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Nicotinamide attenuates the injury-induced decrease of hippocalcin in ischemic brain injury

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HIGHLIGHTS

- Nicotinamide protects brain tissues against cerebral ischemic injury.
- Nicotinamide prevents brain injury-induced decrease of hippocalcin.
- Nicotinamide prevents glutamate exposure-induced decrease of hippocalcin in HT22 cells.

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ABSTRACT

Nicotinamide is an important cofactor in the prevention of brain damage during focal cerebral ischemia. Hippocalcin is a calcium buffer protein that modulates intracellular calcium concentration and attenuates apoptosis. In this study, we investigated whether nicotinamide modulates hippocalcin expression during cerebral ischemia. Male Sprague–Dawley rats were treated with vehicle or nicotinamide (500 mg/kg) 2 h after the onset of middle cerebral artery occlusion (MCAO) and cerebral cortex tissues were collected 24 h after MCAO. Nicotinamide treatment decreased infarct volume in the cerebral cortex of MCAO-operated animals. Our proteomic approach revealed a decrease in hippocalcin expression in vehicle-treated animals during MCAO, which was attenuated by nicotinamide treatment. We used RT-PCR and Western blot analyses to demonstrate that nicotinamide clearly restored the injury-induced decrease in hippocalcin expression. Glutamate toxicity also decreased hippocalcin levels in cultured hippocampal cells, while nicotinamide treatment prevented the glutamate exposure-induced decrease in hippocalcin levels. These results suggest that nicotinamide modulates hippocalcin expression in cerebral ischemic injury and consequently contributes to the prevention of neuronal cell death.

Nicotinamide, the amide form of niacin (vitamin B3), is an essential precursor of nicotinamide adenine dinucleotide (NAD), which is required for cellular function and energy metabolism (16). Nicotinamide acts as a broadly neuroprotective agent against various stimuli including oxidative stress, free radicals, and cerebral ischemia [5–7,10,26,27]. Cerebral ischemia leads to an excessive increase in intracellular calcium and results in an excitotoxic effect [8,9,25]. An increase in the intracellular calcium level leads to the production of free radicals, which in turn damage DNA, ultimately causing neuronal cell death [3,15,25]. Thus, the homeostasis of intracellular calcium is very important for normal neuronal physiological performance [1,9].

Hippocalcin belongs to a family of neuronal calcium-sensor proteins (NCS) that are dominantly expressed in the hippocampus [23]. Hippocalcin interacts with the neuronal apoptosis inhibitor protein (NAIP) and protects neurons against calcium-induced cell death [17,20]. Hippocalcin also counteracts caspase-12 activity and controls neuronal viability. Thus, hippocalcin is considered to contribute to neuroprotective functions by regulating intracellular Ca^{2+}. A previous study demonstrated that nicotinamide attenuates glutamate-induced calcium overload [24]. Although previous studies found that nicotinamide has a neuroprotective effect, the neuroprotective mechanism of nicotinamide has not been fully elucidated. Here we investigated the influence of nicotinamide on hippocalcin expression in a focal cerebral ischemia animal model.

Sprague–Dawley rats (220–230 g, $n = 40$, male) were purchased from Samtako Co. (Animal Breeding Center, Korea). Animals were randomly divided four groups as follows: vehicle + sham group, nicotinamide + sham group, vehicle + middle cerebral artery occlusion (MCAO) group, and nicotinamide + MCAO group ($n = 10$ per

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group). All animal experiments followed a protocol approved by the Institutional Animal Care and Use Committee (IACUC) at the Gyeongsang National University. Animals were maintained on 12 h light/dark cycle with free access to food and water. Nicotinamide (Sigma, St. Louis, MO, USA) was dissolved in normal saline for the vehicle. A single dose of nicotinamide (500 mg/kg) or vehicle was given via the intraperitoneal injection at 2 h after MCAO [27].

MCAO was carried out as previously described method [18]. Before the surgical operation began, animals were anesthetized with sodium pentobarbital (100 mg/kg, i.p.). Then through a midline incision, the right common carotid artery, internal carotid artery, and external carotid artery were exposed. A 4/0 nylon monofilament with its tip rounded by heat was inserted into the external carotid artery and advanced through the internal carotid artery until it reached the origin of the middle cerebral artery and thus blocking blood flow to the cortex. The sham-operated animals underwent the same surgical process, except for arterial blockade. The brain tissues were collected at 24 h after the onset of MCAO. Brains were rapidly cut into coronal sections (2 mm thick) by a brain matrix. The sections were stained with a solution of 2% 2,3,5-triphenyltetrazolium chloride (TTC; Sigma) at 37 °C for 20 min, and the cross-sectional areas in each brain slice were measured with Image-ProPlus 4.0 software (Media Cybernetics, Silver Spring, MD, USA). The ischemic lesion percentage of each slice was calculated by the ratio of the infarction area to the whole slice area.

For the proteomic study, the right cerebral cortices were homogenized in lysis buffer (8 M urea, 4% CHAPS, ampholytes, and 40 mM Tris–HCl) followed by centrifugation at 16,000 × *g* for 20 min at 4 °C. Bradford method (Bio-Rad, Hercules, CA, USA) was used to determine total protein concentration. For the separation of protein, two-dimensional (2D) gel electrophoresis was used. Isoelectric focusing (IEF) was carried out through immobilized pH gradients (IPG) gel strips (pH 4–7 and pH 6–9, 17 cm, Bio-Rad), and the sample buffer (8 M urea, 2% CHAPS, 20 mM DTT, 0.5% IPG buffer, and bromophenol blue) was used for rehydration for 13 h. The protein samples (100 μg) were loaded on IPG strips, and IEF was carried out following a multi-step protocol: 250 V (15 min), 10,000 V (3 h), and then 10,000–50,000 V using Protean IEF Cell (Bio-Rad). After equilibration of the IEF strips, the second dimension was performed using gradient gels (7.5–17.5%) and the strips were applied to gradient gels for SDS gel electrophoresis. The gels were loaded on Protein-II XI electrophoresis equipment (Bio-Rad) at 5 mA for 2 h and followed by 10 mA for 10 h at 10 °C. After gel electrophoresis, the gels were fixed in solution of 12% acetic acid and 50% methanol for 2 h and subsequently washed with 50% ethanol for 20 min. The gels were washed with deionized water and stained with a silver solution (0.2% silver nitrate, 0.75 mL/L formaldehyde) for 20 min, and washed with deionized water. The gel was developed in a solution of 0.2% sodium carbonate, 0.5 mL/L formaldehyde and the reaction was stopped using 1% acetic acid. The gel images were scanned with Agfar ARCUS 1200^{TM} (Agfar-Gevaert, Mortsel, BEL). The PDQuest 2-D analysis software (Bio-Rad) was used for the analysis of the protein spots. The gel pieces containing the desired protein spots were manually cut out from the gels and processed for MALDI-TOF. The gel particles were digested in trypsin-containing buffer, and the extracted. Peptides were analyzed in a Voyager-DE^{TM} STR biospectrometry workstation (Applied Biosystem, Forster City, CA, USA) for MALDI-TOF mass spectrometry. Proteins were identified using the search programs MS-Fit and ProFound program, and the databases SWISS-PROT and NCBI were used to identify protein sequence.

The total RNA of the right cerebral cortices was isolated with Trizol Reagent (Life Technologies, Rockville, MD, USA). Total RNA (1 μg) from each sample was reverse-transcribed into complementary DNA with superscript ||| first-strand system using RT-PCR (Invitrogen, Carlsbad, CA, USA) based on the manufacturer's protocol. The following primers were used: hippocalcin primer (207 bp), forward primer: 5′-ACGCCAACTTCTTCCCCTATG-3′, reverse primer; 5′-AGCCATCAGCGTCTTTGTTT-3′; actin primer (238 bp), forward primer: 5′-GGGTCAGAAGGACTCCTACG-3′, reverse primer; 5′-TTTCACTGCGGCTGATGTAG-3′. The PCR was carried out as follows: 5 min at 94 °C, 30 s at 94 °C, 30 s at 54 °C, 1 min at 72 °C, and 10 min at 72 °C. The samples were amplified for 30 cycles. PCR products were loaded on a 1% agarose gel was visualized under UV light.

Western blot analysis was performed as previously described method [12]. Total protein (30 μg per sample) of right cerebral cortices were applied to each lane on 10% SDS–polyacrylamide gels. After electrophoresis and immunoblotting, the poly-vinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA) were blocked with skim milk for 1 h to block non-specific reaction. PVDF (Millipore, Billerica, MA, USA) membranes were washed in tris-buffered saline containing 0.1% Tween-20 (TBST) for 10 min. The membranes were incubated with anti-actin (diluted 1:1000, Sigma) and anti-hippocalcin (diluted 1:1000, Abcam, Cambridge, UK) as primary antibodies at 4 °C for 15 h. After the membranes were washed with TBST, the membranes were incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (1:5000, Pierce, Rockford, IL, USA) and the ECL Western blot analysis system (Amersham Pharmacia Biotech, Piscataway, NJ, USA) was used for detection according to the manufacturer's protocol.

Mouse hippocampal HT22 cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Hyclone Laboratory, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin (Hyclone Laboratory) as a previously described general method [11,19]. The cells were seeded onto 60-mm tissue culture dishes at a density of 100,000 cells per dish and grown overnight prior to initiating the drug treatments. Glutamate (Sigma) was treated with a final concentration of 5 mM and cells were incubated for 24 h. Nicotinamide (0.1 and 1 mM) was added 30 min before the addition of glutamate. Cell viability was determined through the measurement of cellular metabolism of 3-(4,5-dimethyldiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). MTT solution (5 mg/mL) was added to each well of 96-well plate and cells were allowed to be maintained for 4 h at 37 °C. The solubilization solution (20% sodium dodecyl sulfate and 50% dimethylformamide) was added and absorbance was measured at 570 nm. Cell viability was expressed as a percentage of neuroprotection vs. vehicle set at 100%. The cultured cells were fixed in 4% paraformaldehyde and washed three times with PBS, blocked with 5% normal goat serum (Jackson Immuno Research Labs, West Grove, PA, USA) for 1 h. The cells were incubated overnight with anti-hippocalcin antibody at 4 °C (diluted 1:100, Abcam) and followed by incubation with a fluorescein isothiocyanate (FITC)-conjugated secondary antibody (1:200, Jackson Immuno Research Labs). Fluorescein images were examined using a fluorescence microscope (AXIO, Carl Zeiss Corporation, Thornwood, NY, USA).

The intensity analysis was carried out using SigmaGel 1.0 (Jandel Scientific, San Rafael, CA, USA) and SigmaPlot 4.0 (SPSS Inc., Point Richmond, CA, USA). All data are expressed as mean ± S.E.M. The results in each group were compared by one-way analysis of variance (ANOVA) followed by Student's *t*-test. A value of $P < 0.05$ was considered statistically significant.

We confirmed the neuroprotective effect of nicotinamide against cerebral ischemic injury using TTC staining (Fig. 1A and B). Nicotinamide treatment reduced infarct volume in MCAO-operated animals compared to vehicle + MCAO animals. The infarct regions were 35.15 ± 3.45% and 13.25 ± 1.75% in the vehicle + MCAO and nicotinamide + MCAO animals, respectively (Fig. 1B). Using a proteomics approach, hippocalcin protein spots were detected as differentially expressed proteins in the cerebral cortices of vehicle- and nicotinamide-treated animals during MCAO. The hippocalcin protein spot was decreased in vehicle + MCAO animals, whereas the decrease in hippocalcin was attenuated in nicotinamide + MCAO