Cardiovascular responses to substance P in the nucleus tractus solitarii: microinjection study in conscious rats

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Abdala, Ana Paula L., Andrea S. Haibara, and Eduardo Colombari. Cardiovascular responses to substance P in the nucleus tractus solitarii: microinjection study in conscious rats. Am J Physiol Heart Circ Physiol 285: H891–H898, 2003. First published May 8, 2003; 10.1152/ajpheart.00869.2002.—The cardiovascular effects of substance P (SP) microinjections in the nucleus tractus solitarii (NTS) were evaluated in conscious rats. We chose this model because it is an effective way to access some of the cardiovascular effects of neurotransmitters in the NTS without the inconvenience of blunting pathways with anesthetic agents or removing forebrain projections by decerebration. The cardiovascular responses to SP injections were also evaluated after chronic nodose ganglionectomy. We found that, in conscious rats, SP microinjections into the NTS induced hypertension and tachycardia. Unilateral and bilateral SP injections into the NTS caused a slow increase in blood pressure and heart rate that peaked 1.5–5 min after injection and lasted for 20–30 min. Nodose ganglionectomy increased the duration of the pressor and tachycardic effects of SP and enhanced the pressor response. These data show that SP in the NTS is involved in pressor pathways. The supersensitivity to SP seen after nodose ganglionectomy suggests that vagal afferent projections are involved in those pressor pathways activated by SP in the NTS.

NUCLEUS TRACTUS SOLITARI (NTS) are the sites of termination of primary visceral sensory fibers, which convey information from cardiac vagal receptors, baroreceptors, and chemoreceptors (5). These nuclei are densely innervated by substance P (SP)-like immunoreactive (SP-LI) nerve terminals, some of which originate from the vagus and glossopharyngeal nerves (9, 11, 12, 18). SP-LI is also found in the nodose and petrosal ganglia (12, 45). SP binding sites are present in the dorsal motor nucleus of the vagus (DMNX). The distribution of neuropeptide-1 (NK-1) receptors differs throughout different parts of the DVC. The highest density was detected at intermediate levels of both NTS and DMNX (1). Part of the content of SP in the NTS also derives from trigeminal afferents and terminal projections from the medullary raphe nuclei (30, 40, 43).

It has been suggested that SP is a neurotransmitter or neuromodulator of baroreceptor and chemoreceptor afferents in the NTS (30). For instance, electrical stimulation of aortic depressor nerves elicited a threefold increase in SP release in the NTS (26). In addition, SP postsynaptically potentiated glutamate-induced currents in DMNX neurons (23). It was suggested that SP might be released with glutamate as a cotransmitter (30).

In an autoradiographic study, no change in SP binding was detected in the DVC after unilateral nodose ganglionectomy or vagotomy (17). This would suggest that SP might not be a major neurotransmitter in these pathways. In contrast, later studies have shown increases in the number of SP binding sites after nodose ganglionectomy (36) or transection of the carotid sinus nerve (8).

Previous attempts to elucidate SP function in the NTS using microinjection techniques in the anesthetized rat have produced contradictory results. Some studies have reported hypotension and bradycardia (10, 20), whereas others have shown an increase in blood pressure and tachycardia (13) or no effect at all (39). It is possible that some of this variation is due to the presence of anesthetic agents. For example, the pressor response elicited by glutamate microinjection into the NTS of conscious rats is reversed after anesthesia (25). It is unknown whether the response to SP microinjections into the NTS is influenced by anesthetic agents.

To clarify the functional effects of SP in the NTS without the influence of anesthetic agents, in this study, we used a microinjection technique in nonanesthetized rats to determine 1) the cardiovascular effects elicited by unilateral and bilateral SP microinjections; and 2) whether chronic unilateral nodose ganglionectomy would lead to increased sensitivity to ipsilateral SP microinjection into the NTS.

MATERIALS AND METHODS

SP was obtained from Research Biochemical International (Natick, MA) and dissolved in O2- and CO2-free distilled water. The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
Male Wistar rats (250–300 g body wt) were obtained from the central animal house of the Universidade Federal de São Paulo-Escola Paulista de Medicina. All protocols were approved by the Medical Ethics Committee of the Universidade Federal de São Paulo. The rats were housed in individual cages in a controlled environment and had free access to commercial rat chow and tap water.

**Nodose ganglionectomy.** Five days before the experiments, the animals were anesthetized with 1.5–2% halothane diluted in 100% oxygen. The right nodose ganglion was exposed through a ventral midline cervical incision, retracted caudally to expose the entire ganglion, transected at the rostral and caudal poles, and removed. The layers of muscle were sutured. After surgery, the animals received intramuscular injections of penicillin G (24,000 IU) and streptomycin (10 mg). Sham-operated rats were subjected to the same procedure on the same day, but the ganglion was left intact. The aim of the nodose removal was to determine the importance of pathways in the NTS receiving vagal projections to the pressor responses elicited by SP because it is known that NTS also receives SP-containing projections from other nuclei in the central nervous system.

**Brain cannulas and microinjection.** Four days before the experiments, the animals were anesthetized with tribromoethanol (250 mg/kg ip), placed in a stereotaxic apparatus, and received bilateral guide cannulas in the direction of the intermediate NTS with the use of the stereotaxic coordinates used by Paxinos and Watson (33) and a technique used by Colombari et al. (6). A partial occipital craniotomy was performed to expose the cerebellum, through which two 15-mm-long stainless steel guide cannulas (23 gauge) were introduced in a perpendicular direction 14.0 mm caudal to the bregma, 0.5 mm lateral to the midline, and 7.9 below the skull surface of the bregma. The tip of the guide cannula aimed 1.0 mm above the NTS. The cannulas were fixed to the skull with methyl methacrylate polymer and screws and sealed with a 15-mm-long stainless steel occluder until the start of the experiment. To inject SP, a 33-gauge needle connected by polyethylene-10 (PE-10) tubing to a 1-μl syringe (Hamilton; Reno, NV) was used. The needles were 1.0 mm longer than the guide cannulas. On the day of the experiments, the placement of the cannula was functionally confirmed by microinjection of glutamate (L-Glu) (5 nmol/100 nl), which elicited a pressor response and bradycardia, as reported previously (6, 25).

**Arterial cannulas and recording of blood pressure.** Twenty-four hours before experiments, rats were again anesthetized with (1.5–2%) halothane diluted in (100%) oxygen and instrumented with PE-10 arterial cannulas. The cannulas were filled with heparinized saline (50 U/ml), introduced through a femoral artery, advanced into the lower abdominal aorta, sealed with a steel pin, and then tunneled subcutaneously at an exit point to the nape of the neck.

For experiments, the arterial cannula was connected to a pressure transducer (model P23 ZL, Statham Gould; Oxnard, CA). Pressure was recorded on a polygraph (model 7D, Grass Instruments; Quincy, MA). Mean arterial pressure (MAP) was determined by feeding the signal into the 7D-driver amplifier with the half-amplitude frequency adjusted to 0.5 Hz. Heart rate (HR) was monitored by means of a cardiotoxicometer (model 7P44C, Grass Instruments) triggered by the systolic pressure rise.

The arterial pressure and heart rate of conscious freely moving rats were monitored before and after bilateral or unilateral 100-nl microinjections of SP or distilled water (vehicle). Not more than three doses of SP were microinjected in the same animal. Unilateral and bilateral microinjections were performed in different animals. When more than one dose was tested in the same animal, the doses were randomized and microinjections were performed at least 1 h apart.

**Histology.** After the experiments were completed, the injection site was marked with 100 nl of 2% Evans blue dye. Animals were then anesthetized and perfused transcardially with 1% buffered formalin. The brains were removed, placed in formalin for 48 h, followed by 20% sucrose solution for an additional 48 h. The brains were frozen in a cryostatic microtome (Jung Histoslide 2000, Leica Instruments) and 40-μm-thick coronal sections of the medulla oblongata were cut. Sections were mounted on slides and counterstained with 1% neutral red (24).

**Statistics.** Results are expressed as means ± SE (n = no. of animals). Data were analyzed by one- or two-way analysis of variance, followed by Dunnett’s or Tukey’s posttest with statistical software (SigmaStat for Windows, version 2.0, SPSS; Corte Madera, CA). Values of P < 0.05 were considered significant.

**RESULTS**

**SP microinjections into NTS of conscious rats.** Unilateral SP microinjections into the NTS of conscious normotensive rats (basal MAP = 116 ± 2 mmHg; basal

![Fig. 1. Typical recordings of changes in pulsatile and mean arterial blood pressure (AP) and heart rate (HR) after injection of vehicle and substance P (SP) (1.0 nmol/100 nl) into the nucleus tractus solitarii (NTS) of a conscious rat.](http://ajpheart.physiology.org/)
HR = 332 ± 7 beats/min; n = 37) increased MAP and HR, whereas microinjections of vehicle did not cause visible changes in these parameters (Fig. 1). Figure 2 summarizes the cardiovascular changes 5 min after the moment of injection, when the changes were maximal. Doses <0.1 nmol had no effect (P > 0.05). With higher doses, MAP started to rise after 1–2 min, and reached a plateau within 5 min (Fig. 1). The pressor response to 1.0 nmol of SP (Fig. 3B) was still detectable 10 and 20 min after the microinjection (+18 ± 3 and +14 ± 3 mmHg, respectively; P < 0.05). The duration of the response decreased proportionally with smaller doses, 10 min for 0.5 nmol of SP and 5 min for 0.2 nmol (+19 ± 5 and +9 ± 5 mmHg, respectively, P < 0.05). The tachycardic response induced by SP microinjection began within 1–2 min, reached a plateau within 5 min, and ended after 10 min for 0.5 and 1.0 nmol of SP (Fig. 3B). No behavioral responses were observed at any moment after unilateral injections of SP. Cardiovascular effects elicited by unilateral SP microinjections at a dose close to the ED50 (0.3 nmol) was completely blocked by previous injection of the NK-1 antagonist WIN-51708 (5 nmol/100 nl) at the same site (+18 ± 3 vs. +3 ± 1 mmHg and +33 ± 7 vs. −2 ± 6 beats/min, P < 0.05, n = 5). Baseline MAP and HR were not altered by unilateral WIN-51708 microinjections.

Fig. 3A summarizes cardiovascular changes after unilateral and bilateral microinjections of 0.1 nmol of SP into the NTS. Although this dose had no detectable effect in unilateral microinjections, it significantly increased MAP at 1.5 and 5 min when microinjected bilaterally (+29 ± 6 and +21 ± 4 mmHg, respectively). After the bilateral SP injections, the rats usually initiated exploratory behavior, followed by grooming, which never lasted >2 min.

Bilateral SP microinjections (1 nmol in 100 nl) in conscious normotensive rats (basal MAP = 114 ± 2 mmHg and basal HR = 324 ± 14 beats/min; n = 12) induced greater pressor responses that reached the peak more rapidly (Fig. 3B). MAP increased within 10 s after microinjections and reached a plateau within 1.5 min (+43 ± 4 mmHg, P < 0.05). The duration of the pressor response did not differ from that after unilateral microinjection. HR responses reached a peak 5 min after bilateral SP microinjections and did not differ from unilateral microinjections. However, the duration of tachycardic responses was greater for bilateral microinjections (Fig. 3B).

Fig. 3B compares the cardiovascular effects of unilateral and bilateral microinjections of 1.0 nmol of SP into the NTS of conscious rats. *P < 0.05, different from basal value (Dunnett); †P < 0.05, different from the corresponding time for unilateral microinjection (Tukey).
Effects of nodose ganglionectomy on SP-induced cardiovascular changes. Removal of the right nodose ganglion potentiated the pressor response to unilateral microinjection of SP (0.2 and 1.0 nmol) into the ipsilateral NTS (Fig. 4, B and C). Injection of 0.1 nmol of SP significantly increased blood pressure in rats submitted to ganglionectomy, and not in controls, but the difference between the groups was not significant (Fig. 4A).

Removal of the nodose ganglion did not alter the peak HR response (5 min) induced by 1.0 nmol of SP; however, tachycardic response was potentiated 10 and 20 min after the microinjection (Fig. 4C).

Control microinjections of distilled water (100 nl) into the NTS of animals submitted to nodose ganglionectomy (n = 7) or sham surgery (n = 4) failed to change MAP and HR (P > 0.1). Removal of the nodose ganglion did not change basal MAP and HR (119 ± 4 mmHg and 345 ± 10 beats/min) compared with sham-operated animals (116 ± 6 mmHg and 341 ± 21 beats/min).

Histological analysis. All animals included in this study had injection sites located in the NTS, centered at the level of the area postrema (Fig. 5, A, C, and D). In a few additional rats, injection sites were located in the DMNX, hypoglossal nucleus, adjacent areas outside the NTS (Fig. 5B), and the fourth ventricle (not shown). Injections into these sites did not cause cardiovascular changes. The centers of injection sites and spread of the injectate (100 nl) in animals that underwent nodose ganglion removal (Fig. 5C) were similar to those from animals submitted sham surgery (Fig. 5D).

DISCUSSION

The present study describes the cardiovascular effects elicited by SP microinjections into the NTS of rats in absence of anesthetic agents. Because chronic deafferentation of cardiovascular input to the NTS causes an increase in SP binding sites (8, 36), we also determined whether this morphological response could be translated into increased sensitivity to SP microinjection into the NTS of conscious rats.

The present study shows that in the conscious rat, microinjections of SP (0.1–1.0 nmol/100 nl), either unilaterally or bilaterally, induced increase in blood pressure and HR that could be detected for a relatively long period of time (at least 20 min). Lower doses of SP (0.001 and 0.01 nmol/100 nl) had no cardiovascular effects. The pressor/tachycardic effect of a dose close to the ED$_{50}$ was blocked by previous injection of a NK-1 antagonist as reported previously (44). Most studies in anesthetized animals using low doses of SP (0.00007–0.06 nmol) have reported short lasting hypotension (4,
10, 14, 15, 20, 27), whereas higher doses (0.07–7.0 nmol) induced long lasting pressor responses or had no effect (13, 27, 39). A recent study (2) in a preparation free of anesthetic agents reported increase in perfusion pressure (vasoconstriction) after SP microinjection into the NTS (0.1–100 pmol/40 nl). Taken together, these results suggest that SP effects might depend on the dose microinjected and are also influenced by the presence of anesthetic agents. A similar pattern of response is observed for l-Glu microinjections, which induce hypotension in anesthetized rats and hypertension in conscious ones (6, 25). It has been suggested that a coexistence of SP and l-Glu in primary baroreceptor and chemoreceptor afferents in the NTS (34). There is evidence that the pressor response to l-Glu in conscious rats depends on the integrity of chemoreflex pathways (7). It is possible that chemoreflex pathways are blunted in anesthetized animals, and, as a consequence, the effect of l-Glu on baroreflex pathways prevails.

The difficulty in interpreting data from microinjection studies in the NTS is due to the considerable overlap of different modality inputs. Although the NTS can be subdivided based on the cytoarchitecture and neurochemistry of certain cell groups, visceral afferent inputs that terminate in various NTS subnuclei have loose somatotopic organization (32). Consequently, local microinjections may affect multiple pathways simultaneously and the presence of anesthetic agents may affect these circuits differentially.

Even without the influence of anesthetic agents, one might consider that microinjections having effects on multiple circuits could produce results that are difficult to interpret. However, a peculiarity in the arrangement of the biochemical machinery related to tachykinin release and degradation raises the possibility that even endogenously released SP may activate different circuits lying in the vicinity. SP is believed to be inactivated in the central nervous system mainly by enzymatic degradation. It has been suggested that the principal enzyme responsible for this action is the neutral endopeptidase 3.4.24.11 (30). Interestingly, neutral endopeptidase 3.4.24.11 labeling is largely confined to the border of the NTS where light to moderate staining was observed in the region ventral to the area postrema (21). This could explain the long-lasting effects of higher doses of SP in the NTS because enzymatic degradation of the exogenous peptide would oc-

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Fig. 5. Schematic drawings of consecutive coronal sections from the medulla oblongata showing the intermediate region of the NTS. Hatched areas represent the spread of injectate (100 nl). A: overlap of unilateral injection sites from five representative rats. B: misplaced injection sites (n = 4). C: overlap of unilateral injection sites from five representative rats submitted to removal of the nodose ganglion. D: overlap of unilateral injection sites from five representative rats submitted to sham surgery. Numbers at the right indicate the distance from the bregma (in mm). AP, area postrema; sol, tractus solitarius; X, dorsal motor nucleus of the vagus nerve; XII, hypoglossal nucleus.
inhibitory interneurons (32). Injections of SP into the tractus solitarius elicited larger excitatory postsynaptic potentials in NTS neurons in the working heart-brain stem preparation, which is free of anesthetic agents, showed that ANG II potentiated Glu-induced currents (23). In the NTS, interactions between SP and Glu, a putative neurotransmitter of baroreceptor afferents (38), have been observed in central terminals from peripheral sensory receptors (30). In DMNX neurons, SP postsynaptically potentiated Glu-induced currents (23). In the NTS, 70% of the neurons excited by SP but not by angiotensin II (ANG II) were also excited by Glu, suggesting the coexistence of these two neurotransmitters in primary baroreceptor and chemoreceptor afferents (34). It is well known that SP accounts for some of the ANG II actions in the NTS (31). A microinjection study of the effects of ANG II on cardiorespiratory reflexes in the working heart-brain stem preparation, which is free of anesthetic agents, showed that ANG II potentiated sodium cyanide-induced bradycardia and this effect was abolished with the use of the NK-1 receptor antagonist (31). Electrophysiological studies showed that stimulation of the tractus solitarius elicited larger excitatory postsynaptic potentials in NTS neurons in the presence of ANG II, and this effect was also sensitive to NK1 receptor blockade (19). The authors suggested that the ANG II-induced enhancement of chemoreflex bradycardia was mediated with SP and that the potentiating actions of SP on nonbaroreceptive NTS neurons could cause an inhibition of the baroreceptor reflex via inhibitory interneurons (32). Injections of SP into the medial NTS also potentiated pressor reflex responses induced by isometric muscle contractions in cats (41). There is evidence that the activation of ergoreceptors induces release of SP in the medial NTS (42). A recent study (2) in a preparation free of anesthetic agents showed that nociceptive attenuation of baroreceptor reflex depends on endogenous release of SP acting on GABAergic neurons in the NTS. Both studies provide evidence for a participation of endogenous SP in pressor pathways in the NTS. However, another recent study in spontaneously hypertensive rats (SHR) suggested the participation of endogenously released SP in postexercise hypotension (4). Taken together, these data suggest that the SP-induced pressor/tachycardic response observed in the present study may be related to the potentiating action of this peptide on nonbaroreceptive pathways (possibly those receiving chemoreceptor input), resulting in inhibition of the baroreflex and overriding possible potentiating effects of SP in this same pathway.

The present study showed that removal of the right nodose ganglion potentiated the cardiovascular responses to injection of SP into the NTS. Deafferentation of vagal input was achieved by the removal of the nodose ganglion and thus the cell bodies of origin of visceral afferents of the vagus nerve. This procedure aims the reduction in the content and release of neurotransmitters (including SP) in the NTS, as a consequence receptors expression increases. Although other interventions, such as transection of the vagus caudal to the ganglion also lead to reduction in the release of neurotransmitters, they may not reduce significantly their content (17).

Biosynthesis of tachykinins is likely to be confined to the perikaryon and axonal transport is the sole source of SP in the nerve terminals (30). Thus, removing or killing cells of the nodose ganglion, or transection of their rostral axons, can reduce SP content in the NTS (12, 16, 27). Neuroanatomic studies have shown supersensitivity of SP receptors in the NTS either after nodose ganglionectomy or transection of the carotid sinus nerve. There is no evidence of any changes in SP content or binding sites in the NTS contralateral to the deafferentation (8, 36).

Supersensitivity may not be the only phenomenon associated with the increased tachycardic response after nodose ganglionectomy observed in this study. This procedure also removes efferent vagal fibers that originate in the nucleus ambiguus. Vagotomy reduces SP binding sites in nucleus ambiguous, suggesting that SP receptors located in this nucleus could be diminished by chromatolytic changes that occur after peripheral axotomy (17). If SP is simultaneously potentiating baroreflex and nonbaroreflex-related pathways and the latest inhibits the former overriding its effects as suggested above, then a reduction in vagal output could be responsible for the enhancement in the SP-induced tachycardic effect observed in this study. However, this does not exclude an effect on circuitry mediating cardiovascular and respiratory reflexes.
In summary, the present study shows that SP microinjections into the NTS of conscious rats increases blood pressure and heart rate. This opposes the effects observed in previous studies with anesthetized rats, considering similar doses and volumes injected. The tachycardic effect suggests an inhibition of baroreflex related pathways, probably via a potentiating action in nonbaroreflex pathways. In anesthetized animals, those pathways could be blunted, resulting in different responses to SP microinjection. We also observed supersensitivity to SP microinjection into the NTS of conscious rats 5 days after vagal deafferentation. This data further supports the hypothesis that SP has an active modulatory function in baseline conditions in normotensive animals.

Provided that neuropeptides are usually associated with long-term modulation of different integrative regions of the central nervous system that receive peripheral sensory input, dysfunctions in these modulatory systems may be associated with some chronic neurogenic diseases. For example, SHR have increased number of SP binding sites in some regions of the central nervous system, including the DVC (37). SP-like immunoreactivity is increased in the caudal NTS of SHR (28, 29). Other than this, there is little information concerning a possible contribution of SP to the pathogenesis of hypertension. Therefore, further studies in nonanesthetized animals will be necessary for a better understanding of the role of SP as a modulator of cardiovascular reflexes and as a possible contributing factor for chronic cardiovascular diseases.

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DISCLOSURES

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