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dl-3-n-Butylphthalide Prevents Neuronal Cell Death after Focal Cerebral Ischemia in Mice via the JNK Pathway

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Abstract

dl-3-n-Butylphthalide (NBP) has shown cytoprotective effects in animal models of stroke and has passed clinical trials as a therapeutic drug for stroke in China. Hence, as a potential clinical treatment for stroke, understanding the mechanism(s) of action of NBP is essential. This investigation aimed to delineate the cellular and molecular mechanism of NBP protection in neuronal cultures and in the ischemic brain. NBP (10 M) attenuated serum deprivation-induced neuronal apoptosis and the production of reactive oxygen species (ROS) in cortical neuronal cultures. Adult male 129 S2/sv mice were subjected to permanent occlusion of the middle cerebral artery (MCA). NBP (100 mg/kg, i.p.) administered 2 hrs before or 1 hr after ischemia reduced ischemia-induced infarct formation, attenuated caspase-3 and caspase-9 activation in the ischemic brain. TUNEL-positive cells and mitochondrial release of cytochrome c and apoptosis-inducing-factor (AIF) in the penumbra region were reduced by NBP. The pro-apoptotic signaling mediated by phospho-JNK and p38 expression was down-regulated by NBP treatment in vitro and in vivo. It is suggested that NBP protects against ischemic damage via multiple mechanisms including mitochondria associated caspase-dependent and -independent apoptotic pathways. Previous and current studies and recent clinical trials encourage exploration of NBP as a neuroprotective drug for the treatment of ischemic stroke.

Keywords

dl-3-n-butylphthalide; Ischemic stroke; Apoptosis; Caspase; AIF; Cytochrome C; Mitochondria; MAP kinase

1. Introduction

dl-3-n-butylphthalide (NBP) is a synthesized compound based on the pure component, l-3-n-butylphthalide, originally extracted from the seeds of *Apium graveolens* Linn (Chang and

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Wang, 2003). It is a chiral molecule that has three different stereo isomers known as l-, dl- and d-NBP. All isomers have shown neuroprotective effects against hypoxia-induced damage (Yan and Feng, 1998). The neuroprotective effects of NBP and possible mechanisms have been explored before (Chong and Feng, 1999; Deng and Feng, 1997; Liu and Feng, 1995; Peng et al., 2008a; Peng et al., 2008b). NBP has protective effects that reduce ischemia-induced brain damage and neuronal cell death, improve cerebral blood flow, decrease brain edema and preserve the blood brain barrier (Chong and Feng, 1999; Deng and Feng, 1997; Liu and Feng, 1995; Yan and Feng, 1998; Zhang et al., 2006). NBP also shows beneficial effects in attenuating -amyloid-induced cell death in neuronal cultures and improving cognitive impairment in an animal model of Alzheimer's disease (Peng et al., 2008a; Peng et al., 2010). In addition, NBP has anti-apoptotic, anti-platelet, anti-thrombotic and anti-inflammatory properties (Chang and Wang, 2003; Peng et al., 2008b; Xu and Feng, 2000). However, the molecular mechanism of the effects of NBP remains obscure. For potential clinical applications, the verification of NBP effects in different stroke models is necessary.

Mitochondrial dysfunction is related to necrotic and apoptotic cell death. In apoptosis, mitochondria play a key role in both caspase-dependent and -independent apoptotic processes. The release of cytochrome c and apoptosis-inducing factor (AIF) from mitochondria to the cytosol is a clear indication of mitochondrial dysfunction. Cytosolic cytochrome c and AIF then lead to caspase activation and AIF nuclear translocation, which are key steps in caspase-dependent and -independent apoptotic cascades, respectively (Kluck et al., 1997; Susin et al., 1999).

Mitogen-activated protein kinases (MAPK) respond to extracellular stimuli and regulate various cellular activities, such as gene expression, mitosis, differentiation, and apoptosis (Kuida and Boucher, 2004). c-Jun N-terminal kinase (JNK) and p38 MAP kinase are important members of the MAPK superfamily and key modulators of apoptosis (Nishina et al., 2004; Xia et al., 1995). Whether NBP affects the MAPK pathway has not been explored.

In this study, we examined the neuroprotective effects of dl-NBP against apoptosis in cultured neurons and against ischemia-induced brain damage and cell death in a mouse model of focal ischemic stroke. We also studied the protective action of NBP on mitochondrial integrity and provided novel evidence that reduced mitochondrial damage and JNK/p38 activation contribute to the NBP anti-apoptotic effect.

2. Results

Neuroprotective effects of NBP in cortical neuronal cultures

To focus on the effect of NBP on apoptotic cell death, we first confirmed the neuroprotective action of NBP on serum deprivation (SD)-induced cell death in cortical neuronal cultures (Fig. 1A and 1B). SD is a typical apoptotic insult to neurons (Galli and Fratelli, 1993; Yu et al., 1997). The insult caused over 30% of cell death in the first 24 hrs (Fig. 1B). The cell body shrinkage and caspase-3 activation in these cells is consistent with the apoptotic nature of the cell death (Fig. 1A and 1C). NBP (10 μ M) co-applied with SD showed inhibitory effects on caspase-3 cleavage/activation compared to SD control groups (Fig. 1C and 1D). NBP decreased ROS production 24 hrs after the onset of SD (Fig. 2A and 2B). In an attempt to understand the intracellular signaling pathway that may mediate the NBP effect, we found that the pro-apoptotic phosphorylation of JNK was attenuated by co-applied NBP (Fig. 2C and 2D).

NBP attenuated infarct formation and cell death after ischemic stroke

Adult male 129S2/Sv mice were subjected to sham operation or right middle cerebral artery (MCA) permanent occlusion plus 10-min ligation of bilateral common carotid artery (CCA) (Wei et al., 2005). NBP administration (100 mg/kg, i.p. 1 hr after MCA occlusion) showed a protective effect of attenuating infarct volume assessed 24 hrs after MCA occlusion (Fig. 3). As a positive control, MK-801 (3 mg/kg, i.p. 15 min before stroke) reduced about 50% of infarct formation. A comparable protective effect was obtained with NBP treatment (Fig. 3).

TUNEL-positive cells in the ischemic core and peri-infarct regions were detected at different time points (6, 12 and 24 hrs) after ischemia. TUNEL-positive cells started to appear 6 hrs after stroke in the ischemia control group, while TUNEL staining was not detected in NBP treated mice until 12 – 24 hrs after ischemia. At each time point tested, there were fewer TUNEL-positive cells in the NBP group as compared to the sham-treated controls (Fig. 4C). Significantly less cell death was identified in the NBP group at 12 and 24 hrs after ischemia.

NBP reduced caspase-3 and caspase-9 activation in the ischemic brain

In immunohistochemical experiments, activated caspase-3 staining was seen in the peri-infarct region. NBP administration reduced caspase-3 cleavage/activation by 37% ($P<0.05$) 24 hrs after ischemic stroke compared to that in sham-treated controls (Fig. 5A–5C). Activated caspase-9 was detected using Western blot in the ischemic cortex and it was attenuated by NBP administration (100 mg/kg, i.p. 2 hrs before and immediately after ischemia). Western blotting showed 33% less caspase-9 cleavage compared to that in the control group ($P<0.05$; Fig. 5D and 5E). Consistently, in the Western blot assay, NBP also attenuated caspase-3 cleavage/activation (43% reduction, $P<0.05$; Fig. 5F).

NBP prevented mitochondrial damage after stroke

Cytochrome c release from mitochondria was detected using Western blot 24 hrs after ischemia. There was significantly less cytochrome c in the cytosol of the NBP group cells compared to controls (Fig. 6). Consistently, more cytochrome c was likely retained in the mitochondria of NBP-treated animals (Fig. 6). To evaluate the NBP effect on caspase-independent apoptosis, we also examined ischemia-induced AIF release from mitochondria. NBP significantly reduced AIF in cytosolic compartments while mitochondrial AIF was significantly higher in the NBP group than the control group (Fig. 6).

NBP decreased JNK and p38 activation

The JNK/p38 pathway has been linked to apoptosis (Nishina et al., 2004; Xia et al., 1995). Using Western blot, we detected no difference of total JNK expression between sham-treated and stroke/NBP groups. However, NBP treatment significantly reduced JNK phosphorylation at 24 hrs after stroke compared to the control group (Fig. 7A and 7B). In addition, NBP also reduced p38 expression (Fig. 7C and 7D).

3. Discussion

In the present study, we demonstrate the neuroprotective effect of NBP treatments *in vitro* and in ischemic animals. NBP treatment attenuated ischemia-induced infarct formation in the cerebral cortex and decreased the number of TUNEL positive cells in cell cultures and the stroke penumbra region after cerebral ischemia. NBP reduced the activation of caspase-3 and caspase-9, two key steps in apoptosis. This effect supports the idea that NBP is an effective anti-apoptosis reagent (Dong, 1999). The new evidence of caspase-9 being blocked by NBP indicates that NBP not only inhibits the downstream “executive” caspase-3 activation, but is also able to block the upstream apoptotic cascade activity. This is

consistent with the observation that NBP reduces cytochrome c release from mitochondria, which presumably prevents formation of the apoptosome composed of cytochrome c, apaf-1 and caspase-9.

A previous study showed that dl-NBP was able to maintain mitochondria membrane fluidity (MMF) and Na^+, K^+ -ATPase activity, preventing mitochondria swelling and vacuolation after cerebral ischemia (Xiong, 2000). The same group showed that NBP reduced ischemia-induced intracellular Ca^{2+} accumulation, inflammation, lipid peroxidation, and superoxide radical formation, NBP might also act directly on mitochondrial complex IV to increase its activity (Chong, 1999; Lin, 1996; Xiong, 1999; Xu, 2000). Our experiments show the novel evidence that NBP is able to attenuate both cytochrome c and AIF release from mitochondria, suggesting that NBP is not only protective against ischemia-induced morphological changes but also affect the release of apoptotic factors from mitochondria. Cytochrome c and AIF lead to caspase-dependent and caspase-independent apoptotic pathways, respectively. Both of these pathways are activated during ischemic stroke (Galluzzi et al., 2009) and are suppressed by NBP.

The present investigation provides the novel information that NBP can regulate JNK and p38 activation in cultured neurons and the ischemic brain. Since the expression and activation of JNK and p38 kinases are highly relevant to ischemic damage, this observation indicates a new mechanism for its neuroprotective activity. Thus, previous and current evidence suggests that NBP has comprehensive neuroprotective effects mediated by multiple mechanisms, which are believed to be an essential property for an effective treatment of stroke.

The present investigation tested NBP administration before ischemic insult and 1 hr after MCA occlusion. The pretreatment shows the preventive action of NBP against an ischemic attack. Since NBP has been shown to be highly tolerant to humans, it is possible that NBP can be taken as a preventive drug by human populations with a high risk for ischemic stroke. A previous investigation showed that dl-NBP was effective in reducing infarct volume administered 2 hrs after MCA occlusion in rats (Lin, 1996). Another group reported that, in a hypertensive thrombosis rat model induced by photochemical reaction, NBP orally administered 24 hrs after stroke (75 mg/kg $\times$ 2 times each day for 10 days) reduced the focal thrombi and infarct area (assessed using H&E staining) 10 days after stroke (Huang, 2005). The post-stroke treatment of NBP suggests that NBP may be used for acute and subacute treatments of stroke. The therapeutic window for the post stroke treatment, however, requires further investigation.

In human studies of 60 to 299 cases carried out in China, dl-NBP (200 mg, 3–4 times/day $\times$ 14–20 days, oral administration) improved neurological NIHSS (NIH Stroke Survey) score without severe adverse effect (Chen, 2006; Cui, 2005; Ye, 2006). In a very recent report, the authors collected data from randomized controlled clinical trials in China, and compared dl-NBP with placebo or open control in patients with acute ischemic stroke. Twenty-one trials involving 2,123 patients were included, of which 2 were placebo-controlled and 19 were open-label controlled. Meta-analysis of 10 trials ($n=958$), in which neurological deficits were assessed by CSS, suggested that there were significant differences favoring NBP in the improvement of neurological deficits score during the treatment period. Meta-analysis of 6 trials ($n=590$), in which neurological deficits were assessed by NIHSS, also favored NBP. Adverse events were reported in 13 trials. Gastrointestinal discomfort (1.7%~8%) and abnormal liver function including abnormal ALT (1.4%~17.5%) and abnormal AST (1.9%~8.82%) were the two most common observations. No severe adverse events were reported. Numbers of dead and dependent patients at the end of follow up (at least three months) were not reported in the 21 included trials. Quality of life was not assessed in any

of the trials. The authors concluded that dl-3-butylphthalide can improve the neurological function after acute ischemic stroke and appears to be safe (Wang, 2010). It was suggested that further study is needed to confirm NBP effects for lowering rates of death and dependency (Wang, 2010). This is exceptionally exciting and encouraging information from stroke clinical trials that have so far rarely delivered positive results. Certainly, these results will need to be further verified by independent investigators in different countries. More recent information suggested that l-NBP is more potent than d- and dl-isomers in neuroprotection against cerebral ischemia (Peng, 2005). We suggest that future studies are warranted to focus on the neuroprotective effects of l-NBP. Basic, translational, and clinical investigations are needed to better understand the mechanism of its neuroprotective effects and to establish the therapeutic window for delayed administration of NBP treatment after a stroke attack.

4. Materials and Methods

Materials

Synthesized dl-3-n-butylphthalide (NBP, Butylphthalide or Dinbente) was a generous gift from Shijiazhuang Pharmaceutical Group Ouyi Pharma Co., Ltd, (Shijiazhuang, China). It was dissolved in DMSO (Sigma, St. Louis, MO) before dilution in the culture medium. The final concentration of DMSO was 0.2%. Control tests were performed with 0.2% DMSO. See below for information about antibodies and other reagents.

Neuronal cell cultures

The preparation of nearly pure cortical neurons was previously described (Wang et al. 2003). Briefly, neurons were harvested from the cortex of gestational 14–16 day mouse embryos. The cortex was dissociated in trypsin and plated on poly-D-lysine and laminin coated tissue culture dishes. Cells were maintained in neurobasal media (Invitrogen, Carlsbad, CA) with B27 supplement and L-glutamine until the time of the experiment. Cytarabine (Ara-C) was added on the third day to prevent the proliferation of glial cells.

In vitro apoptosis model

Serum deprivation (SD) was accomplished by exchanging the media for B27-free media through a series of half media changes. Control cultures were treated by sham washes with serum containing media (Yu et al., 1997). NBP was added upon the last wash. Cell death was assessed by LDH release into the media. 50 μ l of media was harvested and assayed using an LDH activity kit (Roche, Basel, Switzerland).

Western blot analysis of cultured cells

For western blot analysis, proteins were isolated from cortical neurons of different experimental groups. Cells were washed twice with ice-cold PBS and lysed in a medium containing 50 mM Tris-HCl (pH 8.8), 150 mM NaCl, 2 mM EDTA, 1% SDS, 2 mM sodium orthovanadate, 1% NP-40, 1% sodium deoxycholate, 20 μ g/ml leupeptin, 20 μ g/ml aprotinin, and 1mM phenylmethanesulfonyl fluoride (PMSF). Cells were scraped from the dish, vortexed, held on ice for 45 minutes and spun at 14,000g for 25 min to remove cell debris. Supernatant was collected and protein concentration was determined using the bicinchoninic acid assay (Pierce, Rockford, IL). Equal amounts of protein were suspended in sample buffer and boiled for 5 min. Cell lysates were resolved on SDS-PAGE using gradient gels (6–15%). Proteins were transferred to a PVDF membrane (Amersham, UK), blocked with 7% milk in TBST buffer (20 mM Tris, 137 mM NaCl and 0.1% Tween 20) and incubated with primary antibody (anti-caspase-3 antibody, 1:1000; Millipore Billerica, MA) overnight. After washing 3 times with TBST buffer, membranes were incubated with

alkaline phosphatase-conjugated secondary antibody (1:5000; Promega, Madison WI) in TBST-3% evaporated milk. The membranes were then washed 3 times with TBST buffer and 3 times with TBS buffer before color development with BCIP/NBT substrate colorimetric method.

Detection of ROS production

Cellular production of ROS was assessed with dihydroethidium (DHE) (Sigma, St. Louis, MO) as described previously (Wang et al 2003). DHE is a non-fluorescent membrane permeable derivative of the DNA intercalating dye ethidium. DHE can be oxidized by superoxide and hydroxyl radicals and upon oxidation fluoresces red and intercalates into the cellular DNA. Cellular ROS can therefore be assessed as the difference in cells with red fluorescence among treatment groups. After 24 hrs cells were treated with 5 μ M DHE and 1:20,000 Hoechst 33342 for 5 min at 37°C and then imaged immediately. Cells were imaged under a fluorescent microscope at 40 $\times$ magnification. For each experiment, treatment groups were run in duplicate culture dishes and at least six fields were assessed for each dish. The results are then compiled from different independent experiments.

Middle cerebral artery occlusion in mice

All animal experiments and surgery procedures were approved by the University Animal Research Committee and met NIH standards. Adult male 129S2/Sv mice (25 – 20g; Harlan, Indianapolis, IN) were subjected to sham operation or surgery procedures modified from a previously described rat protocol (Wei et al., 2005). In brief, animals were anesthetized by 4% chloral hydrate intraperitoneal (i.p.) injection, and the right middle cerebral artery (MCA) was permanently ligated by a 10-0 suture (Surgical Specialties CO., Reading, PA). This was accompanied by a 10-min ligation of bilateral common carotid arteries (CCA). During surgery and recovery periods, body temperature was monitored and maintained at 37.0 $\pm$ 0.5°C using a temperature control unit and heating pads. NBP (100 mg/kg; Hebei Zhongrun Pharmaceutical Co, LTD, Shi Jia Zhuang, China) was dissolved in 0.5% Tween-80 solution and administered intraperitoneally 2 hrs before and right after the CCA reperfusion. Animals were euthanized by decapitation at different time points after ischemic stroke. The brain was immediately removed and mounted in optimal cutting temperature compound (Sakura Finetek USA, Inc., Torrance, CA) at –80°C for further processing.

Triphenyltetrazolium chloride (TTC) staining of infarct volume measurement

One day after MCA occlusion, animals were killed with an overdose of pentobarbital (100 mg/kg) followed by intracardiac perfusion of 200 ml 0.9% NaCl. The brains were then sliced into 1-mm coronal sections. Cortical infarct volume was morphometrically measured after staining with 2% 2, 3, 5-triphenyltetrazolium chloride (TTC, Sigma, St Louis, USA) in phosphate-buffered saline (pH 7.4) at 37°C for 20 min and then stored in 10% neutral-buffered formalin. The cross-sectional area of the TTC-unstained region was determined with the use of an image analyzer (DUMAS, Drexel University). The indirect method (subtraction of residual right hemisphere cortical volume from cortical volume of the intact left hemisphere) was used for infarct volumes calculation.

TUNEL staining of cell death measurement

A TUNEL staining kit (DeadEnd™ Fluorometric TUNEL system, Promega) was used to visualize cell death in 10- μ m coronal frozen sections together with NeuN. After 10-min fixing with 10% buffered formalin phosphate (Fisher Scientific, Pittsburgh, PA) and pretreatment with –20° C ethanol: acetic acid (2:1) and 0.2% Triton X-100, the brain sections were incubated in an equilibration buffer as instructed by the kit. The TdT enzyme and nucleotide mix were then added at proportions specified by the kit for 75 min at room

temperature. The slides were washed with the provided 2× SSC washing buffer. To identify neuronal death, slides were then incubated with the NeuN primary antibody (1:250, Chemicon) overnight. After PBS washes and incubation with Cy3-conjugated anti-mouse IgG (1: 1000, Jackson ImmunoResearch, West Grove, PA), the slides were incubated in Hoechst 33342 (1:20000, Molecular Probes, Carlsbad, CA) for 5 min to stain the nucleus before mounted with ProLong Antifade mounting medium (Molecular Probes, Carlsbad, CA) for observation.

Western blot analysis of in vivo tissues

For each group, 3 animals were used to collect the ipsilateral tissue samples from the lesion border. Proteins were extracted using the NE-PER Nuclear and Cytoplasmic Extraction Kit (Pierce Biotechnology, Rockford, IL). Protein concentrations were determined by bicinchoninic acid assay (Pierce Biotechnology). The samples were kept frozen at -80°C until assessment. Samples of 50 μg proteins were electrophoresed on a 6–15% gradient gel as described above. Membranes were blocked with Tris-buffered saline containing 0.1% Tween-20, pH 7.6%, 7% milk at room temperature for 2 hrs and incubated overnight at 4°C with mouse anti-cleaved caspase-3 (1:500, Millipore Billerica, MA), anti-phosphoralted JNK (1:1000; Cell Signaling, Danvers, MA), anti-p38 (1:1000; Cell Signaling, Danvers, MA), rabbit anti-cytochrome *c* polyclonal antibody (1:200; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or anti-AIF antibody (1:200; Santa Cruz Biotechnology, Inc.) Mouse anti- β -actin (Sigma, St. Louis, MO) was used as the protein loading control. The blots were washed in 0.5% Tris-buffered saline containing 0.1% Tween-20 and incubated with alkaline phosphataseconjugated anti-rabbit or anti-mouse IgG antibody (Promega, Madison, WI) for 2 hours at room temperature. Finally, membranes were washed with Tris-buffered saline containing 0.1% Tween-20 followed by 3 washes with Tris-buffered saline. The signal was detected by the addition of Bromo-chloro-indoryl phosphate/nitro blue tetrazolium (BCIP/NBT) solution (Sigma), and quantified and analyzed by the imaging software Photoshop Professional (Adobe® Photoshop® CS 8.0). The intensity of each band was first measured and then subtracted by the background. The expression ratio of each target protein was then normalized against β -actin.

Immunofluorescence staining

Anesthetized animals were perfused with 10 U/ml heparin and subsequently with 4% formaldehyde in 0.1 M PBS, pH 7.4. The brains were removed, postfixed for 12 h, sectioned at 25 μm on a vibratome, or coronal fresh frozen sections of 10- μm thick were sliced using a cryostat vibratome (Ultapro 5000, St Louis, MO). After the slides were completely air dried, slices were fixed in 10% buffered formalin phosphate for 10 min (fresh frozen sections), followed by treatments in a -20°C ethanol: acetic acid (2:1) solution for 12 min, in 0.2% Triton-100 for 5 min and washed by PBS 3 times between each step. Slides were blocked in 1% gelatin from cold water fish (Sigma) diluted in PBS at room temperature for 1 hour, and subsequently incubated with primary antibodies diluted in PBS overnight at 4°C . The primary antibodies were anti-NeuN (Millipore Billerica, MA), anti-cleaved caspase-3 (Millipore Billerica, MA), anti-phospho-JNK (Cell Signaling, Danvers, MA), and anti-p38 (Cell Signaling, Danvers, MA).

After rinsing with PBS, brain sections were treated with secondary antibodies Alexa Fluor 488 anti mouse, anti-rabbit or anti-goat IgG (Molecular Probes, Carlsbad, CA), Cy3-conjugated anti-mouse or anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA) for 1.5 hrs at room temperature. Hoechst 33342 was applied to stain the nuclei of the cells. The brain sections were mounted and coverslipped, imaged and photographed under a fluorescent microscope (BX51, Olympus, Japan) and laser scanning confocal microscope

(Carl Zeiss Microimaging, Inc., Thornwood, NY). As a negative control, sections were incubated without primary antibodies.

Quantification of immunocyto staining positive cells

The immunostaining positive cells were counted in ischemic penumbra region. For each staining, counting was performed on 3 randomly selected non-overlapping 20× fields per 10-μm or 25-μm thick section. Cell counts for each animal were performed in the same volume of control and NBP treated brains. Design-based stereology and systematic random sampling were employed to ensure accurate and non-redundant cell counting. Every section under analysis was a minimum distance of 150 μm from the next, and a total of 6 sections that spanned the entire infarct region of interest were randomly selected for cell counting. For each section under analysis, the region of interest in the ipsilateral hemisphere and the corresponding location was selected in the contralateral hemisphere. Similarly, age-matched sham control brain sections were selected at random from similar areas of the cortex. Cell counts were performed using ImageJ (National Institutes of Health, Bethesda, MD), compiled, and analyzed. Cell counts were performed without knowledge of experimental treatments.

Statistical Analysis

Student's two-tailed t-test was used for comparison of two experimental groups. Multiple comparisons of more than two groups were performed using one-way analysis of variance (ANOVA) by a post-hoc Tukey test. Changes were identified as significant if *P* was less than 0.05. Mean values were reported together with the standard error of mean (Mean ± SEM).

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Figure 1. Apoptotic cell death and NBP protection in cortical neuronal cultures

Serum deprivation-induced apoptotic cell death was tested in pure neuronal cultures of 7–8 days in vitro. **A** and **B**. NBP (0.1–10 μM) was co-applied to the medium with SD and cell death was assessed using LDH release measurement 72 hrs later. Phase contrast photos in **A** illustrate conditions of cortical neurons in 72 hrs after sham control, SD and SD plus 10 μM NBP. The bar graph in **B** summarizes the toxicity data, showing a significant neuroprotective effect of NBP at 10 μM. N=3 independent assays of different cultures. #. $P < 0.05$ compared with controls. **C**. Cleaved caspase-3 (17 kDa) was detected using Western blot. SD increased caspase-3 cleavage while it was reduced by co-applied 10 μM NBP. β-Tubulin was used as loading control; the faint bands under SD and SD plus NBP were due to less protein loads. This was corrected in data analysis as shown in normalized density in figure **D**. **D**. Summary of caspase-3 experiments. N=3 independent assays.

Figure 2. NBP effects on ROS production and p-JNK activity in apoptotic neurons

ROS production was measured using DHE nuclear fluorescence. **A.** Phase contrast photos, Hoechst nuclear staining (blue) and DHE fluorescent activity (red) was performed 24 hrs after SD. Cortical neurons treated with 10 μ M NBP showed fewer ROS-positive cells than the SD group alone. **B.** The ROS changes compared to controls in SD and SD + 10 μ M NBP groups. **C** and **D.** The phosphorylation of JNK after 24-hr SD was assessed using Western blot, which was reduced by co-treatment with 10 μ M NBP. N=4, *, $P < 0.05$ compared to controls.

Figure 3. Protective effect of NBP against ischemia-induced brain infarct formation

Adult mice were subjected to MCA and CAA occlusion. TTC staining was performed 24 hrs after ischemia to inspect infarct formation. **A.** Representative brain coronal sections showing the ischemia-induced infarction in the ipsilateral cortex (white). **B.** The bar graph summarizes the measurement of infarct volume. As a positive control, MK-801 (3 mg/kg, i.p. 15 min before ischemia) showed about 50% reduction of infarct volume. NBP (100 mg/kg, i.p. 1 hr after ischemia) similarly attenuated ischemia-induced infarct formation. N=10; *, $P < 0.05$ vs. sham control.

Figure 4. Cell death and NBP antagonism in the ischemic cortex

Cell death represented by TUNEL staining was evaluated in the ischemic core and peri-infarct regions 6, 12 and 24 hrs after stroke. **A** and **B**. TUNEL-positive cells 24 hrs after stroke. **C**. Summary of TUNEL-positive cells at different time points after ischemia. NBP treatment (see text and Figure 1 legend) significantly reduced cell death at 12 and 24 hrs after the onset of ischemia. Blue color is Hoechst 33324 staining of cell nuclei. The Insert image in B is an enlarged area to show the double staining. N=7, *, P<0.05 compared with controls.

Figure 5. Apoptotic cell death and NBP protection in vivo after ischemic stroke

Caspase activation was assessed as an indication of apoptosis 24 hrs after focal ischemia in mice. **A** and **B**. In the penumbra of the ischemic brain sections, immunostaining of cleaved caspase-3 (red) showed significant apoptotic cell death. Blue was Hoechst 33342 staining of cell nuclei (blue). NBP treatment (100 mg/kg, i.p. 2 hr before and immediately after ischemia) reduced caspase-3 positive cells. **C**. Summary of caspase-3 immunostaining data. NBP reduced apoptotic cells by 40%. $N=5$, $^* P<0.05$ compared to control. **D**. Western blot of penumbra regions of caspase-9 and cleaved caspase-3 24 hrs after ischemia. **E** and **F**. Summaries of caspase-9 (**E**) and caspase-3 (**F**) Western blot analysis. NBP treatment markedly attenuated caspase activations. $N=5$, $^* P<0.05$ compared with controls.

Figure 6. Cytochrome C and AIF release from mitochondria

In the penumbra region, cytochrome C and AIF in the mitochondrial and cytosolic compartments were tested using Western blot 24 hrs after ischemia. **A.** Representative cytochrome C and AIF bands with and without NBP treatment. **B – C.** Summarized data of cytochrome C in the cytosol and mitochondria. There was significantly less cytochrome C release into the cytosol in the NBP-treated brain. **D – E.** Summarized data of AIF in the cytosol and mitochondria. NBP treatment attenuated AIF in the cytosol while AIF increased in the mitochondria. This is consistent with the idea that NBP prevents AIF release from mitochondria. *. $P < 0.05$ compared with controls.

Figure 7. NBP inhibited JNK/p38 pathway

Total and phosphorylated JNK and p38 expression in penumbra were measured 24 hrs after ischemia using Western blot. **A.** Representative gels of Western blot analysis. **B.** Ratio of phospho-JNK and total JNK with and without NBP treatment. The value was markedly lower after NBP treatment. **D.** Summarized data of p-38 expression with and without NBP treatment. NBP significantly decreased p-38 levels. N=5, *. $P < 0.05$ compared with controls.