NO generation and action during changes in salt intake: roles of nNOS and macula densa

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Wilcox, Christopher S.; Xiaolin Deng; and William J. Welch. NO generation and action during changes in salt intake: roles of nNOS and macula densa. Am. J. Physiol. 274 (Regulatory Integrative Comp. Physiol. 43): R1588–R1593, 1998.—Micropuncture studies of single nephrons have shown that macula densa solute reabsorption via a furosemide-sensitive pathway activates nitric oxide (NO) generation via neuronal NO synthase (nNOS). This pathway is enhanced during salt loading. We investigated the hypothesis that changes in NO generation via nNOS in the macula densa contribute to changes in whole kidney NO generation and action during alterations in salt intake. Groups of rats (n = 6–10) were equilibrated to high-salt (HS) or low-salt (LS) diets and were administered a vehicle (Veh), 7-nitroindazole (7-NI; a relatively selective inhibitor of nNOS), or furosemide (F; an inhibitor of macula densa solute reabsorption) with volume replacement. Compared with LS, excretion of the NO metabolites, NO$_2$ plus NO$_3$ (NOX) was increased during HS (LS: 9.0 ± 0.5 vs. HS: 15.7 ± 0.8 µmol/24 h; P < 0.001), but this difference was prevented by 7-NI (LS: 7.4 ± 1.3 vs. HS: 9.4 ± 1.6 µmol/24 h; NS). During nonselective blockade of NOS with N$^G$-nitro-L-arginine methyl ester (L-NAME), renal vascular resistance (RVR) increased more in HS than LS (HS: +160 ± 17 vs. LS: +83 ± 10% P < 0.001). This difference in response to nonselective NOS inhibition was prevented by pretreatment with 7-NI (HS: +28 ± 6 vs. LS: +34 ± 8% NS) or F with volume replacement (HS: +79 ± 11 vs. LS: +62 ± 4% NS). In conclusion, compared with salt restriction, HS intake increases NO generation and renal action that depend on nNOS and macula densa solute reabsorption.

Differently isoforms of nitric oxide synthase (NOS) are expressed in the kidney in specific segments of the vasculature or nephron where they have discrete physiological functions (24). NOS metabolizes L-arginine to nitric oxide (NO), whose actions in the kidney include vasodilatation of afferent and efferent arterioles and enhancement of the glomerular ultrafiltration coefficient (7), blunting of the tubuloglomerular feedback (TGF)-induced constriction of the afferent arteriole (30), and inhibition of solute absorption in the proximal (6) and distal nephron (21). These actions generally seem appropriate to adapt renal functions to high salt intake, although effects of NO on renin release are complex and controversial (3, 4, 10, 20). Indeed, both a short-term saline infusion (19) and long-term dietary NaCl loading (9) increase renal NO generation, as reflected by increased plasma levels or excretion of the NO metabolites, NO$_2$ plus NO$_3$ (NOX), or the NO messenger compound, cGMP, or increased renal vasodilation during nonselective blockade of NOS with N$^G$-nitro-L-arginine methyl ester (L-NAME). However, the contribution of individual NOS isoforms to these apparent changes in renal NO generation or action with salt intake is currently unclear.

A constitutive, neuronal NOS (nNOS), is heavily expressed in the macula densa cells, where it functions to blunt the expression of the TGF response (30) and to regulate renin secretion (3, 10). Immunocytochemical studies have shown that some nNOS is also expressed in Bowman's capsule, some cells of the thick ascending limb or collecting ducts, efferent arteriole cells (2, 24, 25), and certain renal nerves (2), though not so prominently as in the macula densa. The mRNA for nNOS is expressed predominantly in the macula densa and cortical collecting ducts (20). In single-nephron studies, NO generation in the macula densa is dependent on solute reabsorption, since enhancement of TGF by local micropuncture of NOS inhibitors into the macula densa segment is prevented by coperfusion with furosemide, which blocks macula densa reabsorption via the Na-2Cl-K cotransport process (30). Recently, Brand-Schieber et al. (5) have shown that increases in renal vascular resistance (RVR) induced by nonselective blockade of NOS with N$^G$-nitro-L-arginine (L-NAME) are blunted by blockade of macula densa function with furosemide and volume replacement. They concluded that basal release of NO by the macula densa contributes to maintenance of RVR in the anesthetized rat.

The present studies were designed to test the hypothesis that increased NO generation and action in the kidney during HS intake entails L-arginine metabolism by nNOS and macula densa solute reabsorption via a furosemide-sensitive pathway. This was investigated by contrasting responses of rats adapted to low or high salt intake. Whole animal NO generation was assessed from NOX excretion and the functional role of NO in the kidney from the rise in RVR during infusion of a maximal dose of L-NAME. The role of nNOS was assessed from the response to the relatively specific inhibitor, 7-nitroindazole (7-NI) (1, 14–16), and the role of macula densa solute reabsorption from the effects of furosemide plus volume replacement (5).

**METHODS**

Animal preparation. Male Sprague-Dawley rats (250–350 g) were maintained on standard rat diet (Na content 0.3 g/100 g; Rodent Laboratory Chow 5001, Ralston-Purina, St. Louis, MO) until 8–10 days before study, when they received a special diet with a low-salt (LS) content (Na content 0.03 g/100 g; Teklad, Madison, WI) or the same chow with a high-salt (HS) content (Na content 2.4 g/100 g). During the last 2 days of LS or HS diets, the rats were housed in individual metabolism cages that were thoroughly cleaned daily. For the last 24-h period, urine was collected into a
container with penicillin G (2,000 IU, Bristol-Myers Squibb, Princeton, NJ) and streptomycin (2,000 IU, Sigma) to inhibit bacterial growth. Thereafter, the urine was centrifuged, and an aliquot was stored at ~70°C.

On the experimental day, rats were anesthetized with an intraperitoneal injection of 5-sec-butyl-2-thiobarbituric acid (Inactin, 100 mg/kg; Research Biochemicals International, Natick, MA) and maintained at 37°C on a servo-controlled, heated operating table. Both jugular veins were cannulated; one cannula was used to infuse albumin (6 g/day, Sigma) dissolved in 0.154 M NaCl solution (for HS rats) or 5 g/100 g dextrose solution (for LS rats) at 2 ml/h, and the other was used to deliver drugs. The left carotid artery was cannulated for blood sampling and recording of the mean arterial pressure (MAP) from the electrically damped output of a pressure transducer (Statham model P23, Gould, Oxnard, CA). The abdomen was opened by a midline incision. The left renal artery was cleaned, and a transit time blood flow probe placed around it and connected to a blood flowmeter (Transonic Systems, Ithaca, NY).

After 30 min for equilibration, baseline measurements of MAP and renal blood flow (RBF) were made at 5-min intervals for 15 min. These values were averaged for the basal data. Thereafter, rats received an intravenous infusion of L-NAME (Sigma) at 11.1 µmol·kg⁻¹·min⁻¹ for 20 min. At this time, MAP and RBF were recorded over a 5-min interval for the experimental period. This dose of L-NAME was selected because it produces maximal and stable increases in MAP and RVR that are greater in HS than LS rats (9). The techniques for measurement of RBF with the transit time blood flow probe have been validated in our laboratory (6).

Experimental protocols. There were three groups of LS and HS rats. Rats of group 1 (n = 19) served as a control group. They received intraperitoneal injections of saline vehicle (Veh) at 0 and 12 h during the 24-h period of urine collection and a further injection of vehicle during preparation for surgery on the experimental day. Rats of group 2 (n = 18) received intraperitoneal injections of 7-NI (30 mg/kg) at 0 and 12 h during the 24-h urine collection and a similar injection with anesthesia on the experimental day. This dose of 7-NI was selected, since it produces substantial inhibition of nNOS within 30 min (1, 14, 16, 31) without altering blood pressure (16) or the vasodilator response to acetylcholine (16). In the rat, the effects of 30 mg/kg of 7-NI given intraperitoneally on nNOS are maximal within 30 min, but NOs activity remains >60% inhibited over 3 h and returns to baseline between 4 and 24 h (14). Rats of group 3 (n = 16) were studied only on the experimental day. They received an intravenous injection of furosemide (F; 15 mg/kg) and a maintenance infusion (7 mg·kg⁻¹·h⁻¹). Urinary losses were replaced with a solution whose composition matched urine output during furosemide diuresis (mM: 100 Na, 20 K, and 120 Cl). The rate of intravenous infusion of this solution was adjusted each 10 min to correspond to the rate of urine flow during the prior 10 min. With this protocol, steady values for MAP and RBF could be maintained, and values for hematocrit recorded at the end of the study were not significantly different from the vehicle-treated control group (Veh: 46 ± 1% vs. F + volume replacement: 48 ± 1%; NS).

At the completion of the experiments, rats were killed, and the experimental kidney was removed and weighed.

Chemical methods. NOx was measured by chemiluminescence (Sievers Instruments, Boulder, CO). N02 was catalytically converted to NO3, from which NO was evolved under acid hydrolysis and reacted with O3, which yields photons that were measured in a photomultiplier tube.

Drugs. L-NAME was obtained from Sigma and dissolved in 0.154 M NaCl solution. The Na salt of 7-NI was obtained from Cayman Chemical (Ann Arbor, MI) and dissolved in 0.154 M NaCl. Furosemide was obtained from Astra Pharmaceutical Products (Westborough, MA).

Statistical methods. Data are presented as means ± SE. Some of the perturbations altered the baseline data, and therefore percent changes in response to L-NAME were analyzed. We had previously found that although absolute changes in RVR with L-NAME during LS and HS intakes were dependent on the baseline values of RVR, percent changes were not significantly dependent (9). Therefore, according to the criteria of Kaiser (13), percent changes were analyzed. Between-groups analyses were performed by using ANOVA to assess the effects of salt intake, the effects of pretreatments, and the interaction (i.e., effects of drug pretreatment on response to salt). Where appropriate, post hoc Dunnett's t tests were applied after ANOVA. Within-group analyses were performed, when appropriate, using the paired Student's t-test. Results are considered significant at P < 0.05.

RESULTS

As shown in Table 1, rats of each group had a similar body and experimental kidney weight.

As shown in Table 2, the basal values for NOx excretion in rats adapted to diets with an HS content were about twice as high as those in rats adapted to diets with an LS content. However, during administration of 7-NI to rats of group 2, there was a significant (P < 0.01) reduction in NOx excretion during HS but no significant change during LS. During 7-NI, NOx excretion was no longer significantly different between rats on the two levels of salt intake.

The functional data recorded under anesthesia on the experimental day and the response to L-NAME are shown in Table 3 and Fig. 1. In the basal state, there were no significant differences in the vehicle-treated rats between those receiving LS or HS diets for MAP, RBF, or RVR. For reasons that are not clear, the MAP and RBF seemed rather higher in group 1 whereas those receiving F plus volume replacement (15 mg/kg and 7 mg·kg⁻¹·h⁻¹) during HS intake, whereas those receiving F plus volume replacement had significantly lower values for RBF and higher values for RVR during HS intake, whereas those receiving F plus volume replacement had significantly lower values for MAP and RBF during HS. L-NAME infusion increased the MAP and reduced the RBF of each group (Table 3). In the vehicle-treated animals of group 1, the percent increases in MAP and RVR and decreases in RBF were not statistically different from the vehicle-treated control group (Veh: 46 ± 1% vs. F + volume replacement: 48 ± 1%; NS).

<table>
<thead>
<tr>
<th>Table 1. Body and kidney weights of study groups</th>
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<tbody>
<tr>
<td>Group</td>
</tr>
<tr>
<td>-------</td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td>2</td>
</tr>
<tr>
<td>3</td>
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</tr>
</tbody>
</table>

Values are means ± SE. LS, low-salt intake; HS, high-salt intake; Veh, vehicle; 7-NI, 7-nitroindazole (30 mg/kg ip x 3); F, furosemide + volume replacement (15 mg/kg and 7 mg·kg⁻¹·h⁻¹). There were no significant differences between the groups.
leads to vasoconstriction of the afferent arteriole during potentiates the TGF response (26). TGF activation HS intakes: effects of 7-NI

During F, the L-NAME-induced increase in MAP and reductions in RBF, were all blunted during both levels of salt intake, though to a greater extent during HS. 

Blunting of TGF during HS entails enhanced NO generation via macula densa nNOS. We confirmed previous findings that HS intake enhances excretion of NOx (19). This presumably represents a response to dietary NaCl, since the diets were identical apart from salt intake, and growth of the rats was similar during LS and HS intake, suggesting similar food intakes. Moreover, increased NOx excretion during salt loading is paralleled by increased reabsorption of solute from the macula densa. Enhancement of TGF during microperfusion of NOS inhibitors into the macula densa appears to represent predominantly the response to the inhibition of nNOS in the macula densa rather than endothelial cell NOS (ecNOS) in the afferent arteriolar endothelium. Thus these responses are prevented by inhibition of macula densa solute reabsorption by infusions of cGMP (30), and the response to L-NMMA occurs within 10 s of perfusion of the macula densa, which is presumably too rapid to be a consequence of L-NMMA reabsorption and diffusion through the vessel wall to the site of ecNOS expression on the vascular endothelium (26).

Using a rabbit isolated juxtaglomerular apparatus preparation, Ito and Ren (12) showed that luminal perfusion of the macula densa with L-NAME reduced the diameter of the attached afferent arteriole only if macula densa Na reabsorption was intact. These authors also reported that the endothelium-dependent vasodilator responses to acetylcholine perfused into the macula densa were intact during luminal perfusion of the macula densa with L-NAME. Finally, the relatively selective nNOS inhibitor, 7-NI, is as effective as L-NNA or L-NMMA in enhancing TGF responses when perfused into the macula densa (22, 26).

The TGF response limits renal vasodilation, glomerular filtration, and Na excretion. Therefore blunting of TGF during a HS intake could be an important homeostatic response. Moreover, this response is defective in a rat model of salt-sensitive hypertension (29). Microperfusion of L-NMMA or 7-NI into the macula densa elicits a greater enhancement of TGF during HS than LS intake. Indeed, during LS intake, these NOS inhibitors are not effective (26, 29). These data suggest that blunting of TGF during HS entails enhanced NO generation via macula densa nNOS.

Table 2. Daily excretion of NO2 + NO3 during LS or HS intakes: effects of 7-NI

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Before drug</th>
<th>During drug</th>
<th>P value (before vs. during)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>9</td>
<td>9.0 ± 0.5</td>
<td>15.7 ± 0.8</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>LS</td>
<td>10</td>
<td>7.5 ± 0.6</td>
<td>7.4 ± 1.3</td>
<td>NS</td>
</tr>
<tr>
<td>HS</td>
<td>6</td>
<td>15.4 ± 1.7</td>
<td>9.4 ± 1.6</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>P value</td>
<td>&lt;0.001</td>
<td>NS</td>
<td></td>
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</table>

Values are means ± SE (n, no. of rats) recorded after 5–7 days of LS or HS intake (before drug) and after administration of 7-NI (30 mg/kg ip × 2 over 24 h).

Table 3. Mean arterial pressure, renal blood flow, and renal vascular resistance: basal values during pretreatment and changes with L-NAME

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Salt Intake</th>
<th>MAP, mmHg</th>
<th>RBF, ml·min⁻¹·g⁻¹</th>
<th>RVR, mmHg·ml⁻¹·min⁻¹·g⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Veh</td>
<td>LS</td>
<td>125 ± 5</td>
<td>+27 ± 4</td>
<td>7.5 ± 0.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HS</td>
<td>123 ± 5</td>
<td>+38 ± 4</td>
<td>8.1 ± 0.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>P</td>
<td>NS</td>
<td>&gt;0.05</td>
<td>NS</td>
</tr>
<tr>
<td>2</td>
<td>7-NI</td>
<td>LS</td>
<td>118 ± 4</td>
<td>+14 ± 3*</td>
<td>6.6 ± 0.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HS</td>
<td>107 ± 6</td>
<td>+11 ± 3</td>
<td>5.9 ± 0.4†</td>
</tr>
<tr>
<td></td>
<td></td>
<td>P</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>3</td>
<td>F</td>
<td>LS</td>
<td>113 ± 3</td>
<td>+25 ± 1</td>
<td>7.3 ± 0.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HS</td>
<td>102 ± 4†</td>
<td>+22 ± 2†</td>
<td>6.6 ± 0.3†</td>
</tr>
<tr>
<td></td>
<td></td>
<td>P</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

Values are means ± SE recorded in basal state during pretreatment schedule and absolute changes (Δ) during infusion of L-NAME (11.11 µmol·kg⁻¹·min⁻¹ for 20 min). MAP, mean arterial pressure; RBF, renal blood flow; RVR, renal vascular resistance. Compared with Veh: *P < 0.05; †P < 0.01; ‡P < 0.005.

DISCUSSION

The main new findings of this study are that administration of 7-NI to block nNOS reverses the salt-induced increase in NOx excretion and that administration of 7-NI or furosemide plus volume replacement both prevent the salt-induced enhancement of renal vasoconstriction with L-NAME.

Nine studies have shown that inhibition of NOS in the juxtaglomerular apparatus by microperfusion of NG-monomethyl-L-arginine (L-NMMA), L-NNA, or 7-NI potentiates the TGF response (26). TGF activation leads to vasoconstriction of the afferent arteriole during...
plasma NOX concentration and increased excretion of the NO expression indicator, cGMP (19). During 24 h of 7-NI administration, NOX excretion was reduced by 50% in rats adapted to HS intake but was unaffected in those adapted to LS. During 7-NI, there was no longer a difference in NOX excretion with salt intake, which suggests that much of the increase in NO generation during HS intake can be ascribed to NO generated via nNOS.

Evidence that RVR is modulated differentially by nNOS during HS intake derived from the functional studies. During 7-NI pretreatment, there was a decrease in basal RBF and an increase in basal RVR that were significant only during HS. During furosemide administration plus volume replacement, there was also a reduction in basal RBF, but there were no changes in basal RVR because of an equivalent fall in basal MAP. These data are consistent with the hypothesis that NO generated via nNOS is more important in maintaining renal vasodilation during salt loading.

Recently, Beierwaltes (4) has reported that administration of 7-NI to salt-restricted rats reduces MAP by 5% and RBF by 8%. Very similar effects were seen in this study, but the changes were not significant during LS. This suggests that 7-NI may also have a renal vasoconstricting action during LS intake, though less marked in this study than during HS intake. The study of Beierwaltes (4) indicates that any increase in RVR with 7-NI is unlikely to be secondary to ANG II, since renin secretion was unchanged or reduced by 7-NI, especially during LS.

The results of the vehicle-pretreated group confirm our previous observations that, during nonspecific inhibition of NOS with L-NAME, there is a greater rise in MAP and RVR and a greater fall in RBF in rats adapted to HS compared with LS (9). The increased RVR response to L-NAME during HS probably reflects increased NO generation more than increased NO response, since there were corresponding increases in NOX excretion and, in the macula densa, salt loading does not alter the TGF response to the NO donor compound, SIN-1 (26). We argued that if nNOS were responsible for this differential functional response to L-NAME with salt intake, it should be abolished in animals pretreated with 7-NI. In general, the results support this hypothesis, since, after 7-NI, there was no longer a significant difference in the L-NAME-induced increase in RVR or reduction in RBF in rats at the different levels of salt intake. However, 7-NI pretreatment did blunt the fall in RBF and the increase in RVR in response to L-NAME, even in rats adapted to LS intake, suggesting that nNOS may contribute to whole kidney hemodynamics, even in the absence of salt loading, though not to the same extent as in HS.

It was interesting to note that 7-NI did not raise blood pressure. Indeed, the levels of MAP in the 7-NI-pretreated rats tended to be lower than the vehicle-pretreated controls (Fig. 1). The pressor response to nonselective NOS inhibition in the dog has been related to neural mechanisms, since it is attenuated by ganglionic blockade (23). However, the response to L-NMMA in the rat is not altered by ganglionic blockade or pithing (18). Short-term administration of L-NAME to anesthetized rats reduces sympathetic nerve traffic, although it increases if the baroreflex is interrupted (11). One week of administration of 7-NI increases MAP and TGF in the rat (17). These data indicate the
induced differences in RVR during NOS blockade with macula densa function with furosemide prevented salt—where nNOS is heavily expressed, since interruption of that much of it may derive from the macula densa,
the glomerulus, some thick ascending limb cells, and expressed at the efferent arteriole, Bowman's capsule of nNOS. Probably some of this originates from nNOS of the total NO generated during HS derives from in the present study during HS suggests that about half in MAP and RVR and the decrease in RBF with L-NAME.
Because these might mediate the physiological state characteristic of HS and LS intake. Nonetheless, furosemide with volume replacement blunted the increase in MAP and RVR and the decrease in RBF with L-NAME in the HS rats, which confirms the results of Brand-Schieber et al. (5) in normal salt rats. These effects were not seen in LS rats, which suggests that macula densa NO generation maintains renal vasodilation during normal or salt-loaded conditions but has less effect during LS intake.

In conclusion, NO generation via nNOS is enhanced during HS. HS also enhances NO—dependent renal vasodilation that depends on nNOS and reabsorption via a furosemide—sensitive transport process, probably in the macula densa. These findings highlight the importance of nNOS and macula densa in salt—dependent changes in NO generation and action but do not exclude roles for other NO isozymes.

Perspectives

The 50% reduction in NOX excretion with 7-NI found in the present study during HS suggests that about half of the total NO generated during HS derives from nNOS. Probably some of this originates from nNOS expressed at the effenter arteriole, Bowman's capsule of the glomerulus, some thick ascending limb cells, and other tubular sites (2). Some may also derive from nitrergic neurons and from the brain. Our data suggest that much of it may derive from the macula densa, where nNOS is heavily expressed, since interruption of macula densa function with furosemide prevented salt—induced differences in RVR during NOS blockade with L—NAME.

However, this study provides no direct evidence for the source of the enhanced NO generated during HS intake. Indeed, the expression of nNOS mRNA and protein in the renal cortex and macula densa is actually reversed by selective inhibition of neuronal nitric oxide synthase. Therefore, changes in macula densa NO expression during salt intake may be a response to a local change in NO generation rather than a cause of the change. We have recently shown that a TGF response to local microperfusion of 7-NI into the macula densa can be restored in rats adapted to LS intakes by local microperfusion of L-arginine into the efferent arteriole supplying that nephron's peritubular capillaries (27). Moreover, L-arginine uptake into cells of the loop of Henle via system Y + is enhanced by an HS intake (27). Therefore changes in macula densa NO generation with salt intake may relate more to factors that regulate L-arginine delivery and uptake rather than to nNOS expression.


