Published in final edited form as:

Nitric Oxide. 2007 August ; 17(1): 33–43.

Nitric Oxide Production by Mouse Renal Tubules Can Be Increased by a Sodium-Dependent Mechanism

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Abstract

Renal tubules process large amounts of NaCl that other investigators indicate increases tubular generation of nitric oxide. We questioned whether medullary or superficial cortical tubules would have the greater increase in nitric oxide concentration, [NO], when stressed by sodium and if the sodium/calcium exchanger was involved. Sodium stress in proximal tubules is due to the large amount of sodium absorbed and medullary tubules exist in a hypertonic sodium environment. To sodium stress the tissue, mouse kidney slices were exposed to monensin to allow passive entry of sodium ions from isotonic media and in separate studies, 400 and 600 mOsm NaCl was used. [NO] was measured with microelectrodes. Monensin (10 μ M) caused a sustained increase in medullary and cortical [NO] to ~180% of control and 400 mOsm NaCl caused a similar initial increase in [NO] that then subsided. 600 mOsm NaCl caused a more sustained increase in [NO] of >250% of control. L-NAME strongly attenuated the increased [NO] during sodium stress. The increase in [NO] during NaCl elevation was due to sodium ions because mannitol hyperosmolarity caused ~20% of the increase in [NO]. Entry of sodium during NaCl hyperosmolarity was through bumetanide sensitive channels because the drug suppressed increased [NO]. Blockade of the sodium/calcium ion exchanger strongly suppressed the increased [NO] during monensin, to increase sodium entry into cells, and the elevated NaCl concentration. The data support a sodium - NO linkage that increased NO signaling in proportion to sodium stress by cortical tubules and was highly dependent upon sodium-calcium exchange.

Keywords

Kidney; Tubules; Monensin; Hyperosmolarity; Nitric Oxide

Introduction

There may be a similar nitric oxide generation and sodium ion relationship in the endothelial cells of the small intestine and the tubules of the kidney. When *in vivo* arterioles of the small intestine were exposed to hypertonic 320-380 mOsm NaCl, such as that associated with villus nutrient absorption[1], there was an immediate and sustained increase in nitric oxide concentration, [NO][2;3]. Equivalent mannitol hyperosmolarity caused both much smaller increases in intestinal perivascular [NO] and arteriolar dilation [2-4]. In the intestinal endothelial cells, sodium ions **predominantly** enter the endothelial cells through Na⁺/K⁺/2Cl⁻ cotransport because bumetanide suppressed both increased [NO] and vasodilation [3].

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Once sodium is within the cells, the $\text{Na}^+/\text{Ca}^{+2}$ transporter must be active for increased [NO] to develop and cause the subsequent increase in [NO] and simultaneous vasodilation [3]. A similar set of mechanisms may exist in renal tubules because they have endothelial and neuronal nitric oxide synthase enzymes (eNOS, nNOS) [5;6], a variety of sodium uptake mechanisms, including the $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransporter [7] and Na^+/H^+ cotransporters [8;9], and absorb a great deal of NaCl from both isotonic and hypertonic lumen solutions. In addition, Moeckel et al. [10] have shown that increased extracellular Na^+ from 144 to 262 mM activated Na^+/Cl^- dependent betaine transport by medullary tubular cells, as part of their response to sodium hyperosmotic stress.

A link between tubular absorption of sodium ions and NO generation has been shown with both *in vivo* and *in vitro* preparations. *In vivo* measurement of NO with NO sensitive microelectrodes by Levine et al. [11;12] have shown that sodium exposure caused a rapid increase in tubular lumen [NO] in distal cortical tubules. Using isolated thick ascending limb tubules of the rat, Ortiz et al [13] have shown NaCl increased NO production that suppressed the $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransport to limit further sodium uptake. In this same context, Eitle et al. [14] using *in vivo* split droplet techniques in rat proximal tubules and Rocznik and Burns[9] using isolated rabbit proximal tubules found exogenous NO decreased NaCl coupled fluid transport and cell uptake of sodium. These various studies support the evolving hypothesis that entry of sodium ions into tubular cells initiated a set of mechanisms that used NO to suppress further sodium absorption.

Our primary hypothesis is that renal tubular cells share a mechanism parallel to that found in endothelial cells in that when they are stressed by sodium entry, the exchange of sodium for calcium activates calcium dependent NOS. We also questioned whether cortical tubules, which were predominately proximal tubules by their location immediately beneath the kidney capsule, could respond to sodium stress with increased NO generation as well as medullary tubules that routinely exist in a high sodium environment. The proximal tubules of the cortex use NO as part of a feedback system to limit sodium absorption, as reviewed in 2000 by Liang and Knox [15] and subsequently expanded upon by Levine et al.[12], and Ortiz et al [13] in *in vivo* studies of the effect of sodium on distal cortical tubular NO generation. To have access to the medullary and proximal tubules, slices of mouse kidney in a nutrient physiological fluid at a high oxygen tension were used. Cortical and medullary tubules in slices are reported viable when well oxygenated, as demonstrated by the normal trafficking responses of specific plasma membrane proteins to hormones [16;17]. To cause a situation in which the tubular cells were forced to deal with a sustained influx of sodium ions that could not be stopped by cell processes, the sodium ionophore monensin was applied to the tissue to simulate increased sodium ion uptake from isotonic media. To simulate the hyperosmolarity of the deep medulla, tissue slices from mouse kidney were exposed to hypertonic NaCl solutions. Our initial goal was to study the tubules of the deep medulla since NaCl hypertonicity is a routine and physiological circumstance. For comparison, we also studied superficial cortical tubules. As will be shown, cortical tubules are quite responsive to forced entry of sodium ions using monensin and surprisingly, very responsive to mild and moderate elevations of NaCl. Obviously, superficial cortical tubules do not experience NaCl hypertonic environments, but they experience sodium stress in that they absorb as much as 70% of the filtered sodium load both directly and through sodium coupled glucose transport, as recently reviewed by Aronson [18]. In this context, monensin and elevated NaCl were tools to stress cells with sodium and we will show that nonspecific hyperosmolarity with mannitol caused a much smaller NO response.

Methods

Animal Issues

Male Swiss Webster mice (Harlan Ind., Indianapolis, IN) in the weight range of 20-25 grams were used for all studies. The protocols were approved by the Indiana University Medical School IACUC. The mouse was anesthetized with sodium thiopental at a dosage of 100 mg/kg given intraperitoneally. Once the animal was immobile, it was placed on a warming pad. At surgical depth anesthesia, the lower abdomen was opened along the mid-line and the aorta dissected free. A polyethylene catheter was used to cannulate the abdominal aorta for renal perfusion. The kidneys were perfused *in situ* via the abdominal aorta with 15 ml ice-cold PBS saturated with 95% oxygen/5% carbon dioxide and containing 1% heparin to minimize clotting.

In Vitro Preparation of Kidney Slices

Sagittal section kidney slices for *in vitro* study were cut 150-250 μm thick at the midpoint of the kidney mass. During sectioning, saline ice was used to maintain a $<4^{\circ}\text{C}$ in the vibratome and slices were transferred immediately to ice-cold bicarbonate buffered solution. The bicarbonate based buffer [19] contained 5 mM D-glucose and 0.1 mM arginine, and was pre-equilibrated with 95% O_2 /5% CO_2 . The sectioning procedure was completed within 30-40 min and slices remained viable for 3-5 hours when stored at 4°C in 95% oxygen saturated media. Only slices in which the tissue was intact without tears were used, including an intact tissue section of the entire papilla.

In Vitro Tissue Support

Tissue slices were transferred to an ice cold tissue bath with a 5 ml volume that was perfused with 9-10 ml/min of ice cold oxygenated (95% oxygen/ 5% carbon dioxide) physiological solution. The perfusion media passed over the tissue and was then drained away. To prevent the slice from being washed away by the rapidly flowing media, the slice was held in place by a grid of silk threads connected to a stainless steel support. Once the tissue was correctly placed, the bath was heated to 37°C over a 30 minute period. A circulating water pump with a 900 ml/min flow was used to heat the 112 gm Lucite tissue support. Once all materials were warmed, the partial pressure of oxygen about 1 mm above the tissue was consistently 500-600 mmHg, as measured with an oxygen sensitive microelectrode. The partial pressure of oxygen at the tissue surface was 50-100 mmHg, which represented oxygen consumption by the slice cells. As was previously reported by Yaqoob et al. [20] using suspensions of kidney cortical tubules, reduction of oxygen availability was associated with a large increase in [NO]. To evaluate if sufficient oxygen was available, we determined if elevation or reduction of the bath flow influenced the [NO] during 600 mOsm NaCl, which caused a very large increase in [NO]. So long as the bath flow was at or above 5-6 ml/min for a 5 ml bath volume, the NO response to elevated NaCl concentration was not influenced by raising the bath flow to 20 ml/min. As a routine procedure, bath flow was held at 10 ml/min. We did observe that bath flow lower than 3 ml/min did elevate the resting [NO] and reduced the peak [NO] during 600 mOsm NaCl, presumably in the latter case because inadequate oxygen was available for elevated NO generation.

Measurements of Nitric Oxide

The nitric oxide concentration [NO] was measured with NO sensitive microelectrodes based on techniques developed by Buerk et al [21] and Friedemann et al. [22] and modified by Bohlen [2]. The open tip of the 9-11 μm outer diameter tip of the glass microelectrode was the only site NO could be measured. Therefore, NO can only be measured in cells that the open tip of the microelectrode touched. A carbon fiber (7-8 μm) physically sealed to the glass lumen of microelectrode carried the +0.9V polarization voltage and current generated by NO oxidation.

All microelectrodes were treated with a Nafion membrane to minimize interference from other molecules [22]. The combination of the polarizing voltage and Nafion membrane made the microelectrodes highly selective for NO, as will be confirmed by a variety of tests presented in Results. In past studies of intestinal tissues, we found that by rapidly freezing and thawing tissues did not release molecules that changed baseline microelectrode current as the microelectrode tip entered the tissue [23]. We repeated these tests with kidney slices and again found that after freezing, the microelectrode current did not change when the tissue was impaled, other than a slight transient artifact due to momentary deformation of the electrode tip. These observations indicated that there were no molecules in kidney tissues that can survive freezing, such as amino acids, peptides, nitrite or nitrate compounds, which reacted with the microelectrode and generated a false signal.

Microelectrode current was measured with a Keithley Model 6517A electrometer that also provided the polarization voltage. The output of the electrometer in millivolts per picoampere was recorded using a PowerLab digital chart recorder (AD Instruments) at known [NO] generated in a World Precision Instruments tonometer (Sarasota, FL). The final calibration units were nanomoles of NO per millivolt output of the electrometer. The calibration at [NO] at 0, 600, and 1200 nM with precision NO gases in nitrogen yielded a calibration factor in nanomoles of NO per mv output (or change in picoamperes) of the electrometer. The "0" value for NO in the bathing media was obtained by placing the microelectrode tip the same distance above the support surface as the thickness of a tissue slice at a location upstream in the bath flow from the tissue slice. Essentially the same reference value was also obtained 200 μ m above the tissue surface of a slice because the high oxygen content of the bath neutralized NO molecules diffusing into the bath media.

Histochemical and Western Blot Analysis of NOS

For immunofluorescent staining, kidneys were perfused *in situ* with heparinized PBS followed by 15 ml freshly-prepared 4% paraformaldehyde in PBS, pH 7.4. Kidneys were removed and incubated with gentle agitation in paraformaldehyde/PBS for 90 min at room temperature. After cryoprotection by overnight incubation in 30% sucrose/PBS at 4°C, the kidneys were embedded in tissue-freezing medium (TBS, Durham, NC), and stored at -70 °C. Frozen sections (8 μ m thick) were cut with a cryostat at -20°C, air dried at room temperature and rehydrated in PBS for 30 min. Sections were permeabilized with 0.25% Triton X-100 in PBS for 10 min, quenched with 50 mM NH₄Cl for 10 min, and blocked with 10% normal goat serum in PBS for 3 hours at room temperature. Sections were incubated with primary antibodies overnight at room temperature in 2% serum/PBS at 1:200 dilution. Controls contained no primary antibody. Antibodies for NOS isoforms were polyclonal antibodies raised in rabbits against eNOS (N2643, Sigma-Aldrich, St Louis, MO), nNOS (61-7000, Invitrogen-Zymed, San Francisco, CA), and iNOS (610332, BD Biosciences, San Jose, CA). No cross-reactivity was detected in our previous study using the same sources of antibodies [24]. Sections were washed in three changes of PBS over a 1 hour period, followed by overnight incubation in 2% serum/PBS containing goat anti-rabbit IgG conjugated to Cy5 (Jackson ImmunoResearch, West Grove, PA) at 1:100 dilution. *Lotus tetragonolobus* (LTG) lectin [25] tagged with fluorescein (Vector Laboratories, Burlingame, CA) was also included at this stage at 1:200 dilution to identify proximal tubule cells. Sections were washed in three changes of PBS over a 3 hour period, and mounted in fluoromount-G (Southern Biotechnology, Birmingham, AL) for confocal microscopy. Microscope settings for detection of FITC and Cy5 fluorescence were described previously [26].

For Western blot analysis, kidneys were perfused prior to removal from the animal with heparinized PBS through the abdominal aorta to remove blood. Kidneys were bisected and cortex was carefully separated from outer and inner medulla under a dissecting microscope.

Tissues were homogenized in ice-cold 50 mM mannitol, 5 mM EGTA, 15 mM Tris (pH 7.4), and homogenates were centrifuged for 15 min at 13,000g and 4°C. The supernatants were removed and the protein content was determined with BCA reagent (Pierce, Rockford, IL). Supernatants were mixed with 4x sample buffer and boiled for 5 min before loading on a 7.5% polyacrylamide gel (150 µg protein per lane). After separation, proteins were transferred to nitrocellulose and blotted with rabbit antibodies to eNOS (1/3,000). The blot was stripped and reprobed sequentially with antibodies to nNOS (1/1000) and iNOS (1/1000) [24]. The secondary antibody was goat anti-rabbit IgG conjugated to horseradish peroxidase (1/3,000). Signals were detected with SuperSignal West Pico reagents (Pierce).

Statistics

All data were analyzed using Statistica software (StatSoft, Tulsa, OK). Two way repeated measures ANOVA with a significant difference chosen as $p < 0.05$ was used because each tissue location studied under control conditions was remeasured during the responses to a set of perturbations, including comparisons before and after various forms of pharmacological blockade or fluid composition changes. Where significance was found, a Tukey Least Significant Difference post-hoc test was used for individual comparisons and significance was chosen as $p < 0.05$.

Results

Nitric Oxide Response to Monensin Sodium Loading

The wall of tubules was observed at 200x to direct the microelectrode tip to a specific point of the tubule and the image was recorded to help relocate the same spot as needed in the course of the experiment. In Figure 1, the same site on a tubule was followed for the entire course of the experiment, including exposure to monensin to induce sodium entry into cells followed by KB-R7943 to block the sodium/calcium ion exchanger. The data in Figure 1 illustrate the cortical nephrons generated a much higher resting [NO] than did deep medullary nephrons and had a larger increase in [NO] after monensin was used to increase sodium ion permeability. However, on a relative basis, both regions about doubled their [NO] during monensin exposure. Monensin (10 µM) was applied for 30 minutes before new measurements were taken and remained thereafter.

The increase in [NO] during monensin was thought to be linked to increased accumulation of sodium ions, which were then exchanged for calcium ions by the sodium/calcium exchanger. 50 µM KB-R7943 was used to inhibit the reverse mode activity for exchange of sodium and calcium ions. After 30 minutes of KB-R7943 with monensin still in the media, the [NO] signal of the same tubule was reduced to or slightly below the original resting concentration for both cortical and deep medullary tubules. To verify that the microelectrodes could not respond to KB-R7943 or monensin, either 10 µM monensin or 50 µM KB-R7943 was added during *in vitro* calibration tests of the microelectrodes. The microelectrodes did not respond to either compound. In prior studies, we have also shown that KB-R7943 did not influence our form of NO microelectrode[27].

Nitric Oxide Response to Elevated NaCl Concentration

Elevated NaCl concentration concentrations were used as an alternative means to increase sodium ions entering the tubular cells and to simulate the hyperosmolarity that the medullary tubules would experience in life. Obviously, cortical tubules do not experience appreciable hyperosmolarity, but the comparison to events in the medulla proved to be useful. Starting from isotonic conditions, sufficient sodium chloride solution from a 10 M stock was added to the physiological media to generate 400 and 600 mOsm. A typical set of actual responses are shown in Figure 2, which illustrates continuous recording at constant site on a tubule was

possible over the various perturbations. All the data presented in subsequent figures are from measurements at the same location over time and changing experimental conditions. During 400 mOsm NaCl exposure, the typical NO response by cortical tubules was a rise in NO which began within a few seconds after the hypertonic media reached the tissues. However, the NO response to 400 mOsm NaCl was invariably transient and lasted 2-4 minutes. When isotonic conditions were restored after 400 mOsm, the [NO] again increased, which will be explained in the Discussion. Note in lower panel Figure 2 that during 600 mOsm NaCl exposure, the rise in [NO] is more sustained than occurred with 400 mOsm NaCl, but did decrease to an intermediate concentration greater than control after about 5-7 minutes in most cases.

The upper panel of Figure 3 presents data from 10 experiments (10 slices, 10 mice) of repeated exposure to 600 mOsm NaCl to determine if the 600 mOsm NaCl might be harming the tissue and therefore, its ability to mount an increased [NO] response to hyperosmolarity or return the [NO] to a normal range after exposure to hypertonicity. The same location on a given tubule was followed throughout the experiment. This evaluation was essential to be sure the fundamental [NO] responses were not being damaged by the initial exposure to hypertonicity. As shown, the initial tubular NO response to 600 mOsm NaCl did not appreciably change the following resting [NO] during isotonic conditions. Furthermore, the increase in [NO] to the second challenge of 600 mOsm NaCl was nearly identical to the first response.

To confirm that the elevation of the NO signal during 400 and 600 mOsm NaCl and the subsequent recovery was a biological event and not an artifact of the microelectrode sensor, a variety of tests were performed. The same location on a given tubule was followed throughout the experiment. First, as shown in the lower panel of Figure 3, the large increase in cortical tissue [NO] during 600 mOsm NaCl was strongly suppressed to $37 \pm 8\%$ of the initial NO response in the same location on a tubule by blockade of nitric oxide synthases with 1 mM L-nitro-arginine- methyl ester (L-NAME). In addition, the resting [NO] during L-NAME exposure in isotonic media was reduced to $58 \pm 12\%$ of the initial control [NO]. The second test demonstrated the effects of 600 mOsm on the NO microelectrode calibration. These tests used an *in vitro* calibration chamber for four electrodes chosen at random. The change in electrode current from 0 to 1200 nM NO was 2.9 ± 0.5 picoamperes at 300 mOsm Ringer solution versus 3.0 ± 0.6 picoamperes when the media was raised to 600 mOsm with NaCl. The resting current of the electrode in a nitrogen equilibrated bath changed less than 2% during the elevation of osmolarity to 600 mOsm. The third test was *in vitro* tests of L-NAME effects on the microelectrodes. In these tests of 5 electrodes chosen at random, the NO calibration in the presence of 1 mM L-nitroarginine methyl ester (L-NAME) was $0.98 \pm 0.04\%$ of that for isotonic saline. These data confirm past studies[2;24;28] that our microelectrodes were not sensitive to 1 mM L-NAME.

The average [NO] initial and recovery responses of same cortical tubules to 400 and 600 NaCl mOsm and medullary tubules at 600 mOsm are shown in Figure 4. The same location on a given tubule was followed throughout the experiment. The medullary tubules did not respond appreciably to 400 mOsm NaCl. In the medulla, the [NO] was far lower than in the cortical nephron segments and the responses to 600 mOsm NaCl were by comparison quite small. However, medullary tubule NO responses during elevated NaCl concentration did about double compared to a near tripling in the cortical tissues.

Elevated NaCl Concentration vs. Mannitol

To illustrate that NaCl hypertonicity was more potent to increase [NO] than non-specific hyperosmolarity, the NO responses of the same tubule to 600 mOsm NaCl and 600 mOsm mannitol were measured. The same location on a given tubule was followed throughout the experiment. The data for these paired responses during hyperosmolarity are presented in the upper panel Figure 5 and clearly demonstrated the NO responses were about 4 times larger for

NaCl than equivalent mannitol hyperosmolarity. During the recovery from hypertonic mannitol, a substantially lower [NO] than that during the recovery from 600 mOsm NaCl occurred and was very transient in nature.

To determine the entry pathway for sodium ions during NaCl hyperosmolarity, the [NO] responses were evaluated in before and after 5 μ M bumetanide for 30 minutes to block Na^+ - K^+ - 2Cl^- cotransporter. Many cells of all organs use bumetanide sensitive Na^+ - K^+ - 2Cl^- cotransport to quickly respond to changes in environmental osmolarity [29-31]. The same location on a given tubule was followed throughout the experiment. Bumetanide caused a minor (7%) decrease in resting [NO], but blunted the [NO] response to 600 mOsm NaCl by ~80%. These data are presented in the lower panel of Figure 5 and are based on five slices from 5 mice. Pilot tests of the effects of amiloride blockade demonstrated such small effects as to not be important.

Histochemistry and Western Blot Analysis of Tubular NOS

Figure 6 (top) presents a representative histochemistry image of kidney tissues reacted with the eNOS antibody. Six animals were studied and very similar images were found for all. eNOS was found in the tubules of the cortex and medulla with obvious detection of eNOS in the arterioles and venules. These images are shown to illustrate tubules have substantial eNOS and sources of eNOS in the microvessels are quite small compared to the massive tubular structures. Sections stained for nNOS and iNOS revealed only spotty localized signal in the cortex compared to a more uniform signal in tubules and blood vessels for eNOS. Findings from western blots of three mice using the same antibodies as used for immunocytochemistry indicated small signals for nNOS in both the medulla and cortex. These data are shown in the lower half of Figure 6. To be sure the nNOS antibody worked appropriately, brain samples were also used and a large signal was detected. A larger eNOS signal was detected in the medullary than cortical tissues. Very little iNOS could be detected in either the medulla or cortical tissues.

Role of Sodium-Calcium Exchanger in NaCl Responses

As prior tests indicated NO generated during monensin permeabilization was linked to the sodium/calcium ion exchange (Fig. 1), we questioned if the same mechanism applied to increased [NO] during hypertonic NaCl. KB-R7943 at 50 μ M was used to block the sodium/calcium ion exchanger. The protocol was to measure the basal, hypertonic exposure and recovery NO and then for the same tubular location, add the inhibitor for 30 minutes and repeat the experiment. These paired data are presented in Figure 7. The same location on a given tubule was followed throughout the experiment. As the sodium-calcium exchanger is constantly active, suppression with KB-R7943 lowered the basal [NO] and severely blunted the NO responses to 600 mOsm NaCl and the recovery increase in [NO].

Discussion

A significant concern of the current study was to what extent the NO measurements were in fact nitric oxide. Generalized blockade of all nitric oxide synthases with L-NAME both substantially lowered the resting NO signal and severely depressed the NO response during elevated NaCl concentration (Fig. 3). L-NAME, monensin, and elevated NaCl concentration did not interact with the microelectrodes or change the in vitro sensitivity to NO gas (Results). The next issue was to determine if blood vessels near tubules contributed an appreciable amount of NO. For example, in tissue culture studies with both proximal tubular and endothelial cells, Linas and Repine [32] have shown NO generation, evaluated by cyclic GMP formation, was strongly dependent on the endothelial cells in the co-culture. Their study is of interest because our studies in other microvascular beds have shown elevated NaCl concentration substantially

increased the perivascular [NO] from endothelial cells [2;27;30]. The microelectrode sensor was only sensitive to NO at the open tip plus the tip was about the same width ($\sim 10\ \mu\text{m}$) as the tubular cells. It would actually be difficult to measure NO generated specifically by the microvessels, unless an unusually large vessel or glomerulus was selected, as we have done in the past [24]. Another concern was to what extent tissue sectioning and *in vitro* conditions depressed or artifactually increased the [NO]. As the vast majority of superficial cortical measurements would have been in proximal tubule segments, the resting value near 400 nM seemed suspiciously high in light of Levine's et al. [11;12] tubular lumen [NO] of $\sim 100\text{--}200$ nM measured with microelectrodes in the *in vivo* state in cortical tubules of rats and mice. In addition, Kakoki et al. [7;33] using *in vivo* rat kidneys have found [NO] from microdialysis collections of medullary interstitial fluid to be in the $100\text{--}150\ \mu\text{M}$ range during resting conditions. The differences in concentrations are likely because we measured NO directly on the outer surface of tubules rather than in luminal fluid, where diffusional dilution and some break down of NO reacting with oxygen or other materials would be highly probable in the *in vivo* state. We did find that if the tip of the microelectrode was placed in the lumen of the tubule, the [NO] recorded consistently decreased to below $200\ \mu\text{M}$.

We used monensin to increase isotonic sodium loading of tubular cells similarly to what could happen if sodium transport increased (Fig. 1). The assumption was that if tubular cells behave similarly to our experience with endothelial cells, they would have a substantial NO response to sodium stress. There is evidence that both the medulla and cortex have important NO mechanisms. Zou and Cowley [34] found the rat kidney medulla produced about twice as much NO as did the cortical tissue. Furthermore, the medullary tissue of rats appears to have an unusually well developed ability for calcium dependent NO generation [35]. In terms of blood flow regulation by NOS mechanisms, Kakoki et al. [36] found that combined nNOS blockade and L-NAME treatments reduced cortical and medullary blood flows by similar percentages. Similar data have been found for mice by Mattson and Meister [37] in that cortical blood flow decreased only slightly more (18-24%) than did medullary (13-18%) blood flow after systemic administration of L-NAME. In actual measurements of [NO] shown in Figures 1 and 4, the medullary section of nephrons had a generalized low [NO] at rest compared to that of the cortical nephrons. While monensin sodium loading did increase the [NO] of cortical and medullary nephrons by similar relative amounts, as shown in the lower panel of Figure 1, the absolute increase was much greater in cortical than medullary tubules (upper panel of Fig. 1). In both the medulla and cortical sections of nephrons, the mechanism by which monensin sodium stress increased NO production was linked to removal of sodium ions in exchange for calcium ions. Blockade of the sodium/calcium exchanger with KB-R7943 for 30 minutes lowered the elevated [NO] during ongoing monensin treatment to essentially the natural resting [NO] for both medullary and cortical tubules. We next tested if a different form of sodium stress, NaCl hypertonicity would also increase cortical NO production through a sodium/calcium exchanger mechanism. In the following paragraph, some unique aspects of NaCl hyperosmotic responses will be discussed. For the hyperosmolarity experiments, KB-R7943 again was used to inhibit the sodium/calcium exchanger. Following blockade, the resting [NO] of the same tubule was decreased and the NO response of that tubule during exposure to 600 mOsm NaCl hypertonicity and the overshoot of [NO] during recovery to isotonic conditions were both strongly diminished. We [27;30] have found a quite similar response of reduced resting [NO] and strongly suppressed increased [NO] during elevated NaCl concentration when *in vivo* arterioles of the small intestine were exposed to local application of KB-R7943. We interpreted this behavior in endothelial cells and now in kidney tubules as evidence of sodium/calcium exchange has a role in basal NO production as sodium ions constantly enter cells and an enhanced role when physiological conditions increase the cellular accumulation of sodium ions. The sodium/calcium exchanger has been shown by Schweda et al. [43;44] to be tonically active in the renal vasculature because blockade increases renal resistance independent of renin secretion. Our studies support these earlier studies by Schweda

et al [43;44] because the resting [NO] over tubules was decreased by blockade of the sodium/calcium exchanger.

After finding that monensin and NaCl hyperosmolarity both caused increased NO that was dependent on the sodium/calcium exchanger, we were directed to determine how sodium might enter tubules during NaCl hyperosmolarity. Obviously, cortical tubules do not experience NaCl hyperosmolarity, the elevated NaCl concentration was simply a tool to determine if sodium stress mechanisms are similar with monensin loading of sodium and leakage of sodium ions under high NaCl conditions. 400 mOsm NaCl transiently increased [NO] followed by an additional overshoot during the restoration of isotonic conditions (Fig. 2). A similar pattern, but with a sustained NO response during hypertonicity was found with 600 mOsm NaCl. Mannitol hyperosmolarity was a poor substitute for equivalent elevated NaCl concentration, as shown in Figure 5. However, even with mannitol hyperosmolarity, there was a small overshoot of [NO] during the return to isotonicity. This may indicate that some sodium accumulation occurred with mannitol hyperosmolarity that needed to be dissipated. These results with NaCl and to a lesser extent with mannitol, along with those using the sodium ionophore monensin, support our conclusion that sodium entry into tubular cells is important to increased NO production. The major sodium entry pathway during NaCl hyperosmolarity appeared to be through $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransporters, as shown in the lower panel of Figure 5. [NO] responses to 600 mOsm NaCl were almost eliminated by bumetanide to suppress $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransport. This transport system is used by many cells to respond to hyperosmolarity [29-31], and is expressed in rat proximal tubules [40;41].

The response of tubular cells to sodium entry whether through monensin or NaCl hyperosmolarity was found to be quite different in the medullary and outer cortical tubules. As shown in Figure 1, cortical NO responses during monensin were much larger than occurred in the medulla. The upper panel of Figure 4 compares the NO response of the same cortical tubule to 400 and 600 mOsm NaCl: the lower panel compares NO responses of deep medullary and superficial cortical nephrons in the same tissue slice to 600 mOsm NaCl. Responses of medullary tubules to 400 mOsm NaCl were too small to reach significance and are not shown. The data illustrate the cortical tubules have a large potential to generate NO, whereas deep medullary tubules have much smaller absolute responses, but on a relative basis, did double their [NO]. The smaller basal and response [NO] events in the medulla were not because it lacked NOS enzymes. Western blot analysis of eNOS, nNOS, and iNOS in the medulla and cortex revealed that in absolute terms, the medulla had far more eNOS than did the cortical tissues (Fig. 7). Only trace amounts of the other NOS forms were found. The eNOS data are consistent with observations by Wu et al. [46] and Cowley et al. [42] that the medulla has substantial eNOS capable of generation of NO. However, for the conditions of the current in vitro study, medullary eNOS was not as active as occurred in the superficial cortical nephrons.

In both the renal medullary and cortical tubules, there was an overshoot then substantial recovery of the [NO] with 400 mOsm NaCl and overshoot and less substantial partial recovery of increased [NO] during 600 mOsm NaCl. We questioned whether the decline in [NO] during elevated NaCl was possibly a stress on cells that they could not maintain. However, three factors argue the tubular cells were healthy. First, as shown in the upper panel of Figure 3, repeated exposure to 600 mOsm NaCl caused the same increase in [NO] and did not influence the resting [NO] after hypertonicity. Secondly, as shown in the lower panel of Figure 4, the cells of medullary and cortical tubules generated as high or higher [NO] during recovery from 600 mOsm than their initial response to hypertonic NaCl. Thirdly, shown in the upper panel of Figure 4, the same cortical tubular cells were able to sustain much higher [NO] during exposure to 600 than 400 mOsm NaCl, indicating they were metabolically able to withstand substantial sodium stress. We now believe the partial to substantial recovery of NO during elevated NaCl concentration at both 400 and 600 mOsm reflected the cells were simply expressing a decreased

entry of sodium into the cells as part of feedback suppression of sodium entry into the cells. Studies by Levine et al. [12], Ortiz et al [13], Rocznik and Burns [9], and Eitle et al. [14] have shown NO suppresses transport of sodium ions in a negative feedback scenario and this area has been reviewed by Liang and Knox in 2000 [15]. From our observations, a similar process occurs in tissue slice specimens and is particularly apparent with low sodium stress conditions, such as 400 mOsm NaCl.

Acknowledgements

Supported by Dr. Bohlen's NIH Grant HL-20605 and a Grant-in-Aid to Dr. Kempson from the American Heart Association Midwest Affiliate. N. Thompson and L. Pezzuto were supported by the Medical Student Summer Research Program at IUSM. We thank Drs Carrie Phillips and Stephen Mason for advice and assistance with immunohistochemistry.

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Figure 1.

The upper panel of data are actual NO concentrations measured for deep medulla and outer cortex tissue at rest, during 10 μ M monensin treatment to allow sodium ions to enter the cells, and the combination of monensin and KB-R7943 to suppress the sodium/calcium exchanger. The lower panel presents the same data expressed in percent of control for the resting state. These data demonstrate that the medullary tissue functions at a much lower NO concentration than did the outer cortical cells. However, on a relative basis, the NO generation of medullary cells reacted to sodium entry during monensin treatment just as did cortical cells. For both the medullary and cortical cells, suppression of the sodium/calcium exchanger while monensin was present caused a dramatic decline in NO concentration in about 20-30 minutes. A total of 12 mice were used for these studies and in 9 animals, both a medullary and cortical site in different tissue slices was studied.

Figure 2.

Typical responses of NO signal of cortical renal tubules to 400 and 600 mOsm NaCl solution and the return to isotonic conditions are shown. In every experiment, the NO signal increased transiently during 400 mOsm NaCl solution with substantial recovery to base line values. The response to 600 mOsm NaCl was large and substantially sustained. During the recovery to isotonic conditions, there was always a large transient increase in NO signal after both 400 and 600 mOsm NaCl. In most cases, the transient recovery NO signal was as large or larger than the response during sustained 400 or 600 mOsm NaCl as will be shown in Figure 3.

Figure 3.

The upper graph is a demonstration of NO responses by cortical tubules to repeated exposure to 600 mOsm NaCl within one hour. The data are based on single slices from 10 mice. The results illustrate that neither the resting NO nor response to 600 mOsm changed appreciably following the initial stress of hyperosmolarity. In effect, conditions and responses by the tubules were stable over time and with simple perturbations. The lower graph is to illustrate that the peak NO signal response to 600 mOsm NaCl was indeed from nitric oxide, the resting and peak [NO] to 600 mOsm NaCl were measured, followed by repeated measurements after exposure of the tissue to 1 mM L-NAME for 30 minutes to suppress all forms of nitric oxide synthase. After L-NAME, the resting [NO] was reduced to 57% of control and the peak response to 600 NaCl was 36% of the original normal peak response. Data set based on eight tissue slices with two slices used from each of 4 mice.

Figure 4.

Upper Panel. Mean data for the renal cortical tubule [NO] responses to 400 and 600 mOsm NaCl solutions and the recovery responses during the return to isotonic conditions. The recovery NO after 400 mOsm NaCl was consistently and significantly larger than that at the height of NO response during sustained elevation of NaCl. The NO responses to both sustained 600 mOsm NaCl and its recovery were much larger than those to 400 mOsm. The recovery response to 600 mOsm NaCl was about the same as the increased [NO] during hyperosmolarity. Data is based on studies of 13 animals with one tissue slice per mouse. *Lower Panel.* The panel presents a comparison of [NO] at rest and during 600 mOsm NaCl exposure and recovery for cortical and medullary tubules of the same renal tissue slice. The absolute values of [NO] at rest, during 600 mOsm NaCl, and recovery were much higher in outer cortical than deep

medullary tissue. However, on a relative basis, the [NO] responses of the medulla were about 70-80% of those by the cortical tissues. Data set is based on 5 animals with one tissue slice per mouse and measurement of cortical and medullary tissue in each slice.

Figure 5.

Upper Panel. Comparison NO responses of the same cortical tubule to 600 mOsm NaCl and mannitol hyperosmolarity during the exposure period and recovery to isotonicity. Although 600 mOsm mannitol did cause a significant increase in [NO] compared to control conditions, the responses were much smaller than during 600 mOsm NaCl. There was an overshoot of [NO] during mannitol washout, but it was both very transient and less than half that observed for the same tubule during NaCl hyperosmolarity. Based on 5 animals with one tissue slice per mouse. Lower Panel. In a separate group of 5 mice using one slice per mouse, the effect of 600 mOsm NaCl on tubular NO generation was tested before and after 5 μ M bumetanide for 30 minutes to block $\text{Na}^+ - \text{K}^+ - 2\text{Cl}^-$ cotransporters. There was a small (7%) but significant decline in resting [NO] after bumetanide and the [NO] response to 600 mOsm NaCl decreased to about 20% of the natural response to 600 mOsm NaCl.

Figure 6.

Distribution of eNOS in frozen sections of mouse kidney and in tissue homogenates. eNOS (red) was expressed in proximal tubules, glomeruli and microvessels (top left). Proximal tubules were identified by staining with LTG lectin (green). Staining with antibodies for iNOS and nNOS was not different from non-specific staining observed in the control (no primary antibody). Western blotting (bottom) detected eNOS in both cortical and medullary homogenates with a larger signal for eNOS in the medulla than cortex in the three animals tested. Neither iNOS nor nNOS was detected in significant amounts in renal tissues, but nNOS was detected in the brain tissues of the animal to confirm the nNOS antibody was functional.

Figure 7.

Comparison of NO responses of the same cortical tubule to 600 mOsm NaCl before and after blockade of the sodium/calcium exchanger with KB-R7943. 600 mOsm NaCl caused the expected increase in [NO] compared to control conditions during hypertonicity and the isotonic recovery. KB-R7943 decreased the resting [NO], indicating ongoing sodium/calcium exchange at rest, and all but eliminated the NO responses to elevated NaCl concentration and the isotonic recovery response. Based on 4 animals with one tissue slice per mouse.