GABAergic Excitation of Vasopressin Neurons
Possible Mechanism Underlying Sodium-Dependent Hypertension

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Rationale: Increased arginine-vasopressin (AVP) secretion is a key physiological response to hyperosmotic stress and may be part of the mechanism by which high-salt diets induce or exacerbate hypertension.

Objective: Using deoxycorticosterone acetate-salt hypertension model rats, we sought to test the hypothesis that changes in GABA_A receptor–mediated inhibition in AVP-secreting magnocellular neurons contribute to the generation of Na^+-dependent hypertension.

Methods and Results: In vitro gramicidin-perforated recordings in the paraventricular and supraoptic nuclei revealed that the GABAergic inhibition in AVP-secreting neurons was converted into excitation in this model, because of the depolarization of GABA equilibrium potential. Meanwhile, in vivo extracellular recordings in the supraoptic nuclei showed that the GABAergic baroreflexive inhibition of magnocellular neurons was transformed to excitation, so that baroreceptor activation may increase AVP release. The depolarizing GABA equilibrium potential shift in AVP-secreting neurons occurred progressively over weeks of deoxycorticosterone acetate-salt treatment along with gradual increases in plasma AVP and blood pressure. Furthermore, the shift was associated with changes in chloride transporter expression and partially reversed by bumetanide (Na^+-K^+-2Cl^- cotransporter inhibitor). Intracerebroventricular bumetanide administration during deoxycorticosterone acetate-salt treatment hindered the development of hypertension and rise in plasma AVP level. Muscimol (GABA_A agonist) microinjection into the supraoptic nuclei in hypertensive rats increased blood pressure, which was prevented by previous intravenous V1a AVP antagonist injection.

Conclusions: We conclude that the inhibitory-to-excitatory switch of GABA_A receptor–mediated transmission in AVP neurons contributes to the generation of Na^+-dependent hypertension by increasing AVP release. We speculate that normalizing the GABA equilibrium potential may have some utility in treating Na^+-dependent hypertension. (Circ Res. 2013;113:1296-1307.)

Key Words: arginine vasopressin ■ gamma-aminobutyric acid ■ hypertension ■ NKCC1 ■ sodium ■ supraoptic nucleus

It is well established that excess dietary salt is one of the most powerful risk factors for, and a major cause of, hypertension.1,2 Yet, the mechanisms by which excess salt intake aggravates or produces hypertension remain elusive.3 One possible mechanism is that excess Na^+ increases the circulating levels of arginine-vasopressin (AVP),4 a neurohormone that can increase blood pressure not only through its well-known antidiuretic and vasoconstrictive actions, but also through stimulating the arterial secretion of the potent vasoconstrictor endothelin.5 Consistent with this possibility, the plasma level of AVP is elevated in several animal models of Na^+-dependent hypertension, including the widely used deoxycorticosterone acetate (DOCA)-salt model.6-8 Furthermore, Na^+-dependent hypertension does not develop in the Brattleboro rat,6,9 which genetically lacks AVP, and is suppressed by AVP antagonist.6-9 AVP is synthesized in magnocellular neurosecretory cells (MNCs) in the hypothalamic paraventricular nuclei (PVN) and supraoptic nuclei (SON), and it is released into the general circulation from the nerve terminals of MNCs located at the posterior pituitary. The release of AVP is regulated by sympathetically relayed signals to AVP-secreting MNCs from various sources, including baro- and osmoreceptors.10-11 The major excitatory and inhibitory neurotransmitters involved in this synaptic transmission had been thought to be glutamate and γ-aminobutyric acid...
drinking water for 4 weeks with subcutaneous injections of DOCA (25 mg in 0.4 mL of N,N-dimethyl formamide) every fourth day.21

NO Inhibitor–Induced Hypertension Model
Rats were made hypertensive by having access to drinking water containing L-NAME (60–70 mg/100 mL) for 4 weeks.22

Ethanol-Induced Hypertension Model
To avoid a considerable loss of animals, the ethanol-treated group was submitted to a brief and gradual adaptation period during which rats received 5% ethanol in their drinking water in the first week, 10% ethanol in the second, and 20% ethanol in the third and fourth weeks of experimentation.23 The development of hypertension in the model rats was confirmed by measuring weekly arterial blood pressures at zeitgeber time 1:00 to 4:00 hours (lights-on in the vivarium at zeitgeber time 0:00 hours), with a noninvasive tail-cuff system (model CODA; Kent Scientific Co, Torrington, CT). The animals were acclimated to the restraining device and the tail was heated (32°C) before measurements were started. The average of 10 measurements was taken as the data point for each rat.

Hypothalamic Slice Preparation
Brain slices were prepared as previously described.24 In brief, the animal was anesthetized with urethane (1.25 g/kg IP) and the brain was quickly excised from the skull and submerged in ice-cold artificial cerebrospinal fluid (ACSF; composition in mmol/L: 124 NaCl, 1.3 MgSO4, 3 KCl, 1.25 NaHPO4, 26 NaHCO3, 2.4 CaCl2, and 10 glucose). After being chilled for 1 to 2 minutes, the brain was trimmed to a block containing the hypothalamus. With the use of a vibrslicer (Campden Instruments, United Kingdom), coronal slices (300–450 μm) containing the SON or the PVN were cut from the tissue block in ice-cold ACSF. The slices were transferred to a gas interface recording chamber, which was perfused with warm (34–35°C) aerated (95% O2/5% CO2) ACSF at a rate of 0.5 to 1 mL/min by a peristaltic pump–driven or gravity-fed bath perfusion system.25 Warm (34–35°C) air humidified by 95% O2/5% CO2 gas mixture was continuously blown over the slices to further ensure adequate oxygenation of the brain tissue. Drugs (see below) were applied to the brain slices by including them in the perfusion medium or focally with the Y-tube method.26

Intracellular Electrophysiological Recording
Current or voltage clamp recordings were obtained from neurons in the SON or PVN of hypothalamic slices equilibrated for 1 to 12 hours in the recording chamber. SON was identified as a translucent, triangular area between the fornix and the upper part of the third ventricle. Micropipettes (tip diameter, 1.5–2.0 μm; 3–6 MΩ) pulled from borosilicate tubings (P-97; Sutter Instrument Co, Novato, CA) and filled with gramicidin (50 μg/mL)-containing solution (composition in mmol/L: 143 K-glucolate, 2 KCl, 10 HEPES, and 0.5 EGTA; pH 7.2–7.3) were used for recording in a perforated configuration. The use of low-chloride (2 mmol/L) internal solution along with gramicidin provided a clear readout of when the patch was ruptured, resulting in a whole-cell recording configuration (ie, GABAergic excitatory postsynaptic potential [PSP]/excitatory postsynaptic current). Stable perforated recording condition was usually achieved ≤10 to 25 minutes after seal was formed. Those recordings having steady series resistances (range 30–70 MΩ) and action potential amplitudes of ≥40 mV (measured from spike threshold) were the only ones included in the data pool; the reversal potentials of GABAa receptor–mediated responses were estimated only with recordings that had a series resistance of ≤60 MΩ (typically 30–50 MΩ). The voltage errors resulting from the series resistance were compensated offline for voltage clamp recordings and online for current clamp recordings by using the bridge circuit. We corrected the liquid junction potential before the experiments; we set the pipette potential to ~9 mV just before the formation of patch configuration, knowing that the liquid junction potential was 15.8 mV (at 34.5°C), whereas the perforated patch potential arising from gramicidin perforation was ~6.8 mV. We assumed that the change in resting membrane potential detected when the recording
mode was transformed from perforated to whole-cell configuration represented the perforated patch potential. Neurons sampled in SON and PVN, which responded to a depolarizing current pulse delivered at a holding potential between −70 and −80 mV with repetitive spikes having a delayed onset, were taken as MNCs. The signals from neurons amplified by Axoclamp-2B amplifier (bandwidth filter set at 10 kHz) were digitized and sampled at 50 μs intervals (Digidata1320, pClamp 8.0; Molecular Devices, Sunnyvale, CA).

**Immunohistochemical Identification of Recorded Neurons**
Please find the protocol in the Online Data Supplement.

**Measurement of Plasma Vasopressin and Catecholamine Levels**
Please find the protocols in the Online Data Supplement.

**Western Blot**
Please find the protocol in the Online Data Supplement.

**In Vivo Extracellular Single-Unit Recording and Monitoring of Blood Pressure**
Please find the protocol in the Online Data Supplement.

**Muscimol Microinjection Into the SON**
Before microinjection, rats were deeply anesthetized with urethane (1.25 g/kg IP) and catheterization was performed. One catheter (PE-50 tube) was inserted into the left carotid artery for blood pressure measurement and a second catheter was inserted into the left femoral vein for AVP antagonist injection. The catheterized rats were fixed in a stereotaxic instrument and kept warm (36–38°C) using a heating pad. Then, a 30-gauge injector was lowered into the SON (AP, 1.4 mm caudal to the bregma; ML, 1.8 mm lateral to the midline; DV, 9.3 mm below the skull surface). The GABAA receptor agonist muscimol was injected (1 or 10 nmol in 100 nL ACSF) unilaterally for a period of 60 seconds by injection pump. These doses of muscimol were chosen based on earlier studies demonstrating that microinjection of a similar dose in PVN effectively changed the blood pressure. Experimental procedures were initiated 1 to 1.5 hours after anesthesia onset. Absence of somatic motor reflexes in response to tail pitchting indicated deep anesthesia and analgesia. The baseline blood pressure was monitored for a period of 230 minutes before experimental procedures were performed. After microinjection experiment, Direct Blue dye (0.9% in saline) was injected (100 nL) into the SON. Brains were removed, sectioned (300 μm), and viewed through a light microscope to identify injection sites.

**Intracerebroventricular Infusion of Bumetanide**
The surgery to implant an osmotic minipump (infusion rate, 0.5 μL/h; Alzet, Model 2002; Cupertino, CA) and an infusion cannula (Brain Infusion Kit 2; Alzet) targeting the right lateral ventricle (AP, 1.0 mm caudal to the bregma; ML, 1.6 mm from the midline; DV, 4 mm below the surface of the skull) was performed on the 14th day after the beginning of DOCA-salt treatment. Under anesthesia induced by an intraperitoneal injection of a combination of ketamine (43 mg/kg body weight) and xylazine (8.7 mg/kg body weight), the pump was positioned subcutaneously in the scapular region and attached to the cannula via polyethylene tubing. Before implantation, the pump had been filled with vehicle (ACSF; pH 7.4) or a solution containing bumetanide (500 μmol/L) and placed in warm (36°C) saline overnight for priming.

**Drugs**
Please find the protocols in the Online Data Supplement.

**Statistical Analysis**
Numeric data are expressed as mean±SEM. Student t test and Mann–Whitney U test were used for the comparison of 2 independent data sets with and without normal distribution, respectively. Paired samples were compared with paired t test. One-way ANOVA and pairwise comparison with Holm–Sidak method were performed to compare multiple independent data sets with normal distributions, whereas Kruskal–Wallis 1-way ANOVA on ranks and pairwise comparison with Tukey test or Dunn method were performed to compare data sets without normal distributions. Comparison of multiple data sets collected with repeated measurements was performed with repeated measure of 1-way ANOVA. Fisher exact test was performed to see if there was a contingency between the 2 types of classification. P<0.05 was considered significant. When multiple comparisons were made with Fisher Exact test, the significance level was lowered by dividing the accepted probability value criteria with the number of comparisons.

**Results**

To determine if GABA_A receptor–mediated inhibition is altered in AVP neurons of the DOCA-salt hypertension model rat, we compared the GABA_A receptor–mediated PSPs of putative AVP neurons recorded in the acute hypotalamic slices prepared from Sprague–Dawley rats treated with DOCA-salt for 4 weeks after uninephrectomy (n=17) and untreated intact controls (n=15). The GABAergic PSPs were isolated from glutamatergic ones by including DL-2-amino-5-phosphonopentanoic acid (100 μmol/L; NMDA receptor antagonist) and 6,7-dinitroquinoxaline-2,3-dione (20 μmol/L; non-NMDA receptor antagonist) in the recording medium and monitored at various holding potentials with the use of gramicidin-perforated recording technique, which preserves the [Cl−] of the recorded cell.

**Identification of AVP Neurons**
Many of the putative AVP neurons, sampled from the SON or the lateral magnocellular subnucleus of the PVN, exhibited the electrophysiological characteristics of AVP neurons, including phasic firing patterns (Figure 1A), with little or no inward rectification at membrane potentials between −50 and −130 mV (Figure 1B). After recordings, immunohistochemical analysis (Figure 1C) confirmed that the cells that exhibited the physiological signature of AVP neurons did express AVP neurophysin (21 of 23 cells from 7 rats). In contrast, AVP neurophysin was absent in most of the cells (8 of 9 cells from 6 rats) that exhibited the physiological characteristics of oxytocin neurons, including the absence of phasic firing and strong inward rectification between −50 and −130 mV (Figure 1B). Therefore, for the remainder of this study, we used physiological properties to define MNCs as AVP-expressing neurons and focused our recordings on those cells that exhibited the physiological properties of AVP neurons. We could detect no physiological differences between AVP neurons recorded in SON and PVN regions and, therefore, pooled the resulting data for this study.

**In Control Rats, GABA-Mediated Currents Are Predominately Inhibitory in AVP Neurons**
The GABAergic PSPs detected in AVP neurons (n=44) of control rats (n=15) were predominantly inhibitory (41 of 44 cells), that is, hyperpolarizing relative to action potential threshold, which was −45 mV (Figure 2A, top panel; Figure 2C). A few of the neurons (3 of 44 cells) did show excitatory PSPs (example not shown). These PSPs were always mimicked by the focal application of GABA_A receptor agonist muscimol (10 μmol/L; 10–20 ms; n=44; Figure 2A, top panel) and blocked by bath-applied bicuculline (GABA_B receptor antagonist; 30 μmol/L; n=15, example not shown), indicating that they were
GABA<sub>R</sub>-mediated events. The reversal potential of these GABAergic responses (GABA equilibrium potential, E<sub>GABA</sub>), which was estimated with the currents elicited at various holding potentials by focal application of muscimol (10 μmol/L; 10–20 ms) in the presence of tetrodotoxin (0.5 μmol/L; Figure 2D), was −56±1 mV on average (n=44 cells; range, −27 to −73 mV; Figure 2E). In many of the neurons having GABAergic inhibitory PSPs, E<sub>GABA</sub> was similar to or significantly more positive than the resting membrane potential (−61±3 mV; n=44). Therefore, GABAergic inhibitory PSPs in these cells were either buried in the noise or detected as depolarizing events at resting membrane potential (middle trace of the top panel of Figure 2A). Thus, our physiological data suggest that GABA functions typically as an inhibitory transmitter in AVP neurons of the normal control rat.

In DOCA-Salt–Treated Rats, GABA-Mediated Currents Are Predominately Excitatory in AVP Neurons

In contrast, in the AVP neurons (n=54) of uninephrectomized rats treated with DOCA-salt (n=17), the GABAergic PSPs were almost always excitatory (52 of 54 cells; bottom panel of Figure 2A and Figure 2C). These PSPs were always mimicked by muscimol (10 μmol/L; 10–20 ms; n=54; Figure 2A, bottom panel) and blocked by bicuculline (30 μmol/L; n=15; Figure 2B), again indicating that they were GABA<sub>R</sub>-mediated events. The GABA<sub>B</sub>-receptor antagonist CGP 54626 hydrochloride (1 μmol/L) had no significant effect on the PSPs (n=5, example not shown). The E<sub>GABA</sub> of AVP neurons of DOCA-salt–treated rat averaged −35±1 mV (range, −2.7 to −54 mV; n=54), which was significantly more positive than the action potential threshold and the E<sub>GABA</sub> estimated for the neurons of control rats (P<0.001; Student t test; Figure 2D and 2E). The resting membrane potential and input resistance of AVP neurons were not significantly different between control and DOCA-salt–treated rats (control: −61±3 mV; n=44 versus DOCA-salt: −64±1 mV; n=54; control: 356±19 MΩ; n=44 versus DOCA-salt: 318±14 MΩ; n=51). These results indicate that DOCA-salt treatment causes the E<sub>GABA</sub> to depolarize beyond the action potential threshold in AVP neurons and, therefore, converts GABA<sub>R</sub>-receptor–mediated inhibition into excitation in these cells.

Depolarizing E<sub>GABA</sub> Shift and Resultant Emergence of GABAergic Excitation Occur Over the Span of Several Weeks

Next, we examined the time course of depolarizing E<sub>GABA</sub> shift and resultant emergence of GABAergic excitation in AVP
neurons induced by DOCA-salt treatment. Previous work has shown that DOCA-salt treatment in rat increases arterial pressure and plasma levels of AVP gradually over the span of several weeks. In the present study, we confirmed this (Figure 3A, top panel; Figure 3B) and discovered that progressive increases in arterial pressure and AVP concentration were paralleled by the gradual depolarizing shift of EGABA (Figure 3A, bottom panel) as well as an increase in the proportion of cells showing GABAergic excitation (Figure 3C).

These findings indicate that GABA-related changes in AVP neurons occur gradually over the span of several weeks and may contribute to the development of hypertension by promoting AVP release.

GABAergic Excitation Is Not Found in All Rat Hypertension Models

The depolarizing EGABA shift and resulting GABAergic excitation detected in AVP neurons of the DOCA-salt model rat may be a general response to hypertension or a more specific physiological response to promote AVP release. To examine these possibilities, we investigated GABAergic transmission in AVP neurons of rats made hypertensive by administering ethanol or NO inhibitor (Figure 4A, top panel) and discovered that progressive increases in arterial pressure and AVP concentration were paralleled by the gradual depolarizing shift of EGABA (Figure 3A, bottom panel) as well as an increase in the proportion of cells showing GABAergic excitation (Figure 3C). These findings indicate that GABA-related changes in AVP neurons occur gradually over the span of several weeks and may contribute to the development of hypertension by promoting AVP release.

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GABAergic Excitation Promotes the Release of AVP to Increase Blood Pressure in DOCA-Salt Hypertension Model Rat

GABAergic excitation in AVP neurons may contribute to increased AVP secretion in DOCA-salt model rats. To explore this possibility, we injected the GABAergic receptor agonist muscimol unilaterally into the SON of the rat under urethane anesthesia (Figure 5A, bottom panel) and measured the blood pressure. Muscimol produced no significant changes in blood pressure in control rats, whereas it increased the blood pressure in DOCA-salt hypertension model rats (Figure 5A and 5B), without altering the heart rate (Figure 5A; Online Figure 1A) or plasma levels of catecholamines (Online Figure 1B). In comparison, the GABAergic receptor agonist baclofen injected into the SON (2.25 nmol in 100 nL ACSF) decreased blood pressure in both control and DOCA-salt model rats (Online Figure 1B).
The muscimol-induced increase in blood pressure in DOCA-salt hypertension model rats was blocked by a previous intravenous injection of the V1a AVP receptor antagonist d(CH2)5[Tyr(Me)2, Ala-NH2]AVP (100 μg/kg; Figure 5C and 5D). Alone, this antagonist decreased the systolic blood pressure significantly in a dose-dependent fashion in DOCA-salt model rats, whereas it had no effect in control animals (Online Figure III). These results confirm the previous finding that AVP plays an important role in maintaining hypertension in DOCA-salt model rats. More importantly, they indicate that GABAergic receptor–mediated excitation in AVP neurons promotes AVP release to increase blood pressure in DOCA-salt hypertension model rats.

Depolarized $E_{\text{GABA}}$ Converts Baroreflexive Inhibition Into Excitation in SON Neurons of DOCA-Salt Hypertension Model Rat, Making Increases in Arterial Pressure Promote AVP Release

We assessed the impact of depolarizing $E_{\text{GABA}}$ shift on the reflexive inhibition of AVP neurons in SON by arterial baroreceptor activation using extracellular single-unit recording technique in the anesthetized rat. It is well established that baroreceptors in the aortic arch and carotid sinus buffer acute increases in arterial pressure by inhibiting AVP release from the posterior pituitary and decreasing the heart rate. In control animals, AVP neurons exhibit reflexive inhibition by arterial baroreceptors. In the DOCA-salt model, $E_{\text{GABA}}$ shifts were depolarized, suggesting that the GABAergic afferents to AVP neurons mediate the reflexive inhibition of these cells by arterial baroreceptors. In control animals, AVP neurons exhibit reflexive inhibition by arterial baroreceptors. In the DOCA-salt model, $E_{\text{GABA}}$ shifts were depolarized, suggesting that the GABAergic afferents to AVP neurons mediate the reflexive inhibition of these cells by arterial baroreceptors.
rats (n=3), we found that the activation of baroreceptors by an intravenous injection of the \(\alpha\)-adrenergic agonist phenylephrine (3–9 \(\mu\)g/kg body weight) resulted mostly in the inhibition of SON neurons (11 of 14 neurons; Figure 6A–6C, left panels). In contrast, in the DOCA-salt–treated rats (n=4), baroreceptor activation by phenylephrine mostly excited SON neurons (12 of 16 neurons; Figure 6A–6C, right panels). The proportions of cells showing inhibitory and excitatory responses to phenyephrine injections were significantly different between control and hypertensive rats \((P=0.009; \text{Fisher exact test})\). To assess the functional significance of the conversion of baroreflexive inhibition to excitation in SON neurons of DOCA-salt model rats, we next examined the effect of an acute increase in arterial pressure on plasma AVP concentration. As expected, the blood pressure increase led to a significant rise in AVP concentration in the model rats (Figure 6D). Meanwhile, the increase in arterial pressure had no significant effect on plasma AVP level in control rats. These results suggest that depolarization of \(E_{\text{GABA}}\) in DOCA-salt hypertension model rat impairs the \(GABA_{\alpha}\) receptor–mediated baroreflex in AVP neurons, such that an acute increase in arterial pressure promotes, rather than suppresses, AVP release.

DOCA-Salt Treatment Upregulates NKCC1 and Downregulates KCC2 to Induce the Depolarizing Shift of \(E_{\text{GABA}}\) and Resultant Emergence of GABAeric Excitation

\([\text{Cl}^-]\), the major determinant for \(E_{\text{GABA}}\) and polarity of \(GABA_{\alpha}\) receptor–mediated PSP, is regulated mainly by the \(\text{Cl}^-\)–importing transporter NKCC1 and \(\text{Cl}^-\)–exuding transporter KCC2 in mammalian central nervous system neurons. To see if the levels of these transporters changed in AVP neurons of DOCA-salt model rats, we examined expression levels of NKCC1 and KCC2 in SON, PVN, and hippocampus with Western blots. In SON and PVN tissues, the level of NKCC1 was significantly higher and the level of KCC2 was significantly lower in the DOCA-salt model than in control rats (Figure 7A and 7B). Whereas, in the hippocampus, the levels of Cl transporters were not significantly different between the 2 groups (Online Figure IV A). Also, the levels of NKCC1 and KCC2 in SON and PVN of rats treated with DOCA-salt for only 2 weeks did not differ from those of control rats (Online Figure IVB and IVC). This suggests that the upregulation of NKCC1 and the downregulation of KCC2 in AVP neurons located in the osmoregulatory circuit contribute to the depolarizing \(E_{\text{GABA}}\) shift and the resulting emergence of
GABAergic excitation in the later stages of development of DOCA-salt hypertension.

To test this hypothesis more directly, we next examined the effects of the NKCC inhibitor bumetanide and the KCC2 inhibitor VU0240551 on EGABA and GABAergic response profile. In A VP neurons (n=5) of control rats (n=2), the bath application of bumetanide (10 μmol/L) resulted in a small hyperpolarizing shift of EGABA (Figure 7C, top panel), whereas in cells (n=13) of DOCA-salt model rats (n=8), it caused EGABA to hyperpolarize to a greater extent (Figure 7D, top panel). The effect of bumetanide in these neurons was partially reversible, and it was accompanied by a significant change in GABAergic PSP profile, that is, the ratio of cells showing excitatory PSPs and inhibitory PSPs changed from 11:2 to 1:12 (P<0.001; Fisher exact test).

In meantime, bath-applied VU0240551 (75 μmol/L) caused a significant depolarization of EGABA in A VP neurons (n=10) of the control rat (n=3; Figure 7C, bottom panel). In cells (n=10) of DOCA-salt model rat (n=3); however, it had no significant effect on EGABA (Figure 7D, bottom panel). These effects of VU0240551 were not accompanied by a significant change in GABAergic PSP profile. Thus, these results, coupled with the data from Western blot experiments, indicate that the upregulation of NKCC1 and the downregulation of KCC2 are the molecular mechanisms underlying the depolarizing EGABA shift and the resultant emergence of GABAergic excitation in A VP neurons of established DOCA-salt hypertension model rats.
NKCC1 Plays an Important Role in the Development of DOCA-Salt Hypertension

If this hypothesis is correct, then pharmacologically blocking NKCC1 may hinder the development of hypertension in the DOCA-salt model by suppressing AVP release. To test this possibility, the NKCC blocker bumetanide was infused intracerebroventricularly using osmotic minipump into freely moving rats for a week between days 14 and 21 of DOCA-salt treatment. Infusion of bumetanide delayed the development of hypertension and lowered the plasma level of AVP (Figure 8).

These results provide support for the idea that the upregulation of NKCC1 in AVP neurons contributes to the development of hypertension in DOCA-salt model rats by promoting the emergence of GABAergic excitation and consequent increase in AVP release.

Discussion

Previous studies suggested that hypertension can arise through impairments of inhibitory GABAergic transmission in the central nervous system regions involved in the regulation of blood pressure. Similarly, recent work found that the mechanism that underlies the development of spontaneous hypertension in a line of rats is the upregulation of CI- transporter NKCC1, which then impairs GABA_A receptor-mediated inhibition of presympathetic neurons in the PVN. In the present study, we explored the possibility that in the DOCA-salt hypertension model rat GABAergic inhibition is converted into excitation in AVP neurons to increase the release of AVP and consequently blood pressure. We developed this hypothesis, in part, because of our earlier work that demonstrated that the polarity of GABA_A receptor-mediated transmission in AVP neurons can reverse in response to physiological need. AVP neurons in PVN and SON are heavily innervated by GABAergic afferents, which seem to originate from the perinuclear zones in the adjacent hypothalamus and are stimulated by high-salt intake. In the present study, we found that in AVP neurons of DOCA-salt hypertension model rats GABAergic input evoked depolarizing excitatory responses and that the excitatory action of GABA increased blood pressure by promoting AVP release. GABA was also excitatory in a significant proportion of AVP neurons of the rat with ethanol-induced hypertension, a model dependent on increased AVP secretion. In contrast, GABA remained inhibitory in AVP neurons of the rat made hypertensive by

Figure 7. DOCA-salt treatment increases levels of Na+-K+-2Cl– cotransporter isotype 1 (NKCC1) while downregulating K+-Cl– cotransporter isotype 2 (KCC2) expression in magnocellular neurosecretory cells. A and B, NKCC1, KCC2, and actin bands recognized by Western blot for supraoptic nuclei (SON) and paraventricular nuclei (PVN) tissue samples obtained from control and DOCA-salt hypertensive model rats. Bar graphs show the relative levels of NKCC1 and KCC2 in SON and PVN of control and DOCA-salt model rats. For each experiment, the values were normalized to the average value of the samples collected from control rats. NKCC1 and KCC2 levels were normalized to actin to control for loading. Experiments were repeated 3 times for SON and 5 times for PVN tissues. C and D, Graphs illustrate the impact of NKCC1 inhibitor bumetanide (10 μmol/L) and KCC2 blocker VU2040551 (75 μmol/L) on E_GABA in arginine-vasopressin neurons recorded in hypothalamic slices from control and DOCA-salt hypertension model rats. The symbols connected by lines denote data from the same cells. *P<0.05 for A and B. Values are shown as mean±SEM. *P<0.05 and **P<0.001 (C and D) compared with the value before drug treatment.
administering NO inhibitor. This treatment produced hypertension without increased AVP secretion. These findings suggest that GABAergic excitation in AVP neurons is a general feature of hypertension accompanied by increased AVP release, not an adaptive response to hypertension. Moreover, the findings suggest that one of the mechanisms through which high salt intake, and perhaps excessive alcohol consumption, induces or aggravates hypertension is to bring about GABAergic excitation in AVP neurons and the resultant increase in AVP levels. We examined GABA A receptor–mediated transmission in AVP neurons identified with electrophysiology analysis. In most of the identified AVP neurons, $E_{\text{GABA}}$ was negative to the action potential threshold (which was $\approx -45$ mV), and the GABA A receptor agonist muscimol decreased the firing rate by causing hyperpolarization. These findings indicate that GABA is inhibitory in most AVP neurons as has been reported in most previous studies. One recent study reported that GABA A receptor–mediated transmission produced excitatory responses in AVP neurons in SON and PVN even under control conditions. At present, we cannot offer a satisfactory explanation for these differences but point out that these findings are the exception in the literature.

In normotensive control rats, GABA was mostly inhibitory in AVP neurons. We examined GABA A receptor–mediated transmission in AVP neurons identified with electrophysiological characteristics or post hoc immunohistochemical analysis. In most of the identified AVP neurons, $E_{\text{GABA}}$ was negative to the action potential threshold (which was $\approx -45$ mV), and the GABA A receptor agonist muscimol decreased the firing rate by causing hyperpolarization. These findings indicate that GABA is inhibitory in most AVP neurons as has been reported in most previous studies. One recent study reported that GABA A receptor–mediated transmission produced excitatory responses in AVP neurons in SON and PVN even under control conditions. At present, we cannot offer a satisfactory explanation for these differences but point out that these findings are the exception in the literature.

We found that the activation of GABA A receptors in SON by muscimol microinjection produced no significant changes in arterial pressure in normotensive control rats. In addition, the GABA A receptor–mediated baroreflex triggered by intravenous injection of phenylephrine failed to alter plasma AVP concentration in control animals. If GABA inhibits AVP neurons increases in plasma osmolality, renal sodium and water retention, and sympathetic activity must be involved.

Our results raise the possibility that GABAergic excitation in AVP neurons contributes to the increase in blood pressure by altering the baroreflexive control of these cells. Baroreflex is responsible for the overall tone as well as the acute moment–to–moment regulation of blood pressure by modulating cardiac output and total peripheral resistance. Under resting conditions, baroreceptors respond to acute increases in arterial pressure by activating the vagal parasympathetic center. They also respond to pressure increases by inhibiting the vasocostrictive center of the medulla and suppressing the electric activity of AVP neurons through the release of GABA. The resulting decreases in heart rate, cardiac contractility, and AVP release from the posterior pituitary, along with vasodilation, cause blood pressure to decrease. Using the systemic administration of phenylephrine to interrogate the baroreflex, we discovered that arterial baroreceptor activation led to the excitation, rather than inhibition, of SON neurons in DOCA-salt model rats. Moreover, we found that baroreceptor activation by phenylephrine raised the plasma levels of AVP in DOCA-salt model but not control rats. These phenomena, which presumably resulted from the conversion of GABAergic inhibition to excitation, imply that the baroreflex in AVP neurons no longer functions to buffer blood pressure changes in these hypertensive rats but rather contributes to the generation of hypertension by making increases in blood pressure promote AVP release.

The baroreflexive control of heart rate is significantly suppressed in DOCA-salt hypertension model rats. In the present study, we found that muscimol (10 nmol) injection into the SON of DOCA-salt hypertension model rat resulted in an increase in systolic blood pressure by $\approx 13$ mmHg, with no significant change in heart rate. The absence of heart rate change might be related to the suppressed baroreflexive control of heart rate in the hypertension model rats. In support of this notion, our results (Online Figure V) demonstrate that an increase in systolic blood pressure by 13 mmHg is too small a change to alter the heart rate significantly.
under normal conditions, the activation of GABAergic system should lower blood pressure by suppressing AVP release. The reason for lack of changes in arterial pressure and plasma AVP concentration after the activation of GABAergic system might be that the inhibitory effect of GABAergic transmission on the hormonal output of AVP neurons is already maximal in normal conditions. We speculate that the basal secretory activities of AVP neurons are low in normal conditions in such a way that AVP plays a negligible role in maintaining the arterial pressure. Consistent with this idea, the intravenous injection of V1a receptor antagonist had no significant effect on arterial pressure in control rats unlike DOCA-salt model rats (Online Figure III).

The strength and polarity of GABA<sub>A</sub> receptor–mediated responses of central nervous system neurons are determined predominantly by [Cl<sup>−</sup>], which is regulated mainly by the Cl<sup>−</sup> importer NKCC1 and the Cl<sup>−</sup> exchanger KCC2. We found evidence that the upregulation of NKCC1 as well as the downregulation of KCC2 is responsible for the depolarizing shift of E<sub>GABA</sub> and the resulting emergence of GABAergic excitation in AVP neurons. Direct stimulation by DOCA of mineralocorticoid receptors in MNCs is unlikely to be the reason for the changes in expression levels of Cl<sup>−</sup> transporters because NKCC1 and KCC2 are up- and downregulated, respectively, in MNCs of SON and PVN after the removal of mineralocorticoid by bilateral adrenalectomy in the adult male rat (W.B. Kim, PhD, Y.-B. Kim, BS, H.J. Chung, PhD, and Y.I. Kim, PhD, unpublished data, 2013). The upregulation of NKCC1 in DOCA-salt–treated rats might result from the central action of AVP or oxytocin released from the somata or dendrites of MNCs in response to osmotic stimuli presented by DOCA-salt treatment. A recent study from our laboratory showed that a long-term intracerebroventricular administration of selective oxytocin receptor antagonist partially blocked the NKCC1-dependent depolarizing E<sub>GABA</sub> shift and the resultant emergence of GABAergic excitation in the MNCs of rats subjected to chronic hyperosmotic stress. Meanwhile, the KCC2 downregulation in DOCA-salt–treated rats might arise from the action of brain-derived neurotrophic factor released locally from glia or neurons in PVN and SON. Tropomyosin-receptor-kinase B as well as brain-derived neurotrophic factor mRNA and protein, are present in SON and hyperosmotic stimulus increases the local release of brain-derived neurotrophic factor in SON. Tropomyosin-receptor-kinase B–mediated action of brain-derived neurotrophic factor from microglia was reported to downregulate KCC2 in the lamina I of the spinal cord of the rat with neuropathic pain, enabling GABAergic excitation to occur in neurons located in this spinal region. Still, the mechanisms that underlie the changes in expression of NKCC1 and KCC2 are not known and will require further investigation.

The present data raise the possibility that Cl<sup>−</sup> transporters (NKCC1 and KCC2) can be targeted for the treatment of hypertension. The finding that the NKCC inhibitor bumetanide retarded the development of hypertension in DOCA-salt model rats provides some evidence in support of this hypothesis. With hypertension due to excess consumption of salt and alcohol being a growing problem throughout the world, there remains the need for novel treatment strategies.

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**Disclosures**

None.

**References**


Pharmacological inhibition of this transporter reduced AVP secretion. Increased AVP secretion may be due to an increase in intracellular calcium channels play crucial roles in the glutamate-induced phase shifts of the rat suprachiasmatic circadian clock. 


What Is Known?

- Excess dietary salt is a major risk factor for hypertension.
- Excess salt increases circulating levels of arginine-vasopressin (AVP).
- High AVP can increase blood pressure through a variety of mechanisms.

What New Information Does This Article Contribute?

- High-salt diet combined with mineralocorticoid treatment, which produces hypertension, converts the inhibitory effects of GABAergic input onto AVP secretory cells in the rat hypothalamus into excitatory ones.
- Increased AVP secretion may be due to an increase in intracellular chloride concentration driven largely by the chloride transporter, Na+-K+-2Cl− cotransporter isotype 1.
- Pharmacological inhibition of this transporter reduced AVP secretion and lowered blood pressure in this experimental model of hypertension.

Novelty and Significance

It is well established that consumption of excess dietary salt is associated with hypertension. Yet, the mechanisms by which excess salt intake aggravates or produces hypertension have remained elusive. One possible mechanism is that excess sodium increases the circulating levels of AVP, because previous work has linked increased secretion of this neurotransmitter with raised blood pressure. This previous work raises the question of how a high-salt diet increases the secretion of AVP from hypothalamic neurones. In the present study, we found that under hypertensive conditions induced by a combination of high-salt diet and mineralocorticoid treatment, the normal GABA inhibition of AVP neurones is transformed into excitation, which results in an increase in AVP secretion. This increase in AVP secretion may be because of a shift in chloride homeostasis, which alters how these AVP cells respond to GABA. We speculate that increasing the GABA inhibition of AVP neurones by normalizing the chloride equilibrium potential may be useful in treating salt-dependent hypertension.
GABAergic Excitation of Vasopressin Neurons: Possible Mechanism Underlying Sodium-Dependent Hypertension

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Supplemental Methods

Immunohistochemical (IHC) identification of recorded neurons. Some SON and PVN neurons recorded in slices from the control and DOCA-salt hypertension model rats were injected with biocytin (Sigma) for IHC identification. Biocytin (1.5 mg/ml) was included in the internal solution. Following perforated patch recordings, the membrane patch was ruptured to achieve the whole-cell configuration. Biocytin contained in the recording pipette was allowed to get into the cell for >10 min. On termination of the recording, the slice was fixed in 4% paraformaldehyde in 0.1 M PBS at a pH of 7.4 for 24 h, transferred to 30% sucrose-PBS, then cut into 25–35 μm sections. Fixed slices were incubated for 24 h at 4°C in a rabbit polyclonal antibody against AVP-neurophysin (1:200; Abcam). After the slices had been washed with PBS, a 1:200 dilution of DyLight 488-conjugated goat anti-rabbit secondary antibody (Jackson Immunoresearch) was added with a 1:500 dilution of avidin-AMCA (7-amino-4-methylcoumarin-3-acetic acid; Vector Labs) to label biocytin for 1 h at room temperature. All antibodies and labeling reagents were dissolved in 0.1 M PBS containing 0.3% Triton X-100 and 2% normal goat serum. The sections were examined under a confocal fluorescence microscope for the presence of AVP-neurophysin immunoreactivity and biocytin labeling.

Measurement of plasma vasopressin levels. A total of 0.5-1.0 ml of blood was obtained through the femoral vein catheter or by decapitating the rat and placed into ice-cold tubes containing EDTA (1 mg/ml blood). Samples were placed on ice immediately. Plasma samples were separated by centrifugation at 1,600 × g for 15 min at 4°C and stored at -80°C until analyzed. Plasma vasopressin levels were determined in diluted samples (1:2) by using an Arg⁸-vasopressin enzyme immunoassay (ELISA) kit (Enzo Life Sciences, Plymouth Meeting, PA, USA). The intra- and inter-assay coefficients of variation for the assay for AVP were 8.2% and 9.0%, respectively. The sensitivity was 0.25 pg/ml on average.

Measurement of plasma catecholamine levels. Blood samples were obtained through the femoral vein catheter and placed into ice-cold tubes containing EDTA (1 mg/ml blood). Samples were placed on ice immediately. Plasma samples were separated by centrifugation at 1,600 × g for 15 min at 4°C and stored at -80°C until analyzed. Plasma epinephrine and norepinephrine levels were quantified with a catecholamine ELISA kit (KA1877, Abnova, Taipei City, Taiwan). For competitive ELISA, epinephrine and norepinephrine were extracted with a cis-diol-specific affinity gel, acylated and derivatized enzymatically. The intra-assay coefficients of variation for the assay for epinephrine and norepinephrine were 10.2% and 10.6%, respectively. The sensitivity of epinephrine and norepinephrine were 10 pg/ml and 50 pg/ml, respectively.

Western blot. The SON, PVN and hippocampus were excised from brain slices prepared as above. Since the SON and PVN are very small structures, SON/PVN tissues from two rats were pooled to form a single sample. Each sample was lysed in triple-detergent lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.02% sodium azide, 0.1% sodium dodecyl sulfate, 1% Nonidet P-40, 0.5% sodium deoxycholate, 1 mM phenyl-methylsulfonyl fluoride). After the determination of protein concentration of total tissue lysate with Bradford assay, the tissue lysate was denatured with SDS-reducing buffer and heat (100°C, 5 min). The denatured lysate and prestained molecular size markers were resolved by 8% SDS-PAGE and transferred to nitrocellulose membrane. After being incubated in 5% skimmed milk/Tris-buffered saline for 1 h at room temperature, the membrane was reacted sequentially with primary antibodies [mouse monoclonal anti-NKCC1 (1:2000; Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA), rabbit polyclonal anti-KCC2 (1:2000; Millipore), or rabbit polyclonal anti-β-actin (1:3000; Santa Cruz Biotechnology, CA, USA)] and
HRP-conjugated secondary antibodies (anti-mouse IgG or anti-rabbit IgG; 1:5000; Santa Cruz Biotechnology, CA, USA). The integrated optical density of bands was quantified using the *imageJ* software. Each sample was normalized to the content of β-actin, a constitutively expressed protein.

**In vivo extracellular single-unit recording and monitoring of blood pressure.** We obtained extracellular single-unit recordings from the SON of urethane-anesthetized rats (1.25 g/kg body weight, ip) placed in a stereotaxic instrument and kept warm (36–38°C) with a heating pad. Glass micropipettes (impedance: 10–15 MΩ) filled with 3.0 M-potassium acetate were used as the recording electrodes. The stereotaxic coordinate for the SON was as follows: AP: 1.4 ± 0.2 mm caudal to the bregma, ML: 1.8 ± 0.2 mm lateral to the midline, DV: 9.3 ± 0.2 mm below the skull surface. SON neurons were identified by antidromic activation following stimulation (pulse duration <0.5 ms, intensity <2 mA) of their axon terminals in the posterior pituitary. The signals from neurons amplified by DAM 80 AC differential amplifier (high-pass filter set at 300 Hz, low-pass filter set at 3 kHz, World Precision Instruments, New Haven, CT, USA) were digitized and sampled at 50 μs intervals (Digidata1320, pClamp 8.0). To activate baroreceptors during extracellular single-unit recording, we injected phenylephrine (3–90 μg/kg body weight) through a catheter inserted into the left carotid vein. The blood pressure was monitored through a catheter placed in the left carotid artery. A pressure transducer (BLPR2, World Precision Instruments, USA) and a pressure monitor (model BP-1, World Precision Instruments, USA) coupled to a computerized acquisition system (Digidata 1320, pClamp 8.0) were used for blood pressure recording. The moment-to-moment heart rate was calculated from the inter-pulse interval, using “Mini Analysis” program (Synaptosoft, Fort Lee, NJ).

**Drugs.** We purchased all the drugs and chemicals used in the current study from Sigma-Aldrich, Korea (Seoul, Korea), except muscimol (GABA<sub>A</sub> receptor agonist; Ascent Scientific, Somerset, UK), CGP 54626 hydrochloride (GABA<sub>B</sub> receptor antagonist, Tocris), VU0240551 (KCC2 inhibitor; Tocris), and d(CH<sub>2</sub>)<sub>5</sub>[Tyr(Me)<sub>2</sub>, Ala-NH<sub>2</sub>]<sub>9</sub>AVP (V1a AVP receptor antagonist; gift from Dr. M. Manning at the University of Toledo College of Medicine, Toledo, OH, USA). We prepared the solutions of muscimol, baclofen-HCl (GABA<sub>B</sub> receptor agonist), bicuculline methiodide (GABA<sub>A</sub> receptor antagonist), DL-2-amino-5-phosphonopentanoic acid (AP5, NMDA receptor antagonist) by dissolving these drugs in ACSF, the standard slice perfusion medium, and 6,7-dinitroquinoxaline-2,3-dione (DNQX, non-NMDA receptor antagonist), CGP 54626 hydrochloride and VU0240551 solutions by diluting their dimethyl sulfoxide-based stock solutions with ACSF (final concentrations of dimethyl sulfoxide: 0.001–0.1%). The working solutions of the NKCC inhibitor bumetanide (10 μM for electrophysiology and 500 μM for ICV infusion) were prepared by diluting the stock solution (25 mmol/l in 0.1 mol/l NaOH) with ACSF. The solutions of phenylephrine and V1a AVP receptor antagonist for intravenous (i.v.) injections were prepared by dissolving the agents in 0.9% NaCl solution.
**Online Figure Legends**

**Online Figure I.** Muscimol injection into the SON does not increase sympathetic nerve activity. (A) Lack of effect on the heart rate of muscimol (1 or 10 nmol in 100 nl ACSF) injected into the SON, with and without prior i.v. injection of the V1a AVP receptor antagonist (100 µg/kg body weight). The heart rate was measured immediately before and 16 min after the muscimol injection. In addition, it was measured at 4 and 6 min after 10 and 1 nmol muscimol injections, respectively. At these time points, the muscimol-induced increases in arterial pressure reached their peaks in DOCA-salt hypertension model rats (please see Figure 5B). (B) Lack of the effect of muscimol (10 nmol in 100 nl ACSF) injected into the rat’s SON on the plasma levels of catecholamines. 0.8-ml blood samples were taken at 40 min before and 4 min after the muscimol injection and replaced with the same amount of saline. The double arrowheads in the blood pressure record in the upper panel denote the increase in arterial blood pressure following the muscimol injection. Arrows: times of blood sampling. The numbers in parentheses in (A) and (B) denote the number of rats tested. NS indicates no statistical difference.

**Online Figure II.** Effects on the blood pressure of the GABA_B agonist baclofen injected into the SON. Graph summarizing the effects on the SBP of baclofen (2.25 nmol in 100 nl ACSF) injected into the SON in the control and DOCA-salt model rats. n: number of rats tested. *: P< 0.05, **: P< 0.001 compared to the value at 0 min.

**Online Figure III.** Effect of V1a receptor antagonist on arterial pressure. Three different doses of the drug were i.v. injected at the time points indicated with arrowheads. The traces for arterial pressure in (A) were from 6 different rats. (B) Graph summarizing the effects of V1a receptor antagonist on the SBP in the control and DOCA-salt hypertension model rats. The numbers in parentheses denote the number of rats tested. *: P< 0.05, **: P< 0.001 compared to the value at 0 min.

**Online Figure IV.** Lack of effects of DOCA-salt treatment on the levels of NKCC1 and KCC2 in the hippocampus, SON and PVN of the rat. DOCA-salt was treated for 4 weeks (A) or 2 weeks (B,C) Experiments were repeated three times for the SON and hippocampal tissues and five times for the PVN tissues. NS indicates no statistical difference.

**Online Figure V.** Effect of DOCA-salt treatment on the slope of baroreceptor reflex. Left panel, scatter plots showing the relationship between peak increases in SBP produced by i.v. injections of phenylephrine (3-90 µg/kg body weight) and the baroreceptor reflex-mediated increases in pulse interval in control (n=3) and DOCA-salt model rats (n=4). Linear regression lines were fitted through these points (Control: solid black line; Model: solid grey line). Right panel, bar charts compare the baroreceptor reflex slope (i.e., Δ pulse interval x Δ SBP) between control (n=3) and DOCA-model rats (n=4). The graph in this panel was based on the slopes calculated with the separate sets of data from individual rats. *: P< 0.05.
Online Figure IV

A

Hippocampus

B

SON

C

PVN