Brain Sodium Channels Mediate Increases in Brain “Ouabain” and Blood Pressure in Dahl S Rats

Hao Wang, Frans H.H. Leenen

Abstract—Central infusions of benzamil prevent/reverse salt-induced hypertension in genetic models of salt-sensitive hypertension. Benzamil acts by blockade of ion—presumably sodium—channels. In the present study, we assessed in Dahl salt-sensitive (S) rats on high salt intake whether these channels mediate increases in brain “ouabain” and, thereby, hypertension. Intracerebroventricular (icv) infusions of a low (1.2 μg/kg per hour) or high (4.0 μg/kg per hour) dose of benzamil were given to Dahl S rats on high salt diet (1370 μmol Na⁺/g food) for 2 or 4 weeks. “Ouabain” content was measured using a specific enzyme-linked immunosorbent assay (ELISA). Systolic blood pressure (BP) in Dahl S rats on high salt for 4 weeks increased markedly (188±10 versus 128±4 mm Hg, n=8, P<0.05). Benzamil fully blocked this increase (131±7 mm Hg after the high dose of benzamil). Hypothalamic and pituitary “ouabain” increased significantly (22±7 versus 12±3 and 151±38 versus 69±6 ng/g tissue, respectively, P<0.05) in Dahl S rats on high salt versus regular salt diet for 2 weeks. Benzamil blocked these increases of brain “ouabain” to high salt intake. Similarly, high salt intake for 4 weeks increased hypothalamic (18±2 versus 13±1 ng/g tissue, P<0.05) and pituitary (183±30 versus 78±8 ng/g tissue, P<0.05) “ouabain.” Benzamil also inhibited these increases of brain “ouabain.” Both hypothalamic and pituitary “ouabain” showed significant positive correlations with BP. In contrast, high salt intake did not affect “ouabain” levels in the adrenal gland or plasma in Dahl S rats on high salt for either 2 or 4 weeks. These findings indicate that in Dahl S rats high salt intake only increases brain and not peripheral “ouabain” and that benzamil-blockable brain sodium channels mediate the increases in brain “ouabain” and the subsequent hypertension.

Key Words: ouabain ■ brain ■ blood pressure ■ sodium channels

One decade ago, Hamlyn et al isolated from human plasma an endogenous sodium pump inhibitor structurally similar to the cardiac glycoside ouabain. In genetic models of salt-sensitive hypertension (ie, Dahl salt-sensitive [S] rats and spontaneously hypertensive rats, SHR), a ouabain-like substance (here defined as “ouabain”) in the brain plays a critical role in salt-induced hypertension. In Dahl S rats or SHR, high salt intake increases brain “ouabain,” and blockade of brain “ouabain” prevents both the sympathetic hyperactivity and hypertension. Intracerebroventricular (icv) infusion of Na⁺-rich artificial cerebrospinal fluid (aCSF) also increases brain “ouabain” and blood pressure (BP), and markedly more in Dahl S versus salt-resistant (R) rats or Wistar rats. From these findings we proposed the concept that, in Dahl S rats or SHR on high salt intake, small increases in cerebrospinal fluid (CSF) sodium activate central pathways involving “ouabain,” resulting in sympathoexcitation and hypertension. However, how an increase in CSF sodium may activate brain “ouabain” is not yet clear.

Recent studies have shown that sympathoexcitatory and pressor responses to CSF Na⁺ in normotensive rats are enhanced by the peptide, Phe-Met-Arg-Phe-NH₂ (FMRFamide), and blocked by an amiloride analog, benzamil hydrochloride. FMRFamide is an excitatory neuropeptide, which in rats is present in nerve cells and terminals, especially in the hypothalamus and spinal cord. FMRFamide induces a fast excitatory depolarizing response because of the direct activation of sodium channels, which have been cloned in snails, and can be blocked by amiloride and a more specific analog, benzamil hydrochloride. In rats, excitatory responses to FMRFamide appear to depend on release of ouabain, because blockade of brain “ouabain” by specific antibody Fab fragments prevents sympathoexcitatory and pressor responses to icv FMRFamide. FMRFamide-activated channels appear to play a critical role in several models of salt-dependent hypertension. Icv infusion of benzamil prevents mineralocorticoid-induced hypertension in Wistar rats and salt-induced hypertension in Dahl S rats. In SHR on high salt intake, icv infusion of benzamil results in parallel decreases in renal sympathetic nerve activity and blood pressure. Intravenous infusion of benzamil at the same rates was ineffective, indicating that the above effects of benzamil are indeed a central effect and not due to diffusion into the systemic circulation.

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We hypothesized that, in Dahl S rats, high salt intake through benzamil-blockable brain sodium channels leads to entry of Na⁺ into specific cells resulting in an increase in brain “ouabain” and, thereby, hypertension. To test this hypothesis, we evaluated whether in Dahl S rats, central infusion of benzamil hydrochloride blocks the increases in brain “ouabain” and blood pressure by high salt intake.

Methods

Male 4-week-old Dahl S rats (Harlan Sprague Dawley, Indianapolis, Ind) were housed, 2 per cage, in a climatized room on a 12-hour light/dark cycle at constant room temperature and humidity and given a diet of standard laboratory chow and tap water ad libitum for 1 week before entering the study. At 5 weeks of age (1 day after icv cannulation), the rats were placed on either regular or high sodium diet (rat chow containing either 120 or 1370 μmol Na⁺/g food, Harlan Sprague Dawley, Madison, Wis). Diet was provided for 2 or 4 weeks. All procedures were carried out according to the guidelines of the University of Ottawa Animal Committee for the use and care of laboratory animals.

Icv Cannulation and Infusion of Drugs

After 1 week acclimatization, with the use of a stereotaxic frame (Harvard Apparatus), a 23-gauge right-angled stainless steel cannula was implanted into the left lateral cerebral ventricle and fixed with acrylic cement to the skull of each rat. The cannula was placed 0.4 mm posterior and 1.4 mm lateral to the bregma. The lower end (shorter arm) of the cannula was at a depth of 3.5 mm from dura, and the upper end (longer arm) was connected to an osmotic minipump (Model 2002, Alza, last 14 days) for chronic icv infusion at 0.5 μL/h. The lower end (longer arm) was 0.4 mm posterior and 1.4 mm lateral to the bregma. After 1 week acclimatization, with the use of a stereotaxic frame (Harvard Apparatus), a 23-gauge right-angled stainless steel cannula was implanted into the left lateral cerebral ventricle and fixed with acrylic cement to the skull of each rat. The cannula was placed 0.4 mm posterior and 1.4 mm lateral to the bregma. The lower end (shorter arm) of the cannula was at a depth of 3.5 mm from dura, and the upper end (longer arm) was connected to an osmotic minipump (Model 2002, Alza, last 14 days) for chronic icv infusion at 0.5 μL/h for 14 or 28 days. For the latter duration, all pumps were changed at day 14, and the second pumps contained a higher concentration of benzamil according to the body weights of the rats. Considering the secretion rate of CSF in rats (120 to 320 μL/h), this low rate of icv infusion was not likely to affect CSF pressure. For rats on a regular salt diet, the pumps were filled with artificial CSF (aCSF). For rats on high salt diet, the pumps were filled with vehicle (aCSF), benzamil (1.2 μg/kg per hour), or benzamil (4.0 μg/kg per hour) and implanted subcutaneously on the backs of the rats. For the 14-day experiment, there were 3 groups of rats (n=4/group) and only the high dose of benzamil was used. In the 28-day experiment, there were 4 groups (n=8/group), and both the high and low doses of benzamil were given. The amount of benzamil used reflects “low” and “high” doses, based on previous studies.6

Arterial BP and Heart Rate (HR) Measurements

Thirteen or 27 days after the icv cannulation, carotid arterial cannulation was performed in the afternoon. A PE-50 catheter, filled with heparinized saline (100 IU/mL) was inserted into the left carotid artery. The next morning, the carotid arterial catheters were connected to pressure transducers, and after a rest of approximately half an hour, BP and HR were recorded for 30 minutes with an online computer equipped with AcqKnowledge for Windows (BIOPAC Systems Inc).

Samples Collection and Pretreatment

Blood and tissues were obtained as recently described.6,21 “Ouabain” was extracted by mixing plasma samples with 1 vol of 0.1% trifluoroacetic acid. Tissues were homogenized in 10 volumes of methanol-2 mmol/L ascorbic acid. The homogenate was centrifuged, and the supernatant was dried and reconstituted with 0.1% trifluoroacetic acid. Plasma and tissue extracts were passed through a 200-ng water-equilibrated Sep-Pak C₁₈ column (Waters Corp). “Ouabain” was eluted with 5 mL 25% acetonitrile. The elutes were dried with a vacuum centrifuge and the extracts dissolved using phosphate-buffered saline (PBS, 10 mmol/L, pH 7.4).

Enzyme-Linked Immunosorbent Assay (ELISA) for “Ouabain”

The anti-ouabain antibody was raised from rabbits immunized with the commercially available cardenolide ouabain conjugated with bovine serum albumin. This antibody has a high antibody titer (1:1.6×10⁵) and shows full cross-reactivity with ouabain, 8% cross-reactivity with digoxin, and minimal cross-reactivity with numerous common endogenous steroids and peptides.21 In addition, the “ouabain” was eluted with 25% acetonitrile in water from Sep-Pak C₁₈ cartridges, and common adrenocortical steroids such as corticosterone or aldosterone are too hydrophobic to be eluted at this acetonitrile concentration. “Ouabain” was measured by ELISA as recently described in detail.6,21 Briefly, enzyme immunoassay plates were coated with ovalbumin-ouabain. Fifty microliters of samples or ouabain standards (Sigma Chemical Co) were added to successive wells. Rabbit anti-ouabain antisem (50 μL, dilution of 1:12000) was added to each well. Plates were incubated at 37°C for 2 hours with continuous shaking followed by 4 rinses of 250-μL rinse solution per well. Anti-ouabain antibodies remaining bound to the ovalbumin-ouabain were reacted with 100 μL per well of a 1:1000 dilution of goat anti-rabbit IgG-peroxidase conjugate (Sigma Chemical Co). Unbound anti-rabbit IgG-peroxidase conjugate was washed away by rinsing as described above. The presence of peroxidase enzyme remaining in each well was determined by the absorbance at 450 nm using the Microplate Recorder (Model 3550, Bio-Rad Laboratories Inc). The concentration of “ouabain” in each sample was calculated from the absorbance according to the ouabain standard curve. The assay provides a sensitivity of 0.23 ng/mL plasma and 5.75 ng/g tissue. The average intra-assay coefficient of variation (CV) was 4.7% and the inter-assay CV was 12.3%. The recovery of high and low concentrations of ouabain was 96.3% and 91.4%, respectively.21

Statistical Analysis

Values are expressed as mean±SEM. Differences between groups were evaluated by ANOVA followed by Duncan’s multiple range test. Linear regression analysis was used to study relations between variables. The level of significance was set at a value of P<0.05.

Results

On high sodium intake, body weight gain was less (P<0.05) at both 7 and 9 weeks of age, but treatment with benzamil did not affect this gain (Table).

Systolic, diastolic, and mean arterial pressure increased by 10 to 15 mm Hg in Dahl S rats on high salt for 2 weeks (Table). High salt intake for 4 weeks increased BP by about 45 to 60 mm Hg (P<0.05). Benzamil blocked these increases in a dose-related manner, with most of the inhibition already apparent with the lower dose (Table). HR tended to be higher in Dahl S rats on high salt versus regular salt diet (Table).

After 2 weeks of high salt diet, hypothalamic “ouabain” and pituitary “ouabain” had increased by about 90% and 120%, respectively. Benzamil prevented the increases in hypothalamic and pituitary “ouabain” by high salt intake (Figure 1). After 4 weeks on high salt diet, hypothalamic “ouabain” and pituitary “ouabain” were increased by about 45% and 135%. Benzamil also inhibited these responses. The lower dose of benzamil partially inhibited the increase in hypothalamic “ouabain,” whereas the higher dose of benzamil fully blocked the responses (Figure 2).

In contrast to the clear increases in brain “ouabain,” high salt intake for 2 or 4 weeks caused only minor, 10% to 20%, increases (all NS) in adrenal and plasma “ouabain” levels of
Dahl S rats. Benzamil prevented these modest increases at either dose (Figures 1 and 2).

In Dahl S rats on high salt intake for 4 weeks, both hypothalamic and pituitary "ouabain" showed significant positive correlations with BP, similarly ($r=0.52$ to $0.69$) for systolic, diastolic, or mean arterial pressure (Figure 3). No significant correlations between plasma or adrenal "ouabain" and BP were found.

**Discussion**

The present study provides 2 significant new findings. (1) High sodium intake in Dahl S rats causes hypertension associated with clear increases in hypothalamic and pituitary "ouabain," but not in adrenal and plasma "ouabain." (2) The amiloride analog, benzamil, blocks increases in brain "ouabain" and BP induced by high sodium intake in Dahl S rats in a dose-related manner. These findings indicate that, in Dahl S rats, high salt intake increases brain "ouabain" and not peripheral "ouabain," and that brain benzamil-blockable sodium channels mediate the increases in brain "ouabain" and the subsequent hypertension.

In the present study, using a specific assay for "ouabain," high salt intake caused only minor increases (NS) in peripheral (ie, adrenal and plasma) "ouabain" in Dahl S rats. These findings are consistent with a recent study, wherein, likewise, a specific assay for "ouabain" was used and plasma or urinary...
enhancing Na⁺ entry through sodium channels in the brain. Benzamil blocks responses to both CSF Na⁺ and FMRFamide, but not to ouabain. Blockade of brain “ouabain” by specific Fab fragments also blocks responses to CSF Na⁺ and FMRFamide. The present study shows that icv benzamil also inhibits the increase in brain “ouabain” content in Dahl S rats on high salt intake. One may speculate that benzamil-blockable sodium channels may function as a sensor of CSF Na⁺ and that acute or chronic enhanced Na⁺ entry leads to increases in “ouabain” release and content, ie, open brain benzamil-blockable sodium channels are essential for increased release of “ouabain,” and, if persisting, also cause increases in “ouabain” content in the hypothalamus and pituitary.

Benzamil-blockable brain ion—presumably sodium—channels appear to play a major role in several models of salt-sensitive hypertension. Gomez-Sanchez et al first reported that icv infusions of benzamil prevent mineralocorticoid-induced hypertension in Wistar rats and salt-induced hypertension in Dahl S rats. Nishimura et al demonstrated that icv benzamil normalizes BP in deoxycorticosterone acetate (DOCA)-salt hypertensive rats. Recently, we showed that icv infusion of benzamil markedly decreases renal sympathetic nerve activity, BP, and HR in a dose-related manner in SHR on high salt intake. These antihypertensive effects of benzamil were indeed mediated by central actions because systemic administration at the doses used for icv infusion had no effect on blood pressure. Consistent with the previous study by Gomez-Sanchez et al, the present study shows that icv infusions of benzamil can fully prevent the development of hypertension in Dahl S rats on high salt intake. The pattern of cardiovascular responses in Dahl S rats on high salt intake, such as sympathetic hyperactivity, impairment of baroreflex function, and hypertension, can be mimicked by chronic increases in CSF Na⁺ in Dahl S rats. The sodium-induced sympathetic hyperactivity and hypertension can be prevented by blockade of brain “ouabain” with antibody Fab fragments, which bind ouabain and brain “ouabain” with high affinity. Based on these and the present findings, we postulate that, in Dahl S rats on high salt diet, enhanced Na⁺ entry through benzamil-blockable brain sodium channels increases brain “ouabain” release (and content) and the increased “ouabain” increases sympathetic outflow and BP. In genetically salt-sensitive rats, high salt intake may lead to enhanced Na⁺ entry compared with salt-resistant strains because of (larger) increases in CSF Na⁺ and/or changes in the properties of the sodium channels, eg, by enhanced release of FMRFamide. In genetic models of salt-sensitive hypertension (Dahl S and SHR), development of hypertension on high salt intake appears to critically depend on central mechanisms involving benzamil-blockable ion channels and brain “ouabain.”

In summary, the present results indicate that, in Dahl S rats on high salt intake, benzamil-blockable brain sodium chan-

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**Figure 3.** Relationship between hypothalamic (A) and pituitary (B) “ouabain” levels and mean arterial pressure (MAP) in Dahl S rats on high salt intake and icv vehicle (○), low (△) and high (●) dose of benzamil for 4 weeks.

The “ouabain” level did not change in Dahl S rats on high salt intake for 2 weeks. However, these findings are different from our previous study reporting that plasma sodium pump inhibitors increase in Dahl S rats on high salt. Methodological differences in the method used to detect “ouabain” likely explain these discrepancies. In our previous study, “ouabain” levels in Dahl S rats were quantified by an assay for Na⁺-,K⁺-ATPase enzymatic inhibitory activity. The “ouabain-like” activity measured likely included other Na⁺-,K⁺-ATPase inhibitors, such as marinobufagenin-like factor, proscllaridin A, and bufodienolides. Indeed, the marinobufagenin-like factor was found to increase markedly in plasma and urine of Dahl S rats with either acute or chronic sodium loading.

In contrast to peripheral “ouabain,” high salt intake clearly increased hypothalamic and pituitary “ouabain” contents in Dahl S rats after both 2 and 4 weeks. Both hypothalamic and pituitary “ouabain” showed significant positive correlations with BP after the development of hypertension. In mammals, “ouabain” may have more than one origin, ie, the adrenal cortex, as well as the hypothalamus. The regulation of central and peripheral “ouabain” may involve different stimuli and pathways. The present study and the study by Fedorova et al show that, in Dahl S rats, high salt intake does not affect peripheral “ouabain” and only regulates brain “ouabain” production.

Increases in brain “ouabain” in Dahl S rats on high salt intake were inhibited by chronic icv infusion of benzamil in a dose-related manner. Benzamil is an amiloride analog that inhibits epithelial sodium channels more specifically than amiloride; at least 10 times higher concentrations are required to inhibit Na⁺/H⁺ exchange channels. The peptide FMRFamide enhances the sympathoexcitatory and pressor responses to CSF Na⁺ in normotensive rats presumably by enhancing Na⁺ entry through sodium channels in the brain. Benzamil blocks responses to both CSF Na⁺ and FMRFamide, but not to ouabain. Blockade of brain “ouabain” by specific Fab fragments also blocks responses to CSF Na⁺ and FMRFamide. The present study shows that icv benzamil also inhibits the increase in brain “ouabain” content in Dahl S rats on high salt intake. One may speculate that benzamil-blockable sodium channels may function as a sensor of CSF Na⁺ and that acute or chronic enhanced Na⁺ entry leads to increases in “ouabain” release and content, ie, open brain benzamil-blockable sodium channels are essential for increased release of “ouabain,” and, if persisting, also cause increases in “ouabain” content in the hypothalamus and pituitary.

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In summary, the present results indicate that, in Dahl S rats on high salt intake, benzamil-blockable brain sodium chan-

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nels mediate increases in brain “ouabain” and the subsequent hypertension. Increases in brain “ouabain” are not associated with parallel increases in peripheral “ouabain,” which suggests distinct regulatory mechanisms in the CNS versus the periphery and a paramount role for brain rather than circulatory “ouabain” in salt-sensitive hypertension in Dahl S rats.

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