Neuroendocrine Effects of Dehydration in Mice Lacking the Angiotensin AT1a Receptor

Mariana Morris, Ping Li, Michael F. Callahan, Michael I. Oliverio, Thomas M. Coffman, Susan M. Bosch, Debra I. Diz

Abstract—Angiotensin (Ang) type 1a (AT1a) receptors are critical in the control of blood pressure and water balance. Experiments were performed to determine the influence of dehydration on brain Ang receptors and plasma vasopressin (VP) in mice lacking this receptor. Control or AT1a knockout (AT1aKO) male mice were given water ad libitum or deprived of water for 48 hours. Animals were anesthetized with halothane, blood samples were collected by heart puncture, and brains were processed for Ang-receptor autoradiography with 125I-sarthran (0.4 nmol/L). Dehydration produced an increase in AT1 receptors in the paraventricular nucleus (PVN) and anterior pituitary (AP) in control mice (PVN: 70 ± 16 versus 146 ± 10 fmol/mg protein; AP: 41 ± 7 versus 86 ± 15 fmol/mg protein). No changes were noted in the median preoptic nucleus. The majority of the brain receptors were of the AT1 subtype. There was little or no specific Ang binding in AT1aKO mice and no effect of dehydration. Plasma VP levels were elevated in the halothane-anesthetized animals (>200 pg/mL) with no significant effect of dehydration. A separate experiment was performed with decapitated mice anesthetized with pentobarbital. Dehydration increased plasma VP in control mice, from 3.3 ± 0.6 to 13.3 ± 4.7 pg/mL, whereas no change was noted in the AT1aKO mice, 5.1 ± 0.3 versus 6.1 ± 0.7 pg/mL (water versus dehydration). These results demonstrate a differential response to dehydration in mice lacking AT1a receptors. There was no evidence for AT1 receptors of any subtype in the brain regions examined and no effect of dehydration on VP secretion or brain Ang receptors. (Hypertension. 1999;33[part II]:482-486.)

Key Words: central nervous system ■ hypothalamus ■ vasopressin ■ water balance ■ blood pressure

There are strong links between the renin angiotensin system (RAS) and fluid/electrolyte balance.1–3 Alterations in volume status produced by dehydration, salt loading, or deprivation result in changes in brain and peripheral angiotensin (Ang) receptors.4–8 Dehydration in rats produced an increase in Ang type 1 (AT1) receptors in anterior pituitary (AP) and subfornical organ (SFO), with no change noted in the paraventricular nucleus (PVN).4,5,9 Salt loading caused an increase in SFO and PVN Ang receptors.8,10 Physiological interactions are also observed, such as Ang-mediated drinking and vasopressin responses, sodium-induced potentiation of the pressor action of Ang, and angiotensinergic modulation of the response to dehydration.11–14

The question that has arisen is the role of the specific AT1 subtypes in the responses. Autoradiographic techniques used to measure receptor density do not distinguish between AT1a and AT1b subtypes because of the structural similarity of the receptors.15–18 Likewise, specific pharmacological antagonists are not available. Gene deletion or knockout (KO) methods provide a new way of separating the receptor subtypes in an in vivo model. A comparison of the AT1a and AT1b receptor KO strains supports the idea that AT1a receptors are critical in blood pressure and fluid balance regulation. AT1aKO mice show a reduced blood pressure and a lack of responsiveness to Ang II.19,20 In contrast, deletion of AT1b receptors results in few phenotypic changes.21 AT1aKO mice also show deficits in renal concentrating ability, increases in water intake, and a reduction in stimulus-induced vasopressin secretion.22,23

We proposed to examine osmotic/Ang interactions in the AT1aKO mouse strain to study the mechanisms regulating the AT1 receptor subtypes and their role in the control of vasopressin secretion. With the use of water deprivation as the stimulus, we determined whether osmotic stimulation alters the Ang receptor subtypes (AT1a and AT1b and AT2) and whether the AT1a receptors are involved in dehydration-induced vasopressin secretion.

Methods

Animals
Male mice lacking AT1a receptors for Ang II were bred and maintained in the animal facility of the VA Medical Center in Dayton, Ohio.
Durham. The animals were housed singly with free access to water and food. AT1a genotypes, designated (+ +) for controls and (− −) for the KO-targeted allele, were determined by Southern blot analysis of DNA isolated from tail biopsies.19 There were 4 groups of animals: AT1a KO and control mice with water ad libitum or after 48 hours of water deprivation. The mice were anesthetized with halothane, blood was removed by cardiac puncture, and the brains and pituitaries were frozen on dry ice to use for Ang-receptor autoradiography. In a separate experiment, control and AT1aKO mice were given water ad libitum or dehydrated for 48 hours. The mice were anesthetized with pentobarbital (50 mg/kg IP) and decapitated immediately after the induction of anesthesia (within 5 minutes of injection). Trunk blood was collected for measurement of plasma vasopressin.

Receptor Autoradiography
The autoradiographic technique has been described in detail previously.10,24–26 Tissues were removed from the animal and frozen on dry ice. For storage, tissues were coated in cryoprotectant to protect from desiccation, allowing for optimal cryostat sectioning. Series of adjacent sections (14 μm) were placed on different slides so that adjacent sections could be incubated in each of the conditions described below. To identify the receptor subtypes, 0.4 nmol/L of 125I-[Sar'1-Thr'8]Ang II (2200 Ci/mmol) was incubated both with and without 3 μmol/L unlabeled Ang II, the AT1 antagonist losartan, or the AT2 antagonist PD 123319. To obtain an index of the apparent maximal binding density, total specific binding at this near-saturating concentration of 125I-[Sar'1-Thr'8]Ang II was used for comparisons of density among groups.

Precise quantification of film autoradiograms for bound peptide was carried out with the use of computerized densitometry (Micro Computer Imaging Device; Imaging Research Inc) after exposing 14C standards with the tissue sections. A conversion factor relates 14C to 125I-Ang II in fmol/mg protein.26 Different exposure times were used to obtain accurate readings for high, moderate, and low densities of binding according to the linear range of the film. Measurements were taken from at least 3 different areas in 2 different tissue sections for each condition (ie, in the absence or presence of each competitor). Slides were counter-stained with hematoxylin and eosin or cresyl violet to verify anatomic localization of the binding.

Radioimmunoassay
Plasma was separated and stored frozen (−70°C) until radioimmunoassay measurement. The plasma samples (200 μL) were extracted with acetone and petroleum ether. The lyophilized extract was resuspended in assay buffer, and vasopressin concentrations were measured with the use of a specific and sensitive assay.27

Statistical Analysis
The data are presented as the mean±SEM. ANOVA for multiple groups was used to determine significance (P<0.05) followed by a Newman-Keuls post hoc comparison.
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**Results**

Typical autoradiographs of the PVN and AP illustrate the pattern of Ang-receptor distribution in mice consuming water and after dehydration (Figure 1). In control mice, there is a dense concentration of receptors in PVN and AP. In the AT1aKO mice, there was no significant binding in PVN and only minimal residual binding in the AP. Dehydration increased Ang-receptor density in PVN and AP of control mice with no change noted in these regions of the AT1aKO mice. Quantitative analysis showed that dehydration produced a significant increase in Ang-receptor density in the AP and PVN of control mice, 3.4- and 2-fold differences, respectively (Figure 2). There were no changes noted with dehydration in another brain region, the median preoptic nucleus (MnPO; Figure 2). There was no significant binding in the AT1aKO mice either in the PVN or MnPO, and no increase was observed after dehydration (Figure 2). In the AP, there was low but significant binding (10 ± 2 fmol/mg protein; P<0.01 compared with 0). In all of the tissues examined, the specific binding was displaced almost completely with losartan (Figure 2). There were no changes noted with dehydration in another brain region, the median preoptic nucleus (MnPO; Figure 2). There was no significant binding in the AT1aKO mice either in the PVN or MnPO, and no increase was observed after dehydration (Figure 2). In the AP, there was low but significant binding (10 ± 2 fmol/mg protein; P<0.01 compared with 0). In all of the tissues examined, the specific binding was displaced almost completely with losartan (Figure 1, Table 1). The 85% to 98% competition values were not significantly different from 100%, indicating a predominance of AT1 receptors. However, in the PVN there was 11% ± 4% and 13% ± 3% competition with PD 123319 in the control animals consuming water or after dehydration, respectively. Because the competition with PD 123319 in the PVN was significantly greater than 0, the data support the presence of a small population of AT2 receptors. The proportion of receptors did not change during dehydration. This population of AT2 receptor sites was not detectable in the PVN of the AT1aKO mice, because there was no significant specific binding in the PVN. Plasma vasopressin levels were high in the blood samples collected in mice anesthetized with halothane (Ang-receptor study) with average levels greater than 200 pg/mL (Table 2). For this reason, a second experiment was conducted with AT1aKO and control mice that were decapitated immediately after the induction of pentobarbital anesthesia (Figure 3). Dehydration-induced vasopressin release was observed only in the control animals (P<0.01, watered versus dehydrated) even though osmolality was increased in both groups (P<0.01, watered versus dehydrated).

**Discussion**

Studies of dehydration in mice lacking the AT1a receptor revealed important findings related to the control of Ang-receptor expression and vasopressin secretion. Dehydration produced a marked increase in brain AT1 receptor in control mice but not in AT1aKO mice. This lack of angiotensinergic responsiveness was associated with a deficit in dehydration-induced plasma vasopressin secretion.

The relationship between brain Ang systems in the control of water balance, blood pressure, and vasopressin secretion has been documented using a variety of approaches. Central injection of Ang II or hypertonic saline produces drinking, antidiuresis, an increase in blood pressure, and vasopressin secretion. These effects can be almost completely blocked by AT1 antagonists and AT1 receptor antisense and brain lesions.2,13,28-30 Likewise, the responses to dehydration are thought to involve the RAS because Ang antagonists attenuate drinking.13,31 AT2 antagonists have been reported to block at least a part of the drinking responses,13,14 which would be consistent with our observation of a small population of AT2 receptors in the PVN. Ang is present in the magnocellular neurosecretory nuclei and in pathways connecting the rostral forebrain with the hypothalamus.16,32 Thus, it would be

**TABLE 1. Proportion of Brain AT-1 Receptors in Control Mice**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>PVN</th>
<th>AP</th>
<th>MnPO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water ad libitum</td>
<td>88.5±6.2</td>
<td>85.7±6.3</td>
<td>84.9±15.1</td>
</tr>
<tr>
<td>Dehydration for 48 h</td>
<td>97.5±0.08</td>
<td>92.7±2.6</td>
<td>98.0±1.6</td>
</tr>
</tbody>
</table>

Mean±SEM, percentage of specific displacement with losartan (3 μM).

**TABLE 2. Effect of Dehydration on Plasma Vasopressin (pg/ml) in Halothane-Anesthetized Control and AT1aKO Mice**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Control</th>
<th>AT1aKO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water ad libitum</td>
<td>284.4±30.3</td>
<td>312.6±52.1</td>
</tr>
<tr>
<td>Dehydration for 48 h</td>
<td>368.3±14.3</td>
<td>244.5±15.3</td>
</tr>
</tbody>
</table>

Mean±SEM, blood samples collected by cardiac puncture in anesthetized mice, n=6–7 per group.
predicted that removal of this important system would affect blood pressure, fluid balance, and vasopressin secretion.

Characterization studies of the AT1aKO mouse strains reported lower blood pressures and a lack of pressor responsiveness to Ang II.19,20 Oliverio and colleagues reported alterations in fluid balance in this model, an increase in water intake, a decrease in urine osmolality, and a deficit in the ability to concentrate in response to dehydration.23 The focus of our studies was on the central neuroendocrine changes with the idea that these may also be important in the physiological alterations. In the absence of AT1a receptors, 48 hours of water deprivation produced no change in plasma vasopressin in the face of increased plasma osmolality. The data support the idea that central AT1 receptors in mice are critically involved in osmosis-induced peptide secretion. Furthermore, they show that results obtained with a genetic approach are comparable to those obtained with pharmacological antagonists. For example, the vasopressin response to increased osmolality in isolated hypothalami was blocked by an Ang antagonist25 as was dehydration-induced secretion in the whole animal.34 In this study, plasma vasopressin was used as the end point to evaluate osmotic responsiveness. However, when other indices of the vasopressin axis were measured, there was evidence that osmosensitivity is not lost in AT1aKO mice. Posterior pituitary vasopressin content was reduced after dehydration in AT1aKO animals, and c-Fos expression, an index of neural activation, was observed in neurons in the rostral forebrain and PVN of dehydrated AT1aKO mice.23,35 Further studies are required to determine the nature of the alterations in the hypothalamic vasopressin system in mice lacking the AT1a receptors, particularly the time course of the changes.

Previous studies have shown that dehydration or changes in salt intake in rats alter brain Ang-receptor density in SFO and AP but not PVN.4–6,8,9 The results in mice show that dehydration increased AT1 receptors in the PVN and the AP, with insufficient brain sections in the SFO region to permit analysis. Differences were also noted in the time courses of the receptor changes in mice and rats. In mice, receptor changes were seen after 2 days of dehydration, whereas in rats the earliest time to see changes was after 5 days of dehydration.4 Given the metabolic rate and water turnover in mice (daily intake of 4 to 6 mL per 30 g animal), water deprivation for 48 hours is a severe challenge, probably a greater stimulus than in rats. The reason for the species differences in the localization of the response, PVN and AP, is not known. It may be related to the magnitude of the stimulus, the time course, or a difference in central nervous system organization. Certainly the data support the common theme of interactions between volume status and brain Ang systems, with activation occurring in regions important in blood pressure and volume control.

One of the purposes of the present investigation of the AT1aKO model was to examine the interaction between the Ang-receptor subtypes under control and stimulated conditions. We speculated that in the absence of the AT1a receptor there would be an upregulation of the AT1b receptor or perhaps changes in AT2 receptors. The results provided no direct evidence of such an interaction. There was almost no specific binding in the AT1aKO mice in the brain regions studied, and no change was noted with dehydration. Studies in which in situ hybridization and polymerase chain reaction methods were used for the localization and quantification of the mRNA for the AT1-receptor subtypes showed that AT1b receptors were of a lower density and were expressed in brain, testes, adrenal, kidney, and pituitary.4,7,8,15,18,36 In the rat brain, there is strong evidence for the presence of AT1b mRNA in AP and more controversy regarding its localization in SFO.3,15,18 The question is whether the mRNA is directly translatable into receptor protein. As mentioned previously, in normal animals receptor-binding techniques cannot be used to differentiate the receptor subtypes. However, the AT1aKO animal, which lacks the homologous receptor, should provide a model to study expression of the AT1b receptor. However, there was little evidence for the receptor except in the AP, which showed low levels of binding (10 versus 40 fmol/mg protein, AT1aKO versus control mice). There are physiological studies that indicate that the AT1b receptor is functionally active, as demonstrated by pressor and drinking responses to Ang II after treatment with RAS antagonists in AT1aKO mice.37,38 These results emphasize the usefulness of gene deletion models for the study of physiological function. The present data in the AT1aKO mouse document the complexities that result from the removal of a receptor that is critical in the regulation of cardiovascular and endocrine function. Changes are observed in the response to water deprivation that suggest that the AT1a receptor is important in the osmotic control of vasopressin release. The results further emphasize the interactions between volume status and the central Ang II receptors, with data supporting a role for AT1a rather than AT1b subtypes in mouse brain and pituitary.

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