Vasopressin is a major vasoconstrictor involved in hindlimb vascular responses to stimulation of adenosine $A_1$ receptors in the nucleus of the solitary tract

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ABSTRACT

Our previous study showed that stimulation of adenosine A1 receptors located in the nucleus of the solitary tract (NTS) exerts counteracting effects on the iliac vascular bed: activation of the adrenal medulla and β-adrenergic vasodilation versus vasoconstriction mediated by neural and unknown humoral factors. In the present study we investigated the relative contribution of three major potential humoral vasoconstrictors: vasopressin, angiotensin II and norepinephrine in this response. In urethane/chloralose anesthetized rats we compared the integral changes in iliac vascular conductance evoked by microinjections into the NTS of the selective A1 receptor agonist, N6-cyclopentlyadenosine (CPA, 330 pmol in 50 nl) in intact (INT) animals and following: V1 vasopressin receptor blockade (VX), angiotensin II AT1 receptor blockade (ATX), bilateral adrenalectomy + ganglionic blockade (ADX+GX, which eliminated the potential increases in circulating norepinephrine and epinephrine), ADX+GX+VX and ADX+GX+VX+ATX. In INT animals, stimulation of NTS A1 adenosine receptors evoked typical variable responses with prevailing pressor and vasoconstrictor effects. VX reversed the responses to depressor ones. ATX did not significantly alter the responses. ADX+GX accentuated pressor and vasoconstrictor responses whereas ADX+GX+VX and ADX+GX+VX+ATX virtually abolished the responses. Stimulation of NTS A1 adenosine receptors increased circulating vasopressin over 4-fold (26.4±10.4 vs. 117.0±19 pg/ml). These data strongly suggest that vasopressin is a major vasoconstrictor factor opposing β-adrenergic vasodilation in iliac vascular responses triggered by stimulation of NTS A1 adenosine receptors whereas angiotensin II and norepinephrine do not contribute significantly to the vasoconstrictor responses.
Key words: Purinergic receptors; V₁ receptor blockade; AT₁ receptor blockade; Ganglionic blockade; Adrenalectomy; Lumbar sympathectomy; Iliac vascular conductance.
INTRODUCTION

It is now widely accepted that adenosine operating via $A_1$ and $A_{2a}$ receptors modulates neural cardiovascular control at the level of the nucleus tractus solitarii (NTS) and other brainstem cardiovascular centers (2; 4; 5; 20; 31; 36; 38; 41). Under normal, physiological conditions a natural source of adenosine is ATP released synaptically from neurons as well as from glial cells activated by neighboring neurons (6; 8; 9; 14; 38). This extracellular ATP is catabolized via ectonucleotidases to adenosine which further acts more broadly as a neuromodulator operating via pre or postsynaptic $A_1$ and $A_{2a}$ adenosine receptor subtypes (8; 26; 38; 46). Under pathological conditions such as ischemia, hypoxia, and severe hemorrhage a global release of adenosine from many cell types occurs via the breakdown of intracellular ATP (25; 43; 45). Thus, adenosine which is generated in or released into the extracellular space under physiological or pathological conditions acts not via specific synapses in specific neuronal pathways but rather spatially, reaching all adenosine receptor subtypes in the vicinity; this may resemble signaling via diffusive neurotransmitters/neuromodulators like nitric oxide or carbon monoxide. This spatial aspect of the action of naturally released adenosine seems especially important for the NTS where groups of functionally different neurons usually overlap allowing for "adenosine crosstalk". Specific physiological effects exerted via nonselective, spatial spread of adenosine in the NTS may depend on differential expression of adenosine receptor subtypes on functionally distinct NTS neurons/terminals, as we suggested previously (31; 36).

In the central nervous system adenosine may inhibit or facilitate release of neurotransmitters from synaptic terminals as well as directly inhibit or activate neurons via pre- and postsynaptic $A_1$ or $A_{2a}$ receptors, respectively (8; 26; 31; 36). Since $A_1$ vs. $A_{2a}$ adenosine receptors exert contrasting effects on central neurons/terminals reciprocal effects are usually, although not always, observed in response to selective stimulation of
the two receptor subtypes. NTS A\textsubscript{1} adenosine receptor stimulation predominately yields differential regional sympathoactivation (adrenal>renal>lumbar) and pressor responses (2; 5; 30; 35). In contrast, NTS A\textsubscript{2a} receptor stimulation typically evokes depressor responses accompanied by contrasting regional sympathetic responses: decreases in renal (RSNA), no changes in lumbar (LSNA) and increases in preganglionic adrenal (pre-ASNA) sympathetic nerve activity (2; 5; 16; 32-34). Note that whereas stimulation of NTS A\textsubscript{1} and A\textsubscript{2a} receptors evoke contrasting changes in RSNA and LSNA, both adenosine receptor subtypes activate the sympathetic output to the adrenal medulla (33; 35).

Recent studies from our laboratory showed that the pressor and sympathoexcitatory responses evoked by stimulation of NTS A\textsubscript{1} adenosine receptors are mediated mostly via inhibition of baroreflex mechanisms at the level of the NTS whereas hemodynamic and differential sympathetic responses evoked by stimulation of NTS A\textsubscript{2a} adenosine receptors are mediated mostly via activation of non-baroreflex mechanisms (16; 30; 33-35). It should be stressed that A\textsubscript{1} adenosine receptors may also modulate non-glutamatergic, non-baroreflex mechanisms operating in the NTS. For example, sinoaortic denervation or ionotropic glutamatergic blockade abolished A\textsubscript{1}-adenosine-receptor-mediated increases in RSNA and LSNA whereas this attenuated, but did not abolish, the increases in pre-ASNA (35). The activation of pre-ASNA which persisted after sinoaortic and the glutamatergic blockade was most likely mediated via A\textsubscript{1} adenosine receptor modulation of non-glutamatergic pathways descending into the NTS from higher structures, such as from hypothalamic paraventricular and/or dorsomedial nuclei (12; 28; 42). A\textsubscript{1} adenosine receptors located on these descending nonglutamatergic pathways and/or NTS interneurons could selectively activate the sympathetic output to the adrenal medulla (but not RSNA and LSNA) via disinhibition of direct NTS-RVLM pathways (10). NTS A\textsubscript{1} adenosine receptors may also modulate the
control of heart rate (HR) via both baroreflex and non-baroreflex mechanisms (30; 35). Taken together these observations strongly suggest that A₁ adenosine receptors are differentially located on functionally different NTS neurons/terminals which are mostly, but not exclusively glutamatergic and involved in the baroreflex arch.

Although selective stimulation of NTS A₁ adenosine receptors evokes predominantly pressor responses (2; 5), occasionally biphasic or even depressor responses are observed (35). This variability of the responses is a natural consequence of simultaneous activation of at least two counteracting mechanisms: sympathetic vasoconstriction and β-adrenergic vasodilation mediated via epinephrine released from the activated adrenal medulla. Since β-adrenergic receptors are preferentially located in the muscle vascular (44) bed and both pre-ASNA and LSNA increase upon stimulation of NTS A₁ adenosine receptors it was likely that these two counteracting factors may significantly contribute to the variability of the iliac vascular responses. We confirmed this hypothesis in our recent study by showing that removal of the vasodilatory mechanism (via bilateral adrenalectomy as well as peripheral blockade of β-adrenergic receptors) abolished the variability of the responses normally observed in intact animals and markedly increased the pressor and hindlimb vasoconstrictor responses (21). In contrast, bilateral lumbar sympathectomy tended to increase the vasodilatory component of the responses although the variability still persisted. To our surprise, following combined adrenalectomy plus lumbar sympathectomy a marked, consistent vasoconstrictor component still persisted suggesting that some unknown humoral vasoconstrictor factor(s) are involved (21).

The most likely humoral candidates contributing to the iliac vasoconstriction are vasopressin, angiotensin II and norepinephrine. Since activation of A₁ adenosine receptors may inhibit glutamate release in baroreflex pathway at the level of the NTS
and given that the NTS is a crucial, primary relay station for tonic baroreflex inhibition of vasopressin release (10; 29; 39) it is likely that the activation of A₁ adenosine receptors may inhibit the baroreflex restraint of vasopressin release. When released, vasopressin could evoke powerful peripheral vasoconstriction. However, whether A₁ adenosine receptors are present on those NTS baroreflex neurons/terminals which inhibit the release of vasopressin is unknown. The present study was designed to test this hypothesis.

Since renal sympathetic nerve activity (RSNA) directed to the kidney has been shown to increase following stimulation of NTS A₁ adenosine receptors (35), this may facilitate the renin/angiotensin mechanism leading to humoral vasoconstriction of the hindlimb vasculature mediated via AT₁ angiotensin II receptors (11). In addition, since stimulation of NTS A₁ adenosine receptors increases the activity of various sympathetic outputs (35) it is also possible that circulating norepinephrine released from other synaptic terminals may reach the iliac vasculature and cause vasoconstriction. Therefore, in the present study we investigated the extent to which these three potential humoral vasoconstrictors (vasopressin, angiotensin II and/or norepinephrine) contribute to NTS-A₁-adenosine-receptor-elicited iliac vasoconstriction (21).

MATERIAL AND METHODS

All protocols and surgical procedures employed in this study were reviewed and approved by the institutional Animal Care and Use Committee and were performed in accordance with the Guiding Principles in the Care and Use of Animals endorsed by the American Physiological Society and published by the National Institutes of Health.
Design

This study investigates further the mechanisms responsible for the consistent variability of hemodynamic responses elicited by activation of adenosine A₁ receptors in the NTS (21; 35). Previously we showed that the variability of the pressor/depressor and iliac vasoconstrictor/vasodilator responses is not a simple effect of competitive interactions between sympathetic vasoconstriction vs. β-adrenergic vasodilation but some unknown, powerful, humoral vasoconstrictor factor(s) are also involved (21). Therefore, the present study assessed the relative contribution of potential humoral vasoconstricting factors to the iliac vascular responses evoked by selective stimulation of NTS A₁ adenosine receptors. Experiments were performed on a total of 102 male Sprague Dawley rats. In 63 rats we compared the relative vasoconstrictor effects potentially mediated via vasopressin, angiotensin II, norepinephrine and sympathetic innervation of the hindquarters, the effects normally opposed by simultaneous β-adrenergic vasodilation mediated via activation of the adrenal medulla. In an additional 20 rats, respective time controls were performed and in 6 rats the effectiveness of vasopressin and angiotensin II receptor blockades was assessed. These functional experiments strongly suggested that the major vasoconstrictor factor triggered by activation of adenosine A₁ receptors in the NTS may be vasopressin. Therefore, in an additional group of 13 animals the levels of circulating vasopressin were evaluated before and following microinjections into the NTS of the selective A₁ adenosine receptor agonist, N⁶-cyclopentlyadenosine (CPA) or vehicle control.

Instrumentation and measurements

All the procedures were described in detail previously (4; 19; 21; 32-35). Briefly, male Sprague-Dawley rats (350-400 g, Charles River) were anesthetized with a mixture of α-chloralose (80 mg/kg) and urethane (500 mg/kg, ip), tracheotomized, connected to
a small animal respirator (SAR-830, CWE, Ardmore, PA) and artificially ventilated with 40% oxygen 60% nitrogen mixture. Catheterization of the right femoral artery and vein were performed to monitor arterial blood pressure and infuse drugs, respectively. Arterial blood gases were tested occasionally for appropriate experimental values (Radiometer, ABL500, OSM3). Averaged values measured at the end of each experiment were the following: pH = 7.38±0.01, Po2 = 140.1±3.8 mmHg, and Pco2 = 36.2±0.7 mmHg.

From a mid-abdominal incision, the left iliac artery was exposed. A pulse Doppler blood flow velocity transducer was placed around the artery and connected to the flowmeter (Baylor Electronics). From the same mid-abdominal incision in some animals, bilateral adrenalectomy or lumbar sympathectomy (L1-L6) was performed. The intermesenteric nerves were also severed in sympathectomized animals.

Arterial blood pressure and iliac flow signals were digitized and recorded with an analog-digital converter (Modular Instruments) interfaced to a laboratory computer. The signals were recorded continuously using Biowindows software (Modular Instruments), averaged over 5 second intervals and stored on hard disk for subsequent analysis.

Microinjections into the NTS

Animals were placed in stereotaxic frame with head tilted down at 45º. After the exposure of the brainstem via dissected atlantocipital membrane, the animals were allowed to stabilize for at least 30 min before microinjections. Unilateral microinjections of the selective A1 adenosine receptor agonist CPA (Tocris, 330 pmol) dissolved in 50 nl of artificial cerebrospinal fluid (ACF) were made through multibarrel, glass micropipettes into the medial region of the caudal subpostremal NTS as described previously (4; 19; 32-35). This dose of CPA produced the most consistent, predominantly pressor responses in our previous study (35). The CPA was dissolved in ACF and the pH adjusted to 7.2. In several previous studies we have shown that microinjections of the
same amount of ACF into the same site of the NTS did not markedly affect mean arterial pressure (MAP), HR, RSNA, LSNA and pre-ganglionic adrenal (pre-ASNA) sympathetic nerve activity and vascular flows in iliac, renal and mesenteric arteries (4; 32-35). The changes in all these variables were either not different from zero or smaller than natural, random fluctuations of these variables over the time of measurements. To avoid the effect of desensitization of A₁ adenosine receptors, in all experiments only one dose of the agonist was microinjected into left or right side of the NTS. All microinjection sites were marked with fluorescent dye (DiI, Molecular Probes) and verified histologically (Figure 1) as described previously (4; 19; 32-35).

We believe that the microinjection technique mimics natural, spatial (not strictly synaptic) action of adenosine in the central nervous system as adenosine is naturally produced in the intracellular space by ectonucleotidases from extracellular ATP (released from neurons and glial cells under physiological conditions) (6; 8; 14; 38; 46) or it is directly released into the intracellular space from ischemic/hypoxic neurons and glial cells under pathological conditions (25; 43; 45).

**Experimental protocols**

In a previous study from our laboratory we showed that in addition to sympathetic iliac vasoconstriction and β-adrenergic vasodilation some unknown humoral vasoconstrictor(s) contribute to the consistent variability of hemodynamic responses evoked by selective stimulation of adenosine A₁ receptors located in the NTS (21). This conclusion was based on comparing of the responses observed in intact animals and following four experimental protocols: 1) β-adrenergic blockade, 2) adrenalectomy, 3) lumbar sympathectomy and 4) combined adrenalectomy plus lumbar sympathectomy. In the last experimental condition a powerful iliac vasoconstriction was observed indicating
that non-sympathetic, humoral vasoconstrictors are involved (21). The present study is a direct extension of our previous findings and focuses on the relative contribution to the responses of three potential humoral vasoconstrictors: vasopressin, angiotensin II and norepinephrine. Six experimental protocols were designed, according to the diagrams presented in Figure 2. Data collected in each protocol were compared with responses observed in intact group. In protocols 1 and 2 the contribution of vasopressin and angiotensin II was assessed by comparing hemodynamic responses elicited by stimulation of NTS A<sub>1</sub> adenosine receptors in intact animals with those obtained following selective blockade of vasopressin V<sub>1</sub> receptors and angiotensin II AT<sub>1</sub> receptors via iv. injections of selective antagonists: \([\beta\text{-mercapto-}\beta,\beta\text{-cyclopentylmethylenepropionyl},1\text{-O-Me-Tyr}^2,\text{Arg}^8]\text{-vasopressin}, (20 \mu g/kg, Sigma)\) and losartan (5 mg/kg, Merck Inc.), respectively. To evaluate the potential contribution of circulating norepinephrine to the responses ganglionic blockade (hexamethonium bromide, 25 mg/kg iv, Sigma) was combined with adrenalectomy (protocol 3) and these data and the responses observed previously following adrenalectomy alone (21) are discussed together. This indirect evaluation of norepinephrine contribution to the responses was necessary because total sympathetic denervation is impossible and ganglionic blockade, which prevents secretion of norepinephrine from sympathetic terminals, also impairs/abolishes the effects of activation of the adrenal medulla; thus ganglionic blockade removes \(\beta\)-adrenergic vasodilation simultaneously. Therefore, the appropriate reference point for the responses obtained following the ganglionic blockade (protocol 3) were the responses obtained following adrenalectomy alone which has been already performed in our previous study (21). Protocol 4 removed the combined contribution of norepinephrine and vasopressin to the responses (ganglionic blockade + vasopressin V<sub>1</sub> receptor blockade) whereas protocol 5 removed the combined effect off
all three potential vasoconstrictors considered via ganglionic blockade + vasopressin V₁ receptor blockade + angiotensin AT₁ receptor blockade. Protocols 3-5 were performed in adrenalectomized animals to clarify the experimental conditions by removing any residual adrenal responses which may potentially persist following the ganglionic blockade. Since preliminary results of the above five experimental protocols strongly suggested that only vasopressin has a marked contribution to the responses, in protocol 6 the magnitude of β-adrenergic vasodilation alone, not opposed by major vasoconstrictor factors (sympathetic vasoconstriction and vasopressin) was assessed.

In this protocol bilateral lumbar sympathectomy was combined with blockade of V₁ vasopressin receptors and these data were compared with data following V₁ vasopressin receptor blockade alone (protocol 1) and discussed together with previous data obtained following bilateral lumbar sympathectomy alone (21).

The effectiveness of vasopressin V₁ and angiotensin AT₁ receptor blockades were tested in separate groups of animals (n=3 for each blockade) with iv injections of arginine-vasopressin (50 mU/kg, Sigma) and angiotensin II (300 ng/kg, Sigma), respectively, before and after the blockade. Both blockades remained effective for over 1 hour. Blockade of V₁ vasopressin receptors caused relatively small decreases in MAP and increases in IVC (Table 1) which spontaneously returned toward resting values in approximately 10 min; therefore approximately 10 min after V₁ vasopressinergic blockade the microinjection of CPA was performed in protocols 1 and 6 (Figure 2). However, following blockade of angiotensin AT₁ receptors and ganglionic blockade marked and sustained decreases in MAP and increases in IVC were observed (Table 1). Therefore, in protocols 2-5, where these blockades were performed iv infusions of phenylephrine (PE, Sigma, 200 μg/ml) were used to return the hemodynamic parameters toward baseline, pre-blockade values. Table 2 presents the rates of PE
infusion needed for the compensation. No differences were observed in PE infusion rates between protocols 2-4. However, significantly greater PE infusion rates were required when angiotensin AT$_1$ receptor antagonists, losartan, were combined with ganglionic and V$_1$ vasopressin receptor blockades in protocol 5 (Table 2). During the responses to stimulation of NTS A$_1$ adenosine receptors PE infusion was continued at the same rates as needed to compensate for the altered hemodynamic values in protocols 2-5. The effect of PE infusion on baseline hemodynamic values was estimated in respective time controls for protocols 2-5 (Table 2). In the time-control experiments all procedures except microinjections of CPA were performed in the same time-pattern as in experimental protocols 2-5. Figure 3 shows an example of a time control for the most complex experimental protocol 5. PE infusion rates were similar in the experimental protocols and respective time controls (Table 2).

**Vasopressin assay**

Since the hemodynamic experiments (Protocols 1-6) suggested that vasopressin plays a dominant role in the iliac vascular responses to stimulation of NTS A$_1$ adenosine receptors, in an additional group of animals the effect of microinjections into the NTS of CPA ($n=8$) or respective volume control (50 nl of ACF, $n=5$) on circulating vasopressin were evaluated. We compared the levels of plasma vasopressin measured 30 min before and ~8 min after the microinjections (the average time when maximal hemodynamic responses to stimulation of NTS A$_1$ receptors occur). Arterial blood samples (~1 ml) were slowly withdrawn from the femoral artery into prechilled, heparinized tubes. Blood volume was kept unchanged via simultaneous infusion of the same volume of donor blood into the femoral vein. The samples were immediately placed on ice and centrifuged at 5000 g for 10 min at 4º C. Plasma was collected and stored at -70º C. Plasma vasopressin concentration was assessed via standard
radioimmunoassay procedures in our laboratory as described previously (13; 24; 27). The sensitivity of the vasopressin assay was 0.1 pg/ml and 50% displacement was 4.1 pg/tube. Intra- and inter-assay variability was 7.0% and 13.4%, respectively.

**Data analysis**

Hemodynamic responses were analyzed over a 20 min period following the microinjections, similar to our previous study (21). The responses were quantified as an integration of the differences between the baseline and response values averaged in 1 min periods and summed for 20 min of the response, i.e. when the majority of the responses occur. The integral reflects the predominant trend of the responses despite transient, sometimes large, bidirectional fluctuations in each variable. Because hemodynamic effects evoked by stimulation of NTS A₁ adenosine receptors were variable, often biphasic, or even polyphasic, as we previously reported (21; 35), we used the integral values for the comparison between the experimental groups. The absolute values of blood flow depend to some extent on positioning of the probe around the iliac artery; therefore the comparison between the relative changes in MAP, IBF, and IVC were more reliable. The HR responses, calculated from pulse intervals through the flow probe, were expressed in absolute values (beats/min). Iliac vascular conductance (IVC) was calculated by dividing iliac blood flow (IBF), expressed as a Doppler shift (in Hz) by MAP (in mmHg). In experimental protocols 2-5, where PE was infused to compensate for the decreased MAP and increased IVC the direct effect of PE on baseline hemodynamic variables was evaluated in respective time control experiments and subtracted from experimental data. Specifically, changes occurring in each variable during time-control experiments were integrated for 20 min and subtracted from the respective 20 min integral values obtained in each animal of the experimental groups (protocols 2-5).
One-way ANOVA for independent measures was used to compare hemodynamic responses versus experimental conditions. Differences observed were further evaluated by *t*-test with Bonferroni adjustment for independent measures. Differences between circulating vasopressin levels measured before and after microinjections of ACF or CPA were evaluated using paired *t*-test; the differences in vasopressin levels between the groups (ACF vs. CPA) were evaluated using unpaired *t*-test. The changes in all recorded variables were also compared with zero by means of SYSTAT univariate F test. An *α* level of *P* < 0.05 was used to determine statistical significance.

**RESULTS**

Resting values of MAP, HR, IBF and IVC for each experimental group, measured just before stimulation of NTS A₁ adenosine receptors, are presented in Table 3. The resting MAP and IVC for all the groups where blockades were performed were not different from those for intact animals; this provided reliable comparison between the experimental protocols. The direct effects of ganglionic blockade, and blockade of V₁ vasopressin and AT₁ angiotensin II receptors on all hemodynamic variables are presented in Table 1. The large decreases in MAP and increases in IVC evoked by blockade of AT₁ angiotensin II receptors and/or ganglionic blockade required additional compensation with PE, whereas the small decreases in MAP and increases in IVC evoked by V₁ vasopressin receptor blockade were allowed to partially recover without compensation.

**Effects of V₁, AT₁ and ganglionic blockades on responses to stimulation of NTS A₁ adenosine receptors**

Figure 4 presents examples of the responses evoked by selective stimulation of NTS A₁ adenosine receptors which were observed most often under each experimental
condition. The average integral responses for each experimental group are presented in
Figure 5. In intact animals the typical variability in the responses to stimulation of NTS
A₁ adenosine receptors was observed: the pressor and vasoconstrictor responses
prevailed (Table 4, Figures 4 and 5), although biphasic, polyphasic or, more rarely,
depressor and vasodilatory responses were also observed. As we previously
demonstrated, these patterns and variability of the responses reflected counteracting
effects of β-adrenergic vasodilation vs. sympathetic and humoral vasoconstriction (21;
35). V₁ vasopressin receptor blockade reversed iliac vasoconstrictor responses
observed in the intact group into slight iliac vasodilation (P=0.0001 vs. intact). Blockade
of angiotensin II AT₁ receptors alone did not significantly alter the responses in
comparison to the intact group (P>0.05 for all variables). Elimination of adrenal and
sympathetic neural effects on the iliac vasculature (protocol 3) increased the iliac
vasoconstrictor responses almost 4-fold in comparison to the intact group indicating that
other humoral factor(s) different than circulating norepinephrine play a crucial role in the
iliac vasoconstrictor responses. Subsequent blockade of vasopressin V₁ receptors
(protocol 4) virtually abolished the exaggerated iliac vasoconstriction observed following
adrenalectomy plus ganglionic blockade alone (protocol 3) (Figures 4 and 5) indicating
that the humoral iliac vasoconstriction evoked by stimulation of NTS A₁ adenosine
receptors is mediated mostly via the release of vasopressin. Combined blockade of
neural and all considered humoral factors (adrenalectomy + ganglionic + V₁
vasopressinergic + AT₁ angiotensinergic blockades, protocol 5) had very similar effects
on the responses to that observed in protocol 4 (adrenalectomy + ganglionic + V₁
vasopressinergic blockades); there were no significant differences between these two
groups with respect to MAP, HR and IVC responses (P>0.05 for all comparisons). The
lack of differences between responses observed in protocols 4 and 5 additionally
confirmed that vasopressin is the dominant humoral vasoconstrictor factor triggered by
stimulation of NTS A₁ adenosine receptors. The analysis of absolute values of the
integral responses presented in Table 5 leads to the same conclusions as those based
on the relative responses (Figure 5). The absolute values of the responses, presented
in Table 5, show residual iliac vasoconstrictor effects in protocols 4 and 5. However,
this vasoconstriction was abolished when the respective time control values were
subtracted from the direct experimental values presented in Table 5.

Data collected in protocols 1-5 (Figures 4 and 5) strongly suggested that the
only significant humoral vasoconstrictor contributing to iliac vascular responses evoked
by selective stimulation of NTS A₁ adenosine receptors is vasopressin. Therefore, in
protocol 6 vasopressin and lumbar sympathetic vasoconstrictor components of the
responses were eliminated to unmask β-adrenergic vasodilation alone, not opposed by
neural and humoral vasoconstriction. Following V₁ vasopressin receptor blockade
combined with bilateral lumbar sympathectomy (protocol 6) the iliac vasodilation
response doubled in comparison to that observed following V₁ vasopressin receptor
blockade alone (405.1± 58.4$\Delta$% vs. 195.8±51.2$\Delta$%, P=0.0175).

The above observations are supported by comparison of the frequency of which
we observed increases vs. decreases in hemodynamic variables in each experiment for
all experimental groups (Table 4). In intact animals, decreases in IVC prevailed
whereas following V₁ vasopressinergic blockade the increases in IVC prevailed.
Combined V₁ vasopressinergic blockade and lumbar sympathectomy completely
eliminated iliac vasoconstrictor responses (Protocol 6). AT₁ angiotensinergic blockade
increased the number of pressor and vasoconstrictor events in comparison to the INT
group (Table 4), although there were no significant differences between the averaged
responses observed in this vs. intact groups (Figure 5). Adrenalectomy plus ganglionic
blockade completely eliminated the depressor and iliac vasodilatory responses as
expected (Protocol 3). When V₁ vasopressinergic blockade was added to adrenalectomy and ganglionic blockade (Protocol 4) or all the blockades were performed in adrenalectomized animals (Protocol 5) again variable vasoconstrictor/vasodilatory responses were observed; however, this variability reflected rather random variations of IVC as vascular responses were virtually abolished in these groups (changes in IVC were not different from zero, Figure 5). Prevailing pressor and depressor responses were consistent with prevailing vasoconstrictor and vasodilatory responses in each experimental group. However, the decreases in HR dominated in all experimental groups despite the different experimental conditions.

**Release of vasopressin in response to stimulation of NTS A₁ adenosine receptors**

The above results, obtained via pharmacological blockade approaches, strongly suggested that vasopressin is a dominant vasoconstrictor factor released into the circulation following the selective stimulation of NTS A₁ adenosine receptors. Therefore, in an additional two groups of animals we tested this hypothesis more directly by measuring whether the plasma levels of vasopressin indeed increase as a result of stimulation of NTS A₁ adenosine receptors (Figure 6). The resting levels of vasopressin were similar in both groups (P=0.931) and they were moderately elevated in comparison to vasopressin levels normally observed in intact conscious animals (7; 13; 15; 23; 24) consistent with levels associated with anesthesia and surgical stress (7; 15). Stimulation of NTS A₁ adenosine receptors increased circulating vasopressin levels over 4-fold (P=0.0006). Microinjection of ACF into the NTS also tended to increase the level of circulating vasopressin; however the increase did not reach statistical significance (P=0.083). Notably the difference between vasopressin levels measured across the groups, i.e. following microinjections of CPA vs. ACF was also significant (P=0.041).
DISCUSSION

The present study assessed the relative roles of three major humoral vasoconstricting factors in mediating the responses to stimulation of A_1 adenosine receptors located in the NTS. The release of humoral vasoconstrictor factor(s) had been implied by our previous study which showed that the large iliac vasoconstriction, triggered by stimulation of NTS A_1 adenosine receptors, persisted after bilateral lumbar sympathectomy and adrenalectomy (21). The major finding of the present study is that vasopressin is released into the circulation upon stimulation of NTS A_1 adenosine receptors and that it is the primary humoral factor contributing to the iliac vasoconstriction. The potential release of norepinephrine and angiotensin II did not contribute significantly to the iliac vascular responses.

**NTS A_1 adenosine receptors are involved in control of vasopressin release**

Our previous studies strongly suggested that A_1 adenosine receptors may modulate vasopressin release at the level of the NTS similarly as they modulate baroreflex control of regional sympathetic outputs and HR (30; 35). However, considering differential localization/expression of adenosine receptor subtypes on functionally different NTS neurons (31; 36), it remained unknown if A_1 adenosine receptors are indeed located on these baroreflex neurons controlling vasopressin release. The present study showed that activation of A_1 adenosine receptors in the NTS disinhibits vasopressin release. Therefore, pre- and/or postsynaptic A_1 adenosine receptors are likely located on those NTS baroreflex neurons/terminals which control vasopressin release. This hypothesis based on integrative, systemic data may be confirmed in future studies at the cellular level.

The most likely mechanism responsible for the release of vasopressin into the circulation in response to stimulation of NTS A_1 adenosine receptors is the inhibition of
baroreflex transmission in the NTS resulting in the disinhibition of vasopressin release from hypothalamic nuclei (paraventricular and supraoptic) (10). The role of NTS in baroreflex control of vasopressin release has been well established (29; 39). Bilateral inhibition, anesthetization or lesioning of the NTS, which removes baroreflex mechanisms, results in 10-fold increases of circulating vasopressin levels and vasopressin-mediated hypertension (39). Sinoaortic baroreceptor denervation prevents this response (29). In the present study the unilateral inhibition of NTS baroreflex mechanisms via unilateral activation of $A_1$ adenosine receptors resulted in 5-fold increases in circulating vasopressin.

**Relative roles of vasoactive factors triggered by activation of $A_1$ adenosine receptors in the NTS**

Our previous study showed that activation of $A_1$ adenosine receptors in the NTS evokes neural and humoral iliac vasoconstriction opposed by $\beta$-adrenergic vasodilation (21). The counteracting vascular effects contributed to the variability of the overall pressor/depressor responses with prevailing vasoconstriction and increases in MAP. These conclusions were based on comparison of iliac vascular responses observed in intact animals and following $\beta$-adrenergic blockade, bilateral adrenalectomy, lumbar sympathectomy and combined adrenalectomy plus lumbar sympathectomy. The present study investigated potential humoral vasoconstrictors (vasopressin, angiotensin II and norepinephrine) by assessing the effects of vasopressin $V_1$ receptor and angiotensin $AT_1$ receptor blockade as well as ganglionic blockade performed in adrenalectomized animals. Perhaps the most compelling results were that the blockade of peripheral $V_1$ vasopressin receptors reversed the normal iliac vasoconstriction into marked vasodilation. This vasodilation was further enhanced by adding lumbar
sympathectomy to the V₁ receptor blockade. It is likely that sympathetic nerves have a
smaller role than vasopressin in mediating this iliac vasoconstriction as lumbar
goingly alone, performed in our previous study (21), had a relatively smaller
effect on the IVC responses then V₁ vasopressinergic blockade alone, performed in the
present study. Collectively, these data indicate that vasopressin alone can override
epinephrine induced iliac vasodilation and induce vasoconstriction, but that sympathetic
nerve alone do not. The roles of vasopressin and sympathetic nerves appear additive
as the sum of the individual effects approximate that of the combined effects.
Vasopressin together with increases in LSNA can induce significant vasoconstriction
without which a powerful β adrenergic vasodilation is revealed. We conclude that the
variability often seen in the arterial pressure response to A₁ receptor stimulation is the
direct result of these “push-pull” counteracting mechanisms.

Circulating norepinephrine potentially released upon stimulation of NTS A₁
adenosine receptors likely has little role as ganglionic blockade did not lessen the
intense iliac vasoconstriction seen after bilateral adrenalectomy (21). Probably the
amount of norepinephrine released from sympathetic terminals was too small to exert
measurable effects beyond that of circulating norepinephrine already likely to be
elevated as a result of anesthesia and surgical stress. A previous study from our
laboratory showed that pre-ASNA increased much more than RSNA and LSNA in
response to stimulation of NTS A₁ adenosine receptors (35). It is possible that other
sympathetic outputs responded even less or might even be inhibited with the stimulation.
This could cause the increase of circulating norepinephrine in the present study to be
functionally irrelevant.

Angiotensin II blockade alone markedly decreased baseline MAP and increased
IVC. However the normal pressor and vasoconstrictor responses to microinjections of
CPA did not decrease but rather tended to increase. This indicates that angiotensin II,
similarly as circulating norepinephrine, does not contribute to the iliac vasoconstriction
elicited by stimulation of NTS A₁ adenosine receptors. Although increases of RSNA may
cause an increase in the release of renin from the kidney, (11) the total time from
activation of the renal nerve to the subsequent release of renin and conversion of
angiotensinogen to angiotensin I and then to angiotensin II may have exceeded the time
of the analyzed responses. In addition, losartan used to block AT₁ receptors in this
study most likely crossed the blood-brain barrier and might evoke central effects
counteracting the peripheral actions. Nevertheless, our data show that circulating
angiotensin II had no functional effect as a potential humoral iliac vasoconstrictor
triggered by stimulation of NTS A₁ adenosine receptors.

Vasoactive effects of NTS A₁ vs. A₂a adenosine receptor subtypes

The predominately pressor but often variable responses to selective stimulation
of NTS A₁ adenosine receptors are mediated most likely via inhibition of baroreflex
glutamatergic transmission in the NTS and resetting of baroreflex functions toward
higher MAP (30; 35). In contrast, selective stimulation of NTS A₂a adenosine receptors
evokes decreases in MAP and preferential iliac vasodilation mediated via non-
baroreflex, nonglutamatergic mechanism(s) (16; 33; 34). These responses were
mediated mostly via activation of the adrenal medulla and β-adrenergic mechanism and
to a lesser extent via lumbar sympathetic nerves (19). However, no other humoral
mechanisms were involved as bilateral adrenalectomy and lumbar sympathectomy
abolished the responses (19). How do these two adenosine receptor subtypes
contribute to the responses mediated by adenosine operating in the NTS under
physiological (9; 38) or pathological (25; 37; 43; 45) conditions? It is well known that
microinjections of adenosine into the NTS results in depressor responses similar to
those observed following selective activation of A₂a adenosine receptors (3; 22; 40).
However, both adenosine receptor subtypes may contribute to the depressor responses as they both activate the adrenal medulla and facilitate β-adrenergic vasodilation via baroreflex and unknown, non-baroreflex mechanisms (33; 35). Other components of the responses elicited by NTS A2a receptors (decreases in RSNA and HR) may further contribute to the depressor responses, whereas A1 receptor-mediated sympathoactivation and vasopressin release would oppose these responses. Under physiological conditions adenosine may contribute to the pressor effects evoked by stimulation of the hypothalamic defense area (9; 38) via A1 receptor-mediated inhibition of baroreflex mechanisms and resulting sympathoactivation and vasopressin release (30; 35). In contrast, under pathological conditions naturally released adenosine, acting via both A1 and A2a adenosine receptor subtypes, may contribute to the paradoxical sympathoinhibition and cardiac slowing observed during hypovolemic phase of hemorrhagic shock (37).

Limitations of the method

In the central nervous system adenosine is not released synaptically, but rather produced in the extracellular space via the degradation of ATP which is released at synaptic terminals from neurons as well as released from glial cells activated by extracellular glutamate diffused from nearby active nerve terminals (6; 8; 14; 46). In addition, under pathological conditions adenosine is released into the extracellular space in a global, non-synaptic manner from hypoxic/hypoperfused neurons and glial cells (25; 25; 43; 45). Therefore the natural, spatial (not strictly synaptic) action of adenosine in the NTS, may be simulated well via microinjections of selective agonists of adenosine receptor subtypes. Importantly, although adenosine is released non-selectively in the NTS, it does produce specific differential responses in regional sympathetic outputs and vascular beds as shown by numerous studies from our laboratory (4; 30; 32-35). We
believe that these specific, differential effects evoked by nonspecific, spatial activation of adenosine receptors result from differential location/expression of adenosine receptor subtypes on NTS neurons and synaptic terminals controlling different sympathetic outputs and involved in different mechanisms integrated in the NTS (31; 36). To confirm this hypothesis, based on systemic, integrative approaches, further studies at the cellular level are required.

The experiments were performed in anesthetized animals and these conditions most likely attenuated baroreflex mechanisms which may have contributed to the increased vasopressin levels. In the conscious rat circulating vasopressin level is ~ 2 pg/ml (7; 13; 15; 23; 24; 27). With even limited surgery under anesthesia baseline vasopressin rises to ~6-10 pg/ml (7; 15; 29; 39). In our study after extensive surgery under anesthesia baseline vasopressin levels were ~25 pg/ml. However, despite the increased baseline vasopressin levels, activation of NTS A1 adenosine receptors triggered marked release of vasopressin into the circulation (Figure 6) and a powerful V1 receptor mediated vasoconstrictor effect in the hind limb vasculature was apparent (Figure 5). In contrast, no functional effects of potential, A1-adenosine-receptor-mediated increases in circulating norepinephrine or angiotensin II were observed.

Glucocorticoid deficiency, which may be observed following chronic adrenalectomy, increases circulating levels of vasopressin (18). However, this effect seems to be mediated at the level of transcription of the vasopressin gene and not an immediate release (18). Therefore, in the animals in which the adrenal glands were removed acutely, as in the present study, increased resting levels of vasopressin were most likely due to anesthesia and surgical stress.

The large and sustained changes in resting hemodynamic variables following AT1 angiotensinergic and ganglionic blockades required compensation with iv infusions of phenylephrine to make the relative responses comparable across the experimental
groups. It was extremely difficult to return both MAP and IVC to the pre-blockade levels. Since we tried to compensate most accurately for IVC, the resting MAP, preceding the microinjections into the NTS in the groups where ganglionic blockade was combined with V₁ vasopressinergic and with AT₁ angiotensinergic blockades (Protocols 4 and 5), was slightly lower in comparison to other experimental groups. The long lasting compensatory infusions of phenylephrine in protocols 2-5 could facilitate central baroreflex mechanisms (17). Therefore, the inhibition of enhanced baroreflex activity via stimulation of NTS A₁ adenosine receptors could result in relatively greater pressor and vasoconstrictor responses compared to those observed in intact animals and following V₁ vasopressinergic blockade alone (Protocol 1) and V₁ vasopressinergic blockade combined with lumbar sympathectomy (Protocol 6). In fact, pressor and vasoconstrictor responses tended to increase following AT₁ receptor blockade although this tendency did not reach statistical significance. The pressor and vasoconstrictor responses observed after adrenalectomy combined with ganglionic blockade (Protocol 3) were not different from those observed following adrenalectomy alone in our previous study (21). Finally, these responses (even if slightly exaggerated) were virtually abolished by the vasopressinergic blockade (Protocols 4 and 5). Therefore, the potential central effects of infusions of phenylephrine on responses to stimulation of NTS A₁ adenosine receptors were likely negligible and did not affect the primary conclusions of the present study. The infused phenylephrine activated α₁ adrenergic receptors located on vascular smooth muscles, including those in the iliac vascular bed. The occupation of α₁ adrenergic receptors by an exogenous agonist could mask the effect of circulating endogenous norepinephrine which may be potentially increased following stimulation of NTS A₁ adenosine receptors. The peripheral interactions between simultaneously activated V₁, α₁ and AT₁ receptors could further complicate the comparison of relative roles of
humoral vasoconstrictor factors obscuring the smaller ones. Nevertheless, the marked
effects of vasopressin V$_1$ receptor blockade were evident in experimental groups with
and without phenylephrine compensation (Protocols 1 and 6 vs. Protocols 4 and 5).
This indicates that vasopressin contribution to the iliac vasoconstriction was much larger
than the contribution (if any) of other potential vasoconstrictors (norepinephrine and/or
angiotensin II).

Conclusion
Selective stimulation of NTS A$_1$ adenosine receptors triggers the release of
vasopressin into the circulation, strongly suggesting that A$_1$ adenosine receptors are
likely located on afferent terminals and/or NTS interneurons mediating baroreflex control
of vasopressin release. Vasopressin is a major humoral vasoconstrictor factor
contributing to the iliac vascular responses. The natural variability of MAP and IVC
responses to stimulation of NTS A$_1$ adenosine receptors observed in intact animals is a
result of the simultaneous triggering of sympathetic and vasopressinergic
vasoconstriction counteracted by β-adrenergic vasodilation resulting mainly via
epinephrine release in response to the large increase in adrenal pre-ganglionic
sympathetic activity.
The authors thank Andrew Baumgartner for technical assistance. The authors gratefully acknowledge the generous gift of losartan by Merck Inc. This study was supported by grant HL 67814 from National Institute of Health (O'Leary DS and Scislo TJ), and Merit Review Award by Department of Veterans Affairs (Rossi NF and Scislo TJ).
REFERENCES


28. **Sawchenko PE and Swanson LW.** Immunohistochemical identification of neurons in the paraventricular nucleus of the hypothalamus that project to the medulla or to the spinal cord in the rat. *J Comp Neurol* 205: 260-272, 1982.


**FIGURE LEGENDS**

**Figure 1.** Microinjection sites in the caudal subpostremal NTS for all experiments. Schematic diagrams of transverse sections of the medulla oblongata from a rat brain. NTS, nucleus tractus solitarii; AP, area postrema; c, central canal; 10, dorsal motor nucleus of the vagus nerve; 12, nucleus of the hypoglossal nerve; ts, tractus solitarius; Gr, gracile nucleus; Cu, cuneate nucleus. Scale is shown at the bottom; the number on the left side of the schematic diagram denotes the rostro-caudal position in millimeters of the section relative to the obex according to the atlas of the rat subpostremal NTS by Barraco et al. (1). Microinjection sites were marked with fluorescent dye and are denoted with filled symbols for the pressor responses to CPA and corresponding open symbols for the depressor responses. *A:* microinjections of CPA in intact animals (●, ○), after vasopressin V₁ receptor blockade (VX) (◇), lumbar sympathectomy plus VX (▲, △), and after angiotensin II AT₁ receptor blockade (ATX) (■, □). *B:* microinjections of CPA after bilateral adrenalectomy (ADX) plus ganglionic blockade (GX) (▼), following ADX+GX+VX (○○), after ADX+GX+VX+ATX (□□). In experiments where vasopressin assay was performed the microinjection sites were denoted: + and x for CPA and ACF microinjections, respectively.

**Figure 2.** Time line of how experimental protocols are executed: vasopressin V₁ receptor blockade (VX), lumbar sympathectomy + V₁ receptor blockade (LX+VX), angiotensin II AT₁ receptor blockade (ATX), bilateral adrenalectomy + ganglionic blockade (ADX+GX), adrenalectomy + ganglionic blockade + V₁ receptor blockade (ADX+GX+VX) and adrenalectomy + ganglionic blockade + V₁ receptor blockade + AT₁ receptor blockade (ADX+GX+VX+ATX). Time control experiments were performed for protocols including ATX and/or GX (protocols 2-5) according to the respective diagrams; however microinjections of CPA were omitted.
Figure 3. An example of time control experiment following adrenalectomy plus ganglionic blockade (GX) plus vasopressin blockade (VX) plus angiotensin II AT$_1$ receptor blockade (ATX); no CPA was microinjected in this experiment. The dashed arrow denotes a potential microinjection of CPA and the subsequent 20 min integration of the response. Note that although the infusion of phenylephrine (PE) did not fully compensated for the decrease in mean arterial pressure (MAP) the iliac vascular conductance (IVC) gradually declined constricting iliac vasculature slightly below the baseline level.

Figure 4. Mean arterial pressure (MAP), iliac blood flow (IBF) and iliac vascular conductance (IVC) responses to microinjection of adenosine A$_1$ receptor agonist (CPA, 330 pmol/50 nl) into the subpostremal NTS in intact rats (INT) and following: vasopressin V$_1$ receptor blockade (VX), angiotensin II AT$_1$ receptor blockade (ATX), bilateral adrenalectomy + ganglionic blockade (ADX+GX), adrenalectomy + ganglionic blockade + V$_1$ vasopressin receptor blockade (ADX+GX+VX) and adrenalectomy + ganglionic blockade + V$_1$ vasopressin receptor blockade + AT$_1$ angiotensin II receptor blockade (ADX+GX+VX+ATX). Microinjections of CPA, marked by vertical arrows, were applied ~10 -20 min after the blockades, when baseline levels of all variables stabilized (see Figure 2). Note that VX reversed pressor and vasoconstrictor responses most often observed in INT group into depressor and vasodilatory responses. ADX+GX exaggerated the pressor and vasoconstrictor response. These exaggerated vasoconstrictor responses were virtually abolished following ADX+GX+VX and ADX+GX+VX+ATX.

Figure 5. Integral responses of MAP, IBF and IVC evoked by microinjections of CPA (330 pmol /50 nl) into the caudal subpostremal NTS. Abbreviations as in Figure 4. Data are means ± SE. In groups ATX, ADX+GX, ADX+GX+VX, ADX+GX+VX+ATX respective time control values were subtracted. *, different versus intact group (P<0.05).
#, different versus zero (P<0.05). VX reversed iliac vasoconstrictor responses observed in intact group into vasodilation and virtually abolished exaggerated vasoconstrictor responses observed following ADX+GX.

Figure 6. Comparison of plasma vasopressin levels measured before (Pre) and after (Post) microinjections into the NTS of vehicle (ACF, n=5) or selective A1 adenosine receptor agonist (CPA, n=8). *, differences between pre- vs. post-microinjections (P<0.05); #, differences between the groups (P<0.05). CPA increased circulating vasopressin levels 4-fold whereas ACF did not significantly increase the level of circulating vasopressin.
Table 1. Maximal hemodynamic responses evoked by blockade of V$_1$ vasopressin receptors (VX), AT$_1$ angiotensin II receptors (ATX) and ganglionic blockade (GX).

<table>
<thead>
<tr>
<th>Experimental groups</th>
<th>n</th>
<th>∆% Mean Arterial Pressure, mmHg</th>
<th>∆% Heart Rate, beats/min</th>
<th>∆% Iliac Blood Flow</th>
<th>∆% Iliac vascular Conductance</th>
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</thead>
<tbody>
<tr>
<td>VX</td>
<td>16</td>
<td>-4.7±1.8#</td>
<td>1.2±0.9</td>
<td>10.2±1.6#</td>
<td>15.3±2.7#</td>
</tr>
<tr>
<td>GX</td>
<td>39</td>
<td>-39.7±1.9#</td>
<td>3.1±1.9</td>
<td>16.6±3.3#</td>
<td>99.9±9.9#</td>
</tr>
<tr>
<td>ATX</td>
<td>15</td>
<td>-49.2±2.8#</td>
<td>-13.9±3.5#</td>
<td>8.6±6.1</td>
<td>125.1±18.2#</td>
</tr>
</tbody>
</table>

Data are means ± SE. # P<0.05 vs. zero. Numbers of responses to VX, GX and ATX were combined from those protocols where these blockades were applied as a first pharmacological manipulation: $n_{\text{VX}} = n_{\text{LX}+\text{VX}}$; $n_{\text{GX}} = n_{\text{ADX}+\text{GX}} + n_{\text{ADX}+\text{GX}+\text{VX}} + n_{\text{ADX}+\text{GX}+\text{VX}+\text{ATX}} + n$ of respective controls; $n_{\text{ATX}} = n_{\text{ATX}} + n$ of respective control (see Table 2). The small changes in mean arterial pressure and iliac vascular conductance caused by VX were allowed to return spontaneously toward resting levels, whereas the large, sustained changes in these variables caused by ATX and GX were compensated via iv infusion of phenylephrine (see Table 1).
Table 2. Infusion rates of phenylephrine used to maintain mean arterial pressure and iliac vascular conductance at pre-blockade levels in experimental and respective time control groups in which angiotensin AT₁ receptor blockade (ATX) and/or ganglionic blockade (GX) were performed.

<table>
<thead>
<tr>
<th>Protocol number</th>
<th>Experimental procedure</th>
<th>n</th>
<th>Infusion rate ml/h/kg</th>
<th>Time controls</th>
<th>n</th>
<th>Infusion rate ml/h/kg</th>
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<tbody>
<tr>
<td>2</td>
<td>ATX</td>
<td>10</td>
<td>3.18±0.12*</td>
<td>ATX</td>
<td>5</td>
<td>2.39±0.25*</td>
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<tr>
<td>3</td>
<td>ADX + GX</td>
<td>8</td>
<td>3.02±0.24*</td>
<td>ADX + GX</td>
<td>5</td>
<td>3.20±0.33*</td>
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<td>4</td>
<td>ADX + GX + VX</td>
<td>8</td>
<td>3.45±0.41*</td>
<td>ADX + GX + VX</td>
<td>5</td>
<td>3.94±0.62</td>
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<tr>
<td>5</td>
<td>ADX + GX + VX + ATX</td>
<td>8</td>
<td>6.33±0.24</td>
<td>ADX + GX + VX + ATX</td>
<td>5</td>
<td>5.29±0.62</td>
</tr>
</tbody>
</table>

Data are means ± SE; n = number of rats. * P<0.05 vs. ADX + GX + VX + ATX. Experimental and time control groups following: angiotensin II AT₁ receptor blockade (ATX), bilateral adrenalectomy + ganglionic blockade (ADX+GX), adrenalectomy + ganglionic blockade + V₁ vasopressin receptor blockade (ADX+GX+VX) and adrenalectomy + ganglionic blockade + V₁ vasopressin receptor blockade + AT₁ receptor blockade (ADX+GX+VX+ATX). There were no differences in infusion rates between experimental groups vs. respective time control groups (P>0.05).
Table 3. Resting values of hemodynamic parameters in each experimental group.

<table>
<thead>
<tr>
<th>Protocol number</th>
<th>Experimental procedure</th>
<th>n</th>
<th>Mean Arterial Pressure, mmHg</th>
<th>Heart Rate, beats/min</th>
<th>Iliac Blood Flow, Hz</th>
<th>Iliac Vascular Conductance, Hz/mmHg</th>
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<tr>
<td>1</td>
<td>VX</td>
<td>8</td>
<td>105.3±4.9</td>
<td>359.7±11.2</td>
<td>1091.0±204.2</td>
<td>10.6±2.0</td>
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<tr>
<td>2</td>
<td>ATX</td>
<td>10</td>
<td>95.3±4.9</td>
<td>342.2±8.2</td>
<td>992.8±122.5</td>
<td>10.8±1.5</td>
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<td>ADX + GX</td>
<td>8</td>
<td>93.1±5.1</td>
<td>383.6±11.4</td>
<td>1190.4±167.2</td>
<td>13.2±2.1</td>
</tr>
<tr>
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<td>ADX + GX + VX</td>
<td>8</td>
<td>88.7±1.3</td>
<td>383.3±9.0</td>
<td>1394.0±222.7</td>
<td>15.8±2.7</td>
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<tr>
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<td>ADX + GX + VX + ATX</td>
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<td>88.1±2.1</td>
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<td>1271.0±102.9</td>
<td>14.4±1.1</td>
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<tr>
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<td>LX + VX</td>
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<td>95.8±4.1</td>
<td>340.6±7.0</td>
<td>1336.4±132.8*</td>
<td>14.3±1.7</td>
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<td>(2) Control</td>
<td>(ATX)</td>
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<td>99.3±2.8</td>
<td>330.0±11.8</td>
<td>973.7±173.2</td>
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<td>(ADX + GX)</td>
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<td>84.2±2.8</td>
<td>365.8±17.2</td>
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<td>(4) Control</td>
<td>(ADX + GX + VX)</td>
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<td>88.1±3.5</td>
<td>381.6±7.8</td>
<td>1121.6±141.1</td>
<td>12.7±1.4</td>
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<tr>
<td>(5) Control</td>
<td>(ADX + GX + VX + ATX)</td>
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<td>90.2±4.7</td>
<td>398.9±13.3</td>
<td>1144.6±302.0</td>
<td>12.2±2.4</td>
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Data are means ± SE; n=number of rats. Numbers in parentheses show time controls for respective protocols. Resting values for intact animals and following: vasopressin V₁ receptor blockade (VX), angiotensin II AT₁ receptor blockade (ATX), bilateral adrenalectomy + ganglionic blockade (ADX+GX), adrenalectomy + ganglionic blockade + V₁ receptor blockade (ADX+GX+VX), adrenalectomy + ganglionic blockade + V₁ receptor blockade + AT₁ receptor blockade (ADX+GX+VX+ATX) and lumbar sympathectomy + V₁ receptor blockade (LX+VX).
Table 4. Number of individual experiments where overall increments or decrements were observed for each recorded hemodynamic parameter based on its integral values.

<table>
<thead>
<tr>
<th>Protocol number</th>
<th>Experimental procedure</th>
<th>n</th>
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<th>Heart Rate, beats/min</th>
<th>Iliac Blood Flow, Hz</th>
<th>Iliac Vascular Conductance, Hz/mmHg</th>
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<tbody>
<tr>
<td></td>
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<td>Incr</td>
<td>Decr</td>
<td>Incr</td>
<td>Decr</td>
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<td>3</td>
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<tr>
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<td>7</td>
<td>1</td>
<td>7</td>
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</table>

Number of increments (Incr) and decrements (Decr) observed in intact (INT) animals and following: vasopressin V₁ receptor blockade (VX), angiotensin II AT₁ receptor blockade (ATX), bilateral adrenalectomy + ganglionic blockade (ADX+GX), adrenalectomy + ganglionic blockade + V₁ receptor blockade (ADX+GX+VX), adrenalectomy + ganglionic blockade + V₁ receptor blockade + AT₁ receptor blockade (ADX+...
Table 5. Absolute values of integral changes in MAP, HR, IBF and IVC evoked by microinjections of CPA (330 pmol in 50 nl) into the NTS in each experimental group.

<table>
<thead>
<tr>
<th>Protocol number</th>
<th>Experimental procedure</th>
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<th>Mean Arterial Pressure, mmHg</th>
<th>Heart Rate, beats/min</th>
<th>Iliac Blood Flow, Hz</th>
<th>Iliac Vascular Conductance, Hz/mmHg</th>
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<tbody>
<tr>
<td>1</td>
<td>VX</td>
<td>8</td>
<td>86.0±37.7*</td>
<td>-379.3±96.4*</td>
<td>-1480.3±588.7*</td>
<td>-23.1±7.1*</td>
</tr>
<tr>
<td>2</td>
<td>ATX</td>
<td>10</td>
<td>198.4±47.3*</td>
<td>-70.6±126.0</td>
<td>-1585.8±445.4*</td>
<td>-34.8±6.4*</td>
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<td>3</td>
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<td>312.3±67.1**</td>
<td>-139.0±48.6*</td>
<td>-5758.2±1284.4**</td>
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<tr>
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<td>7.1±46.3</td>
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<td>-2752.3±715.7*</td>
<td>-31.3±11.5*</td>
</tr>
<tr>
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<td>ADX + GX + VX + ATX</td>
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<td>-162.0±71.5*</td>
<td>-502.0±177.3*</td>
<td>2127.6±1489.5*</td>
<td>57.5±11.4*</td>
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<tr>
<td>(2) Control (ATX)</td>
<td></td>
<td>5</td>
<td>80.0±37.6</td>
<td>504.1±91.8**</td>
<td>-303.2±573.6</td>
<td>-9.8±3.6*</td>
</tr>
<tr>
<td>(3) Control (ADX + GX)</td>
<td></td>
<td>5</td>
<td>22.3±36.2</td>
<td>54.5±104.8*</td>
<td>-166.1±364.4</td>
<td>-5.9±3.8</td>
</tr>
<tr>
<td>(4) Control (ADX + GX + VX)</td>
<td></td>
<td>5</td>
<td>15.6±39.7</td>
<td>163.3±54.5**</td>
<td>-1224.1±380.7*</td>
<td>-13.8±5.1*</td>
</tr>
<tr>
<td>(5) Control (ADX + GX + VX + ATX)</td>
<td></td>
<td>5</td>
<td>-11.4±23.7</td>
<td>206.5±61.6**</td>
<td>-2694.9±1428.2</td>
<td>-24.7±13.0</td>
</tr>
</tbody>
</table>

Data are means ± SE. * P<0.05 vs. Intact; # P<0.05 vs. zero. Numbers in parentheses show time controls for respective protocols. Integral changes for intact animals and following: vasopressin V₁ receptor blockade (VX), angiotensin II AT₁ receptor blockade (ATX), bilateral adrenalectomy + ganglionic blockade (ADX+GX), adrenalectomy + ganglionic blockade + V₁ receptor blockade (ADX+GX+VX), adrenalectomy + ganglionic blockade + AT₁ receptor blockade (ADX+GX+VX+ATX), and lumbar sympathectomy + V₁ receptor blockade (LX+VX).
Protocol 1 & 6

\[ \text{VX or LX+VX} \]

\[ \text{20 min integration} \]

Protocol 2

\[ \text{ATX CPA} \]

\[ \text{PE} \]

\[ \text{20 min integration} \]

Protocol 3

\[ \text{ADX+GX CPA} \]

\[ \text{PE} \]

\[ \text{20 min integration} \]

Protocol 4

\[ \text{ADX+GX+VX CPA} \]

\[ \text{PE} \]

\[ \text{20 min integration} \]

Protocol 5

\[ \text{ADX+GX+VX+ATX CPA} \]

\[ \text{PE} \]

\[ \text{20 min integration} \]
Figure 3

MAP (mmHg) vs. TIME (min)

- GX
- VX + ATX
- PE
- ~5 min
- ~15 min
- 20 min integration

IVC (%) vs. TIME (min)
Figure 5

MAP (Δ%)

IBF (Δ%)

IVC (Δ%)

* P<0.05 vs. INT
# P<0.05 vs. Zero
Figure 6

Plasma vasopressin (pg/ml)

<table>
<thead>
<tr>
<th></th>
<th>PRE</th>
<th>POST</th>
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</thead>
<tbody>
<tr>
<td>ACF</td>
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<tr>
<td>CPA</td>
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</tr>
</tbody>
</table>

* * #
Protocol 1 & 6

**VX**
- ~10 min
- 20 min integration

**VX** or **LX+VX**

Protocol 2

**ATX**
- ~10 min
- 20 min integration

Protocol 3

**ADX+GX**

Protocol 4

**ADX+GX+VX**
- ~5 min
- ~15 min
- 20 min integration

Protocol 5

**ADX+GX+VX+ATX**
- ~5 min
- ~15 min
- 20 min integration
MAP (mmHg)

- VX + ATX
- PE

IVC (%)

- GX
- PE
- ~5 min
- ~15 min
- 20 min integration

TIME (min)
* P<0.05 vs. INT
# P<0.05 vs. Zero