Neuronostatin inhibits cardiac contractile function via a protein kinase A- and JNK-dependent mechanism in murine hearts

Yinan Hua,1 Heng Ma,1 Willis K. Samson,2 and Jun Ren1

1Division of Pharmaceutical Sciences & Center for Cardiovascular Research and Alternative Medicine, University of Wyoming, Laramie, Wyoming; and 2Pharmacological and Physiological Science, Saint Louis University School of Medicine, St. Louis, Missouri

Submitted 5 April 2009; accepted in final form 16 June 2009

Hua Y, Ma H, Samson WK, Ren J. Neuronostatin inhibits cardiac contractile function via a protein kinase A- and JNK-dependent mechanism in murine hearts. Am J Physiol Regul Integr Comp Physiol 297: R682–R689, 2009. First published June 24, 2009; doi:10.1152/ajpregu.00196.2009.—Neuronostatin, a newly identified peptide hormone sharing the same precursor with somatostatin, exerts multiple pharmacological effects in gastrointestinal tract, hypothalamus, and cerebellum. However, the cardiovascular effect of neuronostatin is unknown. The aim of this study was to elucidate the impact of neuronostatin on cardiac contractile function in murine hearts and isolated cardiomyocytes. Short-term exposure of neuronostatin depressed left ventricular developed pressure (LVDP), maximal velocity of shortening/relengthening (dL/dtM), and heart rate in Langendorff heart preparation. Consistently, neuronostatin inhibited peak shortening (PS) and maximal velocity of shortening/relengthening (dL/dtM) without affecting time-to-PS (TPS) and time-to-90% relengthening (TR90) in cardiomyocytes. The neuronostatin-elicited cardiomyocyte mechanical responses were mimicked by somatostatin, the other posttranslational product of preprosomatostatin. Furthermore, the neuronostatin-induced cardiomyocyte mechanical effects were ablated by the PKA inhibitor H89 (1 μM) and the Jun N-terminal kinase (JNK) inhibitor SP600125 (20 μM). The PKC inhibitor chelerythrine (1 μM) failed to alter neuronostatin-induced cardiomyocyte mechanical responses. To the contrary, chelerythrine, but not H89, abrogated somatostatin-induced cardiomyocyte contractile responses. Our results also showed enhanced c-fos and c-jun expression in response to neuronostatin exposure (0.5 to 2 h). Taken together, our data suggest that neuronostatin is a peptide hormone with overt cardiac depressant action. The neuronostatin-elicited cardiac contractile response appears to be mediated, at least in part, through a PKA- and/or JNK-dependent mechanism.

SOMATOSTATIN [ALSO NAMED SOMATOTROPIN] release-inhibiting factor or growth hormone inhibiting hormone], is a peptide hormone first found in the hypothalamus. It is also expressed in neuroendocrine organs, gastrointestinal tract, thyroid and adrenal glands, liver, spleen, kidney, prostate, inflammatory, and immune cells (23). Somatostatin has been shown to be involved in the regulatory processes of endocrine system, neurotransmission, cell proliferation, hormone secretion, and cardiovascular function (17). This peptide hormone contains two active isoforms (with 14 amino acids and 28 amino acids) produced by alternative cleavage of a single preproprotein. These two isoforms of somatostatin are capable of activating five related G protein-coupled membrane receptors with different affinity (9, 31, 32). To date, the majority of research on somatostatin has focused on hormonal regulation in gastrointestinal and nervous systems. More recent evidence has suggested the presence of the somatostatin receptors in both human and H9c2 cardiac muscle cells (10, 30). Somatostatin was found to suppress atrial contractile function and blunt atrioventricular conduction (1). Recently, a novel peptide encoded by the somatostatin gene, neuronostatin was identified. Neuronostatin has been shown to regulate neuronal function, blood pressure, food intake, and drinking behavior (28). This novel peptide, like somatostatin, is a posttranslational product of processing of the preprosomatostatin prohormone, suggesting a similar role in the regulation of hormonal and cardiac function. The aim of the present study was to determine the effects of neuronostatin on myocardial and cardiomyocyte contractile function, and to explore the potential mechanisms of action involved in neuronostatin-elicited cardiac contractile response, if any.

MATERIALS AND METHODS

Isolation of murine cardiomyocytes. The experimental procedure used in this study was approved by the University of Wyoming Animal Use and Care Committee (Laramie, WY). Adult female friendly virus B mice (~22 g) were anesthetized with ketamine/xylazine (5:3, 1.32 mg/kg ip). Hearts were rapidly removed and perfused (at 37°C) with the Krebs-Henseleit bicarbonate (KHB) buffer (in mM: 118 NaCl, 4.7 KCl, 1.2 MgSO4, 1.2 KH2PO4, 25 NaHCO3, 10 HEPES, 11.1 glucose, pH 7.4). The hearts were then perfused for 15 min with KHB containing Liberase Blendzyme 4 (Hoffmann-La Roche, Indianapolis, IN). After perfusion, left ventricles were removed and minced. Dispersed cardiomyocytes were filtered through a nylon mesh (300 μm). Extracellular Ca2+ was added slowly and incrementally to 1.25 mM. A total of 12 mice were used for this study (5).

Cell shortening/relengthening. Mechanical properties of cardiomyocytes were assessed using an IonOptix soft-edge system (IonOptix, Milton, MA). Myocytes were placed in a chamber mounted on the stage of an Olympus IX-70 microscope and superfused (~2 ml/min at 25°C) with a KHB buffer containing 1 mM CaCl2. Myocytes were field stimulated at 0.5 Hz. Cell shortening and relengthening were assessed, including peak shortening (PS), time-to-PS (TPS), time-to-90% relengthening (TR90) and maximal velocities of shortening/relengthening (±dL/dt) (5).

Langendorff isolated perfused heart function. Isolated hearts were rapidly excised, washed in an ice-cold arresting solution (120 mM NaCl, 30 mM KCl), and cannulated via the aorta on a 20-gauge stainless-steel blunt needle. Hearts were perfused at 4 ml/min on a Langendorff apparatus using the KHB solution containing (in mM) 118 NaCl, 4.75 KCl, 1.2 KH2PO4, 1.2 MgSO4, 25 NaHCO3, 1.4 CaCl2 and 7 glucose with 95% O2-5% CO2 at 37°C. Platinum electrodes connected to a Grass Instrument stimulus generator were used to pace hearts. Each heart was subjected to 20 min of stabilization, followed by exposure of 0.3, 3, and 30 nM neuronostatin cumulatively. Washout was performed 20 min after the application of the peptide (27).

Western blot analysis. Cardiomyocytes were treated with 0.3 nM neuronostatin for 30, 60, 90, and 120 min followed by protein
extraction using a RIPA lysis buffer. Equal amounts (50 μg protein/lane) of lysates and prestained molecular weight markers were separated on 10% SDS-polyacrylamide gels and transferred onto nitrocellulose membranes. Membranes were incubated for 1 h in a blocking solution containing 5% nonfat milk in Tris-buffered saline (TBS), washed in TBS and incubated overnight at 4°C with the anti-c-jun (1:1,000, Cell Signaling Technology, Boston, MA) and anti-β-actin (1:1,000, Cell Signaling) antibodies. Following incubation with the primary antibodies, blots were incubated with an anti-rabbit IgG HRP-linked antibody (Cell Signaling) at a dilution of 1:2,500 for 1 h at room temperature. Immunoreactive bands were detected by enhanced chemiluminescence autoradiography in accordance with the manufacturer’s instruction. The intensity of the bands was quantitated with a scanning densitometer (Model GS-800, Bio-Rad) coupled with a Bio-Rad PC analysis software (5).

Assessment of mRNA expression by quantitative real-time PCR. cDNA was generated from cardiomyocytes treated with neuronostatin for 30, 60, 90, and 120 min prior to RNA extraction. Quantitative real-time reverse-transcription PCR (qRT-PCR) analysis was performed for c-fos and 18S (used as the housekeeping gene). The experiments were done in triplicate using a QuantiTect SYBR Green real-time PCR kits (Bio-Rad, Hercules, CA). The primer (Integrated DNA Technologies, Coralville, IA) sequences of c-fos were forward: 5′-TTC CTG GCA ATA GCG TGT TC-3′, reverse: 5′-TTC AGA CCA CCT CGA CAA TG-3′ (3), for 18S were, forward: 5′-GTA ACC GTG CCA CCT GCG TGT TC-3′, reverse: 5′-CCA CCT CGA TT-3′, reverse: 5′-CCA TTC AAT CGG TAG TAG CG-3′ (29).

Experimental protocol. Isolated cardiomyocytes were equilibrated for a minimum of 1 h prior to exposure of neuronostatin and somatostatin. Neuronostatin and somatostatin (0.3 pM to 30 nM) were added to the contractile medium at specified concentrations for 20 min before the determination of myocyte contractile function in electrically paced (0.5 Hz) cells. To elucidate whether PKA or PKC plays any role in neuronostatin-induced cardiac response, cells were pretreated with the PKA inhibitor H89 (1 μM) or the PKC inhibitor chelerythrine chloride (1 μM) for 40 min (6); neuronostatin (0.3 nM) or somatostatin (0.3 nM) was then applied for the final 20 min. To assess whether JNK signaling contributes to the neuronostatin-induced cardiac responses, cells were pretreated with the JNK inhibitor (SP600125, 20 μM) for 1 h (33), and neuronostatin (0.3 nM) was introduced for the final 20 min.

Statistical analysis. Data were presented as means ± SE. Statistical analysis was performed with ANOVA using a SigmaPlot statistical software (Jandel Scientific, San Rafael, CA). A P value less than 0.05 was considered to be statistically significant.

RESULTS

Effect of neuronostatin on cardiomyocyte mechanics. Following a 20-min exposure of various concentration (0.0003–30

![Fig. 1. Concentration-dependent effect of neuronostatin and somatostatin (0.0003–30 nM) on cardiomyocyte contractile function in murine cardiomyocytes. A: resting cell length. B: peak shortening (PS, normalized to resting cell length). C: maximal velocity of shortening (+dL/dt); D: maximal velocity of relengthening (−dL/dt); E: time-to-PS (TPS); F: time-to-90% relengthening (TR90). Values are presented as means ± SE, n = 56–66 cells/group; *P < 0.05 vs. control (0 concentration).](http://ajpregu.physiology.org/)
nM) of neuronostatin, the PS amplitude was significantly depressed at the concentration of 0.3 nM or higher. Similarly, ±dL/dt was inhibited by neuronostatin with a threshold between 0.03 nM and 0.3 nM. Neuronostatin exposure did not significantly affect the resting cell length, TPS, and TR90 with the exception of an increased TR90 at the highest peptide concentration (30 nM). For comparison, cardiomyocyte mechanical response of somatostatin, the neuronostatin analog, was also evaluated. Our data revealed a somewhat similar profile in somatostatin (0.0003–30 nM)-elicited cardiomyocyte mechanical responses. The PS amplitude was decreased in response to somatostatin exposure with a threshold between 0.0003 nM and 0.03 nM. ±dL/dt was inhibited by somatostatin, in a manner similar to neuronostatin. Myocyte resting cell length, TPS, and TR90 were not affected by somatostatin in the concentration range tested (Fig. 1).

**Effect of neuronostatin on myocardial contractile function.** Whole heart contractile function assessed by Langendorff perfusion revealed that the left ventricular developed pressure (LVDP), heart rate, and the first derivatives of LVDP (±dP/dt) were significantly suppressed following neuronostatin treatment (0.3–30 nM) (Fig. 2). The representative trace shown in Fig. 2A depicted that the cardiac depressant effect of neuronostatin was able to be partially washed out.

**Effect of the PKA and PKC inhibitors on neuronostatin- and somatostatin-induced cardiomyocyte responses.** It has been shown that somatostatin and its analogs may exert their effects through G protein (7). To examine the potential involvement of PKA and PKC in neuronostatin- and somatostatin-elicited cardiac response, isolated murine cardiomyocytes were pretreated with the PKA inhibitor H89 (1 µM) or the PKC inhibitor chelerythrine (1 µM) for 20 min before the exposure of neuronostatin (0.3 nM) and somatostatin (0.3 nM). Our results indicated H89 abolished neuronostatin-induced inhibitory effects on PS and ±dL/dt without eliciting any effect on cardiomyocyte mechanics itself. H89, by itself or in combination with neuronostatin, did not alter TPS and TR90. None of the neuronostatin-induced cardiomyocyte mechanical responses was affected by the PKC inhibitor chelerythrine. Chelerythrine itself did not affect cardiomyocyte mechanical properties by itself. There was no change in resting cell length in response to

---

Fig. 2. Concentration-dependent effect of neuronostatin (0.3–30 nM) on murine cardiac contractile function using a Langendorff isolated heart perfusion system. A: representative traces illustrating the effects of neuronostatin. B: left ventricular developing pressure (LVDP). C: heart rate. D: maximal velocity of pressure development (+dP/dt). E: maximal velocity of pressure decline (−dP/dt). Values are expressed as means ± SE; n = 3 hearts per group. *P < 0.05 vs. control.
either kinase inhibitor (Fig. 3). To the contrary, chelerythrine abrogated somatostatin-induced inhibitory effects on PS and ±dL/df. H89 failed to affect somatostatin-induced cardiomyocyte mechanical response (Fig. 4). These data suggested that involvement of distinct signaling pathways in neuronostatin- and somatostatin-elicited cardiomyocyte contractile response. The neuronostatin- and somatostatin-induced cardiac contractile responses are likely to be mediated by PKA and PKC pathways, respectively.

Effect of neuronostatin on expressions of early response genes. The early response genes are known to be triggered by certain hormonal or peptide stimulation (4, 12). To better understand the changes in the early response genes in neuronostatin-elicited cardiac contractile response, isolated murine cardiomyocytes were treated with neuronostatin (0.3 nM) for periods of 30 min to 2 h. Total protein was extracted to assess the expression of c-jun, while total RNA was extracted for the real-time assay of c-fos expression. As shown in Fig. 5, neuronostatin exposure elicited a time-dependent induction of c-jun protein and c-fos mRNA expression. The maximal levels of c-jun and c-fos were reached after 90 min of neuronostatin treatment.

Effect of JNK inhibition on neuronostatin-induced cardiomyocyte mechanical response. Given the upregulated c-jun transcriptional factor protein expression, the possible involvement of the c-jun upstream signaling molecule JNK in the neuronostatin-initiated cardiac contractile response was evaluated. Myocytes were pretreated with the JNK inhibitor SP600125 (20 μM) for 40 min before neuronostatin exposure for an additional 20 min. Data shown in Fig. 6 revealed that SP600125 abrogated neuronostatin-induced inhibition on PS and ±dL/df without eliciting any effect on TPS and TR90. SP600125 itself did not show any significant effect on cardiomyocyte mechanics.
**DISCUSSION**

The major finding from this study revealed that the newly identified peptide hormone neuronostatin depresses cardiac contractile function in both whole hearts and cardiomyocytes. In particular, neuronostatin inhibited the LVDP, the first derivatives of LVDP (±dP/dt), heart rate, peak cardiomyocyte shortening, and maximal velocity of cell shortening/relengthening. The neuronostatin-induced cardiac depression resembled that elicited by somatostatin. Furthermore, the neuronostatin-induced cardiac depression may be mediated, at least in part, through the PKA and JNK pathways rather than the PKC pathway. To the contrary, the PKC, but not PKA, pathway is involved in the somatostatin-induced cardiomyocyte contractile response, as reported previously (24, 25). These observations prompt the speculation that the new peptide hormone may exert significant regulatory action on myocardial contractile function.

Following the synthesis of prosomatostatin, the precursor of somatostatin, an enzymatic cleavage process occurs using one or more of the convertase enzymes at basic residues to yield somatostatin and neuronostatin peptides. Neuronostatin is not a cyclic polypeptide but is rather amidated, making it distinctly different from somatostatin (28). Despite the same origin, the regulatory process appears to vary between the two peptide hormones. In our current study, we were unable to identify any major difference in cardiomyocyte contractile response between neuronostatin and somatostatin, with the exception of PS response threshold and prolonged TR90 at the highest concentration of neuronostatin. Both neuronostatin and somatostatin suppress peak shortening and maximal velocities of shortening/relengthening. In an earlier study using rats, McDermott and colleagues (20) found a biphasic response of somatostatin, including a positive response at low doses and a negative contractile response at higher concentrations (20). These in-
In myocytes, we found upregulated role.

At this time, no precise mechanism may be offered to explain the apparent discrepancies between our current study and those reported previously (e.g., monophasic vs. biphasic response favoring an overall negative inotropic response on the hearts.

Physiological responses of somatostatin are mediated through somatostatin receptors coupled with G proteins (16, 34). G proteins activate ion channels and enzymes, such as adenylyl cyclase, phospholipase A2, phosphoinositide 3-kinase, phospholipase C, and guanylate cyclase. The activated ion channels and enzymes are responsible for the activation or degradation of intracellular second messengers, including cAMP, cGMP, inositol 1, 4, 5-triphosphate (IP3), and diacylglycerol, which participate in the regulation of a cascade of physiological and pathological responses through protein kinases and enzymes (11, 19, 21, 22). In our hands, neuronostatin-induced suppression of cardiac contractile function was ablated by inhibition of PKA rather than PKC, suggesting that a PKA- but not a PKC-dependent mechanism in neuronostatin-induced cardiac contractile response. Interestingly, the somatostatin-induced suppression of cardiac contractile function was reversed by the PKC inhibitor but not the PKA inhibitor, suggesting that a PKC- as opposed to PKA-dependent mechanism in the somatostatin-induced cardiac contractile response. This observation suggests that neuronostatin may exert its cardiac action through unique membrane receptors distinctive from the cardiac somatostatin receptor, which is known to be mediated through PKC (24, 25). Furthermore, it is demonstrated that neuronostatin does not stimulate Gi signaling via the somatostatin receptors nor does neuronostatin influence growth hormone release from pituitary cells (28). All these findings support the hypothesis that neuronostatin possesses physiological functions distinct from those of somatostatin, possibly through its own membrane receptors. Interestingly, our study also revealed that the inhibitory effect of neuronostatin on cardiomyocyte contractile function was attenuated by JNK inhibition, suggesting a role of JNK activation in neuronostatin-elicited cardiac contractile response. Somatostatin was recently shown to activate JNK signaling through G proteins (G12 and G13) following binding to the somatostatin receptor (2, 14). Our current finding of the negative cardiac inotropic action of JNK activation agrees with the observation of enhanced smooth muscle contractility in JNK knockout mice (15).

**Perspectives and Significance**

Our current findings suggest that neuronostatin, a newly identified peptide, directly suppresses cardiomyocyte and whole heart contractile function in a concentration-dependent manner. The neuronostatin-elicited cardiac response appears to be mediated through activation of PKA and/or JNK, involving upregulation of early response genes c-jun and c-fos. These data have shed some light on the likely role of neuronostatin in the regulation of not only hormonal and neuroendocrine actions but also cardiovascular homeostasis. In the brain, neu-
Ronostatin is known to increase the mean arterial pressure (28), at least in part via activation of sympathetic nervous system and perhaps via stimulation of the release of vasoactive neurohormones (G. L. C. Yosten and W. Samson, unpublished data). To date, there is no literature describing the direct cardiac effect of neuronostatin, either in vitro or in vivo. Similar to somatostatin, the neuronostatin immunoreactivity has been demonstrated in heart extracts (28). The colocalization of somatostatin immunoreactivity with choline acetyltransferase in nerve terminals surrounding intracardiac ganglia suggested delivery of somatostatin gene product-derived peptides in parasympathetic preganglionic neurons (26). A selective neuronostatin receptor has not yet been identified, however, from biochemical studies (28), and the results reported here favor a G protein-coupled receptor, unique from the somatostatin receptors. Last but not least, neuronostatin may exert pharmacologic actions at concentrations below or equal to those described for other peptides such as adiponectin (5) and members of the adrenomedulin family of peptides (6). It is thus possible that the peptide may exert endogenous physiological effects on the hearts. Given the limited knowledge available for this peptide hormone, further study is warranted to identify the receptors and postreceptor mechanisms involved in the physiological and pathophysiological response of neuronostatin in the cardiovascular system. In particular, it will be important to determine whether neuronostatin exerts cardiac actions in vivo in both the nonfailing and failing heart, as has been demonstrated to be the case for somatostatin (8, 18).

**ACKNOWLEDGMENTS**

We are grateful to Dr. Aaron J. W. Hsueh from Stanford University for providing the synthetic neuronostatin-13.

**GRANTS**

This work was supported in part by National Institutes of Health Grant 5P20 RR016474.
REFERENCES


24. Stroh T, Kreienkamp HJ, Beaudet A. Immunohistochemical distribution of the somatostatin receptor subtype 5 in the adult rat brain: Predomi-

