Renal Oxidative Stress in Medullary Thick Ascending Limbs Produced by Elevated NaCl and Glucose

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Abstract—The effects of NaCl, glucose, and thyroid hormone on the production of superoxide (O$_2^-$) within the renal medulla of Sprague-Dawley rats were examined. Responses of intracellular superoxide [O$_2^-$], in isolated medullary thick ascending limbs (mTALs) were studied using real-time fluorescent microscopy with measurement of the dehydroethidium (DHE) to ethidium (Eth) conversion ratio (Eth/DHE ratio unit). The results demonstrated that elevations of extracellular NaCl (from 152 to 252 mmol/L), D-glucose (from 5 to 25 mmol/L), and triiodo-thyronine (T3; 10 μmol/L) significantly increased [O$_2^-$] levels. Preincubation with superoxide scavenger 4,5-dihydroxy-1,3-benzenedisulfonic acid (1 mmol/L) significantly inhibited these responses. Stimulation with equamolar amounts of choline chloride or L-glucose failed to increase [O$_2^-$], indicating that these O$_2^-$ responses were not determined by changes in osmolality. The responses to NaCl, D-glucose, and T3 were abolished by pretreatment with the Na$^+$/K$^+$-ATPase pump inhibitor ouabain (4 mmol/L) and with Na$^+$/H$^+$-exchanger inhibitor dimethylamiloride (100 μmol/L). We conclude that elevations of extracellular NaCl, D-glucose, or T3 levels can activate both the Na$^+$/K$^+$-ATPase pump and Na$^+$/H$^+$ exchanger in mTAL, which, in turn, is associated with increased intracellular concentrations of superoxide. (Hypertension. 2004;43[part 2]:341-346.)

Key Words: oxidative stress • Na$^+$, K$^+$-transporting ATPase • sodium pump • antiporters • diabetes mellitus • hypertension, sodium-dependent • fluorescence

Evidence of oxidative stress within the kidney has been found in both hypertension and diabetes and may contribute to the pathophysiological changes associated with these diseases. The levels of reactive oxygen species (ROS) are elevated in kidneys of rats with hypertension induced by angiotensin II, in spontaneously hypertensive rats, and Dahl salt-sensitive (Dahl S) rats. It appears that the glomerular sclerosis and tubulointerstitial fibrosis observed in salt-sensitive forms of hypertension may be mediated through pathways of oxidative stress. Similarly, hyperglycemia may induce oxidative stress in renal mesangial cells and be responsible for diabetic nephropathy. Indeed, there are a number of studies indicating that renal oxidative stress contributes to the progression of these pathological states. However, the mechanisms responsible for inducing oxidative stress and the pathways whereby ROS lead to the nephropathies of these diseases remain poorly understood.

Studies in our laboratory have found that the mitochondrial respiratory chain enzymes and NADH oxidase account for the major portion of superoxide production in the renal medulla of rats particularly in the medullary thick ascending limb (mTAL) of Henle. Other studies demonstrated that mTAL exhibits the highest baseline O$_2^-$ levels of any structure within the kidney and that the NAD(P)H oxidase pathway is the major source of angiotensin II-mediated O$_2^-$ production. We and others have also shown that endogenous O$_2^-$ production participates in the normal regulation of renal medullary blood flow and sodium excretion acutely and chronically. Using techniques for fluorescence imaging of intracellular nitric oxide (NO), O$_2^-$, and Ca$^{2+}$ in tubules and vasa recta of microtissue strips obtained from the renal medulla, we have recently established that NO and O$_2^-$ can diffuse from mTAL to the contractile pericytes of the surrounding vasa recta vessels, a process that we have called "tubular-vascular cross-talk."

We hypothesize that increased rates of metabolism in mTAL would increase oxidative stress in mTAL. Each of the 3 stimuli used (NaCl, glucose, and triiodo-thyronine [T3]) were used in an effort to increase metabolism in mTAL in different ways. Isolated medullary tissue strips containing mTAL from kidneys of Sprague-Dawley rats were therefore exposed to elevated concentrations of NaCl, glucose, or T3 to determine intracellular O$_2^-$ responses using dihydroethidium (DHE) and real-time fluorescent microscopy techniques.

Methods

Tissue Fluorescent Imaging

Microtissue strips were dissected from the outer medulla of the left kidney of male Sprague-Dawley rats (170 to 230 g; Harlan, Madison, etc.)
Wis) anesthetized with pentobarbital (60 mg/kg IP). Superoxide (O₂⁻) responses within mTAL epithelial cells were determined using a ratiometric fluorescent imaging technique to determine DHE conversion to ethidium (Eth), as described previously. 5,10,11 Left kidneys were removed and cleared of blood by perfusing with Hanks balanced salt solution (HBSS; Life Technologies) with 20 mmol/L HEPES (Sigma, adjusted to pH 7.4, HBSSH) and 1 mg/mL BSA.

Protocols
All protocols were approved by the Institutional Animal Care Committee. L-arginine (100 mmol/L, Sigma) was added to HBSSH (HBSSH-AG) for physiological measurement of intracellular O₂⁻. Microtissue strips, that were attached to the cover slips with the tissue adhesive Cell-tak (BD Biosciences), were loaded with dihydroethidium (DHE; 50 mmol/L in HBSSH-AG; Molecular Probes) for 1 hour at room temperature and washed twice to remove excess dye. Tissues were incubated for 30 minutes in HBSSH-AG only; HBSSH-AG with 1 mmol/L of O₂⁻ scavenger 4,5-dihydroxy-1,3-benzenedi-sulphonic acid (TIRON, Sigma); HBSSH-AG with 4 mmol/L of the Na⁺/K⁺-ATPase pump inhibitor ouabain (Sigma); or HBSSH-AG with 100 μmol/L of the Na⁺/H⁺-exchanger inhibitor dimethylamiloride (DMA, Sigma).

O₂⁻ responses were imaged in response to superfusion of the tissue strips with the drug vehicle (HBSSH-AG) (152 mmol/L Na⁺, 5 mmol/L d-glucose) followed by responses to 100 mmol/L NaCl (final Na⁺ concentration 252 mmol/L; Sigma); 100 mmol/L choline chloride (ChCl, Sigma); 10 mmol/L d-glucose (final d-glucose concentration 25 mmol/L; Sigma); 10 mmol/L L-glucose (Sigma); and T3 (10 μmol/L, Sigma), all diluted in HBSSH-AG. Agonist stimulation responses were followed by addition of 1 mmol/L of diethyldithiocarbamic acid (DETC, Sigma) to inhibit superoxide dismutase (SOD) or DETC with 500 μmol/L menadione sodium bisulfite (Sigma) to stimulate mitochondrial O₂⁻ release. 8,9 Together, these stimuli served as positive control stimuli to test for dye loading and cell viability.

Statistical Analysis
Values are expressed as mean ± SE. Responses were evaluated using 2-way ANOVA for repeated measurements. A post hoc Bonferroni t test was carried out to determine the significance between vehicle and agonist O₂⁻ responses. A paired t test was used to compare drug vehicle and agonist responses at 250 seconds after the stimulation.

Results
O₂⁻ Responses To Increased NaCl Concentration and the Role of the Na⁺/K⁺-ATPase Pump and Na⁺/H⁺ Exchanger in These Responses
Increasing Na⁺ concentration in the suffusate from 152 mmol/L to 252 mmol/L by adding 100 mmol/L of NaCl to the normal salt solution (Figure 1A) resulted in a significant increase (n=6, P<0.05) in the Eth/DHE ratio of isolated mTAL epithelial cells 66 seconds after stimulation. This response was inhibited by the superoxide scavenger TIRON (1 mmol/L, n=5), indicating that this response was specific to superoxide (O₂⁻). In contrast (Figure 1B), 100 mmol/L of ChCl added to the normal salt solution did not significantly increase the Eth/DHE ratio, which averaged 0.26±0.04 (n=6) compared with the vehicle response of 0.21±0.07 (n=6) at 250 seconds. This compared with a significant increase in the Eth/DHE ratio in response to stimulation with 252 mmol/L Na⁺ solution that averaged 0.44±0.05 (n=6, P<0.05). This series of studies indicated that Na⁺ per se was responsible for the O₂⁻ responses to elevations of NaCl stimulation rather than changes in osmolality. Figure 1C summarizes the O₂⁻ responses obtained at 250 seconds after the stimulation. Preincubation of mTAL with ouabain (4 mmol/L, n=5) and DMA (100 μmol/L, n=5) blocked the 252 mmol/L Na⁺ stimulation of O₂⁻ production. The Eth/DHE responses to 1 mmol/L of DETC with menadione (500 μmol/L) were not inhibited by ouabain or DMA pretreatment, indicating that the effects of these inhibitors on the NaCl responses was not due to the saturation of the dye or the cell death.

O₂⁻ Responses To High Glucose Concentration and the Role of the Na/K-ATPase Pump and Na/H Exchanger in These Responses
Increasing d-glucose concentrations from 5 mmol/L to 25 mmol/L significantly increased the Eth/DHE ratio of
isolated mTAL within 108 seconds (*P* < 0.05, *n* = 5, Figure 2A). As with the NaCl stimulation, this response was also inhibited by O₂⁻ scavenger TIRON (1 mmol/L, *n* = 5). Figure 2B summarizes responses at 250 seconds after stimulation and shows that administration of L-glucose in amounts osmotically equivalent to the 25 mmol/L d-glucose stimulus (20 mmol/L added to 5 mmol/L d-glucose buffer) did not significantly increase the Eth/DHE ratio (0.32 ± 0.04 compared with the vehicle response of 0.26 ± 0.03, *n* = 5). This compares to the response following 25 mmol/L of d-glucose (*n* = 5) that significantly increased the Eth/DHE ratio (0.52 ± 0.07, *n* = 5, *P* < 0.05), confirming that d-glucose specifically stimulated O₂⁻ production independent of changes in osmolality. As shown in Figure 2C, preincubating mTAL with ouabain (4 mmol/L, *n* = 5) or DMA (100 μmol/L, *n* = 5) abolished O₂⁻ production with d-glucose (25 mmol/L) stimulation compared at 250 seconds.

**Figure 2.** A, Time course superoxide responses to high d-glucose concentration (*n* = 5) and inhibition of the response by superoxide scavenger TIRON (1 mmol/L, *n* = 5). B, Comparison of superoxide responses from high d-glucose to identical osmolality solution with L-glucose substitution determined at 250 seconds after stimulation. Vehicle; 5 mmol/L d-glucose buffer (*n* = 5), L-glucose; 5 mmol/L L-glucose buffer with 20 mmol/L of L-glucose (*n* = 5), d-glucose; 25 mmol/L d-glucose buffer (*n* = 5), DETC + MEN; DETC (1 mmol/L) with 500 μmol/L of menadione for positive control. C, Superoxide responses to elevated d-glucose (20 mmol/L increase) concentration with presence of Na⁺/K⁺-ATPase inhibitor ouabain (4 mmol/L, *n* = 5) and Na⁺/H⁺-exchanger inhibitor dimethylamiloride (DMA, 100 μmol/L, *n* = 5) determined at 250 seconds after stimulation; *P* < 0.05 significant from 5 mmol/L d-glucose response.

**Figure 3.** A, Time course superoxide responses to T3 (*n* = 5) and inhibition of the response by superoxide scavenger TIRON (1 mmol/L, *n* = 5). B, Superoxide responses to elevated T3 (10 μmol/L) concentration with presence of Na⁺/K⁺-ATPase inhibitor ouabain (4 mmol/L, *n* = 5) and Na⁺/H⁺-exchanger inhibitor dimethylamiloride (DMA, 100 μmol/L, *n* = 5) determined at 250 seconds after stimulation. DETC + MEN; DETC (1 mmol/L) with 500 μmol/L of menadione for positive control. *P* < 0.05 significant from 152 mmol/L NaCl vehicle response.

**Discussion**

**NaCl, Glucose, and T3 Stimulation of mTAL O₂⁻ Production**

The major question addressed in the present study was whether increased rates of metabolism of mTAL exposed to elevated NaCl and glucose concentrations would increase the production of O₂⁻. The solutes used to stimulate mTAL were those that are known to induce or intensify the renal damage of the outer medulla in hypertension,¹ and diabetes.¹³ High salt intake and enhanced filtration of Na⁺ increase delivery of
Na⁺ load to mTAL, and elevated levels of circulating glucose increase delivery of glucose to mTAL in diabetic states. The present results show that a 100 mmol/L increase of extracellular NaCl and a 20 mmol/L increase of glucose significantly increased intracellular O₂⁻ concentrations within mTAL. These responses were dependent on the Na⁺ or d-glucose per se rather than either Cl⁻ or osmolality because equimolar amounts of ChCl or l-glucose did not increase O₂⁻ levels. T3 stimulation also significantly increased mTAL O₂⁻ production in the present study and was used to confirm that direct stimulation of these transporters could stimulate O₂⁻ production without changes in extracellular solute concentration. As shown, the O₂⁻ responses were dependent on Na⁺/K⁺-ATPase and the Na⁺/H⁺ exchangers because inhibition of either of these prevented the O₂⁻ responses to NaCl and glucose.

Enhancement of NaCl transport in outer medulla has been reported in many types of hypertensive14,15 and diabetic16 rat models, and Na transporters in this region are believed to play an important role in determining the susceptibility to these diseases. A pressor dose of angiotensin II administered to Munich-Wistar rats increased Na⁺/H⁺-exchanger expression in mTAL.15 Na⁺/K⁺-ATPase activity was also found enhanced in the medullary tissue of Milan hypertensive rats.17 Similarly, glucose increases O₂ consumption and Na⁺/H⁺-exchanger activity in proximal tubules of diabetic rat models16 and stimulates the baseline activity of the Na⁺/K⁺-ATPase pump in renal tubules, including mTAL.18 It is believed to be the major substrate driving oxygen consumption and production of ATP and Na⁺/K⁺-ATPase related activity in mTAL.19 Glucose was administered with the assumption that it would be taken up and increase cell metabolism, thereby resulting in increased O₂⁻ production. Although it did indeed have this effect, there is little published information regarding glucose transport in mTAL. The glucose transporter isozyme, GLUT1, has been reported to be localized to mTAL,20 as has GLUT4.21 It is well known that most glucose is reabsorbed in the proximal tubule and that the transcellular flux of glucose in this segment is accomplished by two major classes of transporters: luminal Na-GLUTs that mediate glucose influx and basolateral GLUTs that mediate glucose efflux. GLUT2 in the proximal tubule has a high affinity and is well suited to translocate a large rate of glucose flux in this segment. GLUT1 has a low affinity and its presence in the mTAL may indicate that it could be responsible for the efflux of glucose in this late segment although glucose transport studies have not been carried out in rat mTAL. Taken together, these data would suggest that glucose is transported into and metabolized by mTAL, resulting in the observed production of O₂⁻. T3 has been shown to directly increase Na⁺/K⁺-ATPase in outer medulla of Sprague-Dawley rats22 and Na⁺/H⁺-exchanger activity in renal brush border membranes.23

**Interpretation and Relevance of Results**

Although the intracellular ionic events and the specific molecular mechanisms responsible for stimulating O₂⁻ production in mTAL remains to be determined, there are reasons to believe that the Na⁺/H⁺ exchanger may be the final common pathway required for the generation of O₂⁻ in response to NaCl, glucose, and T3 stimulation. We have recently obtained preliminary evidence that an increase in the outward exchange of H⁺ through the Na⁺/H⁺ exchanger results in O₂⁻ production and this response is dependent on the activity of NAD(P)H oxidase.24 There is evidence in other cell types (ovary and neutrophils) that a protein component (gp91-phox) of NADPH-oxidase serves as a voltage-gated H⁺ pathway.25,26 The present results show that with inhibition of the Na⁺/H⁺-exchanger alone, NaCl and glucose-induced O₂⁻ production was prevented. The dose of the Na⁺/H⁺-exchange inhibitor used in the present study has been reported to have no inhibitory effect on the Na⁺ influx with the Na⁺/K⁺-2Cl⁻ cotransporter.27 This indicates that this Na⁺ influx through the Na⁺/K⁺-2Cl⁻ cotransporter continued to be driven by extrusion of Na⁺ by the Na⁺/K⁺-ATPase pump on the basolateral membrane, while Na⁺/H⁺ exchanger was inhibited with this dose.

Exposure of mTAL to increased extracellular NaCl concentrations would be expected to cause an initial increase of intracellular sodium via both Na⁺/K⁺-2Cl⁻ cotransporter and Na⁺/H⁺-exchanger.19 An increased entry of Na⁺ through the Na⁺/H⁺-exchanger would result in extrusion of H⁺ and raise intracellular pH. Based on our recent observations,22 this would in turn drive an increased production of O₂⁻ via NADH-oxidase within the apical membrane. Similarly, stimulation of Na⁺/K⁺-ATPase pump activity in the basolateral membrane with glucose or T3 would be expected to lower intracellular Na⁺ levels19 and increase the concentration gradient for Na⁺ entry and the extrusion of H⁺ through Na⁺/H⁺-exchanger. O₂⁻ production would thereby be stimulated through the same final NADH-oxidase related common pathway, consistent with the observation that DMA alone inhibited the increased O₂⁻ seen with NaCl, glucose, and T3 stimulation.

The physiological consequences of elevating O₂⁻ levels in the outer medulla have been recently examined. Elevations of medullary O₂⁻ levels produced by chronic medullary interstitial infusion of the SOD inhibitor diethyldithiocarbamic acid (DETC) were found to reduce medullary blood flow26 and produce chronic hypertension.7 Garvin et al have demonstrated using perfused isolated tubules that elevations of NO inhibited the activity of the Na⁺/H⁺ exchanger of mTAL.28 We found that elevations of O₂⁻ in mTAL reduces intracellular NO levels.8 Interaction between NO and O₂⁻ was also shown to regulate NaCl transport in TAL.29,30 Since the present study demonstrates that excess sodium (or glucose) transport in mTAL increases O₂⁻ production, one would anticipate that the related reductions in the bioavailability of NO would increase the activity of Na⁺/H⁺ exchanger and produce yet greater O₂⁻, a positive feedback cycle (Figure 4, tubular oxidative stress cycle). As we have previously proposed, these events could also reduce the medullary bioavailability of NO and thereby reduce medullary blood flow resulting in ischemia with further induction of oxidative stress31 and chronic progressive renal injury (tubular-interstitial vicious cycle, Figure 4).
Although the present results show that oxidative stress is induced by exposure to high extracellular NaCl, glucose, and T3, it is also evident that these stimuli normally do not induce renal injury or hypertension when administered in excess to Sprague-Dawley rats. It appears that the presence of antioxidative enzymes such as SODs and catalases normally buffer these O$_2^-$ actions. There is also evidence from our laboratory that O$_2^-$ produced in mTAL was greatly buffered by NO. However, if medullary NO production was reduced in the medulla, as has been shown in the Dahl S rat model, one would anticipate that an increased sodium load would more readily lead to oxidative stress with reductions of medullary blood flow and greater interstitial fibrosis in the outer medulla, as has been shown to be the case in hypertensive Dahl S rats.

**Perspectives**

The mTAL is a well recognized site of active electrolyte transport, and we have shown that even relatively small physiological increases of extracellular solute (NaCl and glucose) concentrations increases the production of O$_2^-$ within these cells. Furthermore, exposure of mTAL to elevations of hormones (T3 and angiotensin II) similarly increased O$_2^-$ production in mTAL. We have shown in the present studies that O$_2^-$ produced by these solutes is dependent on the Na$^+$/K$^+$-ATPase pump and Na$^+$/H$^+$ exchangers. It would be important to now confirm whether the preliminary evidence is correct, indicating that the gp-91 phox membrane subunit of NADPH oxidase is involved in H$^+$ transport with increases of intracellular pH resulting in the enhancement of O$_2^-$ production. Further studies are required to determine if the Na$^+$/H$^+$ exchanger is the final common pathway and determinant of increased O$_2^-$ production leading to renal injury in salt-induced forms of hypertension and diabetes mellitus.

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**References**


**Figure 4.** Proposed mechanisms of oxidative stress induced by metabolic activation in mTAL. Details are explained in the text of the Discussion and Perspectives sections.
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