Increased central AT₁-receptor activation, not systemic vasopressin, sustains hypertension in ANP knockout mice

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ACKERMANN, Uwe, and Newsha Azizi. Increased central AT₁-receptor activation, not systemic vasopressin, sustains hypertension in ANP knockout mice. Am J Physiol Regulatory Integrative Comp Physiol 278: R1441–R1445, 2000.—We tested the hypothesis that hypertension in atrial natriuretic peptide (ANP) knockout mice is caused in part by disinhibition of angiotensin II-mediated vasopressin release. Inactin-anesthetized F₂ homozygous ANP gene-disrupted mice (−/−) and wild-type (+/+) littermates were surgically prepared for carotid arterial blood pressure measurement (ABP) and background intravenous injection of physiological saline or vasopressin V₂-receptor antagonist (Manning compound, 10 ng/g body wt) and subsequent intracerebroventricular (left lateral ventricle) injection of saline (5 µl) or ANP (0.5 µg) or angiotensin II AT₁-receptor antagonist losartan (10 µg). Only (−/−) showed significant decrease in ABP after intracerebroventricular ANP or losartan. Both showed significant hypertension after intravenous V₂ antagonist, but there was no difference between their responses. We conclude that 1) vasopressin contributes equally to ABP maintenance in ANP-disrupted mice and wild-type controls; 2) permanently elevated ABP in ANP knockouts is associated with increased central nervous angiotensin II AT₁-receptor activation; 3) disinhibition of central nervous angiotensin II AT₁ receptors in ANP-deficient animals does not lead to a significant increase in the importance of vasopressin as a mechanism for blood pressure maintenance.

atrial natriuretic peptide; angiotensin II; central nervous system; blood pressure

IN HEALTHY MAMMALS atrial natriuretic peptide (ANP) is synthesized, stored, and secreted most abundantly by cardiac atrial myocytes (38). Secretion occurs predominantly in response to stress on the atrial wall (17). In some settings, the peptide has a pronounced natriuretic effect, and this use was taken as the hallmark of ANP action. Recent studies in ANP knockouts or transgenics that overexpress the peptide have revealed that ANP is not essential for normal salt balance, even on a high-salt diet (16). However, it exerts a persistent hypertensive effect in transgenic mice in whom increased plasma ANP levels, 5- to 8-fold above normal, are associated with a 25-mmHg decrease in mean arterial blood pressure (ABP) (36). Similarly, mice lacking the pro-ANP gene have a persistent hypertension (16). These blood pressure effects do not arise from ANP-mediated changes in sodium balance (16). They are due to changes in peripheral resistance (2, 23), although isolated peripheral resistance vessels show little response to direct application of ANP (6, 27).

Biologically active receptors for ANP (ANP-A or GC-A receptors) have been localized to large vessels (7, 37), but, with the exception of some vasodilatation in the renal artery and the rabbit facial vein (7), their activation appears to have little effect on whole body vascular resistance. Isolated resistance vessels also show no dilatation after locally applied ANP, except when they have been preconstricted by a-adrenergic agents (6, 27). It is puzzling, therefore, that transgenic mice with a chronic increase in plasma ANP should show life-long hypotension that is due to decreased peripheral resistance (2) and that mice lacking the pro-ANP gene show chronically elevated mean ABP that is also due to changes in total peripheral resistance (23).

We recently sought to determine whether ANP influences either tissue content of the endothelial factors, nitric oxide, endothelin 1 or C-type natriuretic peptide (CNP), or their contribution to blood pressure maintenance. Our results showed no differences among ANP knockouts, ANP transgenics, or wild-type mice (22). Having, therefore, ruled out altered endothelial biology as a major explanation for chronic blood pressure changes in ANP knockouts or transgenics and having seen no differences in their basal plasma renin activity (8), we address in this report the question whether the vasopressin system may be significantly involved in maintenance of a hypertensive state in ANP knockouts.

Vasopressin is synthesized in the cell bodies of magnocellular neurons of the supraoptic and paraventricular nuclei of the hypothalamus. It is transported in axons that project to the posterior lobe of the pituitary. Its release is inhibited by neural input from atrial and arterial stretch receptors (11) and is promoted by several afferents from peripheral sensors or central nervous nuclei (11). Vasopressin release is also promoted by a₁-adrenergic agonists, such as noradrenaline secreted from fibers originating from cell groups in the ventrolateral medulla, or nicotinic agonists, such as acetylcholine from cholinergic neurons adjacent to the supraoptic nucleus (11). Of particular importance are the reports that show angiotensin II-mediated potentiation of vasopressin release as well as suppression of vasopressin release by angiotensin II antagonists (33). These observations are significant because of the known

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The reciprocal relationship between ANP and angiotensin II in the regulation of blood pressure and sympathetic outflow (35) as well as the known inhibitory influence of ANP on vasopressin release (25, 30). Yamada et al. (42) have demonstrated a specific inhibitory action of ANP on angiotensin II-stimulated vasopressin release. These observations led us to hypothesize that the life-long hypertension in ANP knockout mice is caused, at least in part, by disinhibition of angiotensin II-mediated vasopressin release. The experiments described here were designed to answer three questions relevant to the hypertension of ANP-deleted mice: 1) Is lack of central ANP involved? 2) Is central angiotensin II involved? 3) Is peripheral vasopressin involved?

MATERIALS AND METHODS

Animals. Experiments were conducted in anesthetized (Inactin, 100 mg/kg body wt) F2 homozygous ANP gene-disrupted mice (−/−) and the corresponding wild type (+/+) littermates. They were 20–24 wk old and weighed between 20 and 35 g. The ANP gene-disruption model is fully described by J ohn et al. (15). In the mutant (−/−) mice, plasma and atrial ANP levels are undetectable by radioimmunoassay, and ANP-specific atrial granules are absent (15). Our colonies were begun by breeding pairs of both types of mice. The genotype of each animal was confirmed by Southern blot analysis of EcoRI I-digest genomic DNA extracted from tail tissue (15).

Surgical preparation. Once surgical-plane anesthesia was established, the mice were tracheotomized with polyethylene tubing (PE-240; pulled to appropriate diameter) and both the right carotid artery and right external jugular vein were cannulated with PE-50 tubing that had a short length of PE-10 glued to the vessel insertion end. Then the animal was carefully turned so as to rest on its stomach, was placed in a stereotaxic frame, and a small hole was drilled through the skull near bregma so that a 28-gauge stainless steel guide cannula could be epoxy cemented into place 0.2 mm posterior and 1 cm left laterally to bregma and lowered 2.5 mm into the brain. This placed the cannula tip into the left lateral ventricle at the site at which the interventricular foramen to the third ventricle is at its widest (9). Correct placement was ascertained at the end of each experiment by injection of 10 µl of methylene blue (1%). We accepted only animals in which the dye, visualized under a low-power microscope, remained confined to the brain ventricular system.

Protocols. Experimental protocols were begun 30 min after completion of all surgical preparations. Baseline carotid ABP was recorded for 10 min. Then a background intravenous injection was given, and 15 min later, the first of two intracerebroventricular injections (ICV-1) was given (Table 1), followed 5 min later by the second (ICV-2). Ten minutes after ICV-2, the intracerebroventricular injection of methylene blue was given and the experiment was terminated. There were five groups of mice. Each consisted of 10 (−/−) and 10 (+/+), and they were distinguished from one another by the different injections as shown in Table 1.

Drugs. Twenty-eight amino acid rat ANP was purchased from Peninsula Laboratories (Belmont, CA). Sigma Chemical (St. Louis, MO) supplied the vasopressin antagonist [β-mercapto-β-β-cyclopentamethylenepropionyl1, O-Me-Tyr2, Val4, Argβ]-vasopressin (Manning compound), and losartan, a nonpeptide antagonist of angiotensin II AT1 receptors, was generously donated by DuPont-Merck Pharmaceutical.

### Table 1. Intracerebroventricular injections

<table>
<thead>
<tr>
<th>Group</th>
<th>Intracerebroventricular background</th>
<th>ICV-1 (5 µl)</th>
<th>ICV-2 (5 µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.9% saline</td>
<td>0.9% saline</td>
<td>0.9% saline</td>
</tr>
<tr>
<td>2</td>
<td>0.9% saline</td>
<td>0.9% saline</td>
<td>0.5 µg ANP</td>
</tr>
<tr>
<td>3</td>
<td>0.9% saline</td>
<td>10 µg losartan</td>
<td>0.9% saline</td>
</tr>
<tr>
<td>4</td>
<td>AVP V1 antagonist (10 ng/g body wt)</td>
<td>AVP V1 antagonist (10 ng/g body wt)</td>
<td>0.9% saline</td>
</tr>
<tr>
<td>5</td>
<td>AVP V1 antagonist (10 ng/g body wt)</td>
<td>AVP V1 antagonist (10 ng/g body wt)</td>
<td>0.5 µg ANP</td>
</tr>
</tbody>
</table>

IV, intravenous; ICV, intracerebroventricular; AVP, arginine vasopressin; ANP, atrial natriuretic peptide.

Drug dosages. The losartan dosage (10 µg icv) was chosen on the basis of generally employed, maximally effective dosages (5, 10, 14). Intracerebroventricular dosages of ANP have ranged from 0.12 (1) to 5 µg (42). We chose 0.5 µg because it was the lowest dose that led to measurable decreases in ABP in the most susceptible animals (−/−). The dose of arginine vasopressin (AVP) V1 antagonist was determined in pilot experiments. We chose the lowest dose that gave the greatest fall in mean ABP.

Data collection and analysis. ABP was recorded from the carotid artery cannula with an Electromedics microdisplacement transducer (MS20BA07ADS) attached to a BioPac data collection and analysis system (BioPac Systems, Goleta, CA). Heart rate was calculated by the BioPac from the pressure record. Baseline values were taken as the average over the initial 10-min period. The effects of intracerebroventricular injections on blood pressure are reported as changes after ICV-1 or ICV-2 and were calculated as follows. The initial value was taken as the average of the 60-s period preceding injection. The response value was taken as the average over a 60-s period that was centered around the respective peak response. The effects of intravenous injections are also reported as changes. The relevant periods were a 60-s interval immediately before ICV-1 (called “after” in Fig. 3) and an interval of equal duration immediately before the intravenous injection. Between-group comparisons were done by one-way ANOVA, followed by a Bonferroni-corrected t-test when post hoc comparisons were warranted. Adherence to the one-way ANOVA assumption of normally distributed values was tested by applying the Kolmogorov-Smirnov test, and the assumption of equal variances was tested by a Levene median test. Statistical significance was defined as P < 0.05.

RESULTS

ANP-deficient mice were significantly hypertensive with respect to their wild-type littermates (112 ± 8 vs. 94 ± 8 mmHg, means ± SE) but showed no significant difference in basal heart rate (389 ± 20 vs. 428 ± 19 beats/min). Heart rate responses to any of the injections performed in the course of the experiments did not differ between (−/−) and (+/+) and are, therefore, not mentioned further. Intracerebroventricular ANP or losartan had significant hypotensive effects only in ANP-deleted (−/−) mice (Figs. 1 and 2). The onset of these effects was quick (<2 min) and persisted throughout the postinjection period. Both strains showed significant decreases in mean ABP after intravenous injection of vasopressin V1-receptor antagonist (Fig. 3), suggesting involvement of vasopressin in the maintenance of
ABP in both (+/+ and (−/−). However, contrary to our hypothesis, there was no significant difference between them (Fig. 3). Finally, intracerebroventricular injection of ANP caused no significant change in ABP when both central AT1 and peripheral V1 receptors had been inhibited by a preceding injection of the appropriate antagonist (Fig. 4, comparing groups 5 and 4 after ICV-2).

**DISCUSSION**

The involvement of ANP in the central neuromodulation of cardiovascular function was first suggested by Thoren and colleagues (39) and has subsequently been supported by others who have demonstrated that ANP immunoreactivity and binding sites are found in brain structures closely involved in the regulation of ABP, particularly in hypothalamic nuclei, median eminence, septal areas, the AV3V region, and the circumventricular organs (13, 24, 31). This suggests that locally derived as well as plasma-borne natriuretic peptides could exert modulating influences on central cardiovascular regulatory sites. Our present findings (Fig. 1) confirm the report of others that central ANP fails to elicit significant depressor effects at low doses in normal animals (4, 32). On the other hand, the finding that hypotension can be produced in normals by intracerebroventricular doses as high as 5 µg (21) or by a dose of only 0.5 µg in ANP-depleted mice (Fig. 1) implies that ANP is involved in hypotensive responses under some circumstances. We speculate that low doses of exogenous ANP fail to show an additional effect whenever most central receptor sites are already occupied by native ANP. In the absence of native ANP, small amounts of exogenous peptide lead to hypotension.

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**Fig. 1.** Change in mean arterial blood pressure (ABP) observed in F2 homozygous atrial natriuretic peptide (ANP) gene-disrupted mice (−/−) and corresponding wild type (+/+) after intracerebroventricular injection of physiological saline (5 µl) or ANP (500 ng in 5 µl). Data are shown as means (±SE) of a continuous 60-s record, centered about the peak response in each animal. Baseline was taken as mean of 60-s interval preceding injection; *P < 0.05. Δ, change in; ns, not significant.

**Fig. 2.** ΔABP observed in (+/+) and (−/−) after intracerebroventricular injection of physiological saline (5 µl) or AT1-receptor antagonist losartan. Means ± SE; *P < 0.05.

**Fig. 3.** ΔABP observed in (+/+) and (−/−) after intravenous injection of physiological saline or vasopressin V1-receptor antagonist AVPX. Means ± SE; *P < 0.05.

**Fig. 4.** ΔABP observed after intracerebroventricular injection of physiological saline or 500 ng ANP in (+/+) and (−/−) mice that had previously received both vasopressin V1-receptor antagonist [b-mercapto-b-β-cyclopentamethylenepropionyl1-O-Me-Tyr2,Val4,Arg8]-vasopressin (Manning compound) and AT1-receptor antagonist losartan. Means ± SE; *P < 0.05.
The left lateral ventricle is part of the cerebral ventricular system, and substances injected into it will eventually diffuse throughout the cerebrospinal fluid. Its most prominent neighbors are the thalamus and the caudate nucleus of the basal ganglia (28). Its neighbors of potential cardiovascular significance are the paraventricular nucleus, a major site of vasopressin synthesis (18), and two circumventricular organs, subfornical organ and organum vasculosum of the lamina terminalis, both of which are important loci for the modulation of drinking behavior by angiotensin II (41) as well as the modulation, by ANP, of angiotensin II- mediated neuronal excitation (12), water intake (19), or vasopressin release (25, 34, 42). In view of reports that central angiotensin II may be involved as an intermediary of central ANP actions (3, 32) and that ANP and angiotensin II show reciprocal brain expression in their respective receptors in genetic hypertension (29), we tested first whether (-/-) showed elevated central angiotensin II AT1-receptor activation. These receptors are believed to be responsible for the hemodynamic effects of angiotensin II, and they predominate in the hypothalamus (26) and other brain areas with significant influence over cardiovascular control (20). Figure 2 shows that central AT1 activation was a significant part of blood pressure maintenance in (-/-), but not (+/+) mice. However, enhanced peripheral vasopressin-receptor activation was not involved in (-/-) hypertension in these experiments (Fig. 3). Both strains showed significant decreases in ABP immediately after vasopressin V1-receptor antagonism (Fig. 3), but, contrary to our hypothesis, (-/-) animals did not show a significantly greater hypotension after V1 antagonist than did (+/+). Correct interpretation of the role of vasopressin in the hypertension of ANP knockouts may have been complicated by the use of anesthetics in our experiments. Thus, consistent with the demonstration by Yamada et al. (42) in conscious rats, conscious ANP (-/-) mice might have elevated plasma vasopressin levels. In addition, it is possible that the release of vasopressin that occurs with some anesthetic agents (40) might have been enhanced in ANP (+/+) compared with ANP (-/-). This potential clouding of the role of vasopressin can be removed only if the experiments could be conducted in conscious mice.

Finally, we tested whether intracerebroventricular ANP exerted a hypotensive effect above and beyond that which was mediated via central AT1 receptors or peripheral V1 receptors. ANP injected intracerebroventricularly into mice that had previously received a V1 antagonist intravenously and an AT1 antagonist intracerebroventricularly caused no significant fall in ABP in either (-/-) or (+/+)(Fig. 4).

In summary, the results of this study suggest that ABP in ANP-deleted mice is in part maintained by peripheral vasopressin, just as ABP in wild-type controls is partly maintained by that agent. The permanently elevated blood pressure in ANP knockouts is associated with increased central nervous angiotensin II AT1-receptor activation. Its subsequent peripheral mediator is more likely to be enhanced sympathetic nervous activity (23) than elevated vasopressin.

**Perspectives**

Genetically modified animal models have demonstrated a role for ANPs in the long-term regulation of ABP. Whereas the mechanisms of this elevation have not yet been determined, our present results suggest that central nervous antagonism of angiotensin II is involved. The findings corroborate other results from our laboratory in support of the notion that one of the functions of atrial peptides is to suppress efferent sympathetic outflow by antagonizing central nervous angiotensin II actions. Future experiments should be directed at defining the locus of this putative effect, elimination of the confounding influence of anesthesia, and teasing out possible compensatory shifts in the relative importance of other natriuretic peptides such as BNP and CNP.

We are grateful to Dupont Merck Pharmaceutical for generous donation of losartan and to Dr. C. Pang (Queen’s Univ.) for providing the initial ANP knockout mice breeding pairs.

The study was supported by grants from Ciba-Geigy, Canada (now Novartis) and the University Research Incentive Fund of the Province of Ontario.

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Received 9 September 1999; accepted in final form 15 December 1999.


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