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Effects of water deprivation and angiotensin II intracerebroventricular administration on brain nitric oxide synthase activity

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Abstract

Intracranial administration of L-arginine causes a reduction of the water intake induced by water deprivation or by intracerebroventricular (i.c.v.) injection of angiotensin II (angiotensin II), through the release of nitric oxide (NO) in the central nervous system. We studied the effects of i.c.v. angiotensin II (120 ng/rat) in association with i.c.v. L-arginine (2.5–10 μg/rat) on blood pressure. We also studied the effects of both peripheral and central angiotensin II injection (1.5–6 mg kg^{-1} i.p. and 30–120 ng rat^{-1} i.c.v., respectively) on NO synthase activity in the cortex, diencephalon and brainstem, after water deprivation (24 h), conditions producing activation of the renin-angiotensin system. L-arginine dose dependently antagonized the increase in blood pressure induced by i.c.v. angiotensin II ($P < 0.001$). Peripheral administration of angiotensin II produced a dose-dependent reduction of NO synthase activity in the brainstem and cortex ($P < 0.001$), but not in the diencephalon. Water deprivation produced similar effects on brain NO synthase activity. Angiotensin II i.c.v. injection caused NO synthase activity reduction in all brain regions studied ($P < 0.001$). Our findings suggest that NO and angiotensin II could play opposite roles in brain regulation of blood pressure and drinking behaviour.

Keywords: Nitric oxide (NO); Angiotensin II; Nitric oxide (NO) synthase; Blood pressure; Fluid balance; Water deprivation

1. Introduction

The existence of an L-arginine/nitric oxide pathway in the central nervous system (CNS) has been shown and nitric oxide synthase, the enzyme responsible for nitric oxide formation, has been found in the brain (Garthwaite, 1991), where it was found to be widespread in neurons (Griffith and Stuehr, 1995).

When released, nitric oxide diffuses into the cells, interacting fundamentally with guanylate cyclase (Zhang and Snyder, 1995). In the CNS, it may participate in long-term potentiation (Schumann and Madison, 1991) and plays a role in the development of the nervous system (Bredt and Snyder, 1994). Other evidence indicates that this substance is implicated in the mechanisms of neurotoxicity in response to glutamate (Dawson et al., 1991, 1993).

It has been also shown that pharmacological manipulations of the L-arginine/nitric oxide pathway modifies both fluid and food ingestive behaviour. In particular, the administration of inhibitors of NO synthase reduces the intake of food in mice and in rats (Morley and Flood, 1991; Squadrito et al., 1993). Instead, brain nitric oxide formation has antidipsogenic effects in rats in which drinking behaviour is stimulated by central injection of the dipsogenic agent, angiotensin II, or by water deprivation (Calapai et al., 1992, 1994), a condition inducing changes of either intracellular or extracellular components and increases in plasma renin activity and angiotensin II concentration (Yamaguchi, 1981; Barney et al., 1983). The dipsogenic action of angiotensin II is also antagonized by a previous central injection of vasoplegic agents such as nitroprusside (Elghozi et al., 1977). The increase in blood

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pressure is another biological effect occurring when angiotensin II is centrally injected in the conscious rat and a central angiotensinergic system is involved either in the drinking response following water deprivation, or in the blood pressure increase induced by i.c.v. injection of angiotensin II (Rohmeiss et al., 1995). Furthermore, positive angiotensin II immunoreactivity is present in neurons and fibers connecting the brainstem, hypothalamus and cortex (Lind et al., 1985). Angiotensin II is thus considered to have a neurotransmitter role in neural pathways mediating the regulation of thirst and the cardiovascular system (Yang et al., 1992). Since it is well known that nitroprusside is a nitric oxide donor (Moncada et al., 1991) it could be hypothesized that its antagonism by angiotensin II is due to nitric oxide formation in the brain.

In the light of these findings, we investigated the possible interaction between brain nitric oxide and angiotensin II in the central regulation of blood pressure. With the aim to investigate further the possible relation between nitric oxide and angiotensin II, we also evaluated the effects of water deprivation and peripheral and central injection of the peptide on NO synthase activity of the cortex, diencephalon and brainstem.

2. Material and methods

2.1. Animals

Adult male Sprague–Dawley rats weighing 280–320 g were used. The animals were housed at a constant temperature of 22 + 2°C under a 12/12 h light–dark cycle (lights on at 6:00 a.m.), with free access to Purina rat chow pellets and tap water, unless otherwise stated.

2.2. Intracerebroventricular administration

Stainless steel guide cannulas (o.d. = 0.66 mm) were inserted seven days before the experiments. The rats were anesthetized with chloral hydrate (400 mg kg, i.p.) and placed in a stereotaxic instrument (Stellar, Stoelting). Cannulas were implanted in the left lateral cerebral ventricle (stereotaxic coordinates: AP = 1 mm behind the bregma, L = 2.5 mm from the midsagittal suture and V = 2 mm from the dura) according to the Paxinos and Watson atlas (Paxinos and Watson, 1986). Injections into the lateral ventricle (5 μl) were made with a 30-gauge injector temporarily inserted into the guide cannula and protruding 2 mm beyond the cannula tip. Injections were carried out over a period of 1 min.

2.3. Arterial blood pressure

Animals with cannulas implanted in the lateral cerebral ventricle were used to monitor blood pressure. Briefly, animals with a previously implanted cannula in the left lateral cerebral ventricle, had anaesthesia induced and maintained with isoflurane (5% and 2–3%, respectively) mixed with oxygen and nitrous oxide, and a catheter (PE 50) inserted into the left common carotid artery, externalised and secured at the back of the neck. After surgery, these rats were returned to their cages, where they were allowed to recover from anesthesia (about 15 min). After 24 h, the arterial catheter was connected to a pressure transducer. The pressure pulse triggered a cardiotachometer, and arterial blood pressure was displayed on the channels of a polygraph. Angiotensin II (120 ng), L-arginine (2.5–10 μg), D-arginine (10 μg) or artificial cerebrospinal fluid (CSF) was injected i.c.v. in a volume of 5 μl per rat. The i.c.v. injector was inserted 30 min before drug administration to record stable blood pressure. Arterial blood pressure was recorded in conscious, freely moving rats and is reported either as systolic blood pressure or mean arterial blood pressure in mm Hg.

2.4. Nitric oxide synthase activity

To measure NO synthase activity in the frontal cortex, diencephalon and brainstem, the following experiments were conducted. One group of animals was deprived of water but not of food, then killed after various times of deprivation (0, 8, 16 and 24 h). A second group of animals was treated with intraperitoneal (i.p.) administration of various doses of angiotensin II (1.5, 3 or 6 mg kg) or with vehicle and killed 1 h after angiotensin II injection. A third group was treated with i.c.v. administration of different doses of angiotensin II or with artificial cerebrospinal fluid (CSF) and killed 30 min after treatment. Another experiment was carried out on normohydrated and water deprived animals treated with captopril (100 mg kg^{-1} i.p.) or with the vehicle for two days before brain NO synthase activity evaluation and killed 2 h after the last administration. All groups were killed under light anesthesia with ether, by decapitation. The brain was rapidly removed and brain areas quickly dissected out and immediately frozen in liquid nitrogen.

Tissues were homogenized at 4°C in 4 volumes of Hepes buffer 20 mM pH 7.2 containing 320 mM sucrose, 1 mM DL-dithiotreitol, 10 μg ml soybean trypsin inhibitor, 2 μg ml aprotinin and 10 μg ml leupeptin. The homogenates were centrifuged at 100,000 × *g* for 30 min at 4°C. The supernatants, i.e., the cytosolic fractions containing NO synthase activity, were stored at −70°C until use. Protein concentration in the cytosolic fraction was measured spectrophotometrically according to Bradford using bovine serum albumin as standard (Bradford, 1976).

NO synthase activity was evaluated by measuring the rate of conversion of L-[U−^{14}C]arginine to [U−^{14}C]citrulline according to Salter et al. (1991) and expressed as nmol min g tissue. Briefly, an aliquot of the cytosolic

Table 1
Effects of intracerebroventricular (i.c.v.) injection of CSF (control), L-arginine, D-arginine, angiotensin II, L-arginine + angiotensin II and D-arginine + angiotensin II on systolic blood pressure in conscious freely moving rats

Treatment	Systolic blood pressure
Control	121.0 ± 3.2
L-arginine (10 μg)	120.0 ± 3.0
Angiotensin II (120 ng)	151.0 ± 3.2
L-arginine (2.5 μg) + angiotensin II (120 ng)	152.0 ± 3.3
L-arginine (5 μg) + angiotensin II (120 ng)	139.5 ± 3.0[a]
L-arginine (10 μg) + angiotensin II (120 ng)	129.0 ± 3.3[b,c]
D-arginine (10 μg) + angiotensin II (120 ng)	153.2 ± 3.7

[a] $P < 0.05$ vs. angiotensin II.
[b] $P < 0.01$ vs. angiotensin II.
[c] $P < 0.05$ vs. L-arginine (5 μg) + angiotensin II (120 ng).
Each value represents the mean ± S.E. for five or six animals.

fraction (100 mg of protein) was preincubated for 5 min at 37°C in 50 mM potassium phosphate buffer pH 7.2 containing 60 mM L-valine, 120 μM NADPH, 1.2 mM L-citrulline, 1.2 mM $MgCl_2$ and 0.24 mM $CaCl_2$ in the presence of drug or vehicle. The samples were then incubated for 10 min at 37°C with L-[U $-^{14}$C]arginine (150,000 dpm) and 20 μM L-arginine. The reaction was stopped by the addition of 1.0 ml of a mixture of H_2O/Dowex-50 W 1:1 v/v (200-400, 8% cross-linked Na^+ form). The Na^+ form of Dowex-50 W was prepared by washing four times the H^+ form of the resin with 1 M NaOH and then with bi-distilled water until the pH was less than 7.5. The resin was settled by centrifugation (11,000 × g for 3 min) in a microfuge (Beckman, Microfuge 11) and an aliquot of the supernatant was taken for scintillation counting (4 ml Pico-Aqua; Packard 1500). The activity of Ca^{2+}/dependent NO synthase was determined from the difference between the [U $-^{14}$C]citrulline produced by control samples and that in samples containing 1 mM EGTA; the activity of the Ca^{2+}/independent enzyme was determined from the difference between the [U $-^{14}$C]citrulline produced by samples containing 1 mM EGTA and that in samples containing 1 mM EGTA plus 1 mM N^G-monomethyl-L-arginine (L-NMMA).

2.5. Statistical analysis

All statistical procedures were performed using an SPSS statistical software package release 6.1.3 (SPSS, Chicago, IL, USA). The data were evaluated with a One-way analysis of variance (ANOVA) and the Scheffé post-hoc test for multiple comparisons. The data are expressed as the means ± standard error. Statistical significance was set at $P < 0.05$.

3. Results

3.1. Arterial blood pressure

Angiotensin II i.c.v. administration (120 ng rat) caused a marked increase in systolic blood pressure in conscious freely moving rats compared with that of CSF-treated animals. Pretreatment with L-arginine i.c.v. injection, given 30 min before angiotensin II, produced a dose-dependent reduction the of blood pressure increase ($P < 0.001$). The stereoisomer, D-arginine, neither reduced nor changed the pressor effects induced by angiotensin II i.c.v. administration (Table 1; Fig. 1).

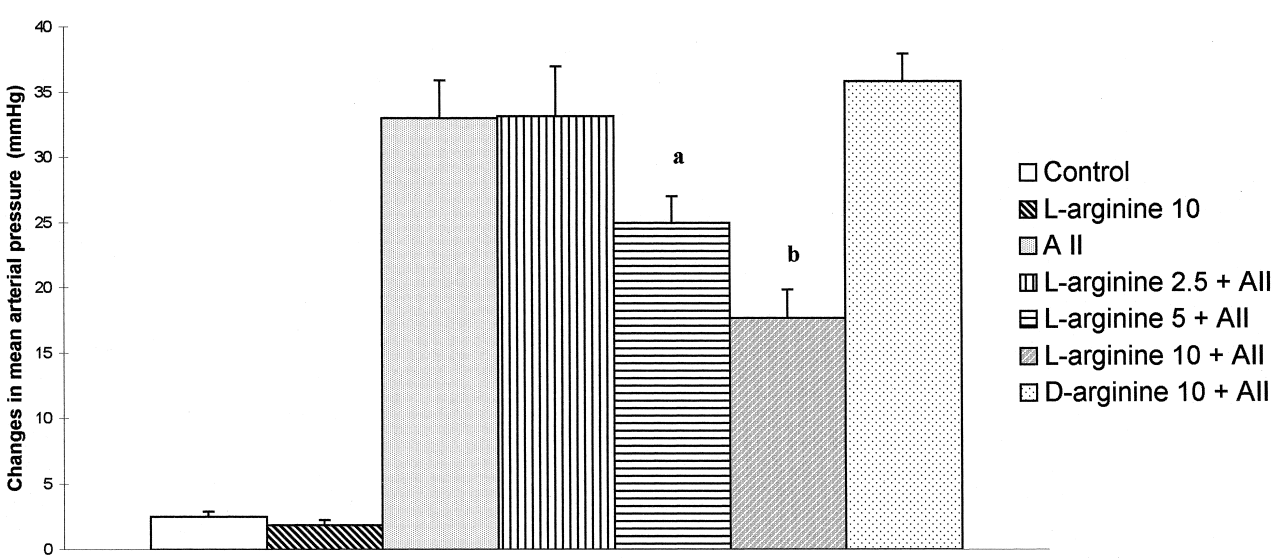

Fig. 1. Effects of i.c.v. injection of CSF (control), L-arginine (μg), angiotensin II (120 ng), L-arginine (μg) + angiotensin II (AII; 120 ng) and, D-arginine (μg) + angiotensin II (AII; 120 ng) on mean arterial pressure in conscious freely moving rats. Each column represents the mean ± S.E. for five or six animals. [a] $P < 0.001$ vs. AII; [b] $P < 0.05$ vs. L-arginine 5 + AII.

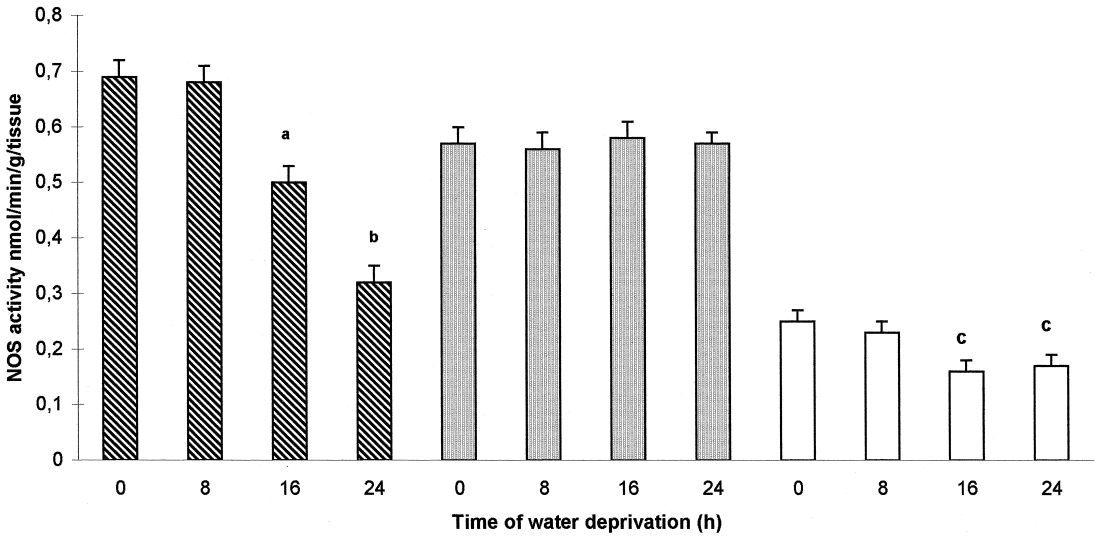

Fig. 2. Effects of water deprivation (0, 8, 16 and 24 h) on brain nitric oxide synthase activity in the frontal cortex, diencephalon and brainstem of rat. Black columns = frontal cortex; grey columns = diencephalon; white columns = brainstem. Each column represents the mean ± S.E. for five or six animals. [a]$P < 0.05$ vs. 0 h; [b]$P < 0.001$ vs. 0 h; [c] = $P < 0.05$ vs. 0 h.

3.2. Nitric oxide synthase activity

NO Ca^{2+}-independent synthase activity was detectable in the brains of rats in any of the groups studied. Water deprivation (24 h) induced a significant reduction of neuronal NO synthase activity in the brainstem and frontal cortex ($P < 0.001$). In the frontal cortex of water deprived rats, after 16 and 24 h of water deprivation, there was a of 27% and 54% reduction, respectively. In the brainstem, the neuronal NO synthase activity reduction after 16 and 24 h of water deprivation was 36% and 32% of the baseline values (0 h), respectively. The effect in the frontal cortex seemed time-dependent, becoming evident at 16 h after the beginning of deprivation. Diencephalic neuronal NO synthase activity did not differ from baseline values (Fig. 2). The results were similar for animals treated with an-

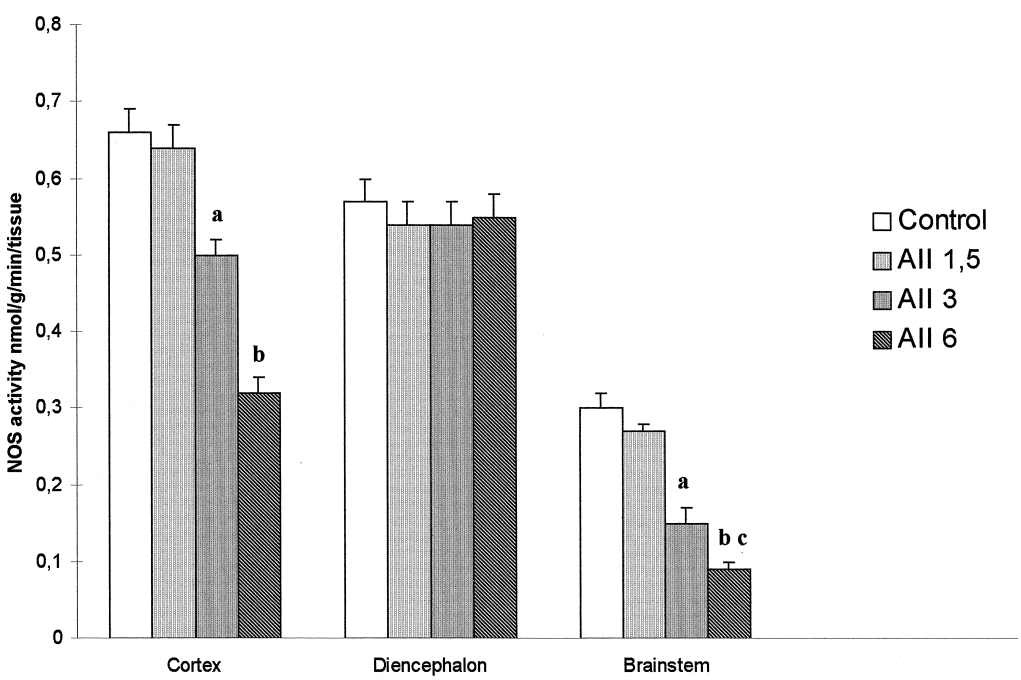

Fig. 3. Effects of subcutaneous injection of angiotensin II (AII; 1.5–6 mg kg^{-1}) on brain nitric oxide synthase activity in the frontal cortex, diencephalon and brainstem of rats. Each column represents the mean ± S.E. for five or six animals. [a]$P < 0.001$ vs. control; [b]$P < 0.001$ vs. AII 3; [c]$P < 0.05$ vs. AII 3.

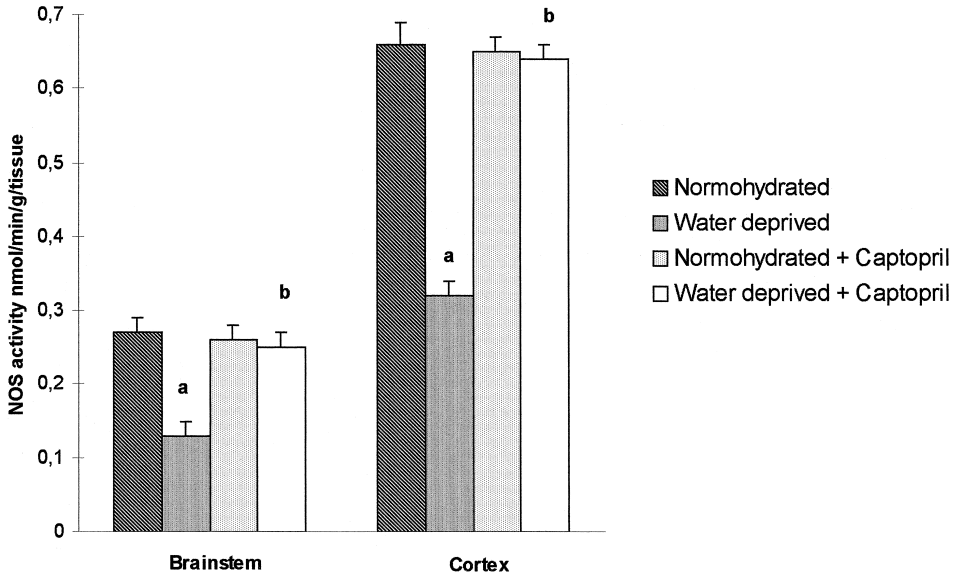

Fig. 4. Effects of intraperitoneal injection of captopril (100 mg kg^{-1}) on brain nitric oxide synthase activity in the brainstem and frontal cortex of normohydrated and water deprived rats. Each column represents the mean ± S.E. for five or six animals. [a]$P < 0.001$ vs. normohydrated; [b]$P < 0.001$ vs. water deprived.

giotensin II systemically. Systemic administration of the octapeptide reduced in a dose-dependent manner the neuronal NO synthase activity in the cortex (24% and 51% with doses of 3 and 6 mg kg^{-1}, respectively) and in the brainstem (50% and 70% with doses of 3 and 6 mg kg^{-1}, respectively) (Fig. 3). The water deprivation-induced NO synthase activity reduction in either cortex or brainstem was abated by peripheral captopril (100 mg kg^{-1}, i.p.) injection (Fig. 4).

Intracerebroventricular administration of different doses of angiotensin II (30–120 ng rat^{-1}) showed to reduced neuronal NO synthase activity in all the areas examined. The effect was present starting with the dose of 60 ng rat^{-1} and clearly dose-dependent after injection of 120 ng

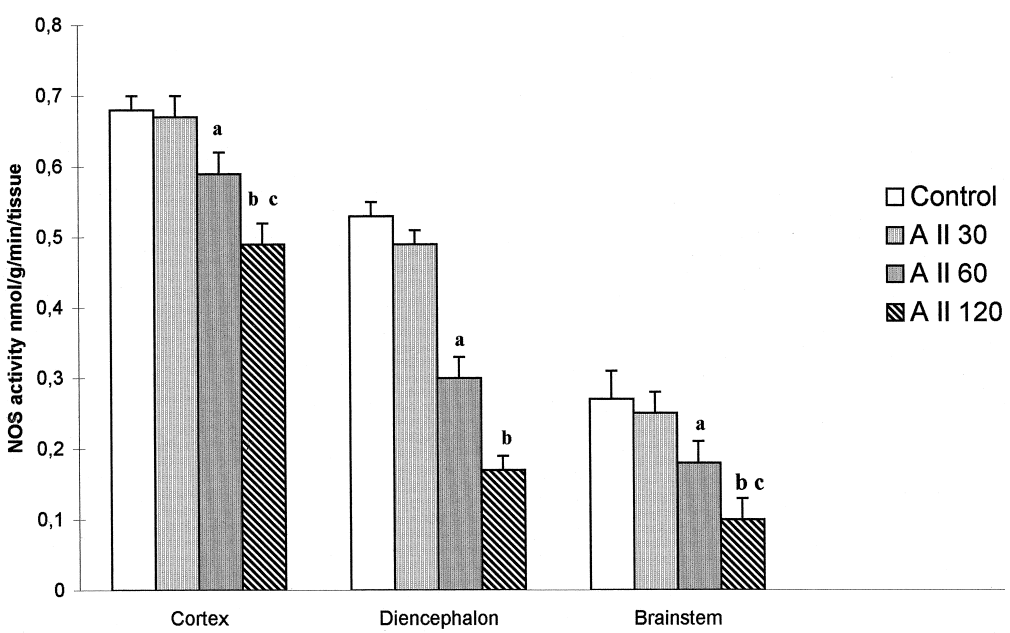

Fig. 5. Effects of i.c.v. injection of angiotensin II (AII; 30–120 ng) on brain nitric oxide synthase activity in the frontal cortex, diencephalon and brainstem of rats. Each column represents the mean ± S.E. for five or six animals. [a]$P < 0.05$ vs. control; [b]$P < 0.001$ vs. control; [c]$P < 0.05$ vs. AII 3.

of angiotensin II. The percentage reduction following the highest dose of angiotensin II i.c.v. injection was of 28%, 68% and 63%, in the frontal cortex, diencephalon and brainstem, respectively (Fig. 5).

4. Discussion

Nitric oxide is implicated in several central nervous system functions including neuroendocrine functions (Costa et al., 1993), long-term potentiation (Haley et al., 1992) and ingestive behaviour (Calapai and Caputi, 1996; Stricker-Krongrad et al., 1996).

An involvement of nitric oxide in the central control of drinking behaviour has been suggested (Calapai et al., 1992; Zhu and Herbert, 1997), but it is not well defined. Brain nitric oxide formation in the rat, particularly in the preoptic area, modulates drinking behaviour induced by intracranial administration of angiotensin II or by water deprivation (Calapai et al., 1994). More exactly, water intake, stimulated either by water deprivation or by angiotensin II i.c.v. injection, is reduced by central pretreatment with the precursor of nitric oxide L-arginine, while it returns to normal when inhibitors of NO synthase are given together with L-arginine (Calapai et al., 1992).

Three isoforms of nitric oxide synthase, the enzyme responsible nitric oxide formation, have been localized and purified. One is the neuronal constitutive form and the others are the constitutive and inducible endothelial form (Förstermann and Kleinert, 1995). The various isoforms of NO synthase are, for the most part, distributed in cells and tissues according to their apparent function (Fukuto and Chauduri, 1995). All the isoforms catalyze five-electron oxidation of a guanidino nitrogen of the amino acid, L-arginine, producing nitric oxide and citrulline (Marletta, 1994). The neuronal form of NO synthase is both calmodulin and Ca^{2+}-dependent (Bredt and Snyder, 1990).

Emerging evidence indicates that nitric oxide in the brain could be implicated in the mechanisms regulating the cardiovascular system. Findings of Togashi et al. indicate that nitric oxide formed in the brain could participate in the regulation of arterial blood pressure by reducing the sympathetic tone of peripheral resistance vessels (Togashi et al., 1992). Furthermore, studies carried out in anesthetized rats showed that NO donors, i.c.v. injected cause a fall in blood pressure, whereas administration of inhibitors of NO synthase by the same route produces a rise in blood pressure (Cabrera and Bohr, 1995). On the other hand, it has been observed that i.c.v. injection of L-arginine exerts pressor actions through stimulation of the brain renin-angiotensin system and peripheral nervous system (Nishimura et al., 1997). Also, a tonic excitatory role for nitric oxide in the central regulation of blood pressure in the spinal cord has been suggested (Lee et al., 1996). In our experiments, centrally injected (i.c.v.) doses of L-arginine, 10-fold lower than those showing pressor action, failed to change blood pressure. However, the same treatment prevented the increase in blood pressure induced by i.c.v. injection of angiotensin II. This effect was not observed when L-arginine was replaced by the stereoisomer, D-arginine. Since L-arginine, but not D-arginine, is the substrate for NO synthase, this findings suggests that the antagonism by L-arginine of central angiotensin II pressor effects could be due to nitric oxide formation in the brain.

It has been shown that mean arterial blood pressure and heart rate are not significantly changed, but that plasma osmolality, renin activity, angiotensin II and vasopressin concentrations increase following 24 h of water deprivation (Brooks, 1992). The blood-borne angiotensin II formed during water deprivation, enters the circulation, successively acting on receptor sites in brain areas lacking a blood-brain barrier, where it may act as a neuromodulator or neurotransmitter (Steckelings et al., 1992). Our experiments showing that captopril, an inhibitor of angiotensin converting enzyme (Barnes et al., 1992), can abate the water deprivation-induced reduction of NO synthase activity in the cortex and brainstem, suggest that peripheral angiotensin II released during the water deprivation period may be responsible for this effect.

Angiotensin II, given s.c., reduced NO synthase activity in cortex and brainstem. Similar results were obtained when neuronal NO synthase activity was measured in water-deprived rats. This latter experiment showed that water deprivation can induce a reduction of neuronal NO synthase activity. These data, according to our previous observations, confirm that nitric oxide may have a role in the regulation of fluid ingestion.

At this point, one could hypothesize that the antagonism of nitric oxide to the angiotensin II dipsogenic action is related to the opposite vascular effects of the two substances. This hypothesis cannot be absolutely excluded. However, it has been shown that intracranial angiotensin II-induced drinking can be dissociated from its pressor effects and that these angiotensin II central actions are not always linked (Fitzsimons and Kucharczyk, 1978). Thus, nitric oxide and angiotensin II effects on drinking and blood pressure may not be inter-dependent. Furthermore, the i.c.v. injection of L-arginine alone did not change arterial blood pressure suggesting that the interaction between the two compounds cannot be simply a vascular one. A possible explanation is that brain nitric oxide formation antagonizes the blood pressure increase induced by centrally injected angiotensin II through a neurogenic interaction influencing the control of arterial blood pressure.

Administration of angiotensin II into the cerebral lateral ventricle produced a reduction of neuronal NO synthase activity, not only in the cortex and brainstem, but also in the diencephalon. In addition, the diencephalic NO synthase activity reduction was greater than that in the other two brain regions examined. One explanation could be that water deprivation produces several effects including an-

giotensin II induction but that some competing biochemical process may offset the angiotensin II effects in the diencephalon. Another possible explanation for the differences in neuronal NO synthase activity following water deprivation or central angiotensin II injection is that angiotensin II formed during water deprivation stimulates a different kind of receptor other than those involved in the effect caused by intracranial administration of the substance.

Our data indicate that a relationship between nitric oxide and angiotensin II exists in the brain. It is particularly evident that angiotensin II seems to act as a direct or indirect inhibitor of brain NO synthase. Moreover, the interactions we found between nitric oxide and angiotensin II indicate that they could have opposite roles in the brain in the regulation of drinking and blood pressure.

In conclusion, there are several lines of evidence showing that nitric oxide interacts with different neurotransmitters, but this is the first report of an influence of angiotensin II on brain nitric oxide formation. This interaction may influence the central regulation of blood pressure and fluid balance.

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