Increased intracellular pH at the macula densa activates nNOS during tubuloglomerular feedback

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Background. The macula densa senses increasing NaCl concentrations in tubular fluid and increases afferent arteriole tone by a process known as tubuloglomerular feedback (TGF). Nitric oxide (NO) production by macula densa neuronal nitric oxide synthase (nNOS) is enhanced by increasing NaCl in the macula densa lumen, and the NO thus formed inhibits TGF. Blocking apical Na+/H+ exchange with amiloride augments TGF and mimics the effect of nNOS inhibition. We hypothesized that increasing NaCl in the macula densa lumen raises macula densa intracellular pH (pHi) and activates nNOS.

Methods. The thick ascending limb and a portion of the distal tubule with intact macula densa plaque adherent to the glomerulus were microdissected and perfused. Macula densa perfusate was changed from low (10 mmol/L) to high NaCl solution (80 mmol/L) to mimic the conditions that induce TGF. Osmolality of both solutions was 180 mOsm, so that changing the solutions did not alter cell volume.

Results. Macula densa pH_i increased significantly from 7.0 ± 0.5 to 7.8 ± 0.6 when the perfusate was changed from low to high (P < 0.05; N = 5). When amiloride was added to inhibit Na+/H+ exchange, the increase in pH_i during TGF was blocked (N = 5). Fluorescence intensity of DAF-2, an NO-sensitive dye, increased by 28.8 ± 4.1% after increasing luminal NaCl (N = 5), indicating an increase in NO production. In the presence of Na+/H+ exchanger inhibitor amiloride or the nNOS inhibitor 7-NI, the increase in NO induced by switching the macula densa perfusate from low to high was blunted. To study whether changes in pH_i can directly alter NO production, we used nigericin, a K+/H+ ionophore, to equilibrate luminal and intracellular pH. When macula densa pH_i was raised from 7.3 to 7.8 in the presence of 10−5 mol/L nigericin in the low NaCl solution, fluorescence of DAF-2 in the macula densa increased by 17.9 ± 1.3% (P < 0.01; N = 5). In the presence of 7-NI, the increase in NO induced by raising pH_i was blocked (N = 5).

Conclusion. We concluded that macula densa pH_i increases during TGF, and this increase in pH_i activates nNOS.

Key words: macula densa, pH_i, nNOS, tubuloglomerular feedback.
METHODS
Isolation and microperfusion of the rabbit afferent arteriole and attached macula densa

We used methods similar to those described previously to isolate and microperfuse the thick ascending limb (TAL) of the loop of Henle and attached macula densa [15, 20–22]. Briefly, young male New Zealand white rabbits (1.5 to 2.0 kg) were anesthetized with ketamine (50 mg/kg, IM), and given an injection of heparin (500 U, IV). The kidneys were removed and sliced along the corticomedullary axis. Slices were placed in ice-cold minimum essential medium (MEM; Gibco, Grand Island, NY, USA) containing 5% bovine serum albumin (BSA; Sigma, St. Louis, MO, USA), and dissected under a stereomicroscope (SZH; Olympus, Tokyo, Japan). 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 (TE2000-S, Nikon, Yuko, Japan). The TAL was cannulated with an array of glass pipettes [15]. Another pipette was used to hold and stabilize the glomerulus. The sample was arranged so that each macula densa cell could be clearly visualized on the edge of the glomerulus. The macula densa was perfused with physiologic saline consisting of (in mmol/L) 10 HEPES, 1.0 CaCO3, 0.5 K2HPO4, 4.0 KHCO3, 1.2 MgSO4, 5.5 glucose, 0.5 Na acetate, 0.5 Na lactate, and either 80 (high NaCl) or 10 NaCl (low NaCl). The pH of the solution was 7.4. The final concentration of Na/Cl was 81/80 mmol/L in the high-NaCl solution and 11/10 mmol/L in the low-NaCl solution. We used mannitol to adjust the low-NaCl solution to the same osmolality as the high-NaCl solution (180 mOsm). The bath consisted of MEM containing 0.15% BSA and was exchanged continuously at a rate of 1 mL/min. In the TGF experiments, a single superficial Af-Art and its intact glomerulus were microdissected together with adherent tubular segments consisting of portions of the thick ascending limb of the loop of Henle, macula densa, and early distal tubule. Both the Af-Art and the end of either the distal tubule or thick ascending limb were cannulated with an array of glass pipettes as described previously [22, 23]. Microdissection and cannulation were completed within 60 minutes at 8°C, after which the bath was gradually warmed to 37°C for the rest of the experiment. Once the temperature was stable, a 30-minute equilibration period was allowed before taking any measurements. The imaging system consisted of a microscope (TE2000-S, Nikon), digital CCD camera (IEEE 1394, Hamatsu Photonics K.K., Hamatsu, Japan), and optical filter changer (Lambda 10–2, Sutter Instruments, Novato, CA, USA). Images were displayed

and analyzed with SimplePCI imaging software (Compix, Tualatin, OR, USA).

Measurement of NO

A cell-permeable fluorescent NO indicator, 4,5-diaminofluorescein diacetate (DAF-2 DA), was used to detect NO production in macula densa cells. The cells were loaded with 10 μmol/L DAF-2 DA in 0.5% dimethyl sulfoxide (DMSO) plus 0.1% pluronic acid from the tubular lumen for 30 to 40 minutes, and then washed for 10 minutes. DAF-2 was excited at 490 nm with a xenon arc light, and emitted fluorescence was recorded at wavelengths of 510 to 550 nm. Square-shaped regions of interest were set inside the cytoplasm of macula densa cells, and mean intensity within the regions of interest was recorded every 5 seconds. Relative changes in DAF-2 fluorescence intensity (F) were calculated with the delta changes in F divided by basal fluorescence intensity (Frest). Relative changes (%) = (F − Frest) × 100/Frest.

Measurement of cell volume

To measure changes in volume, macula densa cells were loaded with 5 μmol/L calcein dissolved in 0.1% DMSO from the tubular lumen for 10 to 15 minutes, and then washed for 10 minutes. Calcein was excited at 490 nm with a xenon arc light, while emitted fluorescence was recorded at wavelengths of 510 to 550 nm. The fluorescent signal was recorded for at least 5 minutes after switching solutions. Regions of interest were set inside the cytoplasm of macula densa cells, and the mean intensity within the regions of interest was recorded every 5 seconds. Relative changes in calcein fluorescence intensity were calculated using the equation: (F − Frest) × 100/Frest. When the macula densa was perfused with low-NaCl solution, calcein intensity in the macula densa was 158.2 ± 11.0 arbitrary units (au). When the perfusate was switched to high NaCl, calcein intensity was 157.6 ± 11.1 au. After the perfusate was switched back to low NaCl, intensity was 157.6 ± 10.9 au. We found no significant differences (N = 6), indicating that there were no significant changes in cell volume when changing from the low NaCl to the high NaCl solution with the same osmolality. Since DAF-2 is a fluorescent dye excited at a single wavelength, its emission signals are affected significantly by changes in cell volume in addition to other factors [24, 25]. In the present study, we found that after increasing the low-NaCl solution to the same osmolality as the high-NaCl solution, cell volume always returned to baseline within 5 minutes when the macula densa NaCl solution was switched either from low to high or high to low. The constant macula densa cell volume made it possible to measure intracellular macula densa NO with DAF-2.
Once the TAL was perfused, macula densa cells were loaded with 5 μmol/L BCECF-AM from the tubular lumen at 37 ± 1°C for 15 minutes, and then washed for 10 minutes. Intracellular dye was excited alternately at 450 and 500 nm, and fluorescence emissions were recorded at 540 nm. Ratiometric measurements of the images (450/500 nm) were recorded and analyzed with the imaging system. The ratio was calibrated as described previously [26]. The pH calibration solutions contained (in mmol/L) 95 KCl, 5 NaCl, 30 N-methyl-D-glucamine, 2.5 NaH2PO4, 1.5 MgSO4, 5 glucose, 2 CaCl2, 25 HEPES, and 0.01 nigericin, and were titrated to selected pH values between 6.4 and 8.0. Nigericin was prepared as a 10 mmol/L stock in methanol and diluted in calibration solution just prior to use. All solutions had an osmolality of 290 mOsm adjusted with mannitol.

**Chemicals**

DAF-2 DA, calcein, BCECF-AM, and pluronic acid were obtained from Molecular Probes (Eugene, OR, USA). 7-nitroindazole (7-NI) was obtained from Cayman Chemicals (Ann Arbor, MI, USA). All other chemicals were purchased from Sigma.

**Statistics**

Data were collected as repeated measures over time under different conditions. We tested only the effects of interest, using a paired t test or analysis of variance (ANOVA) for repeated measures. When using ANOVA, the three pairwise comparisons were then evaluated using three paired t tests. Significance was judged as less than 0.05 or an adjusted value using Hochberg’s method for multiple testing.

**RESULTS**

Since we previously showed that inhibition of luminal NHE mimicked the effect of nNOS inhibition [19], we first investigated whether blocking luminal NHE activity with DMA would prevent the increase in NO production. NO was measured using the fluorescent dye DAF-2. When the macula densa perfusate was switched from low to high NaCl, DAF-2 intensity increased by 28.8 ± 4.1% (from 111.6 ± 12.7 to 140.3 ± 13.3 au; P < 0.01 vs. low NaCl; N = 9) (Fig. 1), indicating a significant increase in macula densa NO. After 5-(N,N-dimethyl) amiloride hydrochloride (DMA) (10^-4 mol/L) was added and the perfusate switched from low to high NaCl, macula densa NO...
DAF-2 intensity increased by 10.2 ± 3.1% (from 144.7 ± 12.3 to 157.3 ± 11.9 au; $P < 0.05$ vs. low NaCl; $P < 0.01$ vs. control period; $N = 5$).

Since the combination of DAF-2 and NO is irreversible [24], DAF-2 intensity at the macula densa perfused with low NaCl was different in the absence and presence of DMA (111.6 ± 12.7 and 144.7 ± 12.3, respectively). To make sure that the varying increase in NO in the presence and absence of DMA was not due to different basal intensities, we washed the DMA from the lumen for 20 minutes, and then switched luminal NaCl from low to high. DAF-2 intensity increased by 22.4 ± 1.3% (from 190.6 ± 16.4 to 233.2 ± 19.7 au; $P < 0.01$ vs. low NaCl; $N = 4$). Comparing the first and last response without DMA, the increase in DAF-2 intensity at the macula densa showed no significant difference (Fig. 2). Thus, macula densa NO increased during TGF, and the NHE inhibitor DMA significantly attenuated this increase.

To make sure the increase in DAF fluorescence was due to NO derived from macula densa nNOS during the TGF response, we used 7-NI (10$^{-5}$ mol/L). During the control period, when we switched the tubular perfusate from low to high NaCl, macula densa DAF-2 fluorescence increased by 38.4 ± 3.9% (from 80.5 ± 9.2 to 107.4 ± 10.1 au; $P < 0.01$ vs. low NaCl). When 7-NI was added to the perfusate, macula densa DAF-2 fluorescence increased by 12.1 ± 1.9% during the TGF response (from 110.2 ± 10.6 to 121.1 ± 8.1 au; $P < 0.01$ vs. control TGF) (Fig. 3). These data indicate that the increase in DAF-2 fluorescence was due to activation of nNOS and production of NO in the macula densa.

Since DMA blocked the increase in NO production induced by increasing luminal NaCl, we next investigated the role of changes in macula densa pHi. To study changes in pHi of macula densa cells influenced by tubular NaCl, we loaded the cells with BCECF-AM and obtained a control response by switching the tubular perfusate from low to high NaCl. Macula densa pHi increased significantly by 0.6 ± 0.1 (from 7.0 ± 0.5 to 7.6 ± 0.6; $P < 0.01$; $N = 5$). When the perfusate was first switched back to low NaCl and DMA (10$^{-4}$ mol/L) added, then 20 minutes later switched to high NaCl with 10$^{-4}$ mol/L DMA, changes in pH i at the macula densa were blocked (from 7.07 ± 0.5 to 7.09 ± 0.5; $N = 5$) (Fig. 4). These data indicate that the changes in macula densa pHi induced by increasing luminal NaCl concentration were initiated by the NHEs at the apical membrane.

To study whether the increase in macula densa pHi was responsible for stimulating NO production, we measured NO concentration while the macula densa was perfused with low-NaCl solution and altered pHi using nigericin.
Fig. 4. Changes in macula densa pH induced by altering tubular NaCl concentration. Increasing tubular NaCl concentration significantly raised MD pH, and this increase was blocked by amiloride (*P < 0.01 vs. low). Low, low NaCl; High, high NaCl; L+DMA, low NaCl plus amiloride; H+DMA, high NaCl plus amiloride.

Fig. 5. Effect of macula densa pH on nNOS activity. Raising macula densa pH from 7.3 to 7.8 significantly increased NO concentration in the macula densa (*P < 0.01 vs. pH 7.3). 7-NI blocked the increase in NO induced by raising pH (N = 5).

When the macula densa was perfused with pH 7.3 low-NaCl solution with 10⁻⁵ mol/L nigericin, the DAF-2 intensity was 118.3 ± 9.8 au. After pH was increased to 7.8, DAF-2 intensity increased 17.9 ± 1.3% (from 118.3 ± 9.8 au to 139.4 ± 11.4 au; *P < 0.01; N = 5). These data suggest that increase macula densa pH enhances NO production. To show that the increase in NO production was due to stimulation of nNOS, we repeated the above experiment except that 7-NI was first added to the perfusate 20 minutes prior to switching pH. In the presence of 7-NI, the increase in NO induced by raising macula densa pH was blocked (from 164.4 ± 1.7 to 166.9 ± 3.1 au; N = 5) (Fig. 5). These data suggest that raising macula densa pH increases NO generation via nNOS. To see if 7-NI, per se, has any effect on macula densa pH, the macula densa was first perfused with high-NaCl solution and then 7-NI (10⁻⁵ mol/L) was added to the luminal side for 20 minutes and to the bath for another 20 minutes. Macula densa pH was measured at baseline, with 7-NI in the lumen and with 7-NI in both lumen and bath. The results showed that there were no significant pH changes caused by 7-NI.

To study the effects of changes in osmolality on TGF, we compared the mannitol-adjusted solution with unadjusted NaCl solution, and studied TGF response using afferent arteriole and tubule double-perfusion methods. We found that when the luminal NaCl solution was switched from low (unadjusted osmolality 81 mOsm) to high (180 mOsm), the diameter of the afferent arteriole was reduced (TGF) by 3.3 ± 0.5 μm. In mannitol-adjusted solution, when luminal NaCl was switched from low (180 mOsm) to high, the TGF was 2.9 ± 0.8 μm (N = 5). There was no significant difference between them.

DISCUSSION

We showed previously that inhibition of luminal NHE mimicked the effect of nNOS inhibition on TGF [19]. These data implied that changes in macula densa pH may regulate NO production. However, we did not directly measure changes in pH or NO. In the present study we examined the relationship between NHE activity, macula densa pH, and NO production by macula densa nNOS. We found that increasing luminal NaCl augmented NO production, and that inhibition of luminal NHE with DMA significantly attenuated this increase. These data directly show that luminal membrane NHE plays a role in the increase in NO production caused by increasing the NaCl concentration in the lumen of the macula densa.

It is important to note that we used DAF-2 to measure macula densa NO. The fluorescent intensities measured in the control period and the DMA-treatment period in the experiments described above were necessarily different [24]. Although initial DAF fluorescence differed between the two periods, the inhibition caused by DMA was not due to the different basal intensities of the fluorescent dye. When we washed the DMA from the lumen and then switched NaCl a third time, the increase in NO measured by an increase in DAF fluorescence was not significantly different from the first period. Increasing luminal NaCl would be expected to activate NHE and alkalinize the macula densa [9]. Therefore, next we investigated changes in macula densa pH, while increasing luminal NaCl and the effect of DMA. We found that increasing tubular NaCl concentration raised macula densa pH from 7.0 ± 0.5 to 7.6 ± 0.6. This increase was totally inhibited by DMA, similar to other reports [8, 9]. These data suggested a direct link between pH and NO production at the macula densa.

To directly test whether the increase in pH caused by increasing luminal NaCl could augment NO...
generation in the macula densa, we added nigericin, a K⁺/H⁺ ionophore [27, 28], to perfusion solutions of different pH [27]. We found that raising pHi from 7.3 to 7.8 independent of changes in NaCl significantly increased macula densa NO levels. To make sure the increase in DAF fluorescence was due to an increase in NO production rather than an effect of pH on the dye, we tested the ability of 7-NI to block this increase. We found that the nNOS inhibitor 7-NI blocked the alkalization-induced increase in DAF fluorescence. These data suggest that the increase in DAF fluorescence induced by the rise in macula densa pHi was due to augmented NO generation in the macula densa. Production of NO by nNOS is reported to be strongly pH-dependent, and NOS activity increases 5-fold with alkalization across a physiologically relevant range [16–18], exhibiting maximal activity at pH 8 [16]. We found that when we increased tubular NaCl, macula densa pHi increased via luminal NHE. The increased pH, directly activated nNOS and increased NO generation. Our finding also offers an explanation for our earlier data [14], which showed that the nNOS inhibitor 7-NI constricted the afferent arteriole only when the macula densa was perfused with high-NaCl solution, and had no effect on diameter when perfused with low NaCl, since the macula densa NO concentration was higher only when the macula densa was perfused with high NaCl. Ichihara et al also found that the afferent arteriolar response to increased distal delivery was augmented during nNOS inhibition [12, 13]. However, Wright and Schnermann [29] found that amiloride had no effect on TGF. The reasons for this discrepancy are probably due to the different species, methods, or amiloride isoforms used in the experiments. For example, 1 mmol/L amiloride inhibited NHE activity by only 36% [30]; however, 300 μmol/L ethilsisopropyl amiloride inhibited NHE activity by 99.5% [8]. In addition, other kidney cells might behave differently from macula densa cells in response to NaCl loading, as reported recently by Levine et al [31], who found that blocking loop NaCl transport with furosemide significantly increased net NO from the entire loop.

The mechanism by which pH regulates macula densa nNOS is unknown. However, it might involve NADPH uncoupling from nNOS at low pH, resulting in inactivation of nNOS and inhibition of NO production [17]. The NADPH uncoupling also induces O₂⁻ generation by nNOS, which further diminishes the bioavailability of NO [17].

The fact that the increase in NO could be totally inhibited by 7-NI also suggests that the changes in DAF-2 intensity were not influenced by changes in pHi within the pH range of the present study. The fluorescence of DAF-2 is comparatively stable in media above pH 7, but its fluorescence substantially decreases below a pH of 7. If, for example, intracellular pH dropped to 6.5, the DAF-2T intensity would decrease by almost 50% as an effect of pH [24, 25].

In the present study we only examined the activity of macula densa luminal NHE; the basolateral NHE was not analyzed. At the macula densa, the luminal membrane expresses the NHE2 isoform and is amiloride-sensitive, while the basolateral side expresses the NHE4 isoform and is osmolality-sensitive [17]. Their physiologic function and mechanisms of regulation remain unclear.

Although our data indicate that pHi is a major activator of macula densa nNOS when luminal NaCl is increased, it may not be the only activator. We found that the increase in NO could not be entirely inhibited by DMA, and that an increase in luminal NaCl caused a 28.8% to 38.4% increase in macula densa NO, whereas an increase in macula densa pHi only increased NO by 17.9%. Other factors, such as changes in intracellular calcium concentration, Na-K-2Cl cotransporter activity, and cell membrane tension could also be involved. Although TGF and macula densa nNOS are both activated by changes in luminal NaCl, the initial mechanisms involved are largely distinct. TGF activation depends primarily on the furosemide-sensitive Na-K-2Cl cotransporter, whereas nNOS is activated mainly via the amiloride-sensitive NHE.

We found that macula densa pHi increased when NaCl at the macula densa increased from 10 to 80 mmol/L, and this in turn activated nNOS, increasing NO production. This is in agreement with our earlier report, in which we measured intracellular NO concentration corrected by changes in cell volume measured with calcein [15]. Kovacs et al [32] studied changes in macula densa NO by measuring fixed points outside the macula densa, and found that NO seemed to increase only at markedly elevated NaCl concentrations. However, this method is inevitably influenced by changes in cell volume, since the cell volume is changing while the measuring point is fixed, so that the distance from the measuring point to the macula densa would change accordingly. Probably that is one of the reasons why they failed to detect the NO increase within the physiologic NaCl changes.

Increases in tubular NaCl concentration from 10 to 140 mmol/L have been reported to cause swelling of macula densa cells, as shown either by directly measuring their length [33, 34] or quantitatively measuring cell volume with calcein [15], which makes it difficult to use DAF-2 to measure NO [24]. We found that after increasing the low-NaCl solution to the same osmolality as high NaCl, cell volume always returned to baseline within 5 minutes when the macula densa NaCl solution was switched either from 10 to 80 or from 80 to 10 mmol/L. This suggests that, like many other types of cells [35–37], macula densa cells are capable of regulatory volume changes.
CONCLUSION

We found that increasing luminal NaCl concentration raises macula densa pHi by activating luminal NHE. The increased macula densa pHi activates nNOS and induces NO production.

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