Neuronostatin acts in brain to biphasically increase mean arterial pressure through sympatho-activation followed by vasopressin secretion: the role of melanocortin receptors

Gina L. C. Yosten, Alicia T. Pate, and Willis K. Samson

Department of Pharmacological and Physiological Science, Saint Louis University, Saint Louis, Missouri

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Yosten GLC, Pate AT, Samson WK. Neuronostatin acts in brain to biphasically increase mean arterial pressure through sympatho-activation followed by vasopressin secretion: the role of melanocortin receptors. Am J Physiol Regul Integr Comp Physiol 300: R1194–R1199, 2011. First published February 16, 2011; doi:10.1152/ajpregu.00849.2010.—Neuronostatin is a recently described neuropeptide that is derived from the somatostatin preprohormone. We have shown previously that neuronostatin led to a biphasic, dose-related increase in mean arterial pressure when injected into the lateral cerebroventricle of adult, male rats. Because neuronostatin depolarized both magnocellular and parvocellular, paraventricular nucleus neurons in hypothalamic slice preparations, we hypothesized that neuronostatin elevated mean arterial pressure first by stimulating sympathetic nervous system activity followed by the release of a pressor hormone, specifically vasopressin. We found that the first phase of neuronostatin-induced increase in mean arterial pressure was reversed by pretreatment with phentolamine, indicating that phase 1 was, indeed, due to an increase in sympathetic activity. We also found that centrally injected neuronostatin led to a dose-related increase in vasopressin secretion in a time course consistent with the peak of the second phase. Furthermore, the second phase of arterial pressure elevation was reversed by pretreatment with a vasopressin 1 receptor antagonist, indicating that phase 2 was likely due to an increase in vasopressin secretion. We previously have shown that the anorexigenic and antidipsogenic effects of neuronostatin were reversed by pretreatment with the melanocortin 3/4 receptor antagonist, SHU9119, so we evaluated the ability of SHU9119 to reverse the effects of neuronostatin on MAP and vasopressin secretion. We found that SHU9119 abrogated the second phase of neuronostatin-induced increase in MAP and neuronostatin-induced vasopressin secretion, indicating that neuronostatin acts through the central melanocortin system to increase vasopressin release, ultimately leading to an elevation in MAP.

blood pressure; vasopressin; melanocortins

Somatostatin is an important neuroendocrine peptide that was discovered in 1972 when its ability to inhibit growth hormone secretion from the anterior pituitary was first noted (23). Because somatostatin-14 and the N-terminally extended form of somatostatin, somatostatin-28, comprised only a small portion of the prehormone, it was speculated for many years that the somatostatin preprohormone coded for the sequence of an additional, biologically active peptide (7, 21). On the basis of bioinformatic analysis of conserved sequences and potential enzymatic cleavage sites, we predicted the existence of a peptide in the C-terminal region of the somatostatin preprohormone (20). This potential peptide sequence was highly conserved between human, rodent, and even goldfish, and on the basis of dibasic residues (potential cleavage sites), it had a predicted size of either 6, 11, 13, or 19 amino acids. The 19-amino acid form of the predicted peptide was synthesized, and an antibody was generated that was used to purify the endogenous peptide from porcine pancreas and spleen. Mass spectrometry and amino acid analyses revealed the native, mature form of the peptide, neuronostatin, to be 13 amino acids in length.

Although neuronostatin and somatostatin are produced from the same preprohormone, the peptides differ in structure and in biological activity. For instance, even though neuronostatin is produced in the same tissues as somatostatin, the peptides undergo differential posttranslational processing (neuronostatin is C-terminally amidated, while somatostatin is cyclized by a disulfide bond), and neuronostatin failed to activate any of the five endogenous somatostatin receptors (20). Unlike somatostatin, neuronostatin did not alter either basal or growth hormone-releasing hormone or ghrelin-stimulated growth hormone release from cultured anterior pituitary cells (20).

When injected into the lateral cerebroventricle, neuronostatin induced a potent, dose-related decrease in both food and water intake in adult, male rats. We recently have shown that the anorexigenic and antidipsogenic effects of neuronostatin are dependent upon central melanocortin receptors, as neuronostatin-induced anorexia and adipsia were reversed by pretreatment with the melanocortin 3/4 receptor antagonist, SHU9119 (26). Centrally administered neuronostatin also led to a biphasic, dose-related increase in mean arterial pressure (MAP), with the initial phase corresponding to the first 10 min (phase 1) and the second phase corresponding to minutes 11 through 45 postinjection (phase 2) (20). Because neuronostatin directly depolarized both magnocellular and parvocellular neurons in hypothalamic slice preparations of the paraventricular nucleus (PVN), we hypothesized that the biphasic increase in MAP induced by neuronostatin was due to an increase in sympathetic activity during phase 1, followed by the release of a pressor hormone, specifically AVP, during phase 2.

Materials and Methods

All procedures and protocols were approved by the Saint Louis University Animal Use and Care Committee. Adult, male rats were housed under controlled conditions (lights on 0600–1800; 23–25°C) with free access to food and water. Animals were anesthetized with a mixture of ketamine (60 mg/ml; Ketaset, Fort Dodge Animal Health, Fort Dodge, IA) and xylazine (8 mg/ml; TranquiVed, VedCo, Saint Joseph, MO) at a dose of 0.1 ml per 100 g body wt, as previously described (25). A stainless-steel cannula (23 gauge, 17 mm) was implanted into the right lateral cerebroventricle using a stereotaxic device. Rats were observed for at least 4 days following surgery to

Address for reprint requests and other correspondence: G. L. C. Yosten, Dept. of Pharmacological and Physiological Science, Saint Louis Univ., Saint Louis, MO 63104, USA (e-mail: gyosten@slu.edu).

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ensure recovery to presurgery weight. Placement and patency of the cannula was confirmed by the dipsoogenic effect of ANG II (50 pmol).

For cardiovascular experiments, an additional cannula [polyethyl-
ene (PE)-50] was implanted into the left carotid artery, as previously described (25). The cannula was filled with heparinized saline (200 U/ml in 0.9% NaCl) and exteriorized between the shoulder blades. The following day, rats were habituated to a quiet room for at least 2 h, and then the carotid cannula was connected to a Digi-Med blood pressure analyzer (BPA) 400 (Micro-Med, Louisville, KY). The BPA-400 collects blood pressure and heart rate data and transmits the information to the Digi-Med System Integrator (DMSI). The PC-based DMSI software integrates the data and calculates MAP, as well as pulse pressure at user-determined intervals. Baseline MAP and heart rate (HR) were recorded for at least 30 min at 1-min intervals.

Four protocols were implemented. To test the activity of different forms of neuronostatin, rats were treated intracerebroventriculatly with vehicle or vehicle containing 300 pmol of neuronostatin-13, neuronostatin-19, scrambled neuronostatin (neuronostatin-scr), or nonamidated neuronostatin (NA-neuronostatin). Neuronostatin-scr was synthesized by reversing the amino acid order of neuronostatin-13. MAP and HR were recorded for at least 45 min at 1-min intervals. To test the hypothesis that phase 1 is due to increased sympathetic activity, rats were pretreated with the α-adrenergic antagonist, phentolamine (10 mg/kg), or saline vehicle, delivered intra-arterially (25).

After a 15-min stabilization period, rats were injected intracerebroventricularly with either saline vehicle or vehicle containing 300 pmol of neuronostatin. MAP and HR were recorded at 1-min intervals for at least 45 min. To determine whether phase 2 is due to an increase in vasopressin secretion, rats bearing intracerebroventricular and carotid cannulas were implanted with an additional cannula in the right jugular vein, as previously described (9). Rats were pretreated with saline or a vasopressin receptor antagonist (β-mercaptopo-β cyclopentamethylenepropionyl1, OEt-Tyr2, Val4, Arg8-vasopressin) (8) administered via the jugular catheter. The dose utilized for these studies (20 μg/kg) was determined in preliminary experiments to block a pressor dose of intra-arterially injected AVP. Animals were allowed to stabilize, and then were injected with either saline vehicle or vehicle containing 300 pmol icv neuronostatin or 20 ng/kg ia vasopressin, and MAP and HR were recorded for 45 min at 1-min intervals. To evaluate the involvement of the central melanocortin system in the hypertensive actions of neuronostatin, rats were pretreated with either saline or the melanocortin 3/4 receptor antagonist, SHU9119 (300 pmol icv) (25), and then treated with either saline or 300 pmol icv neuronostatin. MAP and HR were recorded for 45 min. All MAP and HR data are represented as a change from preinjection baseline (average of the 5 min before treatment).

For the determination of plasma vasopressin levels, animals bearing intracerebroventricular cannulas were habituated to a quiet room for at least 1 h. Two protocols were implemented. In the first, rats were injected with either saline vehicle or vehicle containing 100 or 300 pmol icv neuronostatin. Rats then were killed by rapid decapitation at either 0, 5, 15, 30, or 45 min postinjection, and trunk blood was collected into heparinized tubes. In the second protocol, rats were pretreated intracerebroventricularly with either saline vehicle or 300 pmol SHU9119. Ten minutes later, rats were treated intracerebroventricularly with either saline vehicle or 300 pmol neuronostatin-13. Thirty minutes later, rats were decapitated, and trunk blood was collected into heparinized tubes. All trunk bloods were extracted using C-18 HyperSep columns (Thermo Scientific, Rockville, MD). Vasopressin levels were determined by radioimmunoassy, as previously described (19). Because several assays were required to make all plasma measurements, a common serum pool was used for extraction and recovery control in each assay, and these unknowns, as well as the standard peptide added as a control, were used to calculate extraction efficiency (greater than 85% in all cases) and interassay coefficients of variability (in total less than 7.0%).

Phentolamine was purchased from Sigma Aldrich (St. Louis, MO). All peptides were purchased from Phoenix Pharmaceuticals (Burlingame, CA). Doses of neuronostatin (20), phentolamine (25), and SHU9119 (25) were determined from previous experiments. For all experiments, the vehicle used was sterile saline (0.9% NaCl).

Blood pressure data were analyzed using a nonparametric test (Mann Whitney U). Differences between vasopressin levels were analyzed using a t-test when assessing two groups or ANOVA with Scheffe’s multiple comparison when comparing multiple groups. A nonparametric statistic was used for MAP experiments because MAP data were transformed to reflect change from preinjection baseline to account for natural differences in resting MAP between animals (28).

RESULTS

In our previous experiments, we determined that neuronostatin-13 led to a biphasic increase in MAP, with 300 pmol neuronostatin-13 yielding the greatest change from baseline pressures (20). We characterized the two phases of neuronostatin-13-induced increase in MAP as phase 1 (corresponding to the first 10 min following injection) and phase 2 (corresponding to minutes 11–45) (Fig. 1, inset). To determine the specificity of neuronostatin-13 to exert this hypertensive activity, we also tested the ability of three other forms of neuronostatin to alter MAP. Animals were administered saline vehicle, 300 pmol neuronostatin-13, full-length neuronostatin (neuronostatin-19, 300 pmol) (20), scrambled neuronostatin-13 (300 pmol), or 300 pmol nonamidated neuronostatin-13. Both neu-
ronostatin-13 and neuronostatin-19 led to biphasic, significant increases in MAP (Fig. 1). However, scrambled neuronostatin and nonamidated neuronostatin-13 failed to significantly alter MAP. Neuronostatin-13, scrambled neuronostatin-13, and nonamidated neuronostatin-13 all failed to induce any significant changes in HR (Supplemental Fig. S1). Neuronostatin-19,
however, significantly increased HR during phase 2 compared with saline-injected controls.

To test the hypothesis that the first rise (phase 1) in mean arterial pressure (MAP) induced by neuronostatin was due to an increase in sympathetic activity, rats were pretreated intravenously with the alpha adrenergic antagonist, phentolamine. Central administration of neuronostatin-13 (without pretreatment) led to an increase in MAP during both phase 1 and phase 2, while rats pretreated with phentolamine responded to neuronostatin during phase 2, but not during phase 1. Data were analyzed using a Mann-Whitney U-test (*P < 0.05, **P < 0.01. Comparisons were made as follows: saline/saline vs. saline/neuronostatin; phentolamine/saline vs. phentolamine/neuronostatin).

Fig. 2. Phase 1, but not phase 2, of neuronostatin-induced increase in MAP is reversed by pretreatment with phentolamine. Rats were pretreated intravenously with either saline vehicle or 10 mg/kg phentolamine. After a 15-min stabilization period, rats were injected with saline vehicle or vehicle containing 300 pmol icv neuronostatin. Rats pretreated with saline prior to neuronostatin administration exhibited elevations in MAP during both phase 1 and phase 2, while rats pretreated with phentolamine responded to neuronostatin during phase 2, but not during phase 1. Data were analyzed using a Mann-Whitney U-test (*P < 0.05, **P < 0.01. Comparisons were made as follows: saline/saline vs. saline/neuronostatin; phentolamine/saline vs. phentolamine/neuronostatin).

To ascertain the involvement of AVP in the second rise (phase 2) of MAP induced by neuronostatin-13, we began by determining plasma levels of AVP after central administration of neuronostatin-13. Rats that were treated intracerebroventricularly with 300 pmol neuronostatin-13 exhibited a rise in MAP during phase 2 (minutes 11–25) in phentolamine-pretreated rats. No significant trends in HR change were observed (Supplemental Fig. S2).

To ascertain the involvement of AVP in the second rise (phase 2) of MAP induced by neuronostatin-13, we began by determining plasma levels of AVP after central administration of neuronostatin-13. Rats that were treated intracerebroventricularly with 300 pmol neuronostatin-13 exhibited a significant elevation in plasma AVP levels at 30 min postinjection (Fig. 3), which is consistent with the peak of phase 2 of neuronostatin-induced increase in MAP observed in previous experiments (20). The effect of neuronostatin-13 on plasma AVP levels appeared to be dose-related when compared at 30 min.

To further test the hypothesis that phase 2 is due to an increase in AVP secretion, we tested the ability of a vasopressin 1 receptor (V1) antagonist to reverse the increase in MAP during phase 2. We first had to determine a suitable dose of the V1 antagonist to ensure that it could block the hypertensive effect of a pressor dose of AVP. Therefore, we pretreated animals intravenously with either saline vehicle or 20 ng/kg V1 antagonist. Five minutes later, animals were administered either saline vehicle or 20 ng/kg ia AVP. Animals that were pretreated with saline prior to AVP exhibited a significant elevation in MAP that resolved within 5 min (Fig. 4A). However, no significant alterations in MAP were observed in animals that were pretreated with V1 antagonist before administration of AVP.

Having determined an effective dose of V1 antagonist, we pretreated rats with either saline vehicle or V1 antagonist intravenously before treatment with either saline or 300 pmol icv neuronostatin. Pretreatment with V1 antagonist did not significantly alter baseline MAP (Supplemental Table S1). Animals that received saline before injection of neuronostatin-13 demonstrated an elevation in MAP during both phase 1 and phase 2, as expected. However, rats that were treated with V1 antagonist before intracerebroventricular administration of neuronostatin-13 exhibited a rise in MAP in phase 1, but not during phase 2 (Fig. 4B). No significant trends in change in HR were observed (Supplemental Fig. S3).

We previously have shown that the anorexigenic and antipsychois effects of neuronostatin were reversed by pretreatment with the melanocortin 3/4 receptor antagonist, SHU9119 (26), indicating that those effects of the peptide were dependent upon the central melanocortin system. Because central melanocortin receptors are known to be involved in cardiovascular regulation in addition to appetite control, we pretreated animals intracerebroventricularly with either saline vehicle or vehicle containing 300 pmol SHU9119 (25) before intracerebroventricular administration of either saline or 300 pmol neuronostatin. Rats that received saline vehicle before injection...
with neuronostatin exhibited an increase in MAP during both phase 1 and phase 2 (Fig. 5). Pretreatment with SHU9119 did not significantly alter baseline MAP (Supplemental Table S1). Animals that were pretreated with SHU9119 prior to treatment with neuronostatin exhibited an elevation in MAP during phase 1, but not during phase 2. Data were analyzed using a Mann-Whitney U-test (comparisons were made as follows: saline/saline vs. saline/neuronostatin; SHU9119/saline vs. SHU9119/neuronostatin; *P < 0.05).

FIG. 5. Melanocortin receptor antagonism reverses phase 2 of neuronostatin-induced increase in MAP. To determine whether central melanocortin system was involved in mediating the cardiovascular effects of neuronostatin, we pretreated animals intracerebroventricularly with the melanocortin 3/4 receptor antagonist, SHU9119 (300 pmol) prior to intracerebroventricular injection of either saline or 300 pmol neuronostatin. Animals that received SHU9119 before neuronostatin exhibited a significant elevation in MAP during phase 1, but not during phase 2. Data were analyzed using a Mann-Whitney U-test (comparisons were made as follows: saline/saline vs. saline/neuronostatin; SHU9119/saline vs. SHU9119/neuronostatin; *P < 0.05).

Table 1. Melanocortin antagonism reverses the effect of neuronostatin on plasma vasopressin levels

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>Saline Control</th>
<th>300 pmole Neuronostatin-13</th>
<th>300 pmole SHU9119</th>
<th>Neuronostatin-13 plus SHU9119</th>
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<tr>
<td>Saline Control</td>
<td>31†</td>
<td>1.78 ± 0.31</td>
<td>5.85 ± 0.73*</td>
<td>1.59 ± 0.39</td>
<td>2.18 ± 0.75</td>
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<tr>
<td>SHU9119</td>
<td>13</td>
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<tr>
<td>Neuronostatin-13</td>
<td>7</td>
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Values are expressed as means ± SE. Plasma AVP levels are given as picograms AVP per milliliter of plasma. *P < 0.001 vs. saline control, ANOVA, Scheffe’s multiple comparisons. †Data are the same as those are presented in Fig. 3.

DISCUSSION

Neuronostatin is produced in the hypothalamus and, when exogenously applied, it depolarized both magnocellular (projecting to posterior pituitary) and parvocellular (projecting to brain stem autonomic centers) neurons in hypothalamic slice preparations of the PVN (20). Neuronostatin also led to a biphasic increase in MAP when injected intracerebroventricularly into adult male rats (20). Because of the depolarizing effect of neuronostatin on at least two populations of neurons in the PVN, we hypothesized that neuronostatin exerted its effects by activating the central melanocortin system.
effect on MAP by two separate mechanisms. We found that phase 1 of the neuronostatin-induced increase in MAP was reversed by pretreatment with phenolamine and that phase 2 was abrogated by pretreatment with a vasopressin 1 receptor antagonist. We also found that centrally injected neuronostatin led to a dose-related increase in plasma vasopressin levels in a time course consistent with the second phase of neuronostatin-induced hypertension. Together, these data indicate that neuronostatin can increase MAP both by increasing sympathetic nervous system activity and by initiating vasopressin release from the posterior pituitary.

It is interesting to note that while neuronostatin induced AVP secretion, this effect did not attain significance until 30 min after central injection of the peptide. The observed delay is likely due to a combination of factors. First of all, neuronostatin may act in different brain sites to exert its effects on the sympathetic nervous system and vasopressin secretion, and the neurons responsible for mediating the stimulation of vasopressin release may be at a more distant location from the site of injection. Secondly, because of the rapid clearance of AVP (11), it may take several minutes for enough AVP to accumulate in blood to overcome those mechanisms and reach plasma levels capable of inducing a pressor response (14). Neuronostatin-induced sympathoactivation also likely affects the release of AVP from the posterior pituitary, since an increase in arterial pressure can inhibit AVP secretion (17). The latter explanation is more plausible, since vasopressin can be rapidly released in response to orthostatic changes (12). However, in our experiments, an initial increase, not a decrease as in hypotensive events, was observed, and thus, the baroreflex activation of AVP secretion would not have occurred.

We also show that the hypertensive effect of neuronostatin during phase 2 was reversed by pretreatment with the melanocortin 3/4 receptor antagonist, SHU9119. This result was unexpected, since it has been well documented that melanocortin agonists increase sympathetic nervous system activity (2, 4, 10, 15), and the hypertensive effect during phase 1 was abrogated by phentolamine pretreatment. However, to our knowledge, only one study thus far has documented that melanocortin agonists can stimulate vasopressin release (3). Our data confirm that a direct or indirect connection between the central melanocortin system and magnocellular vasopressin neurons exists, since SHU9119 reversed the effect of neuronostatin not only on MAP during phase 2, but also on plasma vasopressin levels. Several reports indicate that proopiomelanocortin (POMC) neurons modulate oxytocin neuron activity (1, 18); therefore, it is conceivable that POMC neurons synapse on not only oxytocin neurons, but vasopressin neurons as well.

We reported that, according to bioinformatic analysis, neuronostatin could potentially exist as either a 6-, 11-, 13-, or 19-amino acid peptide and that the endogenous form of the peptide is likely 13 amino acids (20). Because the long form of the peptide (neuronostatin-19) produced an effect on MAP that was similar to the effect neuronostatin-13, the N-terminus of the peptide is probably not critical for ligand recognition of the receptor. Although the receptor is currently not known, activation of the receptor likely requires the peptide to be posttranslationally modified to be C-terminally amidated, since treatment with a nonamidated form of neuronostatin-13 did not elevate MAP in our animals. It was also clear that the effect of neuronostatin on MAP was not a nonspecific effect, since administration of the scrambled neuronostatin-13 peptide sequence did not significantly alter MAP.

**Perspectives and Significance**

This work provides evidence for an interaction, either direct or indirect, between POMC neurons and magnocellular vasopressin neurons. This interaction is supported by at least one study, in which melanocortin agonists led to an increase in vasopressin secretion in hypothalamic explants (3). In sharp contrast to the relative scarcity of data connecting POMC and vasopressin neurons, a large body of literature confirms the interaction of POMC and oxytocin neurons (1, 13, 18, 22, 27), specifically placing oxytocin as a potential downstream mediator of melanocortin signaling. For example, the anorexigenic effect of a melanocortin agonist was reversed by pretreatment with the oxytocin receptor antagonist, OVT (27). However, the interaction between POMC and oxytocin neurons does not appear to be ubiquitous. We have shown previously that the hypertensive and anorexigenic effects of another neuropeptide, nesfatin-1, are reversed by intracerebroventricular pretreatment with either SHU9119 or OVT, suggesting that nesfatin-1 acts through the central melanocortin system to activate caudally projecting oxytocin fibers, eventually leading to an increase in MAP and a decrease in food intake (27). Neuronostatin appears also to depend upon the central melanocortin system to exert its activities (26). However, neuronostatin does not require the presence of central oxytocin receptors to inhibit food intake (26) but instead appears to activate vasopressin neurons. How can two peptides that are both dependent upon the central melanocortin system exert their effects through separate downstream effectors? We propose that these two neuropeptides, nesfatin-1 and neuronostatin, activate functionally distinct subpopulations of POMC neurons with differing synaptic partners. Indeed, several lines of evidence suggest that POMC neurons are not a homogenous population of cells, but instead are a heterogeneous mixture of several POMC subtypes that differ on the basis of receptor and neuropeptide expression profiles and anatomic distribution within the arcuate nucleus (5, 6, 16, 24).

In addition to identifying the exact site of action of neuronostatin in the brain, future studies must address the issue of the potential physiological relevance of endogenous neuronostatin in terms of central control of cardiovascular function.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the authors.

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