Increase of sodium delivery stimulates the mitochondrial respiratory chain
H$_2$O$_2$ production in rat renal medullary thick ascending limb

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Ohsaki Y, O’Connor P, Mori T, Ryan RP, Dickinson BC, Chang CJ, Lu Y, Ito S, Cowley AW, Jr. Increase of sodium delivery stimulates the mitochondrial respiratory chain H$_2$O$_2$ production in rat renal medullary thick ascending limb. Am J Physiol Renal Physiol 302: F95–F102, 2012. First published October 5, 2011; doi:10.1152/ajprenal.00469.2011.—The mitochondria-rich epithelial cells of the renal medullary thick ascending limb (mTAL) reabsorb nearly 25% of filtered sodium (Na$^+$) and are a major source of cellular reactive oxygen species. Although we have shown that delivery of Na$^+$ to the mTAL of rats increases superoxide (O$_2^-$) production in mTAL, little is known about H$_2$O$_2$ production, given the lack of robust and selective fluorescent indicators for determining changes within the whole cell, specifically in the mitochondria. The present study determined the effect of increased tubular flow and Na$^+$ delivery to the mTAL on the production of mitochondrial H$_2$O$_2$ in mTAL. H$_2$O$_2$ responses were determined in isolated, perfused mTAL of Sprague-Dawley rats using a novel mitochondrial selective fluorescent H$_2$O$_2$ indicator, mitochondria peroxo yellow 1, and a novel, highly sensitive and stable cytosolic-localized H$_2$O$_2$ indicator, peroxyfluor-6 acetoxymethyl ester. The results showed that mitochondrial H$_2$O$_2$ and cellular fluorescent signals increased progressively over a period of 30 min following increased tubular perfusion (5–20 nl/min), reaching levels of statistical significance at ~10–12 min. Responses were inhibited with rotenone or antimycin A (inhibitors of the electron-transport chain), polyethylene glycol-catalase and by reducing Na$^+$ transport with furosemide or ouabain. Inhibition of membrane NADPH-oxidase with apocynin had no effect on mitochondrial H$_2$O$_2$ production. Cytosplastic H$_2$O$_2$ (peroxyfluor-6 acetoxymethyl ester) increased in parallel with mitochondrial H$_2$O$_2$ (mitochondria peroxo yellow 1) and was partially attenuated (~65%) by rotenone and completely inhibited by apocynin. The present data provide clear evidence that H$_2$O$_2$ is produced in the mitochondria in response to increased delivery of Na$^+$ to the mTAL, and that whole cell H$_2$O$_2$ levels are triggered by the mitochondrial reactive oxygen species production. The mitochondrial production of H$_2$O$_2$ may represent an important target for development of more effective antioxidant therapies.

reactive oxygen species; mitochondria; kidney; flow

THE REABSORPTION OF NACL IN the medullary thick ascending limbs (mTAL) of Henle normally accounts for 25–30% of the filtered load. The rate of metabolism and cell density of mitochondria of these epithelial cells is highest among all of the nephron segments and among the highest in the body, including the heart (2, 4, 14, 24). Increased delivery of Na$^+$ and mechanical stretch of isolated perfused mTAL results in stimulation of mTAL superoxide (O$_2^-$) production through a PKC-α related pathway (21), which, in turn, enhances the rate of Na$^+$ reabsorption (1, 5, 22, 23, 33). O$_2^-$ production appears to be linked to cell metabolism and Na$^+$ transport in the mTAL, as seen by the increases of reactive oxygen species (ROS) that occur in response to increased luminal flow and Na$^+$ delivery (1).

NADPH-oxidase is a primary source of O$_2^-$ in the mTAL, accounting for nearly 50% of O$_2^-$ production, while the other 50% appears to come from mitochondrial sources, as estimated indirectly using pharmacological agents to inhibit activity of the oxidase (e.g., diphenyleneiodonium and apocynin) and by uncoupling the electron transport chain with dimetironen (38). Little is known about sites of production of H$_2$O$_2$ in the mTAL, although evidence suggests that H$_2$O$_2$ plays an important functional role in the region of the outer medulla. There is evidence in Sprague-Dawley (SD) rats that local excess production of H$_2$O$_2$ within the medulla of the kidney would produce hypertension. Specifically, chronic renal medullary infusion of a SOD inhibitor into the single remaining kidney of nephrectomized SD rats resulted in increased interstitial H$_2$O$_2$ concentrations and produced hypertension (28). Acute interstitial infusion of H$_2$O$_2$ was shown to reduce medullary blood flow and sodium excretion in a dose-dependent manner that was reversible by catalase (7). Chronic renal medullary infusion of H$_2$O$_2$, which increased medullary interstitial H$_2$O$_2$ concentrations threefold, produced chronic hypertension (28). H$_2$O$_2$ was found to be elevated in the renal interstitium of Dahl salt-sensitive rats compared with controls, and interstitial infusion of catalase significantly reduced H$_2$O$_2$ levels and greatly attenuated the salt-induced hypertension and medullary tubulointerstitial fibrosis and capillary injury (37). These studies have indicated that elevations of H$_2$O$_2$ in the renal medulla contribute to blood pressure salt-sensitivity and renal injury. The present study was, therefore, designed to elucidate mechanisms and sources of H$_2$O$_2$ production in the mTAL of the outer medulla.

Two highly sensitive, novel fluorescent probes were utilized: one that could selectively detect H$_2$O$_2$ in the mitochondria of living cells [mitochondria peroxo yellow 1 (MitoPY1)] (10) and the other [peroxylfluor-6 acetoxymethyl ester (PF6-AM)] that detected whole cell H$_2$O$_2$ (12). The goal of the study was to determine the effect of increased tubular flow and sodium delivery on the mitochondrial and whole cell H$_2$O$_2$ production in mTAL of the rat kidney. The results provide the first direct evidence that increases in the delivery of Na$^+$ to the mTAL results in increased production of mitochondria H$_2$O$_2$, which,
in turn, stimulates membrane NADPH-oxidase, yielding an overall increase of intracellular H$_2$O$_2$.

**METHODS**

*Preparation of Rat Renal mTAL*

Male SD rats (Harlan Sprague Dawley, Madison, WI), weighing 180–220 g, were maintained on a commercially available pelleted diet (5001, Purina Mills, Gray Summit, MO) with free access to water. Renal mTAL were isolated from SD rats, as previously reported (30). Rats were anesthetized with pentobarbital sodium (50 mg/kg ip), and the kidneys were perfused to clear the blood with 10 ml of chilled (4°C) Hanks’ balanced salt solution (HBSS) containing 20 mM HEPES (HBSS-H, pH 7.4) and 1 mg/ml bovine serum albumin. This maintained metabolism and O$_2$ consumption at a minimum. Renal microtissue strips were dissected from the outer medulla, and this thin strip of tissue from the inner stripe of the outer medulla containing mTAL was placed on a glass coverslip coated with the tissue adhesive Cell-Tak (BD Biosciences, Bedford, MA) for fluorescence imaging, as our laboratory has previously described (1, 31). The dissecting bath was exposed to room air (21% O$_2$; 159 mmHg), and, when the mTAL were transferred to the imaging chamber, they were maintained at 37°C (Warner Instruments) throughout the protocol, with the perfusion chamber and bath solution exposed to room air (21% O$_2$). Thus during periods in which mTAL would be hypoxic (HBSS-H flushing and before dissection), they were kept cool to limit metabolism, and they were well-oxygenated relative to in vivo conditions during all protocols. From each kidney, generally three to four separate coverslips were prepared containing isolated mTAL. This enabled different treatments of mTAL from the same rats. However, we did not repeat the same treatment on the same rat, so each number (e.g., N = 6) represents a separate rat in all cases. All protocols were approved by the Institutional Animal Care and Use Committee of the Medical College of Wisconsin.

**Fluorescence Imaging of H$_2$O$_2$**

Levels of mitochondrial H$_2$O$_2$ were detected by measuring the intensity of the activated MitoPY1, a novel fluorescent probe for imaging H$_2$O$_2$ levels specifically within the mitochondria of living cells (10). Tissue strips containing mTALs were incubated with MitoPY1, as described in the protocol below, and the coverslips were placed in a heated imaging chamber maintained at 37°C (Warner Instruments) during the experiment. Fluorescence images of changes in the mitochondrial H$_2$O$_2$ level probed by MitoPY1 were obtained using a Leica DMI 6000B inverted microscope equipped with a ×63/1.20 water immersion objective lens (HCX PL APO) and Leica TCS-SP5 laser scanning confocal microscope (Leica, Exton, PA). MitoPY1 was excited at 514 nm, and emission signal from 530 to 590 nm was acquired every 2 min. A series of confocal images were scanned in 2-μm increments and summed for an image representing a three-dimensional projection of the entire 50-μm section. Three-dimensional projected images of each time point were analyzed using LAS AF software. As illustrated in Fig. 1A, MitoPY1 colocalizes with mitochondria in isolated mTAL epithelial cells, demonstrating the high density of this organelle in the mTAL.

Levels of intracellular H$_2$O$_2$ were detected by measuring the intensity of PF6-AM, as described in the protocol below. Fluorescence images of changes in the intracellular H$_2$O$_2$ level probed by PF6-AM were obtained using a Nikon TE-2000U inverted microscope equipped with a ×60/1.1 water immersion objective lens and a high-resolution digital camera (Photometrics Cascade 512B Roper Scientific, Tucson, AZ) (1). Excitation was provided by a Sutter DG-4 175-W xenon arc lamp (Sutter Instrument, Novato, CA) at alternating wave lengths, and emission control was achieved using a Lambda 10 optical filter changer (Sutter Instrument). PF6-AM was excited at 480 nm, and 510/40-nm band-pass emission was acquired every 10 s. As illustrated in Fig. 1B, PF6-AM clearly localizes in the cytoplasm and in the nucleus, although we cannot exclude the possibility that it may...
also enter the mitochondria or other subcellular organelles, such as endoplasmic reticulum.

Fluorescence intensity of all of these images was quantified over an area of ~10–15 mTAL cells using MetaFluor imaging software (Universal Imaging, Downingtown, PA), as our laboratory has reported previously (9, 30, 31).

**Microperfusion of mTAL**

Glass pipettes (Drummond Scientific, Broomail, PA) were pulled to an internal diameter of 8–14 μm. The tip of these micropipettes was beveled and smoothed and mounted on a micromanipulator (World Precision Instruments) on the microscope stage. As our laboratory has previously described (1), a micropipette was inserted into the open lumen of the mTAL, and the tubule was perfused with the desired solution and flow rate using a Nano Pump A1400 (World Precision Instruments, Sarasota, FL).

**Protocols**

**Microperfusion of mTAL to determine effects of tubular flow and NaCl concentration on mitochondrial H₂O₂ production.** To determine mitochondrial H₂O₂ level, mTALs were incubated with 5 μM MitoPY1 in HBSS-H for 1 h at 37°C (10). The tissues were washed twice to remove excess dye and then mTAL were microperfused with HBSS-H at a flow rate of 5 nl/min during a baseline period and then increased to 20 nl/min. Some mTALs were pretreated with 200 U/ml polyethylene glycol-conjugated catalase (200 μM/l), rotenone (10 μM/l), antimycin A (1 μM/l), apocynin (1 mmol/l), or vehicle by adding the specific solution to the fluid in the chamber. In time control studies, tubules were perfused at 5 nl/min throughout the study with vehicle in the chamber solution.

Other mTALs were microperfused using a special pipette that enables rapid exchange of perfusion solution to change Na⁺ concentration ([Na⁺]), and tubules were perfused at a fixed rate of 15 nl/min. Perfusate containing either 0 or 60 mmol/l [Na⁺] was infused during a baseline period and then changed to a perfusate of 0, 60, or 143 mmol/l [Na⁺]. Osmolality of perfusate was adjusted by addition of choline chloride, so that solutions had equal osmolality. Mitochondrial H₂O₂ responses were measured using MitoPY1 fluorescence. At the end of experiments, all mTALs were treated with 1 mM H₂O₂ as a positive control, with removal from analysis of mTALs that did not respond.

**Whole cell H₂O₂ production in response to changes in luminal flow and NaCl.** To compare global H₂O₂ levels vs. mitochondrial H₂O₂ levels, we utilized the recently developed cytosolic-localized fluorescent H₂O₂ probe, PF6-AM (12). To determine intracellular H₂O₂ levels, mTALs were incubated with 5 μM PF6-AM (12) in HBSS-H for 20 min at room temperature. After loading, mTALs were washed twice to remove excess dye and microperfused with HBSS-H at a low flow rate of 5 nl/min during a baseline period, then the flow rate was increased to 20 nl/min, and responses were determined over the next 30 min. During the experiments, mTALs were treated with rotenone (10 μM/l), apocynin (1 mmol/l), or vehicle by introducing the solution to the chamber. In time control studies, tubules were perfused at 15 nl/min throughout the study. All mTAL preparations were treated with 1 mM H₂O₂ at the end of the study as a positive control to ensure responsive mTAL preparations.

**Statistical Analysis**

Data are expressed as means ± SE. The data were analyzed statistically by two-way repeated-measures ANOVA, and multiple comparisons were performed by using Tukey’s test. Statistical analyses were performed using the SigmaPlot 11 (Systat Software, San Jose, CA). Differences were considered significant at a P < 0.05.

**RESULTS**

**Increased Luminal Flow to mTAL Increases Mitochondrial H₂O₂ in mTAL**

MitoPY1 was loaded to mTAL to identify the effect of luminal flow on mitochondrial H₂O₂ production in mTAL (Fig. 2). mTAL, which were microperfused at a low flow rate of 5 nl/min through the entire experiment as a time control, showed no increase of MitoPY1 intensity. In contrast, when the luminal perfusion was increased from 5 to 20 nl/min, MitoPY1 intensity was significantly increased compared with time control. Treatment of the mTAL with the cell-permeable H₂O₂ scavenger, polyethylene-conjugated catalase (200 U/ml) significantly attenuated the increase of mitochondrial H₂O₂ in mTAL induced by the higher flow and was different from the time control. The attenuation of the MitoPY1 signal responses with catalase indicates the specificity of the dye and provides validation that an increase in luminal flow significantly elevates H₂O₂ concentrations within the mitochondria of the epithelial cells of the mTAL.

**Increased Concentration of Luminal Na⁺ Concentration Increases Mitochondrial H₂O₂ Production in mTAL**

To determine if changes in luminal [Na⁺] would stimulate mitochondrial H₂O₂ production independent of changes in luminal flow, mTAL were microperfused at a moderate but constant flow rate of 15 nl/min. Mitochondrial H₂O₂ responses were then determined following increases from 0 to 60 mM, or 60 to 143 mM [Na⁺], and compared with a time control response with perfusate maintained at 60 mM [Na⁺] (Fig. 3). A small progressive rise of the MitoPY1 signal was observed in both the tubules perfused at 15 ml/min, with [Na⁺] fixed at either 0 or 60 mM. Increase of the perfusate [Na⁺] concentration from 0 to 60 mM (0–60) resulted in no significant change in the rate of mitochondrial H₂O₂ production. However, when

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**Fig. 2. The effect of perfusate flow rate on mitochondrial H₂O₂ production was determined in mTAL tissue strips. Perfusate flow rate was maintained at 5 nl/min in a time control group for the entire period (●; n = 6), changed to 20 nl/min after 10 min in another group with vehicle in the bath (○; n = 6), and changed to 20 nl/min with 200 U/ml of polyethylene glycol (PEG)-catalase in the bath (●; n = 6). Mitochondrial H₂O₂ is expressed as the mean ± SE of the change in intensity of the fluorescent dye MitoPY1. Significant differences across time and compared with vehicle group (P < 0.05).**
[Na\(^+\)] was raised from 60 to 143 mM, a significant increase in the production of mitochondrial H\(_2\)O\(_2\) was observed, indicating that a threshold of Na\(^+\) must be achieved to markedly increase levels of mitochondrial H\(_2\)O\(_2\).

**Evidence That Observed Increases of Mitochondria H\(_2\)O\(_2\) Concentrations in Response to Increased mTAL Flow Emanate From the Mitochondria**

Preincubation of mTAL with either a mitochondrial respiratory chain complex I inhibitor (10 \(\mu\)M rotenone) or a complex III inhibitor (1 \(\mu\)M antimycin A) abolished the increases in mitochondrial H\(_2\)O\(_2\) in response to increased mTAL flow compared with vehicle control (Fig. 4). Inhibition of the membrane NADPH-oxidase activity with apocynin (1 mM) had no significant influence on the observed increases of mitochondria H\(_2\)O\(_2\) in response to increased luminal flow in the mTAL (Fig. 5). These data indicate that all of the observed increases of mitochondrial H\(_2\)O\(_2\) associated with increases of tubular flow emanated from the mitochondria itself and not from NAD(P)H oxidase.

**Importance of Increased Na\(^+\) Flux in Signaling Increased Mitochondrial H\(_2\)O\(_2\) Production in Response to Increased mTAL Tubular Flow**

Increased luminal flow results in both an increased delivery of Na\(^+\) to the mTAL, while also stretching the lumen diameter and increasing the wall shear stress. To ascertain the contribution of Na\(^+\) flux to mitochondrial H\(_2\)O\(_2\) production, the microperfused mTAL were pretreated with the Na\(^+\)-K\(^+\)-2Cl\(^-\) (NKCC2) cotransporter inhibitor furosemide (100 \(\mu\)M) or with the Na\(^+\)-K\(^+\)-ATPase inhibitor ouabain (4 mM). Both of these compounds significantly attenuated the mitochondrial H\(_2\)O\(_2\) production stimulated by luminal flow, as shown in Fig. 6, indicating the importance of Na\(^+\) transport in this process.

**Changes in Total Intracellular H\(_2\)O\(_2\) Concentrations With Increased mTAL Tubular Perfusion**

Total intracellular H\(_2\)O\(_2\) responses were determined using the cytosolic-localized H\(_2\)O\(_2\) dye PF6-AM. Figure 7, top, shows that total intracellular H\(_2\)O\(_2\) concentrations were significantly increased when mTAL luminal flow was increased...
from 5 to 20 nl/min compared with the vehicle control (5 to 5 nl/min). In Fig. 7, bottom, it can be seen that pretreatment of mTAL with the complex I inhibitor rotenone (10 μmol/l) partially eliminated this response (~65% reduction; \(P<0.05\)), while pretreatment with apocynin (1 mmol/l) completely eliminated the responses to increased tubular flow compared with vehicle. As discussed below, these data indicate an important interaction between activation of mitochondria by \(\text{Na}^+\) transport and cell membrane NAD(P)H-oxidase \(\text{H}_2\text{O}_2\) production.

**DISCUSSION**

This study provides the first direct evidence that physiological increases of mTAL tubular flow and associated \(\text{Na}^+\) transport stimulate mitochondrial \(\text{H}_2\text{O}_2\) production. The data indicate that this, in turn, may stimulate an increased production of \(\text{H}_2\text{O}_2\) from the membrane NADPH-oxidase pathway, thereby enhancing an overall increase of intracellular \(\text{H}_2\text{O}_2\) levels. The application of the novel mitochondria-specific \(\text{H}_2\text{O}_2\) fluorescent dye (MitoPY1) has enabled, to our knowledge, the first in vitro characterization of mitochondrial oxidative stress using a normal physiological stimulus. The mitochondrial source of the \(\text{H}_2\text{O}_2\) production is confirmed by the data presented in Figs. 4 and 5. First, it was seen that the response of the Mito-PY1 dye was not different in isolated mTAL in the absence of or with pretreatment with apocynin (Fig. 5). Second, inhibition of the mitochondrial electron transport chain at complex I with rotenone or complex III with antimycin A both completely abolished any increase of \(\text{H}_2\text{O}_2\) (MitoPY1) in response to increased mTAL perfusion (Fig. 4). If \(\text{H}_2\text{O}_2\) from extramitochondrial sources contributed to the signal, we would not expect to have observed complete inhibition and even a reduction in the signal, as seen in Fig. 4. Together these data indicate that the observed increases of mitochondrial \(\text{H}_2\text{O}_2\) did not emanate from sources outside the mitochondria and were not produced by the cell membrane.

While \(\text{O}_2^-\) produced from a single reduction of molecular oxygen is likely the primary ROS produced in mitochondria, \(\text{O}_2^-\) is quickly converted to \(\text{H}_2\text{O}_2\), a membrane-permeable ROS, by MnSOD (40). The present results are consistent with recent observations that ANG II stimulation increases both mitochondrial \(\text{H}_2\text{O}_2\) (determined using MitoPY1) and whole cell \(\text{H}_2\text{O}_2\) (determined using PF6-AM), a response that was partially inhibited by rotenone (27).

**Evidence of Feed-Forward \(\text{H}_2\text{O}_2\)-\(\text{H}_2\text{O}_2\) From Mitochondria to Membrane NADPH Oxidase**

NAD(P)H-oxidase stimulated \(\text{O}_2^-\) production is greatest in the mTAL and cortical thick ascending limb (26). Increases of mTAL perfusion and/or tubular stretch increase mTAL \(\text{O}_2^-\) generation, and this, in turn, has been found to enhance the rate of \(\text{Na}^+\) reabsorption (1, 5, 16, 22, 23, 33). The contribution of...
mitochondria to total ROS production in these events is supported by observations that increases of mitochondrial H$_2$O$_2$ concentrations were required to achieve a robust increase of cellular H$_2$O$_2$ (see Fig. 7, top). Inhibition of mitochondrial H$_2$O$_2$ production by rotenone (Fig. 7, bottom), as visualized by the lack of changes of MitoPY1 fluorescence with increased luminal flow, resulted in a significant reduction (~65%) of the whole cell H$_2$O$_2$ (PF6-AM fluorescence) response to the change in flow. The whole cell H$_2$O$_2$ response to increased tubular flow and Na$^+$ delivery was completely eliminated by pretreatment of mTAL with apocynin. We propose that the small, but statistically significant, increase of PF6-AM fluorescence in the presence of apocynin serves to feed forward to stimulate membrane NAD(P)H oxidase production of H$_2$O$_2$. It is not possible to quantitatively compare the relative changes in fluorescent responses between the two dyes, since the absolute actual intracellular concentrations can only be estimated and depend on many different physical attributes of the cell and of the dyes. The inhibition of mitochondrial respiration with rotenone would be expected to result in an increase in glycolytic metabolism, which may stimulate membrane NADPH oxidase. However, it is more likely that the mitochondria drives this response via mitochondrial H$_2$O$_2$ production, as supported by results obtained by others in aortic endothelial cells (3, 15, 35). The present study provides evidence that mitochondrial H$_2$O$_2$ is produced and escapes in sufficient amounts to serve as the signaling molecule to enhance NADPH oxidase, either directly or via yet unidentified intermediate signaling pathway(s). Our data are consistent with these earlier observations in which the vascular endothelial cells were stimulated with high, nonphysiological concentrations of ANG-II or H$_2$O$_2$ (2, 15, 35). H$_2$O$_2$ is thought to escape to the cytoplasm, probably through the opening of the prolonged (high-conductance) mitochondrial permeability transition pore (19, 41) and then act via c-Src to stimulate NADPH oxidase (3, 39). Whatever the precise mechanism, the present data provide clear evidence that the increased cellular levels of H$_2$O$_2$ are triggered by changes in mitochondria that are driven by increased Na$^+$ transport in the mTAL.

In contrast, pretreatment of mTAL with apocynin had no effect on mitochondrial H$_2$O$_2$ responses to increased tubular flow (Fig. 5), indicating that H$_2$O$_2$ produced from membrane NADPH oxidase does not feed-forward to stimulate mitochondrial H$_2$O$_2$ production. Evidence of dual feed-forward loops (one from cell membrane ROS production that stimulates mitochondrial ROS, and the other from mitochondrial ROS stimulating the membrane NADPH oxidase) has been obtained using vascular endothelial cells, which were stimulated with high, nonphysiological concentrations of ANG-II or H$_2$O$_2$ (3, 15, 35). These studies found that membrane NADPH-oxidase-derived O$_2^-$ increased mitochondrial ROS by opening mitochondrial K$^+$ channels, which then further activated the membrane NAD(P)H oxidase, indicating the presence of a vicious cycle (3, 15, 35). This feed-forward vicious cycle has been proposed to contribute to ANG-II-induced vascular dysfunction and hypertension.

Although no evidence for a vicious cycle was found in our mTAL that were stimulated with changes in luminal flow and Na$^+$, it should be recognized that the present studies were designed to expose the mTAL only to changes of luminal flow and Na$^+$ delivery within the physiological range. A vicious cycle would not be expected to be revealed under these circumstances, since this would produce a highly unstable system and disrupt normal homeostatic processes. It is reasonable to expect, however, that, in hypertensive states such as rats chronically treated with ANG-II or in Dahl salt-sensitive rat strains, a vicious cycle may occur as ROS production is driven to very high levels. Protection from damaging effects of mitochondria ROS production was recently demonstrated by the scavenging of mitochondrial O$_2^-$ with mito-TEMPO in ANG-II-induced hypertension in mice and in transgenic mice overexpressing mitochondrial SOD2 (13).

**Functional Relevance of Relationship of Na$^+$ Transport to mTAL-H$_2$O$_2$ Production**

Nearly 20–30% of filtered NaCl in the kidney are reabsorbed in the mTAL, where Na$^+$ is uniquely reabsorbed independent of H$_2$O$_2$, enabling the formation of the urinary concentration gradient. About 50% of Na$^+$ mTAL reabsorption is paracellular due to the positive lumen electrical gradient generated by apical K$^+$ conductance, whereas the remaining 50% is transcellular (4). Na$^+$ enters through the apical membranes of the mTAL via NKCC2 cotransporters (~75%) and apical Na$^+$/H$^+$ exchangers (NHE3) (~25%). The driving force for Na$^+$ reabsorption by mTAL is provided by basolateral Na$^+$/K$^+$/ATPase, which extrudes Na$^+$ from the cell. The high rates of metabolism required for this transport of Na$^+$ are accommodated by a high density of mitochondria (4, 24).

It is evident from the present study, in which ouabain and furosemide inhibited mitochondrial ROS production, that an increase in mTAL Na$^+$ transport is required to stimulate an increase in mitochondrial H$_2$O$_2$ production. The precise mechanism responsible for signaling the mitochondria is unknown. Interestingly, however, only a change in [Na$^+$] from 60 to 149 mM stimulated mitochondrial H$_2$O$_2$, not a change in [Na$^+$] from 0 to 60 mM, consistent with previous observations in our laboratory in which whole cell changes of O$_2^-$ were determined (1). These observations are not easily explained based on the reported kinetics of the NKCC2 cotransporter obtained from perfused rat mTAL (17, 20, 36) or based on the kinetics determined for the three major isoforms of the NKCC2 cotransporter identified in the apical membrane of murine mTAL. When expressed in Xenopus oocytes, NKCC isoforms exhibited EC$_{50}$ values for Na$^+$, ranging from 3 to 21 mM (34). Since these concentrations are well below in vivo tubular mTAL [Na$^+$], ranging from 60 to 160 mM (4), these data indicate that, even under normal conditions of mTAL Na$^+$ delivery, these key transporters of Na$^+$ reabsorption would be operating above saturation levels. Yet it is well recognized that the net transport rate of the mTAL is exceedingly high, ranging from 87 to 870 pmol·mm$^{-1}$·min$^{-1}$ (4), and provides the mTAL segment with its remarkable capacity to “buffer” NaCl loads (17). The isolated mTAL ceases to reabsorb NaCl when the perfusate contains only 50 mmol/l Na$^+$ and/or if the flow rate is very low (17).

Studies by Lee and McDonough (25) may explain the wide range of transport rates that appear in the literature and provide direct evidence that an increased luminal flow and Na$^+$ delivery are related to an insertion of Na$^+$ transporters in the mTAL. The net increase of Na$^+$ transport by these mechanisms could explain the effects of high luminal [Na$^+$] and delivery upon
ROS production in the present study. That 60 mM Na\(^+\) was unable to evoke a response of the mitochondria to produce H\(_2\)O\(_2\) (Fig. 3) suggests that some threshold may be required to initiate these changes in mTAL tubular Na\(^+\) transport. One would expect in vivo that a high-salt diet would likely be to promote production of H\(_2\)O\(_2\), since Na\(^+\) levels entering the mTAL are greater than isotonic and close to that of the interstitial space (17). The present study shows that increased Na\(^+\) transport was the overall signal that stimulated mitochondrial H\(_2\)O\(_2\) production in the mTAL, although exactly what relays this signal to the mitochondria remains unknown. It is interesting that high-salt intake appears to differentially regulate surface NKCC2 expression in mTAL of Dahl S and Dahl R rats via phosphorylation of NKCC2 (18). Dahl S rats increased surface NKCC2 without affecting total NKCC2 expression or phosphorylated-NKCC2, while Dahl R rats showed the opposite response. An increase in NKCC2 activity in salt-sensitive strains could thereby exacerbate the stimulatory effect on mitochondrial H\(_2\)O\(_2\) production.

The functional consequences of increased H\(_2\)O\(_2\) production in the mTAL and elevations of cellular H\(_2\)O\(_2\) are beginning to emerge, but much remains to be resolved. Increased intracellular production of H\(_2\)O\(_2\), as initiated by increased mitochondrial ROS production and amplified by a feed-forward stimulation of membrane NADPH oxidase, could be important not only in cellular energetics and renal injury, but also in the overall regulation of medullary vasa recta blood flow. H\(_2\)O\(_2\) is a more stable and diffusible molecule than O\(_2\)\(^{•−}\) and could more efficiently target the capillaries that surround the mTAL, as our laboratory has demonstrated with nitric oxide (NO) and O\(_2\)\(^{•−}\), when tissue NO concentrations are very low (8, 30). As detailed in the Introduction, H\(_2\)O\(_2\) has been shown to serve as a vasoconstrictor of the vasa recta capillaries and, when infused into the medullary interstitial space, reduces medullary blood flow and sodium excretion and increases arterial blood pressure (7). In pathological states of salt sensitivity as in the Dahl salt-sensitive rat model, where medullary tissue H\(_2\)O\(_2\) concentrations are elevated and outer medullary tissue injury is prevalent (37), the effects of medullary H\(_2\)O\(_2\) appear to be of great consequence.

Comments Related to the Fluorescent Dyes

As with any study utilizing fluorescent probes to measure local cellular events, it is important to have a high level of confidence of both the localization and specificity of the probes. Since mTAL are highly susceptible to oxidative stress (8) and the production of a variety of ROS, it was critical to utilize an H\(_2\)O\(_2\)-selective probe for the present study. It has been challenging to develop organelle-targeted small molecules to detect specific ROS in living cells (11, 42). MitoPY1 represents a new type of fluorophore for imaging mitochondrial ROS, as developed and validated by Dickinson and Chang (10). Designed with a chemo-specific boronate switch (6, 29), the probe is specific for H\(_2\)O\(_2\) over NO, O\(_2\)\(^{•−}\), and hydroxyl radical (10). MitoPY1 preferentially localizes to the mitochondria (Fig. 1A) due to a triphenylphosphonium targeting group that takes advantage of the pH gradient that is specific to the mitochondria (32). MitoPY1 was initially validated in proof-of-principle experiments involving a neurodegenerative disease model and other pathological stress conditions in model cell lines.

PF6-AM is also a recently designed probe with lipophilic acetoxyethyl esters groups that allow the dye to pass readily through cell membranes into the cytoplasm (12). Once in the cell, esterases de-protect the acetoxyethyl ester groups, resulting in a dianionic form of PF6 that is membrane impermeable and is thereby trapped in the cell, where it can respond to intracellular H\(_2\)O\(_2\) levels. Because it is so well trapped compared with first- and second-generation boronate probes, the PF6 dye has greater sensitivity. In the present study, this enhanced cellular retention (Fig. 1B) not only provided greater sensitivity, but also enabled us to track the changes of cellular H\(_2\)O\(_2\) over prolonged periods of time, which were necessary to characterize the slowly developing H\(_2\)O\(_2\) responses (15–30 min) within the mTAL following the increase of luminal flow. More generally, the application of multiple H\(_2\)O\(_2\)-specific fluorescent probes with similar structures but different cellular localizations provides a powerful approach to elucidate the roles of ROS generation with organelle resolution.

Perspectives

The present study demonstrates that increased delivery of Na\(^+\) to the mTAL can increase mitochondrial H\(_2\)O\(_2\) and intracellular H\(_2\)O\(_2\) in isolated mTAL epithelial cells and thereby may contribute to salt-induced hypertension and renal injury. Others have shown that mitochondria-targeted antioxidants, such as mito-TEMPO, can have significant beneficial antihypertensive effects. The disappointing effects of vitamin E and other antioxidant dietary supplementation on ROS-driven degenerative processes of chronic diseases, such as aging, diabetes, and hypertension, have been puzzling, and many efforts have been made to explain these failures. One common explanation is that these agents have not been targeted to sites of ROS generation and never reach sufficient concentrations to achieve a therapeutic impact. The present study indicates that the mitochondria of the mTAL may be one such important site for which more effective therapeutic approaches could be targeted.

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DISCLOSURES

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AUTHOR CONTRIBUTIONS

Author contributions: Y.O., R.P.R., B.C.D., and Y.L. performed experiments; A.W.C.J. conception and design of research; A.W.C.J. drafted manuscript; P.M.O., T.M., B.C.D., C.J.C., and A.W.C.J. interpreted results of experiments; R.P.R., B.C.D., C.J.C., Y.L., S.I., and A.W.C.J. approved final version of manuscript; C.J.C., and A.W.C.J. edited and revised manuscript; Y.O., P.M.O., T.M., R.P.R., B.C.D., C.J.C., Y.L., S.I., and A.W.C.J. performed experiments; A.W.C.J. conception and design of research; A.W.C.J. drafted manuscript.

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