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ORIGINAL PAPER

Low [NaCl]-induced neuronal nitric oxide synthase (nNOS) expression and NO generation are regulated by intracellular pH in a mouse macula densa cell line (NE-MD)

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Abstract Changes in the luminal NaCl concentration ([NaCl]) at the macula densa (MD) modulate the tubuloglomerular feedback (TGF) responses via an affect on the release of nitric oxide (NO). This study was performed in a newly established mouse macula densa cell line (NE-MD) to investigate the effects of lowering [NaCl] on the neuronal NO synthase (nNOS) protein expression and L-arginine (Arg)-induced NO release. Expression of nNOS protein and release of NO were evaluated by Western blot analysis and an NO-sensitive electrode, respectively. Intracellular pH (pH_i) was monitored by the BCECF assay. Although there was weak staining of the nNOS protein expression, L-Arg-induced NO generation was negligible in normal (140 mM NaCl) solution. Both were significantly ($P < 0.05$) increased either in the presence of furosemide (12 μM), an inhibitor of the Na^+–K^+–$2Cl^-$ cotransporter, or in a low (23 mM) Cl^- solution. Furosemide- and low Cl^--induced NO generation was completely inhibited by 50 μM 7-nitroindasole (7-NI), a nNOS inhibitor. Moreover, these increases were significantly ($P < 0.05$) inhibited by the addition of 100 μM amiloride, an inhibitor of the Na^+/H^+ exchanger, or by its analogue 5-(*N*)-ethyl-*N*-isopropyl amiloride (EIPA), and also at a lower pH of 7.1. Furthermore, nNOS expression and NO release were not stimulated in as low as 19 mM Na^+ solution. In conclusion, low [Cl^-], but not low [Na^+] in the lumen at the MD, increased nNOS protein expression and NO generation. Changes in the luminal [NaCl] may modulate the TGF system via an effect on the NO generation from the MD.

Keywords Neuronal nitric oxide synthase · Macula densa · Intracellular pH · Na^+/H^+ exchanger · Tubuloglomerular feedback

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Introduction

Kidney macula densa (MD), a small group of distal tubule cells next to the cortical thick ascending limb of Henle's loop (TAL), is faced to the same glomerulus from which it originated, and is located between the afferent and efferent arterioles [1]. The MD cells uniquely express neuronal nitric oxide synthase (nNOS), a brain-type of NOS, in the kidney, and generate NO in the presence of L-arginine (L-Arg). Although it is considered to be "constitutively" expressed, the nNOS gene may also be transcriptionally regulated in brain [2, 3] and in kidney [4]. Several lines of evidence suggest that the expression of nNOS protein and mRNA in MD cells is regulated by low salt intake [5–7] and stimulated by the addition of furosemide (a loop diuretic), an inhibitor of the Na^+–K^+–$2Cl^-$ cotransporter [7, 8]. The NO generated in the kidney results in natriuresis and diuresis [9].

Our previous study showed that the nNOS protein expressed in a newly established mouse macula densa cell line (NE-MD) was increased time-dependently in the presence of 12 μM furosemide [10]. This finding, i.e., furosemide-induced expression of nNOS protein, is consistent with the view that the nNOS in MD cells plays an important role in these changes, with high distal flow

(or high [NaCl] at the MD) inhibiting NO production, and simulation of low distal flow by furosemide stimulating NO production [5, 7, 11]. Thus, the NO released from the MD modulates the tubuloglomerular feedback (TGF) system [12]. On the other hand, our finding is apparently inconsistent with previous reports, as follows: (1) high salt intake increases the NO generation produced by nNOS, compared with salt restriction [13]. (2) In the short-term regulation of the TGF system, increasing luminal [NaCl] increased nNOS activity [14] and NO production [15]. (3) A short-term saline infusion [16] as well as long-term (2 weeks) dietary NaCl loading [17] increases renal NO generation, as reflected by increased plasma levels or excretion of the NO metabolites, such as NO_2 and NO_3. These apparent discrepancies in the cellular mechanisms of [NaCl]-dependent nNOS protein expression and NO generation require clarification.

MD cells have unique characteristics in Na^+ and water transport along the nephron. They express the Na^+–K^+–$2Cl^-$ cotransporter and the Na^+/H^+ exchanger with relatively high water permeability at the luminal membrane [18–21], although the latter is not consistent with the finding of low water permeability of the MD luminal membrane [22]. Part of the Na^+ entry into MD cells is mediated via the Na^+–K^+–$2Cl^-$ cotransporter (NKCC2) (80%) and the Na^+/H^+ exchanger (NHE2) (20%). Thus, changes in luminal [NaCl] may cause changes in both luminal NaCl entry and intracellular pH (pH_i) in the MD. Recently, in isolated and perfused rabbit kidney tubules, pH_i was increased upon either the addition of furosemide [23] or lowering of the luminal [Cl^-] [24] via activation of luminal NHE. In contrast, blocking NHE in the luminal membrane of MD significantly enhanced the TGF response [25].

In this study, to resolve the apparent discrepancies of [NaCl]-dependent NO generation in the MD, we have examined whether nNOS protein expression and L-Arg-induced NO generation in NE-MD cells are (1) furosemide-dependent, (2) pH-sensitive, (3) [Na^+]-dependent, and/or (4) [Cl^-]-dependent.

Materials and methods

Cell culture

A kidney tubule cell line of macula densa, NE-MD, was recently established in our laboratory [10] and was used for the present studies at passages of 5–20. Briefly, NE-MD cells were derived from the cultured kidney cells of SV40 transgenic mice with an nNOS-EGFP transgene. When NE-MD cells became confluent (typically in 7–10 days), they were maintained by subculture. NE-MD cells were grown in RITC80-7 medium (Iwaki, Tokyo) supplemented with 5% FBS (Invitrogen, Carlsbad, CA), 10 μg/ml transferrin (Roche Diagnostics, Indianapolis, IN), 0.08 U/ml insulin (Novo Nordisk, Copenhagen, Denmark), 10 ng/ml recombinant human EGF (Wakunaga pharmaceutical Co. Ltd, Osaka), and 50 U/ml penicillin G/50 μg/ml streptomycin (Invitrogen). Cells were maintained at a permissive temperature (33°C) for the expression of SV40 temperature-sensitive large T-antigen gene, in humidified 5% CO_2 atmosphere.

Reagents

Furosemide and 2′,7′-bis-(2-carboxyethyl)-5-carboxyfluorescein diacetoxymethyl ester (BCECF-AM) were purchased from Sanofi Aventis (Tokyo) and Molecular Probes (Eugene, OR), respectively. Tris (hydroxymethyl) aminomethane [2-amino-2-hydroxymethyl-1,3-propanediol] was from Wako Pure Chemical Industries (Osaka). All other reagents, such as 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), amiloride hydrochloride, 5-(*N*-ethyl-*N*-isopropyl)-amiloride (EIPA), dimethyl sulfoxide (DMSO), and S-Nitriso-*N*-acetyl-D,L-penicillamine (SNAP) were purchased from Sigma–Aldrich Inc (St Louis, MO).

Experimental solutions

A normal solution (Na^+ Ringer) was composed of (in mM): 140 NaCl, 5.0 KCl, 1.0 $CaCl_2$, 1.0 $MgCl_2$, 5.5 D-glucose, 1.0 Na pyruvate, and 10 HEPES (pH 7.4 with NaOH) (292 mOsm/kgH_2O). Ion composition of 0 Na^+ solution was the same as a normal solution except for Na^+ (NaCl was totally replaced with choline Cl). Isosmotic low salt media were made by mixing appropriate volumes of normal saline with free salt medium. The mixing ratios for low (1/4 and 1/10) salt solutions were 1:3 and 1:9 (normal : free salt solution), respectively. Ionic compositions of various extracellular solutions are shown in Table 1. In some experiments, 12 μM furosemide and/or 100 μM amiloride (or EIPA) was added to the medium throughout the pre-incubation and NO-measurement. Osmolality was measured by a freezing point osmometry (Fiske's one-ten osmometer, Fiske Associates, Norwood, MA).

Measurement of nitric oxide (NO)

Culture medium was replaced and pre-incubated with various types of ionic solutions (Table 1) with or without amiloride or its analogue for 2–5 h until the NO-measurement. The release of L-Arg-induced NO from the NE-MD cells was directly measured using an NO meter with an associated NO sensor [ISO-NO nitric oxide sensor,

Table 1 Ionic composition

Component	Na^+ Ringer	Low Na^+		Low Cl^-	
	Na^+ R	1/10 Na^+	1/4 Na^+	1/10 Cl^-	1/4 Cl^-
Na^+	144.5	18.5	39.5	144.5	144.5
$Choline^+$	–	126	105	–	–
K^+	5	5	5	5	5
Cl^-	149	149	149	23	44
$Gluconate^-$	–	–	–	126	105
Mg^{2+}	1	1	1	1	1
Ca^{2+}	1	1	1	1	1
HEPES	10	10	10	10	10
Osmolality (mOsm/ kgH_2O)	293	292	293	291	292

Solutions are expressed in mM. pH was adjusted to 7.4 by the addition of NaOH

World Precision Instruments (WPI), Sarasota, FL] [26]. The size of the electrode (ISO-NOP200) was 200 μm in diameter. The ISO-NO sensors were amperometric. This redox current is proportional to the concentration of NO in the sample. In solution, this sensor can measure NO concentrations as low as 1 nM.

For calibration, SNAP was used in combination with a catalyst, copper sulfate, to generate a known quantity of NO in solution. The signal slowly declines in this method because the generated NO is quickly oxidized to nitrite and nitrate, which are no longer detected by the probe. The calibration curve can be simply constructed by plotting the magnitude of the signal in picoamperes versus the final concentration of SNAP for that particular signal. A fresh stock solution of SNAP was prepared at the beginning of every day. In this study, the bathing solution always contained 1 mM D-arginine to avoid an unexpected shift of the baseline upon addition of the concentrated L-arginine solutions.

Measurement of intracellular pH

The optical equipment for the dual-excitation image analysis (480 nm/450 nm) consisted of a fluorescence microscope (Axiovert135, Zeiss, Germany), a CCD Camera (C6790, Hamamatsu Photonics, Hamamatsu), and an Image Processor (Argus Hisca, Hamamatsu Photonics, Hamamatsu), and was used throughout the study. Briefly, NE-MD cells were incubated in a nearly normal solution (pH adjusted with Tris) containing 2 μM BCECF-AM at 37°C for 20 min, rinsed twice, and maintained in a warmed chamber at 37°C until the optical measurement. Calibration of intracellular pH was routinely performed at the end of each record by the high-K^+ nigericin method [27].

Western blot analysis

Using methods similar to those described previously [10], NE-MD cells were lysed in a TBS buffer containing 24 mM Tris, 136 mM NaCl, 2.6 mM KCl, and 2 mM leupeptin (Sigma–Aldrich) adjusted to pH 7.4. Large tissue debris and nuclear fragments were removed by centrifugation (800 rpm) for 5 min at 4°C. The supernatant was isolated and then diluted in sodium dodecyl sulfate (SDS) sample buffer with 6% β-mercaptoethanol. Proteins were denatured in boiling water for 10 min, separated by polyacrylamide gel electrophoresis, and transferred to a polyvinylidene difluoride membrane (PVDF) (Bio-Rad Laboratories, Hercules, CA). The blots were blocked for 30 min with 5% nonfat dry milk in Tris-buffered saline with 0.05% Tween 20 (TBS/T), followed by incubation overnight at 4°C with the rabbit polyclonal anti-human nNOS antibody (1:1000, Cayman Chemical Co, Ann Arbor, MI) or the mouse monoclonal anti-rabbit GAPDH antibody (1:5,000, Abcam Inc., Cambridge, MA). After washing with TBS/T, blots were incubated with a horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (GE Healthcare, Buckinghamshire, UK) or goat anti-mouse secondary antibody (GE Healthcare) for 2 h at room temperature. The blots were then washed with TBS/T and subjected to visualization using ECL kits (GE Healthcare).

Statistical analysis

Results are presented as the means ± SE of the experiments. Significance was determined by Student's *t*-test. Significance was set at $P < 0.05$.

Results

NO generation in cultured NE-MD cells

L-Arg-induced NO generation was measured by using an NO sensing-electrode in NE-MD cells. NO generation was negligible when the cells were incubated in a normal (140 mM NaCl) solution without furosemide (Fig. 1a, top). In contrast, it was highly increased when the cells were incubated with furosemide (12 μM) for 2 h (Fig. 1a, middle). The NO value was expressed as the mean of the 6-min rise after the addition of L-Arg to the bath. Furosemide-induced NO generation was completely abolished in the presence of 50 μM 7-nitroindasole (7-NI), a relatively specific nNOS inhibitor (Fig. 1a, bottom). These results are summarized in Fig. 1b. Further, NO generation was negligible even in the furosemide-treated NE-MD cells when D-arginine ($n = 1$), L-lysine ($n = 1$), or

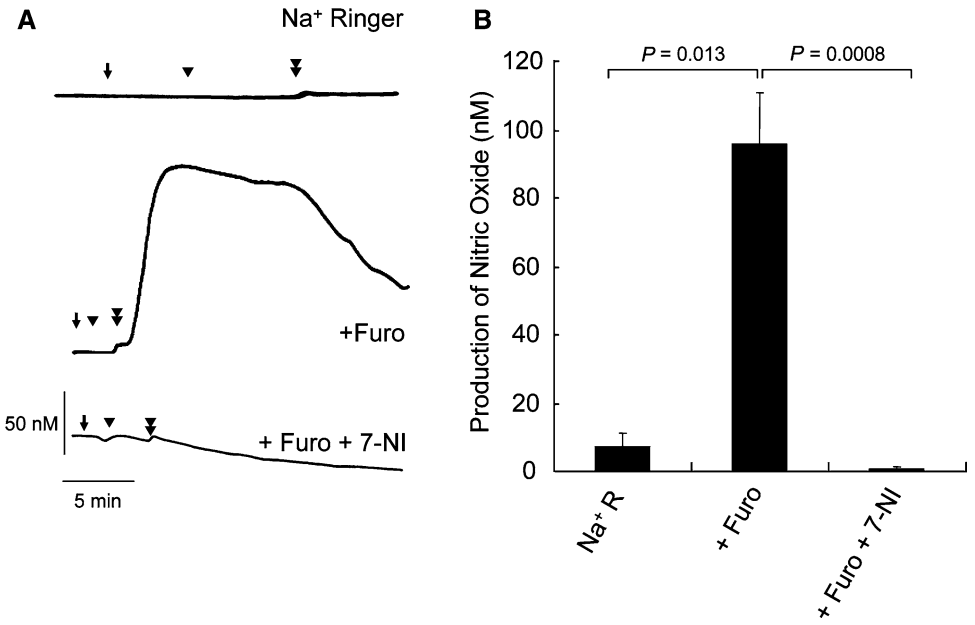


Fig. 1 L-Arg induced NO release from NE-MD cells. **a** Representative traces of NO release in normal solution (Na^+ Ringer, *top*), furosemide-treated (+Furo, *middle*), and furosemide-treated in the presence of 7-nitroindasole (+Furo+7-NI, *bottom*). *Arrows* and *arrowheads* indicate the addition of normal solution and stock solutions of L-Arg, respectively. *Single* and *double arrowheads*, respectively, indicate the final concentrations of 0.2 and 1.0 mM L-Arg after dilution. **b** The NO concentrations released are 7.5 ± 3.6 (Na^+ Ringer), 95.4 ± 15.2 (+Furo), and 1.0 ± 0.3 nM (+Furo+7-NI) in the presence of 1 mM L-Arg ($n = 4$, each). Statistical significance compared with the control values are indicated in the figure

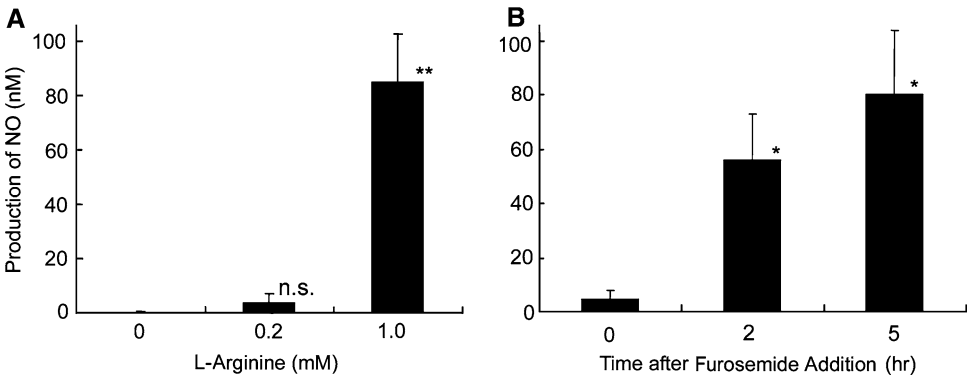


Fig. 2 Dose-dependent and time-dependent NO release from NE-MD cells pre-incubated with furosemide. **a** (Dose-dependent): NE-MD cells were incubated in the presence of furosemide for 5 h. The NO values are 1.6 ± 1.3 (0 mM L-Arg (control), $n = 4$), 6.5 ± 2.7 (0.2 mM L-Arg, $n = 4$), and 79.7 ± 24.1 nM (1 mM L-Arg, $n = 4$). **b** (Time-dependent): L-Arg (1 mM)-induced NO release from the pre-treated NE-MD cells: 4.6 ± 3.0 (0 h (control), $n = 3$), 56.0 ± 16.6 (2 h, $n = 4$), and 79.7 ± 24.1 nM (5 h, $n = 4$). * and ** are $P < 0.05$ and $P < 0.02$, respectively, compared with the values of control; *ns* not significant

L-glutamate ($n = 1$) was added to the bath solution (data not shown).

In the next series of experiments, L-Arg-induced NO generation was measured in NE-MD cells pre-incubated for 5 h with furosemide (12 μM) (Fig. 2a). NO generation was significantly ($P < 0.02$) and dramatically increased at 1.0 mM L-Arg, but not at 0.2 mM. Secondly, L-Arg (1 mM)-induced NO generation was measured in NE-MD cells pre-incubated with furosemide for 0, 2, and 5 h (Fig. 2b). It increased time-dependently through the period of measurement. In summary, L-Arg-induced NO generation increased concentration-dependently and time-dependently. In this study hereafter, NO generation was examined in the NE-MD cells pre-treated for 2 h under the various conditions.

Effects of low [Cl^-] and low [Na^+]

L-Arg (1 mM)-induced NO generation was significantly increased when NE-MD cells were incubated in a low

[Cl^-] solution. The increase was partially inhibited in the presence of 100 μM amiloride, a Na^+/H^+ exchanger inhibitor, and is completely inhibited by the addition of 50 μM 7-NI. These results are summarized in Fig. 3, suggesting that L-Arg-induced NO generation may be pH-sensitive.

On the other hand, L-Arg-induced NO generation was slight when the cells were incubated in a low [Na^+] solution (Fig. 4a, top). No further decrease was observed in the presence of 50 μM 7-NI (Fig. 4a, bottom). These data are summarized in Fig. 4b. The results suggest that NO generation from the low [Na^+] pretreated NE-MD cells may be inhibited by the intracellular acidification caused by the inhibition of Na^+/H^+ exchanger because of a lowering of the [Na^+] in the bath.

NO generation is pH-sensitive

Acute and chronic effects of pH_i on L-Arg-induced NO generation were examined in the furosemide-treated NE-MD cells with or without EIPA, an analogue of amiloride (a Na^+/H^+ exchanger inhibitor). L-Arg (1 mM)-induced NO generation was significantly decreased in the presence of EIPA. In contrast, it was acutely (approximately 20 min) recovered after the removal of EIPA (Fig. 5).

In the next series of experiments, L-Arg-induced NO generation was measured under different pH conditions. Compared with NO generation of control at pH 7.4 (Fig. 6a, middle), it was significantly decreased at pH 7.1 (Fig. 6a, bottom) and was unchanged at pH 7.6 (Fig. 6a, top). These results are summarized in Fig. 6b.

Intracellular pH measurement

Intracellular pH (pH_i) was measured in the NE-MD cells pre-loaded with BCECF-AM by using an Argus Hisca device (Methods). In this study, there was no significant difference in the initial pH_i of NE-MD cells with or without furosemide. However, pH_i was decreased in response to a stepwise decrease in extracellular [Na^+] (Fig. 7a). Values of decreased pH_i were estimated to be approximately 7.0 and 7.1, respectively, at Na^+ concentrations of 17.5 and 35 mM (Fig. 7b). Further, pH_i was quickly decreased upon the addition of EIPA to the normal (140 mM Na^+) solution (Fig. 8a). The half-decreased dose (apparent EC_{50}) was 0.9 μM (Fig. 8b).

Western blotting

To determine if intracellular acidification was responsible for the inhibitory effect on the furosemide-induced nNOS protein expression, confluent NE-MD cells were incubated in normal salt, normal salt plus furosemide (12 μM), and normal salt containing furosemide plus EIPA or DMSO for 2 h. Compared with normal salt (Na^+ Ringer), incubation with furosemide (2 h) increased the level of nNOS protein expression (+12 μM furosemide). Interestingly, the level was decreased in the presence of EIPA, but was unchanged in the presence of the vehicle (DMSO) (Fig. 9a, left four blots). Moreover, we determined whether a reduction in Na^+ or Cl^- was responsible for the low [NaCl] effect on nNOS protein expression. NE-MD cells were incubated in various types of low salt media for 2 h (Table 1). Low Cl^-

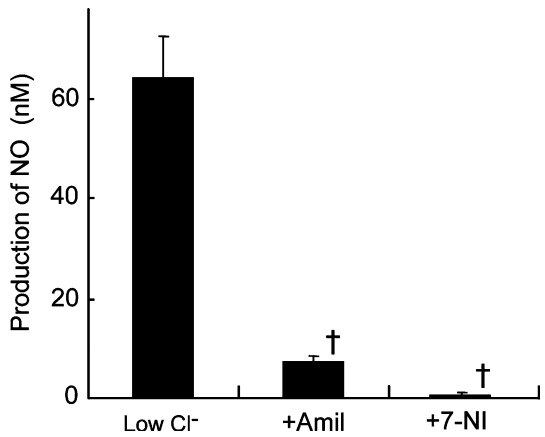


Fig. 3 L-Arg-induced NO release from NE-MD cells treated with low [Cl^-] for 2 h. The NO concentrations released are 75.6 ± 11.8 (low Cl^-, $n = 5$), 8.0 ± 1.4 (+Amil, $n = 4$), and 0.3 ± 0.2 nM (+7-NI, $n = 4$) in the presence of 1 mM L-Arg. $^{\dagger}P < 0.01$, compared with the value of control (low Cl^-)

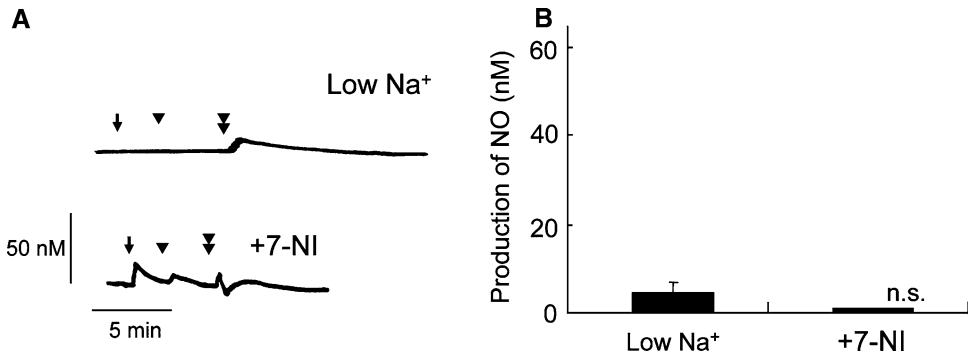


Fig. 4 L-Arg-induced NO release from NE-MD cells incubated with low [Na^+] for 2 h. **a** Representative traces of NO release from the NE-MD cells in a low [Na^+] (control) and in the presence of 7-NI. *Arrows* and *arrowheads* indicate the same meanings shown in Fig. 1. **b** The NO released is 4.0 ± 2.8 (low Na^+, $n = 4$) and 0.5 ± 0.2 nM (+7-NI, $n = 4$) in the presence of 1 mM L-Arg

media stimulated the level of nNOS protein expression slightly (at 1/4 [Cl^-]) and significantly (at 1/10 [Cl^-]). In contrast, the level of nNOS protein expression was unchanged in low Na^+ media (Fig. 9a, right four blots). Although nNOS protein expression was significantly increased in the presence of furosemide and at 1/10 [Cl^-], it was not in low Na^+ media. Further, furosemide-induced nNOS protein expression was significantly ($P < 0.02$) inhibited in the presence of EIPA. These results are summarized in Fig. 9b.

Discussion

In this study, it was demonstrated in a newly established mouse macula densa cell line (NE-MD) [10] that furose-

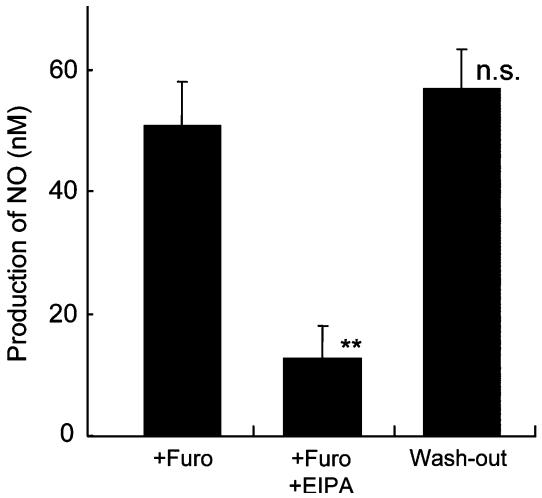


Fig. 5 Effects of EIPA on NO production in furosemide-pretreated NE-MD cells. The NO released is 50.9 ± 7.4 (+Furo, $n = 6$), 12.6 ± 5.4 (+Furo+EIPA, $n = 3$), and 57.1 ± 6.4 nM (wash-out, $n = 3$). $^{**}P < 0.02$, *ns* not significant (vs. +Furo)

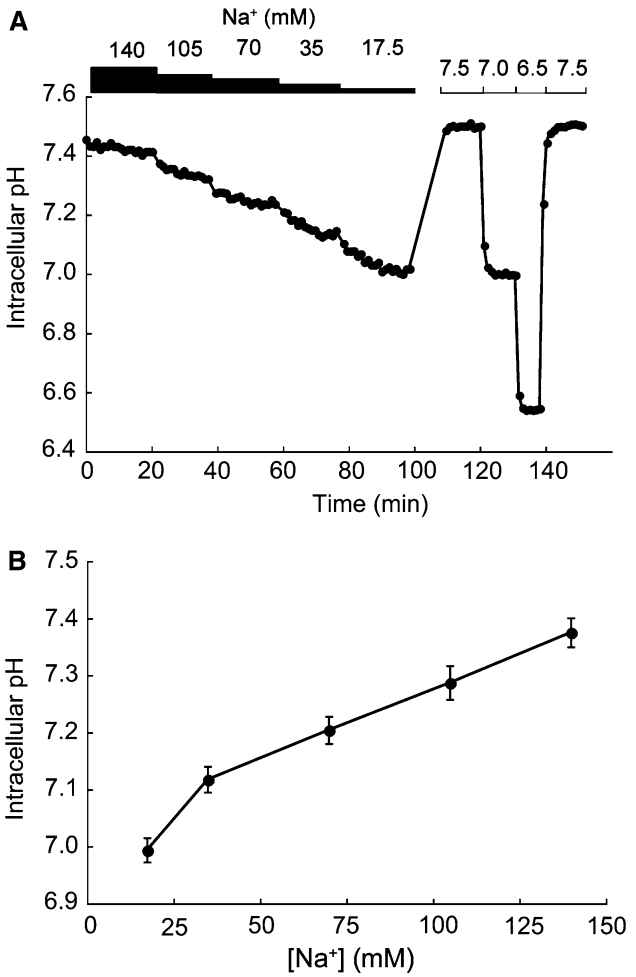
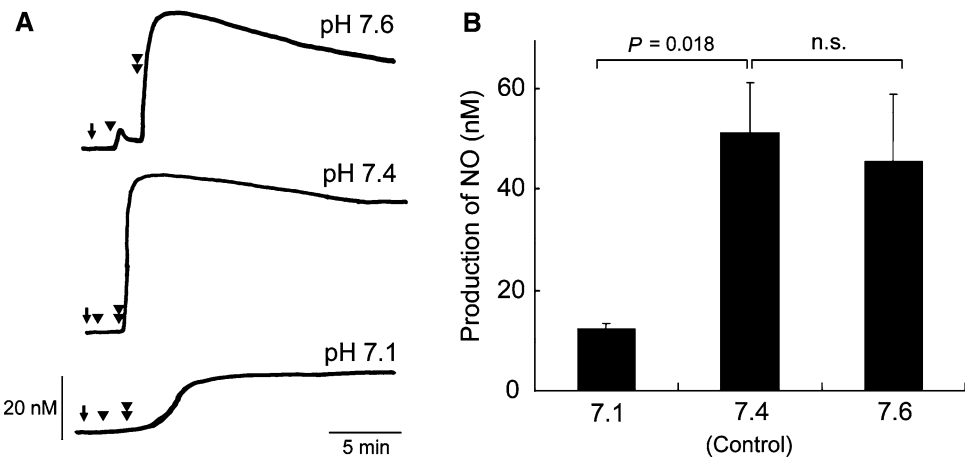


Fig. 7 The effects of Na^+ removal on pH_i in NE-MD cells. **a** Representative tracing of the pH_i changes induced by the reduction of Na^+ from the bathing solution and its calibration at the end of the trace. Concentrations of the remaining [Na^+] in the bathing solution are indicated at the top (*left*). Values at the top (*right*) show pH of the extracellular solutions for the calibrations. **b** pH_i-[Na^+] curve. *Bars* indicate the standard errors. $n = 7$ each

Fig. 6 L-Arg-induced NO release at different pH levels in furosemide-pretreated NE-MD cells. **a** Representative traces of NO release at pH 7.6 (*top*), 7.4 (*middle*), and 7.1 (*bottom*). *Arrows* and *arrowheads* indicate the same meanings shown in Fig. 1. **b** NOs released are 12.1 ± 1.1 (pH 7.1), 51.1 ± 10.0 (pH 7.4), and 45.5 ± 13.6 nM (pH 7.6) in the presence of 1 mM L-Arg ($n = 3$, each column). Statistical significance was obtained by comparison with control values (pH 7.4)



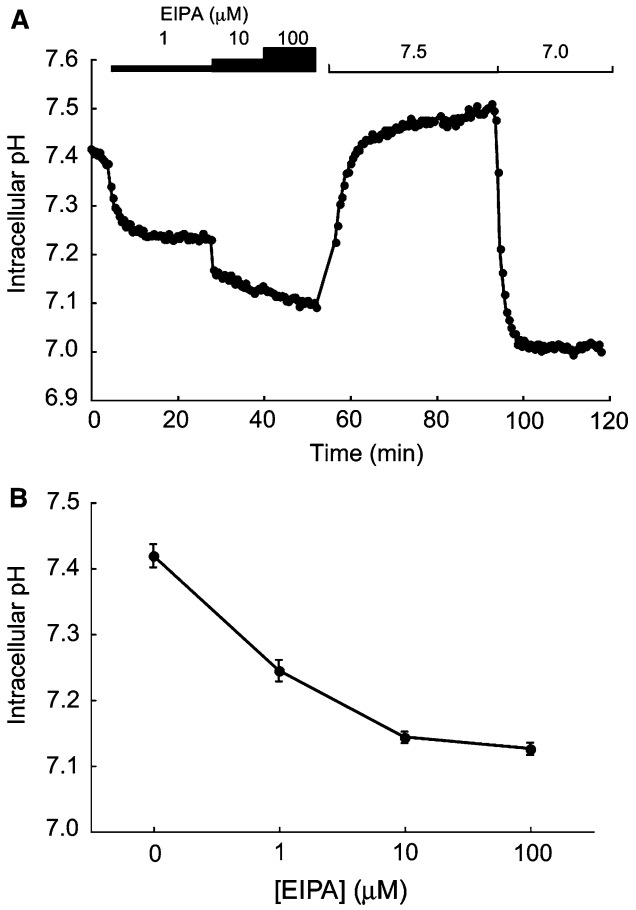


Fig. 8 Effect of EIPA on pH_i of NE-MD cells. **a** Representative tracing of the pH_i change induced by the addition of EIPA to the bath and its calibration at the end. Concentrations of EIPA in the bathing solution are indicated at the top (*left*). **b** EIPA dose-inhibition curve; $n = 6$ each

mide (an inhibitor of Na^+–K^+–$2Cl^-$ cotransporter) and low [Cl^-], but not low [Na^+], induced increases in nNOS protein expression and L-Arg-induced NO generation. These results strongly suggest that (1) inhibition of NaCl entry at the luminal membrane of MD cells increases nNOS protein expression and its activity, and (2) the intracellular acidification induced by the inhibition of Na^+/H^+ exchanger because of either amiloride or the low luminal [Na^+] failed to increase nNOS protein expression and activity. Changes in luminal [NaCl] have at least two different and independent effects on the NO generation in MD. First, low [NaCl] in the lumen may stimulate nNOS protein expression by decreasing NaCl entry into MD cells [28]. Second, low [Na^+] may cancel or inhibit the intracellular signals of the nNOS protein expression process by decreasing the pH_i. Thus, the overall signals of low [NaCl] at MD to stimulate nNOS protein expression appear to be negligible. These findings and the possible intracellular mechanisms are consistent with a previous report that inhibition of luminal Na^+/H^+ exchangers (NHE) on MD cells augments the TGF system [25]. On the other hand, acute increase in NO generation after the removal of EIPA (Fig. 5) is, from another angle, inconsistent with the finding of the Western blotting analysis (Fig. 9). It is speculated that instantaneous alkalinization (or normalization) in the pH_i may stimulate NO generation, although nNOS protein expression was never induced under the pretreated conditions of either furosemide plus EIPA or low Na^+ media. Thus, the present study may provide several valuable leads to help solve the apparent discrepancies in [NaCl]-dependent nNOS expression and NO generation among different researchers [5, 7, 11, 13–16].

In this study, pH_i in NE-MD cells was measured using the fluorescent probe BCECF-AM and was calibrated by a unique method [27]. Initial measurements of pH_i (control) were conducted in the medium of a normal solution without EIPA, an inhibitor of the Na^+/H^+ exchanger. The slope of the relationship between pH_i in a log scale and extracellular [Na^+] in [mM] was almost linear in the range of 40–145 mM, but was steeper in the range of 10–40 mM. The pH_i obtained in NE-MD cells was 7.0–7.2 in 10–70 mM [Na^+] and was approximately 7.2–7.4 in 70–145 mM [Na^+]. These values are similar to those obtained in a variety of epithelial cells, including MD [23]. According to previous studies, luminal [NaCl] at the MD varied between 25 and 75 mM [29, 30]. These observations suggest that pH_i in MD cells may quickly vary up and down in response to normal physiological changes in [NaCl] [23]. It should be noted that NOS activity is strongly pH-sensitive [23, 28, 31].

We speculate that pH_i in NE-MD cells may be controlled by the functional activity and the expression level of NHE isoforms in the cell membrane [19, 32]. Interestingly, after adding EIPA to the medium, pH_i was dose-dependently decreased. The inhibitory potency (K_i) of EIPA was determined to be approximately 1 μM. This is consistent with the previous finding that luminal NHE isoform in the macula densa was NHE2 [19]. Moreover, there was no significant difference in the initial pH_i when NE-MD cells were incubated with or without furosemide. This suggests that NHE2 may not be functional under the control conditions (140 mM NaCl). However, when NE-MD cells were pre-incubated in low Cl^- with 140 mM Na^+, L-Arg-induced NO generation was significantly increased, and this was reversed in the presence of amiloride. This may suggest that decreases in intracellular [Na^+] and [Cl^-] due to an inhibitory effect on Na^+–K^+–$2Cl^-$ cotransport, which presumably increases proton efflux through the activation of NHE and stimulates the nNOS protein expression. Overall effects of low [Cl^-] stimulate the L-Arg-induced NO generation in MD cells.

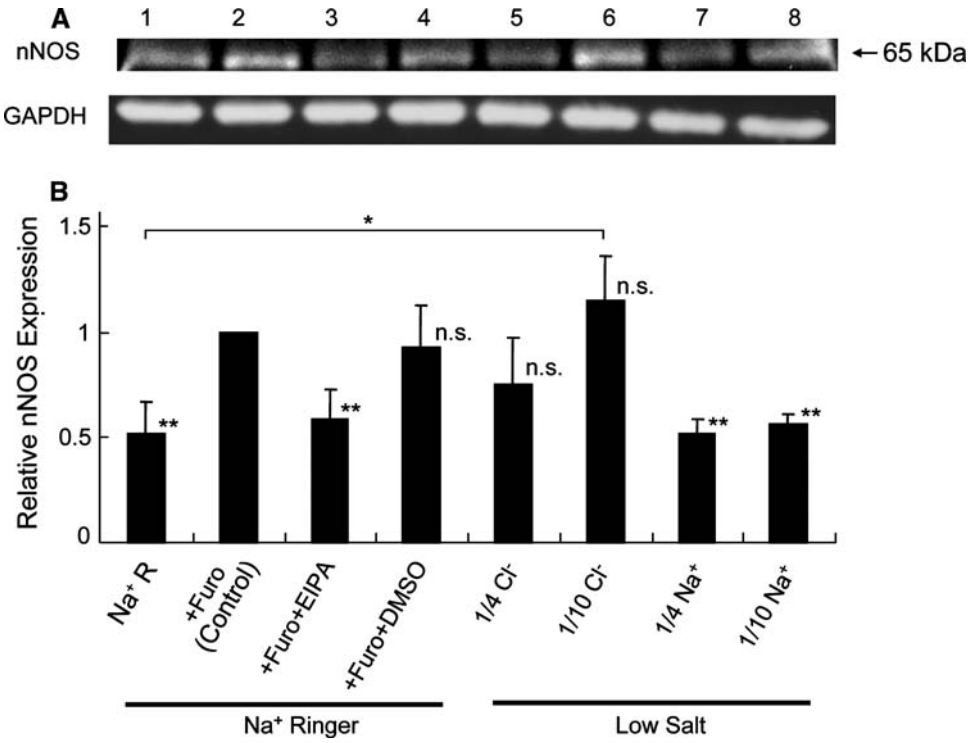


Fig. 9 Expression of nNOS protein in NE-MD cells pre-treated under various conditions (Western blotting). **a** Representative staining of nNOS protein expression (from *left* to *right*) in Na^+ Ringer (lane 1), furosemide-treatment (lane 2), furosemide-treatment plus EIPA (lane 3), furosemide-treatment plus DMSO (lane 4), 1/4 Cl^- (lane 5), 1/10 Cl^- (lane 6), 1/4 Na^+ (lane 7), and 1/10 Na^+ (lane 8). Various levels of a ~65-kDa band were observed. Staining of corresponding GAPDH was used for normalization; 2 μg protein in each lane. **b** The relative staining values are 0.52 ± 0.15 (Na^+ Ringer), 1 [+Furo (control)], 0.59 ± 0.14 (+Furo+EIPA), 0.93 ± 0.21 (+Furo+DMSO), 0.75 ± 0.22 (1/4 Cl^-), 1.16 ± 0.21 (1/10 Cl^-), 0.51 ± 0.08 (1/4 Na^+), and 0.57 ± 0.04 (1/10 Na^+). $^{**}P < 0.02$ compared with control. $^{*}P < 0.05$ compared with Na^+ Ringer (1st column). The experimental number is 4 except for lanes 3 and 4 of $n = 3$

In the kidney, NO has many physiological roles in the regulation of renal hemodynamics, including the glomerular filtration rate and medullary blood circulation via relaxation of vascular tone [7, 11, 20]. The net effect of NO generated in the kidney results in natriuresis and diuresis [9]. A part of the function of the kidney macula densa (MD), control of the body fluid volume in response to acute and chronic alterations in salt balance [7, 11], may depend on the [NaCl]-dependent NO release from the MD. The present study suggests that L-Arg-induced NO generation in MD cells can be influenced by at least two processes in response to changes in luminal [NaCl]. First, MD cell pH varies along with changes in the extracellular [NaCl]. A low flow rate along the distal tubule, including the MD region, is associated with low luminal [Na^+] and probably acidified MD cells, while high flow rates result in high luminal [Na^+] and alkalinized MD cells. Second, nNOS protein expression was stimulated by inhibition of NaCl entry through Na^+–K^+–$2Cl^-$ cotransport in the presence of furosemide. However, under physiological conditions (25–75 mM NaCl, [29, 30]), an increase in nNOS protein expression did not occur, and NO generation was negligible in the present study. This is probably due to intracellular acidification, while a higher luminal [Na^+] in proportion to the high flow rates alkalinizes the MD cells. At this point, NO generation would be instantaneously stimulated upon changes in the pH_i, if the nNOS protein expression were sufficient. The net effect of increased luminal [NaCl] is alkalinization of the pH_i in MD via activation of the luminal Na^+/H^+ exchanger. Thus, higher [NaCl]-dependent NO release from the macula densa is induced by the acute normalization of pH_i. The low luminal [Na^+] failed to induce the nNOS protein expression probably because of the accompanying acidification in the cell. It is still uncertain whether prolonged low [NaCl] conditions may induce the nNOS protein expression or not.

In conclusion, low [Cl^-] as well as addition of furosemide in the lumen at the MD increased nNOS protein expression and L-Arg induced NO generation. Changes in the luminal [NaCl] may modulate the TGF system via an effect on the NO generation from the MD.

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