NAP, a Femtomolar-Acting Peptide, Protects the Brain Against Ischemic Injury by Reducing Apoptotic Death

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Background and Purpose—We sought to determine the cerebroprotective potential of NAP, a synthetic octapeptide related to vasoactive intestinal peptide. Activity-dependent neuroprotective protein mediates some of the protective effects of vasoactive intestinal peptide. The neuroprotective NAP sequence is derived from activity-dependent neuroprotective protein.

Methods—Spontaneously hypertensive rats underwent permanent middle cerebral artery occlusion by craniotomy and electrocoagulation. After dose-response and time-course experiments, the animals were injected with NAP (3 μg/kg) or vehicle intravenously 1 hour after stroke onset. Another group of rats was injected with the d-amino acid isomer of NAP (D-NAP) and served as a negative control. Rats were examined for motor and behavioral deficits 24 hours to 30 days later, and infarct volumes were determined. The effect of NAP administration on apoptotic death was determined by terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) and caspase-3 stainings.

Results—NAP significantly reduced motor disability and infarct volumes compared with vehicle or D-NAP when tested at 24 hours after stroke onset (9.67 ± 1.4% versus 17.04 ± 1.18% and 19.19 ± 1.9% of hemispheric volume, respectively; P < 0.05). NAP given 4 but not 6 hours after permanent middle cerebral artery occlusion still conferred significant neuroprotection (infarct volume 10.9 ± 3.9% of hemispheric volume; P < 0.05 versus vehicle). Long-term studies demonstrated that infarct volumes and disability scores remained significantly lower after 30 days in NAP-treated animals. NAP significantly reduced the number of apoptotic cells.

Conclusions—Our results indicate that the durable cerebroprotection by NAP involves antiapoptotic mechanisms. (Stroke. 2002;33:1085-1092.)

Key Words: animal models ▪ cerebral ischemia ▪ neuropeptides ▪ neuroprotection ▪ vasoactive intestinal peptide ▪ rats

NAP, an 8-amino acid peptide (NAPVSIPQ = Asn-Ala-Pro-Val-Ser-Ile-Pro-Gln) derived from activity-dependent neuroprotective protein (ADNP), was recently discovered.1–4 The finding of NAP originated from studies on the peptidergic neurotransmitter vasoactive intestinal peptide (VIP).5,6 VIP binds to glial cells and activates a myriad of secondary effects including enhanced synthesis of neuroprotective proteins, such as the NAP-containing ADNP.1

NAP (microgram per kilogram doses) exhibited neuroprotection in vivo.1,2,7,8 In apolipoprotein E–deficient mice, daily subcutaneous NAP injections for the first 2 weeks of life accelerated the acquisition of developmental reflexes and prevented cholinergic deficits and short-term memory loss.1 In a rat cholinotoxicity model, daily intranasal NAP application produced significant improvements in short-term spatial memory.2 In a mouse paradigm of traumatic head injury, a single NAP injection dramatically reduced edema formation and mortality and facilitated recovery.3 In a model of fetal alcohol syndrome, fetal death was inhibited by NAP treatment.4

In vitro, NAP (femtomolar concentrations) protected neurons against toxicity associated with oxidative stress,3,9 glucose deprivation,10 the β-amyloid peptide, N-methyl-D-aspartate, the envelope protein of HIV (gp120), and electric blockade.1 The activities of NAP have been partly associated with cGMP formation, the control of nitric oxide production,11 and immunomodulation (tumor necrosis factor-α),7 signaling pathways that are key to cellular survival.
Taken together, the multifaceted neuroprotective activities attributed to NAP offer potential efficacy in conditions associated with multifactorial neuronal damage, such as stroke.

Materials and Methods

Peptide Synthesis

NAP was custom synthesized by SynPep or Peninsula. The integrity of the peptide was ascertained by high-performance liquid chromatography (HPLC) (C-18, 5 μm, 250×4.6 mm) (Merck), by N-CHO capillary electrophoresis (Beckman Coulter Inc), and by MALDI (matrix-assisted laser desorption/sonization) mass spectroscopy analysis, indicating 90% to 98% purity. Biological activity was determined by cell culture experiments.1,9,10

Animals

Thirteen-week-old male spontaneously hypertensive rats (SHR) were obtained from the Tel-Aviv University animal facility. The use of SHR allows for highly reproducible brain damage in the model for ischemia used below. Rats were housed in the animal care facility in compliance with institutional guidelines and were given free access to food and water until the night before surgery and at all times after recovery form anesthesia. Animals were weighed before the surgery and daily thereafter until the time of euthanasia.

Surgical Procedure

Permanent middle cerebral artery occlusion (PMCAO) by craniotomy and electrocoagulation was used as before.12,13 After anesthesia with intraperitoneal pentobarbital (60 mg/kg), the left femoral artery was cannulated for invasive measurement of blood pressure (Biopac MP 100-TSD104A, Biopac Systems). Temperature, heart rate, and oxygen saturation were monitored throughout the experiment. Rats were not maintained under any specific conditions. For the first 24 hours and before drug administration with a laser-Doppler probe (LDF TSD144, Biopac Systems) in selected animals. Blood flow in the area surrounding the infarct was monitored before, during, and after PMCAO for up to 2 hours after drug administration with a laser-Doppler probe (LDF TSD144, Biopac Systems) in selected animals. The rats were placed in a tiltable stereotaxic head holder under a surgical microscope. The middle cerebral artery was exposed to its junction with the inferior cerebral vein, where it was occluded and cut by electrocoagulation as it was carefully traced off the brain surface.

Drug Administration and Evaluations

All motor, behavioral, and histological assessments were performed blindly by one of the authors.

The first set of experiments included a pilot dose-response trial. NAP was injected to the tail vein (intravenously) at concentrations of 0.3, 3, 30, or 150 μg/kg (n=5 per dose) 1 hour after PMCAO. Vehicle-treated rats (n=5) served as one control group, and another group of animals (n=3) was injected with the D-amino acid isomer of NAP (D-NAP). In a confirmatory study, rats were given NAP at doses of 1, 3, or 10 μg/kg, and infarct volumes and motor deficits were compared with those seen in vehicle-treated animals. The second experiment included a time course of NAP 3 μg/kg administered at 1, 4, or 6 hours after PMCAO (n=5 per time point). On the basis of the results of these 2 preliminary studies, we used a concentration of 3 μg/kg given 1 hour after PMCAO for all subsequent experiments. For the short-term evaluation of infarct volume, we used 10 NAP-injected animals and 10 vehicle-treated rats. For long-term experiments, we used 14 vehicle-injected rats as a control group and 14 NAP-treated rats as the treatment group.

Motor Evaluation

Rats were examined at different time points after PMCAO with the use of a motor disability scale.12,13 Animals were scored 1 point for each of the following parameters: ability to perform forelimb extension while momentarily suspended by the tail, hind limb flexion when pulled from the table, and rotation to the paretic side against resistance. Additionally, 1 point was scored for circling motion to the paretic side when attempting to walk, 2 points for failure to walk out of a circle 30 cm in diameter within 20 seconds, and 3 points for inability to walk. Thus, an animal with a maximal deficit scored 6 points, and an animal with no deficit scored 0 points.

Behavioral Tests

To allow for maximal possible recovery of sensory and motor deficits, before testing the animals were evaluated with a modified adhesive removal test 25 to 30 days after surgery.14,15 In this test, a small, round, colored sticker is applied to the paretic forepaw, and the time required to discard the sticker is measured. This test evaluates the animal’s ability to sense the sticker, visually detect it, and discard it with the use of its normal forepaw.

Infarct Size

Brains were cut into 2-mm-thick slices and stained with 2,3,5-triphenyltetrazolium chloride (TTC 2% solution in PBS) for 8 hours and preserved in 3.7% formaldehyde. Slices were photographed online with an image acquisition system (Lis 700, Aplitec). Image analyzing software (Sigmascan- Pro, SPSS) was used for the estimation of the infarct volume. Infarct areas were measured in 7 consecutive slices starting from the frontal pole. The results are expressed as a percentage of the noninfarcted hemisphere at 24 hours after PMCAO (short term): infarct volume = [(left hemisphere–(right hemisphere–right infarct))/left hemisphere]×100. When measured 30 days after PMCAO, infarct volumes were determined by subtracting the volume of the infarcted hemisphere from that of the normal hemisphere and dividing the outcome by the volume of the normal hemisphere: infarct volume = [(left hemisphere–right hemisphere)/left hemisphere]×100. The infarct size obtained with this method contains both the actual infarct and tissue changes secondary to atrophy and therefore may overestimate the infarct volume.

Central Nervous System Permeability

NAP, propyl 3-3,4-[3H] (American Radiolabeled Chemicals Inc), 2.5 Ci/mmol was injected into SHR (n=6) 1 hour after PMCAO (4×1010 disintegrations per minute [dpm/kg]). Fifteen, 30, and 60 minutes after injection, rats were perfused and killed, and tissues were solubilized (100 mg/1 mL Luma Solve, Lumac). Radioactivity was determined after the addition of Optiflour (10 mL/100 mg, Packard) in a beta scintillation counter. Radioactive NAP accumulation was also assessed in untreated rats as above. Thirty minutes after intravenous injection of 10×1010 dpm/kg, brains were removed, the cortical hemispheres were homogenized, and postmitochondrial supernatant was subjected to HPLC (RP18, 12.5 mm/4 mm, Merck), with a linear gradient of acetonitrile 3.75% to 60% in 0.1% trifluoroacetic acid.2 Eluted fractions were assessed for radioactivity in Optiflour as above.

Detection of Apoptosis

Animals were killed at 24, 48, or 72 hours after PMCAO. Slices 6 μm thick were grouped into sets of 3: the first set was stained with hematoxylin and eosin (H&E); the second set was stained with terminal deoxynucleotidyl transferase–mediated dUTP nick 3′-end labeling (TUNEL) (Neurolutes, R&D Laboratories), and biotinylated nucleotides were detected with a streptavidin-conjugated horseradish peroxidase and diaminobenzidine; and the third set of slides was stained with a monoclonal caspase-3 antibody (Santa Cruz Biotechnology clone sc-7148). For caspase immunohistochemistry, brains were fixed in 4% formaldehyde, and positive cells were detected with the use of fluorescein isothiocyanate–conjugated goat antimouse IgG (Jackson) and fluorescent microscopy (Olympus, model BH-2). Control slides were stained only with the primary or the secondary antibody to ensure staining specificity.

Infarct diameters were determined on the H&E slices, and 2 slides from areas bregma –0.2 to bregma +0.2 were evaluated per animal. TUNEL- and caspase–positive cells were systematically enumerated in each rat, on consecutive slides, with the use of infarct...
coordinates obtained from the H&E staining in zones representing the infarct core and penumbra. The core zone was at the center of the cortical border of each slide and the penumbral zones at the upper and lower edges of the cortical boundary zone between normal and infarcted brain (See Figure 4A for details). The relative percent reduction in the number of positive cells in the NAP-treated rats was calculated by the following formula: 
\[
\frac{1 - \text{(number of positive cells in NAP-treated rats/number of positive cells in vehicle-treated rats)}}{100}.
\]

Statistical Evaluation
Evaluation was performed with the Sigma Stat package (SPSS). Student’s t test and the Mann-Whitney test were performed for comparison of groups and scores. One-way ANOVA with Newman-Keuls or Dunnert’s correction and ANOVA on repeated measures were used to compare multiple groups and day-to-day intragroup changes, respectively.

Results
NAP Reduces Motor Disability and Infarct Volumes After PMCAO in a Dose- and Time-Dependent Manner
Body temperature, blood pressure (mean±SD arterial pressure=127±4.5 mm Hg), heart rate (270.5±3.3 bpm), and oxygen saturation (98.1±0.55%) were stable throughout the surgical procedure and afterward and did not differ between the various study groups. Measurements were performed at baseline and 30 and 60 minutes after surgery. Cerebral blood flow in the peri-infarct area dropped to 22.1±1.2% and 23.3±2.5% of baseline values in NAP- and vehicle-treated rats, respectively, after PMCAO and did not change after drug administration (24.2±3.1% and 23.4±2.2% of baseline

Figure 1. A, Motor disability scores observed with different doses of NAP. Rats were injected with different doses of NAP (0.3, 3, 30, and 150 μg/kg; n=5 per dose) or vehicle intravenously 1 hour after PMCAO. Motor disability scores were determined 24 hours after PMCAO. B, Infarct volumes observed with different doses of NAP. The same experiment as in panel A was conducted. Infarct volumes were determined 24 hours after PMCAO after staining brain slices with TTC. C, Motor disability scores observed at different times of administration of NAP. Rats were injected with NAP (3 μg/kg; n=5 per point) or vehicle (n=5) at different time points after PMCAO. Motor disability was assessed 24 hours after PMCAO. D, Infarct volumes observed with different times of administration of NAP. The same experiment described in panel C was conducted. Infarct volumes were determined 24 hours after PMCAO.
Figure 2. A, Motor disability scores in NAP- and vehicle-treated animals at different time points after PMCAO. Rats were injected with NAP (3 μg/kg; n=14) or vehicle (n=14) 1 hour after PMCAO. Motor disability scores were determined on different days after PMCAO. Results were compared with those observed in vehicle-treated rats. B, Adhesive-removal test in NAP- and vehicle-treated rats at different time points after PMCAO. Experiments were performed as described in Materials and Methods. Note that animals treated with NAP (n=14) performed better in this test in the first 3 days of its application, but the performance equaled that of vehicle-treated rats (n=14) thereafter. C, Long-term assessments of infarct volumes in NAP- and vehicle-treated rats. Rats were injected with NAP (3 μg/kg) or vehicle 1 hour after PMCAO. Infarct volumes were determined 24 hours (n=10 per experimental group) and 30 days after PMCAO (n=14 per experimental group.)

* - P < 0.05

* - P < 0.05 vs. Vehicle
values in the NAP and vehicle groups, respectively). Animal weights did not differ between the groups at any time point before or after the stroke, as monitored up to 30 days after PMCAO (290±19 g).

In animals injected with 3 μg/kg NAP, motor functions measured 24 hours after PMCAO were significantly improved compared with vehicle-treated animals (motor disability score 0.7±0.2 versus 1.9±0.3, respectively; P<0.05; Figure 1A). At the same time point, an approximately 50% reduction in infarct size was observed in NAP-treated animals (Figure 1B) compared with rats treated with vehicle or D-NAP (infarct size 9.67±1.4% versus 17.04±1.18% and 19.19±1.9% of hemispheric volume, respectively; P<0.05). A dose-response curve indicated that animals treated with either 30 or 150 μg/kg of NAP also had smaller infarcts than those observed in vehicle- or D-NAP–treated rats, but the infarct size was not reduced any further than that observed in rats treated with 3 μg/kg. The results of the confirmatory dose-response study showed that 3 μg/kg is indeed the optimal dose since infarct volumes and motor disabilities were significantly reduced in animals treated with either 3 or 10 μg/kg but not in animals treated with 1 μg/kg compared with vehicle (motor scores 1.7±0.5, 2±0, and 2.33±0.6 versus 3.6±1.2, respectively, and infarct volumes 9.5±1.4%, 9.97±3.97%, and 20.4±1.22% versus 18.8±4.2% of hemispheric volume, respectively).

A time-course NAP administration experiment showed that motor activity was improved (Figure 1C) and infarct volumes (Figure 1D) were significantly smaller in rats injected up to 4 hours after PMCAO (10.9±3.9% of hemispheric volume; P<0.05 versus vehicle). The infarct volumes were also slightly reduced in animals treated 6 hours after PMCAO (14.6±0.15% of hemispheric volume; P>0.05), but this reduction failed to reach statistical significance (Figure 1D).

On the basis of the dose-response curve and the time-course results, further experiments were performed with 3 μg/kg NAP injected 1 hour after PMCAO.

**Long-Term Effects of NAP Treatment**

Motor disability scores were significantly lower in NAP-treated rats than those obtained in vehicle-injected rats throughout the 30 experimental days (Figure 2A). In both groups there was a slight improvement in motor functions in the first 5 days after PMCAO, which was followed by an insignificant motor deterioration. After 10 days, motor disability reached a plateau in both groups.

On the adhesive removal test, 26 days after PMCAO, a significantly shorter latency to removal of the tape was evident in the NAP-treated animals on the first through the third days of testing. At later time points all tested animals improved (Figure 2B), and on days 29 and 30 after PMCAO, there were no differences in the performance of animals in both groups.

Lesion volumes remained smaller in NAP-treated rats than those observed in vehicle-treated animals 30 days after the stroke (8.05±2.6% versus 20.64±2.23% of hemispheric volume; P=0.0007; Figure 2C). When compared with the lesion volumes observed in animals killed 24 hours after PMCAO, the lesions at 30 days showed an insignificant reduction in size in animals injected with NAP but increased slightly in the vehicle-treated animals, which is explained by the fact that the lesion-size measurements at day 30 also included secondary postischemic atrophy. However, the differences in lesion volumes between days 1 and 30 after PMCAO did not reach statistical significance in either group (P=0.8 in the NAP group and P=0.12 in the vehicle group).

**Brain Penetration**

NAP-associated radioactivity was detected in the brain as early as 15 minutes after injection following PMCAO and was maintained for at least 1 hour in the ischemic tissue, suggesting tissue accumulation (Figure 3). To further assess brain distribution, radioactive NAP was injected (intravenously) in male rats, and radioactivity was measured also in the hippocampus and midbrain, indicating homogeneous distribution 30 minutes after application. HPLC analysis suggested that at least 17% of the radioactive material detected in the cortex, 30 minutes after injection, was intact NAP. To assess dose-related NAP penetration, we injected (intravenously) escalating doses of radioactive NAP (5×10⁴ to 2×10⁵ dpm/g), and results showed an almost linear increase in NAP penetration in the cortex and cerebellum, although to a somewhat lesser degree than the increased input (2- to 3-fold output versus 4-fold input).

**Effects on Apoptosis**

TUNEL- or caspase-3–positive cells were present mainly in the core and penumbral zones and were essentially not detectable, with the sensitivity used here, in the contralateral
hemisphere or in areas of the ipsilateral hemisphere that were not directly involved by the infarct.

At 24 hours after surgery, the percentages of TUNEL-positive cells in the core and penumbra of NAP-treated animals represented at least half of vehicle-treated values (Figure 4B and Figure 5C, 5D, and 5F). This NAP protective effect was attenuated at later time points after PMCAO but remained significant. In all animals, the number of TUNEL-positive cells increased between 24 and 72 hours after PMCAO. Similar to the TUNEL counts, the number of
caspase-3–positive cells was significantly lower in NAP-treated rats than in vehicle-injected rats (Figure 4C and Figure 5A, 5B, and 5E). The overall reduction in apoptotic cells in NAP-treated animals, detected by both methods, ranged from approximately 50% to 65% and persisted throughout all time points examined, in both the core and penumbral zones.

Discussion

The present study demonstrated that a single postinjury (up to 4 hours) injection of NAP had durable protective effects (infarct size, sensory and motor reflexes) in a model of focal irreversible cerebral ischemia. NAP appeared to produce cerebroprotection by reducing apoptosis.

Previous experiments detected intact NAP in the mature rat brain2 and in the embryonic mouse nervous system8 30 to 60 minutes after intranasal or intraperitoneal administration, respectively. In comparison to other peptides, these results imply increased stability.2,8 In this study intravenously injected NAP reached the brain, eliminating the problem of drug availability, which limits the effectiveness of many neuroprotective drugs. It is hypothesized that exogenous NAP may provide a trigger for a protective cascade that otherwise cannot be fully activated under ischemic conditions.

The protective effect of NAP reached a maximal value at 3 μg/kg, corroborating previous experiments that have shown NAP to be active at femtomolar concentration.1 The specificity of NAP actions is further emphasized by the failure of its D-isomer to produce neuroprotection, signifying stereospecificity and inferring receptor-mediated mechanisms. Higher doses of NAP also produced a reduction in infarct size, but this was not accompanied by a similar reduction in motor disability. More studies will be needed to corroborate and explain these results, which could be related to a somewhat limited NAP access to the brain and/or the mechanistic aspects of the action of NAP and/or the relatively low sensitivity of the motor scale used.1,10,11

Of note, while motor abilities in vehicle-treated rats failed to reach the NAP-associated improvements, behavioral deficits, assessed in the adhesive removal test, attained the same levels in control and NAP-treated animals after a few testing days. This could be explained by several mechanisms, including a more rapid recovery rate for sensory impairments, activation of secondary sensory centers in the brain, and a lesser degree of involvement of the cortical areas examined with the adhesive removal test (parieto-occipital) in the stroke model used.

The present experiments have shown that NAP does not alter systemic blood pressure, cerebral blood flow temperature, or other physiological parameters, excluding these pathways as possible protective mechanisms.

The durability of the salutary effects shown by NAP, as well as its putative trophic capabilities, alludes to a possible antiapoptotic effect exerted by this compound. Indeed, we were able to demonstrate a significant reduction in the number of apoptotic cells in rats treated with NAP. The TUNEL method detects DNA breakdown that occurs in apoptosis. However, such changes may also occur in cells undergoing necrotic death.16,17 To increase sensitivity and specificity for apoptosis detection, TUNEL detection was accompanied by immunohistochemical staining with a monoclonal antibody to caspase-3. Because the antibody used in the present experiment detects caspase-3 in general and is not specific for the activated form, it could be claimed that its presence is not indicative of an active apoptotic process. However, caspase-3–positive cells were detected essentially only in the infarct tissue and not in the contralateral hemisphere or in noninfarcted areas of the ipsilateral hemisphere or in sham-operated animals (R.R. Leker, MD, et al, unpublished data, 2001). In the staining scheme used, the infarct area was systematically divided into zones of equal dimensions, which were individually set for each rat independently of infarct volume, with predetermined areas of core and penumbra. This method reduces the possibility that the smaller number of apoptotic cells detected in the NAP-treated animals results from an overall decrease in the infarct volume and favors a primary antiapoptotic effect for NAP. This effect could be mediated through modulation of cGMP and nitric oxide concentrations11 that have been implicated in caspase-associated apoptosis.18,19

The exact step in the apoptotic cascade influenced by NAP remains unknown. However, the reduction in the number of caspase-3–positive cells suggests an upstream site of involve-
ment. Furthermore, since NAP has other potential anti-ischemic effects, it may reduce major apoptotic triggers. Such potential targets include inhibition of N-methyl-D-aspartate toxicity, reduction of inflammatory cytokine production, and reduction in oxidative stress.

In conclusion, our findings suggest that the synthetic octapeptide NAP is cerebroprotective in a model of focal irreversible ischemia, partially by reducing the number of cells dying from apoptosis. Further experiments in different models of cerebral ischemia will be needed to determine the potential of this molecule in future therapeutic trials in humans.

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