Effect of sodium delivery on superoxide and nitric oxide in the medullary thick ascending limb

Michiaki Abe, Paul O’Connor, Mary Kaldunski, Mingyu Liang, Richard J. Roman, and Allen W. Cowley, Jr.

Department of Physiology, Medical College of Wisconsin, Milwaukee, Wisconsin

Submitted 17 October 2005; accepted in final form 15 February 2006

A recent study in our laboratory found that most of renal injury in hypertension induced by ANG II occurs in the outer medulla and is secondary to an elevation in renal perfusion pressure (RPP) (35). Chronic servo-control of RPP reduced the degree of renal interstitial fibrosis and tubular injury in the outer medulla by 75% (35). Previous studies also indicated that renal interstitial fibrosis and capillary injury occur first in the outer medulla and juxtamedullary glomeruli in many different forms of experimental hypertension including Dahl S rats (21), ANG II-induced hypertension (35), and the two-kidney, one-clip model of hypertension (15).

The mechanisms whereby elevations of RPP lead to injury in the renal medulla are yet unknown, but it is recognized that acute increases of RPP inhibit proximal tubular Na⁺ reabsorption and increase Na⁺ delivery to the medullary thick ascending limb (mTAL) (9, 14, 22, 40). Furthermore, enhancement of NaCl transport in the outer medulla has been found in Dahl S rats (19) and in diabetes (2). An increase in Na⁺ delivery to mTAL would be expected to enhance Na⁺ transport and this could increase production of superoxide (O₂⁻). To examine this possibility, thin tissue strips were dissected from the outer medulla of Sprague-Dawley rats and the mTALs were micropерfused in vitro with solutions containing different Na⁺ concentrations and at different flow rates. The production of nitric oxide (NO) and O₂⁻ by cells in the perfused mTAL segment was determined utilizing the fluorescent dyes 3-amino-4-aminomethyl-2',7'-difluorofluorescin diacetate FM (DAF) (12, 23, 33) and dihydroethidium (DHE), respectively (33, 34). The results of these studies indicate that physiological increases of both luminal Na⁺ concentration at a fixed flow rate or increased flow rate at a fixed luminal Na⁺ concentration increase the production of superoxide O₂⁻ and reduce levels of NO in mTAL.

MATERIALS AND METHODS

General

Experiments were performed on male Sprague-Dawley rats weighing 170–230 g (Harlan, Madison, WI). The rats were maintained ad libitum on water and a standard pellet diet (cat. no. 5001 Purina Mills containing 0.9% Na) in the Animal Resource Center of the Medical College of Wisconsin. All protocols were approved by the Institutional Animal Care and Use Committee.

Preparation of Medullary Tissue Strips

Rats were anesthetized with pentobarbital sodium (50 mg/kg ip). A PE-50 catheter was inserted from the femoral artery into the aorta to a site below the left renal artery. The aorta was ligated above the left renal artery and the left kidney was perfused with 15 ml of modified Hanks’ balanced salt solution (HBSS: Life Technologies) with 20 mmol/l HEPES (Sigma, adjusted to pH 7.4, HBSSH) and 1 mg/ml BSA. The renal artery was then ligated, the left kidney excised, and the outer medulla quickly isolated and transferred into a Petri dish filled with ice-cold HBSSH + BSA. The petri dish was mounted on a cooled microscope stage and maintained at 4°C during dissection. Microdissection was performed using a Leica M3Z stereomicroscope. A piece of outer medullary tissue containing one to three mTALs with interspersed vasa recta and collecting ducts was microdissected using Dumont #5 forceps and mounted on 15-mm-round glass coverslips coated with Cell-tak tissue adhesive (BD Biosciences).

Microperfusion of mTAL

In vitro perfusion of mTAL entailed a number of key steps: 1) flow calibration of the micropuncture pipette system; 2) development of a microscope stage with an attached micromanipulator to enable near and far focusing with no change in the relationship of the tubule to the pipette; 3) construction of a micropipette system with a fluid exchange...
pipette to allow rapid change of the perfusate without removing the pipette from the lumen of the tubule; 4) rapid microdissection of the medullary tissue without the use of collagenases to obtain thin tissue strips containing mTAL that retained the normal proximity of local tissue structures (connective tissues etc.) in the ex vivo state (33, 34); 5) anchoring of tissues on the coverslips to minimize movement of mTAL during perfusion and real-time fluorescence measurements; and 6) image analysis using a high-magnification water immersion lens (from ×60 to ×90) to detect and measure changes of NO or O$_2^-$ signals from individual mTAL cells (see Figs. 1, top, and 3, top).

Glass pipettes (Narishige) were pulled to an internal diameter of 3–5 μm, the tip smoothed and beveled, and mounted on a micromanipulator (World Precision Instruments) that was secured to the microscope stage. A micropipette was inserted into the open lumen of the tubule using a microinjector (World Precision Instruments) that was secured to the microscope stage. A micropipette was inserted into the open lumen of the mTAL and the tubule was perfused with the desired solution. The microscope stage. A micropipette was inserted into the open lumen of the tubule that was fitted with a concentric fluid exchange pipette to enable rapid change of the perfusate without removing the micropipette from the lumen of the tubule; pipette to allow rapid change of the perfusate without removing the micropipette from the lumen of the tubule; pipette to allow rapid change of the perfusate without removing the micropipette from the lumen of the tubule; pipette to allow rapid change of the perfusate without removing the micropipette from the lumen of the tubule; pipette to allow rapid change of the perfusate without removing the micropipette from the lumen of the tubule; pipette to allow rapid change of the perfusate. The microperfusion pipette was fitted with a concentric fluid exchange pipette to enable rapid exchange of the intraluminal perfusate.

Before each study, the microperfusion pipettes were calibrated and the relationship between the height of the reservoir chamber (cm) and the flow rate (nl/min) was established by measuring the rate of filling of constant bore capillary tubes. Although physiological flow rates within mTAL cannot be directly measured, one can obtain an estimate of the normal physiological range from measurements of late proximal tubular to early distal flow rates (28). Late surface proximal tubular flows typically average 20 nl/min and flow to the early distal tubule is ~5 nl/min. Therefore, 5 and 20 nl/min were chosen as the “low” and “high” perfusion rates for the mTAL in the present studies. A moderate flow rate of 15 nl/min was used in studies in which the Na$^+$ concentration of the luminal fluid was altered.

Fluorescence Imaging

Fluorescence measurements were made by using a Nikon TE2000 inverted microscope with a ×60 water-immersion (numerical aperture 1.2) objective lens and the integrated ×1.5 magnification. The signal was detected using a high-resolution digital camera (Photometrics Cascade 512B, Roper Scientific, Tucson, AZ). Excitation was provided by a Sutter DG-4 175-W xenon arc lamp (Sutter Instruments, Novato, CA) at alternating wavelengths. Emission control was accomplished using a Lambda 10 optical filter changer (Sutter Instrument) equipped with a broad array of filters for varying wavelengths and bandpass widths for both single and multiple dye imaging. Levels of NO were detected by measuring the intensity of the activated DAF excited at 480 nm with the emission signal isolated at 535 nm and collected every 5 s (45). DHE was excited by dual wavelengths at 380 and 480 nm with the emission signals of Eth isolated at 445 and 605 nm. The signals were collected at 3-s intervals and recorded as the Eth/DHE ratio. This system therefore allowed imaging and quantitation of DAF fluorescence for measurement of NO and ratiometric quantitation of ditydrothidium fluorescence for O$_2^-$ as we previously described (12, 33, 34).

Tubular perfusion experiments were performed within a RC43C quick change chamber closed perfusion bath (Warner Instrument, Hamden, CT). This imaging chamber was mounted on the stage of the inverted microscope that allowed the superfusion of the control buffer and in vitro microperfusion of mTAL specimens. Temperature was maintained at 37°C using a Warner TC-344B controller. Fluorescence intensity of the signaling dyes was quantified over an area of ~10–15 mTAL cells using MetaFluor version 6.1 imaging software (Universal Imaging, Downingtown, PA) running on a Pentium 4 with a high-quality video card.

Protocols

O$_2^-$ responses to changes in luminal Na$^+$ concentration in mTAL. To maintain normal NO production during the experimental protocol, the tubular perfusate contained physiological levels of L-arginine (100 μmol/l, Sigma) added to HBSS (HBSS-AG). As described previously (12, 33, 34), tissues were incubated with 50 μmol/l DHE fluorescent dye for 30 min in either 1) HBSS-AG alone, 2) HBSS-AG with 1 mmol/l of O$_2^-$ scavenger, 4,5-dihydroxy-1,3-benzene-disulfonic acid (Tiron, Sigma), or 3) HBSS-AG with 4 mmol/l of the Na-K-ATPase pump inhibitor ouabain (Sigma). The coverslip was then placed on the microscope stage and tissue, excited at alternating wavelengths, signals were collected at 3-s intervals and recorded as the ratio of Eth/DHE fluorescence intensities. Fluorescent signals were recorded for ~200 s while the tubule was perfused with solution containing 60 mmol/l concentration of Na$^+$. The perfusion solution within the micropipette was then rapidly exchanged (~5 s) to a perfusate containing 149 mmol/l concentration of Na$^+$ and fluorescent signals were recorded for another 200 s. At the end of each experiment, a positive control was performed by addition of 1 mmol/l of diethylthiocarbamic acid (DETC; Sigma) to the bath to inhibit superoxide dismutase (SOD) and 500 μmol/l menadione sodium bisulfite (Sigma) was also added to stimulate mitochondrial O$_2^-$ release (33, 34). Only tissues which exhibited greater than 50% increase in signal intensity from the last time point were included in the final analyses. On average, we collected responses from five to seven tubules with a success rate of 40% based on the number of tubules that demonstrated this robust response to DETC + menadione.

NO responses to changes in luminal Na$^+$ concentration. In these experiments, the tubules were loaded for 30 min at room temperature with 10 μmol/l of the fluorescent triazolo-fluorescein analog, intracellular activated form of DAF-FM (DAF) for measurement of intracellular levels of NO. In one group of mTAL, responses to an acute increase in the concentration of Na$^+$ in the luminal perfusate from 60 to 149 mmol/l were studied at a constant tubular perfusion rate of 15 nl/min. In another group, mTAL responses to a decrease in perfused Na$^+$ concentration from 149 to 60 mmol/l were studied at a constant perfusion rate of 15 nl/min. As a positive control, DETC NONOate was added to the preparation following completion of each experiment and yielded, on average, a 50% increase in signal intensity. We collected responses from five to seven tubules with a success rate of greater than 80% as based on the number of tubules that demonstrated this robust response to DETC NONOate.

O$_2^-$ responses of mTAL to changes in luminal flow from 5 to 20 nl/min at a fixed Na$^+$ concentration of either 60 or 149 mmol/l. The Eth/DHE responses of mTAL to increases of luminal flows from 5 to 20 to 50 nl/min were determined. One group of mTALs were perfused with a 60 mm Na$^+$ solution and another group with 149 mmol/l. As changes in luminal flow rate altered the shape of the cells and the diameter of the tubular cells, after each step change in flow the fluorescent window over each of the cellular regions being analyzed was readjusted so the same area was encompassed in the analysis. Moving the focus of the region of interest resulted in instantaneous changes in the signals which appeared as spikes in the signal, which were removed as artifacts. These spikes cannot account for a steady increase in signal intensity from the last time point were included in the final analyses. On average, we collected responses from five to seven tubules with a success rate of greater than 80% as based on the number of tubules that demonstrated this robust response to DETC NONOate.
Eth/DHE ratios remained constant when the tubules were perfused with the 60 mM Na+ indicating little or no production of O2· (slope = 0.02 ± 1.1 O2· × 10⁻⁹/s). Within 10 s after an increase in the concentration of Na+ of the perfusate, the slope of the Eth/DHE ratios increased significantly (1.3 ± 0.4 O2· × 10⁻⁹/s). Pretreatment of the tissue with the O2· scavenger Tiron completely inhibited this response as determined in perfused mTAL in tubules from five separate rats (data not shown). Stimulation of O2· production was a result of the increase in the luminal Na+ as the osmolalities of low- and high-sodium perfusate were adjusted to be identical using choline chloride. The Na+ concentration-induced increases in O2· production were dependent on an increase in the activity of the Na⁺-K⁺-ATPase activity as the DHE responses to elevations in luminal Na+ concentrations were completely inhibited in mTAL pretreated with 4 mmol/l ouabain (−0.4 ± 0.1 O2· × 10⁻⁴/s).

**NO Responses to Changes in Luminal Na⁺ Concentration in mTAL Perfused at a Constant Flow Rate of 15 nl/min**

Figure 2 summarizes the DAF responses obtained from five mTALs before and after an increase in the Na+ concentration of the luminal perfusate. Perfusion of mTAL with the solution containing 60 mM Na+ concentration resulted in a progressive increase in the DAF intensity indicating that there is a continuous production of NO under these conditions (1.1 ± 0.3 NO × 10 U/s). Within 10 s after increasing luminal Na+ concentration to 149 mM, the rate NO production decreased as indicated by the significant decrease in the slope of the DAF intensity curve (0.5 ± 0.1 NO × 10 U/s; P < 0.05). This was not a result of DAF quenching, nor an inability of the dye to respond to NO, because the DAF signal within all of the mTAL responded to NO, because the DAF signal within all of the mTAL increased significantly following addition of the NO donor DETA NONOate to the bath significantly (data not shown). The fall in the production of NO in response to increased luminal Na+ concentration paralleled the increases in O2· production shown in Fig. 2. Conversely, as shown in Fig. 3, when mTALs were perfused initially with the high-Na+ solution (149 mM) and then the perfusate was abruptly reduced to 60 mM Na+, the DAF signal sharply increased, indicating that
Sodium transport was responsible for this O$_2$ delivery increase from 5 to 20 nl/min was nearly the same as that observed in the perfusion rates of both 5 and 20 nl/min flows, indicating the Na$^+$ contribution of Na$^+$ contribution to NO production. When luminal flow rate was increased from 5 to 20 nl/min, there was an increase in the rate of NO production. This response was significantly reduced in the presence of the NO scavenger carboxy-PTIO.

O$_2^\cdot$ Responses to Increases in Luminal Flow Rate from 5 to 20 nl/min

Eth/DHE responses in five mTAL to increasing luminal perfusion rates at normal and elevated concentrations of luminal Na$^+$ are summarized in Fig. 4. O$_2^\cdot$ production as reflected by changes in Eth/DHE ratio remained stable when mTALs were perfused at a rate of 5 nl/min with a perfusate Na$^+$ concentration of 60 mM (0.0 ± 0.1 O$_2^\cdot$ × 10$^{-4}$/s; n = 5). When luminal flow rate was increased from 5 to 20 nl/min, the slope of the Eth/DHE signal significantly increased (slope 1.1 ± 0.2 O$_2^\cdot$ × 10$^{-4}$/s). No further increase was observed when flow was increased to 50 nl/min (data not shown). This response tended to be reduced (albeit not statistically significant) when tissues were preincubated with ouabain in the bath. In contrast, when mTALs were perfused with 149 mM Na$^+$ concentration at a flow rate of 5 nl/min, a progressive increase in O$_2^\cdot$ production was observed as seen by a positive slope of the Eth/DHE ratio (slope 1.0 ± 0.1 O$_2^\cdot$ × 10$^{-4}$/s; n = 5). Pretreatment of tubules with ouabain totally inhibited this production of O$_2^\cdot$ as indicated by the slope (0.1 ± 0.1 O$_2^\cdot$ × 10$^{-4}$/s; P < 0.05) of the Eth/DHE ratio, indicating that mTAL sodium transport was responsible for this O$_2^\cdot$ production. In mTAL preincubated with ouabain and perfused with 149 mM Na$^+$, there was a significant reduction in the Eth/DHE ratio at perfusion rates of both 5 and 20 nl/min flows, indicating the contribution of Na$^+$ transport at each of these flow tubular flow rates. However, in mTAL preincubated with ouabain and perfused with luminal Na$^+$ concentrations of 149 mM, the rate of increase in O$_2^\cdot$ production when luminal flow was increased from 5 to 20 nl/min was nearly the same as that observed in the absence of ouabain as by the slope of the Eth/DHE ratio (1.2 ± 0.2 O$_2^\cdot$ × 10$^{-4}$/s; P < 0.05). The mTALs were all treated with DETC + menadione at the completion of each protocol as a positive control to ensure adequate loading of the cells and the absence of dye leakage (not shown). Taken together, the results summarized in Fig. 4 indicate that increases in sodium delivery with high tubular flow rates increase O$_2^\cdot$ production but have no effect when luminal Na$^+$ concentration is high and transport is saturated. The time control studies in which six mTALs (n = 3 rats) were perfused at 5 nl/min with 60 mM Na$^+$ showed that Eth/DHE ratios remained constant over a period of 600 s, indicating little or no production of O$_2^\cdot$ (slope 0.05 ± 0.04 O$_2^\cdot$ × 10$^{-4}$/s).

Fig. 3. Reduction of sodium in the perfusate from 149 to 60 mM at a fixed flow rate of 15 nl/min resulted in a marked increase in NO production (triangles). Top: representative mTAL response is shown in the DAF-FM fluorescent image. The administration of the NO scavenger carboxy-PTIO (circles) significantly reduced the slope of NO production (#P < 0.05). When luminal flow rate was increased from 5 to 20 nl/min, NO production was attenuated (slope decreased by 40%; 1.6 ± 0.7 ÷ 10 U/s; P < 0.05; n = 6), and no further change was seen when the perfusion rate was increased further to 50 nl/min (data not shown). In contrast, at a luminal Na$^+$ concentration of 149 mM, the production of NO was greatly attenuated at the low perfusion rate (5 nl/min; 0.7 ± 0.4 ÷ 10 U/s) and no significant change in the rate of NO production was observed when the perfusion rates was increased to 20 (1.2 ± 0.3 ÷ 10 U/s; n = 5) and then to 50 nl/min (data not shown). Also shown in Fig. 5 is the change in the appearance of the epithelial cell layer in the perfused tubule as the lumen diameter expands with the increased pressure and flow. The time control studies in which six mTALs (n = 3 rats) were perfused at 5 nl/min with 60 mM Na$^+$ showed that the rate of production of NO over the period of the study was constant with a slope not significantly different from that shown in Fig. 5 but extended over a time period of 600 s (slope 2.94 ± 0.6 NO ÷ 10 U/s).

Fig. 4. Response of O$_2^\cdot$ production as measured by the change in the ratio of ethidium to dihydroethidium (Eth/DHE) to an increase in flow rate at low sodium (60 mM; ○) and high sodium (149 mM; ●) is summarized. O$_2^\cdot$ production was measured at 5 nl/min at both sodium levels, and then after the flow was increased to 20 nl/min. In separate perfused tubules, ouabain was added to the bath and the same flow change and sodium levels were applied (▲ and ●).

DISCUSSION

The present study examined whether an increase in the delivery of Na$^+$ to the mTAL directly increases production of...
superoxide (\(\text{O}_2^•\)) and secondarily reduces NO bioavailability in this nephron segment. It is recognized that salt-sensitive-induced forms of hypertension are associated with renal damage (14, 20, 32) and that renal interstitial fibrosis and capillary injury occur first in the outer medulla and juxtamedullary glomeruli. The underlying mechanisms responsible for this renal injury remain unclear, and it was this question that our studies have begun to address. Increases in RPP are known to inhibit proximal tubular Na\(^+\) reabsorption (9, 14, 22, 40), which in turn increases the tubular flow rate and delivery of NaCl through the loop of Henle to the mTAL. There is also evidence of chronic downregulation of apical Na\(^+\) transporters in the proximal tubular apical membranes of SHR (30, 44), consistent with an increased delivery of Na\(^+\) to the mTAL. Because active transport of the Na\(^+\) reabsorption with the loop of Henle is almost completely dependent on metabolic function and Na-K-ATPase activity at the mTAL (13), we hypothesized that increased delivery of Na\(^+\) to mTAL in hypertension would increase the production of O\(_2^•\) in this tubular segment. Given these observations, it is of particular relevance to determine whether an increase in the delivery of Na\(^+\) in outer medullary tubular segments could produce an increase in the production of oxygen free radicals (O\(_2^•\)).

Our previous work using superfused microdissected strips from the outer medulla indicated that O\(_2^•\) production in nonperfused tubules increased when the Na\(^+\) concentration of the bath was increased from 150 to 250 mM (34). It was unclear, however, the extent to which the changes in bath NaCl concentrations altered Na\(^+\) concentrations in the lumen of these nonperfused tubules and when the increase in O\(_2^•\) reflected changes in tubular transport. Moreover, the physiological significance of these findings remained in question as it had yet to be determined whether tubular delivery of NaCl within the physiological range could have the same effect on O\(_2^•\) production.

To address these questions, microperfusion techniques were therefore developed that enabled luminal perfusion of mTAL tubular segments within thin tissue strips obtained from the outer medulla. The mTALs in these studies were perfused with solutions using a range of Na\(^+\) concentrations and flow rates were estimated to occur when RPP is altered from 100 to 150 mmHg (40). Development of the novel techniques for microperfusion of mTAL while carrying out real-time fluorescence imaging studies to determine intracellular changes of NO and O\(_2^•\) has significantly advanced our understanding of the relevance of tubular Na\(^+\) delivery to the production of O\(_2^•\) and NO in the mTAL. These ex vivo tubular perfusion techniques in tissue strips have enabled the relative rates of O\(_2^•\) and NO production to be determined in the perfused mTAL preparation under conditions of low and high luminal Na\(^+\) concentrations at fixed perfusion rates, or conditions of low and high luminal perfusion rates at fixed Na\(^+\) concentrations. Time-resolved production of O\(_2^•\) and NO could thereby be computed by regression analyses using the rates of change of the respective fluorescent signals.

Because a double-barrel pipette was not used to seal the cut end of the tubule, it was initially a concern whether using a single-barreled pipette would perfuse the tubule only in the forward direction. Although we cannot be certain that some backleak was not occurring in this studies, it was found using dye infusions that the only time that a backward puff of dye from the cut end of the lumen was observed was at the moment perfusion pressure was elevated momentarily to open the collapsed lumen and start the perfusion. Once the tubule was opened and flowing, the height of the perfusion syringe was immediately set at the height required to delivery the desired flow based on each pipette’s calibration data. This change in lumen diameter as perfusion was started was quite visible as was a change in diameter when flow rates were increased from 5 to 20 nl/min as seen in Fig. 5. The presence of the pipette within either the cut perfused end of the tubule appeared to offer sufficient resistance to enable only the forward movement of the perfusate. This technique would not be useful to precisely determine epithelial transport rates of sodium and other solutes where the precision of these flow rates would be of vital importance. However, we were able to consistently observe changes in the fluorescent indicators for O\(_2^•\) and NO in response to the changes in Na\(^+\) concentrations and tubular flow rates. The question being addressed in these studies was whether O\(_2^•\) and NO would respond to physiological changes in sodium delivery, and the results indicate that small variations in the expected perfusion rates as determined from our calibrations did not obscure the conclusions of this study.

Effect of Changes in Perfusate Na\(^+\) Concentration and Flow Rate on O\(_2^•\) Production in mTAL

Because mTAL of intact kidneys are not accessible for direct micropuncture studies, it is necessary to extrapolate the range of normal tubular Na\(^+\) concentration and flow rates based on analyses of tubular fluid obtained from either the late proximal tubules, the tip of the papilla, or the early distal tubule. It has been determined in rats that the Na\(^+\) concentration of luminal

---

Fig. 5. Response of NO production (DAF-FM fluorescence) to a change in flow rate at a sodium concentration of 60 mM (triangles) and 149 mM (circles) is summarized in this figure. The rate of NO production when the tubule was perfused with 60 mM Na\(^+\) was significantly reduced when the perfusion rate was increased from 5 to 20 nl/min. In contrast, the rate of NO production in tubules perfused with 149 mM Na\(^+\) was significantly less (P < 0.05) when the perfusion rate was 5 nl/min and did not change when the flow rate was increased from 5 to 20 nl/min. Top: typical expansion of lumen and flattening of epithelial cells observed as the pressure and flow increase.
fluid collected from Henle’s loop at the tip of the papilla is 344 meq/l H2O (20). Na+ transport in the ascending thin limb is passive so the concentration of NaCl reaching the early portion of mTAL is about equal to the interstitial Na+ concentrations in the regions of the lower and outer medulla (8, 32). The interstitial Na+ concentration at the inner medullary junction has been reported as 220–240 meq/l in rat (41). However, Na+ concentrations determined in the early distal tubule of rats by micropuncture have been found in the range from 30 to 60 meq/l (31). Burg and associates (1, 16) microperfused isolated rat mTAL with solutions of varying Na+ concentrations and compared the perfusate Na+ concentrations with those of the collected fluids to determine Na+ reabsorption. Perfusion of solutions containing 75–159 meq Na+/l into mTAL resulted in significant increases in tubular Na+ reabsorption. Secretion of Na+ was observed when mTALs were perfused with sodium concentrations in the range of 49–52 meq/l. Na+ concentrations of 50–74 meq/l were assumed to establish a limiting Na+ concentration gradient in which the rate of active Na+ transport in mTAL is counterbalanced by back-flux of sodium. Because this value is close to Na+ concentrations seen in distal tubules (31), a value of 60 mM was chosen as the lower limit of Na+ concentration normally found in mTAL and 149 mM was chosen as the higher value, similar to that typically delivered to mTAL (1, 37, 38).

The present studies provide the first evidence that an increase in the luminal Na+ concentration in the physiological range from 60 to 149 mM/l increases the production of O2·− in mTAL. This response occurs rapidly, within 10 s after changing of the Na+ concentration of the perfusate. Similar rapidity of O2·− production has been reported within renal tissue in mitochondria isolated from myocytes by Loschen et al. (29). It was not due to an osmotic effect as osmolality of the high and low perfusate solutions was adjusted with choline chloride to have the same osmolality. The response indeed appears to have been driven by the change in the Na+ concentration of the perfusate because no change in O2·− was seen when the Cl− of the perfusate was increased with choline substituted for sodium. Furthermore, the Na+-driven O2·− responses were clearly dependent on an increase in the activity of the Na+/K+-ATPase as they were completely blocked by pretreatment of the mTAL with ouabain. Previously, we found that Na+-induced increases in O2·− production within nonperfused mTAL were also inhibited by ouabain and by the Na+/H+ exchanger inhibitor dimethylaminolide. Blockage of Na+/H+ exchange also reduces Na+ transport in the mTAL (31) which is consistent with the idea that the increase in O2·− production is directly proportional to sodium transport and oxygen utilization by the mTAL (34). On the other hand, amiloride also increases intracellular pH by blocking Na+/H+ exchange-dependent outflow of H+ in rat thick ascending limb cells (26), which in turn may increase production of O2·− by increasing the activity of NAD(P)H oxidase.

As indicated above, when the mTAL is perfused with 60 mM NaCl, the rate of active Na+ transport in mTAL is counterbalanced by back-flux of sodium (1, 16). It was in this state that we found the production of O2·− to be minimal. A steady generation of NO (DAF) was also observed under these conditions as indicated by the positive slope in Fig. 2. Consistent with these observations, there was a significant increase in NO production when the 149 mM perfusate was reduced to 60 mM (Fig. 3). These data indicate that when the tubule is perfused with a low-Na+ perfusate, there is a significant amount of NO production in mTAL. This is consistent with known interactions of O2·− and NO (17). This production is not limited by L-Arg availability, which was present at concentrations of 100 μmol/l in the perfusate. Because obtaining ratiometric fluorescence measurements is not possible when a single wavelength dye is used, it remains possible that changes in cell geometry or volume could have influenced the observed DAF fluorescent signals. However, in the present studies when luminal Na+ concentration was increased from 60 to 149 mM, cell volumes would be expected to remain unchanged as the tonicity of the perfusate of the bath remained constant. Flattening of the DAF slope was observed when Na+ was increased from 60 to 149 mM and was reduced when the perfusate sodium concentration was reduced from 149 to 60 mM. These responses were significantly reduced in the presence of the NO scavenger carboxy-PTIO demonstrating that DAF was closely tracking changes in intracellular NO during these maneuvers. We conclude, therefore, that NO production falls when luminal Na+ concentration is increased and rises when luminal Na+ concentration is reduced.

Finally, it is evident one looks at the combined results of the DHE and DAF responses to increases in luminal Na+ concentration that the intracellular O2·− and NO production of mTAL change reciprocally with each other. When tubular Na+ concentration was increased from 60 to 149 mM/l, O2·− production increased (Fig. 1) and the rate of NO production decreased (Fig. 2). Our findings are consistent with those of Ortiz and Garvin (36), who recently reported that decreases in O2·− concentrations with the SOD mimetic tempol increased NO production in the TAL.

Role of Change in Wall Tension and Shear Stress on the Balance Between NO and O2·− in mTAL

In increased wall tension and shear stress were created by raising the perfusion pressure and therefore the flow rates of the perfused mTAL. The effects of increasing perfusion rate on mTAL O2·− production were negligible when the tubule was perfused with a high concentration of Na+ (149 mM). These are conditions in which the rate of production of O2·− would already be strongly stimulated as Na+ concentrations along the mTAL would remain above the Km for maximal Na+ transport and not fall to levels sufficient to limit transport. In contrast, when the tubule was perfused with a low-Na+ concentration (60 mM), O2·− production increased significantly when flow rates were increased from 5 to 20 nl/min (see increased slope of the Eth/DHE ratio in Fig. 4).

These responses may simply reflect changes in tubular Na+ transport and oxygen consumption of the tubule because at low flow rates the Na+ concentration may fall below 60 mM and be increased when flow rate is elevated. However, the data also indicate that mechanical factors may contribute to these responses. Na-K-ATPase activity of the mTAL is the highest of all nephron segments (13, 16) and, as shown in our study, the production of Na+-induced O2·− was inhibited by pretreatment of the mTAL with ouabain in the bath. As seen in Fig. 4, however, under conditions of high tubular Na+ concentrations...
EFFECT OF Na\textsuperscript{+} DELIVERY ON NO AND O\textsubscript{2}\textsuperscript{-} IN mTAL

(149 mM) even in the presence of ouabain, an increase of tubular flow from 5 to 20 nl/min increased in O\textsubscript{2}\textsuperscript{-}. Although the contribution of Na\textsuperscript{+} transport to O\textsubscript{2}\textsuperscript{-} production was clearly reflected by the reduction in the Eth/DHE ratio at perfusion rates of both 5 and 20 nl/min, the increase in the slope of the Eth/DHE ratio with increased tubular perfusion is not inhibited by ouabain and therefore reflects a potential mechanical component of increased in O\textsubscript{2}\textsuperscript{-} production in these studies.

The mechanisms connecting mechanical effects to O\textsubscript{2}\textsuperscript{-} production were beyond the scope of the present study. However, it is clear that the epithelial cells of mTAL are geometrically altered when pressure and flow are increased in perfused tubules as seen by a flattening of the cells and an increase in lumen diameter. These changes in addition to the effects of flow and shear stress on these cells together could influence cell function. The effects of flow and shear stress on O\textsubscript{2}\textsuperscript{-} production were first studied in blood vessels in which oscillatory shear stress stimulated O\textsubscript{2}\textsuperscript{-} production via NAD(P)H oxidase (18). The ability of mechanical stresses to influence production of free radicals of isolated, perfused tubules was also observed by Ortiz et al. (39), who reported that luminal flows in cortical TAL of 20–25 nl/min increased NO secretion to the luminal fluid secondary to translocation of eNOS. It could be expected that similar mechanically induced responses can occur in the mTAL. However, these effects were apparent only when either mTAL Na\textsuperscript{+} transport was inhibited by ouabain or when the tubule was perfused with low concentrations of Na\textsuperscript{+} (60 mM).

Relationship of Hypertension to Oxidative Stress and Injury in the Outer Medulla

It is recognized that diets high in sodium lead to increases in blood pressure in many forms of hypertension (1, 10, 42, 43) and that “salt-sensitive” individuals are effectively treated with restriction of salt intake. In the general population, diuretic therapy remains one of the primary treatments for hypertensive patients (7). Despite the wide recognition of the impact that salt intake can have on arterial blood pressure and renal end-organ damage frequently seen with salt-induced forms of hypertension (1, 21, 35), the underlying mechanisms responsible for this renal injury remain unclear.

Increases of RPP are known to inhibit proximal tubular Na\textsuperscript{+} reabsorption (9, 14, 22, 40), which in turn increases the tubular flow rate and delivery of NaCl through the loop of Henle to the mTAL. It is known that 25% of the filtered Na\textsuperscript{+} is absorbed along Henle’s loop (24, 25) and Na\textsuperscript{+} reabsorption with the mTAL is almost completely dependent on metabolic function and Na-K-ATPase activity (13). The present results have shown that increased luminal delivery of Na\textsuperscript{+} to mTAL can increase the production of O\textsubscript{2}\textsuperscript{-} in this tubular segment. The intracellular source and mechanisms related to the Na\textsuperscript{+}-induced O\textsubscript{2}\textsuperscript{-} production remain to be determined, but based on the results of other recent studies, it appears that the Na\textsuperscript{+}/H\textsuperscript{+} exchanger in the apical membrane may be the final pathway required for the generation of O\textsubscript{2}\textsuperscript{-} in response to NaCl (46) and that the outward movements of H\textsuperscript{+} ions activate NAD(P)H oxidase to produce O\textsubscript{2}\textsuperscript{-} in mTAL cells (27).

To conclude, it has been shown previously that O\textsubscript{2}\textsuperscript{-} produced in mTAL can diffuse to surrounding vasa recta microvessels when the bioavailability of NO is low (33) and that increased O\textsubscript{2}\textsuperscript{-} and H\textsubscript{2}O\textsubscript{2} reduce medullary blood flow (5). Based on the present results, it is possible that sustained increases in the delivery of Na\textsuperscript{+} to mTAL could initiate a cascade of events leading to oxidative stress and fibrotic injury of the outer medulla as seen early in many forms of hypertension.

ACKNOWLEDGMENTS

The authors thank G. Slocum for expert assistance with the microscopy and M. Skelton for careful review of the manuscript.

GRANTS

This work was supported by National Institutes of Health National Heart, Lung, and Blood Institute Grants HL-29587 and HL-49219.

REFERENCES

EFFECT OF Na⁺ DELIVERY ON NO AND O₂⁻ IN mTAL


