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The Raf-1 inhibitor GW5074 and the ERK1/2 pathway inhibitor U0126 ameliorate PC12 cells apoptosis induced by 6-hydroxydopamine

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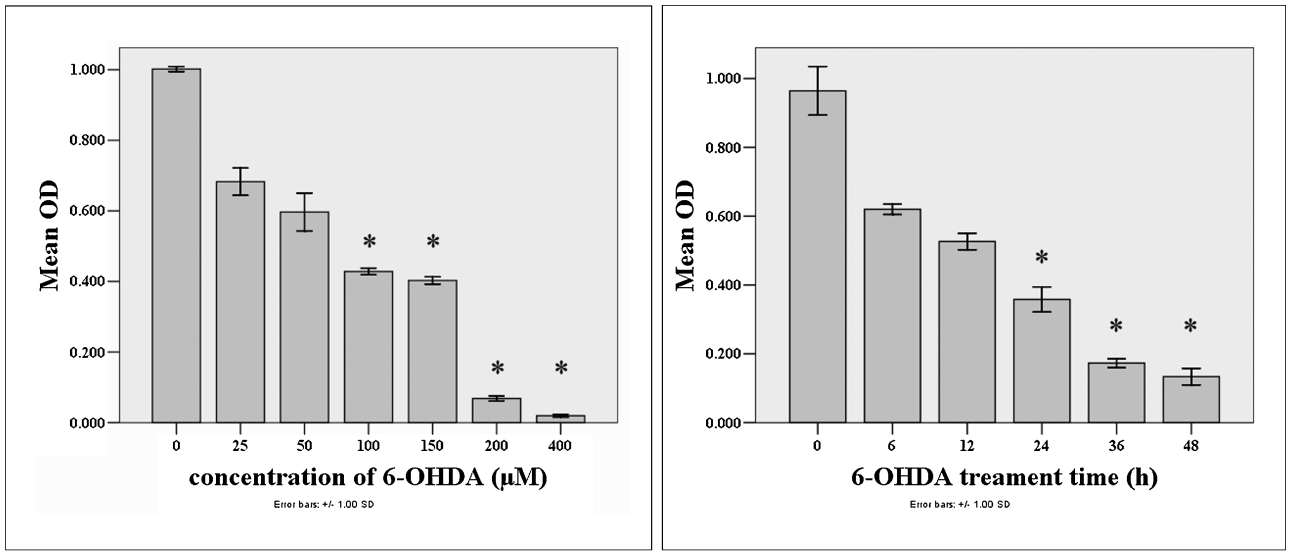
6-Hydroxydopamine (6-OHDA) is a widely used dopaminergic neurotoxin that leads to cell apoptosis *in vivo* and *in vitro*, and is a widely accepted experimental model of neurodegeneration in Parkinson's disease. However, the molecular mechanisms responsible for 6-OHDA-induced cell apoptosis are unclear. We found that the treatment of PC12 cells with 6-OHDA resulted in a significant decrease in cell viability and elevated apoptosis as detected by MTT assay, Hoechst 33258 staining, and flow cytometry. In addition, 6-OHDA induced a time-dependent phosphorylation of ERK1/2 at Thr-202/Tyr-204 and of Raf-1 at Ser-338, but a decreased level of Raf-1 phosphorylation at Ser-259. Phosphorylation of ERK1/2 at Thr-202/Tyr-204 and Raf-1 at Ser-338 were inhibited by the Raf-1 inhibitor GW5074, while the ERK1/2 pathway inhibitor U0126 decreased phosphorylation of ERK1/2. Furthermore, 6-OHDA-induced PC12 cells apoptosis was suppressed by GW5074 and U0126. Our results suggest that GW5074 and U0126 act as neuroprotants against 6-OHDA toxicity in PC12 cells by modulating Raf-1/ERK1/2 signaling systems.

1. Introduction

Parkinson's disease (PD) is a chronic age-related neurodegenerative disease characterized by the progressive death of dopaminergic (DA) neurons, particularly dopaminergic neurons of the ventral midbrain, and by the presence of Lewy bodies in the substantia nigra pars compacta (SNpc) (Fan et al. 2009; Ruffels et al. 2004; Lang and Lozano 1998). The main clinical symptoms of PD are severe motor disturbances including uncontrollable tremor, postural imbalance, slow movement, and rigidity, leading to a significantly reduced quality of life for PD patients. Although the etiology of PD is not fully clear, recent evidence suggests that sustained activation of the mitogen-activated protein kinase pathway may play an important role in the pathogenesis of PD (Gómez-Lázaro et al. 2008; Miller et al. 2007; Chen et al. 2009).

Mitogen-activated protein kinases (MAPKs) are important mediators of signal transduction from the cell surface to the nucleus, and are critical regulators of proliferation, differentiation, and apoptotic cell death. Mammals express at least three distinct groups of MAPKs, the extracellular signal regulated protein kinases 1/2 (ERK1/2), c-Jun N-terminal kinases (JNKs), and p38 MAPKs, along with multiple specific upstream activating kinases. The ERK1/2 group is an ubiquitous regulator of cell growth, proliferation, and differentiation, but a number of studies also suggest that activation by specific external signals can trigger cell death (Chen et al. 2008; Mebratu and Tesfaigzi 2009; Grewal et al. 1999). In the ERK pathway, the MAPKKK called Raf kinases phosphorylate and activate MAPK/ERK kinase (MEK1/2), which in turn phosphorylates and activates ERK1/2. The Raf family of serine/threonine protein kinases, central members of the MAPK pathway, comprise three isoforms, A-Raf, B-Raf, and Raf-1 (C-Raf), each having three conserved regions: two N-terminal regulatory domains (CR1 and CR2) and a C-terminal catalytic kinase domain (CR3). Raf-1 is universally expressed in mammalian cells and is phosphorylated and activated by both Ser/Thr and Tyr kinases, while A-Raf and B-Raf exhibit more restricted expression patterns (Zang et al. 2008; Chong et al. 2003; Leevers et al. 1994). Moreover, Raf/ERK1/2 pathway activation is critical for the induction of apoptotic cell death in some tumors and neurodegenerative diseases (Houben et al. 2007; Wang et al. 2000; El-Ashry et al. 1997; Tanaka et al. 2002; Zhu et al. 2002).

To obtain additional insight into the molecular mechanism of PD, we applied the rat pheochromocytoma (PC12) cell line, which provides an established neuron-like system that can be used as a cellular model for PD (Fu et al. 2010). 6-OHDA, a catecholaminergic neurotoxin that selectively destroys dopaminergic neurons, is proven to be a useful tool to produce PD models. In this study, 6-hydroxydopamine induced the expression and phosphorylation of Raf-1 and ERK1/2 in apoptotic PC12 cells, while inhibition of Raf-1 and ERK1/2 phosphorylation markedly increased PC12 cell survival, indicating that phosphorylation of Raf-1 and ERK1/2 participated



(A) (B)

Fig. 1: Effect of 6-OHDA on OD of PC12 cells assessed by MTT. 6-OHDA induces concentration- and time-dependent cell death. (A) Concentration course plot of OD after 24 h 6-OHDA of PC12 cell cultures as assessed by MTT. (B) Time dependence of 6-OHDA-evoked PC12 cell death was carried out for 6, 12, 24, 36, and 48 h in 100 μM. Data represent the mean ± SEM of three independent experiments. *: $p < 0.05$

in the signaling pathways leading to 6-OHDA-induced apoptosis in PC12 cells. These results define Raf-1/ERK1/2 as possible signaling factors in the pathogenesis of PD.

2. Investigations and results

2.1. 6-OHDA-induced cell death in PC12 cells

To determine the effect of 6-OHDA, we measured cell viability using an MTT mitochondrial function assay. When the PC12 cells were incubated with increasing amounts of 6-OHDA for 24 h, the OD value decreased in a concentration-dependent manner. The 50% lethal dose of 6-OHDA was about 107 μM for 24 h (Fig. 1A); thus, the dose of 100 μM 6-OHDA was used in all the experiments. As shown (Fig. 1B) by the MTT assay, 6-OHDA induced the decline of PC12 cell viability in a time-dependent manner, which is consistent with previous studies on PC12 cells apoptosis (Hanrott et al. 2006).

2.2. 6-OHDA-induced phosphorylation of ERK1/2 in PC12 cells

6-OHDA induced mitochondrial ERK activation in B65 cells (Kulich et al. 2007) and DA caused a strong increase in p-ERK1/2 in rat striatal neurons (Chen et al. 2009). In order to understand the underlying mechanisms of 6-OHDA-induced cytotoxicity and the role of the MAPK signaling, we examined the effect of 6-OHDA on the phospho-activation status of ERK1/2. In this study, ERK1/2 phosphorylation in response to 100 μM 6-OHDA for 2 to 48 h was estimated by Western blotting using a phosphospecific ERK1/2 (Thr-202/Tyr-204) antibody. Indeed, 6-OHDA increased p-ERK1/2 immunoreactivity (Fig. 2A), with peak levels reached after 2, 6, and 24 h treatment that was sustained for 48 hours ($p < 0.05$) as indicated by densitometric analysis of Western blots (Fig. 2B).

2.3. 6-OHDA-induced phosphorylation of Ser338 and dephosphorylation of Ser259 Raf-1 in PC12 cells

Phosphorylation of ERK1/2 is regulated by the upstream kinase Raf-1. We therefore hypothesize that 6-OHDA activation of ERK1/2 can result from upregulation of Raf-1 protein or enhanced Raf-1 phosphorylation in PC12 cells. To investigate the phosphorylation state of Raf-1 in PC12 cells, phospho-specific antibodies that recognize the phosphorylated Ser-259 and Ser-338 of Raf-1 were employed. Stimulation of PC12 cells with 100 μM 6-OHDA produced a marked increase in Raf-1 Ser-338 phosphorylation (Fig. 2A, 2C) but generated gradual reduction of Raf-1 Ser-259 phosphorylation in a time-dependent manner (Fig. 2A, 2D). These results suggest the involvement of Raf-1 in ERK1/2 activation by 6-OHDA and are consistent with previous studies of AD (Echeverria et al. 2008) and other neurodegenerative diseases.

2.4. ERK1/2 phosphorylation and apoptosis by 6-OHDA as attenuated by U0126 and GW5074

To determine whether Raf-1/ERK1/2 activation is directly involved in the apoptosis induced by 6-OHDA, we investigated the effect of Raf-1 and MEK inhibitors on 6-OHDA-induced ERK1/2 and Raf-1 phosphorylation. In Fig. 3, 6-OHDA-induced ERK1/2 phosphorylation in PC12 cells was blocked by U0126. GW5074 also significantly suppressed ERK1/2 phosphorylation. U0126, a MEK inhibitor, did not show any effect on Raf-1 Ser-338 phosphorylation, whereas GW5074, a Raf-1 specific inhibitor, inhibited Raf-1 Ser-259 and Ser-338 phosphorylations.

Pre-incubation with both U0126 and GW5074 showed a protective effect against PC12 cell apoptosis induced by 6-OHDA (Fig. 4A, 4B), which is measured by the decrease in MTT, nuclear fragmentation, and condensation in PC12 cells. Cell viability was significantly decreased following 24 h exposure to 100 μM of 6-OHDA. Treatment with GW5074 and U0126 prior to 6-OHDA exposure significantly increased cell viability. Hoechst33258 staining showed that GW5074 and U0126 reduced chromatin condensation in 6-OHDA-treated PC12 cells (Fig. 4B). These results strongly suggest that Raf-1/ERK1/2 is involved in 6-OHDA-induced apoptosis.

To distinguish early stage apoptotic cells from all cells and further confirm and quantify apoptosis in PC12 cells induced by 6-OHDA, cells were stained with Annexin V-FITC/PI and then analyzed by flow cytometry (Fig. 4C). A similar pattern of neuroprotection was observed using Annexin V-FITC/PI staining. The AnnexinV-FITC/PI staining showed that PC12 cells

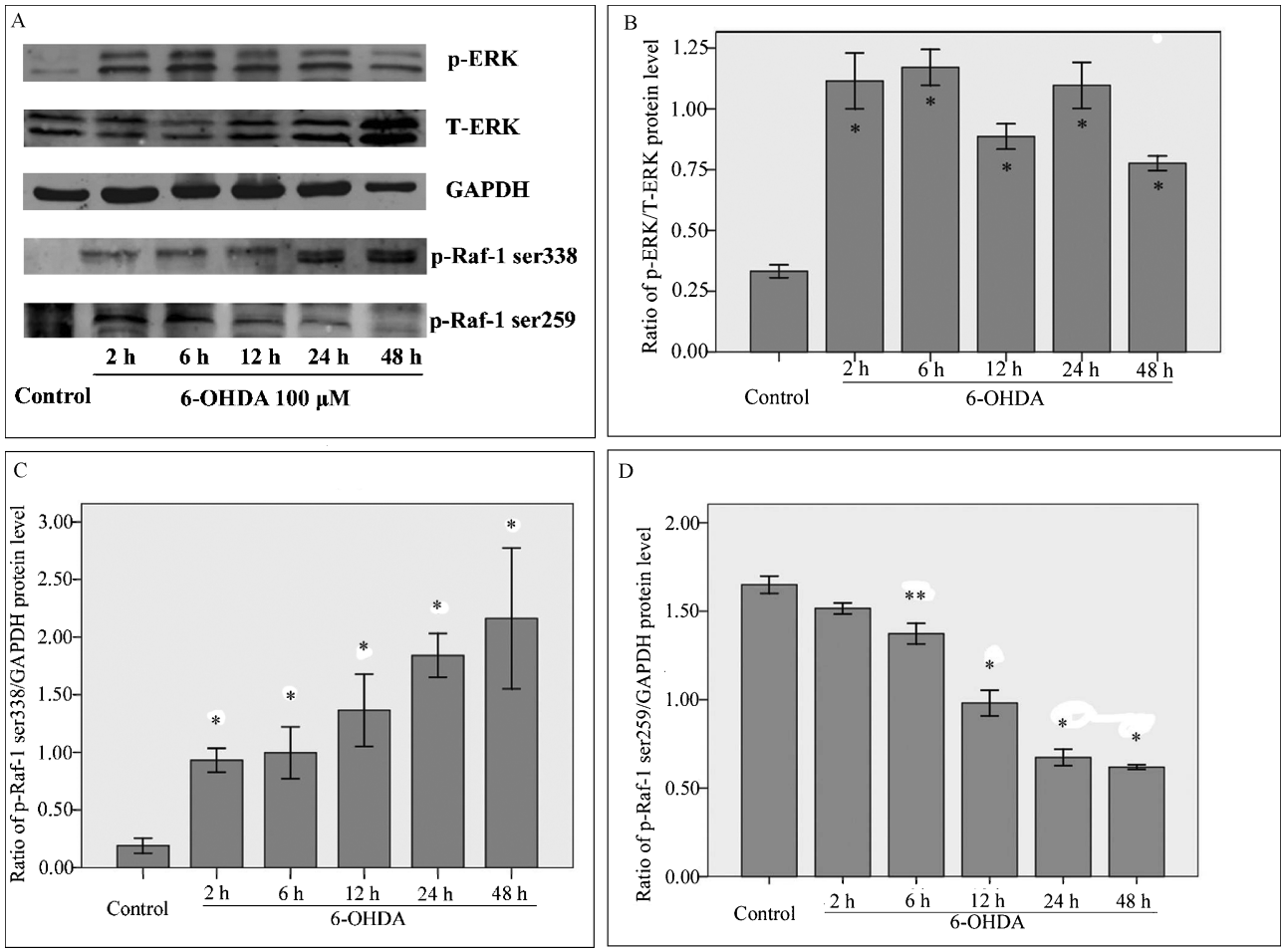


Fig. 2: Time course of 6-OHDA-induced phosphorylation of ERK1/2, Raf-1 ser338, and dephosphorylation of Raf-1 ser259 in PC12 cells. (A) Representative Western blot analysis of ERK1/2 and Raf-1 expression in PC12 cells at various times (2, 6, 12, 24, and 48 h) of 6-OHDA 100 μM treatment. The blots were probed for GAPDH as a loading control. (B, C, D) Combined results represent the mean ± SEM from three independent experiments. Data are presented as the ratio of phospho-ERK1/2 to total ERK1/2, phospho-Raf-1 (ser338) to GAPDH, and dephospho-Raf-1 (ser259) to GAPDH in the graph. Asterisk indicates a P-value less than 0.01 vs. control. Double-asterisk indicates a P-value less than 0.05 vs. control

treated with 100 μM 6-OHDA mainly generated apoptosis more than necrosis following 6-OHDA treatment. Pretreatment with GW5074 and U0126 decreased the percentage of early apoptotic cells.

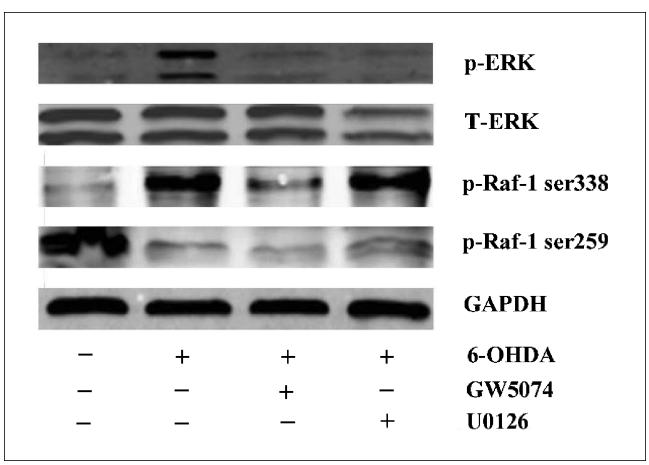


Fig. 3: Effects of ERK inhibitor (U0126, 10 nM) and Raf-1 inhibitor (GW5074, 10 nM) on the phosphorylation of ERK, Raf-1 of PC12 cells induced by 6-OHDA. Representative Western blot analysis of ERK1/2 and Raf-1 phosphorylated expression in PC12 cells with various inhibitors of 6-OHDA treatment. The amount of GAPDH was measured as an internal control. 6-OHDA-induced ERK1/2 phosphorylation in PC12 cells was blocked by U0126. GW5074 also significantly suppressed ERK1/2 phosphorylation. But 6-OHDA-induced Raf-1 phosphorylation at ser338 was not inhibited by the pretreatment with U0126

3. Discussion

In agreement with results from our previous report (Fan et al. 2009), we demonstrated that 6-OHDA induced apoptosis in PC12 cells in the present work. We described the expressions and phosphorylations of Raf-1 and ERK1/2 in 6-OHDA-induced PC12 cell apoptosis. 6-OHDA stimulated time-dependent Raf-1/ERK1/2 phosphorylated activation in PC12 cells in 100 μM. Raf-1/ERK1/2 pathway, through the phosphorylation of Raf-1 and ERK1/2, may partly play a role in PC12 cell death caused by 6-OHDA.

MAPK pathways are regulated through a series of phosphorylation steps in a three-component module: MAPKs are activated by MAPK kinases (MAPKK) on dual residues of threonine and tyrosine, and MAPKKs are in turn phosphorylated by MAPKK kinases (MAPKKK) on dual residues of serine/threonine. ERK1/2 is two isoforms of extracellular signal-regulated kinase (ERK) that belong to the family of mitogen-activated protein kinases (MAPKs). ERK1/2, often activated by various stimuli, is highly involved in cell proliferation, migration, differentiation and death. When considering the ERK cascade, these levels correspond to Raf-1, MEK1/2, and ERK1/2. Prolonged activation of ERK1/2 is associated with neuronal cell apoptosis in response to massive production of reactive oxygen species (ROS) in the hippocampal cell line (Cagnol et al. 2006). Sustained activation of ERK1/2 induced by hyperexcitation and focal ischemic injury may activate cell death programs (Stanciu et al. 2000). The MEK inhibitor U0126 can promote the survival of HT22 cells and primary neuronal cell cultures challenged with glu-

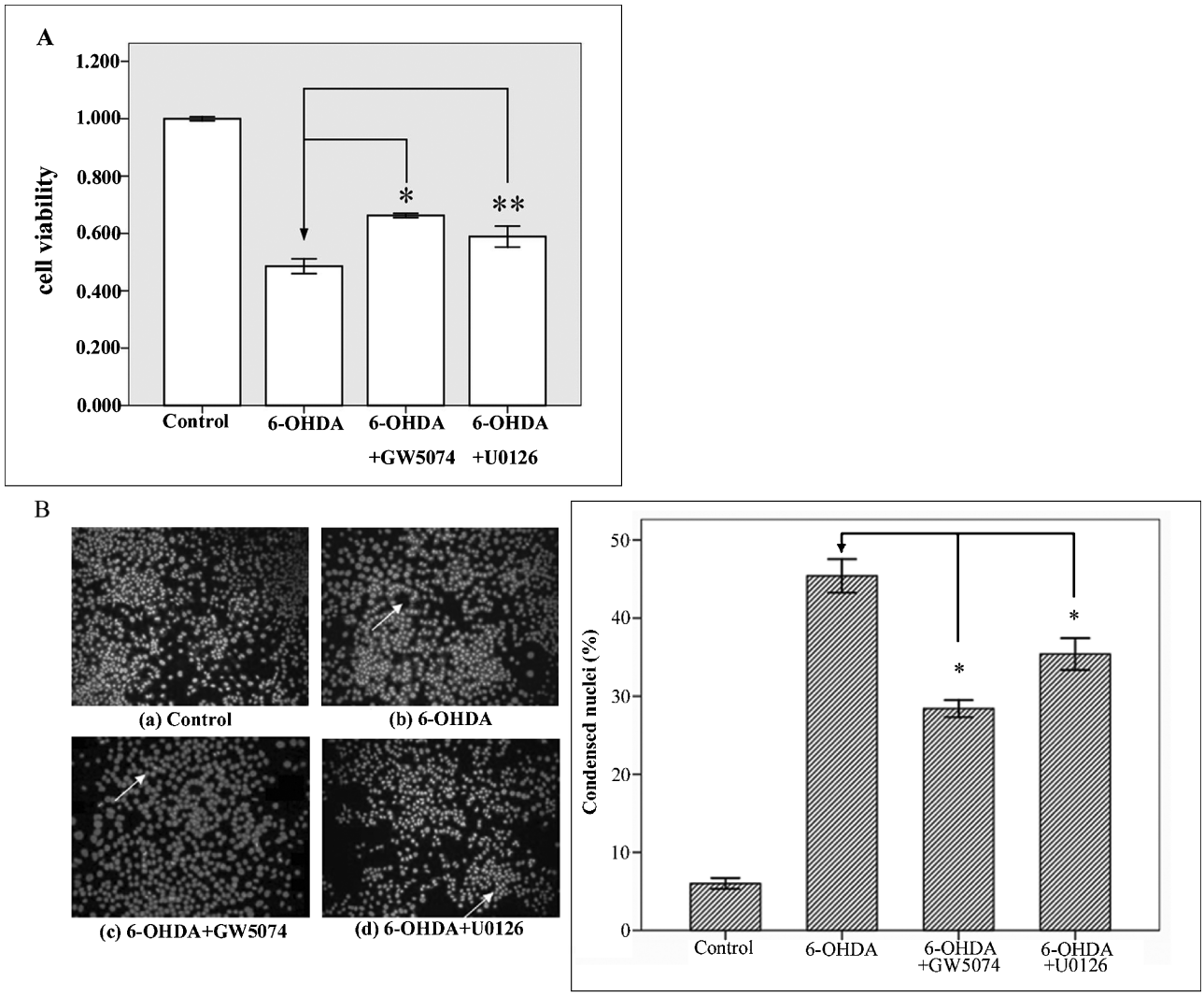


Fig. 4: Effects of GW5074 and U0126 on 6-OHDA-induced PC12 cells apoptosis. (a) Control PC12 cells; (b) PC12 cells treated with 6-OHDA at 100 μM for 24 h; (c) PC12 cells treated with 6-OHDA and 10 nM GW5074; (d) PC12 cells treated with 6-OHDA and 10 nM U0126, (A) Cell viability was evaluated by MTT assay. U0126 and GW5074 could significantly inhibit 6-OHDA-induced cell death. Asterisk indicates a P-value less than 0.01 vs. 6-OHDA alone. Double-asterisk indicates a P-value less than 0.05 vs. 6-OHDA alone. Data are expressed as mean ± SEM from three independent experiments. (B) Effect of GW5074 and U0126 on 6-OHDA-induced chromatin condensation. PC12 cells were stained with Hoechst33258 after incubation for 24 h with or without 100 μM 6-OHDA and observed under fluorescence microscopy. Hoechst33258 staining showed that U0126 and GW5074 suppressed chromatin condensation in the 6-OHDA-treated PC12 cells. Arrows point to the cells that show chromatin condensation (magnification, × 200). Asterisk indicates a P-value less than 0.05 vs. 6-OHDA alone. Data are expressed as mean ± SEM from five independent experiments. (C) Quantitative percentage of apoptotic PC12 cells treated with 6-OHDA, GW5074, and U0126. The percentage of apoptotic and necrotic PC12 cells was determined by Annexin V-FITC/PI staining method. GW5074 and U0126 decreased the percentage of early apoptotic cells

tamate or hypoxia (Satoh et al. 2000). Pharmacological ERK inhibition by intravenous injection of U0126 is also found to elicit neuroprotection against forebrain ischemia (Namura et al. 2001). Furthermore, persistent ERK phosphorylation is detected in degenerating populations of neurons affected by Parkinson's or Lewy body disease (Zhu et al. 2003). Thus, ERK-induced apoptosis might be implicated in some degenerative disorders affecting specific types of neurons. Consequently, in this paper, we confirmed that sustained phosphorylations of ERK1/2 are involved in 6-OHDA-induced apoptosis in PC12 cells (Fig. 2A, 2B).

Raf-1, a well-known member of the Raf serine/threonine kinase family known to phosphorylate MEK, has been shown to participate in ERK signaling cascade. This may be particularly significant as previous studies of neural tissue have identified B-Raf as the main isoform in ERK activation by oestradiol (Toran-Allerand et al. 1999). Raf-l is expressed ubiquitously and its complex activation involves the dephosphorylation of the inhibitory site Ser-259 and phosphorylation at the activation site Ser-338. Among all Raf kinases, Raf-1 is of special interest in relation to neurodegenerative disorders due to its capacity to trigger neuroinflammation (Mei et al. 2006). After stimulation, Raf-l is recruited to the membrane by Ras, where it activates ERK1/2, which in turn phosphorylates and activates ERK. To examine whether Raf-1 might play a crucial role in 6-OHDA-induced PC12 cell apoptosis, GW5074 (Raf-1 inhibitor) was used. Raf-1 is associated with Ras-GTP and then is activated by additional modifications such as phosphorylation at Ser-338. Activated Raf-1 triggers sequential activation of downstream molecules (Zang et al. 2002). Thus, the phosphorylation of Raf-1 at Ser-338 is a critical step in Raf-1 activation. We further examined Raf-1 Ser-338 phosphorylation in response to 6-OHDA stimulation in PC12 cells using an anti-phospho-Raf-1 antibody targeting the pSer-338 form. When cells were treated with 100 μM 6-OHDA for various times, phosphorylation at Raf-1 Ser-338 increased within 2 h and was maintained for at least 48 h, while phosphorylation of Raf-1 at Ser-259 decreased (Fig. 2A, 2C, 2D). In addition, we found that GW5074 and U0126 both inhibited 6-OHDA-induced ERK1/2 phosphorylation. However, we also found that 6-OHDA-induced Raf-1 phosphorylation at Ser-338 was not inhibited by the pretreatment with U0126 (Fig. 4A). Using the selective Raf-1 inhibitor, GW5074, ERK1/2

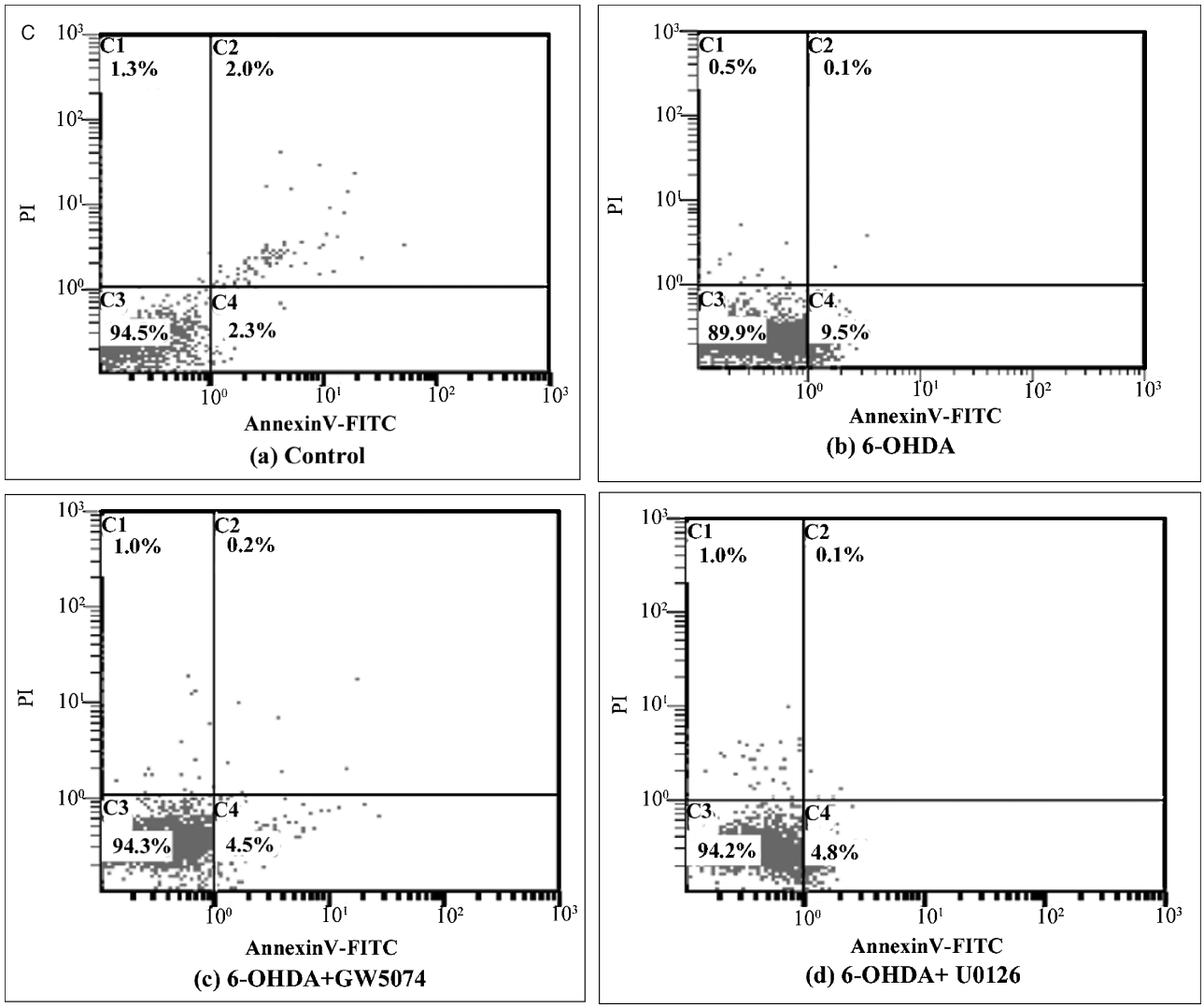


Fig. 4: *Continued*

activation was reversed which indicates that this activation is dependent on Raf-1. Furthermore, the dependence of 6-OHDA-induced ERK1/2 activation on MEK1/2 was previously been shown, as the MEK inhibitor, U0126, completely abrogated ERK1/2 activation. In addition, pre-incubation with U0126 or GW5074 lowers the percentage of apoptotic PC12 cells challenged by 6-OHDA cell viability analysis, Hoechest 33258 assay and Annexin-V FITC analysis by FCM *in vitro* (Fig. 4). The inhibitory effect of GW5074 or U0126 on the phosphorylation level of ERK1/2 may be due to the fact that it binds MEK1/2 in a noncompetitive manner, thereby inhibiting the catalytic activity of some enzymes (Duncia et al. 1998). Although we cannot rule out the possibility that 6-OHDA activates ERK1/2 via an alternative 6-OHDA-sensitive pathway, these results indicate that Raf-1 is the upstream molecule of ERK1/2 in 6-OHDA-induced ERK1/2 activation leading to cell apoptosis. Of course, more experiments are required to fully elucidate this signaling pathway. Taken together, these results establish that 6-OHDA-induced Raf-1/ERK1/2 activation proceeds through the classical MAPK cascade in PC12 cells.

Several lines of evidence accrued in this study suggest that apoptosis stimulated by 6-OHDA in PC12 cells is mediated by the activation of the Raf-1/ERK1/2 pathway. First, 6-OHDA activates the Raf-1/ERK1/2 pathway and induces apoptosis in a both dose- and time-dependent manner. Second, ERK1/2 pathway inhibitor U0126 and Raf-1 inhibitor GW5074 attenuate ERK1/2 activation accompanied with decreased PC12 cell apoptosis stimulated by 6-OHDA, respectively. Although the detailed mechanism underlying apoptosis induced by 6-OHDA remains unclear, the current study suggests that an extrinsic pathway stimulated by the activation of Raf-1/ERK1/2 is closely correlated with this process.

In summary, we have demonstrated that 6-OHDA activates the Raf-1/ERK1/2 signal pathway. 6-Hydroxydopamine stimulation not only phosphorylates ERK1/2 and Raf-1(Ser-338), but also dephosphorylates Raf-1(Ser-259), consequently leading to apoptosis and death of PC12 cells. In addition, we also confirm that U0126 and GW5074 are partially protective against 6-OHDA-induced cell toxicity. Collectively, our present study indicates novel pharmacological targets for the clinical treatment of PD.

4. Experimental

4.1. Materials and reagents

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and horse serum were obtained from Invitrogen (Carlsbad, CA, USA). 6-OHDA, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT), U0126 [1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)butadiene] (ERK1/2 pathway inhibitor), and GW5074 (Raf-1 inhibitor) were all obtained from Sigma-Aldrich Co. (Poole, Dorset, UK). PhosSTOP Phosphatase Inhibitor Cocktail Tablets and Complete Protease Inhibitor Cocktail Tablets were from Roche. The antibodies to ERK1/2(L34F12, mouse monoclonal), phospho-ERK1/2 (Thr-202/Tyr-204, rabbit polyclonal), and phospho-Raf-1 (Ser-338, rabbit polyclonal)

were purchased from Cell Signaling Tech, Inc. Antibody used to detect phospho-Raf-1 (Ser-259, rabbit polyclonal) was obtained from Santa Cruz Biotechnology, Inc. The apoptosis detection kit with Annexin V-FITC and propidium iodide was purchased from Beyotime Institute of Biotechnology (China). The IRDyeTM800 conjugated anti-rabbit, IRDyeTM700 conjugated anti-mouse second antibodies, and anti-GAPDH mouse monoclonal antibodies were kind gifts from Dr. Meng Xiangning. Other chemicals were of analytical grade from standard commercial sources.

4.2. Cell culture and treatments

Undifferentiated PC12 cells from the rat pheochromocytoma cell line, a generous gift from Dr. Fan Ying, were grown in DMEM supplemented with 5% (v/v) horse serum and 5% (v/v) fetal calf serum at 37 °C under a humidified atmosphere of 95% air and 5% CO_2. After adherence and ~80% confluence, cells were planted on plates at a density of 2×10^5 cells/ml. Cells were treated with 6-OHDA at different concentrations (25, 50, 100, 150, 200, and 400 μM) and then cultured for different times (2, 6, 12, 24, 36, and 48 h). Pharmacologic inhibitors were added prior to 6-OHDA: GW5074, 10 nM for 60 min and U0126, 10 nM for 60 min. GW5074 and U0126 were dissolved in dimethyl sulfoxide (DMSO). Experiments were carried out using cells between passages 10 and 18.

4.3. Cell viability

The MTT assay was employed to assess cell viability. The working stock of MTT was 5 mg/ml in phosphate-buffered saline (PBS). The solution was added to the culture medium at a dilution of 1: 10. PC12 cells were planted in a 96-well plate (5000 cells/well). After treatment with 6-OHDA and/or pharmacologic inhibitors, cells were incubated in the medium with MTT for 4 h at 37 °C. The medium was then aspirated, and the dark-brown formazan crystals formed were dissolved in DMSO. Measurement of the optical densities of the samples at 570 nm was conducted using a microplate reader. Cell viability was indicated as the percentage of OD value to that of control cells (with no treatment of 6-OHDA). All experiments were carried out in triplicate.

4.4. Measurement of apoptotic cell death

4.4.1. Apoptosis assay using Hoechst 33258 staining

PC12 cells were planted on sterile cover glasses placed in 6-well plates. After inhibitor or/and 6-OHDA treatment, chromatin state was analyzed by staining cells with the dye Hoechst 33258 (Beyotime, China). Cultures were fixed, rinsed three times with PBS and then stained with 0.5 mg/ml of Hoechst 33258 for 5 min in the dark at room temperature (RT). After three rinses with PBS, cell staining was examined under a fluorescent microscope (IX71, Olympus, Japan). Uniformly stained nuclei were scored as healthy, viable cells. Shrunken, condensed, or fragmented nuclei were scored as apoptotic. Data were expressed as percentages of the condensed nuclear number to the total number.

4.4.2. Apoptosis assay using flow cytometry

The level of apoptosis was analyzed using an Annexin V-FITC/propidium iodide (PI) apoptosis detection kit. Flow cytometry analysis was performed on a Becton Dickenson FACScan^{TM} flow cytometer. Briefly, cells in different treatment groups were washed in cold PBS and resuspended in a binding buffer. Cell concentration was adjusted to approximately 10^6/ml. The cells were incubated with 5 μl annexin V/FITC and 10 μl PI for 15 min in the dark at RT, resuspended in 400 μl binding buffer, and then analyzed by flow cytometry. Cells negative for both annexin V and PI staining were defined normal cells; annexin V-positive and PI-negative stained cells were early apoptotic cells; both annexin V and PI -positive stained cells were necrotic or late apoptotic cells; and PI-positive and annexin V-negative stained cells were fragmentation for cells.

4.5. Western blotting analysis

Cells were lysed in buffer containing 20 mM Tris HCl (PH: 7.5), 1 mM EDTA, 1% NP40, 140 mM NaCl, 50 mM NaF, PhosSTOP Phosphatase Inhibitor, and Complete Protease Inhibitor Cocktail. The 70 μg of the total protein assayed by BCA protein assay kit (Beyotime) was separated on 10% and 12% SDS-PAGE and then transferred to PVDF membrane (Millipore). The membranes were blocked for 1 h in 5% BSA and TBS. The 0.1% Tween-20 were washed 3 times for 5 min each, with 15 ml of TBS-T and incubated in 10 ml primary antibody dilution (the antibody to total ERK1/2 dilution 1:1000, phospho-ERK1/2 dilution 1:1000, phospho-Raf-1 dilution 1:500, GAPDH dilution 1:5000) buffer with gentle agitation overnight at 4 °C. Membranes were washed with TBS-T 3 times for 10 min and incubated with the corresponding IRDye 800 or IRDye 700-labeled IgG secondary antibody in the dark for 40 min at RT. Following another 3 washes with TBS-T for 5 min, the membranes were scanned by an Odyssey infrared imaging system (LI-COR). Quantification of immunoreactive bands was performed using Scion Image software.

4.6. Statistical analysis

Data were expressed as mean ± SEM, and statistical significance was assessed by one-way analysis of variance with subsequent Turkey tests. All analyses were carried out using SPSS software (13.0 for Windows), $P < 0.05$ were considered significant.

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