Selective Sensitization by Nitric Oxide of Sympathetic Baroreflex in Rostral Ventrolateral Medulla of Conscious Rabbits

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Abstract—Nitric oxide (NO) deficiency in the rostral ventrolateral medulla (RVLM) has been implicated in impaired baroreflex control in hypertensive and heart failure animals. However, the role of local NO in normal baroreflex regulation remains unclear. This study aimed to examine the role of NO in tonic and baroreflex control of blood pressure (BP) in the RVLM of conscious rabbits. Microinjections of NO donors, S-nitroso-N-acetylpenicillamine and sodium nitroprusside (5 to 20 nmol), or NO itself (20 to 200 pmol) into the RVLM dose-dependently increased BP. Bilateral microinjections of an NO synthase (NOS) inhibitor NG-nitro-L-arginine methyl ester (L-NAME; 10 nmol), its inactive enantiomer D-NAME, or soluble guanylate cyclase (sGC) inhibitors, 1-H-[1,2,4]oxadiaolo[4,3-a]quinoxalin-1-one (ODQ, 250 pmol) and methylene blue (10 nmol), into the RVLM did not affect resting BP, heart rate, or renal sympathetic nerve activity (RSNA). However, L-NAME, methylene blue, and ODQ decreased RSNA baroreflex gain by 42% to 55%, whereas D-NAME did not affect this reflex. Co-microinjections of L-NAME and superoxide scavenger tempol (20 nmol) decreased RSNA baroreflex gain by 37±8%. Microinjections of a neuronal NOS (nNOS) inhibitor, 7-nitroindazole (500 pmol), into the RVLM decreased RSNA baroreflex gain by 42±12%, without altering resting BP, heart rate, or RSNA. Local administration of inducible NOS (iNOS) inhibitors, S-methylisothiourea (0.25 nmol) and aminoguanidine (0.25 and 2.5 nmol), affected neither resting nor baroreflex parameters. These results suggest that nNOS-derived NO facilitates sympathetic baroreflex transmission in the RVLM at least in part via a sGC-dependent, superoxide-independent mechanism. However, local nNOS and iNOS play little role in the tonic support of BP in conscious rabbits. (Hypertension. 2005;45:901-906.)

Key Words: baroreflex ▪ blood pressure ▪ brain ▪ nitric oxide ▪ rabbits

Nitric oxide (NO) is a free radical messenger that plays a key role in a wide range of biological functions involved in central cardiovascular control.1 In particular, NO is important in the modulation of neurotransmitter release induced by stimulation of ionotropic glutamate receptors by excitatory amino acids (EAAs).2 This stimulation activates NO synthase (NOS), thereby increasing postsynaptic production of NO and its diffusion to presynaptic sites, where NO modulates the release of EAAs, GABA, and other transmitters via a soluble guanylate cyclase (sGC)-dependent mechanism.2,3

One brain region where NO may be of primary importance in cardiovascular control is the rostral ventrolateral medulla (RVLM), which is thought to be a final common pathway for regulating sympathetic activity.4 Microinjections of NO donors into this region, unless given in high doses,5 increase blood pressure in a sGC-dependent manner in conscious6,7 and anesthetized animals.5,8,9 By contrast, local administration of NOS and sGC inhibitors decrease or block the hypertensive responses to EAAs6,10 and also attenuate various cardiovascular reflexes.11,12 Importantly, NO may also play a role in the tonic support of blood pressure in the RVLM.9,13 In particular, it has been shown that NO derived from the neuronal (nNOS) and inducible (iNOS) isoforms of NOS exerts, respectively, excitatory and inhibitory actions in the RVLM,9 and the balance between these actions is shifted toward excitation in hypertensive animals.14

Recent evidence suggests that NO deficiency in the RVLM may underlie impaired baroreflex regulation in such disease states as hypertension and heart failure.15,16 However, the role of NO in normal baroreflex regulation in the RVLM remains elusive, because this messenger has been found to have little effect on baroreflexes in control anesthetized animals.11,16 Given that anesthesia and surgical stress may be limiting factors in studying the effects of NO signaling,17,18 the current study aimed to determine the effect of NO on sympathetic and cardiac baroreflexes in the RVLM of conscious, chronically instrumented rabbits. The role of local nNOS and iNOS in the tonic and reflex control of blood pressure was also examined in this study.
Methods

Surgical Procedures
The experiments were performed in 22 conscious New Zealand White and multicolored (English strain) rabbits, weighing 2.3 to 3.4 kg, and bred and housed at the Baker Heart Research Institute. All procedures were approved by the Alfred Medical Research and Education Precinct Animal Ethics Committee. Two weeks before the experiments, all rabbits were implanted with guide cannulae for bilateral microinjections into the RVLM, and 1 week later a bipolar electrode for recording renal sympathetic nerve activity (RSNA).19 On the day of the experiment, a central ear artery and marginal ear vein were catheterized under local anesthesia.

Sympathetic and Cardiac Baroreflexes
During the experiment, pulsatile arterial pressure and integrated RSNA were continuously sampled at 500 Hz using an analog to digital data acquisition card. The beat-to-beat mean arterial pressure (MAP), heart rate (HR), and integrated RSNA were detected online using a LabVIEW program. The baroreflex was assessed using intravenous bolus injections of phenylephrine and sodium nitroprusside.20 MAP, RSNA, and HR from individual rabbits were averaged over 2-second intervals and fitted to a sigmoid logistic function to produce the RSNA–MAP and HR–MAP curves using a nonlinear regression program.21 Because voltages recorded from RSNA electrodes vary considerably between animals, in each experiment the values were normalized to the upper plateau of the control baroreflex curve, which was taken to equal 100 normalized units (nu), as described previously.19

Microinjections Into the RVLM of Conscious Rabbits
The microinjections into the RVLM were made, at a volume of 100 nL, through a stainless steel injector (O.D. 315 μm), which extended 7.0 mm beyond the guide cannula and was connected via polyethylene SP8 tubing to a 250-μL Hamilton syringe. Concentrated (≈2 mmol/L) solutions of NO were prepared on the day of the experiment by dissolving NO in deoxygenated distilled water and were stored in gas-tight glass vials, as described previously.22 The gas space above the NO solution was filled with NO gas and closed with a pierceable, self-sealable cap (Alltech Associates, Australia). Immediately before microinjection, the cap was pierced by the injector and the NO solution was back-filled from the injector tip. The NO gas from the headspace was used to make a 1-mm-long gas bubble inside the injector to separate the NO solution from distilled water filling the microinjecting system.

In each rabbit, the location of injection sites within the RVLM was confirmed functionally (using microinjections of 3 to 5 nmol of glutamate) and histologically, as described previously.19,20,21 Baroreflexes were evaluated before and 10 to 20 minutes after bilateral microinjections of 10 nmol of a NOS inhibitor Nω-nitro-arginine methyl ester (l-NAME), its inactive enantiomer d-NAME, sGC inhibitors, methylene blue (MB) and 1-H-1,2,4-oxadiaolo[4,3-a]quinoline-1-one (ODQ; 250 pmol), or co-microinjections of l-NAME and superoxide scavenger tempol (20 nmol). In additional experiments, baroreflexes were tested before and after microinjections of an nNOS inhibitor 7-nitroindazole (7-NI, 500 pmol) and iNOS inhibitors, S-methylisothiourea (SMT; 250 pmol) and aminoguanidine (AG; 250 and 2500 pmol). Each rabbit was subjected to 1 to 2 different treatments per experiment in 3 to 4 experiments separated by 1 to 2 days. In the case of 2 treatments on the same experimental day, a 3-hour period was allowed between treatments and full recovery of baroreflex function was observed before proceeding to the next treatment. The order of treatments was randomized between and within experiments. The selected doses of other drugs were based on preliminary experiments and on previous studies from other laboratories.5,8,10 All drugs were obtained from Sigma and dissolved, except 7-NI, SMT, and ODQ, in Ringers solution (Baxter). 7-NI, SMT, and ODQ were dissolved in 10% dimethyl sulfoxide in Ringers.

Statistical Analysis
All values are expressed as mean±SEM. Data were analyzed by repeated measures ANOVA. Orthogonal partitioning of the sums of squares was used for determination of the effects of test agents on resting and reflex parameters.23 Statistical significance was set at a value of P<0.05.

Results
Microinjection of NO and NO Donors Into the RVLM
Unilateral microinjections of aqueous NO solution (20, 100, and 200 pmol; n=3 to 4 for each dose) into the functionally identified pressor region of the RVLM evoked a rapid and dose-dependent increase in MAP (Figure 1A). The hypertensive effect of NO was confined to the RVLM and was strongly correlated with that of glutamate (linear regression analysis: R=0.88, P<0.01, n=6). Similarly, microinjections of NO donors, S-nitroso-N-acetylpenicillamine (SNAP), and sodium nitroprusside (5, 10, and 20 nmol, unilaterally; n=4 to 6) into the RVLM but not adjacent regions of the ventrolateral medulla dose-dependently increased MAP (Figure 1B). Control microinjections of light-decomposed SNAP24 or N-acetylpenicillamine (20 nmol; n=5 to 6) into the RVLM had little effect on hemodynamic parameters (Figure 1B). Microinjection of a sGC inhibitor MB (10 nmol)
into the RVLM decreased the hypertensive effect of SNAP by 39\% (n=5, P<0.05; Figure 1B).

Microinjection of L-NAME and Co-microinjection of L-NAME and Tempol Into the RVLM

Bilateral microinjections of L-NAME (10 nmol, n=9), but not D-NAME (10 nmol, n=7), into the RVLM selectively attenuated the RSNA baroreflex gain. Co-injections of tempol (20 nmol, n=5) did not alter the effect of L-NAME on the baroreflex. Circles on baroreflex curves represent resting values. Dots on right panels represent the gain values obtained from each rabbit before and after treatment. *Significantly different from corresponding control responses.

Microinjection of sGC Inhibitors Into the RVLM

Bilateral microinjections of sGC inhibitors, MB (10 nmol, n=6) and ODQ (250 pmol; n=5), into the RVLM did not change resting hemodynamic or sympathetic parameters (Table and Figure 4). Similarly to L-NAME, MB and ODQ decreased the RSNA baroreflex gain by 42\% and 55\%, respectively (P<0.01), without altering the range or upper plateau of this reflex (Figure 4). The HR baroreflex gain was not different before and after microinjection of MB (−7.4±1.0 bpm/mm Hg and −8.5±1.4 bpm/mm Hg, respectively) or ODQ (−6.5±0.8 bpm/mm Hg and −5.8±2.1 bpm/mm Hg, respectively).
bpm/mm Hg, respectively). In 3 additional rabbits, microinjection of ODQ just rostral to the RVLM (at the level of the caudal facial nucleus) had little effect on the RSNA and HR baroreflex gain (average change: \(-4 \pm 16\%\) and \(-7 \pm 20\%\), respectively).

The pressor response to unilateral microinjection of glutamate into the RVLM (+35±4 mm Hg, n=8) was attenuated by 36±8% after MB (P<0.01). Likewise, local pre-injections of ODQ decreased the pressor effect of glutamate from 31±4 mm Hg to 15±6 mm Hg (n=4; P<0.05).

Microinjection of nNOS and iNOS Inhibitors Into the RVLM

Bilateral microinjections of a nNOS inhibitor 7-NI (500 pmol, n=6), iNOS inhibitors, SMT (250 pmol, n=6), and AG (250 pmol and 2.5 nmol; n=3), or vehicle (10% dimethyl sulfoxide, n=4) into the RVLM did not alter resting hemodynamic and sympathetic parameters (Table and Figure 5A). Microinjections of SMT into the RVLM did not change statistically the RSNA baroreflex parameters (Figure 5B). Likewise, the RSNA baroreflex gain was not different before and after local microinjections of 250 pmol of AG (\(-8.8 \pm 3.9\) nu/mm Hg and \(-9.1 \pm 2.9\) nu/mm Hg, respectively). The higher dose of AG also had little effect on the sympathetic gain (\(-9.5 \pm 3.5\) nu/mm Hg and \(-10.9 \pm 2.7\) nu/mm Hg, before and after injection, respectively). The HR baroreflex remained unaltered after microinjections of either SMT or AG into the RVLM (not shown). Conversely, microinjection of 7-NI into the RVLM decreased the RSNA baroreflex gain by 43±10% (P<0.01), without affecting the operational range of this reflex (Figure 5B). In 2 other rabbits, microinjection of 7-NI just rostral to the RVLM did not alter the gain of the RSNA baroreflex (not shown). Bilateral microinjections of vehicle into the RVLM did not alter the RSNA baroreflex (Figure 5B) or the HR baroreflex (data not shown).

**Discussion**

This study demonstrates for the first time that NO in the RVLM is important in the normal regulation of arterial baroreflexes. The current results thus extend recent experimental findings that overexpression of NOS in the RVLM improves impaired baroreflex function in such pathological states as hypertension and heart failure.\(^{15,16}\) The present data also suggest that although NO exerts predominately excitatory actions in the RVLM of conscious rabbits, these actions play little role in the tonic support of blood pressure under normal physiological conditions. This minor role is unlikely to be attributable to opposing effects of nNOS-derived and iNOS-derived NO on RVLM vasomotor neurons.

In the current study, microinjection of NO donors, SNAP and sodium nitroprusside, dose-dependently increased blood pressure. The excitatory effect of SNAP was dependent on the presence of releasable NO group, because light-decomposed SNAP and N-acetylpenicillamine (the non-S-nitrosylated starting material for SNAP synthesis)\(^{22}\) did not change blood pressure. Furthermore, the distinct pressor effect of microinjections of NO itself strongly indicates its excitatory role in the RVLM of conscious normal rabbits.
This finding is in line with most previous studies, which reported that NO donors, unless given in high doses, evoke neuronal excitation in the RVLM.\textsuperscript{5,6,8,9} The present results indicate that nNOS-derived NO in the RVLM serves to sensitize the baroreflex and thus to increase the sympathetic response to a given change in blood pressure.\textsuperscript{21} Previous studies from this laboratory have shown that blockade of ionotropic EAA receptors in the RVLM by kynurenic acid attenuates the gain, but not the operational range of the RSNA baroreflex in rabbits.\textsuperscript{21} The modulatory action of NOS inhibitors on the RSNA baroreflex in the current study is remarkably similar to these earlier findings. In view of an essential role of the NO/sGC pathway in glutamatergic transmission in the RVLM,\textsuperscript{5,6,10} as also confirmed in the current study, these similarities suggest that this pathway may underlie the EAA-driven modulation of the sympathetic baroreflex. The selective inhibitory effect of the sGC blockers, ODQ and MB, on the RSNA baroreflex gain is in line with this possibility.

Apart from the activation of sGC, NO can readily react with superoxide (O$_2^-$) to form peroxynitrite.\textsuperscript{3} Peroxynitrite in turn can stimulate, in a SOD-dependent manner, the release of GABA in neuronal cultures,\textsuperscript{3} thereby providing an alternative way for NO to influence neurotransmission. However, this mechanism is unlikely to play a role in normal baroreflex modulation by NO in the RVLM, because local injections of SOD mimetics, tempol and tiron, did not alter baroreflexes in conscious rabbits.\textsuperscript{25} Further, in the present study, local co-injections of l-NAME and tempol decreased the RSNA baroreflex gain in the same extent, as did l-NAME alone, suggesting that NO controls this reflex independently of O$_2^-$. It does not appear that this was simply caused by the inability of co-injections to change the NO/O$_2^-$ balance in the RVLM, because co-administration of l-NAME altered the tempol-dependent tachycardic response to stress.\textsuperscript{25} Thus, given the rapid inactivation of NO by O$_2^-$, the mechanism of this functional independence remains to be determined. It has been suggested that transferring NO to target neurons is mediated in part by stabilizing agents, such as S-nitrosothiols, which are resistant to inactivation by O$_2^-$.\textsuperscript{26} The recent finding that S-nitrosoglutathione mimics excitatory actions of NO donors in the RVLM of conscious rats\textsuperscript{7} indicate that such a stabilizing mechanism may be important in NO signaling in this region.

In the current study, microinjections of NOS and sGC inhibitors into the RVLM did not alter the HR baroreflex. This finding is in contrast to the inhibitory effect of kynurenic acid on this reflex observed in the same preparation earlier.\textsuperscript{21} Together these data indicate that the EAA-sensitive, but NO/sGC-insensitive, mechanism may specifically modulate the cardiac baroreflex in the RVLM. It is plausible that this signaling mechanism is also O$_2^-$-insensitive, because local microinjections of tempol alone\textsuperscript{25} or tempol and l-NAME did not alter the HR baroreflex. The present results thus suggest that sympathetic and cardiac baroreflexes are independently regulated by distinct signaling systems in the RVLM.

Recent studies by Chan et al.\textsuperscript{9,27} indicate that nNOS and iNOS tonically exert opposing actions on RVLM vasomotor neurons in anesthetized rats. Specifically, these investigators have shown that microinjections of low doses of several nNOS inhibitors, including 7-NI, into the RVLM decrease blood pressure, whereas local injections of iNOS inhibitors, and in particular SMT and AG, evoke pressor responses.\textsuperscript{9,27} However, these opposing tonic influences are unlikely to take place in the RVLM of conscious, chronically instrumented...
rabbits because SMT, AG, and 7-NI did not alter resting blood pressure or RSNA. The dose of SMT and AG used in the present experiments was the same as that which produced a sustained pressor response in the aforementioned studies. Further, the dose of 7-NI was similar to that which selectively inhibited nNOS in the RVLM of anesthetized rats. This dose has also been shown to effectively attenuate the EAA-evoked increase in blood pressure and extracellular NO levels in the RVLM of cats. In addition, local microinjections of 7-NI at the dose as low as 5 pmol also did not change blood pressure in pilot experiments. The disparity between the studies, apart from species differences, may relate to the influence of anesthesia and surgical stress, which can rapidly activate iNOS. Another important difference is the use of artificial ventilation in the previous works, which has been shown to increase excitatory drive in the RVLM via activating vagal and chest wall afferents. Because NO acts largely via presynaptic modulation of transmitter release, the difference between the studies suggests that its effect may vary according to the functional state of afferent inputs to RVLM presympathetic neurons. In particular, the current data indicate that the effect of nNOS-derived NO on blood pressure depends on the activity of the inputs from baroreceptor afferents. By contrast, local iNOS-derived NO does not appear to affect blood pressure in conscious rabbits either at rest or during baroreceptor stimulation. In summary, the current results suggest that nNOS-derived NO facilitates synaptic baroreflex transmission in the RVLM, at least in part, via a sGC-dependent, O2−-independent pathway in conscious rabbits. In conjunction with previous findings, these results also indicate that this NO-dependent modulation of the sympathetic baroreflex can be initiated by activation of EAA receptors in the RVLM. The present data, however, suggest that NOS inhibition in the RVLM has little effect on resting blood pressure in conscious normal rabbits and this cannot be attributed to opposing actions of nNOS-derived and iNOS-derived NO on the local vasomotor neurons.

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References
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