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Insect peptide CopA3-induced protein degradation of p27Kip1 stimulates proliferation and protects neuronal cells from apoptosis

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ABSTRACT

We recently demonstrated that the antibacterial peptide, CopA3 (a D-type disulfide dimer peptide, LLCIALRKK), inhibits LPS-induced macrophage activation and also has anticancer activity in leukemia cells. Here, we examined whether CopA3 could affect neuronal cell proliferation. We found that CopA3 time-dependently increased cell proliferation by up to 31 ± 2% in human neuroblastoma SH-SY5Y cells, and up to 29 ± 2% in neural stem cells isolated from neonatal mouse brains. In both cell types, CopA3 also significantly inhibited the apoptosis and viability losses caused by 6-hydroxy dopamine (a Parkinson disease-mimicking agent) and okadaic acid (an Alzheimer's disease-mimicking agent). Immunoblotting revealed that the p27Kip1 protein (a negative regulator of cell cycle progression) was markedly degraded in CopA3-treated SH-SY5Y cells. Conversely, an adenovirus expressing p27Kip1 significantly inhibited the antiapoptotic effects of CopA3 against 6-hydroxy dopamine- and okadaic acid-induced apoptosis, and decreased the neurotropic effects of CopA3. These results collectively suggest that CopA3-mediated protein degradation of p27Kip1 may be the main mechanism through which CopA3 exerts neuroprotective and neurotropic effects.

1. Introduction

CopA3 is a 9-mer disulfide dimer peptide (LLCIALRKK) derived from the amino acid sequence of the α-helical region of the 43-amino-acid insect peptide, Coprisin, which was originally isolated from the Korean dung beetle, *Copris tripartitus* [1]. We previously demonstrated that CopA3 exhibits antibacterial activity against various pathogenic bacteria [2], has selective anticancer effects on human leukemia cells [3], and shows anti-inflammatory effects in the gut [2]. CopA3 also inhibits activation of murine macrophages caused by LPS exposure [4]. However, although the biological activity of CopA3 has been examined in a variety of cellular systems [2,3], no previous study has examined its action on neurons. Here, we assessed whether CopA3 treatment could increase neuronal cell proliferation or show neuroprotective effects.

There are numerous neurodegenerative diseases, including, Alzheimer's disease [5] and Parkinson's disease [6]. Alzheimer's disease, which is a progressive neurodegenerative disease that represents the most common form of dementia in the elderly population, is characterized by the presence of senile plaques, neurofibrillary tangles, and the loss of cholinergic neurons in the basal forebrain [5]. Parkinson's disease is characterized by the progressive loss of dopaminergic neurons in the pars compacta of the substantia nigra, resulting in bradykinesia and postural instability [6]. At present, there is no cure for Alzheimer's or Parkinson's disease, and the causes and factors underlying their progression are not well understood.

Various natural products have been studied for their ability to prevent the inception and progression of neurodegenerative disorders, including Alzheimer's and Parkinson's disease [7]. Plant species such as Ginseng and Celastrus paniculatus have been discussed as potential sources for agents capable of controlling these diseases, and numerous metabolites are currently being evaluated for their potential to control the symptoms, development, and/or progression of neuronal disorders [7]. Some peptides, such as NAP peptide (activity-dependent neuroprotective protein-derived

Abbreviations: MNSCs, mouse neural stem cells; OA, okadaic acid; 6-OHDA, 6-hydroxy dopamine; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

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peptide), have also been shown to have neuroprotective functions [8].

In the present study, we show that CopA3 has neurotropic effects on SH-SY5Y human neuroblastoma cells and neural stem cells isolated from mouse brains. Surprisingly, CopA3 significantly inhibited the apoptosis and cell viability losses of SH-SY5Y and mouse neural stem cells exposed to 6-hydroxy dopamine and okadaic acid. These results suggest that CopA3 may be a drug candidate for the treatment of neurodegenerative diseases, such as Alzheimer's and Parkinson's disease.

2. Materials and methods

2.1. Synthesis and structural determination of the dimer peptide, CopA3

The insect-derived CopA3 peptide (D-type) was synthesized by AnyGen (Gwang-ju, South Korea) [2], 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). To form the interchain disulfide bond, the synthetic peptide was dissolved in acetonitrile–H_2O (50/50) solution and then oxidized in an aqueous 0.1 M NK_4HCO_3 solution (pH 6.0–6.5) for 24 h.

2.2. Cell culture and reagents

Human neuroblastoma SH-SY5Y cells were maintained in DMEM containing 10% FBS (Invitrogen, Carlsbad, CA, USA), in a 37 °C humidified incubator with 5% CO_2. The polyclonal antibody against caspase-3 was obtained from Cell Signaling Technology (Beverly, MA, USA). The polyclonal antibodies against p27Kip1, c-Src, survivin, PTEN and acetylated tubulin 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, serine/threonine phosphatase inhibitor (okadaic acid; OA), 6-hydroxy dopamine (6-OHDA) and transcription inhibitor (cycloheximide) were purchased from Sigma–Aldrich (St. Louis, MO, USA). The p27Kip1 adenovirus was obtained from Vector Biolabs (Philadelphia, PA, USA).

2.3. Cell viability

SH-SY5Y cells (3×10^3 cells/well) were treated with CopA3 (10 μg/ml) for 1 h, exposed to medium, OA (20 nM) or 6-OHDA (100 μM) for 12 h, and then incubated with MTT dye for 2 h. The solubilization reagent (DMSO) was added, and absorbance was determined at 570 nm (model 3550; Bio-Rad, Mississauga, Canada) [3].

2.4. Cell proliferation kinetics and BrdU cell proliferation assay

SH-SY5Y cells (5×10^3 cells/well) were seeded in 6-well plates and incubated with CopA3, and cell numbers were monitored using an IncuCyte live-cell imaging system (Essen BioScience, Ann Arbor, MI, USA) [9]. The imaging system was placed in a CO_2 incubator, and images were acquired every 3 h over 4 days. Confluence measurements were obtained at each time point using the IncuCyte cell density detection software. The proliferation of CopA3-treated cells was also measured based on the rate of DNA synthesis, using a BrdU Cell Proliferation Assay (Roche, Indianapolis, IN, USA), according to the manufacturer's instructions [10]. Briefly, cells (1×10^4 cells/well) were seeded to a 96-well microplate, treated with or without CopA3 for 48 h, and then further cultured with the BrdU mixture for 24 h. The cells were then fixed, incubated with the anti-BrdU antibody for 1 h, and then incubated with HRP-conjugated goat anti-mouse IgG for 1 h. Absorbances at 450 nm and 540 nm were determined using a microplate reader.

2.5. Isolation of mouse neural stem cells (MNSCs)

MNSCs were isolated from the cerebral striatum of neonatal (postnatal day 2) mouse brains. This study was approved by the Animal Care and Use Committee of Daejin University (Pocheon, South Korea). To maintain the cells in an undifferentiated proliferative state, we cultured the cells as free-floating neurospheres in serum-free DMEM/F12 (Invitrogen, CA, USA) containing 1X B27 (Invitrogen), FGF2 (20 ng/ml, Peprotech, Rocky Hill, NJ, USA), and EGF (20 ng/ml, Peprotech) [11]. The MNSCs were propagated for 1–2 passages in 96-well plates (10^4 cells/well).

2.6. Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) assay

Cells were treated with CopA3 for 1 h, exposed to OA or 6-OHDA for 12 h, and then fixed with 4% paraformaldehyde for 20 min. Cells with fragmented nuclear DNA were detected by TUNEL assay (Promega, Madison, WI, USA), according to the manufacturer's instructions [2].

2.7. 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 rat p27Kip1 (sense, 5-TTC TTTTCACTTCGGGCTGT-3 and antisense, 5-CACAAAACATGCC ACTTTGG-3; 370 bp product). Actin was amplified as the internal control. The PCR reactions were conducted with the optimal number of cycles (94 °C for 1 min, 58 °C for 1 min, and 72 °C for 1 min).

2.8. Statistical analysis

The results are presented as mean values ± SEM. Data were analyzed using the SIGMA-STAT professional statistics software program (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. CopA3 enhances cell proliferation in human neuroblastoma cells

Since we previously demonstrated that the synthesized insect peptide, CopA3 (a D-type disulfide dimer peptide, Fig. 1A), inhibits LPS-induced activation of human macrophages and has anticancer activity in human leukemia cells, we herein assessed whether CopA3 could affect neuronal cell proliferation. Human neuroblastoma SH-SY5Y cells were exposed to CopA3, and cell numbers were assessed using an IncuCyte live-cell imaging system at the indicated time points. As shown in Fig. 1B, CopA3 caused a marked time-dependent increase in cell numbers, with the maximum increase in cell number seen at 10 μg/ml CopA3. BrdU cell proliferation assays revealed that 10 μg/ml CopA3 yielded a 31 ± 2% increase in cell proliferation compared to the medium control. In contrast, CopA3 treatment did not induce cell proliferation in human colonic epithelial (HT29) cells (Fig. 1C).

We previously showed that a high concentration (150 μg/ml) of CopA3 caused human leukemia cell-specific apoptosis among

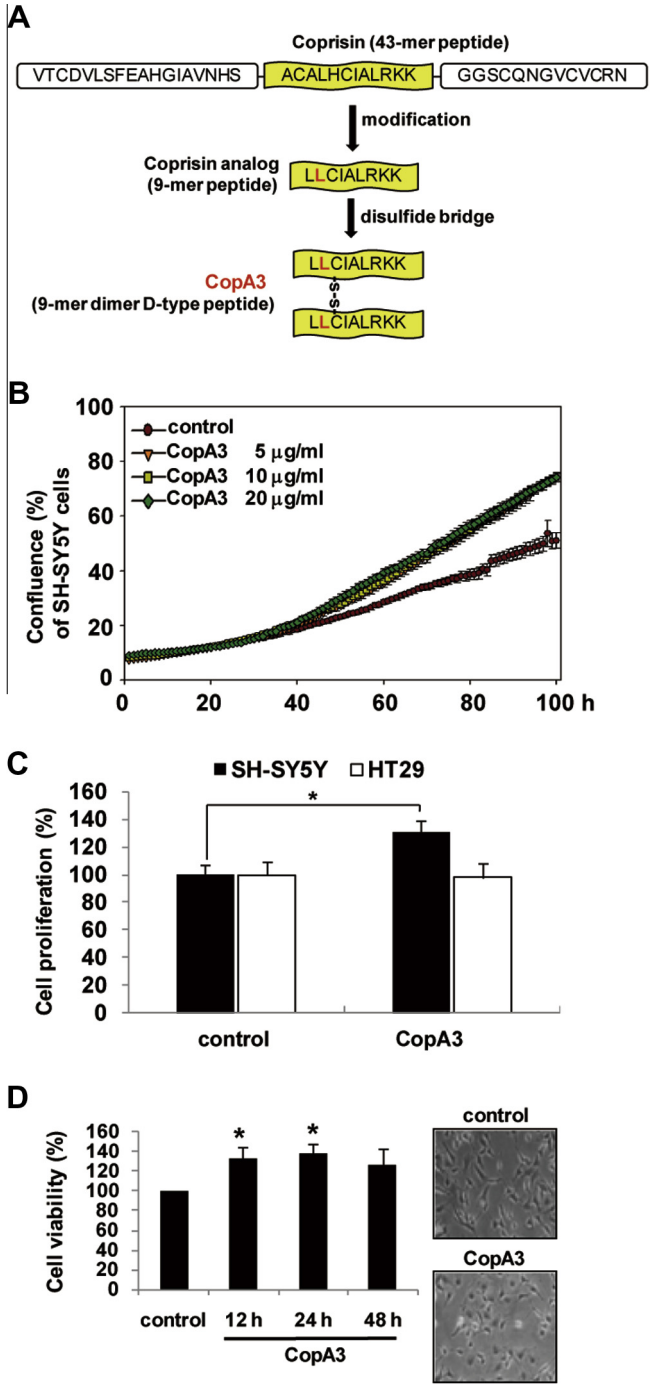

Fig. 1. The insect peptide, CopA3, increases the proliferation of human neuroblastoma cells. (A) Synthesis and dimerization of CopA3 (D-type) by disulfide bond formation. (B) Human neuroblastoma SH-SY5Y cells were treated with medium (control) or CopA3 for the indicated durations, and cell proliferation was measured with an IncuCyte live-cell imaging system. The results represent the means ± SEM of three experiments performed in triplicate. (C) SH-SY5Y cells and human colonic epithelial cells (HT29) were incubated with medium (control) or CopA3 (10 μg/ml) for 72 h, and cell proliferation was measured by BrdU uptake experiments (*$p < 0.005$). (D) SH-SY5Y cells were incubated with medium (control) or CopA3 for the indicated durations, and cell viability was measured by MTT assays. The bars represent the means ± SEM of three experiments performed in triplicate (*$p < 0.005$). Light microscopic images (100×) of SH-SY5Y cells incubated for 48 h with CopA3 (right panel).

AML-2, Jurkat and U937 cells [3]. However, we found that CopA3 treatment enhanced the cell viability of SH-SY5Y cells (Fig. 1D, left panel). Microscopic image analysis also revealed that CopA3 treatment did not alter the typical shapes of the neuronal cells (Fig. 1D, right panel), suggesting that CopA3 may have proliferative effects on neuronal cells without apparent cellular toxicity.

3.2. CopA3 inhibits SH-SY5Y cell apoptosis caused by okadaic acid and 6-hydroxy dopamine

Based on the neurotropic effects of CopA3, we next assessed whether CopA3 could have neuroprotective activities. Okadaic acid (OA) has been shown to act as an Alzheimer's disease-mimicking agent in various neuronal cell lines [13,14] and animal models [15]. Similarly, 6-hydroxy dopamine (6-OHDA) causes neurodegeneration in an *in vitro* model of Parkinson's disease [16,17]. Here, we assessed the protective effects of CopA3 in OA- or 6-OHDA-treated SH-SY5Y cells. As shown in Fig. 2A, OA treatment (20 nM) [13,14] caused a marked loss of viability in SH-SY5Y cells, but this was completely abrogated by pretreatment with CopA3 (10 μg/ml) for 1 h. Similarly, a marked loss of cell viability was seen in SH-SY5Y cells incubated with 6-OHDA (100 μM) [16] for 12 h, but this was also significantly inhibited by CopA3 pretreatment (Fig. 2A).

Next, we assessed whether CopA3 inhibited OA- or 6-OHDA-induced neuronal cell apoptosis. SH-SY5Y cells were incubated with CopA3 for 1 h and treated with OA or 6-OHDA for 12 h, and then immunoblotting was used to measure caspase-3 activation (a hallmark of apoptosis) [2]. Treatment with OA or 6-OHDA alone significantly induced caspase-3 activation, but these inductions were significantly blocked by CopA3 pretreatment for 1 h (Fig. 2B). The results of our TUNEL assay confirmed these findings, showing that CopA3 protected against OA- or 6-OHDA-induced apoptosis in SH-SY5Y cells (Fig. 2C, lower panel). Propidium iodide (PI) staining was used to visualize the cell nuclei (Fig. 2C, upper panel).

3.3. CopA3 triggers p27Kip1 protein degradation to mediate neural cell proliferation

Given our observations that CopA3 has neurotropic and antiapoptotic activities, we next sought to identify some of the intracellular molecules responsible for mediating these effects of CopA3. As shown in Fig. 3A, we observed that p27Kip1, a cyclin-dependent kinase inhibitor that negatively regulates cell proliferation [18], was markedly downregulated in CopA3-treated SH-SY5Y cells. In contrast, other signaling molecules, such as c-Src, survivin, PTEN and acetylated tubulin, were unaltered following CopA3 treatment.

We next assessed whether this downregulation occurred at the transcriptional level. However, RT-PCR analysis showed CopA3 treatment did not reduce the level of p27Kip1-encoding mRNA in SH-SY5Y cells (Fig. 3B). We thus next assessed whether p27Kip1 protein degradation was altered. SH-SY5Y cells were treated with a transcription inhibitor (cycloheximide) for 2, 4 or 8 h in the presence or absence of CopA3, and the protein levels of p27Kip1 were examined. As shown in Fig. 3C, the protein half-life of p27Kip1 in SH-SY5Y cells was ~6 h, which is similar to that observed in other cells [19]. In SH-SY5Y cells treated with CopA3, however, the half-life was ~2 h, indicating that CopA3 downregulates p27Kip1 through rapid protein degradation. Since p27Kip1 is known to be predominately degraded by proteasome-dependent protein degradation [18], we speculate that CopA3-mediated protein degradation of p27Kip1 in neural cells may occur via proteasome-dependent degradation.

Next, we assessed whether adenovirus-mediated overexpression of p27Kip1 inhibits the neurotropic effects of CopA3. SH-SY5Y cells were infected with a p27Kip1-encoding adenovirus (1×10^7 PFU/ml) or a control GFP-adenovirus for 24 h, treated with CopA3 for 24, and then assessed for cell proliferation by PI staining and FACS analysis. As shown in Fig. 3D, cells infected with control

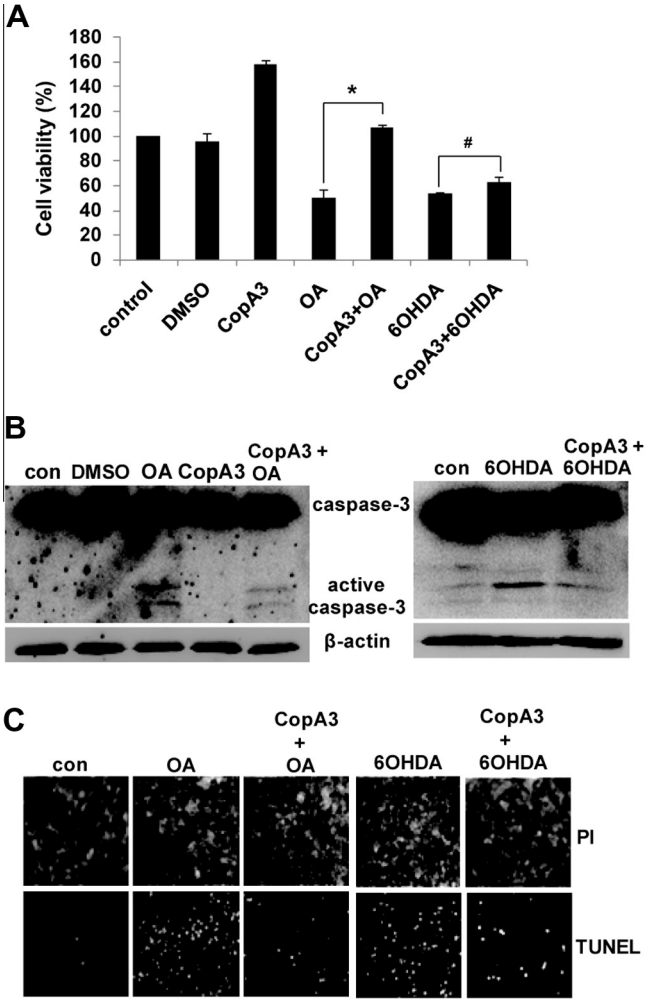

Fig. 2. CopA3 inhibits apoptosis and viability loss in SH-SY5Y cells. (A) SH-SY5Y cells (10^5 cells/well) were pretreated with CopA3 (10 μg/ml) for 1 h and then incubated with medium (control), DMSO, okadaic acid (OA, 20 nM), OA plus CopA3, 100 μM 6-hydroxy dopamine (6-OHDA) alone, or 6-OHDA plus CopA3 for 12 h, and cell viability was measured by MTT assays ($^{*}p < 0.005$ and $^{\#}p < 0.05$). (B) SH-SY5Y cells were pretreated with CopA3 for 1 h and then incubated with medium (con), DMSO, OA alone, OA plus CopA3, 100 μM 6-OHDA alone, or 6-OHDA plus CopA3 for 12 h. Cell lysates were subjected to 10% polyacrylamide gel electrophoresis and blots were probed with antibodies against caspase-3 and β-actin. The presented results are representative of three independent experiments. (C) DNA fragmentation in cells treated as described above was measured by TUNEL assays. Propidium iodide (PI) staining was used to visualize cell nuclei.

Adeno-GFP virus and treated with CopA3 showed an increased number of cells in S and G2/M phases. In contrast, CopA3 treatment had no effect in cells infected with the p27Kip1-expressing adenovirus. In cells infected with control Adeno-GFP virus, OA- and 6-OHDA-induced apoptosis were markedly blocked by CopA3 pretreatment for 1 h (Fig. 3E). In cells overexpressing p27Kip1, in contrast, CopA3 treatment had no effect on OA- and 6-OHDA-induced apoptosis (Fig. 3E). These results suggest that CopA3-induced neural cell proliferation and protection against cell-damaging agents may require the downregulation of p27Kip1. Previous studies have shown that p27Kip1 can constrain the proliferation of neural progenitor cells in adult brain under homeostatic and ischemic conditions [20], and the regulation of its half-life has been associated with Alzheimer's disease [18]. Thus, our present results suggest that CopA3 has neurotropic and neuroprotective effects via its ability to trigger the rapid degradation of the p27Kip1 protein.

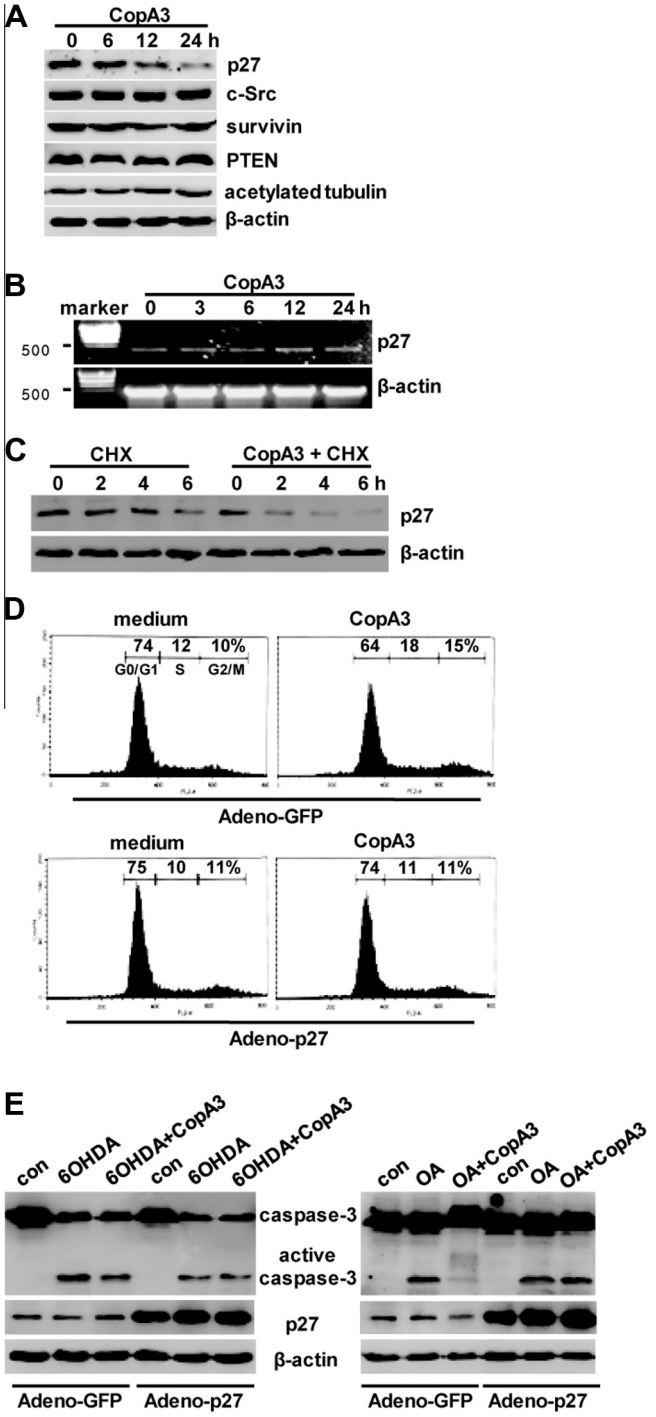

Fig. 3. CopA3 downregulates p27Kip1 by shortening its protein half-life. (A) SH-SY5Y cells (10^5 cells/well) were treated with CopA3 for the indicated durations. Cell lysates were subjected to 10% polyacrylamide gel electrophoresis and blots were probed with antibodies against p27Kip1 (p27), c-Src, survivin, PTEN, acetylated tubulin and β-actin. The presented results are representative of three independent experiments. (B) Total RNA was isolated from CopA3-treated cells, cDNA was synthesized, and p27Kit1 and β-actin were amplified by PCR (see Section 2). The results shown are representative of three separate experiments. (C) Cells were incubated with cycloheximide alone (CHX, 100 μM) or CHX plus CopA3 for the indicated times. (D) Cells infected with a p27Kip1 adenovirus (1×10^7 PFU/ml) or a control GFP adenovirus (1×10^7 PFU/ml) for 24 h were incubated with CopA3 for 46 h, and cell cycle measurements were performed using PI staining and FACS analysis. The results shown are representative of three separate experiments. (E) Cells infected with a p27Kip1 adenovirus or a control GFP adenovirus for 24 h were incubated with medium (con), 20 nM okadaic acid (OA) alone, OA plus CopA3, 100 μM 6-hydroxy dopamine (6OHDA) alone, or 6OHDA plus CopA3 for 12 h. The results shown are representative of three separate experiments.

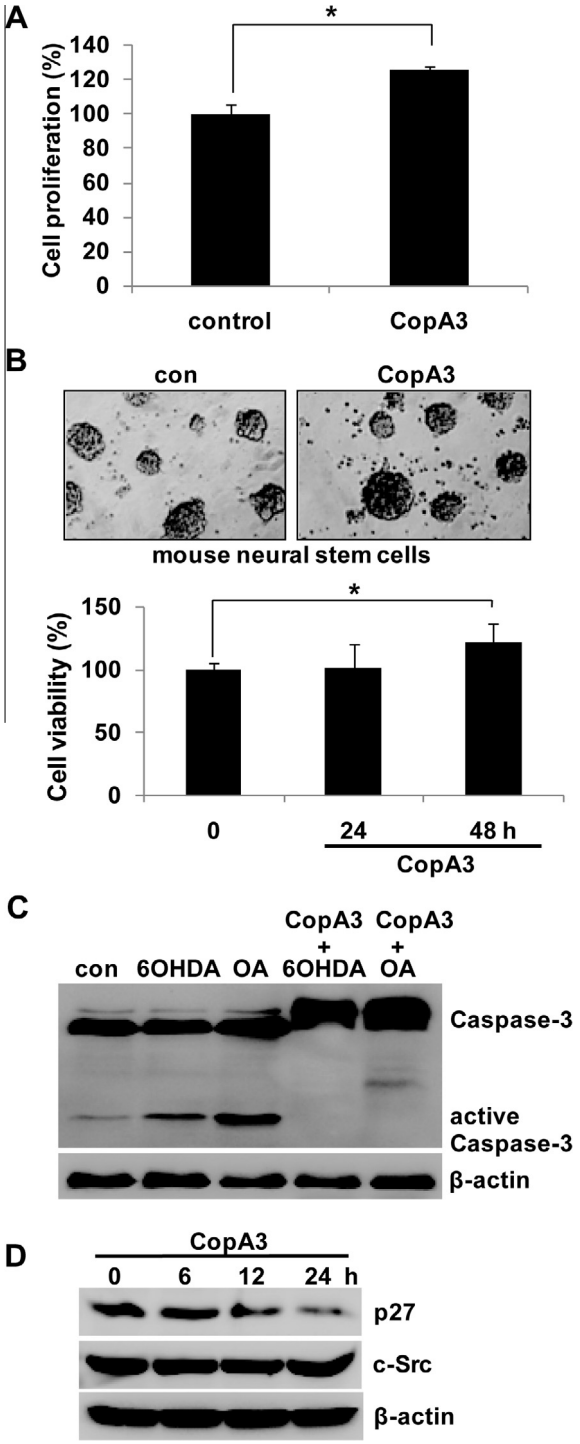

Fig. 4. CopA3 increases the proliferation of mouse neural stem cells (MNSCs) and protects them against 6-hydroxy dopamine- and okadaic acid-induced apoptosis. (A–C) MNSCs were isolated from neonatal mouse brains and maintained as free-floating neurospheres in serum-free medium containing 1X B27, FGF2 (20 ng/ml), and EGF (20 ng/ml). (A) MNSCs (10^4 cells/well) were seeded in a 96-well plate and incubated with 10 μg/ml CopA3 for 48 h, and cell proliferation was measured by BrdU uptake experiments. The bars represent the means ± SEM of three experiments performed in triplicate (*$p < 0.005$). (B) MNSCs were incubated with CopA3 for the indicated durations, and cell viability was measured by MTT assays (lower panel, *$p < 0.01$). Light microscopic images (100×) of neurospheres after 48 h incubation (upper panel). (C) MNSCs were pretreated with CopA3 (10 μg/ml) for 1 h and then incubated for 12 h with medium (con), 100 μM 6OHDA alone, 20 nM OA alone, 6OHDA plus CopA3, or OA plus CopA3. Immunoblots were probed with antibodies against caspase-3 and β-actin. The presented results are representative of three independent experiments. (D) MNSCs were treated with CopA3 for the indicated durations, and blots were probed with antibodies against p27Kip1, c-Src and β-actin.

Additionally, given our observations that CopA3 can block the apoptosis caused by both OA and 6-OHDA, which cause neural cell stress through different mechanisms [14,16], we speculate that CopA3 may directly block one or more of the common apoptotic pathways (e.g., caspase activation).

3.4. CopA3 has protective and proliferative effects on mouse neural stem cells (MNSCs)

Since we found that CopA3 inhibited apoptosis and increased cell proliferation in SH-SY5Y cells, we next assessed its effects on neural stem cells isolated from the cerebral striata of neonatal mouse brains [11]. Mouse neural stem cells (MNSCs) were maintained in an undifferentiated proliferative state and exposed to 10 μg/ml CopA3 for 72 h, and BrdU uptake experiments were used to measure cell proliferation. Consistent with the results in SH-SY5Y cells, MNSCs exposed to CopA3 for 72 h showed a 29 ± 2% increase in cell proliferation compared to the medium control (Fig. 4A). Moreover, 10 μg/ml CopA3 did not have any apparent toxic effect on MNSCs (Fig. 4B, lower panel). Neurosphere, a free-floating cluster of proliferative neural stem cells, formed well in both control and CopA3-treated cultures (Fig. 4B, upper panel). Lastly, we assessed whether CopA3 inhibited the OA- or 6-OHDA-induced apoptosis of MNSCs. MNSCs were incubated with 10 μg/ml CopA3 for 1 h, treated with OA or 6-OHDA for 12 h, and then subjected to immunoblotting for measurement of caspse-3 activation. Both OA and 6-OHDA caused caspase-3 activation, but these activation levels were significantly inhibited by pretreatment with CopA3 (Fig. 3C). Consistent with our results in SH-SY5Y cells, the expression of p27Kip1 was markedly reduced in CopA3-treated MNSCs (Fig. 4D), suggesting that specific downregulation of p27Kip1 is critical for the effects of CopA3 on cell proliferation and apoptosis in neuronal cells.

In terms of other peptides with neuroprotective functions, the NAP peptide has minimal effects on vital organ functions in animals [8], while showing neuroprotective effects against Alzheimer's disease [21,22], hypoxic-ischemic brain injury [23] and hypoxia-induced seizures [24]. It was also shown to enhance neuronal development [25]. Another short amino peptide, humanin, protects neurons from amyloid β-induced toxicities and other stresses [26]. Finally, low-molecular-weight peptides have lower antigenicities compared to higher-molecular-weight agents, making it notable that CopA3 is a small (9-mer) peptide. In sum, our results together with previous findings suggest that the CopA3 peptide may be a potential drug candidate for the treatment of neurodegenerative diseases, such as Alzheimer's and Parkinson's disease.

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