Salt-sensitive splice variant of nNOS expressed in the macula densa cells

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Lu D, Fu Y, Lopez-Ruiz A, Zhang R, Juncos R, Liu H, Manning RD Jr, Juncos LA, Liu R. Salt-sensitive splice variant of nNOS expressed in the macula densa cells. Am J Physiol Renal Physiol 298: F1465–F1471, 2010. First published March 24, 2010; doi:10.1152/ajprenal.00650.2009.—Neuronal nitric oxide synthase (nNOS), which is abundantly expressed in the macula densa cells, attenuates tubuloglomerular feedback (TGF). We hypothesize that splice variants of nNOS are expressed in the macula densa, and nNOS-β is a salt-sensitive isoform that modulates TGF. Sprague-Dawley rats received a low-, normal-, or high-salt diet for 10 days and levels of the nNOS-α, nNOS-β, and nNOS-γ were measured in the macula densa cells isolated with laser capture microdissection. Three splice variants of nNOS, α-, β-, and γ-mRNAs, were detected in the macula densa cells. After 10 days of high-salt intake, nNOS-α decreased markedly, whereas nNOS-β increased two- to threefold in the macula densa measured with real-time PCR and in the renal cortex measured with Western blot. NO production in the macula densa was measured in the perfused thick ascending limb with a fluorescent dye DAF-FM. When the tubular perfusate was switched from 10 to 80 mM NaCl, a maneuver to induce TGF, NO production by the macula densa was increased by 38 ± 3% in normal-salt rats and 52 ± 6% (P < 0.05) in the high-salt group. We found 1) macula densa cells express nNOS-α, nNOS-β, and nNOS-γ, 2) a high-salt diet enhances nNOS-β, and 3) TGF-induced NO generation from macula densa is enhanced in high-salt diet possibly from nNOS-β. In conclusion, we found that the splice variants of nNOS expressed in macula densa cells were α-, β-, and γ-isoforms and that enhanced level of nNOS-β during high-salt intake may contribute to macula densa NO production and help attenuate TGF.

nitric oxide; tubuloglomerular feedback; high-salt diet

MACULA Densa cells serve as a distal nephron sensor that detects changes in tubular fluid composition and transmits information to afferent arteriolar smooth muscle cells [tubuloglomerular feedback (TGF)] and renin-containing granular cells (21). Nitric oxide (NO) is one of the most important factors that regulate TGF. This NO, which sets the sensitivity of the TGF system, is mainly generated by neuronal NO synthase (nNOS) that is abundantly expressed in the macula densa cells (20, 41). Expression of nNOS in the macula densa is modulated by salt intake; a high-salt diet decreases nNOS expression, whereas a low-salt diet increases it (3, 29, 33). However, this pattern of expression of nNOS is contrary to what one would expect, because NO activity is increased, rather than decreased, during a high-salt diet (8, 26, 28, 35). Indeed, increasing either salt intake or delivery to the macula densa elevates macula densa NO levels and attenuates TGF in vivo and in vitro (16, 18, 38). Although the reasons for this discrepancy between NO activity and expression of nNOS are not known, several possibilities exist including 1) increased activity of the nNOS enzyme, 2) an alternative source of NO, and 3) the presence of distinct splice variants of nNOS that might not all be detected by the methods used in the previous studies.

Alternate splicing can produce several nNOS mRNA variants and protein isoforms, such as nNOS-α, nNOS-β, nNOS-γ, and nNOS-μ (the latter is only expressed in myocytes) (4, 9, 14, 36). nNOS-β, which normally has a low level in the kidney, becomes more abundantly expressed during renal injury (30). Because nNOS-β has similar activity to that of nNOS-α (it has 82% the activity of nNOS-α) (4, 14), net nNOS activity will depend on the sum of the two isoforms, and thus an increase in nNOS-β can significantly increase NO activity, even in the setting of decreased nNOS-α. Given that the antibodies used in several of the previous studies to assess nNOS expression may have only detected nNOS-α and not the other isoforms, it is possible that the discrepancy between nNOS activity and level during high-salt intake may be due to an increase in nNOS-β isoforms. Therefore, it is important to determine the level of the different nNOS splice variants in the kidney and macula densa, and their changes during diverse physiologic conditions such as during changes in sodium intake, to understand their role in regulating TGF and kidney function. In the present study, we hypothesized that dietary salt causes differential level of nNOS splice variants in the macula densa, which in turn modulates TGF. Specifically, we tested whether a high-salt diet increases the nNOS-β, which contributes to the enhanced NO production and blunted TGF observed during salt loading.

METHODS

Animals

All procedures and experiments were approved by the Institutional Animal Care and Use Committee at the University of Mississippi Medical Center. Male Sprague-Dawley (SD) rats, aged 8–10 wk, were divided into three groups: group 1 was maintained on a normal-salt diet (0.3% NaCl) with tap water (n = 5), group 2 received a low-salt diet (0.03% NaCl) with tap water (n = 5), and group 3 was fed a high-salt diet (8% NaCl) with 0.45% NaCl in their drinking water (n = 5). After 10 days, the rats were anesthetized with ketamine (50 mg/kg ip) and xylazine (50 mg/kg ip) and the kidneys were removed. We separated the cortex and medulla for total RNA from one kidney. The other kidney was used for laser capture microdissection (LCM).

Assessment of nNOS Isoforms in the Renal Cortex and Macula Densa

LCM. LCM was used to isolate macula densa cells as we previously described (42). Briefly, kidneys from SD rats were removed and...
The kidneys were removed and sliced along the corticomedullary axis. A single superficial intact glomerulus was microdissected together with adherent tubular segments consisting of portions of the TAL, macula densa, and early distal tubule. Using a micropipette, the sample was transferred to a temperature-regulated chamber mounted on an inverted microscope (Nikon). The TAL was cannulated with an array of glass pipettes. The macula densa was perfused with physiologic saline consisting of (in mM) 10 HEPES, 1.0 CaCl₂, 0.5 K₂HPO₄, 4.0 KHCO₃, 1.2 MgSO₄, 5.5 glucose, 0.5 Na acetate, 0.5 Na lactate, and either 80 or 10 mM NaCl. The pH of the solution was 7.4. Mannitol was used to match the osmolality of the low-salt solution with the high-salt solution (180 mosmol/kg H₂O). The bath consisted of MEM (containing 1.8 CaCl₂, 0.814 MgSO₄; 5.33 KCl, 127.24 NaCl, 1.01 NaH₂PO₄; 5 NaHCO₃; 5.56 glucose; 0.28 l-arginine, 10 HEPES, 10 mM NaOH plus different amino acids and vitamins) and was exchanged continuously at a rate of 1 ml/min. The imaging system consisted of a digital CCD camera, xenon light (Shutter Instruments), and optical filter changer (Shutter Instruments). Images were captured and analyzed with NIS Elements imaging software (Nikon).

Measurement of NO in isolated perfused macula densa. We measured NO production in macula densa cells using a cell-permeable fluorescent NO indicator 4-aminomethylaminono-2,7’-difluorofluorescein diacetate (DAF-FM) as described previously (17, 18). Briefly, the macula densa cells were loaded with 10 μM DAF-FM in 0.5% dimethyl sulfoxide plus 0.1% pluronic acid from the tubular lumen for 30 to 40 min and then washed for 10 min. DAF was excited at 490 nm with a xenon light, and the emitted fluorescence was recorded at wavelengths of 510 to 550 nm. Square-shaped regions of interest (ROIs) were set inside the cytoplasm of macula densa cells and mean intensity within the ROIs was recorded every 5 s for 5 min. NO production was calculated based on the percentage changes to the basal fluorescence intensity of DAF.

Statistics

Data are expressed as means ± SE. Data were analyzed using either a t-test or ANOVA for repeated measures with post hoc analysis using the Fisher LSD method. Data were considered to be significantly different if P < 0.05.

RESULTS

nNOS Splice Variants in the Macula Densa

We first determined whether the macula densa cells isolated with LCM expressed splice variants of nNOS. As shown in Fig. 1, macula densa cells were easily identified by their anatomic location and morphology. As shown in Fig. 2, the macula densa of normal SD rats expressed all three splice variants; nNOS-α was detected at 534 bp, nNOS-β at 480 bp, and nNOS-γ at 160 bp. To confirm that the amplified products were in actual fact nNOS-α, nNOS-β, and nNOS-γ, we purified the PCR products with a Qiagen Purified Kit and then sent them to Seqwright for sequencing. The sequences of the PCR products provided additional support that these were α-, β-, and γ-splice variants of nNOS (see supplemental data; the online version of this article contains supplemental data).

Effect of Dietary Salt on nNOS Splice Variants of the Macula Densa

We investigated whether salt intake altered the level of the nNOS splice variants. For this, we first measured nNOS level, using real-time PCR, in the macula densa isolated with LCM of SD rats fed either a normal-, high-, or low-salt diet for 10 days (Fig. 3). As shown in Fig. 3, the high-salt diet had a modest
effect on nNOS-α level (it decreased it to $0.39 \pm 0.08$ U), whereas it greatly increased nNOS-β (it increased almost 3-fold; $2.94 \pm 0.17$ U). On the other hand, the low-salt diet increased the nNOS-α ($2.40 \pm 0.11$ U; $n = 5$) but had no significant effect on nNOS-β. No changes were observed in levels of nNOS-γ during low and salt intake (data not shown).

Next, we determined whether salt intake affected the protein levels of nNOS splice variants in the renal cortex. We used COOH-terminal nNOS antibodies to detect all isoforms of nNOS. We detected three bands at 160, 136, and 125 kDa, corresponding to nNOS-α, -β, and -γ, respectively, with Western blot (Fig. 4). High-salt diet significantly increased expression of nNOS-β ($1.89 \pm 0.5$ U) and decreased nNOS-α ($0.72 \pm 0.2$ U; $P < 0.05$ vs. control, $n = 5$), but no significant effect on nNOS-γ ($0.84 \pm 0.1$ U).

Effect of Dietary Salt on TGF-Induced NO Production in Macula Densa

Because the high-salt diet caused reciprocal changes in the nNOS-α and nNOS-β splice variants (nNOS-β increased but nNOS-α decreased), we next investigated whether these changes in nNOS splice variant level resulted in a net change in the NO levels in the macula densa. For this, we tested whether TGF-induced changes (by acutely raising luminal NaCl from 10 to 80 mM) in NO levels in the macula densa are different in rats maintained on a high-salt diet compared with a normal-salt diet. The NO generated during the TGF response is what modulates the TGF response (16, 18, 19, 25, 27). Figure 5 shows images of a representative experiment in a rat on a normal-salt diet. As depicted by the DAF fluorescence...
NO levels increase markedly when TGF is activated by raising the luminal NaCl to 80 mM. Figure 6 shows the average data of the experiments. The acute TGF response increased the macula densa NO levels by 38% in the animals maintained on a normal-salt diet. This TGF-induced increase in NO was accentuated in the animals fed a high-salt diet; NO increased by 52% during TGF (n = 5). Taken together, our data suggest that a high-salt diet induces increases in nNOS-β, which is associated with augmented TGF-induced increases in NO.

### DISCUSSION

The principal findings of our study are 1) macula densa cells express nNOS-α, nNOS-β, and nNOS-γ, 2) a high-salt diet enhances nNOS-β, and 3) NO generation from the macula densa during TGF is enhanced during high-salt intake possibly from nNOS-β. Our findings suggest that enhanced nNOS-β during high-salt intake may contribute to macula densa NO production and help attenuate TGF.

NO production in the kidney can come from all three isoforms of NOS: endothelial, inducible, and neuronal NOSs. However, the macula densa predominantly expresses nNOS (20, 41), whose activity is enhanced during TGF (18) by elevated intracellular pH (16). Evidence of its importance in modulating macula densa function, TGF, and consequently blood pressure is provided by the findings that 1) selective blockade of nNOS in the macula densa amplifies the TGF response (13, 16, 19, 24) and induces hypertension (22) and 2) the activity of nNOS in the macula densa is impaired in several strains used in experimental models of hypertension such as Dahl salt-sensitive, Milan, and spontaneously hypertensive rats (11, 12, 37). Consequently, there has been much interest in elucidating the mechanisms that regulate nNOS activity in the macula densa. The recent identification of several splice variants of nNOS in other tissues raised the possibility that they may be important in the kidney as well. Smith et al. (30) evaluated which splice variants are present in the kidney and found that the renal cortex expresses nNOS-α and nNOS-β but not nNOS-γ.
While renal cortical nNOS is predominately expressed in the macula densa cells (20, 41), it is also found in Bowman’s capsule, proximal tubules, TAL, efferent arteriole, collecting duct, and certain renal nerves (1, 32, 34). Because the focus of our studies is on the mechanisms by which nNOS modulates TGF, our aim was to determine the splice variants expressed by the macula densa cells. We used LCM to isolate macula densa cells from rat kidney as described previously (42) and applied RT-PCR techniques to identify the splice variants expressed by these cells (due to the limitation of number of cells captured with LCM, we could not extract enough protein for Western blot analysis). We found that all three isoforms of nNOS, α, β, and γ, are expressed in the macula densa. Thus, our present study provides the first evidence that macula densa cells express the α-, β-, and γsplice variants of nNOS.

There are several lines of evidence suggesting that nNOS plays an important role in modulating renal function during high-salt intake. First, rats on a high-salt diet have higher plasma levels and increased renal excretion rates of nitrite/nitrates (8, 26, 28, 35) and increased cGMP levels (28), suggesting that NO activity is higher during high-NaCl intake. Second, we recently found that acutely increasing NaCl concentration enhances NO production by the macula densa (16, 18). Third, inhibiting nNOS in the macula densa in vitro with l-NMMA or 7-nitroindazole (7-NI) augments TGF responses to a greater extent in animals on a high-salt diet (38, 40). Moreover, NOS inhibitors have a greater effect on renal blood flow (RBF), glomerular filtration rate (GFR), and renal vascular resistance in animals fed a high-salt diet (6, 28, 35). Because many of these effects are blocked by 7-NI, a selective inhibitor of nNOS, they are likely due to nNOS (39). Finally, there is evidence in normal and hypertensive humans that compared with low-salt intake, a high-salt diet is associated with an elevation in GFR, RBF, sodium and cGMP excretion (2, 23) and that these effects were significantly enhanced following l-arginine administration, suggesting that they may be due to the increased NO production, including NO in the macula densa. All of these data suggest that chronic NaCl challenges at the macula densa enhance NO generation. However, the level of nNOS at the macula densa in these conditions is in the opposite direction; that is, protein and mRNA level of nNOS decreases (3, 29, 33). The results of our present study may provide an answer for the above conflicting data. Many of the previous studies may utilize NH2-terminal antibodies against nNOS, but these would likely only identify the nNOS-α splice variant (4, 14), which decreases during a high-salt diet. Our study suggests that the increase in the nNOS-β splice variant may account for the increase in NO generation during salt loading. This contention assumes that the nNOS-β splice variant has NOS activity, which is supported by several lines of evidence. First, in vitro studies showed that the nNOS-β is catalytically active, demonstrating 80% of the full-length nNOS activity when transfected into COS cells (5). Second, nNOS-β in the brain cortex and striatum has been reported to increase two- to threefold challenges at the macula densa.
in nNOS-α knockout mice (7). Third, citrulline, which is formed by NOS stoichiometrically with NO (30), colocalized with nNOS-β (7). Finally, Hutt et al. (10) demonstrated that nNOS-β mediates a major portion of penile erection in nNOS-/- mice; they found that nNOS-α-/-, eNOS-/-, and double NOS-/- animals retain erectile function, while complete nNOS-deficient mice lacking nNOS-α, -β, and -γ do not (9, 36). Thus, nNOS-β in the macula densa is likely to be a functional enzyme just like in other tissues. Therefore, these studies taken together with ours suggest that nNOS-β may be responsible for the increase in macula densa NO generation, and the consequent blunting of TGF, and increased GFR and RBF observed during a high-salt diet. Of note, nNOS-γ did not change with any of the salt diets used and exhibits only 3% of nNOS activity in transfection studies (4); thus, it seems unlikely that this splice variant contributes much to salt-induced increases in NO generation. The sodium was given in the food and water with an objective of achieving a total sodium intake of 25 meq/day. This is equivalent to the sodium intake per grams of body weight achieved in a previous study done by one of the authors (31). The amount of sodium intake in the present study, when body weight is factored in, is equivalent to 5.8 g/day in a 70-kg human. The average human sodium intake is 3.4 g/day (data from the CDC) with a large percentage of people eating 10 g/day. Therefore, the sodium intake is high but within the range of human sodium intake.

The pathophysiologic influence of salt-induced increases in nNOS-β is not fully established but may be significant. This is because NO appears to be an important modulator of TGF during fluctuations in salt intake. This contention is supported by previous in vivo micropuncture studies that showed that inhibiting macula densa NO production, by perfusing it with L-NAME or 7-NI, enhances TGF to a greater extent during a high-salt diet compared with during low-salt intake (38, 40). In addition, acutely increasing NaCl delivery to the macula densa increases NO production from nNOS (16, 18). Thus, these data suggest that blunting of TGF during high-salt intake requires enhanced NO generation via macula densa nNOS. Our current study raises the possibility that salt-induced increases in nNOS-β may be the primary source of the increase in NO and the subsequent blunting of TGF during salt loading. Consequently, abnormalities in this mechanism may lead to abnormalities in renal salt handling and hypertension. Indeed, there is support for this notion from several studies. For instance, long-term blockade of nNOS (with 7-NI) leads to hypertension in SD rats and salt-sensitive hypertension in Dahl salt-resistant rats (22, 31), and as mentioned before, several experimental models of experimental hypertension have abnormal nNOS activity in the macula densa (11, 12, 37). Thus, nNOS-β may be an important regulator of TGF and renal hemodynamics and abnormalities in its regulation may contribute to salt-sensitive hypertension. However, further studies are needed to clarify the role of nNOS in modulating salt-induced changes in TGF because not all studies find accentuation of TGF during nNOS inhibition. For instance, Ren et al. (25) found that TGF responses were similar and were equally potentiated by 7-NI in rabbits fed a high- vs. low-salt diet. The reasons for this discrepancy are not clear, but may be due to the different species, preparations, or conditions.

In summary, the present study demonstrates the presence of nNOS-α, nNOS-β, and nNOS-γ in the macula densa cells, and specific increases in renal cortical nNOS-β during high-salt intake. The nNOS-β in the macula densa cells is likely to be a salt-sensitive isoform, thus mediating the increase in macula densa NO production observed during high-salt intake. Thus, inappropriate level and function of nNOS-β may contribute to salt-sensitive hypertension. Manipulations of the level and function of nNOS-β might be a new target for the treatment of salt-sensitive hypertension.

GRANTS

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DISCLOSURES

No conflicts of interest are declared by the authors.

REFERENCES


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