EXPERIMENTAL STUDY

Evaluation for roles of nitric oxide generated in the anteroventral third ventricular region in controlling vasopressin secretion and cardiovascular system of conscious rats

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Abstract

Objective: To examine local actions of nitric oxide (NO) on the neural mechanisms controlling the release of vasopressin (AVP) and the cardiovascular system in the anteroventral third ventricular region (AV3V), a pivotal area for autonomic functions, and to pursue the problem of whether it may have any role in the AVP and cardiovascular responses evoked by plasma hypertonicity or by increased prostaglandin E₂ (PGE₂) in the AV3V – one possible factor implicated in osmotic responses.

Methods: We infused NO-related agents into the AV3V, its adjacent area, the nucleus of the vertical limb of the diagonal band (VDB), or into the lateral cerebral ventricle of conscious rats, monitoring effects on plasma AVP, osmolality, sodium, potassium and chloride, arterial pressure and heart rate in the presence or absence of an osmotic or PGE₂ stimulus. The infusion sites were determined histologically.

Results: Infusion of L-arginine, the substrate of NO synthase (NOS), into the AV3V structures such as the median preoptic nucleus and periventricular nucleus produced dose-related increases in plasma AVP, arterial pressure and heart rate 5 or 15 min later, whereas infusion of D-arginine (which is not a substrate for NO synthesis) was without significant effect on these variables. Plasma osmolality or electrolytes were not changed by these treatments. The AV3V infusion of sodium nitroprusside (SNP), a spontaneous releaser of NO, also induced dose-dependent augmentations of plasma AVP, without evoking remarkable alteration in the cardiovascular parameters. The infusion of L- or D-arginine into the VDB affected none of the variables significantly. When applied intracerebroventricularly, L-arginine caused only increases in plasma AVP, whereas SNP caused only reductions in arterial pressure, leaving other variables at stable values. The effects of AV3V L-arginine on plasma AVP and the cardiovascular variables were abolished by N^G-nitro-L-arginine methyl ester (L-NAME), a potent inhibitor of NOS, applied 15 min before. In contrast, infusion of L-NAME to the AV3V did not exert a significant effect on the responses of plasma AVP or cardiovascular variables to AV3V application of PGE₂ or i.v. infusion of hypertonic NaCl. The infusion of L-NAME alone did not affect plasma variables including AVP, although it tended to increase basal arterial pressure and heart rate.

Conclusion: These results suggest that NO generated in or near the AV3V may act to enhance AVP release, arterial pressure and heart rate, but it may not play an essential role in eliciting the responses of these variables to osmotic or PGE₂ stimuli.

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Introduction

Nitric oxide (NO) is a non-conventional neuromodulator produced from L-arginine by NO synthase (NOS) (1, 2). The presence of NOS in the paraventricular (PVN) and supraoptic (SON) vasopressin (AVP) neurons and its

increase in these neurons or the neurohypophysis by salt loading or dehydration suggest possible involvement of NO in regulating the secretion of AVP (3–5). However, the role of NO is not certain, as reflected by conflicting data obtained to date through both *in vivo* and *in vitro* studies (6–9). Such discrepancies could be

explained by the possibility that NO may act at various cerebral sites to induce diverse effects, because NOS or its mRNA is distributed throughout the brain (1, 2). In order to understand roles of NO in the release of AVP and other phenomena, therefore, it seems necessary to clarify its actions on discrete regions exhibiting NOS activity.

Neurons in the PVN and SON are innervated by those in the anteroventral third ventricular region (AV3V), comprising structures such as the organum vasculosum laminae terminalis, periventricular preoptic nucleus, and the median and medial preoptic nuclei (10–12). The AV3V lacks a complete blood–brain barrier, shows sensitivity to various factors such as interleukin (IL)-1 β , prostaglandin (PG) E₂ and hypertonicity (11–14), and participates in various autonomic functions, including AVP release, cardiovascular actions, febrile response and drinking behavior (10–12). In addition, histochemical studies have demonstrated the presence of NOS and its mRNA in AV3V neurons (15, 16). The possibility that NO formed in this region may have physiological significance has been suggested by the report that local application of L-arginine into the preoptic area (POA) changed the drinking behavior of rats (17). To our knowledge, however, roles of AV3V NO in controlling the release of AVP and the cardiovascular system have not yet been investigated.

We have recently indicated that stimulation of PGE₂ receptors in the AV3V elicits AVP-releasing pressor and tachycardiac responses in rats, and AVP-releasing and pressor actions evoked by plasma hypertonicity may be mediated by AV3V PGE₂ (18, 19). It is known that there may be a functional correlation between PGE₂ and NO. For example, NO can activate cyclooxygenase to promote generation of PGE₂ (20, 21), and the febrile response to the application of PGE₂ to the POA was blocked by an NOS inhibitor administered concomitantly (22). Therefore, if AV3V PGE₂ contributes to the AVP and pressor responses to the plasma hypertonicity, NO might also be implicated in these effects. In agreement with this view, Liu *et al.* (23) have reported that the intracerebroventricular (i.c.v.) injection of a potent NOS inhibitor (24, 25), N^G-nitro-L-arginine methyl ester (L-NAME) – a treatment that may allow the drug to act on the AV3V (26) – attenuated the effect of an osmotic stimulus on the water intake of rats. However, systemic infusion of L-NAME was without significant effect on osmotic AVP secretion in rabbits (27), and excitation of PVN neurons by i.c.v. IL-1 β (which is capable of stimulating synthesis of both PGE₂ (28) and NO (29, 30)) was prevented not by L-NAME (31), but by a prostaglandin inhibitor (13). Accordingly, it may also be postulated that NO formed in the AV3V may not have a significant role in the AVP and cardiovascular responses to PGE₂ or hyperosmolality.

The purpose of the present study was to examine the actions of NO on AV3V neural mechanisms for release of AVP and cardiovascular function, and to pursue the

problem of whether NO generated in the AV3V may contribute to the AVP and cardiovascular responses to PGE₂ and an osmotic load. In conscious rats, we first tested the effects of the infusion to the AV3V of L-arginine, its biologically inactive stereoisomer D-arginine and a spontaneous releaser of NO (32), sodium nitroprusside (SNP) on basal plasma AVP concentrations, osmolality, electrolytes, heart rate and arterial pressure. As these solutions were hypertonic, AV3V infusion of a hypertonic NaCl solution was also performed to compare effects. We then examined the influence of the AV3V administration of L-NAME on the responses of those variables to the application of L-arginine or PGE₂ to the AV3V or the i.v. infusion of hypertonic NaCl. Drugs administered into the AV3V may exert their effects by diffusion into the cerebral ventricle or adjacent tissue, therefore, we also studied the effects of administering the NO-related agents into the ventricle or the nucleus of the vertical limb of the diagonal band (VDB) containing NOS activity (1, 2, 33).

Materials and methods

Animals and surgery

Wistar male rats weighing 300–360 g were anesthetized with pentobarbital sodium (50 mg/kg i.p.) and placed in a stereotaxic apparatus (Type SR-6; Narishige, Tokyo, Japan). After a scalp incision was made, the lambda and bregma were located and the skull was leveled. A hole was drilled in the skull, and stainless-steel guide cannulae of 0.5 mm outer diameter (Eicom, Kyoto, Japan) were lowered to just above the AV3V or VDB, or into a lateral cerebral ventricle. Coordinates of the tips were 0.3 mm posterior to the bregma, on the midline and 6.6 mm below the surface of the skull for AV3V cannulae, 0.7 mm anterior to the bregma, on the midline and 6.0 mm below the surface of the skull for VDB cannulae, and 0.8 mm posterior to the bregma, 1.5 mm right from the midline and 3.7 mm below the surface of the skull for the ventricular cannulae (34). The cannulae implanted were fixed on the skull with small screws and dental resin. After surgery, rats were injected i.m. with Penicillin G (30 000 U). The rats were kept in individual cages and food and water were available *ad libitum*.

Seven or 8 days after surgery, the rats were catheterized with PE-50 tubing in the left femoral artery and right jugular vein under ether anesthesia. When loading of hypertonic or isotonic saline was to be conducted, the left femoral vein was also catheterized with the tubing. The tubes were filled with heparinized saline (240 U/ml), sealed, exteriorized and secured at the back of the neck. In using the rats in the current study, we adhered to the Guiding Principles for the Care and Use of Animals approved by the Council of the Physiological Society of Japan.

Experiments

Experiments were performed 1 or 2 days after the vascular catheterization mentioned above. The rats were transferred into individual plastic boxes ($8 \times 8 \times 25$ cm) that permitted them freedom of movement. After approximately 60 min, the arterial line was connected to a disposable pressure transducer (Omeda, Tokyo, Japan) connected to an AP-600G amplifier (Nihon Kohden, Tokyo, Japan). Output signals from the amplifier were introduced to a personal computer system through a PowerLab/4s (AD Instruments Japan, Tokyo, Japan) and processed for electronic recordings and analyses of arterial pressure and heart rate. In parallel with the electronic recordings, signals of arterial pressure from the AP-600G amplifier and those of heart rate from an AT-601G pulse counter (Nihon Kohden) connected to the amplifier were recorded on paper charts, using an RTA-4100 recorder (Nihon Kohden). A preceding period of 30–60 min was necessary for these cardiovascular parameters to stabilize. The rats were then given one of the following treatments: [1] AV3V infusion of a $1 \mu\text{l}$ solution containing 0, 0.57 (100 μg) or 1.3 μmol (225 μg) L- or D-arginine; [2] AV3V infusion of $1 \mu\text{l}$ isotonic saline (0.15 mol/l) containing 0, 0.08 (25 μg), 0.17 (50 μg) or 0.34 μmol (100 μg) SNP or infusion of $1 \mu\text{l}$ hypertonic saline (0.77 mol/l); [3] i.c.v. infusion of $1 \mu\text{l}$ isotonic saline containing 0 or 0.34 μmol SNP or infusion of the $1 \mu\text{l}$ solution containing 1.3 μmol L- or D-arginine; [4] VDB infusion of $1 \mu\text{l}$ isotonic saline or of the $1 \mu\text{l}$ solution containing 1.3 μmol L- or D-arginine; [5] AV3V infusion of $1 \mu\text{l}$ isotonic saline containing 0 or 0.74 μmol (200 μg) L-NAME and AV3V infusion of $1 \mu\text{l}$ isotonic saline containing 0 or 12.8 nmol (4.5 μg) PGE₂, or infusion of the $1 \mu\text{l}$ solution containing 1.3 μmol L-arginine; [6] AV3V infusion of $1 \mu\text{l}$ isotonic saline containing 0 or 0.74 μmol L-NAME and i.v. infusion of isotonic or hypertonic (2.5 mol/l) saline. Time intervals between the first and second AV3V infusions in treatment [5] were 15 min, and those between the AV3V infusion and the onset of the i.v. infusion in treatment [6] were 10 min.

The intracranial infusion was carried out at a rate of $1 \mu\text{l}/\text{min}$ for 1 min, using 30 gauge stainless-steel tubing that protruded 1.0 mm (for AV3V and VDB infusion) or 0.3 mm (for i.c.v. infusion) beyond the ends of the guide cannulae and was connected to a microsyringe via polyethylene tubing. Approximately 1 min elapsed before the infusion tubing was withdrawn, to permit movement of drug away from its tip. The i.v. infusion was conducted at a rate of 0.1 ml/kg per min for 30 min through the femoral venous line. For these intracranial and i.v. infusions, a Harvard pump (model 11, South Natick, MA, USA) was used.

In rats undergoing treatments [1] to [4], blood samplings were conducted 5 min before and 5 min and 15 min after the intracranial infusion. A 3 ml blood

sample was taken from the femoral arterial line over a 30-s period during which 3 ml of donor blood was returned through the jugular venous line. In rats undergoing treatments [5] and [6], the first blood samples were obtained, as described above, 10 min after the AV3V infusion of L-NAME or vehicle, at a time corresponding to 5 min before the second AV3V infusion or to 0 min before the i.v. infusion. Further blood samples were taken 5 min and 15 min after the second AV3V infusion, or 15 min and 30 min after the onset of the i.v. infusion. The donor blood was prepared on the day of experiment by collection (into a heparinized beaker (150 U/rat)) of the trunk blood issuing after decapitation of normal rats anesthetized lightly with diethyl ether.

The blood samples were divided into two fractions immediately after collection. A 2 ml aliquot was mixed with 100 μl of a solution containing 125 mmol/l disodium EDTA and 25 mmol/l *o*-phenanthroline for subsequent measurements of plasma AVP. The remaining blood was used to check plasma osmolality and electrolytes. The separation of plasmas was performed by centrifugation at 2500 *g* at 2 °C for 15 min. The plasma samples were stored at –25 °C until required for use.

Chemicals

L-Arginine and L-NAME hydrochloride were purchased from Research Biochemicals International (Natick, MA, USA). D-Arginine and SNP were obtained, respectively, from Wako Pure Chemical (Osaka, Japan) and Sigma (St Louis, MO, USA). PGE₂ was kindly supplied by Ono Pharmaceutical (Osaka, Japan). Solutions of L- and D-arginine were prepared as follows: 174 mg or 435 mg were added, respectively, to 750 μl or 1000 μl 0.9% NaCl, followed by the addition of 1000 μl 1 mol/l HCl or 945 μl 2.5 mol/l HCl respectively. The HCl rendered the arginines soluble and neutralized the solutions to pH 7.4–7.7. SNP and PGE₂ were dissolved in 0.9% NaCl. All drug solutions were freshly made on the day of the experiments. The doses of L-NAME and PGE₂ used in this study were selected by reference to previous reports (16, 18, 19, 23, 35).

Histological analyses

After each experiment was terminated, the rats were killed by i.v. injection of a lethal dose of pentobarbital sodium. Trypan blue solution (2%) was administered i.c.v. or into the AV3V or VDB, utilizing the same infusion tubing as used for the experiments. The brain was removed. The validity of the i.c.v. infusion performed during the experiments was confirmed by cutting the brain manually and inspecting the spread of the dye in the ventricular system. When infusion sites in the AV3V and VDB were to be examined, the brains were kept in 10% formalin solution for at least 2 weeks.

Figure 1 Effects of topical infusion of L-arginine 0.6 μmol (▲, $n=4$) or 1.3 μmol (■, $n=5$), D-arginine 1.3 μmol (▼, $n=8$), or isotonic saline (○, $n=9$) into the AV3V on plasma AVP (PAPV), systolic arterial pressure (SAP) and heart rate (HR). Each solution (1.0 μl) was infused over a period of 1 min from -1 to 0 min. SAP and HR were measured every 30 s, and averaged for 5-min periods preceding blood samplings at -5, 5 and 15 min for determinations of plasma variables, including AVP. ^a $P<0.05$ compared with -5 min; ^b $P<0.05$ compared with 5 min; ^c $P<0.01$ compared with saline (○); ^d $P<0.05$, ^e $P<0.01$ compared with group receiving 1.3 μmol D-arginine (▼); ^f $P<0.01$ compared with group receiving 0.6 μmol L-arginine (▲).

Afterwards, serial coronal sections of approximately 40 μm thickness were cut with a freezing microtome. The sections were mounted on glass slides and stained with 0.1% cresyl violet. The infusion sites were judged according to the tissue injury made by the tubing, using the brain atlas (34) as a reference.

Measurements

Plasma AVP was extracted with acetone and petroleum ether and determined by radioimmunoassay. Synthetic [Arg⁸]vasopressin (Protein Research Foundation, Osaka, Japan) was used as the reference standard. Tracer-labelled [¹²⁵I]AVP was supplied from Mitsubishi Kagaku (Tokyo, Japan). The assay procedures have been described in detail elsewhere (36). Briefly, cross-reaction

of the AVP antiserum with AVP-related peptides such as lysine vasopressin, arginine vasotocin, deamino-D-AVP and oxytocin was respectively $3.2 \times 10^{-1}\%$, $8.9 \times 10^{-3}\%$, $2.6 \times 10^{-2}\%$ and less than $5.9 \times 10^{-6}\%$. The recoveries (means $\pm$ S.D.) of synthetic [Arg⁸]vasopressin added to a plasma pool were $57.2 \pm 7.4\%$ ($n=10$) at doses of 1.8–3.6 pmol/l and $54.3 \pm 6.4\%$ at doses of 9.1–36.4 pmol/l ($n=30$). The values given for plasma AVP were corrected for losses during the extraction, with a mean recovery of 55.8%. The within- and between-assay coefficients of variation for AVP assay were 10.1 and 13.9% respectively. The lower limit of detection of the AVP assay was 0.46 pmol/l.

Plasma osmolality was estimated by freezing-point depression (Dai-ichi Kagaku OM-6040, Kyoto, Japan). Plasma sodium and potassium were determined by flame photometry (Hitachi 775-A, Tokyo, Japan), and plasma chloride, by electrometric titration (Hiranuma CL-7, Tokyo, Japan). Systolic arterial pressure and heart rate were measured every 30 s from electronic or paper records, and averaged for 5-min periods preceding blood samplings.

Statistics

All data are expressed as means $\pm$ S.E.M. Within-group comparisons were carried out using a one-way analysis of variance (ANOVA) with repeated measures (5% level of significance), unless stated otherwise. Between-group comparisons were performed using a two-way ANOVA (5% level of significance). Subsequently to these analyses, comparisons of means were conducted by Scheffe's *F*-test and by Duncan's multiple-range test respectively to detect statistically significant within-group and between-group differences.

Results

Effects of AV3V infusion

AV3V infusion of isotonic saline did not significantly affect plasma AVP, osmolality, sodium, potassium and chloride, arterial pressure or heart rate throughout the experiments (Fig. 1). The infusion of 0.6 μmol L-arginine tended to increase slightly the plasma AVP, arterial pressure and heart rate 5 or 15 min later, whereas infusion of 1.3 μmol L-arginine led to prominent augmentations in these variables, peak responses appearing at 5 min (Fig. 1). The values of these variables 5 min after the administration of 1.3 μmol L-arginine were significantly greater than those observed in the group infused with the same dose of D-arginine. Although the infusion of D-arginine caused small increases in arterial pressure and heart rate, the values of these variables were not significantly different from those of the group receiving isotonic saline infusion. The administrations of L- or D-arginine did not change plasma osmolality, sodium, potassium or

Figure 2 Infusion sites in the AV3V of L-arginine 1.3 μmol ($\bullet$, $n=5$), D-arginine 1.3 μmol ($\circ$, $n=8$), SNP 168 nmol (Δ , $n=5$) or 336 nmol ($\blacktriangle$, $n=6$), hypertonic saline 770 mmol/l (∇ , $n=5$) and PGE₂ (12.8 nmol ($\blacktriangledown$, $n=8$)). Panels A–E correspond to coronal brain sections at distances from the bregma of +0.2, –0.26, –0.3, –0.4 and –0.8 mm respectively. 3V, 3rd ventricle; ac, anterior commissure; ADP, anterodorsal preoptic nucleus; AVPe, anteroventral periventricular nucleus; HDB, nucleus of the horizontal limb of the diagonal band; MPOL, medial preoptic nucleus, lateral part; MnPO, medial preoptic nucleus; MPA, medial preoptic area; MPOC, medial preoptic nucleus, central part; MS, medial septal nucleus; ox, optic chiasm; Pe, periventricular hypothalamic nucleus; StHy, striohypothalamic nucleus; VDB, nucleus of the vertical limb of the diagonal band; VMPO, ventromedial preoptic nucleus.

chloride significantly. Infusion sites of 1.3 μmol L- or D-arginine in these rats included the median preoptic nucleus, medial POA, periventricular nucleus, anteroventral periventricular nucleus, ventromedial preoptic nucleus and anterodorsal preoptic nucleus (Fig. 2).

The ability of 1.3 μmol L-arginine to enhance plasma AVP, arterial pressure and heart rate was also evident in rats receiving pre-administration of isotonic saline, the vehicle for L-NAME, 15 min before the arginine. When 0.74 μmol L-NAME was pre-administered, however, the effects of L-arginine were abolished and the values for plasma AVP, arterial pressure and heart rate detected at

Figure 3 Effects of pre-administration of L-NAME on the plasma AVP (PVP) response to AV3V infusion of L-arginine or PGE₂. The first infusion (1.0 μl) of 0.74 μmol L-NAME or saline and the second infusion (1.0 μl) of 1.3 μmol L-arginine, 12.8 nmol PGE₂ or saline were conducted over 1-min periods from –16 to –15 min and from –1 to 0 min respectively. $\circ$, saline+saline ($n=10$); Δ , saline + L-arginine ($n=6$); $\square$, saline + PGE₂ ($n=8$); $\bullet$, L-NAME + saline ($n=4$); $\blacksquare$, L-NAME+PGE₂ ($n=6$); $\blacktriangle$, L-NAME + L-arginine ($n=5$). ^a $P<0.05$ compared with –5 min; ^b $P<0.01$ compared with saline + saline ($\circ$); ^c $P<0.05$, ^d $P<0.01$ compared with saline + L-arginine (Δ); ^e $P<0.01$ compared with L-NAME + saline ($\bullet$).

5 or 15 min were not statistically different from those in the group receiving L-NAME and isotonic saline (Fig. 3, Table 1). The infusion sites of L-NAME and L-arginine in these rats were identified in the median preoptic nucleus, medial POA and periventricular nucleus.

The AV3V infusion of SNP resulted in dose-dependent increases in plasma AVP, with maximal responses at 5 min (Fig. 4). Plasma osmolality, sodium, potassium and chloride were not affected significantly. In some SNP groups, arterial pressure or heart rate at 15 min were slightly but significantly greater than the respective basal values at –5 min (Table 2). However, these values were not statistically different from those of the vehicle group, except for heart rate at 15 min in the group receiving 336 nmol SNP. It was verified that infusion sites of SNP in these rats had been in the median preoptic nucleus, periventricular nucleus and medial POA (Fig. 2).

When the AV3V sites depicted in Fig. 2 were infused with 1 μl hypertonic saline (770 mmol/l, 1540 mosmol/kg), the sodium concentration of which was equal to and the osmolality of which was relatively large compared with that of the solution containing

Table 1 Cardiovascular parameters in the rats for which plasma AVP responses are shown in Fig. 3.

Group	Systolic arterial pressure (mmHg)			Heart rate (beats/min)		
	– 5 min	5 min	15 min	– 5 min	5 min	15 min
SAL + SAL (<i>n</i> = 10)	115 ± 3	121 ± 4	115 ± 4	377 ± 21	414 ± 26	403 ± 29
SAL + L-ARG (<i>n</i> = 6)	121 ± 3	143 ± 3 ^{a,d}	132 ± 3 ^d	396 ± 10	452 ± 21 ^a	433 ± 12
SAL + PGE ₂ (<i>n</i> = 8)	113 ± 3	141 ± 3 ^{a,d}	147 ± 4 ^{a,d}	383 ± 11	523 ± 29 ^{a,c}	474 ± 24 ^{a,c}
L-NAME + SAL (<i>n</i> = 4)	130 ± 2 ^d	133 ± 3	127 ± 3 ^c	470 ± 11 ^d	460 ± 13	466 ± 25 ^c
L-NAME + L-ARG (<i>n</i> = 5)	130 ± 5	136 ± 3	135 ± 4	437 ± 7	455 ± 10	454 ± 13
L-NAME + PGE ₂ (<i>n</i> = 6)	124 ± 4	141 ± 5 ^a	155 ± 4 ^{a,b,e}	448 ± 31	511 ± 25	514 ± 17

SAL, saline; L-ARG, L-arginine.

^a *P* < 0.05 compared with – 5 min; ^b *P* < 0.05 compared with 5 min; ^c *P* < 0.05, ^d *P* < 0.01 compared with SAL + SAL; ^e *P* < 0.01 compared with L-NAME + SAL.

336 nmol SNP in 1 µl (770 mmol/l, 1172 mosmol/kg), plasma AVP increased significantly 5 and 15 min later. However, the responses were fairly small relative to those elicited by the administration of 336 nmol SNP (Fig. 4). The infusion of hypertonic saline also caused a pressor response (Table 2), whereas plasma osmolality and electrolytes remained unchanged.

Figure 4 Effects of AV3V infusion of SNP 84 nmol (▲, *n* = 6), 168 nmol (■, *n* = 5) or 336 nmol (▼, *n* = 6), or isotonic (○, *n* = 9) or hypertonic (●, *n* = 5) saline on plasma AVP (PAVP). Each solution (1.0 µl) was infused over a period of 1 min from – 1 to 0 min.^a *P* < 0.05 compared with – 5 min; ^b *P* < 0.05 compared with 5 min;^c *P* < 0.05, ^d *P* < 0.01 compared with isotonic saline (○); ^e *P* < 0.05,^f *P* < 0.01 compared with hypertonic saline (●); ^g *P* < 0.01 compared with group receiving 168 nmol SNP (■).

Effects of i.c.v. infusion

Administration of 1.3 µmol L-arginine into the lateral cerebral ventricle caused AVP-increasing responses (Fig. 5). Although the arterial pressure was slightly increased 5 min later, the values were not statistically different from those of the D-arginine group, and were significantly smaller (*P* < 0.05) than those of the group given 1.3 µmol L-arginine into the AV3V (Fig. 1). The i.c.v. infusion of L-arginine did not exert a significant effect on heart rate, plasma osmolality, sodium, potassium or chloride. In the group receiving D-arginine i.c.v., all the monitored variables remained unaltered. I.c.v. application of 336 nmol SNP elicited a depressor response 5 min later, without changing plasma AVP and other variables. These effects of SNP on plasma AVP and arterial pressure were different from those evoked by its application to the AV3V.

Effects of VDB infusion

After the VDB application of 1.3 µmol L-arginine, slight increases were observed in arterial pressure and heart rate. Values of these variables, however, were similar to those in the D-arginine group (Fig. 6). Plasma AVP, osmolality and electrolytes did not change after the application of the two agents. The infusion sites of L- and D-arginine were verified to have been situated in the areas of the VDB and medial septum, distances of which from the bregma ranged from 0.2 to 1.0 mm, according to the brain atlas of Paxinos & Watson (34).

Effects of PGE₂ and hyperosmolality in the presence or absence of L-NAME

In rats receiving pre-administration of vehicle, infusion of PGE₂ into the median preoptic nucleus and periventricular nucleus (Fig. 2) led to marked increases in plasma AVP, arterial pressure and heart rate, without affecting plasma osmolality, sodium, potassium and chloride. Such responses of plasma AVP and arterial pressure were not altered by the pre-administration of L-NAME (Fig. 3, Table 1). Although increases in heart

Table 2 Cardiovascular parameters in the rats for which plasma AVP responses are shown in Fig. 4.

Group	Systolic arterial pressure (mmHg)			Heart rate (beats/min)		
	– 5 min	5 min	15 min	– 5 min	5 min	15 min
SAL (<i>n</i> =9)	120 ± 4	121 ± 4	126 ± 4	395 ± 12	420 ± 13	452 ± 14
HT-SAL (<i>n</i> =5)	116 ± 4	135 ± 2 ^{a,c}	130 ± 4 ^a	379 ± 16	405 ± 7	433 ± 16 ^a
SNP (84 nmol) (<i>n</i> =6)	123 ± 5	129 ± 4	133 ± 3	390 ± 8	425 ± 13	441 ± 27 ^{a,b}
SNP (168 nmol) (<i>n</i> =5)	119 ± 4	118 ± 3	129 ± 6 ^{a,b}	410 ± 13	439 ± 14	457 ± 20 ^a
SNP (336 nmol) (<i>n</i> =6)	121 ± 4	117 ± 4 ^d	129 ± 5 ^b	385 ± 11	414 ± 24	393 ± 24 ^c

SAL, saline; HT-SAL, hypertonic saline.

^a *P*<0.05 compared with – 5 min; ^b *P*<0.05 compared with 5 min; ^c *P*<0.05 compared with SAL; ^d *P*<0.05 compared with HT-SAL.

rate induced by PGE₂ were rendered insignificant (*P*>0.05) by L-NAME treatment – possibly because of an increase in its basal value at –5 min – the values at 5 and 15 min were similar to those of the group receiving vehicle plus PGE₂ (Table 1).

The i.v. infusion of isotonic saline in rats receiving AV3V infusion of vehicle did not significantly affect any of the variables monitored. This was also the case when

L-NAME was administered into the AV3V before the infusion of isotonic saline (Fig. 7).

When hypertonic saline was infused i.v. after AV3V administration of vehicle, plasma AVP augmented progressively, in parallel with increases in plasma osmolality, sodium and chloride (Fig. 7). Plasma concentrations of sodium at 0, 15 and 30 min were 139.6 ± 0.7 mmol/l, 147.4 ± 0.9 mmol/l (*P*<0.01 compared with 0 min or i.v. isotonic saline infusion (142.8 ± 0.9 mmol/l)) and 151.0 ± 1.0 mmol/l

Figure 5 Effects of i.c.v. infusion of L-arginine 1.3 μmol (▲, *n*=6), D-arginine 1.3 μmol (●, *n*=3), SNP 336 nmol (■, *n*=4), or saline (○, *n*=5) on plasma AVP (PAVP), systolic arterial pressure (SAP) and heart rate (HR). Each solution (1.0 μl) was infused over a period of 1 min from – 1 to 0 min. SAP and HR were measured every 30 s, and averaged for 5-min periods preceding blood samplings at – 5, 5 and 15 min for determinations of plasma variables, including AVP. ^a*P*<0.05 compared with – 5 min; ^b*P*<0.05 compared with 5 min; ^c*P*<0.05, ^d*P*<0.01 compared with saline (○); ^e*P*<0.01 compared with D-arginine (■).

Figure 6 Effects on plasma AVP (PAVP), systolic arterial pressure (SAP) and heart rate (HR) of the topical infusion of L-arginine 1.3 μmol (■, *n*=6), D-arginine 1.3 μmol (▲, *n*=5), or saline (○, *n*=4) into the nucleus of the vertical limb of the diagonal band. Each solution (1.0 μl) was infused over a period of 1 min from – 1 to 0 min. SAP and HR were measured every 30 s, and averaged for 5-min periods preceding blood samplings at – 5, 5 and 15 min for determinations of plasma variables, including AVP. ^a*P*<0.05 compared with – 5 min; ^b*P*<0.05 compared with saline (○).

Figure 7 Effects of AV3V infusion of L-NAME on the responses of plasma AVP (PAVP), plasma osmolality (POsm) and systolic arterial pressure (SAP) to the i.v. infusion of saline 0.15 mol/l or hypertonic saline 2.5 mol/l. L-NAME 0.74 μ mol or its vehicle (saline) was infused (1 μ l) over a period of 1 min from -11 to -10 min preceding the i.v. infusion (0.1 ml/kg body weight per min) of saline or hypertonic saline started at 0 min. ^a $P < 0.01$ compared with 0 min; ^b $P < 0.05$ compared with 15 min; ^c $P < 0.05$, ^d $P < 0.01$ compared with saline AV3V + saline i.v. ($\circ$, $n = 5$); ^e $P < 0.05$, ^f $P < 0.01$ compared with L-NAME AV3V + saline i.v. ($\bullet$, $n = 5$); ^g $P < 0.05$ compared with saline AV3V + hypertonic saline i.v. (Δ , $n = 8$). $\blacktriangle$, L-NAME AV3V + hypertonic saline i.v. ($n = 6$).

($P < 0.01$ compared with 0 min or i.v. isotonic saline infusion (142.8 ± 1.1 mmol/l)) respectively, and those of chloride at 0, 15 and 30 min were 99.8 ± 1.0 mmol/l, 108.1 ± 0.9 mmol/l ($P < 0.01$ compared with 0 min or i.v. isotonic saline infusion (101.0 ± 1.7 mmol/l)) and 111.1 ± 0.7 mmol/l ($P < 0.01$ compared with 0 min or i.v. isotonic saline infusion (100.3 ± 1.8 mmol/l)) respectively. Arterial pressure was also enhanced by hypertonic saline (Fig. 7), whereas significant alteration was not caused in heart rate or plasma potassium ($P > 0.05$). AV3V application of L-NAME for which infusion sites had been located in the median preoptic nucleus and periventricular nucleus did not influence the responses of plasma AVP, osmolality and electrolytes to the osmotic load (Fig. 7).

Although the pressor action of hypertonic saline was not significant ($P > 0.05$) after the L-NAME treatment, it was noticed that basal arterial pressure at 0 min was significantly large in comparison with that of the group infused with vehicle before the osmotic load, the phenomenon by which the pressor action might have been obscured.

As mentioned above, AV3V administration of L-NAME tended to increase basal levels of arterial pressure and heart rate. When the data at -5 min in Table 1 and 0 min in Fig. 7 were analyzed collectively because they reflected the values 10 min after the L-NAME treatment, basal arterial pressure and heart rate were 120 ± 2 mmHg (pre-infusion value 121 ± 2 mmHg) and 400 ± 5 beats/min (pre-infusion value 397 ± 7 beats/min) respectively, in rats given vehicle infusion, and 128 ± 2 mmHg ($P < 0.01$ compared with pre-infusion value (118 ± 1 mmHg) by paired t -test or compared with vehicle group by Duncan's test) and 437 ± 8 beats/min ($P < 0.01$ compared with pre-infusion level (397 ± 6 beats/min) or vehicle group), respectively, in rats given L-NAME infusion.

Discussion

Over recent years, histochemical studies have demonstrated the existence of NOS in neural cell bodies and fibers of the AV3V (15, 16), the region important for autonomic functions (10–12). However, the physiological significance of NO generated in this area has scarcely been investigated yet, despite various observations suggesting significant roles of cerebral NO in controlling autonomic functions (1, 2). The present study was undertaken to explore roles of AV3V NO in AVP secretion and cardiovascular function in the presence or absence of an osmotic stimulus or its related factor, PGE₂ (18, 37).

We demonstrated that AV3V application of L-arginine augmented plasma AVP, arterial pressure and heart rate in basal conditions (Fig. 1). The effects on the first two variables were the same as those induced by i.c.v. application of this agent (9), supporting the view that action sites of drugs given into the cerebral ventricle may include the AV3V (26). As the increase in plasma AVP appeared shortly after the L-arginine infusion in the absence of AVP-releasing factors such as increased plasma osmolality or sodium and reduction of blood pressure, it may be attributable to the ability of the agent to promote AVP secretion. The solution containing L-arginine at the dose of 1.3μ mol in 1 μ l showed hyperosmolality of about 1.8 osmol/kg. Because AVP-releasing mechanisms in the AV3V possess osmo- or sodium-sensitivity (38), as we verified in the present study (Fig. 4), it could be argued that the AVP-enhancing ability of the AV3V infusion of L-arginine may have been due to the hyperosmolality of the solution. However, substances such as L-arginine that are capable of easily penetrating the cell membrane are

known to be ineffective as osmotic stimuli (39). In fact, AV3V infusion of the D-arginine solution (1.3 μmol in 1 μl), which had the same osmolality as the L-arginine solution and which cannot be used for NO synthesis, was not able to increase plasma AVP or the cardiovascular variables (Fig. 1). Moreover, the responses of these variables to AV3V L-arginine were abolished by the potent NOS inhibitor, L-NAME (25). Considering the current results together with the presence of NOS or its mRNA in the AV3V (15, 16), it seems that NO synthesized from L-arginine may have been responsible for the AVP-enhancing, pressor and tachycardiac effects evoked by the AV3V infusion of the agent. This idea is supported in part by the result obtained with SNP, the drug capable of generating NO without depending on NOS activity (32): AV3V infusion of the SNP solution (336 nmol in 1 μl) resulted in remarkable augmentation of plasma AVP, and the AVP response was markedly large compared with that elicited by the administration of hypertonic NaCl (770 nmol in 1 μl), the osmolality of which was relatively greater and the sodium concentration of which was equal (Fig. 4). The AVP-releasing activity of SNP has also been reported by an *in vitro* study using a brain slice preparation (7). Unlike the effects of AV3V L-arginine, AV3V SNP did not cause conspicuous pressor and tachycardiac actions (Table 2). Although the reason for this discrepancy is not certain, differences in the sites and periods of generation of NO (40) may be indicated, in addition to a possible influence of the cyanate moiety of SNP.

The VDB is also known to contain NOS activity (1, 2, 33). This region is adjacent to the AV3V and may be involved in the regulation of the release of AVP associated with blood pressure changes (41). Therefore, it could be suspected that L-arginine applied into the AV3V might have diffused into this area to elicit the effects. However, infusion of L-arginine into the VDB affected none of the variables monitored (Fig. 6). This result makes such a possibility unlikely and suggests a negligible role of VDB NO in controlling the release of AVP or the cardiovascular system. Conversely, the fact that an AVP-enhancing effect was brought about not only by AV3V L-arginine but also by i.c.v. L-arginine might arouse a suspicion that the agent given into the AV3V diffused into the ventricular system to produce an AVP-releasing effect through actions on other periventricular areas. It seems certain, however, that the AV3V possesses AVP-releasing mechanisms sensitive to NO, because the effect of SNP on AVP was observed only when it was applied into the AV3V (Fig. 4). L-Arginine did not induce pressor or tachycardiac effects in the case of the i.c.v. infusion. Therefore, it seems reasonable to speculate that the AVP and cardiovascular responses to AV3V L-arginine may have been caused primarily by NO action on the AV3V.

The AV3V neurons receive afferent inputs arising from the brainstem catecholaminergic neurons, and innervate neurons in the PVN and SON that send axons

to the neurohypophysis and brainstem cardiovascular areas, in addition to those in the parabrachial nucleus and mesencephalic central gray (11, 12, 42–44). NO has been demonstrated to potentiate basal and evoked release of dopamine, noradrenaline and acetylcholine from presynaptic nerve endings in several brain regions (45); these are the neurotransmitters capable of inducing AVP release and pressor action when liberated into the AV3V or i.c.v. (46–50). Glutamate release may also be potentiated by NO (51). In addition, NO is known to function as one mediator in intracellular signaling pathways triggered by stimulation of N-methyl-D-aspartate, α -adrenergic or cholinergic receptors that have been shown to reside in the AV3V (20, 24, 52). Taken together, these observations could be presumed to indicate that NO generated from L-arginine or SNP given into the AV3V may have exerted the cardiovascular and pituitary effects by affecting release of those neurotransmitters or intracellular events triggered by them.

We have confirmed our previous finding that AV3V infusion of PGE₂ leads to remarkable increases in plasma AVP, arterial pressure and heart rate (Fig. 3, Table 1). It is probable that PGE₂ receptors in the AV3V may have been responsible for these phenomena, because they are abundant in this region (14) and the agent applied into the i.c.v. or the neighboring structures such as the medial septal nucleus, anterior commissure or VDB were not effective (50). The effects of the prostanoid were not affected significantly by pre-treatment with L-NAME (Fig. 3, Table 1). This result is in agreement with the data that excitation of PVN neurons in response to a stimulus (IL-1 β), the effect of which may be dependent on stimulation of PGE₂ synthesis in periventricular areas, was not influenced by i.c.v. L-NAME (31). The possibility that the dose of L-NAME used may have been large enough to inhibit NOS activity is suggested by the current observation that the treatment with L-NAME abolished the AV3V actions of L-arginine, and by previous reports that the drug injected centrally at doses similar to those we used brought about maximal inhibition of NOS activity over a 6-h period (25) and prevented hypotension-evoked AVP secretion throughout a 30-min experimental period (35). Therefore, it appears that NOS in the AV3V may not be involved in the AVP and cardiovascular responses to PGE₂. Although POA application of L-NAME has been reported to block the febrile response to PGE₂ (22), cerebral mechanisms underlying the thermogenic action of PGE₂ may be different from those implicated in the AVP and cardiovascular responses to the prostanoid. Similarly, AV3V application of L-NAME was without significant effect on the responses of AVP and arterial pressure to i.v. infusion of hypertonic saline (Fig. 7). This result supports the observation of Goyer *et al.* (27) that L-NAME infused into the peripheral circulation because of its ability to cross the blood–brain barrier (25) did not alter such responses in rabbits. The

fact that the AV3V infusion of L-NAME influenced neither the effects of plasma hypertonicity nor those of PGE₂, one cerebral factor engaged in the osmotic responses (18, 37), suggests that NOS activity in the AV3V may not have an essential role in regulating AVP release and arterial pressure in the hyperosmotic state. In contrast to this view, Liu *et al.* (23) have reported inhibition of osmotic drinking by i.c.v. L-NAME, suggesting a possible role of periventricular NOS activity in the osmosensitivity. However, those investigators did not locate the sites of action of this drug.

In conclusion, we suggest that NO synthesized in the AV3V may not be implicated in the AVP-releasing and cardiovascular responses to AV3V PGE₂ or plasma hyperosmolality, despite its potential to facilitate hormone secretion and cause pressor and tachycardiac actions. At present, the physiological role of AV3V NO remains obscure. To clarify this problem, further studies are necessary.

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