Neuroprotectin D1 attenuates brain damage induced by transient middle cerebral artery occlusion in rats through TRPC6/CREB pathways

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Received January 3, 2013; Accepted May 31, 2013

DOI: 10.3892/mmr.2013.1543

Abstract. Neuroprotectin D1 (NPD1) may serve an endogenous neuroprotective role in brain ischemic injury, yet the underlying mechanism involved is poorly understood. In the present study, we aimed to investigate whether intracerebroventricular (ICV) injection of NPD1 is neuroprotective against transient focal cerebral ischemia. We also sought to verify the neuroprotective mechanisms of NPD1. Rats subjected to 2 h ischemia followed by reperfusion were treated with NPD1 at 2 h after reperfusion. PD98059 was administered 20 min prior to surgery. Western blot analysis was performed to detect the protein levels of calpain-specific aII-spectrin breakdown products of 145 kDa (SBDP145), transient receptor potential canonical (subtype) 6 (TRPC6) and phosphorylation of cAMP/Ca^{2+}-response element binding protein (p-CREB) at 12, 24 and 48 h after reperfusion. The immunoreactivity of p-CREB and TRPC6 was measured by quantum dot-based immunofluorescence analysis. Infarct volume and neurological scoring were evaluated at 48 h after reperfusion. NPD1, when applied at 2 h after reperfusion, significantly reduced infarct volumes and increased neurological scores at 48 h after reperfusion, accompanied by elevated TRPC6 and p-CREB activity, and decreased SBDP145 activity. When mitogen-activated protein kinase kinase (MEK) activity was specifically inhibited, the neuroprotective effect of NPD1 was attenuated and correlated with decreased CREB activity. Our results clearly showed that ICV injection of NPD1 at 2 h after reperfusion improves the neurological status of middle cerebral artery occlusion (MCAO) rats through the inhibition of calpain-mediated TRPC6 proteolysis and the subsequent activation of CREB via the Ras/MEK/ERK pathway.

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Key words: neuroprotectin D1, neuroprotection, middle cerebral artery occlusion, TrpC, CREB, rat

Introduction

Acute ischemic stroke, the most common form of stroke, can produce an irreversibly injured core, in which cell death is rapid and not salvageable, and an ischemic penumbra, where tissue is damaged but potentially salvageable (1,2). The penumbra has a limited life span and appears to undergo irreversible damage within a few hours unless reperfusion is initiated and/or neuroprotective therapy is administered (3). Therefore, the early rapid recovery of cerebral blood flow and effective neuroprotective treatment in cerebral ischemia remains vital. However, to date, there remains no established treatment for lessening ischemic brain injury.

Brain injury after focal cerebral ischemia develops from a series of pathological processes, including excitotoxicity, peri-infarct depolarizations, ionic imbalance, oxidative stresses and apoptosis (1,2,4,5). Although these mechanisms have been implicated in ischemic neuronal death, Ca^{2+} overload remains the central focus. Cerebral ischemia causes significant glutamate release and exposure to high levels of glutamate leads to the overactivation of N-methyl-D-aspartate receptors (NMDARs), causing Ca^{2+} overload, which leads to calpain activation (5-7). The activation of calpain leads to proteolysis of transient receptor potential canonical (subtype) 6 (TRPC6) channels. TRPC6 channels play a critical role in promoting neuronal survival against focal cerebral ischemia (8). TRPC6 activates cAMP-response element binding protein (CREB) through the Ras/MEK/ERK pathway, and contributes to TRPC6-mediated CREB activation, resulting in neuronal survival (9). Activation of calpain leads to TRPC6 degradation and contributes to neuronal damage in ischemia (8). Therefore, inhibition of TRPC6 degradation to preserve neuronal survival may be a new therapeutic strategy against ischemic brain damage.

NPD1, a stereospecific derivative of docosahexaenoic acid (DHA) formed through a lipoxygenase enzyme that acts on free DHA (10,11), reduces infarct volume at 48 h after reperfusion (12). However, the precise mechanism responsible for the

neuroprotective activity of NPD1 has yet to be fully elucidated. Potential mechanisms explaining how NPD1 may serve an endogenous neuroprotective role include reducing apoptosis, inhibiting leukocyte infiltration and pro-inflammatory gene expression, and binding toxic peroxides (12-16). However, previous studies have not unequivocally confirmed the effects of intracerebroventricular (ICV) injection of NPD1 on TRPC6/CREB-mediated neuroprotection.

The present study was performed to investigate whether ICV injection of NPD1 at 2 h after reperfusion has a neuroprotective effect, and to verify whether NPD1 improves neurological status through inhibition of calpain proteolysis of TRPC6, subsequently inducing CREB activation via the Ras/MEK/ERK pathway.

Materials and methods

Animals and surgical procedures. Male Sprague-Dawley rats, weighing 200-250 g, were purchased from Hunan weasleyg scene of experimental animals Co., Ltd. Experimental protocols were approved by the committee of experimental animals of Tongji Medical College and conformed to internationally accepted ethical standards (Guide for the care and use of laboratory animals; NIH Publication 80-23, revised 1978). The animals were anesthetized with 10% chloral hydrate (400 mg/kg, i.p.). Transient focal cerebral ischemia was produced by intraluminal occlusion of the right middle cerebral artery (MCA) for 2 h. Briefly, the right carotid artery was exposed to separate the external carotid artery and the internal carotid artery. The external carotid artery was occluded at the level at which the MCA branches out and a 4-0 monofilament nylon suture (Beijing Sunbio Biotech Co. Ltd., Beijing, China) with a rounded tip was introduced through the internal carotid artery until mild resistance was felt. Two hours later, the filament was gently removed for the reperfusion (reperfusion confirmed by laser Doppler). Sham surgery rats were treated similarly, although the filament was not advanced to the origin of the MCA. The body temperature was maintained at 37.5±0.5˚C with a temperature-controlled heating pad attached to a rectal probe during surgery. Continuous laser-Doppler flowmetry (Perimed PF5000, Stockholm, Sweden) was used to monitor regional cerebral blood flow (rCBF) in the cortex supplied by the MCA to ensure accurate occlusion and reperfusion. Animals that showed a CBF reduction <70% were excluded from the experimental group, as well as animals that died after ischemia induction. In a separate experiment, physiological parameters (cranial temperature, arterial pH, $PaCO_2$ and PaO_2) were monitored and analyzed (n=6). Arterial blood samples were obtained 5 min prior to ischemia (baseline), 60 min following ischemia, and 12, 24 and 48 h following reperfusion for blood gas analysis.

Animal groups and treatments. Rat ICV injection was performed under anesthesia with a stereotaxic instrument using a microsyringe pump. A scalp incision was made and a burr hole was opened in the right parietal skull, 1.8 mm lateral and 1.0 mm posterior to the bregma. A syringe was inserted into the brain to a depth of 4.2 mm below the cortical surface. Drugs or vehicle were injected slowly (0.5 μl/min) into the right ventricle.

All treatments were administered in a blinded manner. The rats were randomly divided into four groups, and each group was again divided into three subgroups (n=12 per subgroup) according to the time of reperfusion (12, 24 and 48 h after reperfusion). The experimental groups and subgroups were as follows: i) Sham surgery (Group S; subgroup S12, S24 and S48); ii) middle cerebral artery occlusion (MCAO; Group I; subgroup I12, I24 and I48); iii) ischemia combined with NPD1 treatment (Group N; subgroup N12, N24 and N48) and iv) ischemia combined with NPD1 plus PD98059 (MEK inhibitor) treatment (Group P; subgroup P12, P24 and P48). Another 27 rats were randomly divided into three groups: i) Sham surgery (Group S); ii) MCAO (Group I); iii) ischemia combined with PD98059 treatment (Group M).

NPD1 (100 ng/μl; Cayman Chemical Company, Ann Arbor, MI, USA) was dissolved in ethanol. PD98059 (1.5 mg/ml; Sigma, St. Louis, MO, USA) was prepared in 1% DMSO (Sigma). NPD1 (5 μl) or ethanol (5 μl) was injected slowly (0.5 μl/min) into the right ventricle at 2 h after reperfusion. PD98059 (0.5 ml, i.p.) or DMSO (0.5 ml, i.p.) was also administered to rats 20 min prior to the surgery.

Measurement of infarct volume. The extent of infarction was measured with 2,3,5-triphenyl-tetrazolium chloride (TTC). At 48 h after reperfusion, rats were deeply anesthetized with 10% chloral hydrate, and the brains were rapidly removed, washed in phosphate-buffered saline (PBS) at room temperature and frozen at -20˚C for 10 min. Brain tissue from an area 4 mm anterior and 6 mm posterior to the bregma was cut into five serial 2 mm coronal sections. The sliced brain tissues were stained with 2% TTC (Amresco, Solon, OH, USA) for 30 min at 37˚C in the dark followed by overnight immersion in 4% paraformaldehyde in 0.1 M PBS, pH 7.4, at 4˚C. The infarcted tissue remained unstained (white), whereas normal tissue was stained red. The extent of ischemic infarction was traced and the integrated volume was calculated using Image J software (NIH Image). Infarct volume was calculated by adding the infarction areas of all sections and multiplying by slice thickness. To compensate for the effect of brain edema, the corrected infarct volume was calculated as follows: percentage of corrected infarct volume = {[total lesion volume - (ipsilateral hemisphere volume - contralateral hemisphere volume)]/contralateral hemisphere volume} x 100.

Neurological test. Neurological evaluation of motor sensory functions was carried out at 48 h after reperfusion. An 18-point scale of neurologic deficit scores was used for evaluation of neurologic behavior (17). The scores were assessed in a blinded fashion. The scale was based on the following six tests: i) spontaneous activity; ii) symmetry in the movement of four limbs; iii) forepaw outstretching; iv) climbing; v) body proprioception; and vi) response to vibrissae touch. The score assigned to each rat at completion of the evaluation equaled the sum of all six test scores. The final minimum score was 3 and the maximum was 18.

Lysis and protein content determination. All the rats were sacrificed by decapitation at 12, 24 and 48 h after reperfusion. Slices containing maximal ischemic damage were selected (from an area between 3 and 6 mm posterior to the frontal

Table I. Physiological parameters.

Time point	Temperature (˚C)	PaO_2 (mmHg)	$PaCO_2$ (mmHg)	Arterial pH
Baseline	37.2±0.2	96.6±5.4	38.6±5.9	7.37±0.08
Ischemia, 60 min	37.7±0.3	92.1±6.4	37.3±3.8	7.36±0.05
Reperfusion, 12 h	37.5±0.2	98.9±5.1	38.1±4.1	7.35±0.10
Reperfusion, 24 h	37.1±0.1	94.8±5.7	35.6±4.3	7.38±0.12
Reperfusion, 48 h	37.3±0.2	91.7±7.2	37.8±5.2	7.39±0.13

Results are presented as the mean ± SEM (n=6). PaO_2, arterial oxygen tension; $PaCO_2$, arterial carbon dioxide tension.

pole). The tissues were immediately frozen in liquid nitrogen and stored at -80˚C. Total protein extraction was performed using a commercially available kit (KGP250; Nanjing Keygen Biotech Co. Ltd., Nanjing, China). Nuclear protein extraction was performed using the ProteoJET cytoplasmic and nuclear protein extraction kit (Fermentas International, Glen Burnie, MD, USA). Protein concentrations were determined using the BCA protein assay kit (Beyotime, Jiangsu, China).

Western blot analysis for aII-spectrin, TRPC6 and p-CREB. Protein samples from total or nuclear fractions were boiled for 10 min in 1X sample buffer (Beyotime) prior to loading onto a Tris-HCl gel. Equal amounts of total protein extracts or nuclear protein extracts were separated by SDS-PAGE and transferred onto polyvinylidene difluoride membranes by electrophoresis, and membranes were blocked with 5% non-fat milk in TBST (0.1% Tween-20 in TBS) for 1 h at room temperature. Membranes were then incubated overnight at 4˚C with either a mouse monoclonal anti-aII-spectrin (1:1000; Enzo Biochem, New York, USA), rabbit polyclonal anti-TRPC6 (1:1000; Abcam, Cambridge, MA, USA), rabbit polyclonal anti-p-CREB (1:1000; Cell Signaling Technology, Beverly, MA, USA), rabbit polyclonal anti-Lamin B1 (1:500; Bioworld Technology Inc., St. Louis, MN, USA) or mouse monoclonal anti-GAPDH antibody (1:100; Proteintech Group, Inc., Chicago, IL, USA) followed by horse radish peroxidase (HRP)-conjugated goat anti-mouse IgG antibody (1:3000; Proteintech Group, Inc.) or anti-rabbit IgG antibody (1:5000; Proteintech Group, Inc.). Labeled proteins were detected using the Chemi-Doc Imaging System (Bio-Rad, Hercules, CA, USA). Protein bands were quantified by Image Lab™ image acquisition and analysis software (Bio-Rad). Western blots were repeated three times using samples prepared from three different rats for each experimental condition studied.

Quantum dot-based immunofluorescence. At 12, 24 and 48 h after reperfusion, rats (n=3 for each group) were anesthetized with 10% chloral hydrate and infused through the left ventricle with cold saline as a vascular rinse followed by a fixing solution containing 4% paraformaldehyde in PBS. The brains were removed and fixed overnight in 4% paraformaldehyde in PBS at 4˚C. The brains were then blocked and embedded in paraffin. Paraffin-embedded brains were cut into 4-μm sections according to standard procedures. Paraffin sections (n=3 for each group) were incubated overnight with antibodies against TRPC6 (1:100; Abcam) and p-CREB (1:100; Cell Signaling Technology) at 4˚C after being blocked with 2% bovine serum albumin (BSA). The samples were then incubated with a biotinylated secondary antibody at 37˚C for 30 min. Paraffin sections were then incubated with streptavidin-conjugated QDs605 (1:100, Wuhan Jiayuan Quantum Dot Co., Ltd., Wuhan, China) after being blocked with 2% BSA. TRPC6- and p-CREB-positive cells were measured at x200 magnification per visual field in the cortex, three visual fields per section in three brain sections. Fluorescent signals were detected with a fluorescence microscope (BX51; Olympus, Tokyo, Japan). The acquisition and quantitative analysis of images was performed with a multi-spectral imaging system (Nuance Fx; CRi, Hopkinton, MA, USA).

Statistical analysis. For all quantitative analysis of data, measurements were made with the experimenter blinded to the treatment group. GraphPad Prism (version 5 for Windows; GraphPad Software, La Jolla, CA, USA) software was used for all statistical analyses. Results are presented as the means ± SEM. The neurological score data comparison was analyzed using the Kruskal-Wallis test followed by the post hoc Dunn's test. For all other measurements, one-way analysis of variance (ANOVA) followed by Newman-Keuls multiple comparison test was used. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Physiological parameters. No statistical significance was noted among different time points for any of the physiological parameters, including cranial temperature and blood gas (Table I).

NPD1 significantly reduces infarct volumes in ipsilateral ischemic hemispheres at 48 h after reperfusion. After ischemic/reperfusion injury, the white-stained infarct area was prominent in the MCAO group, and almost the entire MCA area appeared infarcted. By contrast, NPD1-treated rats had significantly reduced infarct volumes compared with the MCAO group ($P<0.01$). After application of PD98059, the infarct volume was significantly increased compared with the NPD1-treated group (Fig. 1A and B; $P<0.01$).

NPD1 promotes functional recovery at 48 h after reperfusion. Sham surgery rats did not have any deficits. Statistical analysis confirmed that NPD1-treated animals had significantly greater neurological scores than the MCAO group ($P<0.01$). After

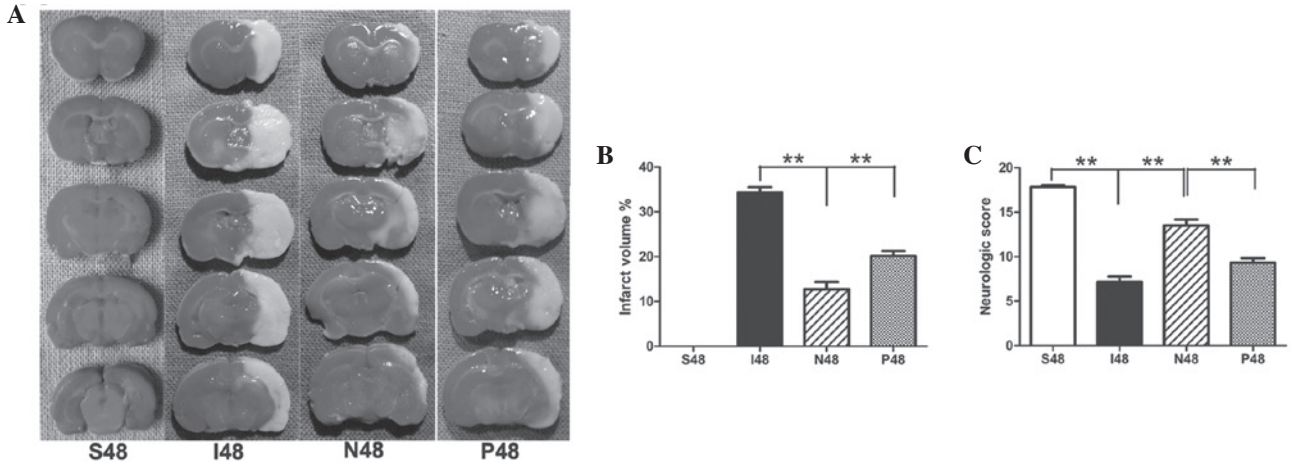

Figure 1. NPD1 reduced infarct volumes in the ipsilateral cortex and promoted functional recovery at 48 h after reperfusion. (A) TTC staining of representative coronal sections. (B) Quantification of infarct volumes. NPD1-treated rats had significantly reduced infarct volumes compared with the MCAO group (P<0.01). After application of PD98059, the infarct volume was significantly increased compared with the NPD1-treated group (P<0.01). Bars represent the means ± SEM (n=6). (C) Quantification of neurologic scores. NPD1-treated animals had significantly greater scores than the MCAO group. PD98059 administered 20 min prior to the surgery attenuated NPD1-induced functional recovery. Bars represent the means ± SEM (n=6). **P<0.01. NPD1, neuroprotectin D1; MCAO, middle cerebral artery occlusion; TTC, 2,3,5-triphenyltetrazolium chloride

treatment with PD98059, the neurological scores were significantly decreased compared with the NPD1-treated group (Fig. 1C; P<0.01).

NPD1 inhibits the formation of calpain-specific aII-spectrin breakdown products (SBDP145). The sham surgery group presented little SBDP145. Quantitative analysis confirmed that the protein levels of SBDP145 in the MCAO group were gradually increased during the experimental time course. Compared with the sham surgery group, the protein levels of SBDP145 in the MCAO group were significantly increased as early as 12 h after reperfusion indicating early calpain activity (P<0.05); this significant increase was also present at 24 h (P<0.01) and 48 h (P<0.01). When MCAO rats were treated with NPD1, the protein levels of SBDP145 were significantly decreased at 24 h (P<0.01) and 48 h (P<0.01). In addition, NPD1 attenuated the calpain-specific fragment of aII-spectrin, but had no effect on caspase-3 and its cleavage activity on aII-spectrin (Fig. 2).

NPD1 inhibits calpain-mediated TRPC6 channel degradation. In the MCAO group, the TRPC6 protein level was also gradually decreased during the experimental time course (Fig. 3A and B). Compared with the sham surgery group, the TRPC6 level in the MCAO group was significantly decreased at 12 h (P<0.01), 24 h (P<0.01) and 48 h (P<0.01). When MCAO rats were treated with NPD1, the protein levels of TRPC6 were significantly increased at 24 h (P<0.05) and 48 h (P<0.01). Immunofluorescence analysis showed the cytomembrane staining pattern of TRPC6 in neurons of the cerebral cortex and the immunofluorescence analysis obtained similar results as the western blot analysis (Fig. 3C and D).

PD98059 exerts no effect on ischemic stroke in rats at 24 h after reperfusion. To study the effect of PD98059 in stroke-induced rats, PD98059 was administered 20 min prior to surgery. Notably, after application of PD98059, no statistical significance was noted in the protein levels of p-CREB between group I and group M (Fig. 4A). There was no significant difference between the two groups during the whole process of ischemia and reperfusion by measuring the infarct volumes and neurological scores (Fig. 4B and C).

NPD1 maintains phosphorylation of CREB through inhibition of TRPC6 degradation. In the MCAO group, p-CREB was gradually decreased during the experimental time course (Fig. 5A and B). Compared with the sham surgery group, p-CREB in the MCAO group was significantly decreased at 12 h (P<0.01), 24 h (P<0.01) and 48 h (P<0.01). Compared with the MCAO group, p-CREB in the NPD1-treated group was significantly higher than that at 24 h (P<0.05) and 48 h (P<0.01). As expected, administration of PD98059 20 min prior to surgery leads to a significantly decreased p-CREB level compared with the NPD1-treated group at 12 h (P<0.05), 24 h (P<0.05) and 48 h (P<0.01). Immunofluorescence staining also showed the same results, and immunoreactivity appeared as nucleus labeling, with no labeling within the cytoplasm or cell membrane (Fig. 5C and D).

Discussion

Our results strongly demonstrate that NPD1, when applied by the ICV route at 2 h after reperfusion, significantly reduced infarct volumes measured by TTC staining. We observed that the decreased infarct volumes obtained with NPD1 were mirrored by enhanced functional recovery. These protective effects are comparable with the observations of Marcheselli *et al* (12), who revealed that NPD1 administered continuously by ICV perfusion reduced the infarct volume by 50% at 48 h after reperfusion. In our study, application of NPD1 with PD98059 20 min prior to surgery led to infarct volumes that were significantly increased, and the neurological scores were significantly decreased. These results demonstrated that ICV injection of NPD1, at very low doses (500 ng, ICV), effectively reduced cerebral ischemic injury in rat models.

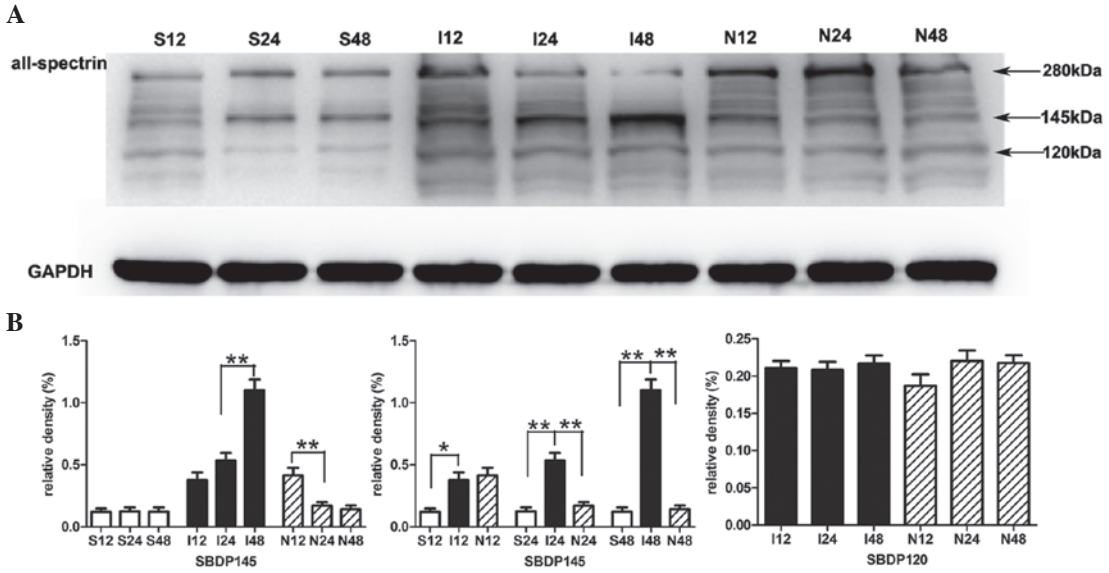

Figure 2. Calpain activation assessed by the protein levels of SBDP145. (A) Western blot analysis of SBDP145 expression at 12, 24 and 48 h after reperfusion. Total protein extracts were prepared from the ipsilateral cortex. Identical amounts of protein (50 μg) were applied to each lane of the SDS-PAGE gel (6% polyacrylamide gel). GAPDH was used as an internal reference. A 145-kDa band corresponding to SBDP145 protein was clearly detected. (B) Densitometric analysis of the protein levels of SBDP145 (n=3). Application of NPD1 significantly reduced the protein levels of SBDP145 at 24 h (P<0.01) and 48 h (P<0.01). In addition, NPD1 attenuated calpain-specific fragment of aII-spectrin, but had no effect on caspase-3 and its cleavage activity on aII-spectrin. Values are the means ± SEM. *P<0.05 and **P<0.01. NPD1, neuroprotectin D1.

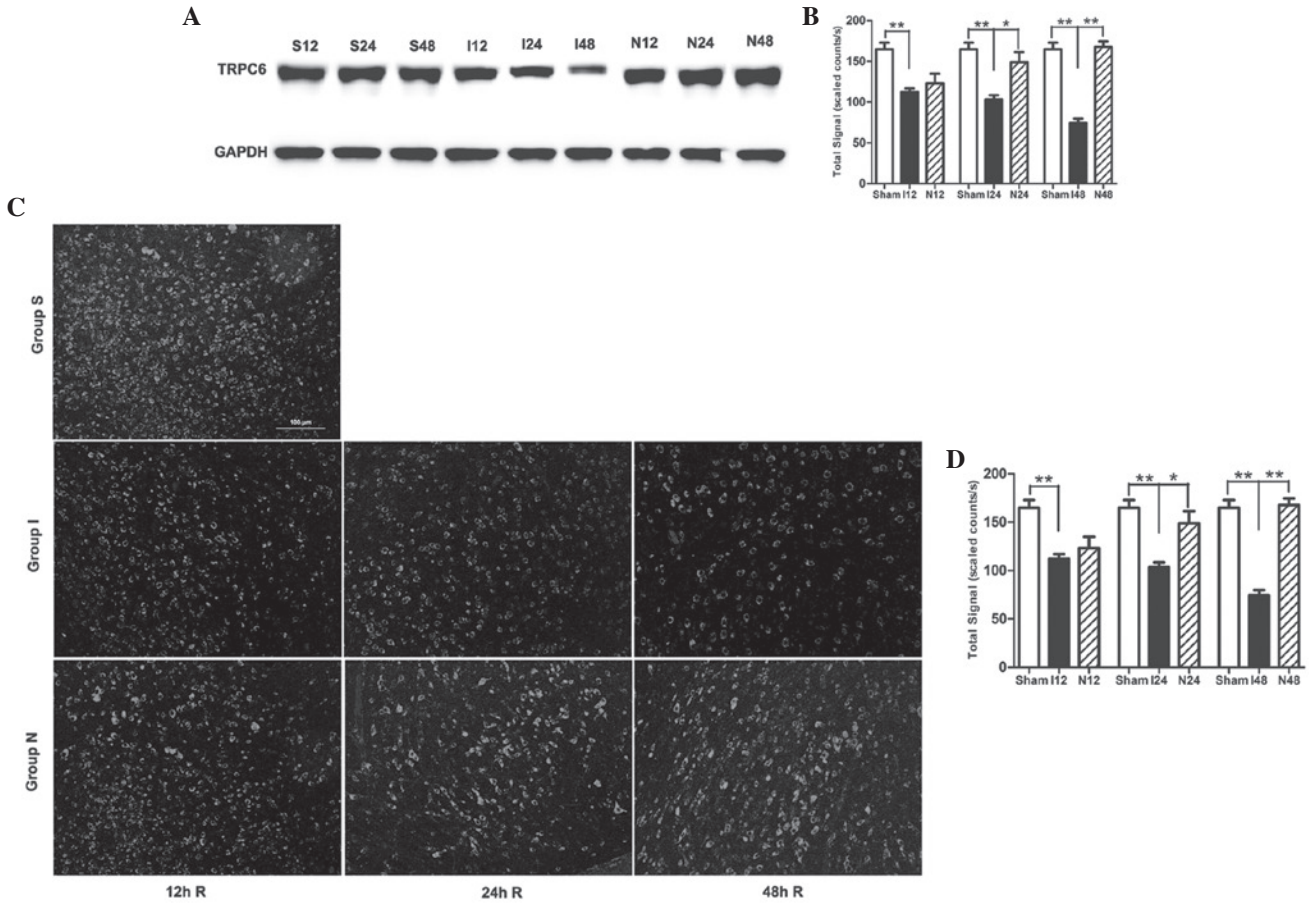

Figure 3. Effect of NPD1 administered at 2 h after reperfusion on TRPC6 expression at 12, 24 and 48 h after reperfusion. (A) Western blot analysis of TRPC6. Total protein extracts were prepared from the ipsilateral cortex. Identical amounts of homogenate protein (50 μg) were applied to each lane of the SDS-PAGE gel (8% polyacrylamide gel). GAPDH was used as an internal reference. A 106-kDa band corresponding to TRPC6 protein was clearly detected. (B) Densitometric analysis of the protein levels of TRPC6 (n=3). Application of NPD1 significantly reduced the protein levels of TRPC6 at 24 h (P<0.05) and 48 h (P<0.01). (C) TRPC6 immunoreactivity (x200). Staining was present within the cell membrane. (D) Quantification of the levels of TRPC6 (n=3). Values are the means ± SEM. *P<0.05; **P<0.01. NPD1, neuroprotectin D1; TRPC6, transient receptor potential canonical (subtype) 6.

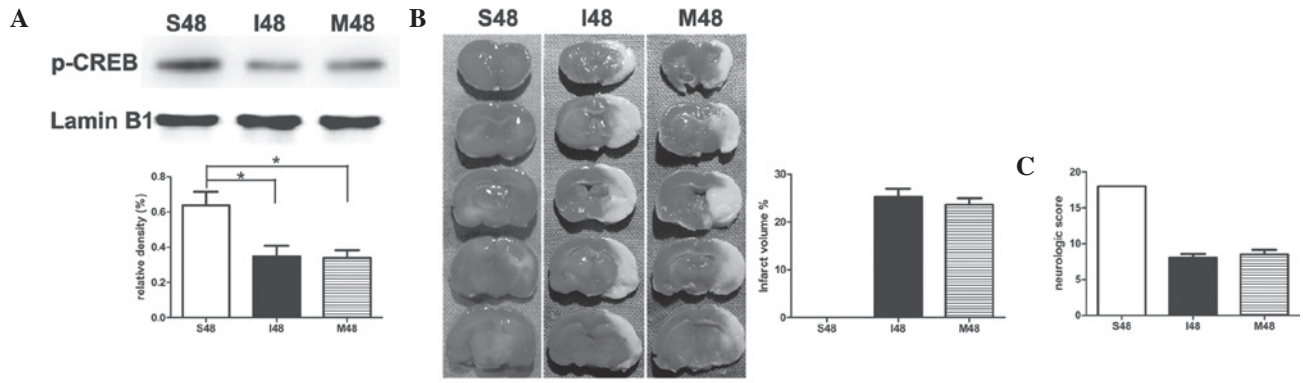

Figure 4. Effect of PD98059 on the stroke rats at 48 h after reperfusion. (A) Western blot analysis of p-CREB expression (n=3). Nuclear protein extracts were prepared from the ipsilateral cortex. Quantification of p-CREB assessed by western blot analysis was normalized to the expression level of Lamin B1. Application of PD98059, no statistical significance was noted in the protein levels of p-CREB in MCAO rats. (B) Representative TTC staining of the cerebral infarct in the rat brain (n=6). (C) Quantification of neurological scores (n=6). There was also no significant difference between group I and group M by measuring the infarct volumes and neurological scores. Values are the means ± SEM. *P<0.05 vs. sham surgery. p-CREB, phosphorylated cAMP-response element binding protein; MCAO, middle cerebral artery occlusion; TTC, 2,3,5-triphenyltetrazolium chloride.

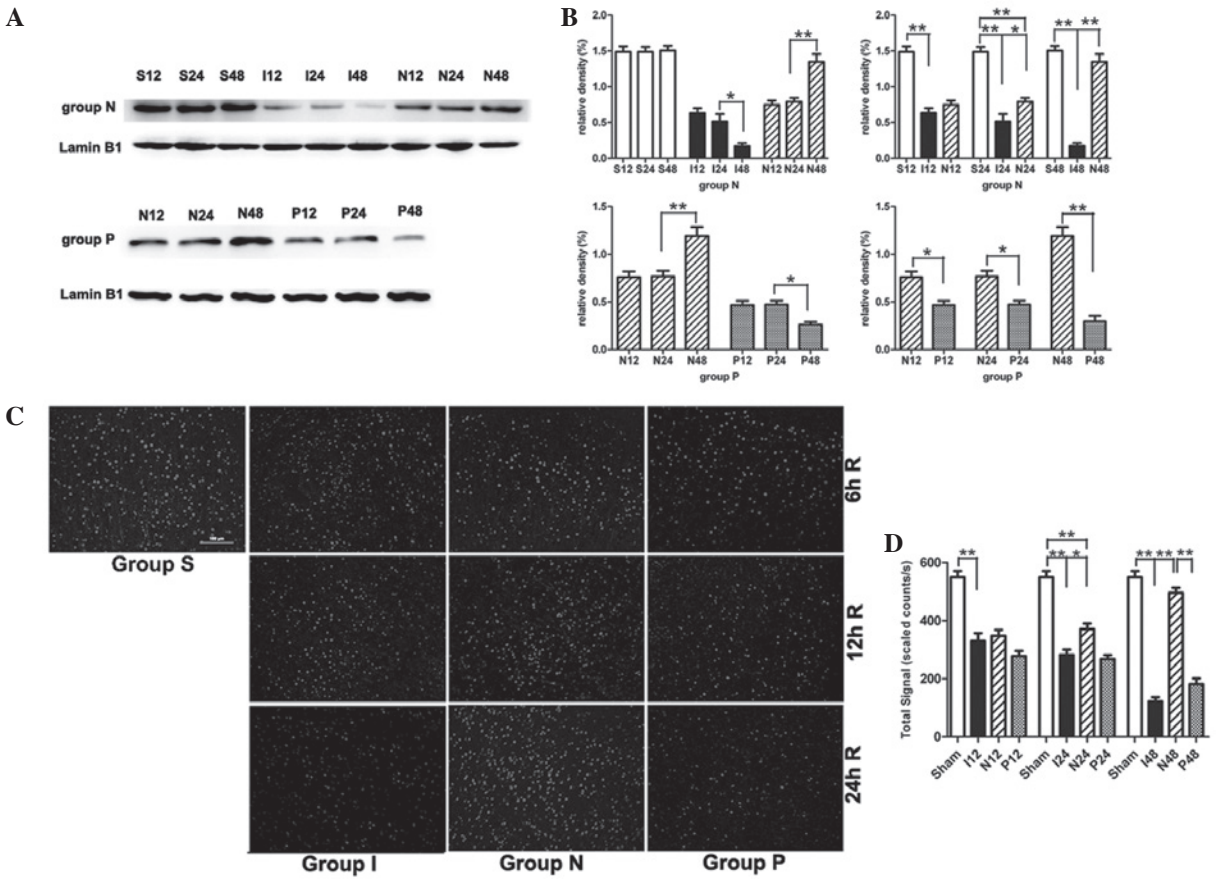

Figure 5. Effect of NPD1 administered at 2 h after reperfusion on the levels of p-CREB at 12, 24 and 48 h after reperfusion. (A) Western blot analysis of p-CREB expression. Nuclear protein extracts were prepared from the ipsilateral cortex. Identical amounts of homogenate protein (60 μg) were applied to each lane of the SDS-PAGE gel (10% polyacrylamide gel). Lamin B1 was used as an internal reference. A 43-kDa band corresponding to p-CREB protein was clearly detected. (B) Densitometric analysis of the protein levels of p-CREB (n=3). Application of NPD1 significantly increased the protein levels of p-CREB in MCAO rats at 24 h (P<0.05) and 48 h (P<0.01). Following administration with PD98059 20 min prior to the surgery, the p-CREB level was significantly decreased at 12 h (P<0.05), 24 h (P<0.05) and 48 h (P<0.01) compared with the NPD1-treated group. (C) P-CREB immunoreactivity (x200). Staining was present within cell nucleus. (D) Quantification of the levels of p-CREB (n=3). Values are the means ± SEM. *P<0.05; **P<0.01. NPD1, neuroprotectin D1; p-CREB, phosphorylated Ca^{2+}-response element binding protein; MCAO, middle cerebral artery occlusion.

There are several mechanisms, including excitotoxicity, ionic imbalance, peri-infarct depolarizations, oxidative stresses and apoptosis (1,2,5) that have been implicated in ischemic neuronal death. Ca^{2+} overload remains the most critical mechanism. The NMDA receptor is an important excitatory neurotransmitter receptor in the brain, which has been

reported as the pivotal player for Ca^{2+} overload in response to cerebral ischemia. A large number of *in vitro* and *in vivo* studies have suggested that NMDA receptor antagonists are effective in ischemic neuronal death. Pharmacological agents that block glutamate release or glutamate-mediated postsynaptic excitability may reduce neural degeneration in stroke rats (18,19). However, clinical trials examining the treatment of stroke using NMDA antagonists have all failed and have caused severe side effects (20).

Calpains are intracellular calcium-dependent cysteine endopeptidases, which are activated by NMDARs-mediated cytosolic Ca^{2+} overload (8,21). Under physiological conditions, calpain activity is likely to be stimulated by transient localized increases in cytosolic Ca^{2+} and tightly regulated by the presence of an endogenous inhibitor calpastatin. By contrast, the increase in cytosolic Ca^{2+} during cerebral ischemia overwhelms endogenous regulatory systems resulting in pathological calpain activity (6,22). Calpain inhibitors provide varying degrees of neuroprotection in animal models (22,23). aII-Spectrin, the most well-studied target of calpain and caspase, is an abundant cytoskeletal protein that is specifically cleaved by calpain into 150/145-kDa, and is also specifically cleaved by caspase-3 into 150/120-kDa fragments. These characteristics make aII-spectrin cleavage a useful tool to evaluate the activity of calpains and caspase-3 (24,25). In our study, brain samples from sham surgery rats presented very little SBDP145, whereas MCAO rats had elevated levels of SBDP145 in the cortical regions of the ipsilateral hemisphere in the first 48 h post-injury. NPD1 treatment significantly reduced SBDP145 formation and made it recover to basal levels at 24 h. However, NPD1 treatment had no effect on the formation of caspase-3-specific aII-spectrin breakdown products of 120kDa (SBDP120) in cerebral ischemia. Therefore, our results strongly demonstrate that NPD1, when applied at 2 h after reperfusion, specifically inhibited calpain (not caspase) activation, which induced resistance to ischemia and reperfusion injuries.

The transient receptor potential (TRP) channel was first identified in *Drosophila melanogaster* (26) and is a subfamily of the nonselective cation channels permeable to Ca^{2+}. TRPC6 channels are present in numerous cell types, including neurons (27,28). TRPC6 protein in neurons in ischemia was specifically downregulated by calpain proteolysis (8). Channels formed by the TRP family of proteins have a variety of biological functions. For example, TRPC3 and TRPC6 are involved in brain-derived neurotrophic factor (BDNF)-mediated growth cone turning, neuron survival and spine formation (9,29). TRPC6 also promoted dendritic growth via the CaMKIV-CREB-dependent pathway (30). A previous study provided evidence that TRPC6 was specifically degraded in transient ischemia and this degradation occurred prior to and during neuronal cell death, and that increases in its protein level or activity prevented neuronal death. Therefore, the conventional conception about treatment of ischemic brain damage with NMDA receptor antagonists may have to be renovated. However, inhibition of calpain proteolysis of TRPC6 may protect animals from ischemic brain damage (8). TRPC6 channels play a critical role in promoting neuronal survival against focal cerebral ischemia and calpain-mediated downregulation of TRPC6 contributes to ischemic brain injury (8). In our study, the levels of TRPC6 proteins in the MCAO group were greatly decreased at 12 h after reperfusion and the reduction in TRPC6 protein levels remained prominent at 24 and 48 h, in support of the observations of Du *et al* (8). NPD1 treatment significantly enhanced the protein levels of TRPC6 at 24 and 48 h. In addition, NPD1 significantly reduced infarct volumes and enhanced functional recovery at 48 h. Therefore, our results indicated that inhibition of calpain proteolysis of TRPC6 by NPD1 protects rats from ischemic brain damage.

In cortical neurons, entry of Ca^{2+} results in calcium-dependent activation of ERK, which in turn activates CREB transcriptional pathways to support neuronal survival (9,30,34). Phosphorylation of serine-133 in CREB allows it to contact its co-activator, CREB-binding protein/p300, and is necessary for its activation. The CREB activation is a critical event in neuroprotection against ischemic injury (35,36). Overexpressing TRPC6 markedly increased CREB phosphorylation and CREB-dependent transcription (9). Blocking TRPC6 degradation maintained phosphorylation of CREB and greatly prevented ischemic brain damage. In our study, the protein levels of p-CREB significantly increased in the NPD1-treated group at 24 h and recovered to the level of the sham surgery group at 48 h. When MEK activity was specifically inhibited by PD98059, the neuroprotective effect of NPD1 was attenuated and correlated with decreased CREB levels. These results clearly demonstrated that the activation of CREB through the MEK pathway is a pivotal downstream effector for the neuronal protective effect of TRPC6. Taken together, these results suggested that NPD1 blocked calpain-mediated TRPC6 channel degradation and stimulates the Ras/MEK/ERK pathway that converges on CREB activation, and contributed to neuroprotection.

Unlike the intravenous and intraperitoneal routes, the ICV route of administration is a useful experimental method to study the effects of chemicals or cellular grafts in the ventricular compartment of the brain following focal ischemia (37,38). In the present study, it is noteworthy that ICV injection of NPD1 at 2 h after reperfusion very rapidly attenuated ischemic cerebral injury within 12 h of reperfusion. This rapid effect may be a result of the ICV route. The doses of NPD1 (500 ng) administered into the lateral ventricle were very low, consistent with one previous study (12), suggesting that ICV injection of NPD1 is a cost-effective and highly efficient method in cerebral ischemia.

In conclusion, our results suggest that ICV injection of NPD1 at very low doses (500 ng) significantly reduces calpain-mediated TRPC6 channel degradation, and stimulates the Ras/MEK/ERK pathway that converges on CREB activation and rapidly attenuates ischemic cerebral injury during the acute period of ischemic stroke. Therefore, ICV administration of NPD1 following cerebral ischemia as a neuroprotective treatment may confer clear advantages and provides theoretical support for the use of NPD1 in ischemic stroke management during the acute or subacute period.

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