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Neurotropic and neuroprotective activities of the earthworm peptide Lumbricusin

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ABSTRACT

We recently isolated a polypeptide from the earthworm *Lumbricus terrestris* that is structurally similar to defensin, a well-known antibacterial peptide. An 11-mer antibacterial peptide (NH_2-RNRRWCIDQQA), designated Lumbricusin, was synthesized based on the amino acid sequence of the isolated polypeptide. Since we previously reported that CopA3, a dung beetle peptide, enhanced neuronal cell proliferation, we here examined whether Lumbricusin exerted neurotropic and/or neuroprotective effects. Lumbricusin treatment induced a time-dependent increase (~51%) in the proliferation of human neuroblastoma SH-SY5Y cells. Lumbricusin also significantly inhibited the apoptosis and decreased viability induced by treatment with 6-hydroxy dopamine, a Parkinson's disease-mimicking agent. Immunoblot analyses revealed that Lumbricusin treatment increased ubiquitination of $p27^{Kip1}$ protein, a negative regulator of cell-cycle progression, in SH-SY5Y cells, and markedly promoted its degradation. Notably, adenoviral-mediated over-expression of $p27^{Kip1}$ significantly blocked the antiapoptotic effect of Lumbricusin in 6-hydroxy dopamine-treated SH-SY5Y cells. These results suggest that promotion of $p27^{Kip1}$ degradation may be the main mechanism underlying the neuroprotective and neurotropic effects of Lumbricusin.

1. Introduction

Common neurodegenerative diseases include amyotrophic lateral sclerosis [1], Alzheimer's disease [2], and Parkinson's disease [3]. Among them, Parkinson's disease is characterized by the progressive loss of dopaminergic neurons, resulting in tremors, bradykinesia, and postural instability [3]. There is currently no cure for Parkinson's disease, and the factors responsible for mediating the progression of Parkinson's disease are not well understood in detail. To date, a number of natural products have been evaluated for their ability to control the symptoms, development, and progression of neuronal disorders [4]. Among the sources of natural products that have received attention for their potential treatment of neuronal disorders are ginseng and *Celastrus paniculatus*. NAP peptide, derived from ADNF (activity-dependent neuroprotective protein), is also known to have neuroprotective functions [5]. A number of studies have also demonstrated that antimicrobial peptides isolated from insects have a broad spectrum of biological properties, including anticancer, anti-inflammatory and neurotropic/neuroprotective activities. Notable among these is CopA3 peptide, a 9-mer (LLCIALRKK) D-type disulfide-linked dimeric peptide isolated from the Korean dung beetle, *Copris tripartites* that has been shown to possess neurotropic and neuroprotective properties in addition to its antibacterial activity.

Earthworms are known to have evolved strong immune defense systems against invading microorganisms in the environment, and antimicrobial peptides isolated from earthworms [6], such as lumbricin-I, have been demonstrated to possess bactericidal activity against microorganisms [7]. In a recent study, we injected the

Abbreviations: SH-SY5Y, neuroblastoma cells; 6-OHDA, 6-hydroxy dopamine; MTT, 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide; FACS, fluorescence-activated cell sorter; DMSO, dimethyl sulfoxide; p21, $p27^{Kip1}$; IP, immunoprecipitation; GFP, green fluorescent protein.

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earthworm *Lumbricus terrestris* with lipopolysaccharide (LPS) to induce immune responses and isolated candidate genes that were highly upregulated compared to controls using GeneFishing technology. Among the isolated genes was one predicted to encode a polypeptide that is structurally similar to the antimicrobial peptide defensin. An 11-mer peptide containing the sequence possibly responsible for antimicrobial activity (NH_2-RNRRWCIDQQA) was synthesized based on the sequence of this polypeptide. This peptide, named Lumbricusin, was tested for possible effects on neuronal cell proliferation and neuroprotection, given previous reports of the neuroprotective activity of the insect peptide CopA3 [8]. Here, we report that Lumbricusin exerted a neurotropic effect on SH-SY5Y human neuroblastoma cells and significantly inhibited the apoptosis and decreased viability induced by 6-hydroxy dopamine. Our findings suggest the potential of Lumbricusin as a drug candidate for the treatment of Parkinson's disease.

2. Materials and methods

2.1. Synthesis of Lumbricusin

The Lumbricusin peptide was synthesized by AnyGen (Gwangju, South Korea) [9], purified by reverse-phase high-performance liquid chromatography (HPLC) using a Capcell Pak C18 column (Shiseido, Japan), and eluted with a linear gradient of water–acetonitrile (0–80%) containing 0.1% trifluoroacetic acid (45% recovery). The identity of the peptide was confirmed by electrospray ionization (ESI) mass spectrometry (Platform II; Micromass, Manchester, United Kingdom).

2.2. Cell culture and reagents

Human neuroblastoma SH-SY5Y cells were maintained in Dulbecco's modified Eagle medium (DMEM; Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS). Cells were cultured in a 37 °C humidified incubator with 5% CO_2 [8]. Polyclonal antibodies against phospho-ERK1/2 and caspase-3 were obtained from Cell Signaling Technology (Beverly, MA, USA). Polyclonal antibodies against $p27^{Kip1}$, c-Src, PTEN (phosphatase and tensin homolog), and H-Ras were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The β-actin antibody, propidium iodide (PI), 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye, MG132, bafilomycin A1, 6-hydroxy dopamine (6-OHDA), and cycloheximide were purchased from Sigma Aldrich (St. Louis, MO, USA). A $p27^{Kip1}$-expressing adenovirus was obtained from Vector Biolabs (Philadelphia, PA, USA).

2.3. Cell viability

SH-SY5Y cells (3×10^3 cells/well) were pretreated with Lumbricusin (10 μg/ml) for 1 h, exposed to medium (control) or 6-OHDA (100 μM) for 12 h, and then incubated with MTT dye for 2 h. The solubilization reagent was added, and absorbance was determined at 570 nm in a microplate reader (model 3550; Bio-Rad, Mississauga, Canada) [10].

2.4. BrdU cell proliferation assay

The proliferation of Lumbricusin-treated cells was measured based on the rate of DNA synthesis using a BrdU Cell Proliferation Assay (Roche, Indianapolis, IN, USA), according to the manufacturer's instructions [11]. Briefly, SH-SY5Y cells (1×10^4 cells/well) were seeded in a 96-well microplate, treated with or without Lumbricusin for 36 h, and then further cultured with the BrdU mixture for 12 h. The cells were then fixed, incubated with the anti-BrdU antibody for 1 h, and incubated with horseradish-peroxidase (HRP)-conjugated goat anti-mouse IgG for 30 min. Absorbance at 450 nm was determined using a microplate reader.

2.5. DNA fragmentation analysis for apoptosis

Cells were treated with medium, Lumbricusin, 6-OHDA (100 μM), or Lumbricusin plus 6-OHDA for 48 h, and harvested by centrifugation. The treated cells were incubated at 4 °C for 1 h in PBS containing 50 μg/ml PI and 0.1% Triton X-100. PI fluorescence was measured by fluorescence-activated cell sorter (FACS) analysis (FACScan; Becton Dickinson, Lincoln Park, NJ, USA).

2.6. RNA isolation and semi-quantitative RT-PCR

RNA was prepared using the TRIzol reagent (Life Technologies, Gaithersburg, MD, USA), and reverse transcription (RT) was performed as previously described [12]. The resulting product (1 μl) was amplified with primers specific to human $p27^{Kit1}$ (sense, 5-TTCTTTTCACTTCGGGCTGT-3 and antisense, 5-CACAAAACATGCCAC TTTGG-3; 370 bp product). Actin was amplified as an internal control. Polymerase chain reactions (PCRs) were conducted with an optimal number of cycles of 94 °C for 1 min, 58 °C for 1 min, and 72 °C for 1 min.

2.7. Statistical analysis

The results are presented as the mean ± SEM. Data were analyzed using the SIGMA-STAT software package (Jandel Scientific Software, San Rafael, CA, USA). Analyses of variance with protected *t*-tests were used for intergroup comparisons.

3. Results and discussion

3.1. The earthworm peptide Lumbricusin increases proliferation of human neuroblastoma cells

Since we previously demonstrated that the insect-derived CopA3 peptide induced neuronal cell proliferation [8], we sought to determine whether the earthworm peptide Lumbricusin (NH_2-RNRRWCIDQQA) (Fig. 1A) also enhanced neuronal cell proliferation. To explore this possibility, we exposed human neuroblastoma SH-SY5Y cells to 10 μg/ml Lumbricusin for 48 h and measured the levels of DNA synthesis using a BrdU cell proliferation assay. Lumbricusin treatment (10 μg/ml) increased SH-SY5Y cell proliferation by 51% compared to the medium control (Fig. 1B). However, Lumbricusin treatment did not affect the proliferation of human colonic epithelial (HT29) cells or human adrenal carcinoma (SW13) cells (Fig. 1B), suggesting that the proliferative effect of Lumbricusin is specific to neuronal cells. As shown in Fig. 1C, PI staining and FACS analysis also revealed that Lumbricusin caused marked proliferation of neuronal cells (~21% increase in S-phase cells compared to medium controls). Since the insect peptide CopA3 at a high concentration (150 μg/ml) caused apoptosis in AML-2, Jurkat and U937 human leukemia cells [10], we next assessed whether Lumbricusin was cytotoxic toward neuronal cells. To explore this, we exposed SH-SY5Y cells to Lumbricusin (10 μg/ml) for 12, 24, 48 and 72 h, and measured cell viability by MTT assay. As shown in Fig. 1D, Lumbricusin treatment slightly increased the viability of SH-SY5Y cells rather than inducing cell toxicity (Fig. 1D, upper panel). A microscopic image analysis also revealed that Lumbricusin did not affect the typical morphology of neuronal cells (Fig. 1D, lower panel). These results suggest that, similar to the insect peptide CopA3, the earthworm peptide Lumbricusin also possesses neurotropic activity.

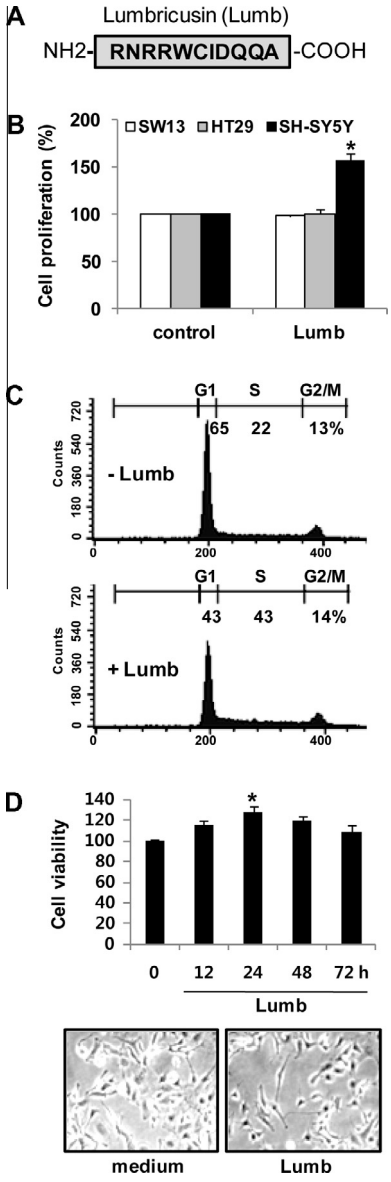

Fig. 1. The earthworm peptide Lumbricusin enhances neural cell proliferation. (A) Amino acid sequence of Lumbricusin. (B) Human neuroblastoma SH-SY5Y cells (10^5 cells/well) were treated with medium (control) or Lumbricusin (10 μg/ml) for 48 h, and cell proliferation was assessed by measuring BrdU uptake. The results represent the means ± SEM of three experiments performed in triplicate (*$p < 0.005$). (C) SH-SY5Y cells were treated with medium (−Lumb) or Lumbricusin (+Lumb) for 48 h, and DNA synthesis levels were measured by PI staining and FACS analysis. (D) SH-SY5Y cells were incubated with Lumbricusin (10 μg/ml) for the indicated durations, and cell viability was measured by MTT assay. The bars represent the means ± SEM of three experiments performed in triplicate (*$p < 0.005$). *Lower panel:* Light microscopic images (100×) of SH-SY5Y cells after 48 h incubation with Lumbricusin.

3.2. Lumbricusin blocks 6-OHDA-induced apoptosis in SH-SY5Y cells

Since we found that Lumbricusin has neurotropic activity, we next assessed whether Lumbricusin is also neuroprotective. 6-OHDA has been used as an *in vitro* and *in vivo* model of Parkinson's disease [13,14] in cultured cell lines and animals. Therefore, we investigated the neuroprotective activity of Lumbricusin using a 6-OHDA neuronal apoptosis model. As shown in Fig. 2A, 6-OHDA (100 μM) treatment [15,16] markedly decreased the viability of SH-SY5Y cells, but this was completely abrogated by pretreatment with Lumbricusin (10 μg/ml) for 1 h. Next, we assessed whether Lumbricusin blocked neuronal cell apoptosis induced by 6-OHDA. To this end, cells were incubated with Lumbricusin for 1 h and then treated with 6-OHDA for 12 h; thereafter, cells with fragmented DNA were identified and quantified by PI staining and FACS analysis. Compared to control cells exposed to medium, 6-OHDA-stimulated cells exhibited a 34% increase in sub-G1 populations (apoptotic cells). Notably, as shown in Fig. 2B, this increase was significantly decreased by Lumbricusin pretreatment (7%). We also measured caspase-3 activity (a hallmark of apoptosis) [9] in cells treated as described above by immunoblot analysis. Treatment with 6-OHDA alone significantly induced caspase-3 activation; this effect was also markedly blocked by Lumbricusin pretreatment for 1 h (Fig. 2C). To exclude the possibility that Lumbricusin inhibits the response of SH-SY5Y cells to 6-OHDA through direct binding to 6-OHDA, we pretreated cells with Lumbricusin for 1 h, removed the medium that possibly contained Lumbricusin peptide by washing cells three times with fresh medium, and then exposed cells to 6-OHDA for 12 h to induce neural cell apoptosis. As shown in Fig. 2D, Lumbricusin significantly inhibited 6-OHDA-induced neural cell apoptosis independent of direct binding of Lumbricusin to 6-OHDA. These results collectively suggest that the earthworm-derived peptide Lumbricusin exerts neurotropic and neuroprotective effects by causing alterations in the intracellular signaling of neuronal cells.

3.3. Lumbricusin promotes proteasome-mediated $p27^{Kip1}$ degradation

We next attempted to identify intracellular signaling pathways that mediate the neurotropic and neuroprotective effects of Lumbricusin. To accomplish this, we treated SH-SY5Y cells with different concentrations of Lumbricusin and assessed changes in signaling molecules known to regulate cell proliferation by immunoblot analysis. As shown in Fig. 3A, Lumbricusin treatment induced a marked, concentration-dependent reduction in the protein levels of $p27^{Kip1}$, a cyclin-dependent kinase inhibitor that negatively regulates cell proliferation [17]. However, other signaling molecules, including c-Src, PTEN (phosphatase and tensin homolog), phospho-ERK1/2 (extracellular signal-regulated kinase 1/2) and H-Ras, were not changed by treatment with Lumbricusin. The decrease in $p27^{Kip1}$ induced by Lumbricusin treatment (10 μg/ml) in SH-SY5Y cells was also time dependent. Lumbricusin treatment did not reduce $p27^{Kip1}$-encoding mRNA in SH-SY5Y cells (Fig. 3C), indicating that this downregulation did not occur at the transcriptional level. To determine whether Lumbricusin altered $p27^{Kip1}$ protein degradation, we treated SH-SY5Y cells with the translation-inhibitor cycloheximide (100 μM; to prevent de novo protein synthesis) for 2, 4 or 8 h in the presence or absence of Lumbricusin, and examined the levels of $p27^{Kip1}$ protein. As shown in Fig. 3D, the half-life of $p27^{Kip1}$ protein in untreated SH-SY5Y cells (~6 h) was significantly shortened in cells treated with Lumbricusin (<2 h). We also assessed whether MG132, a proteasome inhibitor [18], blocks Lumbricusin-induced $p27^{Kip1}$ degradation by pretreating SH-SY5Y cells with 10 μM MG132 for 1 h and then exposing cells to Lumbricusin; $p27^{Kip1}$ degradation was monitored by immunoblot analysis. As shown in Fig. 3E, Lumbricusin-induced downregulation of $p27^{Kip1}$ was completely blocked by MG132 treatment. However, bafilomycin A1, a lysosomal inhibitor [18], was without effect. These results indicate that the marked decrease in $p27^{Kip1}$ protein induced by Lumbricusin is dependent on the proteasomal protein degradation pathway, consistent with previous reports that $p27^{Kip1}$ downregulation is predominately mediated by proteasome-dependent protein degradation [17]. We also found that compared to control cells exposed to medium, ubiquitination of $p27^{Kip1}$ was markedly increased in SH-SY5Y cells treated with Lumbricusin (Fig. 3F).

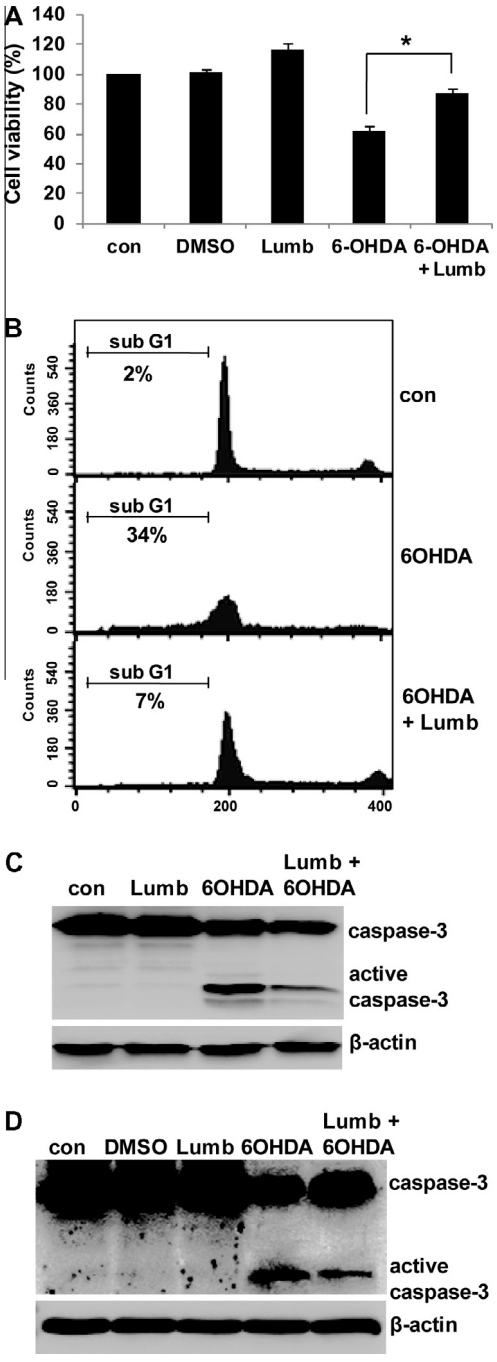

Fig. 2. Lumbricusin inhibits 6-OHDA-induced apoptosis and loss of viability of SH-SY5Y cells. (A) Human neuroblastoma cells (10^5 cells/well) were pretreated with Lumbricusin (10 μg/ml) for 1 h and then incubated with medium (control), vehicle (dimethyl sulfoxide [DMSO]), 100 μM 6-OHDA alone, or 6-OHDA plus Lumbricusin (Lumb) for 12 h, and cell viability was measured by MTT assay (*$p < 0.005$). (B) SH-SY5Y cells were pretreated with Lumbricusin (10 μg/ml) for 1 h and then incubated with medium (con), 100 μM 6-OHDA alone, or 6-OHDA plus Lumbricusin (Lumb) for 48 h. Cells with fragmented DNA were identified and quantified by PI staining and FACS analysis. (C) SH-SY5Y cells were pretreated with Lumbricusin (Lumb) for 1 h and then incubated with medium (con), 100 μM 6-OHDA alone, or 6-OHDA plus Lumbricusin (Lumb) for 12 h. Proteins in cell lysates were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) on 10% gels, and blots were probed with antibodies against caspase-3 and β-actin. The results presented are representative of three independent experiments. (D) Cells were pretreated with Lumbricusin for 1 h, residual Lumbricusin was removed by washing cells three times with fresh medium, and then cells were exposed to 6-OHDA for 48 h. Proteins in cell lysates were resolved by SDS–PAGE on 10% gels, and blots were probed as described in (C).

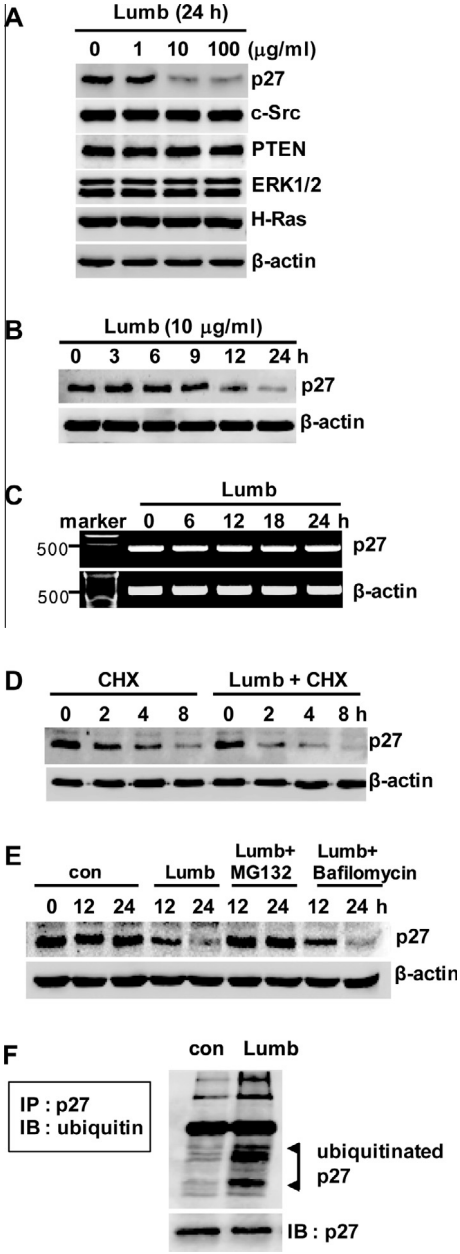

Fig. 3. Lumbricusin causes rapid degradation of $p27^{Kip1}$ protein. (A) SH-SY5Y cells (10^5 cells/well) were treated with different concentrations of Lumbricusin (Lumb). Proteins in cell lysates were resolved by SDS–PAGE on 10% gels, and blots were probed with antibodies against $p27^{Kip1}$ (p27), c-Src, PTEN, phospho-ERK1/2, H-Ras, and β-actin. The results presented are representative of three independent experiments. (B) Lumbricusin decreases $p27^{Kip1}$ protein in a time-dependent manner. (C) cDNA was synthesized from total RNA isolated from cells treated with Lumbricusin, and $p27^{Kit1}$ and β-actin were amplified by PCR (see Section 2). The results shown are representative of three separate experiments. (D) Cells were incubated with cycloheximide alone (CHX; 100 μM) or CHX plus Lumbricusin for the indicated times. (E) SH-SY5Y cells were pretreated with MG132 (10 μM) or bafilomycin A1 (100 nM) for 1 h and then exposed to Lumbricusin (Lumb) for the indicated durations. (F) SH-SY5Y cells were pretreated with medium or Lumbricusin (Lumb) for 12 h, after which lysates were prepared and immunoprecipitated (IP) with an anti-$p27^{Kip1}$ antibody (p27). The immunoprecipitates were resolved by SDS–PAGE on 10% gels, and blots were probed (IB) with antibodies against ubiquitin and $p27^{Kip1}$. The results shown are representative of three separate experiments.

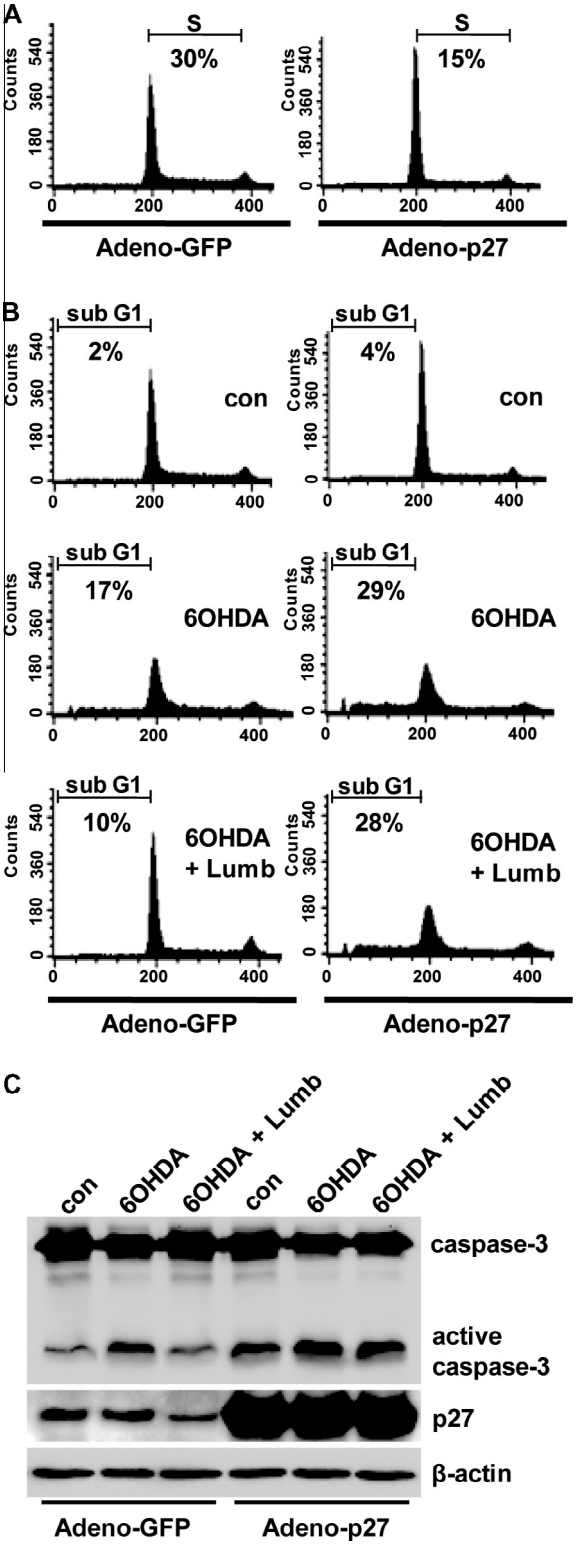

Fig. 4. Overexpression of $p27^{Kip1}$ blocks the neuroprotective effect of Lumbricusin. (A) SH-SY5Y cells (10^5 cells/well) were infected with a $p27^{Kip1}$-expressing adenovirus (1×10^7 PFU/ml) or a control GFP adenovirus (1×10^7 PFU/ml) for 24 h, and then the cell-cycle distribution was analyzed by PI staining and FACS analysis. The results shown are representative of three separate experiments. (B) Cells were infected with a $p27^{Kip1}$ adenovirus (right panels) or a control GFP adenovirus (left panels) for 24 h and then incubated with medium (con), 100 μM 6-OHDA alone, or 6-OHDA plus Lumbricusin (Lumb; 10 μg/ml) for 12 h. The level of apoptosis was measured by PI staining and FACS analysis. (C) Cells were infected with a $p27^{Kip1}$ adenovirus or a control GFP adenovirus for 24 h, then incubated with medium (con), 6-OHDA, or 6-OHDA plus Lumbricusin for 12 h. Cell lysates were resolved by SDS–PAGE on 10% gels, and blots were probed with antibodies against caspase-3, $p27^{Kip1}$ (p27), and β-actin. The results presented are representative of three independent experiments.

3.4. Overexpression of $p27^{Kip1}$ blocks the protective and proliferative effects of Lumbricusin

Finally, we assessed whether adenoviral-mediated overexpression of $p27^{Kip1}$ inhibited neuronal cell proliferation. To do this, we infected SH-SY5Y cells with a $p27^{Kip1}$-expressing adenovirus (1×10^7 PFU/ml) or a control green fluorescent protein (GFP)-expressing adenovirus for 24 h and then measured cell proliferation by PI staining and FACS analysis. As shown in Fig. 4A, infection with adenovirus expressing $p27^{Kip1}$ reduced the number of cells in S phase (~15%) reflecting proliferative cells compared to that in cells infected with control Adeno-GFP virus (~30%). We also investigated whether adenoviral-mediated overexpression of $p27^{Kip1}$ inhibited the neuroprotective effect of Lumbricusin. As shown in Fig. 4B (left panels), 6-OHDA-induced apoptosis was increased in cells infected with control Adeno-GFP virus, an effect that was markedly blocked by pretreatment with Lumbricusin for 1 h. However, in cells overexpressing $p27^{Kip1}$, Lumbricusin treatment did not inhibit 6-OHDA-induced apoptosis (Fig. 4B, right panels). As expected, 6-OHDA-induced activation of caspase-3 was observed in cells infected with control Adeno-GFP virus, and this increase was also markedly inhibited by Lumbricusin treatment (Fig. 4C). However, the activation of caspase-3 was not blocked by Lumbricusin treatment in cells overexpressing $p27^{Kip1}$ (Fig. 4C). We also found that $p27^{Kip1}$ overexpression alone cause marked activation of caspase-3 in the absence of 6-OHDA. There are a number of reports that $p27^{Kip1}$ inhibits proliferation of neural progenitor cells in the adult brain under normal and ischemic conditions [19]. Regulation of the half-life of $p27^{Kip1}$ protein is also associated with neurodegenerative disease [17]. These findings are consistent with a previous report that the insect peptide CopA3 exerts a neuroprotective effect through degradation of $p27^{Kip1}$ [8].

Taken together, the results presented here indicate that, similar to the insect peptide CopA3, the earthworm-derived peptide Lumbricusin also induces neural cell proliferation and protects against a cell-damaging agent. Many peptides that exert both proliferative and protective effects have been reported. For example, the peptide Humanin is known to protect neurons from amyloid β-induced toxicities [20]. The NAP peptide also exerts neuroprotective effect against Alzheimer's disease [21,22] as well as hypoxic-ischemic brain injury [23]. Like the insect peptide CopA3, earthworm-derived Lumbricusin, a small (11-mer) peptide, may also be a potential drug candidate for the treatment of Parkinson's disease. The notion that low-molecular-weight peptides have lower antigenicities than larger peptides lends further credence to this possibility [8].

Conflict of interest

The authors declare that they have no conflict of interest to disclose.

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