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The myth of nitric oxide in central cardiovascular control by the nucleus tractus solitarii

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Abstract

Considerable evidence suggests that nitroxidergic mechanisms in the nucleus tractus solitarii (NTS) participate in cardiovascular reflex control. Much of that evidence, being based on responses to nitric oxide precursors or inhibitors of nitric oxide synthesis, has been indirect and circumstantial. We sought to directly determine cardiovascular responses to nitric oxide donors microinjected into the NTS and to determine if traditional receptor mechanisms might account for responses to certain of these donors in the central nervous system. Anesthetized adult Sprague Dawley rats that were instrumented for recording arterial pressure and heart rate were used in the physiological studies. Microinjection of nitric oxide itself into the NTS did not produce any cardiovascular responses and injection of sodium nitroprusside elicited minimal depressor responses. The S-nitrosothiols, S-nitrosoglutathione (GSNO), S-nitrosoacetylpenicillamine (SNAP), and S-nitroso-D-cysteine (D-SNC) produced no significant cardiovascular responses while injection of S-nitroso-L-cysteine (L-SNC) elicited brisk, dose-dependent depressor and bradycardic responses. In contrast, injection of glyceryl trinitrate elicited minimal pressor responses without associated changes in heart rate. It is unlikely that the responses to L-SNC were dependent on release of nitric oxide in that 1) the responses were not affected by injection of oxyhemoglobin or an inhibitor of nitric oxide synthesis prior to injection of L-SNC and 2) L- and D-SNC released identical amounts of nitric oxide when exposed to brain tissue homogenates. Although GSNO did not independently affect blood pressure, its injection attenuated responses to subsequent injection of L-SNC. Furthermore, radioligand binding studies suggested that in rat brain synaptosomes there is a saturable binding site for GSNO that is displaced from that site by L-SNC. The studies suggest that S-nitrosocysteine, not nitric oxide, may be an interneuronal messenger for cardiovascular neurons in the NTS.

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Over the past 15 years a new concept of signal transduction has arisen as a result of studies suggesting that the radical nitric oxide (NO·), generated by constitutive nitric oxide synthase (NOS), may participate in endothelium-derived relaxation (1), inhibition of platelet aggregation (2), and interneuronal transmission of signals (3). A soluble gas passing freely through cell membranes to act at the cytoplasmic enzyme soluble guanylate cyclase (sGC), which served as a "receptor" (4), was considered extraordinary (5,6) but gained wide acceptance (6). Now there are numerous studies that support an important role for NO· in synaptic transmission both in the central and in the peripheral nervous system (3,7). While the understanding of signal transduction mechanisms underlying such transmission is incomplete, activation of sGC with formation of cyclic GMP (cGMP) is known to play a role (8).

Earlier studies suggested that NO· is released by central neuronal processes that are stimulated by activation of the N-methyl-D-aspartate (NMDA) receptor (9,10) and that NMDA receptor activation is associated with activation of sGC and formation of cGMP (11). We have previously shown that NMDA receptors within the nucleus tractus solitarii (NTS) are integral to the baroreceptor reflex (12), and others have suggested that nitroxidergic mechanisms may play a role in cardiovascular control through the NTS (13-17). We have sought to test this hypothesis and determine if NO· released from donor compounds elicits cardiovascular responses when administered into the NTS, the primary site of termination of vagal and glossopharyngeal cardiovascular afferent nerves.

Table 1 - Effects of NO· donors injected into the NTS.

	Depressor	No effect	Pressor
SNC	++		
SNP	±		
NO·	±		
GSNO		+	
SNAP		+	
GTN			±

In our initial studies we assessed effects of unilateral microinjection (25-50 nl) into the NTS of S-nitrosocysteine (SNC), an S-nitrosothiol known to release NO·. The compound elicited cardiovascular responses that were qualitatively similar to those produced by injection of glutamate or NMDA into the NTS (12,18,19). These findings have been replicated in conscious rats (20,21). Our results seemed consistent with the hypothesis, but we studied the effects of microinjecting other NO· donors at homologous sites in the NTS. Regardless of the doses used (10-1000 pmol), microinjection of S-nitrosoglutathione (GSNO), S-nitrosoacetylpenicillamine (SNAP), sodium nitroprusside (SNP) or glyceryltrinitrate (GTN) into the NTS did not produce responses like those produced by S-nitrosocysteine (250 pmol). In fact, glyceryltrinitrate actually elicited small pressor responses and never depressor responses. Microinjection of a concentrated solution of NO· itself did not significantly alter either arterial pressure or heart rate (see Table 1). We confirmed that the injectate contained NO· by making similar injections from the same injection system into a closed tube filled with nitrogen and then assessing the presence of NO· in the tube by the chemiluminescence technique (22).

Because SNC might be acting through a receptor independent of the release of NO·, we sought to determine if responses following microinjection of S-nitroso-L-cysteine (L-SNC) differed from those elicited by S-nitroso-D-cysteine (D-SNC). L-SNC elicited dose-dependent (10 to 250 pmol) transient depressor and bradycardic responses that were significantly greater than those elicited by injection of the dextro-isomer D-SNC at the same site (23). We found that identical amounts of NO· were released from L- and D-SNC when exposed to homogenates of whole brain tissue. Therefore, the different responses elicited by L- and D-SNC were not the result of release of different amounts of NO· by the two isomers. Note that

S-nitrosothiols were always freshly prepared by standard techniques (24) prior to each experiment. Synthesis was confirmed by spectrophotometry.

To further test if NO· released by L-SNC into the extracellular space mediated responses to the S-nitrosothiol, we studied the effects produced by L-SNC before and after injection of oxy- (Fe^{2+}) hemoglobin at the same site in NTS. Doses of 5 (N = 5) and 40 (N = 6) pmol oxyhemoglobin had no effect on responses to L-SNC (250 pmol). The 40-pmol dose would have been sufficient to scavenge as much as 160 pmol of NO· and should have significantly reduced responses to L-SNC were they the result of actions of NO·.

Because cleavage of an S-nitrosothiol yields not only nitrogen monoxide but also a disulfide, we sought to determine if cystine, the disulfide product of SNC breakdown, was reponsible for the actions of L-SNC. Microinjection of cystine (50-500 pmol; N = 4) into the NTS did not elicit any significant cardiovascular responses. However, microinjection of the parent thiol, L-cysteine, (N = 6) produced dose-dependent (threshold dose 50 pmol; maximally effective dose 500 pmol) cardiovascular responses like those produced by L-SNC. Unlike responses to L-SNC (250 pmol), whose effects were not altered by prior injection of excitatory amino acid antagonists, responses to L-cysteine (200 pmol) were abolished by injection of kynurenic acid into the NTS. The dose of 1 nmol kynurenic acid selectively blocks responses to ionotropic excitatory amino acid agonists (12,19).

If L-SNC were acting in some way through the NMDA receptor complex, it might have led to activation of sGC in target cells through synthesis of NO· (10). We found that responses to L-SNC were significantly reduced by prior microinjection of methylene blue (250 pmol), which inhibits sGC and formation of cGMP (25). Bilateral microinjection of methylene blue into the NTS also attenuated the Bezold Jarisch reflex elicited by intravenous infusion of serotonin (26). However, methylene blue may also inhibit NOS (27). Therefore, we sought to determine if the blockade of NOS by injection of L-nitroarginine methyl ester (L-NAME; 1 µmol) into the NTS altered responses to L-SNC (250 pmol) injected at the same site up to 60 min later. The inhibitor had no effect on responses to L-SNC (250 pmol).

In further studies we confirmed that mechanisms for biosynthesis of NO· are present in the NTS (28). The presence of NOS in NTS neurons and terminal fields was demonstrated by 1) staining for NADPH diaphorase with nitroblue tetrazolium that has been shown to correlate generally with other methods for visualizing the location of NOS (29-31); 2) immunohistochemical methods with an antibody (bNOS antibody, Transduction Labs, Lexington, KY, USA) to rat neuronal NOS, and 3) *in situ* hybridization for NOS mRNA with a cDNA probe kindly provided by Dr. David Bredt. Such evidence would provide indirect support for the synthesis of S-nitrosothiols that rapidly form by nitrosation of thiol groups by NO^+, one of the redox products of NO· itself (32,33). Direct evidence for the synthesis of S-nitrosothiols by the brain has also been provided by a recent report (34). The physiological relevance of nitroxidergic pathways in the NTS has been suggested by others who have shown that NOS is reduced in the NTS after removal of a nodose ganglion (14,17). Thus, vagal afferents, some of which contribute to cardiovascular reflex transmission, may have the capacity to synthesize NO·, and by implication, S-nitrosothiols.

We are seeking to determine if these S-nitrosothiols bind at specific sites in the CNS. Because SNC is very labile and cysteine is not currently available as a [^{3}H]-labeled precursor, we have used [^{3}H]-GSNO as a more stable (35) labeled ligand. Using established radioreceptor binding assays, we identified specific, saturable binding of [^{3}H]-GSNO to crude synaptic membrane fractions from whole brain homogenates (36). Unlabeled GSNO displaced binding of the radiolabeled ligand.

L-SNC competed with [^{3}H]-GSNO for binding but to a lesser extent than did GSNO (36).

Thus, [^{3}H]-GSNO may bind to the same site as L-SNC but may have a different affinity for the binding site. However, because there was significant breakdown of SNC during incubation for displacement experiments, it is likely that our experiments underestimated binding affinity of SNC at the GSNO binding site.

In contrast to displacement effected by L-SNC, agonists and antagonists acting at glutamate receptors, including L-glutamate, CNQX, 2-AP-5, AP-3, and MK801, did not displace [^{3}H]-GSNO from its binding site. Thus, [^{3}H]-GSNO binding sites differ from glutamate receptors. This result agrees with the lack of effect of kynurenic acid on cardiovascular responses elicited by L-SNC in the NTS.

Since GSNO did not elicit cardiovascular responses when injected into the NTS but competed with SNC for binding, we sought to determine if GSNO may be an endogenous antagonist for S-nitrosothiol binding sites. Our preliminary studies show that responses to SNC injected into the NTS are significantly reduced by microinjection of GSNO at the same site.

Most studies of nitroxidergic mechanisms in the NTS have used indirect means to assess the physiologic relevance of these mechanisms in cardiovascular reflex control. For example, L-arginine injected into the NTS has been shown to decrease arterial pressure and heart rate (16). Because L-arginine is the substrate upon which NOS acts to synthesize NO· this study was interpreted as supporting a role for NO· in cardiovascular control by the NTS. However, as previously mentioned, an S-nitrosothiol formed in the NTS as a result of NO· synthesis could well have been responsible for the responses seen. There are abundant thiols in biological tissues (37) to support such a biosynthetic mechanism. Others have promoted a role for NO· in the NTS because of the actions of NOS inhibitors administered into the nucleus (13,38), but the difficulty in measuring authentic NO· and SNC in biological tissues (34) makes the direct application of these findings to NO· as the end product of NOS risky as well.

Our studies suggest that cardiovascular and autonomic effects elicited by administration of NO· precursors or inhibitors of NOS may relate to effects on production of S-nitrosothiols such as SNC. SNC may exert its action through binding to a site on target cell membranes. In contrast, it is acknowledged that classic receptors for NO· itself do not exist. Thus for NO· it is unclear how specificity of action might occur. Mathematical models suggest that NO· may diffuse from 100 to 1000 µm from a point source (5,39,40), which therefore could affect 2 million or more synapses (5). Specificity of action could occur if NO·-generating, NOS-positive nerve elements are juxtaposed to nerve elements that contain sGC and form cGMP (4). Indeed, presynaptic terminals containing one of the enzymes are often adjacent to postsynaptic membranes that contain the other (4), but exceptions have been described (31,41).

In fact, if NO· diffuses great distances from its site of release, close apposition of the source with the site of action would be unnecessary. On the other hand, a terminal poised next to a target cell membrane would be consistent with release of a transmitter, perhaps an S-nitrosothiol, that acts at a more traditional receptor. If this were true, NO· could play a role both as a freely diffusable agent in solution and as a constituent of a more classic transmitter.

The lability of NO· would be one factor limiting the extent of its effects. With a half life generally estimated to be only a matter of seconds (42), NO· forms peroxynitrites, NO^+ and NO^- (33,43). One of the potential mechanisms of action of NO· relies upon a one-electron transfer and formation of the nitrosonium NO^+ ion which, unlike NO·, readily nitrosylates thiols to form S-nitrosothiols (32,33). This nitrosylation of thiols may provide another avenue for specific actions of NO·. For example, nitrosylation of the cysteine redox site in the ion channel linked to the NMDA receptor (44) may be the mechanism by which NO· affects that channel (45). On the other hand, thiols, particularly cysteine, glu-

tathione, and protein thiols, are major components of biological systems where they are found in concentrations of 1-10 mM (37). Thus, rapid formation of S-nitrosothiols could be expected upon synthesis of NO· (37). The S-nitrosothiols could concentrate in the cell of origin, the extracellular fluid, and in target cells as well. At any one of these sites they could serve as a reservoir of NO· (46). Recent studies have confirmed that the brain synthesizes S-nitrosothiols when provided with native thiols (34). S-nitrosothiol synthesis would naturally occur in regions where NO· itself is synthesized through actions of NOS on L-arginine (47).

These endogenous S-nitrosothiols may participate in cell to cell signaling independent of their release of NO· (48). Like NO· these compounds are also labile but, unlike NO·, they may be stored and released in response to stimuli (49). The compounds produce many of the same physiological responses as NO· but their physiological activity is not related to the speed with which they release NO· (48). Some S-nitrosothiols pass quickly through cell membranes (24) and, like NO·, activate sGC (24,49), but membrane transport is not essential for their action (24).

In conclusion, although our studies do not rule out a role for NO· itself in transmission in the NTS, they do suggest a possible direct role for S-nitrosothiols in addition to being an NO· donor. We conjecture that S-nitrosothiols, acting at distinct binding sites, participate in transmission of cardiovascular reflex signals in the NTS of rats. It is unclear whether S-nitrosothiols contribute similarly to signal transduction at other central sites.

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