected to Western blot analysis. $^{*}P<0.01$, $n=3$–6/group.

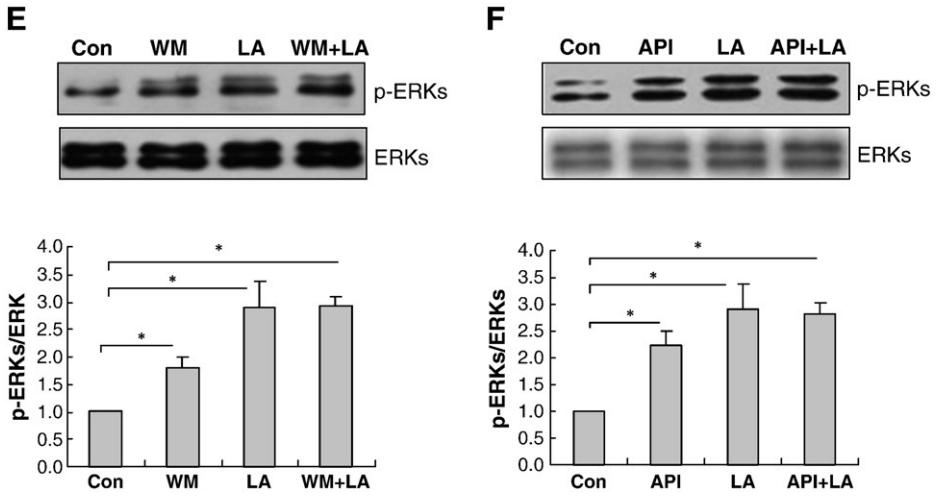

Fig. 3 (*continued*).

representative images of DCF fluorescence in cells treated with or without 500 μM LA. DCF fluorescence was stronger in LA-treated cells than in untreated cells.

Because excessively high levels of ROS are detrimental to the cell, we used a MTT assay to examine whether higher concentrations of LA could inhibit cell proliferation. As shown in Fig. 5B, 500 μM of LA did not significantly modify cell proliferation when compared with time-matched controls at all measured time points. However, proliferation was significantly lower in cells treated with 1000 or 1500 μM LA when compared with time-matched controls ($P<0.01$).

To determine the role of ROS production in LA-induced neurite outgrowth, we treated N2a cells with two antioxidants, NAC or DPI, 30 min prior to LA administration. Fig. 5C shows that either NAC or DPI administration significantly prevented LA-induced ROS production. Importantly, LA-induced neurite outgrowth was completely abolished by both NAC and DPI pretreatment (Fig. 5D). Fig. 5E shows that NAC administration significantly decreased LA-induced phospho-ERK levels by 69.3% when compared with LA-treated, NAC-untreated cells. These data suggest that LA-induced neurite outgrowth requires moderate ROS production, which serves as a signaling molecule for the activation of ERK.

3.6. LA-induced increases in neurite length in primary neurons is inhibited by antioxidant and ERK inhibitor

Finally, we examined whether antioxidant or ERK inhibitor could suppress the observed LA-induced increase in neurite length in primary neurons (Fig. 1B). Primary hippocampal neurons were pretreated with the antioxidant, NAC, or the MEK/ERK inhibitor, PD, 30 min prior to LA exposure. As shown in Fig. 6, treatment with either NAC or PD blocked the LA-induced increase in neurite length ($P<0.01$). The upper panel shows representative neurite morphology of cells treated with LA for 36 h in the presence or absence of NAC or PD.

4. Discussion

The primary findings of the present study are that treatment with LA promotes neurite outgrowth in both primary hippocampal neurons and mouse neuroblastoma N2a cells. Moreover, LA stimulation activated both PI3K/Akt and ERK signaling. However, the inhibition of ERK activation, but not PI3K/Akt, abolished LA-stimulated neurite growth. We confirmed that LA-induced ERK activation was mediated by production of ROS, which serves as a signaling molecule. Inhibition of ROS production via antioxidant administration completely abolished LA-induced neurite outgrowth and ERK activation. Our results suggest that the observed LA-induced neurite outgrowth was mediated by the activation of ERK signaling through a ROS-dependent mechanism.

It has long been noted that axonal and synaptic damage can precede the loss of neuronal cell bodies by months [21,22]. Recently, it has become clear that the loss of major synapses and axons occurs long before the appearance of symptoms in most, and possibly all, neurodegenerative diseases, including Alzheimer's, Parkinson's, Huntington's, motoneuron and prion diseases [21,22]. We have previously reported that LA treatment prevents drug-induced neurite loss and neuronal death in N2a cells [11], suggesting that neurite protection could be a potential mechanism mediating LA-induced neuroprotection. Indeed, we demonstrate in the present study that prolonged LA exposure of both primary hippocampal neurons and N2a cells significantly enhances neurite outgrowth. Recent studies have shown that LA has neuroprotective properties in both patients and animal models with various neural diseases, including pain disorders [4], Alzheimer's disease [5], Parkinson's disease [6] and diabetic polyneuropathy [23]. Interestingly, LA has been considered as a novel therapeutic approach for multiple sclerosis and other chronic inflammatory diseases of the central nervous system [24]. The mechanisms of LA's protective effects involve inhibiting T-type calcium channels in the pain pathway [4], reducing oxidative damage and improving cognitive performance [25,26]. In addition, LA may inhibit ASK1/Daxx signaling [6] and activate PI3K/Akt or PI3K/PKG/ERK pathways [5,11]. However, nothing is known regarding whether these signaling pathways are involved in LA-induced neurite outgrowth.

The activation of the PI3K/Akt signaling pathway has been reported to play a role in mediating neuronal differentiation [12–14] and in protecting neurons from drug-induced damage [5,11]. In the present study, we observed that LA administration significantly increased the levels of phospho-Akt in N2a cells, suggesting that LA stimulation activated the PI3K/Akt signaling pathway. However, pharmacological inhibition, either by WM to inhibit PI3K activity or by API to prevent Akt phosphorylation, did not affect LA-induced neurite outgrowth. These data indicate that PI3K/Akt signaling activation is not involved in LA-induced neurite outgrowth in N2a cells. Interestingly, we observed that PI3K/Akt inhibition significantly increases levels of ERK phosphorylation, indicating that there may be an interaction between PI3K/Akt and ERK. This finding also suggests that the activation of ERK signaling may be involved in LA-induced neurite outgrowth.

Indeed, we observed that LA administration increased levels of ERK phosphorylation in N2a cells, suggesting that LA treatment

activated ERK signaling. To determine the role of ERK activation in LA-induced neurite outgrowth, we treated cells with an ERK-specific inhibitor 30 min prior to LA administration. We observed that administration of the ERK inhibitor prevented LA-induced ERK phosphorylation. More significantly, ERK inhibition completely abolished LA-induced neurite outgrowth in primary neurons and N2a cells. These data provide strong evidence that the activation of ERK is required for LA-stimulated neurite outgrowth. Our observation is supported by other studies showing the involvement of ERK signaling in neuronal differentiation. For example, NGF- and human chorionic gonadotropin (hCG)-stimulated neurite outgrowth in PC12 cells has been shown to activate ERK signaling [15,16]. Moreover, the inhibition of ERK activation prevents both NGF- and hCG-stimulated neurite outgrowth [17]. Collectively, these data suggest that ERK activation is essential for neurite outgrowth. However, the mechanism by which LA activates the ERK signaling pathway is still unclear.

ROS can be generated by multiple sources, including NADPH oxidases, xanthine oxidase, uncoupled NO synthase and mitochondria [27]. NADPH oxidases produce ROS in normoxic conditions, whereas other enzymes produce ROS only following its conversion to a dysfunctional state [27]. DPI is an inhibitor of NADPH oxidases, xanthine oxidase and uncoupled NO synthase [27]. In the present study, we observed that DPI inhibited LA-stimulated ROS production in N2a cells grown in normoxic conditions, suggesting the involvement of NADPH oxidases in LA-induced ROS production.

Numerous studies have shown that LA mediates its antioxidant effects by scavenging reactive oxygen and nitrogen species in vitro. However, there is little evidence showing that this occurs in vivo or

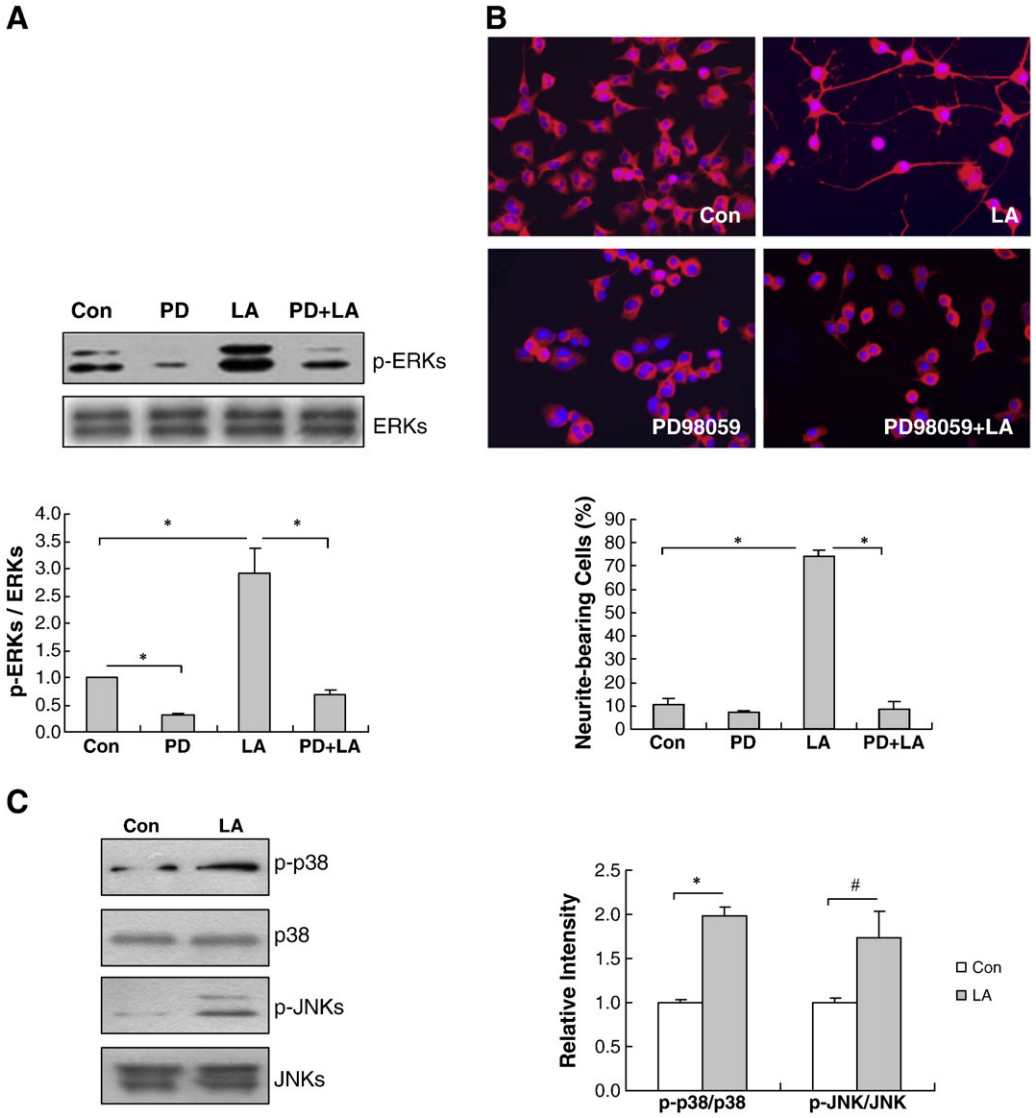

Fig. 4. Inhibition of MEK/ERK abolishes LA-induced neurite outgrowth. (A) PD98059 (PD) suppresses LA-induced activation of ERK. N2a cells were pretreated with PD 30 min prior to LA exposure (500 μM). Thirty minutes following LA stimulation, cellular extracts were prepared and subjected to Western blot analysis. $^{*}P<0.01$, $n=3$–6/group. (B) PD98059 abolishes LA-induced neurite outgrowth. N2a cells were pretreated with PD 30 min prior to LA exposure (500 μM). Sixty hours following LA treatment, morphology was examined and the neurite-bearing cells were counted under a light microscope (200 ×). $^{*}P<0.01$, $n=3$/group. Upper panel shows neurites visualized by immunostaining against β-tubulin-3 ($n=3$/group). (C) LA treatment induces activation of p38 MAPK and JNK. N2a cells were treated with LA (500 μM) for 30 min. Cellular extracts were prepared and subjected to western blot analysis. $^{*}P<0.01$ and $^{\#}P<0.05$, $n=3$/group. (D, E) Inhibition of JNK suppresses LA-induced neurite outgrowth. N2a cells were pretreated with SP600125 30 min prior to LA exposure (500 μM). JNK activation was examined 30 min following LA treatment (D). Morphology was examined and neurite-bearing cells were counted under a light microscope (200×) following a 60-h treatment with LA (E). Neurites were visualized by immunostaining against β-tubulin-3 *(E)*. $^{*}P<0.01$ and $^{\#}P<0.05$, $n=3$/group. (F, G) Inhibition of p38 MAPK has no effect on LA-induced neurite outgrowth. N2a cells were pretreated with SB202190 30 min prior to LA exposure (500 μM). Activation of p38 MAPK was examined 30 min following LA treatment (F). Morphology was examined and neurite-bearing cells were counted under a light microscope (200×) following 60-h treatment with LA (G). Neurites were visualized by immunostaining against β-tubulin-3 *(G)*. $^{*}P<0.01$, $n=3$/group.

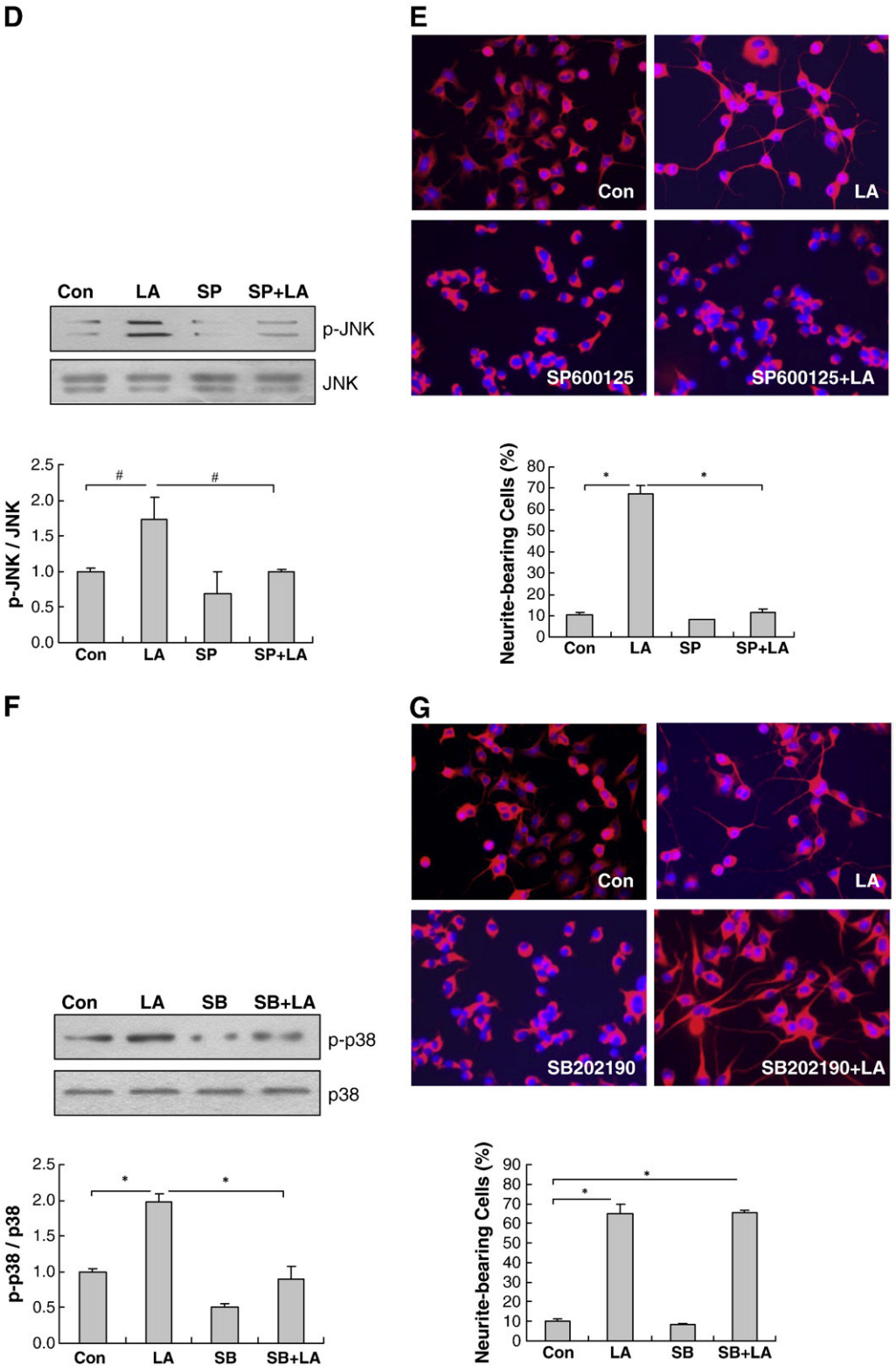

Fig. 4 (*continued*).

that radical scavenging contributes to the primary mechanism of action of LA [28,29]. Mounting evidence indicates that LA may exhibit antioxidant and prooxidant properties [30,31]. The mechanisms underlying the positive and negative effects of LA on ROS production are not yet clear. However, Dicter et al. suggest that LA acts as a powerful antioxidant only following long-term incubation, whereas it may function as a prooxidant following short-term incubation and rapid uptake by tissue or cells [32]. The beneficial role of LA supplementation on the organismal and cellular levels could be due to its mild prooxidant activity, which could, in turn, lead to cellular adaptation against oxidative stress by increasing redox capacity (e.g., glutathione synthesis) [31,32]. Indeed, the LA-mediated suppression

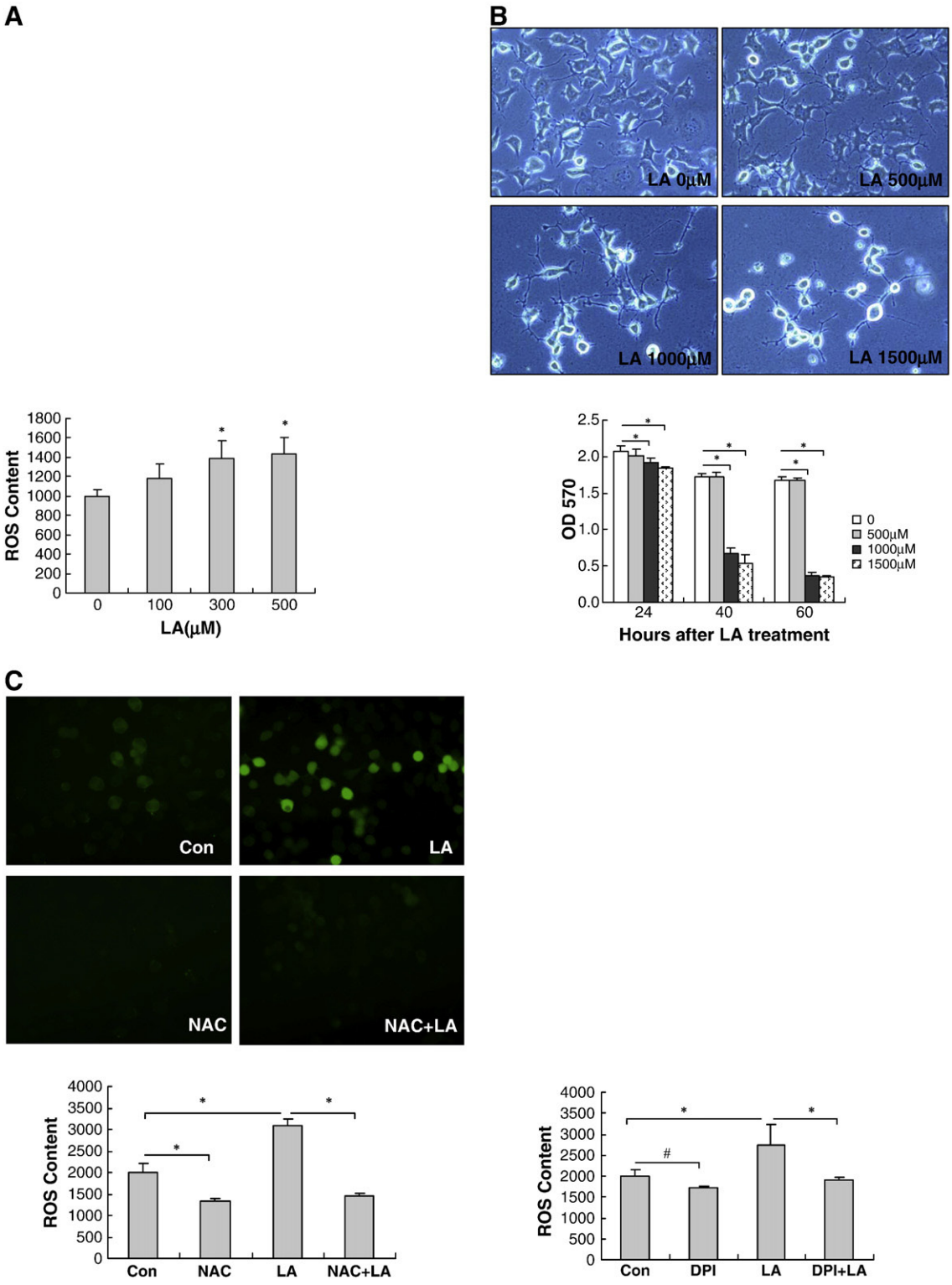

Fig. 5. LA-induced ROS generation is required for LA-induced neurite outgrowth. (A) LA increases ROS generation. ROS levels were determined using the DCFH assay following treatment with LA (0, 100, 300 and 500 μM) for 30 min. $^{*}P<0.01$ vs. 0 μM group, $n=4$/group. (B) Effect of LA on cell proliferation. Cell proliferation was measured using a MTT assay. N2a cells were treated with LA at different concentrations (500, 1000 or 1500 μM). Untreated cells served as controls. The MTT assay was performed at 24, 40 and 60 h following LA treatment, respectively. Note that treatment with 500 μM LA did not significantly alter cell proliferation. Upper panel shows the representative morphology of N2a cells treated with LA for 48 h. $^{*}P<0.01$, $n=5$/group. (C) Antioxidants abolish the LA-induced ROS generation. Cells were treated with two antioxidants, NAC and DPI, 30 min prior to LA administration (500 μM). ROS levels were determined using a DCFH assay following 30 min treatment with LA. Upper panel shows representative images of DCF fluorescence in N2a cells that treated with LA for 30 min in the presence or absence of NAC. $^{*}P<0.01$ and $^{\#}P<0.05$, $n=4$–6/group. (D) Treatment with antioxidants abolishes LA-induced neurite outgrowth. N2a cells were treated with NAC or DPI 30 min prior to LA administration (500 μM). Sixty hours following LA treatment, morphology was examined and neurite-bearing cells were counted under a light microscope (200×). $^{*}P<0.01$, $n=3$–4/group. Upper panel shows neurites visualized by immunostaining with β-tubulin-3 ($n=3$/group). (E) Treatment with antioxidants inhibits LA-induced activation of ERK. N2a cells were pretreated with NAC 30 min prior to LA administration. The levels of phospho-ERK were examined 30 min following LA administration. $^{*}P<0.01$ and $^{\#}P<0.05$, $n=4$–7/group.

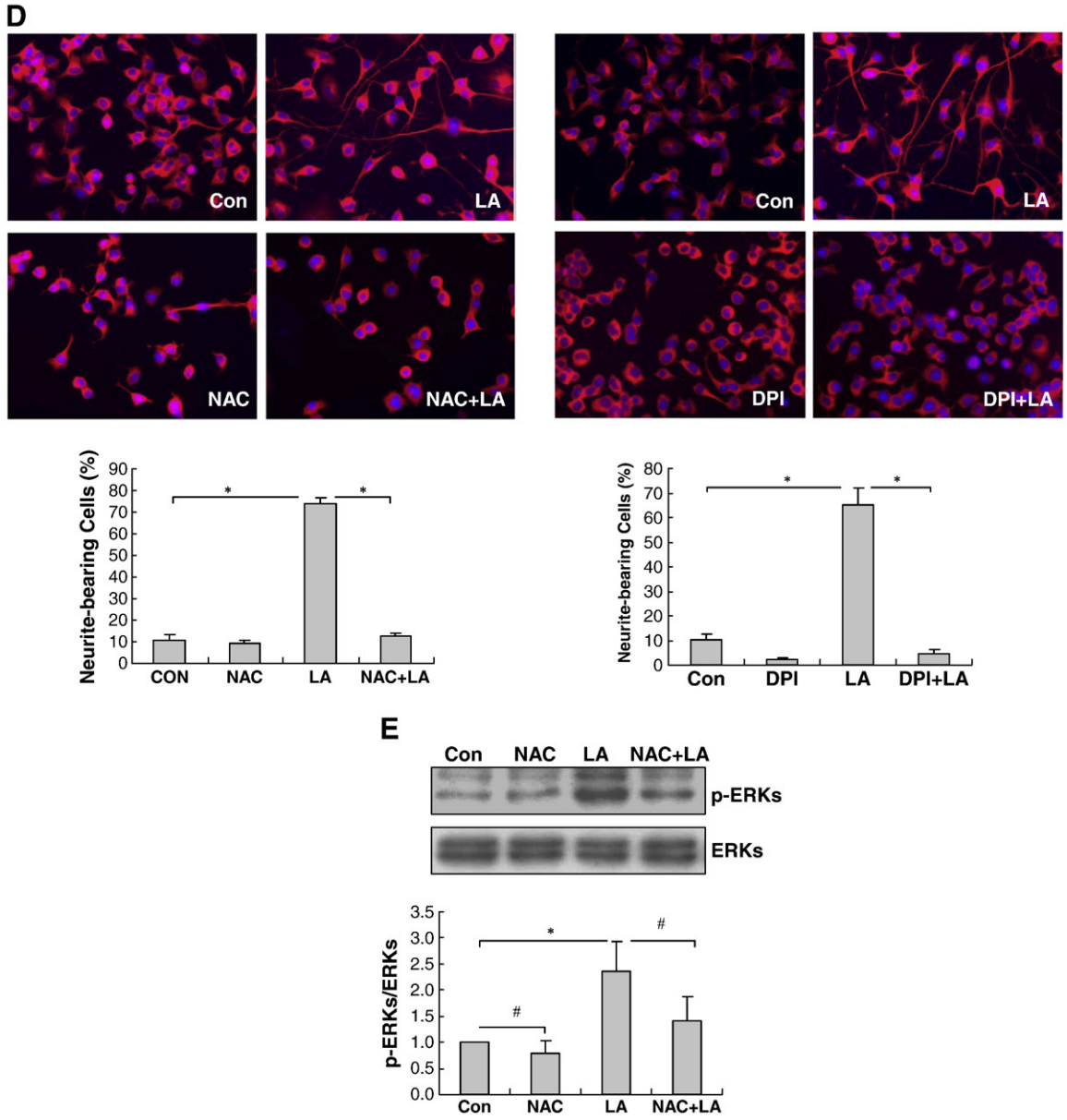

Fig. 5 (*continued*).

of 6-hydroxydopamine- induced ROS generation and apoptosis was shown to require pre-incubation of PC12 cells with LA for 12–24 h [31]. Recent evidence suggests that the therapeutic and anti-aging effects of LA are due to its modulation of signal transduction and gene transcription, both of which improve the antioxidant status of the cell. Paradoxically, this likely occurs via prooxidant mechanisms, not by radical scavenging [28–30]. ROS has not only been shown to be toxic but also to function as a signaling molecules [28,32,33]. We and others have reported that the administration with LA moderately increases cellular ROS levels [11,32,34]. These moderately enhanced ROS levels were positively correlated with an LA-mediated improvement of neuronal survival [11,35,36]. In the present study, we observed that LA administration induces transient moderate ROS production in N2a cells. However, treatment of the cells with antioxidants prior to LA administration significantly attenuates LA-induced ERK activation. More importantly, administration of antioxidants completely abolished LA-stimulated neurite outgrowth in primary neurons and N2a cells. These data suggest that LA induces the moderate production of ROS, which in this content serve as signaling molecules and lead to the activation of ERK. The activation of this pathway results in neurite outgrowth. Our observation is supported by other studies [1,18–20]. For example, NGF- and $CoCl_2$-induced neurite outgrowth in PC12 cells requires the production of ROS [19,20]. When this evidence is considered as a whole, it suggests that transient, moderate generation of ROS by LA is required for LA-stimulated neurite outgrowth, which is mediated through the activation of ERK signaling.

In summary, this study demonstrates that LA stimulates neurite outgrowth in both primary hippocampal neurons and mouse neuroblastoma N2a cells. The mechanisms by which this occurs involve the LA-induced activation of ERK signaling through a ROS-dependent mechanism.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

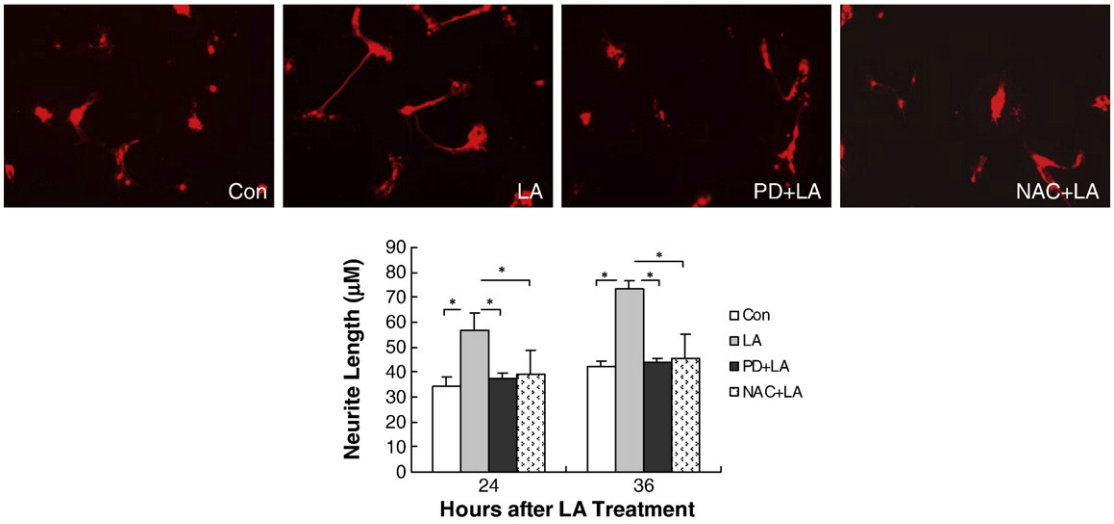

Fig. 6. LA-induced increases in neurite length of primary hippocampal neurons is inhibited by antioxidant and ERK inhibitor. Primary hippocampal neurons were pretreated with the antioxidant, NAC, or the MEK/ERK inhibitor, PD, 30 min prior to LA exposure. Neurite length was measured following incubation with LA (500 μM) for 24 and 36 h. The upper panel shows representative neurite morphology visualized by β-tubulin-3 immunostaining of neurons treated with LA for 36 h. $^{*}P<0.01$, $n=3$/group.

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