Acute and chronic increases in osmolality increase excitatory amino acid drive of the rostral ventrolateral medulla in rats

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Water-deprived rats exhibited decreased plasma volume and elevated osmolality by intravenous infusion of 5% dextrose in water (5DW). Nous infusion of isotonic saline or after normalization of plasma water-deprived rats after normalization of blood volume by intravenous infusion of hypertonic saline. In conclusion, acute and chronic increases in osmolality increase sympathetic tone to some vascular beds, increases in subjects with hypertension (17, 39). Similarly, a chronic decrease in body fluids due to sodium or water depletion is associated with increased activity of sympathetic nerves (2, 11, 16, 45). Recent studies have probed brain mechanisms by which changes in sympathetic tone are effected. The rostral ventrolateral medulla (RVLM) has received attention because its importance in supporting basal sympathetic activity. Indeed, lesions or acute blockade of the RVLM cause the activity of many sympathetic nerves to cease (for reviews, see Refs. 14 and 40).

Evidence to support the hypothesis that increased activity of the RVLM underlies the elevated sympathetic tone exhibited by hypertensive animals includes the observation that acute inhibition of the RVLM in Goldblatt hypertensive rats decreases arterial blood pressure similarly to ganglionic blockade (6). In addition, whereas blockade of excitatory amino acid (EAA) input to the RVLM by bilateral microinjection of the ionotropic EAA antagonist kynurenic acid (KYN) does not alter arterial pressure in normal animals, in a number of hypertensive models, KYN elicits profound decreases in arterial pressure (6, 23, 24). Similarly, we (9) have recently reported that bilateral microinjection of KYN into the RVLM decreases arterial pressure in a nonhypertensive model of increased sympathetic tone, 48 h water-deprived rats. These data suggest that the increased sympathetic tone and activity of the RVLM during these states is mediated at least in part by increased EAA drive. However, the mechanism by which EAA activity in the RVLM increases is not known.

Part of the difficulty in investigating how EAA drive of the RVLM can change is that the origin of EAA input has not been definitely established and may arise from multiple sites (14, 40). Nevertheless, water deprivation is an excellent model with which to study this question, because factors that initiate the increased sympathetic activity can be identified. Specifically, water deprivation is associated with volume depletion and increases in osmolality. Blood volume depletion may increase sympathetic activity via the baroreceptor reflex, which could act in the brain to increase sympathetic tone. Therefore, one purpose of the present study was to test the hypothesis that the increased EAA drive of RVLM is mediated at least in part by the decreased blood volume. This hypothesis was tested by determining whether the hypotensive response to KYN in water-deprived rats is smaller following normalization of blood volume by intravenous infusion of saline.

Another factor that may mediate increased EAA drive of the RVLM during water deprivation is the hypertonicity. Acute increases in osmolality increase sympathetic tone to some vascular beds (for review, see Ref. 43), and normalization of the high osmolality in conscious, water-deprived rats decreases lumbar sympathetic nerve activity (LSNA) (35). Moreover, acute increases in brain osmolality activate neurons in the paraventricular nucleus that project to the RVLM (43). Finally, we (9) have recently demonstrated that the magnitude of the depressor response to RVLM microinjection of KYN in water-deprived rats is highly correlated to the basal level of osmolality. Therefore, to test the hypothesis that the increased EAA drive of the RVLM observed during water deprivation is due in

SYMPATHETIC NERVES are active in normal resting animals, and this level of basal tone can vary under physiological and pathophysiological conditions (for review, see Ref. 11). For example, sympathetic activity, at least to some vascular beds, increases in subjects with hypertension (17, 39). Similarly, a chronic decrease in body fluids due to sodium or water depletion is associated with increased activity of sympathetic nerves (2, 11, 16, 45). Recent studies have probed brain mechanisms by which changes in sympathetic tone are effected. The rostral ventrolateral medulla (RVLM) has received attention because of its importance in supporting basal sympathetic activity. Indeed, lesions or acute blockade of the RVLM cause the activity of many sympathetic nerves to cease (for reviews, see Refs. 14 and 40).

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part to increased osmolality, the following two experiments were performed. First, we determined whether acute normalization of elevated osmolality in water-deprived rats by intravenous infusion of 5% dextrose in water (5DW) diminishes the KYN response as it decreased LSNA (35). Second, we determined whether an acute elevation of osmolality in water-replete rats increases LSNA and EAA drive of the RVLM, as assessed by the depressor response to bilateral KYN microinjection into the RVLM.

METHODS

Animals

Experiments were performed using male Sprague-Dawley rats (Sasco; Wilmington, MA) weighing ~250–350 g. All rats were housed in a room with a 12:12-h light-dark cycle a minimum of 5 days before experimentation. Rats had free access to food (LabDiet 5001, Richmond, IN). Water-deprived rats were housed singly for 48 h without water; water-replete rats were grouped one to three and were allowed free access to distilled water.

Surgery

Throughout the surgery and experiment, body temperature was maintained at 37 ± 1°C by using a rectal thermistor and a heating pad. Anesthesia was induced with 5% isoflurane in 100% oxygen. A trachea tube was first inserted so that the animals could be artificially ventilated, and a surgical plane of anesthesia was maintained with 2% isoflurane in 100% oxygen.

Femoral arterial and venous catheters were then implanted for arterial pressure measurements and infusions, respectively. In a subset of the rats, a lumbar nerve electrode was implanted as previously described (35). In brief, an abdominal midline incision was made, and the intestines, abdominal aorta, and vena cava were carefully pulled aside to expose the underlying lumbar nerve, which was then dissected free from the surrounding tissue. A bipolar electrode made of Teflon-coated stainless steel wire (A&M Systems; Everett, WA) was positioned under the nerve and secured in position with quick-drying, Teflon-coated stainless steel wire (A&M Systems; Everett, WA) was inserted through a microscope reticule, the movement of a small bubble or Teflon-coated stainless steel wire (A&M Systems; Everett, WA) was inserted through a microscope reticule, the movement of a small bubble or

RVLM Microinjections

Functional identification of the RVLM was made by observing >20 mmHg pressor responses to L-glutamate [1 nmol/100 nl (25, 28, 41)]. Single-barreled glass micropipettes (20–40 μm tip diameter) containing glutamate were positioned as described by Kiely and Gordon (28). Briefly, the tip of the rat’s nose was pushed down until the calamus scriptorius was 2.4-mm posterior to the interaural line. With the use of calamus scriptorius as zero, injections were made as the following: 1.0–0.6 (usually 0.8) mm anterior, 1.8–2.0 mm (usually 1.9) lateral, and 2.8–4.0 mm ventral to the dorsal surface of the medulla. Injections (100 nl) were made over ~3–7 s using either a 1-μl Hamilton syringe held in a micromanipulator or a PicoPump (WPI); the successful microinjection of drugs was verified by watching, through a microscope reticule, the movement of a small bubble or the fluid meniscus (PicoPump) a distance calibrated to be 50 or 100 nl. No more than three penetrations were made per side before the experiment commenced. Glutamate was dissolved in artificial cerebrospinal fluid containing (in mM): 128 NaCl, 2.6 KCl, 1.3 CaCl2, 0.9 MgCl2, 20 NaHCO3, and 1.3 Na2HPO4. KYN was first dissolved in one part 100 mM sodium bicarbonate and then diluted with 9 parts artificial cerebrospinal fluid. The pH of all solutions was corrected to 7.4. Before the pipette was filled with a new drug, it was flushed with aCSF and then with several volumes of the new drug.

At the conclusion of the experiment, ~50 nl of 2.5% Alcian Blue in 0.5 M sodium acetate was injected into the RVLM by using the same pipette and coordinates used for the injections. The brain was removed and placed in 4% formaldehyde in phosphate-buffered saline for at least 48 h. The brain stem was subsequently cut into 50-μm sections using a cryostat; sections were mounted on glass microscope slides and counterstained with neutral red. RVLM injection sites were verified against those previously published, within an area ~500-μm caudal to the caudal end of the facial nucleus and ventral to nucleus ambiguus (Fig. 1) (9).

Experimental Protocols

After the RVLM was identified functionally, ~30 min were allowed for stabilization and collection of baseline data, and then one of the following protocols was performed. In most protocols, rats were first pretreated with a V1 vasopressin antagonist [Manning Compound; d(CH2)3,Tyr(Me)2,Arg4-vasopressin; 5 μg in 100 μl iv] to eliminate effects of changes in vasopressin, subsequent to alterations in blood volume or osmolality, on sympathetic tone and therefore potentially the EAA drive of RVLM and KYN responses.

Protocols in Water-Deprived Rats

These experiments investigated the origin of the increased EAA drive of the RVLM in water-deprived rats. Protocol 1. This protocol tested the hypothesis that decreased blood volume contributes to the increased EAA drive of the RVLM during water deprivation. Water-deprived rats (n = 6) were first pretreated with the V1 antagonist. Ten minutes later, KYN (2.7 nmol in 100 nl) was injected bilaterally into the RVLM: after the first microinjection was completed, the pipette was removed and positioned on the other side; the second injection was then made usually within 1 min of the first injection. One hour later, a blood sample (300 μl) was taken into a heparinized syringe for measurements of plasma sodium and chloride concentrations, plasma protein concentration, and hematocrit. The sample was replaced with an equal volume of isotonic saline. Five minutes later, an intravenous infusion of urethane (1.2 g/kg in 1 ml saline) was then administered over ~30 min; 10 min after the urethane infusion was begun, the gas anesthetic was slowly withdrawn, but artificial ventilation with 100% oxygen was maintained throughout the experiment. After the surgery and the urethane infusion were completed, the rats were allowed to stabilize for about 30–60 min before experimentation. Depth of anesthesia was periodically assessed by confirming the lack of response to tail or paw pinch. Additional urethane (0.2 g/kg) was occasionally administered intravenously as needed.

Protocol 2. This protocol tested the hypothesis that the increased osmolality observed during water deprivation increases the EAA drive of RVLM. This protocol was conducted in water-deprived rats (n = 5) similarly to protocol 1, except that 5DW was infused intravenously (0.2 ml/min for 50 min) instead of saline. In addition, to maintain lowered plasma sodium and chloride levels, a second infusion of 5DW (0.2 ml/min for 50 min) was begun 10 min after the second set of KYN injections. The 5DW infusion rate was selected to normalize

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both osmolality and volume. A larger volume of 5DW was required relative to isotonic saline, because the volume of distribution of water is greater than saline.

**Protocol 3.** To determine the effects of time and preparation on the depressor responses to KYN, a separate set of water-deprived rats (time controls; \( n = 6 \)) was treated similarly as rats in protocols 1 and 2, except that fluids were not infused.

**Protocol 4.** To estimate the volume loss following water deprivation and to determine whether the 5DW and isotonic saline administered in protocols 1 and 2 replenished the volume lost, plasma volume was measured in additional rats. The rats were paired by weight (range 315–380 g); one rat was water deprived for 48 h, whereas the other rat continued with free access to distilled water. Rats were then anesthetized with 2% isoflurane and surgically prepared for microinjection experiments. After surgery, the isoflurane was withdrawn and urethane was administered, as described in Surgery. Approximately 1–3 h later, plasma volume was estimated from the volume of distribution of Evan’s blue dye, as previously described (27). Briefly, after an initial 300-μl blood sample (replaced with equal volume of isotonic saline) was taken, 0.3 ml of Evan’s blue dye (0.5% in isotonic saline) was injected into the venous line and flushed with 0.2 ml saline. Arterial blood samples (150 μl) were then collected 10, 20, 30, 40, and 60 min later, each replaced with an equal volume of saline. The concentration of diluted dye was determined by extrapolating the concentration values obtained for the timed samples back to time 0; plasma volume was calculated as the amount of dye injected divided by the extrapolated concentration.

**Protocols in Water-Replete Rats**

To further investigate the effect of increases in osmolality on sympathetic activity and the EAA input to RVLM, four experiments were performed in water-replete rats.

**Protocol 5.** The purpose of this protocol was to determine whether acute increases in osmolality in anesthetized water-replete rats increase LSNA. Rats instrumented for measurements of arterial pressure, heart rate, and LSNA were used for this experiment. Nitroprusside (70 μg) was injected intravenously to verify the integrity of the nerve recordings, and at least 45 min later the experiment was initiated. The rats were first injected with the V1 vasopressin antagonist, and after 10 min, a blood sample (300 μl) was collected for measurement of plasma sodium and chloride levels, protein, and hematocrit. The sample was replaced with an equal volume of isotonic saline intravenously. Five minutes later, a 10-min infusion of either hypertonic saline (3 M, 0.11 ml/min, \( n = 6 \)) or isotonic saline (0.11 ml/min, \( n = 5 \)) was begun. Five minutes after saline infusion was completed, another blood sample (300 μl) was collected and replaced. Measurements of arterial pressure, heart rate, and LSNA were continued for 3 h, when a final blood sample (300 μl) was collected.

**Protocol 6.** This experiment tested the hypothesis that acute increases in osmolality increase EAA drive of the RVLM, by determining whether bilateral KYN microinjections into the RVLM of water-replete rats decrease arterial pressure after hypertonic saline infusion (\( n = 7 \)) but not isotonic saline infusion (\( n = 6 \)). This protocol was identical to protocol 5 except that rats were prepared for RVLM Fig. 1. Coronal sections through rat medulla illustrating sites of drug microinjections into rostral ventrolateral medulla (RVLM). Top: water-deprived rats receiving isotonic saline (open circle), 5% dextrose in water (5DW, closed circle), or no fluid (open triangle). Bottom: water-replete rats receiving isotonic (open circle) or hypertonic (closed circle) saline. Sections are —11.8 mm from bregma; all injections were within 200 μm from this section.
injections instead of LSNA recordings and four bilateral microinjections of KYN into RVLM were performed at hourly intervals beginning 10 min after intravenous infusion of isotonic or hypertonic saline.

**Protocol 7.** This protocol determined whether acute increases in osmolality increase responsiveness of the RVLM to glutamate. Rats were given the V1 vasopressin antagonist; 10 min later a blood sample (300 μl) was collected and replaced with isotonic saline. Then either hypertonic (n = 7) or isotonic saline (n = 5) was infused as in protocol 5. After an hour, a blood sample (300 μl) was taken, and unilateral glutamate microinjections were begun. Four doses (0.1, 0.5, 1, 5 nmol; 100 nl) were injected in random order. Each dose was injected three times: at the functionally identified coordinates as well as 0.2 mm dorsal and ventral to this spot. Whereas the results of the three injections were similar, the data obtained from the functionally identified site were used for data analysis. When a new dose was given, the pipette was removed from the brain, flushed with aCSF, and filled with the new solution. All injections were separated by at least 5 min.

**Protocol 8.** To determine whether hypertonic saline infusion is associated with changes in vascular reactivity to adrenergic agonists, separate groups of rats were tested with varying doses (1, 3, 6, 10, and 20 μg/kg iv) of phenylephrine. Different doses were given randomly, with at least 5 min between injections (i.e., after blood pressure and heart rate returned to control). After an initial series of phenylephrine injections, a blood sample (300 μl) was collected and the volume replaced with isotonic saline. The V1 vasopressin antagonist was then injected, and after 10 min, hypertonic saline (n = 6) was infused intravenously as described in protocol 4. One hour later, another blood sample was collected and replaced as above, and after a 5-min stabilization period, the phenylephrine injections were repeated.

Pressor responses to phenylephrine injection were also quantified in two separate groups of rats pretreated with both the V1 antagonist and hexamethionium (30 mg/kg). Pressor responses were determined before and after infusion of hypertonic saline (3 M, 0.11 ml/min for 10 min; n = 6) or isotonic saline infusion (0.11 ml/min for 10 min; n = 4). In both groups, hexamethionium (30 mg/kg) was injected again intravenously just before the second series of phenylephrine injections were started. Longer periods (6–9 min) were required between injections to allow pressure and heart rate to return to control.

**Analysis of Blood Samples**

Plasma concentrations of sodium and chloride were measured from whole blood using a Beckman Labyte 810, and hematocrit was measured in duplicate from centrifuged capillary tubes. The tubes were then broken to extract plasma, from which plasma protein concentration was measured using a Hitachi refractometer. Plasma osmolality was measured from two to three replicate 20-μl samples using an Advanced Micro Osmometer (model 3300) after centrifugation of blood using a refrigerated Eppendorf 5402 centrifuge.

**Data Acquisition**

The arterial catheter was connected to a pressure transducer and Grass polygraph for measurements of mean arterial pressure and heart rate. In some experiments, these data were also recorded by a computer (BIOPAC Systems, Santa Barbara, CA). In experiments measuring LSNA, the recording electrode was connected to a Grass differential amplifier (P511). Raw nerve activity was filtered, and frequencies between 100 and 3,000 Hz were then amplified, displayed on an oscilloscope, and fed to a Grass integrator (7P10) where the signal was whole wave rectified and integrated in 1-s bins. LSNA was determined as the average nerve activity over 30 s at given time points. At the end of the experimental protocol, background noise was determined by blockade of ganglionic transmission with hexamethonium (30 mg/kg), and levels of background noise were subtracted from values obtained for LSNA during the experiment. LSNA was normalized to baseline nerve activity before the start of the protocol (% baseline).

**Chemicals**

KYN, glutamate, hexamethonium, and phenylephrine were all obtained from Sigma, and the V1 vasopressin antagonist was obtained from Bachem.

**Data Analysis**

Increases in plasma volume following 5DW or isotonic saline infusion in rats in protocols 1 and 2 were estimated from the decreases in plasma protein concentration and hematocrit using previously described formulas (44). Between-group differences in responses to KYN, glutamate, or phenylephrine were determined using ANOVA for repeated measures. To determine at which times significant differences occurred, a significant time effect or interaction was followed by the post hoc Bonferroni correction. Effects of water deprivation on blood pressure and blood chemistry values were determined using t-tests. P values <0.05 were considered statistically significant.

**RESULTS**

**Basal Values**

Water deprivation increased plasma osmolality and plasma sodium and chloride concentrations (Table 1). Plasma volume was decreased, as indicated by direct measurement and also indirectly by increases in hematocrit and plasma protein concentration (Table 1). Baseline blood pressure levels before administration of the V1 vasopressin antagonist were not different between groups (Table 1). However, within 5 min after administration of the V1 antagonist, blood pressure was decreased more (P < 0.05) in water-deprived (−18 ± 2 mmHg, n = 17) compared with water-replete (−6 ± 1 mmHg, n = 37) rats.

**Normalization of Blood Volume and Osmolality in Water-Deprived Rats**

As illustrated in a representative tracing in Fig. 2, bilateral microinjection of KYN decreased arterial pressure in water-deprived rats. When the results of the first KYN depressor responses were combined among groups of water-deprived rats (protocols 1–3; n = 17), the decrease in pressure averaged 24 ± 2 mmHg (P < 0.05).

To determine whether the KYN response is mediated by the decrease in blood volume, KYN was injected again after

| Table 1. Basal values in water-deprived and water-replete rats |

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<tr>
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<th>Water Replete</th>
<th>Water Deprived</th>
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<tbody>
<tr>
<td>Plasma sodium concentration, meq/l</td>
<td>140.6 ± 0.6</td>
<td>150.3 ± 0.5*</td>
</tr>
<tr>
<td>Plasma chloride concentration, meq/l</td>
<td>109.6 ± 0.5</td>
<td>114.0 ± 0.6*</td>
</tr>
<tr>
<td>Plasma osmolality, mosmol/kgH2O</td>
<td>299.9 ± 0.7</td>
<td>310.8 ± 1.1*</td>
</tr>
<tr>
<td>Hematocrit, %</td>
<td>45.6 ± 0.4</td>
<td>51.5 ± 0.6*</td>
</tr>
<tr>
<td>Plasma protein concentration, g/dl</td>
<td>6.0 ± 0.1</td>
<td>6.6 ± 0.6*</td>
</tr>
<tr>
<td>Mean arterial pressure, mmHg</td>
<td>115 ± 3</td>
<td>121 ± 3</td>
</tr>
<tr>
<td>Plasma volume, ml</td>
<td>13.2 ± 0.4</td>
<td>10.7 ± 0.2*</td>
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Values are mean ± SE. Data, except plasma volume, were obtained from all rats in protocols 1–3; n = 36–42 water-replete rats; n = 14–17 water-deprived rats, except the subset of rats pretreated with hexamethonium (protocol 8). Plasma volume (protocol 4) was measured in 5 additional pairs of water-deprived and water-replete rats. *P < 0.05 compared with water-replete rats.
normalization of blood volume with isotonic saline infusion (protocol 1). Plasma sodium (151.0 ± 0.7 to 150.9 ± 0.5 meq/l) and chloride (114.9 ± 0.8 to 118.6 ± 1.0 meq/l) levels were not significantly altered during the experiment. However, the infusion decreased plasma protein concentration and hematocrit and was estimated to increase plasma volume by 12–15% (Table 2). To assess whether this volume increase was sufficient to replenish losses, the volume decrement secondary to water deprivation was estimated by computing the difference in plasma volume between pairs of weight-matched, water-deprived and water-replete rats (protocol 4). This difference, as a percentage of the plasma volume of water-deprived rats, averaged 25.7 ± 5.8% (range: 14.5–48.0%) and was not significantly different from the volume gain following the isotonic saline infusion. This comparison suggests that the isotonic saline infusion returned the volume of water-deprived rats to normal. On the other hand, in time control animals, plasma sodium (150.0 ± 1.4 to 151.0 ± 0.9 meq/l), chloride (114.7 ± 1.7 to 115.0 ± 1.8 meq/l), protein (6.0 ± 0.1 to 6.1 ± 0.2 g/dl) levels and hematocrit (50.7 ± 1.0 to 50.6 ± 1.2%) levels were not significantly changed.

Fig. 3A illustrates that the magnitude of the depressor responses to KYN was not altered in either rats given isotonic saline or in the time control animals; moreover, the responses were not significantly different between groups. These data do not support the hypothesis that the decreased blood volume mediates the increased EAA drive of RVLM present in water-deprived rats.

To determine whether the KYN response is mediated by hypertonicity, KYN responses were compared before and after reduction of plasma sodium and chloride concentrations by infusion of 5DW. Plasma sodium concentration decreased from 149.9 ± 1.2 to 141.0 ± 1.3 meq/l immediately after the first infusion (P < 0.05) and decreased further (P < 0.05) to 138.3 ± 1.5 meq/l by the end of the experiment. The sodium levels were not significantly changed.

Table 2. Estimated increases in plasma volume following isotonic saline or 5DW infusion in water-deprived rats

<table>
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<th>Isotonic Saline</th>
<th>5DW</th>
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<tr>
<td></td>
<td>Control</td>
<td>5 min</td>
</tr>
<tr>
<td>Plasma protein concentration, g/dl</td>
<td>7.1±0.1</td>
<td>6.2±0.1†</td>
</tr>
<tr>
<td>% Increase in plasma volume calculated from protein</td>
<td>13.7±1.5</td>
<td>11.6±1.9</td>
</tr>
<tr>
<td>Hematocrit, %</td>
<td>53.0±0.7</td>
<td>49.4±1.0†</td>
</tr>
<tr>
<td>% Increase in plasma volume calculated from hematocrit</td>
<td>15.2±1.6</td>
<td>15.3±1.6</td>
</tr>
</tbody>
</table>

Values are means ± SE. % increase in plasma volume estimated from changes in protein and hematocrit and computations previously described and validated (44). Times are minutes after the infusion is completed. *P < 0.05, protein vs. hematocrit estimates; †P < 0.05 compared with control.
values after 5DW infusion were not different from those measured in water-replete rats (Table 1). Similarly, plasma chloride concentration decreased from 111.7 ± 0.6 to 106.2 ± 1.0 meq/l immediately after the infusion (P < 0.05) and then further (P < 0.05) to 103.4 ± 0.7 meq/L. These values were lower than those measured in water-replete rats (P < 0.05; Table 1). The infusion also decreased plasma protein concentration and hematocrit and increased plasma volume (Table 2). The increase in volume computed from hematocrit changes was smaller 80 min after 5DW infusion than the values obtained from changes in plasma protein; however, this value is likely erroneous due to increases in red blood cell volume (20). Nevertheless, the remaining estimates of the volume increment were not significantly different from the estimated decrement following water deprivation, suggesting that volume losses were largely replenished. The volume increase was also not different from that produced by isotonic saline infusion (Table 2), indicating that a comparison of effects of the isotonic saline infusion to the effects of 5DW infusion should reveal the role of osmolality alone in the KYN response.

As shown in Fig. 3B, whereas the KYN response remained stable in the time control rats, it became smaller after 5DW infusion (P < 0.05, ANOVA, time and interaction). Nevertheless, a significant depressor response (−13 ± 2 mmHg; P < 0.05) to KYN microinjection persisted in the water-deprived rats after 5DW infusion. These data indicate that at least part of the increased EAA drive of RVLM in water-deprived rats is secondary to the increased plasma sodium and/or chloride concentrations.

**Acute Increases in Osminality in Water-Replete Rats**

Because the time lines of protocols 5 and 6 were identical, blood chemistry data were combined. As shown in Fig. 4, hypertonic saline infusion increased plasma sodium and chloride concentrations; plasma protein concentration (−0.6 ± 0.1 g/dl) and hematocrit (−4.1 ± 0.3%) decreased (P < 0.05). All of these changes were sustained during the protocol. In contrast, plasma sodium, chloride (Fig. 4), protein (−0.1 ± 0.1 g/dl), and hematocrit (0.1 ± 0.2%) levels did not change significantly in rats that received isotonic saline.

In rats from protocols 5 to 8 not pretreated with hexamethonium, hypertonic saline infusion increased arterial pressure from 106 ± 4 to 117 ± 3 mmHg (P < 0.05, n = 26) by the end of the 10-min infusion; pressure then rapidly returned to control values. Overall, there was no significant effect of hypertonic saline infusion on arterial pressure (Fig. 5). The isotonic saline infusion also had no significant effect on blood pressure either during (115 ± 5 mmHg) to 116 ± 4 mmHg, n = 16 rats from protocols 5 to 7) or after (Fig. 5) the infusion.

Despite significant increases in plasma sodium and chloride concentrations, a depressor response to KYN was not observed immediately following the hypertonic saline infusion (protocol 6, Fig. 4). Interestingly, however, a response gradually developed to reach a maximum at the end of the protocol (Fig. 4, P < 0.05, ANOVA, time and interaction). Similarly, LSNA was not immediately affected by the hypertonic saline infusion (Fig. 5); however, LSNA gradually increased during the 3-h protocol to reach levels 167 ± 19% of control (P < 0.05, ANOVA, time and interaction). Heart rate also tended to rise, but this change did not reach statistical significance. In contrast, neither LSNA (Fig. 5) nor the depressor response to KYN (Fig. 4) was altered following isotonic saline infusion. These data indicate that acute increases in osmolality are associated with increases in LSNA and EAA drive of the RVLM; however, these responses take time to develop.

To determine whether the increased EAA drive was due in part to increased sensitivity of the RVLM to EAA, pressor responses to unilateral microinjection of glutamate were compared in rats before and after receiving hypertonic saline. Glutamate injection produced dose-dependent increases in arterial pressure (Fig. 6; P < 0.05, ANOVA, dose); however, the responses were smaller in the rats after hypertonic saline (P < 0.05, ANOVA, interaction). This decrease was not due to a time effect, because the responses were unchanged after isotonic saline infusion (Fig. 6). To evaluate whether the diminished pressor responses were secondary to changes in vascular responsiveness, the changes in arterial pressure to phenylephrine were also compared. The dose-dependent (P < 0.05) increases in pressure were smaller after hypertonic saline infusion (Fig. 7, P < 0.05, ANOVA, interaction). Because water deprivation increases baroreflex gain (8, 34), which could attenuate pressor responses to phenylephrine, the injections were repeated in other rats after combined V1 vasopressin and ganglionic blockade (Fig. 8). Again, pressor responses to...
phenylephrine were smaller after hypertonic saline infusion 
\((P < 0.05, \text{ANOVA, interaction})\) but not after isotonic saline infusion (Fig. 8).

**DISCUSSION**

Water deprivation is associated with regional increases in sympathetic tone (10, 35, 36) and with increased EAA drive of the RVLM, as indicated by a depressor response to bilateral microinjection of KYN into the RVLM (see Ref. 9 and present study). However, the mechanism for the increased EAA activity is unknown. The major new findings of the present study are that 1) the KYN depressor response was not significantly diminished following acute normalization of blood volume by intravenous infusion of saline; however, 2) the response was reduced, though not eliminated, by acute normalization of plasma sodium and chloride levels, and 3) an acute increase in plasma sodium and chloride levels was associated with a slowly developing increase in LSNA and depressor response to KYN microinjection into RVLM. Collectively, these data suggest that increases in osmolality increase EAA drive of the RVLM.

The present results confirm that blockade of ionotropic EAA receptors in the RVLM in water-deprived, but not water-

[Fig. 5. Changes in lumbar sympathetic nerve activity (LSNA), HR, and MAP in rats before and after infusion of isotonic or hypertonic saline (protocol 5). Control values of MAP were 120 ± 5 mmHg (hypertonic saline group) and 123 ± 5 mmHg (isotonic saline group). Control values of HR were 341 ± 21 beats/min (hypertonic saline group) and 333 ± 10 beats/min (isotonic saline group). *\(P < 0.05\) between groups.

Fig. 6. Increase in MAP in response to unilateral microinjection of glutamate into the RVLM before and after infusion of isotonic (top, \(n = 5\)) or hypertonic (bottom, \(n = 7\)) saline. In rats receiving isotonic saline (top), MAP just before injections averaged 112 ± 6 mmHg before the infusion and averaged 99 ± 6 mmHg after the infusion. In rats receiving hypertonic saline (bottom), MAP just before injections averaged 110 ± 6 mmHg before the infusion and averaged 115 ± 8 mmHg after the infusion. The responses were smaller in the rats after hypertonic saline infusion (\(P < 0.05, \text{ANOVA, interaction}\)) but were not significantly different in rats after isotonic saline infusion.]

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depressor response to KYN. Indeed, using a similar experimental approach as ours, Ramsay et al. (33) showed in dogs that the drinking induced by water deprivation was reduced only by 27% after normalization of blood volume. However, infusion of even greater volumes of isotonic saline in conscious rats failed to significantly decrease renal or lumbar sympathetic nerve activity (35, 36), suggesting that decreased baroreceptor afferent activity is not involved. It remains possible that the decreased volume acts via a mechanism, other than the baroreflex, which is slowly reversible. Thus we conclude that if volume depletion drives, in part, the increased EAA activity in RVLM, then it acts by a mechanism that takes more than 1 h to be reversed by correction of the volume deficit.

The present study also tested the hypothesis that the increased EAA drive of the RVLM in water-deprived rats is mediated by increased osmolality. Acute increases in osmolality produce changes in sympathetic activity that vary in direction and magnitude depending on the site at which the hypertonic solution is administered, on the nerve being studied, and on whether or not the baroreceptors are intact (for review, see Ref. 43). However, in rats, the net effect of rapid systemic increases in osmolality appears to be sympathoexcitation, because the increase in total peripheral resistance produced by hypertonic saline infusion in V1 vasopressin receptor-blocked rats is not present after ganglionic blockade (18). This acute sympathoexcitation can apparently be sustained, because intracerebroventricular infusion of hypertonic saline produces a slowly developing hypertension that is associated with greater depressor responses to ganglionic blockade (31). Water deprivation may be another model of chronic hypertonicity-induced sympathoexcitation. It increases osmolality and is also associated with greater depressor responses to ganglionic blockade (19), as well as increases in adrenal nerve activity (10) and lumbar nerve activity (as a function of the baroreflex maximum) (35), but not renal nerve activity (as a function of baroreflex maximum) (36). Importantly, hypertonicity clearly supports LSNA, because acute normalization of high osmolality in conscious, water-deprived rats decreased LSNA and arterial blood pressure (35). Moreover, we previously observed that the magnitude of the depressor response to KYN into RVLM is highly correlated with the basal level of osmolality (9). Therefore, the present study determined whether normalization of high osmolality would also decrease EAA drive of the RVLM as it decreased LSNA (35).

The depressor response to KYN was reduced soon after 5DW was infused and plasma sodium and chloride levels were lowered. Because the 5DW infusion increased plasma volume similarly to isotonic saline infusion (which did not alter the KYN response), we conclude that hypertonicity contributes to the increased EAA activity in water-deprived rats. Interestingly, however, about half of the depressor response to KYN remained more than 1 h after replenishment of water losses. Thus it also appears that a component of the increased EAA drive is not quickly reversed.

To further investigate the relationship between high osmolality and EAA input to RVLM, the depressor responses to RVLM microinjections of KYN and changes in LSNA were also investigated in water-replete rats after a rapid intravenous infusion of hypertonic saline. Both LSNA and the depressor response to KYN increased after the acute increases in sodium chloride levels. Because water deprivation and hypertonic

![Graph](https://via.placeholder.com/150)

Fig. 7. Increase in MAP in response to intravenous injection of phenylephrine before and after infusion of hypertonic saline. MAP just before injections averaged 108 ± 6 mmHg before the infusion and averaged 105 ± 13 mmHg after the infusion. The responses were smaller in rats after hypertonic saline infusion (P < 0.05, ANOVA, interaction).

![Graph](https://via.placeholder.com/150)

Fig. 8. Increase in MAP in response to intravenous injection of phenylephrine in rats pretreated with a V1 vasopressin antagonist and hexamethonium before and after infusion of isotonic (top, n = 4) or hypertonic (bottom, n = 6) saline. In rats receiving isotonic saline (top), MAP just before injections averaged 54 ± 8 mmHg before the infusion and averaged 58 ± 8 mmHg after the infusion. In rats receiving hypertonic saline (bottom), MAP just before injections averaged 54 ± 3 mmHg before the infusion and averaged 65 ± 2 mmHg after the infusion. The responses were smaller in the rats after hypertonic saline infusion (P < 0.05, ANOVA, interaction), but were not significantly different in rats after isotonic saline infusion.
saline infusion both increase osmolality but have opposite effects on blood volume and most hormones altered by blood volume changes, collectively the results strongly suggest that increases in osmolality increase EAA drive of the RVLM.

The time course of the responses after acute hypertonic saline deserves further comment. Immediately after the infusion, LSNA was not significantly increased and bilateral KYN microinjection into RVLM did not significantly decrease arterial pressure. Weiss et al. (46), using a hypertonic stimulus of similar magnitude, also observed that it took time (about 30 min) for a significant increase in LSNA to be produced. In addition, LSNA increased more rapidly in rats in which arterial baroreceptor afferents had been cut. Thus baroreflex restraint may have prevented an initial increase in LSNA and an early response to KYN after hypertonic saline infusion in the present experiments. However, these investigators (46) also reported that the ultimate increases in LSNA 30 min after hypertonic saline infusion were the same in intact and sinoaortic denervated rats, suggesting that baroreflex restraint waned, presumably due to acute resetting. Because acute resetting is complete within ~15 min (3), the later rises in LSNA and the slowly developing depressor response to KYN observed in the present study may be mediated by a mechanism other than baroreflex resetting.

One potential explanation for the delayed increases in LSNA and in the depressor response to hypertonic saline infusion is that it takes significant time for the hypertonic stimulus to equilibrate in brain tissue. However, brain osmoreceptors likely reside in the circumventricular organs (30, 42) and directly sense changes in extracellular fluid osmolality. Furthermore, sodium concentration in the cerebrospinal fluid rapidly (within minutes) equilibrates after systemic administration of hypertonic solutions (42, 47), making this explanation unlikely. Second, the hypertonicity may induce synaptic plasticity or morphological reorganization (7). Another possibility is that the increased EAA drive of RVLM requires a change in protein expression. Previously, we reported (9) that in water-deprived rats the pressor response to microinjection of glutamate into RVLM is increased, indirectly suggesting an increase in EAA drive. Therefore, in the present study, it was determined whether the increased EAA drive of RVLM to glutamate.

However, multiple other potential brain sites and mechanisms exist. For example, recent evidence (32, 48) supports a role for paravascular neurons in the paraventricular nucleus (PVN) in this response. These neurons are known to project directly to RVLM and spinal cord. Moreover, Toney et al. (43) have shown that acute intracarotid injections of hypertonic saline increase the activity of PVN neurons that can be antidromically stimulated from the RVLM. In contrast, Kantzides and Badoer (26) reported that virtually no RVLM-projecting PVN neurons expressed Fos following systemic increases in osmolality in rats and concluded that these neurons are not activated by a hypertonic stimulus. However, because sympathetic activity was not measured in these studies, it is not known if the stimulus was sympathoexcitatory. Furthermore, because the rats were euthanized 30 min after the hypertonic saline infusion was completed, based on the results of the present study it may be that with more time, Fos expression would have increased. Water deprivation has been associated with increased angiotensin II receptors in the subfornical organ and the PVN (1, 5, 21) and increased release of angiotensin into the PVN (21). Furthermore, blockade of angiotensin AT1 (12) or EAA receptors (4) in the PVN attenuates the changes in sympathetic activity following an acute increase in osmolality. Interestingly, in a study of water-deprived rats, Stocker et al. (37) recently indicated that acute blockade of the PVN by microinjection of muscimol produces a depressor response, which is similar in magnitude to the depressor response we observe following KYN microinjection in the RVLM. Thus we speculate that the increased EAA drive of RVLM originates directly or indirectly from the PVN.

In conclusion, the present studies reveal that acute and chronic increases in osmolality are associated with increased EAA drive of the RVLM. Interestingly, part of the increased EAA activity in water-deprived rats was reduced soon after replenishment of water losses, suggesting that part of the mechanism exhibits a short half-life. However, significant EAA drive remained. In addition, the increased EAA drive of RVLM that followed acute increases in sodium chloride concentrations took hours to develop. Therefore, another mechanism that exhibits longer on- and off-responses, including for example a change in protein expression or synaptic plasticity, may also be involved.

Perspectives

It is noteworthy that two states associated with chronic increases in sympathetic tone, water deprivation and hypertension, both exhibit increased EAA drive of the RVLM. In addition, Mayorov and Head (29) have recently observed that inotropic EAA receptors in the RVLM contribute to the increase in renal nerve activity and blood pressure elicited by air-jet stress. Thus we speculate that in other states associated with elevated sympathetic activity, such as pregnancy, sodium deprivation and congestive heart failure, the increased sympathetic tone may be due at least in part to increased EAA drive of the RVLM. Further studies are required to address this question, as well as others that investigate the roles of other neurotransmitters, including angiotensin II (15, 22) and nitric oxide, and where in the brain the increased EAA activity originates.

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