Adenosine Modulates Cardiovascular Functions Through Activation of Extracellular Signal-Regulated Kinases 1 and 2 and Endothelial Nitric Oxide Synthase in the Nucleus Tractus Solitarii of Rats

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Background—The nucleus tractus solitarius (NTS) is the primary integrative center for baroreflex. Adenosine has been shown to play an important modulatory role in blood pressure control in the NTS. Our previous results demonstrated that adenosine decreases blood pressure, heart rate, and renal sympathetic nerve activity and modulates baroreflex responses in the NTS. We also demonstrated that a nitric oxide synthase (NOS) inhibitor may block the cardiovascular effects of adenosine in the NTS, which suggests interaction between the adenosine receptor and NOS. However, the signaling mechanisms of adenosine that induce nitric oxide release in the NTS remain uncertain. The aim of the present study was to investigate the possible signal pathways involved in the cardiovascular regulation of adenosine in the NTS.

Methods and Results—Adenosine was microinjected into the NTS of urethane-anesthetized male Sprague-Dawley rats. Blood pressure and heart rate decreased significantly after microinjection. The cardiovascular effects of adenosine were attenuated by prior administration of the mitogen-activated protein kinase/extracellular signal-regulated kinase inhibitor PD98059 (6 nmol/60 nL) or an endothelial NOS–selective inhibitor, L-NIO (6 nmol/60 nL); however, the neuronal NOS–specific inhibitor vinyl-L-NIO (600 pmol/60 nL) did not attenuate the cardiovascular effects of adenosine. Western blot and immunohistochemistry studies demonstrated that adenosine induced extracellular signal-regulated kinases 1 and 2 and endothelial NOS phosphorylation in the NTS. Pretreatment with PD98059 diminished the endothelial NOS phosphorylation evoked by adenosine.

Conclusions—These results represent a novel finding that extracellular signal-regulated kinases 1 and 2 is involved in cardiovascular regulation in the NTS. They also indicate that the cardiovascular modulatory effects of adenosine in the NTS are accomplished by activation of mitogen-activated protein kinase/extracellular signal-regulated kinases 1 and 2 and then endothelial NOS. (Circulation. 2008;117:773-780.)

Key Words: adenosine ■ baroreceptors ■ signal transduction ■ nitric oxide ■ brain

The nucleus tractus solitarius (NTS) is the primary integrative center for cardiovascular control and other autonomic functions in the central nervous system. The NTS not only integrates convergent information but itself is the site of substantial modulation. Our previous studies demonstrated that several neuromodulators are involved in cardiovascular control in the NTS, including ATP,1 adenosine,1,2 angiotensin II,3 angiotensin III,4 nitric oxide,5–7 carbon monoxide,8–11 and insulin.12 Among these neuromodulators, adenosine has been shown to play an important role in central cardiovascular control.1,2,13 Microinjection of adenosine into NTS produces slowly developed, long-lasting (minutes), and dose-related decreases in blood pressure (BP) and heart rate (HR).2,14

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Adenosine acts as a neuromodulator at many levels of the central nervous system, including the brainstem, which is the central cardiovascular control center.15,16 The natural precursor of neuromodulator adenosine in the central nervous system is ATP, which is dephosphorylated and rapidly becomes adenosine via the activity of ectonucleotidases on synaptic release.17 Among 4 adenosine receptor subtypes, the A1 and A2A receptor...
subtypes have been demonstrated to play neuromodulatory roles in the NTS.\textsuperscript{15,18,19} The A\textsubscript{1} receptor is expressed in the central nervous system ubiquitously and has been shown to inhibit neuronal activity, whereas the A\textsubscript{3} receptor is expressed at high levels in limited regions of the brain that are primarily linked to adenylyl cyclase activation.\textsuperscript{17,20} In the NTS, activation of the A\textsubscript{1} and A\textsubscript{3} receptors has been demonstrated to elicit dose-related increases and decreases in BP, respectively.\textsuperscript{14,15} Together, these studies suggest that the cardiovascular modulatory effects of adenosine in the NTS are mediated predominantly by the A\textsubscript{3} receptor.

Studies including ours have demonstrated that the depressor and bradycardic effects of adenosine in the NTS were attenuated by a nitric oxide synthase (NOS) inhibitor.\textsuperscript{7,21} In addition, microinjection of nitric oxide (NO) donors into subpostremal NTS evokes a pattern of regional sympathetic responses that increases in preganglionic adrenal nerve activity and decreases in renal, postganglionic sympathetic nerve activity. Interestingly, these sympathetic responses are similar to those evoked by stimulation of the adenosine A\textsubscript{3} receptor in the NTS.\textsuperscript{13} All of these results indicate that the cardiovascular modulatory effects of adenosine in the NTS might be accomplished by NOS activation and subsequent NO release; however, the underlying signal mechanism between adenosine receptor activation and NO production in the NTS remains unclear. Recently, it has been demonstrated that activation of the adenosine A\textsubscript{3} receptor can cause NO release in endothelial cells.\textsuperscript{22,23} In addition, it has been reported that adenosine induces endothelial NOS (eNOS) activation in a Ca\textsuperscript{2+}-insensitive manner that involves extracellular signal-regulated kinase (ERK) 1 and 2 phosphorylation in human umbilical vein endothelial cells.\textsuperscript{24} This raises the possibility that the cardiovascular modulatory effects of adenosine in the NTS might be mediated through activation of ERK1/2 and eNOS.

In the present experiments, we tested whether adenosine could induce ERK1/2 and eNOS activation and whether specific ERK1/2 and eNOS inhibitors are able to inhibit the cardiovascular effects of adenosine in the NTS. In addition, we examined whether a specific ERK inhibitor could attenuate the eNOS activation induced by adenosine in the NTS. Our results suggest that a possible adenosine-ERK-eNOS signaling pathway is well regulated in the modulation of cardiovascular responses to adenosine in the NTS.

**Methods**

**Experimental Chemicals**

All experimental chemicals were purchased from Sigma-Aldrich (St Louis, Mo) except when otherwise noted. Chemicals microinjected into the NTS (eg, adenosine, PD98059, L-NIO, and vinyl-L-NIO) were dissolved in reduced-serum medium (Opti-MEM I; Invitrogen, Carlsbad, Calif).

**Animals**

Male Sprague-Dawley rats (weight 250 to 350 g) were obtained from the National Science Council Animal Facility (Taipei, Taiwan) and housed in the animal room of Kaohsiung Veterans General Hospital (Kaohsiung, Taiwan). The rats were kept in individual cages in a room where lighting was controlled (12-hour light/12-hour dark cycle), and temperature was maintained at 23°C to 24°C. The rats were given normal rat chow (Purina; St. Louis, Mo) and tap water ad libitum. All animal research protocols had been approved by the Research Animal Facility Committee of Kaohsiung Veterans General Hospital.

**Intra-NTS Microinjection and Hemodynamic Measurements**

Rats were anesthetized with urethane (1.0 g/kg IP and 0.3 g/kg IV if necessary). The preparation of animals for intra-NTS microinjection and the methods used to locate the NTS have been described previously.\textsuperscript{5} Briefly, a polyethylene cannula was inserted into the femoral vein for fluid supplementation, and BP was measured via a femoral-artery cannula by pressure transducer and polygraph (Gould, Cleveland, Ohio). HR was monitored by a tachograph preamplifier (Gould). To verify that the needle tip of the glass electrode was exactly in the NTS, l-glutamate (0.154 mmol/60 nL) was microinjected. This would induce a characteristically abrupt decrease in BP (ΔBP ≥35 mm Hg) and HR (ΔHR ≥50 bpm) if the needle tip was located precisely in the NTS.

To investigate whether ERK 1 and ERK 2 participate in cardiovascular responses to adenosine in the NTS, 2 groups of rats (6 rats per group) received microinjection of adenosine (0.77 nmol/60 nL) in the unilateral NTS, and changes in BP and HR were measured. The rats were then allowed to rest for at least 30 minutes until BP and HR returned to basal levels. An MEK inhibitor, PD98059 (6 pmol/60 nL), or vehicle was microinjected into the NTS, then the same dose of adenosine (0.77 nmol) was microinjected into the NTS 10, 30, 60, and 90 minutes after PD98059 or vehicle administration. Cardiovascular parameters were recorded.

To investigate whether eNOS or neuronal NOS (nNOS) also participates in the cardiovascular responses to adenosine in the NTS, similar experimental procedures were performed to study the effects of pretreatment with a selective eNOS inhibitor, L-NIO (l-N\textsuperscript{3}-1-iminoethyl)-ornithine; Calbiochem, San Diego, Calif; 6 mmol/60 mL), and a specific nNOS inhibitor, vinyl-L-NIO (l-N\textsuperscript{3}-1-imino-3-butenyl)-ornithine; Alexis Biochemicals, San Diego, Calif; 600 pmol/60 nL), on the cardiovascular effects of adenosine in the NTS.

**Western Blot Analysis**

Four groups of rats (6 rats per group) were enrolled in this test. Rats received intra-NTS microinjection with vehicle, PD98059, or L-NIO, respectively, 10 minutes before adenosine injection: another group of rats received microinjection with the A\textsubscript{3} receptor agonist CGS21680 (24 pmol/60 nL). Ten to 20 minutes after adenosine injection, rats were euthanized, and brainstems were excised immediately. The NTS was dissected by a micropunch (1-mm inner diameter) from a brainstem slice 1 mm thick at the level of the obex under a microscope. Total protein extract was prepared by homogenization of the NTS in lysis buffer with protease inhibitor cocktail and phosphatase inhibitor cocktail and was incubated for 1 hour at 4°C. Protein extracts (20 μg per sample assessed by BCA protein assay; Pierce) were subjected to 7.5% to 10% SDS-Tris glycerin gel electrophoresis and transferred to a polyvinylidene difluoride membrane (GE Healthcare, Buckinghamshire, UK). The membrane was blocked with 5% nonfat milk in TBS/Tween 20 buffer (10 mmol/L Tris, 150 mmol/L NaCl, 0.1% Tween 20, pH 7.4), incubated with anti-p-ERK1/2(Thr202/Tyr204) antibody (Cell Signaling Technology, Danvers, Mass), anti-ERK1/2 antibody (Cell Signaling Technology), anti-p-eNOS(Thr476) antibody (Cell Signaling Technology), and anti-eNOS antibody (BD Biosciences, San Jose, Calif) at 1:1000 in PBST with 5% BSA, and incubated at 4°C overnight. Peroxidase-conjugated anti-mouse (1:5000) and anti-rabbit antibody (1:5000) was used as secondary antibody. The membrane was detected with an ECL-Plus detection kit (GE Healthcare) on film. The films were scanned by photo scanner (4490, Epson, Long Beach, Calif) and were analyzed with NIH Image densitometry analysis software (National Institutes of Health, Bethesda, Md).

**Immunohistochemistry Analysis**

Adenosine (0.77 nmol/60 nL) was administered into the NTS with or without PD98059 (6 mmol/60 nL) pretreatment. Ten to 20 minutes after adenosine microinjection, brainstems were excised and fixed with 4% formaldehyde. Paraffin-embedded serial sections were cut at 5-μm thickness. The sections were dewaxed, quenched in 3% H\textsubscript{2}O\textsubscript{2}/methanol, antigen-retrieved in citric buffer (10 mmol/L, pH 6.0), blocked in 5% goat serum, and incubated with anti-phospho-
ERK1/2 and nNOS-specific inhibitor, vinyl-L-NIO, on the cardiovascular responses induced by adenosine in the NTS. We investigated the effects of a selective eNOS inhibitor, L-NIO, and an nNOS-specific inhibitor, vinyl-L-NIO, and counterstained with hematoxylin. The sections were then photographed with a microscope equipped with a charge-coupled device camera.

**Statistical Analysis**
Group data are expressed as mean±SEM. The paired Student t test (for comparisons of cardiovascular parameters before and after pretreatment), unpaired Student t test (for control and study group comparisons), or 1-way ANOVA with Sheffé post hoc comparison was applied to compare group differences. Differences with $P<0.05$ were considered significant.

The authors had full access to the data and take full responsibility for its integrity. All authors have read and agree to the manuscript as written.

**Results**

**Effects of MEK Inhibitor on Cardiovascular Responses to Adenosine in the NTS**
To assess whether ERK1 and ERK2 participate in the cardiovascular modulatory effects of adenosine in the NTS, we investigated the inhibitory effect of an MEK inhibitor, PD98059, on the cardiovascular effects of adenosine in the NTS. Microinjection of adenosine into unilateral NTS induced hypotension and bradycardia. The depressor effect of single-dose adenosine lasted for more than 5 minutes, and the bradycardic effect lasted for more than 15 minutes (Figure 1A). These results were consistent with our previous findings. Pretreatment with PD98059 did not elicit significant cardiovascular responses (Figure 1A). Interestingly, the depressor and bradycardic responses of the same dose of adenosine in the NTS were attenuated 10 minutes after PD98059 treatment (−37.9±2.9 versus −21.7±3.1 mm Hg and −78.6±13.2 versus −52.9±12.0 bpm, respectively; $P<0.05$, paired t test; n=6; Figure 1A and 1B). The cardiovascular effects of adenosine in the NTS recovered gradually 30 minutes after PD98059 treatment (Figure 1A). To clarify the specificity of the inhibitory effects of PD98059 in the NTS, we tested the effect of PD98059 on the cardiovascular response of L-glutamate in the NTS. The results revealed that pretreatment with PD98059 did not alter the depressor and bradycardic effects of L-glutamate (Data Supplement Figure I), which is the major neurotransmitter in the NTS.

**Effects of eNOS Inhibitor and nNOS Inhibitor on Cardiovascular Responses to Adenosine in the NTS**
Our previous studies suggested that cardiovascular modulatory responses to adenosine are accomplished through activation of NOS in the NTS. To identify which constitutive NOS contributes to these cardiovascular responses to adenosine in the NTS, we investigated the effects of a selective eNOS inhibitor, L-NIO, and an nNOS-specific inhibitor, vinyl-L-NIO, and counterstained with hematoxylin. The sections were then photographed with a microscope equipped with a charge-coupled device camera.

**Adenosine Induced eNOS Phosphorylation in the NTS**
Using a specific pharmacological inhibitor, we demonstrated that eNOS but not nNOS was involved in the adenosine-mediated hypotensive and bradycardic responses in the NTS.

![Figure 1. The cardiovascular effects of adenosine in the NTS before and after administration of the MEK inhibitor PD98059. A, Representative tracings demonstrate the cardiovascular effects of adenosine (0.77 nmol) in unilateral NTS before and 10 minutes after pretreatment with PD98059 (6 pmol) in anesthetized rats. The depressor and bradycardic effects of adenosine were significantly attenuated by PD98059. As PD98059 washed out, the cardiovascular effects of adenosine recovered. B, Graph reveals the effects of pretreatment PD98059 on mean BP (MBP) and HR after microinjection of adenosine into unilateral NTS. Both the MBP and HR effects of adenosine were significantly attenuated by PD98059. Adenosine was administered 10 minutes after vehicle or PD98059. Values are shown as mean difference±SEM, n=6. *$P<0.05$ vs control group. ADO indicates adenosine; PD, PD98059.](image-url)
We further investigated whether adenosine may induce eNOS phosphorylation in the NTS. The phosphorylation of eNOS was determined by immunoblotting and immunohistochemistry staining with antibodies specifically against phospho-eNOS5177. The results of immunoblotting analysis demonstrated that adenosine could increase eNOS phosphorylation in the NTS (1.9±0.2 versus 1.0±0.3, P<0.05, n=6; Figure 3A and 3B). To confirm that intra-NTS adenosine administration induces eNOS phosphorylation, paraffin sections of the brainstem were subjected to immunohistochemistry staining against phospho-eNOSS1177, which demonstrated that the number of phospho-eNOS–positive cells in the NTS of the adenosine-administered side was much less than on the vehicle-pretreated side (Figure 3C, frame c versus frame d). The immunohistochemistry results were consistent with those of immunoblotting.

**Adenosine Induced ERK1/2 Phosphorylation in the NTS**

PD98059 inhibited the depressor and bradycardic effects of adenosine in NTS, which suggested that ERK1 and ERK2 contribute to cardiovascular responses to adenosine in the NTS. We further investigated whether adenosine can induce ERK1/2 phosphorylation in the NTS. Phosphorylation of ERK1/2 was determined by immunoblotting and immunohistochemistry staining with an antibody specifically against phospho-ERK1/2T202/Y204. Immunoblotting analysis of protein extract of adenosine-treated NTS revealed that ERK1/2 phosphorylation was increased compared with that of the control group (1.8±0.1 versus 1.0±0.1 for ERK1 and 1.8±0.2 versus 1.0±0.1 for ERK2, P<0.05; n=6; Figure 4A and 4B). To confirm that adenosine induces ERK1/2 phosphorylation in situ, paraffin sections of the brainstem were subjected to immunohistochemistry staining with antibodies specifically against phospho-ERK1/2T202/Y204. Figure 4C shows that there were more phospho-ERK1/2–positive neuronal cells in the NTS of the adenosine-administered side than in the control side (frame c versus frame d). The results of immunoblotting and immunohistochemistry staining were consistent and indicated that adenosine can induce ERK1/2 phosphorylation in situ.

**MEK1/2 Inhibitor Attenuated Adenosine-Induced eNOS Phosphorylation in the NTS**

To test whether ERK1/2 may induce eNOS phosphorylation, we investigated the inhibitory effect of PD98059 on eNOS phosphorylation in the NTS. The results from the immunoblotting analysis demonstrated that pretreatment with the MEK inhibitor PD98059 could attenuate adenosine-induced eNOS phosphorylation in the NTS (0.8±0.2 versus 1.9±0.2, P<0.01; Figure 3A and 3B). These results suggested that ERK-eNOS signaling served as the downstream molecules of adenosine-mediated cardiovascular responses in the NTS. This conclusion was further supported by the results of immunohistochemistry staining against phospho-eNOS5177, which demonstrated that the number of phospho-eNOS–positive cells on the PD98059-pretreated side was much less than on the vehicle-pretreated side (Figure 3C, frame g versus frame h).

**Adenosine Activates ERK1/2 and eNOS via the Adenosine A2A Receptor in the NTS**

It was reported that the cardiovascular modulatory effects of adenosine in the NTS are mediated predominantly through the A2A receptor. The above results demonstrated that adenosine could induce ERK1/2 and eNOS phosphorylation in the NTS. Therefore, we tested whether the induction of ERK1/2 and eNOS phosphorylation is via the adenosine A2A receptor in the NTS. Immunoblotting of NTS treated with the A2A agonist CGS21680 revealed increases in ERK1/2 phosphorylation (2.1±0.5 versus 1.0±0.4 for ERK1 and 2.0±0.1 versus 1.0±0.2 for ERK2, P<0.05; n=6; Figure 5A). eNOS5177 phosphorylation also increased in NTS that received CGS21680 microinjection (1.8±0.2 versus 1.0±0.2, P<0.05; n=6; Figure 5B). Together, these results support the idea that signal molecule activation by adenosine in the NTS occurs predominantly through the A2A receptor.

**Discussion**

Adenosine has been demonstrated to play an important role in cardiovascular modulation in the NTS.1 Our previous experiments demonstrated that the depressor and bradycardic effects of adenosine in the NTS were blocked by the nonspecific NOS inhibitor Nω-nitro-L-arginine methyl ester,
which suggested that cardiovascular modulation by adenosine was mediated by activation of NOS and subsequent NO release. Results of the present study confirm and extend our previous observations that eNOS but not nNOS participates in the signal mechanism effect of adenosine on cardiovascular modulation in the NTS. The present data further indicate that ERK1 and ERK2 are the intermediate signal molecules that activate eNOS in the NTS. The present study demonstrated that adenosine-ERK-eNOS signaling exists and plays an important role in modulation of cardiovascular responses to adenosine in the NTS.

The ERK cascade is distinguished by a characteristic core cascade of 3 kinases. The first kinase is a so-called MAP kinase kinase kinase (MAPKKK; Raf-1 and B-Raf), which activates the second kinase, a MAP kinase kinase (MAPKK; MEK), by serine/threonine phosphorylation. MEK in turn activates ERK1/2 by phosphorylation of both threonine and tyrosine residues.25 The ERK cascade was originally discovered to be a critical regulator of cell division and differentiation; however, ERK1 and ERK2, as well as their upstream regulators and many of their downstream targets, are highly expressed in mature neurons.26 This raises the question of whether ERK1 and ERK2 not only regulate neuronal development but also play regulatory roles in mature neurons in response to various physiological stimuli. A number of studies have suggested the regulatory roles of ERK1/2 in mature neurons. First, ERK1 and ERK2 have been demonstrated to be phosphorylated and activated in neurons in response to excitatory glutamatergic signaling, which controls many forms of synaptic plasticity that are thought to underlie higher brain processes such as learning and memory.26 Second, activation of ERK1/2 has been identified in central neurons that respond to specific stimuli, such as nociception, unfamiliar taste, synaptic plasticity, and fear memory consolidation.27–29 Third, peripheral administration of cholecystokinin and hydralazine can induce ERK1/2 phosphorylation in the NTS.30,31 Finally, experiments on rat hippocampus brain slices revealed that a wide variety of G-protein–coupled receptor neurotransmitters, including N-methyl-D-aspartate and adrenergic, dopamine, and muscarinic acetylcholine receptors, activate ERK1/2 by protein kinase A or protein kinase C.32 All of this suggests the possibility of a broad physiological role for the ERK cascade in the adult central nervous system. Indeed, in the present study, we demonstrated that microinjection of adenosine into the NTS induced eNOS phosphorylation in situ and that blockade of ERK1/2 activation by PD98059 attenuated the depressor and bradycardic effects of adenosine (Figure 1). The present study provides the first evidence to demonstrate the ERK1/2 activation is involved in cardiovascular modulation in the NTS.

It would be interesting to know how adenosine activates ERK1/2 in mature neurons. The activated adenosine A2A receptor activates adenyl cyclase, which in turn increases cAMP.17 Interestingly, cAMP may either inhibit or activate

**Figure 3.** Quantitative analysis of phospho-eNOS in the NTS after microinjection of adenosine with or without PD98059. A, Immunoblotting revealed that phosphorylated eNOS was increased in the NTS that received adenosine. Phosphorylation of eNOS was blocked by pretreatment with PD98059 10 minutes before adenosine microinjection. B, Densitometric analysis of immunoblotting of phospho-eNOS showed that adenosine induced an almost 2-fold increase in phospho-eNOS compared with the control group. Phospho-eNOS was decreased by PD98059 pretreatment. Values are shown as mean ± SEM, n = 6. *P < 0.05, control vs adenosine group, #P < 0.01, adenosine vs PD98059 + adenosine group. C, Immunohistochemistry of the brainstem for phospho-eNOS demonstrated microinjection of adenosine into the NTS induced eNOS phosphorylation in situ (c vs d). Pretreatment with PD98059 significantly reduced eNOS phosphorylation in the NTS (g vs h). PD98059 or vehicle was administered 10 minutes before adenosine. Scale bar = 200 μm. ADO indicates adenosine.
ERK1/2 in a cell-specific manner. Emerging new data revealed that the determinant of whether cAMP activates or inhibits the ERK cascade is B-Raf. In B-Raf-expressing cells, cAMP may activate B-Raf by complex mechanisms, and the activated B-Raf subsequently activates the MEK-ERK cascade. A convincing model of how cAMP activates B-Raf is not currently available, but the accumulated data suggest the involvement of RAS, Rap1, Src, protein kinase A, and 14-3-3 proteins. Interestingly, B-Raf is the major RAF isoform expressed in neurons. Therefore, we infer that adenosine activates ERK1/2 in the NTS by activation of the A2A receptor, an increase in cAMP, activation of B-Raf, and the ensuing phosphorylation and activation of MEK.

Figure 4. Quantitative analysis of phospho-ERK1/2 in the NTS with adenosine treatment. A, Phosphorylated ERK1/2 was increased in the adenosine-microinjected group compared with the control group. B, Densitometric analysis of immunoblotting of phospho-ERK1/2 revealed adenosine induced an almost 2-fold increase in ERK1/2 phosphorylation. Values are mean±SEM, n=6. *P<0.05, control vs adenosine group. C, Immunohistochemistry stain of the brainstem for phospho-ERK1/2 demonstrated microinjection of adenosine into the NTS induced ERK1/2 phosphorylation in situ (c vs d). Scale bar=200 μm. ADO indicates adenosine.

Figure 5. Adenosine A2A receptor agonist, CGS21680, induced ERK1/2 and eNOS phosphorylation in the NTS. A, Immunoblotting revealed there were at least 2-fold increases in phospho-ERK1 and phospho-ERK2 in the NTS that received CGS21680. B, CGS21680 also induced an almost 2-fold increase in phospho-eNOS in the NTS. Values are mean±SEM, n=6. *P<0.05, control vs CGS21680 group.
Signaling molecules that regulate eNOS activity include PI3K/Akt, cyclic nucleotide-dependent kinases (protein kinase A and protein kinase G), protein kinase C, and ERKs. In the present study, we demonstrated that PD98059 inhibited eNOS phosphorylation (Figure 3A and 3B). This result suggests that adenosine may phosphorylate eNOS through activation of MEK/ERK cascades in the NTS. This result is further supported by reports that inhibition of ERK1/2 activation attenuated the eNOS phosphorylation that was induced by estrogen and vascular endothelial growth factor. Although it has been reported for years that ERKs could phosphorylate and activate eNOS, the exact signaling mechanisms that couple the activated ERK1/2 to eNOS activation remain uncertain.

eNOS, originally identified in vascular endothelium, is expressed in several nonendothelial cell types, including neurons of various rat brain regions. Although the physiological functions of eNOS in cardiovascular modulation in the NTS remain controversial, several studies, including the present study, demonstrated that eNOS participates in cardiovascular modulation in the NTS. By reverse-transcription polymerase chain reaction, Waki et al demonstrated that eNOS is expressed in several nonendothelial cell types, including neurons of various rat brain regions. Although the physiological functions of eNOS in cardiovascular modulation in the NTS remain controversial, several studies, including the present study, demonstrated that eNOS participates in cardiovascular modulation in the NTS. By reverse-transcription polymerase chain reaction, Waki et al demonstrated that eNOS mRNA is expressed in the NTS of adult Wistar-Kyoto and spontaneously hypertensive rats. Suppression of the endogenous eNOS by overexpression of dominant-negative eNOS in bilateral NTS increased spontaneous baroreceptor reflex gain in conscious Wistar rats and decreased BP in mature spontaneously hypertensive rats.

In the present study, our data revealed that eNOS not only exists in the NTS (Figure 3A and 3C) but that it also mediates cardiovascular modulation by adenosine (Figure 2). Microinjection of adenosine into the NTS induced eNOS phosphorylation in situ (Figure 3C). Scislo et al reported the differential role of NO in regional sympathetic responses to stimulation of NTS A2A adenosine receptor. They demonstrated that the nNOS-selective antagonist TRIM [1-(2-trifluoromethylphenyl) imidazole; 20 nmol/100 nL] reversed normal depressor responses and significantly attenuated the A2A receptor–elicited decreases in HR and renal sympathetic nerve activity. They concluded that the hemodynamic and sympathoinhibitory responses caused by stimulation of the NTS A2A receptor were predominantly mediated by nNOS. Conversely, in the present study, only an eNOS-selective antagonist attenuated the depressor and bradycardic effect of adenosine (Figure 2B), whereas the nNOS-specific antagonist did not (Data Supplement Figure II). TRIM is a potent inhibitor of nNOS (IC50 of 2.8 μmol/L) and a relatively weak inhibitor of eNOS (IC50 1057.5 μmol/L). The concentrations of TRIM used by Scislo and coworkers (200 nmol/L) may have been too high, and therefore, TRIM might have lost its nNOS selectivity and could no longer discriminate which NOS mediated the cardiovascular modulatory effects of adenosine in the NTS. In the present study, we not only demonstrated the pharmacological effects of eNOS inhibitors on cardiovascular parameters (Figure 2), we also demonstrated eNOS phosphorylation in situ in the NTS by immunohistochemistry staining (Figure 3C). These evidences confirm that cardiovascular modulation by adenosine in the NTS is predominantly through eNOS activation, with only a small contribution, if any, of nNOS.

In conclusion, we present a novel adenosine-ERK-eNOS signaling pathway in the NTS that allows us to dissect the adenosine signaling pathway related to the production of NO in the regulation of BP and HR. Our results suggest a possible interaction between ERK1/2 and eNOS in the NTS. The present data demonstrate that adenosine modulates central cardiovascular control via activation of ERK1/2 and its downstream eNOS.

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Disclosures

None.

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**CLINICAL PERSPECTIVE**

The sympathetic nervous system has moved toward center stage in cardiovascular medicine. Studies have demonstrated that sympathetic overactivity characterizes the hypertensive state and participates in the development, maintenance, and progression of elevated blood pressure. The importance of sympathetic overactivity in heart failure progression and mortality and in the development of ventricular arrhythmias is now well established, and a central nervous system–mean arterial pressure (CNS-MAP) set-point theory has recently been proposed. It has been hypothesized that hypertension occurs as the result of a primary shift of the CNS-MAP set point to a higher operating pressure, which results in increased sympathetic nerve activity. The nucleus tractus solitarius (NTS), located at the dorsal part of the brainstem, was discovered to be an important sympathetic nervous system integral center in the central nervous system. Adenosine, a ubiquitously distributed neuregulator, was found to participate in sympathetic activity regulation in the NTS. In the present study, we investigated the signaling mechanism of adenosine with regard to cardiovascular modulation in the NTS and found that the MEK-ERK (mitogen-activated protein kinase–extracellular signal-regulated kinase) cascade, which was originally discovered to be a critical regulator of cell division and differentiation, participates in adenosine-mediated central cardiovascular control. In addition, we also demonstrated that endothelial nitric oxide synthase, which was originally identified in the vascular endothelium, is present and participates in cardiovascular regulation in the NTS. Further investigation of the molecular mechanisms involved in sympathetic nervous activity modulation might elucidate the pathogenesis of the CNS-MAP set-point shift and sympathetic overactivity in essential hypertension.
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