Neuronal Nitric Oxide Synthase: Its Role and Regulation in Macula Densa Cells

GERGELY KOVÁCS,*† PÉTER KOMLÓSI,*† AMANDA FUSON,* JÁNOS PETI-PETERDI,*† LÁSZLÓ ROSIVALL,†‡ and P. DARWIN BELL*

*Nephrology Research and Training Center, Division of Nephrology, Departments of Medicine and Physiology, University of Alabama at Birmingham, Birmingham, Alabama; †International Nephrology Research and Training Center, Institute of Pathophysiology, Semmelweis University, Budapest, Hungary; and ‡Hungarian Academy of Sciences and Semmelweis University Nephrology Research Group, Budapest, Hungary

Abstract. Macula densa (MD) cells detect changes in distal tubular sodium chloride concentration ([NaCl]L), at least in part, through an apical Na:2Cl:K co-transporter. This co-transporter may be a site for regulation of tubuloglomerular feedback (TGF), and recently angiotensin II (Ang II) was shown to regulate the MD Na:2Cl:K co-transporter. In addition, nitric oxide (NO) produced via neuronal NO synthase (nNOS) in MD cells attenuates MD-TGF signaling. This study investigated [NaCl]L-dependent MD-TGF production. Co-transporter activity by NO, and the possible interaction of NO and Ang II. MD cell Na+ concentration ([Na+]i) and NO production were measured using sodium-binding benzofuran isophthalate and 4-amino-5-methylamino-2',7'-difluorescein diacetate, respectively, using fluorescence microscopy. Na:2Cl:K co-transport activity was assessed as the initial rate of increase in [Na+]i when [NaCl]L was elevated from 25 to 150 mM. 10^{-4} M 7-nitroindazole, a specific nNOS blocker, significantly increased by twofold the initial rate of rise in [Na+]i, when [NaCl]L was increased from 25 to 150 mM, indicating co-transporter stimulation. There was no evidence for an interaction between the stimulatory effect of Ang II and the inhibitory effect of NO on co-transport activity, and, furthermore, Ang II failed to alter MD-NO production. NO production was sensitive to [NaCl]L but increased only when [NaCl]L was elevated from 60 to 150 mM. These studies indicate that MD-NO directly inhibits Na:2Cl:K co-transport and that NO and Ang II independently alter co-transporter activity. In addition, generation of MD-NO seems to occur only at markedly elevated [NaCl]L, suggesting that NO may serve as a buffer against high rates of MD cell transport and excessive TGF-mediated vasoconstriction.

Macula densa (MD) cells serve as a distal nephron sensor element detecting changes in tubular fluid composition and transmitting information to afferent arteriolar smooth muscle cells (tubuloglomerular feedback [TGF]) and renin-containing granular cells (1). The entry of sodium chloride into MD cells occurs primarily through a furosemide-sensitive apical Na:2Cl:K co-transporter (NKCC2) and the Na:H exchanger NHE2 (2–4). Alterations in luminal sodium chloride concentration ([NaCl]L) initiate a number of electrochemical processes in MD cells, including changes in intracellular ion concentrations (3,5–7), membrane potential (2,4), and cell volume (8,9). Recently, it has been suggested that MD cells communicate with the underlying mesangial cell/smooth muscle/granular cell juxtaglomerular complex via the basolateral release of ATP, prostaglandin E2, and production of nitric oxide (NO) (10–13).

NaCl entry into MD cells seems to be a necessary step in signal generation by these cells. It is also possible that this step may be a critical regulatory point in controlling MD cell communication. For instance, with the identification of angiotensin II (Ang II) AT1 receptors in MD cells (14), it was found that Ang II at nanomolar concentrations stimulated Na entry and cell alkalization via Na:H exchange in MD cells (3). We have also recently reported that Ang II, at least in the same concentration range, directly stimulates Na:2Cl:K co-transport activity in MD cells (5). This stimulation of NaCl entry into MD cells by Ang II is consistent with Ang II–mediated enhancement of TGF responses (15,16).

There has been considerable interest in the role of NO in TGF and renin secretion. This was initially based on immunohistochemical findings of a high level of neuronal NO synthase (NOS) expression in MD cells (17,18). It has been presumed that the regulation of MD NO production is under the control of MD apical transport, although this point has not been well established. However, there is a wealth of information regarding the role of NO in MD-TGF signaling. Local, intratubular administration of non–isoform-specific NOS blockers or 7-nitroindazole (7-NI), which selectively blocks nNOS, was shown to enhance TGF responses (18–22). NO has also been shown to participate in renin secretion with current evidence indicat-
ing that MD NO production stimulates renin release. Taken together, NO derived from MD cells seems to attenuate TGF responses, whereas it increases renin releases (23–25).

Several reports indicate that there may be an interaction between the NO and Ang II pathways. For instance, Carey et al. (26) observed an Ang II-AT2−-mediated renal vasodilatory-natriuretic pathway that involves NO. Also, four other groups demonstrated involvement of NO in AT1-dependent processes in the kidney (27–30), suggesting that there may be a close interaction between Ang II and NO.

In view of the existence of nNOS in MD cells and that MD NO affects TGF responses and renin release, we tested the hypothesis that NO may modulate apical Na:2Cl:K co-transport in MD cells. In addition, we directly determined the effects of apical transport on MD NO production and also evaluated possible NO–Ang II interactions in the regulation of MD Na:2Cl:K co-transport activity.

Materials and Methods
Preparation of MD
Studies were performed using the isolated-perfused cortical thick ascending limb (cTAL) with attached glomerulus–MD preparation as described in several recent publications (3,5–7,31). Briefly, individual cTAL with attached glomeruli were manually dissected from kidneys of New Zealand white rabbits that weighed 0.5 to 1 kg. The 4°C, isosmotic dissection solution, was a low NaCl Ringer’s solution composed of (in mM) 25 NaCl, 125 N-methyl-d-glucamine (replacing Na+), 125 cyclamate (replacing Cl−), 5 KCl, 1 MgSO4, 1.6 Na2HPO4, 0.4 NaH2PO4, 1.5 CaCl2, 5 d-glucose, and 10 HEPES for buffering pH to 7.4. Preparations were transferred to a thermostated Lucite chamber mounted on a Leitz Fluovert inverted microscope and bathed with an isosmotic modified Ringer’s solution (composition is same as above except that it contained 150 mM NaCl instead of NMDG-cyclamate). The cTAL was cannulated and perfused with the low NaCl Ringer’s solution. Both perfusate and bath solutions were bubbled with 100% O2, pH was adjusted to 7.4, and the bath temperature was maintained at 38°C.

[Na+]i Measurement
[Na+]i of MD cells was measured using dual-excitation wavelength fluorescence microscopy (Photon Technologies Inc., Lawrenceville, NJ) and sodium-binding benzofuran isophthalate (SBFI) as described previously (5,6). Briefly, MD cells were loaded by adding the Na+-sensitive fluorescence probe SBFI-AM (50 µg), dissolved in DMSO containing 5 mg/ml Pluronic F-127 to the luminal perfusate. An adjustable photometer sampling window was positioned over the whole MD plaque, and SBFI fluorescence was measured at an emission wavelength of 510 nm in response to excitation wavelength of 340 nm and 380 nm. After approximately 15 min of loading, an additional 10 min was allowed to elapse for stabilization of fluorescence intensities at both wavelengths. SBFI fluorescence ratios (340/380 nm) were converted to [Na+]i values using an equation that was derived from the calibration procedure described previously (5,6). Briefly, after 10 µM monensin + nigericin was added to both lumen and bath, intratubular and bath [Na+] were increased in a stepwise manner from 0 to 150 mM. Linear regression was applied to transform raw ratios to [Na+]i values. We measured the following parameters: resting [Na+]i in MD cells under control conditions (isosmotic 25 mM luminal and 150 mM bath [NaCl]) and the magnitude (Δ[Na+]i) and initial rate (Δ[Na+]i/Δt) of increases in [Na+]i when [NaCl]L was increased from 25 to 150 mM (calculated from a linear fit using PTI software). Experiments consisted of monitoring SBFI ratio for a period of approximately 100 s to ensure that the ratio was stable, followed by assessing changes in the SBFI ratio during an increase in [NaCl]L. Once the ratio had stabilized, [NaCl]L was then returned to the low [NaCl] solution to assess reversibility. Measurements were obtained under control conditions or with addition of hormones or drugs that were added to the lumen 2 to 3 min before the increase in [NaCl]L.

Intracellular and Extracellular [NO]i Measurements
A recently developed family of fluorescence dyes, diaminofluoresceins (DAF), react specifically with NO to produce intensely fluorescence triazole derivatives without interacting with NO2−, NO−, or other oxygen species, including ONOO−, O2−, and H2O2 (32). To assess NO production in MD cells, we used a newly improved DAF analog, 4-amino-5-methylamino-2',7'-difluorescin diacetate (DAF-FM DA) (33), that is very sensitive to NO (3 nM detection limit). Like all of the DAF, DAF-FM DA binds NO covalently, which results in the highly fluorescent DAF-FM T form. In contrast to other forms of DAF, DAF-FM DA is insensitive to changes in pH (34).

Fluorescence activity of DAF-FM T was measured at an emission wavelength of 530 nm in response to an excitation wavelength of 495 nm. MD cells were loaded with the NO-sensitive dye in a manner similar to SBFI-AM by adding 50 µg of DAF-FM DA to the low [NaCl] perfusate for 20 min. The DAF-FM T fluorescence intensity was normalized to the initial values. With the use of this single wavelength excitation fluorescence probe, any increase in fluorescence of intracellular DAF-FM T represents an increase in MD NO level, whereas any decreases in fluorescence intensity could not be caused by a lowering in NO level as a result of the covalent bond between NO and dye molecules. In MD cells, with reductions in [NaCl]L, we have observed decreases in DAF-FM fluorescence, which we attribute to cell shrinkage and dye quenching. Consequently, this series of studies were limited to testing the effects of agents on MD NO production that should not produce large changes in cell volume, including Ang II, l-arginine, and spermine NONOate. A very important question concerns the effects of changes in [NaCl]L on MD NO production. However, alterations in [NaCl]L produce marked changes in MD cell volume (8,9,35), which can drastically affect the fluorescence intensity of a dye that is located intracellularly. To rule out the confounding effect of changes in cell volume, we developed another approach to test for [NaCl]L-dependent NO production and release from MD. DAF-FM (the nonpermeable form of DAF-FM DA; 2 µM) was continuously administered into both the bath and the luminal solutions to assess changes in extracellular NO levels. Because NO is a gas, it should be readily permeable to cell membranes, and therefore measuring extracellular NO levels should reflect intracellular NO generation. The rate of bath superfusion was reduced to 0.5 ml/min to allow sufficient time for dye–NO interactions. Instead of photometry, these studies were performed with the use of an imaging-based system (Photon Technologies) and a SenSys cooled charged-coupled device. Region of interest windows (ROI) were used to capture fluorescence intensity at the extraglomerular mesangium adjacent to the MD, the apical region of the MD, and at the basolateral region of the cTAL that lies opposite the MD. An ROI was also placed away from glomerular–MD preparation to obtain a value for background intensity, which was then subtracted from the values obtained from the other ROI. Traces from the ROI were normalized to control values obtained in the presence of 25 mM [NaCl]L.
Materials

Angiotensin II (Ang II), L-arginine, 7-NI, and furosemide were purchased from Sigma (St. Louis, MO). Spermine NONOate and Nω-propyl-L-arginine were obtained from Cayman Chemicals (Ann Arbor, MI). The fluorescence dyes SBFI-AM and DAF-FM DA were purchased from Teflabs (Austin, TX) and Molecular Probes (Eugene, OR), respectively.

Statistical Analyses. Data are expressed as means ± SEM. Statistical significance was tested using paired or unpaired t test. Significance was accepted at P < 0.05. N represents the number of preparations.

Results

Effects of 7-NI, L-Arginine, and Ang II on Apical Na: 2Cl:K Co-transport Activity

Under control conditions (25 mM NaCl in the perfusate and 150 mM NaCl in the bath), resting MD [Na\(^+\)] was 23 ± 1.4 mM (n = 45). As depicted in Figure 1 and summarized in Figures 2 and 3, when luminal [NaCl] ([NaCl]\(_L\)) was elevated from 25 to 150 mM, [Na\(^+\)] was increased (ΔNa\(^+\)) by 21.5 ± 1.6 mM (n = 6) at an initial rate (ΔNa\(^+\)/Δt) of 6.8 ± 0.8 × 10\(^{-4}\) mM/s. Consistent with what we reported previously, the magnitude and the rate of this increase was diminished by 80% in the presence of 10\(^{-4}\) M furosemide (data not shown) (5,6). As depicted in Figures 1 and 2, administration of 10\(^{-5}\) M luminal 7-NI, a specific nNOS blocker, substantially increased both ΔNa\(^+\) and ΔNa\(^+\)/Δt. This effect was eliminated by the addition of furosemide. In an effort to stimulate nNOS activity in MD cells, we added 10\(^{-3}\) M L-arginine (substrate of NOS) to the luminal perfusate. Figure 3 shows that 10\(^{-3}\) M luminal L-arginine had no effect on ΔNa\(^+\) or ΔNa\(^+\)/Δt.

Other studies were performed to investigate a possible interaction between NO and Ang II. As shown in Figure 4, 10\(^{-9}\) M (low dose) luminal Ang II increased co-transport activity by approximately 1.5-fold, whereas 10\(^{-6}\) M (high dose) Ang II had no effect on ΔNa\(^+\) and ΔNa\(^+\)/Δt. Combination of either low- or high-dose Ang II with 10\(^{-4}\) M 7-NI did not modify the stimulatory effect of 7-NI, suggesting a lack of interaction of Ang II and NO in regulating apical co-transport activity.

Effect of NO Donors and Ang II on MD NO Level

MD cells were loaded with DAF-FM DA to monitor changes in NO production. As depicted in Figure 5A, under control conditions, the rate of change in fluorescence activity slowly declined over 10 min. This gradual decrease in fluorescence activity reflects both dye leakage (decrease in fluorescence) and the effects of basal NO generation (increase in fluorescence). Administration of 10\(^{-4}\) M Spermine NONOate (an exogenous NO donor) into the bath caused a large increase in fluorescence activity within 3 min (Figure 5, B through D). These data suggest that an elevation in MD NO concentration can be detected in MD cells. As it is shown in Figure 6, however, neither 1 mM L-arginine nor nanomolar or micromolar concentrations of L-arginine had any significant effect on MD NO production.
lar concentrations of Ang II had a significant effect on NO production in MD cells.

**Effect of [NaCl]L and Ang II on MD NO Production**

The nonpermeable form of DAF-FM DA, DAF-FM, was added to the bath and lumen to detect NO release from MD and cTAL cells. As shown in Figure 7 and summarized in Figure 8, there was no significant increase in NO when [NaCl]L was changed from 25 to 60 mM. In contrast, a further increase in [NaCl]L from 60 to 150 mM resulted in the production and release of NO across both MD basolateral and apical plasma membranes, whereas there was no significant NO production and release across the basolateral membrane of the cTAL (Figures 7 and 8). Production of NO from MD was clearly inhibited by 100 μM 7-NI administrated into both the lumen and the bath (Figure 8). Finally, measurements were performed to examine the effect of 10−6 M bath Ang II on MD NO production using extracellular DAF-FM. Ang II had no effect on MD NO production in the presence of 25 mM luminal NaCl, which is consistent with our previous data obtained with the intracellular DAF-FM DA (n = 4; data not shown).

**Discussion**

Alterations in [NaCl]L and osmolality, resulting from changes in flow rate along the TAL, produce a highly complex series of intracellular events at the MD. With an elevation in [NaCl]L, there are increases in MD cell pH, Na+, and Ca2+ (3,5–7). There is also cell swelling; basolateral membrane depolarization; and a host of changes in intracellular enzymes, transporters, and channels (36). All of these events are directly or indirectly linked to the entry of NaCl into these cells and perhaps also to changes in luminal osmolality (cell volume). Previous studies have estimated that there is normally an 80/20% split between Na:2Cl:K co-transport and Na:H exchange for the entry of Na+ across the apical membrane. Therefore, the co-transporter is responsible for the majority of Na+ entry into these cells, and understanding what controls Na:2Cl:K co-transport is important in understanding MD cell signaling.

NO plays a critical role in the regulation of renal blood flow, GFR, renin secretion, and sodium excretion (23–25). In terms of TGF, numerous micropuncture studies have shown that nNOS-specific and nNOS-nonspecific inhibitors (18,20,37,38) acutely enhance TGF responses and feedback sensitivity. However, there are some uncertainties regarding the role of NO in TGF. For example, Ren et al. (39) reported that the MD-NOS system decreased TGF responses through a soluble guanylate cyclase-cGMP-dependent protein kinase pathway, whereas in other work, they suggested that NO produced by eNOS in TAL cells might also inhibit TGF responses (21). In addition, NO is produced by other cells of the juxtaglomerular apparatus, including vascular endothelial cells, and this source of NO may play a role in controlling vascular resistance and renin release. For instance, endothelium-derived NO seems to inhibit renin release via a cGMP-dependent pathway (40), whereas NO derived from MD nNOS may directly stimulate renin secretion (41,42). Furthermore, there seems to be a long-term TGF adaptation to chronic inhibition of nNOS or in nNOS-deficient mice (19,43). In either case, TGF responses seem to be normal when nNOS is chronically inhibited. Thus, the MD-NOS system is complex; however, what has clearly been shown is that acute manipulation of nNOS activity can alter feedback sensitivity or responsiveness.

In a review article, Ortiz and Garvin (44) summarized the effects of NO on tubular ion transport processes. For the cTAL, they reported that NO produced by eNOS decreased net NaCl
flux through inhibition of the Na:2Cl:K co-transporter activity via NO-cGMP-phosphodiesterase II–mediated decreases in cAMP levels (45,46). The regulation of the other major NKCC isoform (NKCC1) is also inhibited by NO as shown in rat smooth muscle cells (47).

Because MD cells express high levels of nNOS and because MD-NO production seems to alter TGF and renin release, we elected to determine whether NO could directly affect MD Na:2Cl:K co-transport. This was accomplished by measuring intracellular \([\text{Na}^+]\) using the fluorescence probe SBFI. As documented in our previous work (5), changes in \([\text{Na}^+]\), in MD cells can serve as an indicator of Na:2Cl:K co-transport activity. We found that addition of 7-NI, a specific inhibitor of nNOS, when added to the lumen, significantly enhanced the magnitude and rate of increase in \([\text{Na}^+]\) obtained with increases in \([\text{NaCl}]_\text{L}\) from 25 to 150 mM. This effect occurred via the co-transporter because the concomitant addition of furosemide reduced the magnitude and rate of change in

\([\text{Na}^+]\) to increased \([\text{NaCl}]_\text{L}\) by >80%. We also investigated the effect of \(5 \times 10^{-4}\) M \(\text{L}-\text{arginine}\), another nNOS-specific inhibitor, and confirmed that it increased co-transporter activity, too (data not shown). In other work, we tested the effects of addition of \(10^{-3}\) M \(\text{L}-\text{arginine}\) added to the lumen on MD Na transport. \(\text{L}-\text{arginine}\), a precursor of NO, did not alter Na transport in MD cells with increases in \([\text{NaCl}]_\text{L}\). This lack of an effect of \(\text{L}-\text{arginine}\) can be interpreted in several ways. The first possibility is that the system \(y^+\), which is usually responsible for \(\text{L}-\text{arginine}\) transport, is not located on the apical membrane, and therefore there is no transport of \(\text{L}-\text{arginine}\) into MD cells. Second, it is possible that there is a high endogenous level of nNOS activity in MD cells that is not substrate limited. On the basis of the work performed by Welch and Wilcox (48) showing the existence of transport \(y^+\) at the apical membrane of MD cells in rats, the latter explanation seems to be the more likely. In previous micropuncture studies in rats on a normal salt intake, \(10^{-2}\) M luminal \(\text{L}-\text{arginine}\) attenuated TGF responses, whereas \(5 \times 10^{-4}\) M was ineffective (18,20). Thus, it is also possible that a higher concentration of \(\text{L}-\text{arginine}\) might have attenuated co-transport activity in our studies. Nevertheless, these studies clearly demonstrate that NO produced by the MD cells suppresses apical Na:2Cl:K co-transport in these cells. That nNOS blockade was able to stimulate markedly co-transporter activity supports a high endogenous level of NO production in MD cells.

In previous studies, we focused on the effects of Ang II on MD cell function because Ang II is a specific positive modulator of TGF responses. Although there is clearly a direct vascular effect of Ang II, it was also shown that MD cells possess \(\text{AT}_1\) receptors (14). Thus, we examined the effects of Ang II on Na/H exchange and Na:2Cl:K co-transport activity in MD cells. We found that this hormone, at least in nanomolar concentrations, activated both of these ion transport processes, whereas Ang II at micromolar concentrations failed to stimulate both Na/H and Na:2Cl:K co-transport. This suggested that the modulator effects of nanomolar Ang II on TGF might occur by directly stimulating the transport rate of NaCl into MD cells. This concept was recently supported by the findings of Wang et al. (16), who reported nanomolar Ang II potentiation of TGF via luminal \(\text{AT}_1\) receptors in MD cells.
Recently, there was work reporting an interaction between NO and Ang II-AT1 receptors in kidney. Stimulation of NO production by Ang II via AT1 receptors in vitro has been described in rat proximal tubule cells (30), cortical collecting duct cells (29), and renal resistance arteries (38). However, it was suggested that AT1A receptors enhance TGF-mediated afferent arteriole constriction, at least in part, through reducing the counteracting modulation by nNOS (27). Because nanomolar but not micromolar Ang II stimulates co-transport activity, we asked the following question: “Is the lack of transport stimulation at micromolar Ang II due to the counteracting or negating influence of enhanced NO production?” In this regard, we found that 7-NI abolished the biphasic effect of Ang II, and so, in the presence of both low and high concentrations of Ang II, there was stimulation of Na:2Cl:K co-transport activity. There are at least two possible explanations for the stimulation of co-transport in the presence of 7-NI and micromolar Ang II. First is that micromolar concentration of Ang II stimulate NO production, which then antagonizes the stimulatory effects of Ang II. This possibility was readily testable using the new fluorescence probes for NO and directly measuring the effects of Ang II on MD NO production. The second possibility is that inhibition of NO production stimulates co-transport activity independent of the effects of Ang II. In this case and for reasons that are not known, high concentrations of Ang II do not lead to stimulation of co-transport activity.

The newly developed NO-sensitive fluorescent dye DAF-FM DA reacts covalently with NO but not with any other reactive oxygen species, resulting in a pH-insensitive, stable fluorescence form: DAF-FM T (33,34). We tested the fluorescence activity of DAF-FM T in MD cells and found that it decreased slightly over time, which was presumably due to a balance between dye leakage and endogenous NO production. Administration of 100 μM Spermine NONOate, a stable NO donor, markedly increased DAF-FM fluorescence activity in MD cells, suggesting that this method is a useful tool for investigation of intracellular NO level in MD cells.

Figure 7. Fluorescence images of an MD preparation in the presence of extracellular DAF-FM at 25 (A), 60 (B), and 150 mM (C) luminal NaCl concentrations. (D) Brightfield image of the same preparation. Blue area represents MD. (E and F) Subtracted images showing the differences in DAF-FM fluorescence when luminal NaCl was changed from 25 to 60 and from 60 to 150 mM, respectively. The red area within the glomerulus is due to the presence of a holding pipette that was used to stabilize the glomerulus at the bottom of the chamber.
mediated decrease in NO production as suggested by Ichihara et al. (27) in AT1A-deficient mice. Again, with the covalent binding of the dye to NO, it is not obvious how one can design experiments to test for a decrease in NO production.

Workers in the area of MD NO–TGF interactions have long maintained that increases in the apical entry of NaCl should be associated with nNOS stimulation and enhanced NO production (25). Thus, we were very interested to determine the effects of increased [NaCl]L on NO production. What we found is that an increase in [NaCl]L resulted in a marked increase in MD cell fluorescence that would be consistent with an increase in NO production. Unfortunately, with a return of [NaCl]L to 25 mM, MD cell fluorescence returned to control values. Because this dye binds covalently to NO, there should have been no return of the signal when [NaCl]L was lowered (data not shown). We interpret the increased fluorescence signal as [NaCl]L was elevated to be the unquenching of the dye as a result of an increase in MD cell volume (9). The decrease in signal with a return to 25 mM [NaCl]L simply reflects cell shrinkage and a quenching of the fluorescence signal. Thus, it was our opinion that this dye could not be used when there are large excursions in cell volume. During this time, there was a very little effect on MD NO levels. However, in a recent study (8), it was demonstrated that $5 \times 10^{-3}$ M l-arginine added to the bath or the lumen increased NO level in MD cells by 17 and 30%, respectively. Importantly, we found that addition of Ang II did not increase MD NO levels at either nanomolar or micromolar concentrations. Thus, these experiments eliminate the possibility that micromolar Ang II fails to stimulate co-transport as a result of augmented MD NO production. Inhibition of NO by 7-NI still enhanced co-transporter activity even in the presence of a high level of Ang II, suggesting an independent action of Ang II and NO. It should be stated that this work does not exclude the possibility that the stimulation of the co-transporter at nanomolar concentrations might involve an Ang II–mediated decrease in NO production as suggested by Ichihara et al. (27) in AT1A-deficient mice. Again, with the covalent binding of the dye to NO, it is not obvious how one can design experiments to test for a decrease in NO production.

Figure 8. Effect of luminal NaCl on the rate of change in DAF-FM intensity at the basolateral and apical regions of the MD as well as at the cortical thick ascending limb. Values are means ± SEM; *P < 0.05 (n = 4)

In our studies, we were surprised to find that the largest increase in MD NO production occurred when [NaCl]L was increased from 60 to 150 mM. The uniqueness of this observation in TGF physiology cannot be overemphasized. Previous work measuring in vivo and in vitro TGF responses, MD basolateral membrane potential, Na:2Cl:K co-transport, [Ca2+]i, and [Na+]i all have found that the dynamic range for changes in these indices reaches the maximum at approximately 60 mM. Thus, the finding that the largest change in NO production occurs when [NaCl]L is >60 mM strongly suggests that the activation of nNOS is not occurring through apical NaCl entry via Na:2Cl:K co-transport. As previously reported (49), the apical Na:H exchanger does not saturate at 60 mM [NaCl]L. However, if further MD cell alkalization up to a [NaCl]L of 150 mM. Whether MD nNOS is activated by Na:H exchanger-induced cell alkalization is clearly an important question that remains to be answered. The relationship between other non-MD forms of nNOS and pH seems to be less than definitive, although this NOS isoform is generally considered to be pH sensitive. However, our studies are, in fact, consistent with recent studies by Garvin and colleagues (50), who reported that modulation of in vitro TGF responses was enhanced using an inhibitor of apical Na:H
exchanger; they suggested that this effect was occurring through MD NO based on concomitant work with 

There are several conclusions that can be gleaned from these studies. First, our data suggest that MD NO, among its other functions, acts as a break or buffer system in MD cells when there are large increases in \([\text{NaCl}]_L\). This idea is consistent with mechanisms in other epithelial cells that result in inhibition of apical electrolyte entry when transport rates are high (51). It might also suggest that on a minute-to-minute basis, NO does not in fact inhibit NO production. This is certainly an area that needs to be addressed experimentally as we further explore the MD cellular mechanisms that are involved in NO production.

Acknowledgments

This work was supported by grants from the National Institute of Diabetes and Digestive and Kidney Diseases (DK-32032), AstraZeneca, and the Hungarian Research Foundation (OTKA 29260, FKFP-290, 1989). This work was also supported by a NATO fellowship (P.D.B.).

G.K. and P.K. are postdoctoral fellows from the Institute of Pathophysiology, Semmelweis University Medical School, Budapest, Hungary.

Special thanks to Martha Yeager for secretarial assistance.

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