Activation of NAD(P)H oxidase by outward movements of H + ions in renal medullary thick ascending limb of Henle

Ningjun Li, Guo Zhang, Fu-Xian Yi, Ai-Ping Zou and Pin-Lan Li

Am J Physiol Renal Physiol 289:1048-1056, 2005. First published Jun 21, 2005;
doi:10.1152/ajprenal.00416.2004

Am J Physiol Renal Physiol 289: F1048–F1056, 2005.
First published June 21, 2005; doi:10.1152/ajprenal.00416.2004.

Activation of NAD(P)H oxidase by outward movements of H^+ ions in renal medullary thick ascending limb of Henle

Ningjun Li, Guo Zhang, Fu-Xian Yi, Ai-Ping Zou, and Pin-Lan Li

Department of Pharmacology and Toxicology, Medical College of Virginia, Virginia Commonwealth University, Richmond, Virginia

Submitted 22 November 2004; accepted in final form 17 June 2005

Li, Ningjun, Guo Zhang, Fu-Xian Yi, Ai-Ping Zou, and Pin-Lan Li. Activation of NAD(P)H oxidase by outward movements of H^+ ions in renal medullary thick ascending limb of Henle. *Am J Physiol Renal Physiol* 289: F1048–F1056, 2005. First published June 21, 2005; doi:10.1152/ajprenal.00416.2004.—The present study was designed to test the hypothesis that the production of superoxide ($O_2^-\cdot$) by NAD(P)H oxidase is coupled to tubular metabolic activity through ionic activation mediated by H^+ movement across cell membrane. Using dual fluorescent microscopic imaging analysis, intracellular $O_2^-\cdot$ levels and pH (pH_i) in renal medullary thick ascending limb of Henle (TALH) cells were simultaneously measured. It was found that intracellular $O_2^-\cdot$ levels in these cells were increased in parallel to the elevation of pH_i by outflow of H^+ induced via NH_4Cl loading followed by rapid removal. This increase in intracellular $O_2^-\cdot$ levels was substantially blocked by an inhibitor of Na^+/H^+ exchanger, methylisobutyl-amiloride (MIA; 100 μM), a chemical SOD mimetic, Tiron (1 mM) or an inhibitor of NAD(P)H oxidase, diphenylene iodonium (DPI; 100 μM). In additional groups of TALHs, a proton ionophore, carbonylcyanide *m*-chlorophenylhydrazone (10 μM) was used to produce H^+ conductance, leading to H^+ flux across cell membrane depending on extracellular pH. The efflux of H^+ increased both pH_i and intracellular $O_2^-\cdot$ levels, but the influx of H^+ did not increase intracellular $O_2^-\cdot$ levels. The H^+ efflux-induced increase in intracellular $O_2^-\cdot$ levels was completely blocked by DPI and another NAD(P)H oxidase inhibitor, apocynin (100 μM). In in invo experiments, renal medullary infusion of MIA (100 μM) was found to significantly decrease the concentrations of H_2O_2 in the renal medullary interstitium. These results suggest that it is the outward movements of H^+ ions that activates NAD(P)H oxidase to produce $O_2^-\cdot$ in TALH cells. This H^+ outflow-associated activation of NAD(P)H oxidase importantly contributes to tissue levels of reactive oxygen species in the renal medulla.

redox signaling; renal tubules; ion transporter; Na^+/H^+ exchanger; superoxide

RECENT STUDIES HAVE indicated that reactive oxygen species (ROS) are importantly involved in intracellular signaling and thereby participate in the regulation of various physiological and pathological processes in a variety of mammalian cells or tissues. There is overwhelming evidence that NAD(P)H oxidase is one of the major sources of superoxide ($O_2^-\cdot$) production in vascular and renal cells under physiological conditions (1, 8, 18, 21, 33, 34, 36, 40, 48). Despite the extensive studies on the role of NAD(P)H oxidase in mediating physiological or pathological activities in different mammalian cells, the mechanisms activating or regulating the activity of this enzyme are still poorly understood.

It had been well documented that NAD(P)H oxidase reduces O_2 to form $O_2^-\cdot$ using NAD(P)H as an electron donor. In phagocytes or phagocyte-like cells, proton efflux is coupled with the production of $O_2^-\cdot$ by NAD(P)H oxidase and that inhibition of proton efflux leads to inhibition of $O_2^-\cdot$ generation (11, 13, 15, 23, 28, 29). This coupling between activation of NAD(P)H oxidase and proton efflux has been indicated to play an important role in the maintenance of intracellular pH (pH_i) by removal of excessive H^+ produced by activated NAD(P)H oxidase (28, 29). However, there is increasing evidence demonstrating that proton efflux not only results from the activation of NAD(P)H oxidase, but also serves as an activator or modulator of this enzyme if primary stimuli act on proton exchangers or channels. For example, in human neutrophils and eosinophils, phorbol myristate acetate (PMA) was found to activate NAD(P)H oxidase and increase H^+ currents and electron currents, and the activation of H^+ currents preceded the significant activation of NAD(P)H oxidase (13, 14). Diphenylene iodonium (DPI), a NAD(P)H oxidase inhibitor, inhibited the enzyme activity but had no effect on the enhanced proton current (13, 14). These results suggest that stimulation of H^+ currents by PMA is its primary effect and NAD(P)H oxidase activation is a later event. Using H^+ ionophore, NAD(P)H oxidase-dependent $O_2^-\cdot$ production was demonstrated to be stimulated by H^+ movements across cell membrane of neutrophils (25). Studies using pulmonary arteries demonstrated that blockade of H^+ channel by Zn^{2+} and Cd^{2+} significantly inhibited the $O_2^-\cdot$ production via NAD(P)H oxidase and blocked hypoxic pulmonary vasoconstriction (12, 30, 31) and that inhibition of carbonic anhydrase changed pH_i and attenuated NAD(P)H oxidase-dependent pulmonary vasoconstriction (16). These reports support the view that outflow of H^+ may importantly contribute to the regulation of NAD(P)H oxidase activity.

Although NAD(P)H oxidase has been reported to play an important role in the regulation of renal function, so far little is known regarding the mechanisms activating or regulating this enzyme in renal tissues or cells, in particular, under physiological conditions. We recently reported that NAD(P)H oxidase-mediated production of $O_2^-\cdot$ was the primary source of intracellular $O_2^-\cdot$ in thick ascending limb of Henle (TALH) and it importantly contributed to the tissue $O_2^-\cdot$ levels in the kidneys (34, 48). This NAD(P)H oxidase-derived $O_2^-\cdot$ production in TALH is related to the metabolic activity in this tubular segment (34, 38). However, there is no evidence elucidating how tubular activity is coupled with NAD(P)H oxidase activation.

Address for reprint requests and other correspondence: N. Li, Dept. of Pharmacology and Toxicology, Medical College of Virginia, Virginia Commonwealth Univ., P.O. Box 980613, Richmond, VA 23298 (e-mail: nli@vcu.edu).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The present study was designed to further test the hypothesis that the production of $O_2^-\cdot$ by NAD(P)H oxidase is regulated by outward movement of H^+ associated with tubular activity in rat TALHs. By simultaneously monitoring the intracellular levels of pH and $O_2^-\cdot$ using dual fluorescence microscopic imaging techniques, we examined the effects of Na^+/H^+ exchanger-dependent H^+ outflow and passive chemical gradient-dependent H^+ influx or efflux on $O_2^-\cdot$ production via NAD(P)H oxidase in isolated TALHs. Using microdialysis analysis, we also determined the contribution of this H^+ outflow-regulated NAD(P)H oxidase activation to renal interstitial ROS levels in in vivo animal experiments. Our results demonstrated that in TALH cells, an outward flow of H^+ associated with Na^+/H^+ exchange or direct efflux was capable of activating NAD(P)H oxidase and enhancing $O_2^-\cdot$ production. This H^+ outflow-activated NAD(P)H oxidase may be one of the important mechanisms determining renal tissue redox status associated with its metabolic activity.

MATERIALS AND METHODS

Animals. Male Sprague-Dawley (Harlan, Madison, WI) rats weighing 250–300 g were maintained on a standard pellet diet (Purina Mills) with free access to water. All animal procedures were approved by the Institutional Animal Care and Use Committee.

Microdissection of nephron segments. The left kidney in anesthetized male Sprague-Dawley rats was flushed with 20 ml of ice-cold PBS (10 mM phosphate, 140 mM NaCl, 2.7 mM KCl, pH 7.4) and then removed and dissected to isolate the outer medulla. The outer medulla was transferred into a temperature-controlled dissection bath (0–4°C). Under a LEICA MZ8 stereomicroscope with dark field illumination, five to eight thin tissue strips were peeled, which mainly contained a single layer of tubules and vessels (37). These strips were used to load fluorescence dyes for simultaneous monitoring of pH_i and $O_2^-\cdot$ in TALHs.

Simultaneous measurement of pH_i and $O_2^-\cdot$ levels in TALHs. The levels of pH_i and $O_2^-\cdot$ were measured by fluorescent imaging analysis as described previously in our studies and by others (34, 44, 46). In brief, renal medullary strips were transferred to a coverslip coated with cell-touch adhesives Cell-Tak (BD Biosciences). After an incubation of 20–30 min, the tissue strips were attached to the coverslips. Then, the coverslips were placed into a recording chamber (Warner Instruments, Hamden, CT) installed on the stage of an inverted microscope (Nikon Diaphot). This glass recording chamber held 1 ml of solution. A buffer change system was built in the chamber that could be flushed to change bath solution. There was no flow-through chamber, and samples were not suffused with buffer during experiments. The recording chamber was filled with modified Hanks' buffered saline solution containing (in mM) 137 NaCl, 5.4 KCl, 4.2 $NaHCO_3$, 3 Na_2HPO_4, 0.4 KH_2PO_4, 1.5 $CaCl_2$, 0.5 $MgCl_2$, 0.8 $MgSO_4$, 10 glucose, 10 HEPES (pH 7.4). After a 30-min equilibration period, the chamber solution was exchanged with 1 ml of Hanks' buffer containing 2,7-*bis*-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxy methyl (BCECF-AM; for pH assay, 10 μM; Molecular Probes, Eugene, OR), dihydroethidium (DHE; for $O_2^-\cdot$ assay, 2 μM; Sigma, St. Louis, MO), and BSA (0.05%). The tissue strips were incubated in this loading solution for 30 min at room temperature and then rinsed three times with Hanks' buffer to remove extracellular DHE and BCECF-AM. DHE can enter the cell and is fluorescent with excitation/emission of 380/435 nm in cell cytoplasm. DHE is oxidized specifically by $O_2^-\cdot$ to yield ethidium (Eth), which binds to DNA and has fluorescence at excitation/emission of 480/610 nm. The increase in Eth-DNA fluorescence is suggestive of $O_2^-\cdot$ production within cells (5, 9). The fluorescence excitation profile of BCEDF is pH dependent (43). BCECF-AM is nonfluorescent and its conversion to fluorescent BCECF via the action of intracellular esterase has been most widely used as fluorescent indicator for pH_i (6, 7, 17, 19).

After dyes were loaded, temperature of the bath solution was adjusted to 37°C by a temperature control system (Warner Instruments) and the tissue strips were equilibrated at 37°C for 30 min. Under microscope with a ×20 phase/fluorescence objective (Nikon Diaphot), TALHs were focused and 5–10 areas with strong fluorescence loading on TALH walls were chosen for continuous recording of pH_i and $O_2^-\cdot$. The excitation light through a filter of 440 ± 10 and 480 ± 10 nm for BCECF, 380 ± 20 nm for DHE, and 490 ± 20 nm for Eth were provided using a high-speed wavelength switcher (Lambda DG-4, Sutter, Novato, CA). Emission light from TALHs was passed through an emission filter of 520 ± 20 nm for BCECF, 430 ± 15 nm for DHE, and 610 ± 25 nm for Eth using a high-speed rotating filter wheel (Lambda 10–2, Sutter). The fluorescence images were recorded by a digital camera (SPOT RT Monochrome, Diagnostic Instruments), and a Metafluor imaging and analysis software (Universal Imaging) was used to acquire, digitize, and store the images for off-line processing and statistical analysis.

Induction of H^+ movements across cell membrane of dissected TALHs. Two methods were used to induce the movement of H^+ across cell membrane in this TALH preparation. For Na^+/H^+ exchange-dependent outflow of H^+, TALHs were loaded with NH_4Cl for 3 min and then washed by NH_4Cl free Hanks' solution. This NH_4Cl loading and removal will rapidly eliminate NH_3 and result in retention of H^+ within the cells. After removal of NH_4Cl, a time-dependent outflow of H^+ occurs, which is primarily dependent on Na^+/H^+ exchanger (NHE) activity. This NH_4Cl prepulse method is widely used in different studies of Na^+/H^+ exchange in renal tubular cells. (6, 17, 19). To inhibit the outflow of H^+ under this condition, MIA, a NHE inhibitor (100 μM), was used. Because Na^+/H^+ exchange is also dependent on extracellular Na^+, adjustment of the bath solution into Na^+-free solution (replace NaCl with *N*-methyl-D-glucamine or KCl) could significantly block H^+ outflow (17, 19, 25, 27), which was used as an additional method to inhibit Na^+/H^+ exchange. To explore the potential roles of other acid/base transporters besides NHE, such as H^+-ATPase (3) and Na/HCO_3 cotransporter (32) in H^+ outflow and $O_2^-\cdot$ production after NH_4Cl prepulse, bafilomycin (5 μM), an inhibitor of H^+-ATPase (17), and 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS; 1 mM), an inhibitor of Na/HCO_3 cotransporter (32), were used.

In addition, a proton ionophore, carbonylcyanide *m*-chlorophenylhydrazone (CCCP), was used to produce H^+ permeability across TALH cell membrane. After an incubation of TALHs with CCCP (10 μM) for 30 min, H^+ permeability or conductance was formed and then a chemical gradient-dependent H^+ influx or efflux was generated by altering pH in the bath solution (25, 26). This H^+ influx or efflux was determined by monitoring pH_i as described above.

The association between $O_2^-\cdot$ production and H^+ movements across cell membrane of TALHs was examined by a parallel monitoring of pH_i and $O_2^-\cdot$. To confirm the Eth fluorescence was $O_2^-\cdot$ specific, a superoxide scavenger, Tiron (20, 47) (1 mM, Sigma), was used to block the changes in Eth fluorescence. To determine whether $O_2^-\cdot$ production was derived from NAD(P)H oxidase, different inhibitors of NAD(P)H oxidase, DPI (100 μM, Sigma) and apocynin (100 μM, EMD Bioscience, La Jolla, CA), were utilized.

Effects of Na^+/H^+ exchange-dependent H^+ movements on renal redox status in in vivo animal experiments. Rats were prepared for renal medullary interstitial infusion and microdialysis as we described previously (10, 48). After being anesthetized with ketamine (Ketaject; 30 mg/kg body wt im; Phoenix Pharmaceutical, St. Joseph, MO) and thiobutabarbital (Inactin; 50 mg/kg body wt ip; Sigma), the rats were placed on a thermostatically controlled warming table to maintain body temperature at 37°C. A catheter was placed in the left femoral vein for a continuous infusion of 0.9% NaCl solution containing 2% albumin at a rate of 1 $ml \cdot h^{-1} \cdot 100$ g body wt^{-1} throughout the experiment to replace fluid loss and maintain a constant hematocrit

(≈40%). For in vivo microdialysis experiments, the left kidney was immobilized by placement of its dorsal side up in a kidney cup. A microdialysis probe (Bioanalytical Systems, West Lafayette, IN) with a 0.5-mm tip diameter, 2-mm dialysis length, and 20-kDa transmembrane diffusion cutoff was gently implanted into the outer medulla (5–5.5 mm in depth) vertically from the dorsal surface. The probe was perfused with PBS containing (in mM) 205 NaCl, 40.5 Na_2HPO_4, and 9.5 NaH_2PO_4 (pH 7.4, 550 mosM) at a rate of 2.0 μl/min throughout the experiment. This microdialysis probe is also constructed with an incorporated infusion line. This infusion line goes through inside the dialysis probe and exits from the tip of the probe. This third cannula was used for renal medullary interstitial infusion during collection of the dialysate when it was implanted into the renal medulla. After a 1.5-h equilibration period, two 30-min control dialysates were collected for the analysis of basal renal interstitial H_2O_2 levels as a marker of ROS, followed by infusion of methylisobutyl-amiloride (MIA; 100 μM, Sigma). At the end of each experiment, H_2O_2 concentrations of dialysate samples (50 μl) were measured with a fluorescence dye (Amplex red reagent) as described below.

A plastic catheter was inserted into the left and right ureters, respectively, for urine collection. Urine samples were used for analysis of water, sodium, and potassium excretion as we described previously (10, 48). At the end of each experiment, animals were euthanized with an excess intravenous dose of pentobarbital sodium (150 mg/kg). The left kidney was excised and the position of the dialysis probe was confirmed. If the probe was positioned incorrectly, the data of these dialysates were discarded.

Fluorescence spectrometric assay of H_2O_2 concentrations. Amplex red is a fluorogenic substrate with very low background fluorescence; it reacts with H_2O_2 with a 1:1 stoichiometry to produce highly fluorescent resorufin (35). Fluorescence spectrometry of renal interstitial H_2O_2 levels was performed using Amplex red (Molecular Probes) as we described previously (10). Briefly, 200 μM Amplex red reagent and 1 U/ml horseradish peroxidase were added to the renal dialysate collected from the study (50 μl), a series of H_2O_2 standard solution in 50 mM sodium phosphate buffer (pH 7.4) and sodium phosphate buffer alone as a control for background fluorescence, and the samples were incubated for 30 min in Falcon 96-well microplates in the dark at room temperature. Fluorescence intensity was measured in an automatic microplate reader (model KC_4, Bio-Tek Instruments, Winooski, VT) at an excitation wavelength of 530 ± 25 nm and an emission wavelength of 590 ± 35 nm. After subtraction of background fluorescence, H_2O_2 concentrations of renal interstitial dialysate were calculated on the basis of a H_2O_2 standard curve generated using H_2O_2 and Amplex red.

Urine and electrolyte analysis. Urine flow rates were determined gravimetrically and used to determine electrolyte excretion rates. Urinary sodium concentrations were measured by a flamephotometer (Buck Scientific, East Norwalk, CT) according to the instruction of the manufacturer. The urinary sodium excretion rate ($U_{Na}V$) was calculated and expressed as micromoles per minute per gram of kidney weight.

Statistical analysis. Data are presented as means ± SE. Significant differences in mean values between and within multiple groups were examined by using ANOVA for repeated measures, followed by a Duncan multiple-range test. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Increased intracellular $O_2^-\cdot$ production coupled with Na^+/H^+ exchange-dependent outflow of H^+ in NH_4Cl-prepulsed TALHs. During the recovery period after washing out NH_4Cl from the batch solution, an outward flow of H^+ in TALHs occurred, as indicated by increase in pH_i that was monitored by fluorescence intensity of BCECF. In parallel, intracellular $O_2^-\cdot$ levels were elevated, as indicated by enhanced Eth fluorescence (a ratio of Eth/DHE fluorescence at 610/430 nm). Intracellular $O_2^-\cdot$ levels were presented as the ratio of Eth/DHE fluorescence compared with the 0 time point when NH_4Cl was washed out. After preloading and washing out 20 mM of NH_4Cl, pH_i was 6.85 ± 0.023 and then slowly recovered to 7.25 ± 0.047 during 4–5 min. It has been well known that this recovery of pH_i is due to H^+ outflow mainly dependent on Na^+/H^+ exchanger activity (6, 7, 17, 19, 27). In parallel to the increase in pH_i, intracellular $O_2^-\cdot$ levels increased, as shown by increase in Eth/DHE ratio from 1 to 1.18 ± 0.025 (units) in TALHs. This increase in $O_2^-\cdot$ production was terminated when pH_i increase reached a plateau (Fig. 1*A*). Although increases in pH_i and $O_2^-\cdot$ were parallel, statistically the increase was earlier in pH_i than that in $O_2^-\cdot$ after washing out NH_4Cl. By comparing with 0 time point after washing out NH_4Cl, increases in pH_i reached statistical significance at 60 s, while $O_2^-\cdot$ levels at 90 s.

In control TALHs without loading NH_4Cl, a parallel recording of both pH_i and $O_2^-\cdot$ was also performed. There were no significant changes observed in the fluorescent intensity of BCECF and Eth in these control TALHs (Fig. 1*B*), which served as time control, indicating that alterations of pH_i and $O_2^-\cdot$ were not due to time-dependent autofluorescence in NH_4Cl-prepulsed tubules.

Production of intracellular $O_2^-\cdot$ dependent on Na^+/H^+ exchange-mediated H^+ outflow in NH_4Cl-prepulsed TALHs. In the presence of a Na^+/H^+ exchanger inhibitor, MIA (100 μM), increase in pH_i was blocked during the recovery period after removal of NH_4Cl, which was accompanied by abolishment of

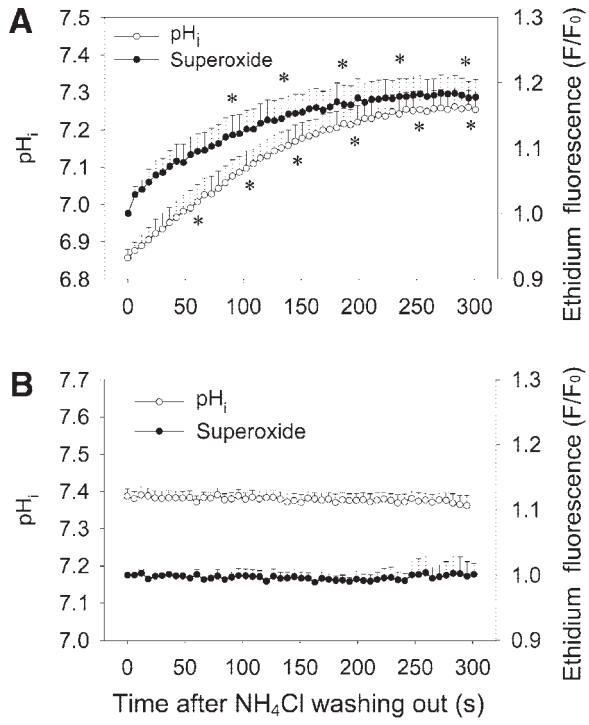

Fig. 1. Outflow of H^+ is coupled to increase in intracellular $O_2^-\cdot$ in NH_4Cl-prepulsed thick ascending limbs of Henle (TALHs). *A*: time-dependent $O_2^-\cdot$ increases parallel to rise of intracellular pH (pH_i). *B*: time control recording of pH_i and $O_2^-\cdot$ in parallel. *$P < 0.05$ compared with the values obtained at 0 s after washing out NH_4Cl ($n = 8$).

increase in the production of intracellular $O_2^-\cdot$ (Fig. 2*A*). Similarly, removal of Na^+ from the bath solution by substituting NaCl for *N*-methyl-D-glucamine (Fig. 2*B*) or KCl (Fig. 2*C*) inhibited Na^+/H^+ exchange (17, 19, 25, 27) and blocked both increases in pH_i and intracellular $O_2^-\cdot$. In the presences of inhibitor of H^+-ATPas or Na/HCO_3 cotransporter, the patterns of increases in pH_i and intracellular $O_2^-\cdot$ levels caused by NH_4Cl prepulse were the same as that in control TALHs (Fig. 3 vs. Fig. 1*A*).

Specific inhibition of $O_2^-\cdot$ increases in NH_4Cl-prepulsed TALHs. To determine whether the Eth fluorescence increase is specific to $O_2^-\cdot$ production, we examined the effect of chemical mimetic of SOD, Tiron, and a selective inhibitor of NAD(P)H oxidase, DPI, on $O_2^-\cdot$ levels during H^+ extrusion after washing out NH_4Cl. First, incubation of TALHs with Tiron (1 mM) specifically blocked the increase in intracellular $O_2^-\cdot$ levels, but it had no effect on the elevation of pH_i induced by removal of NH_4Cl (Fig. 4*A*). The pattern and magnitude of increase in pH_i

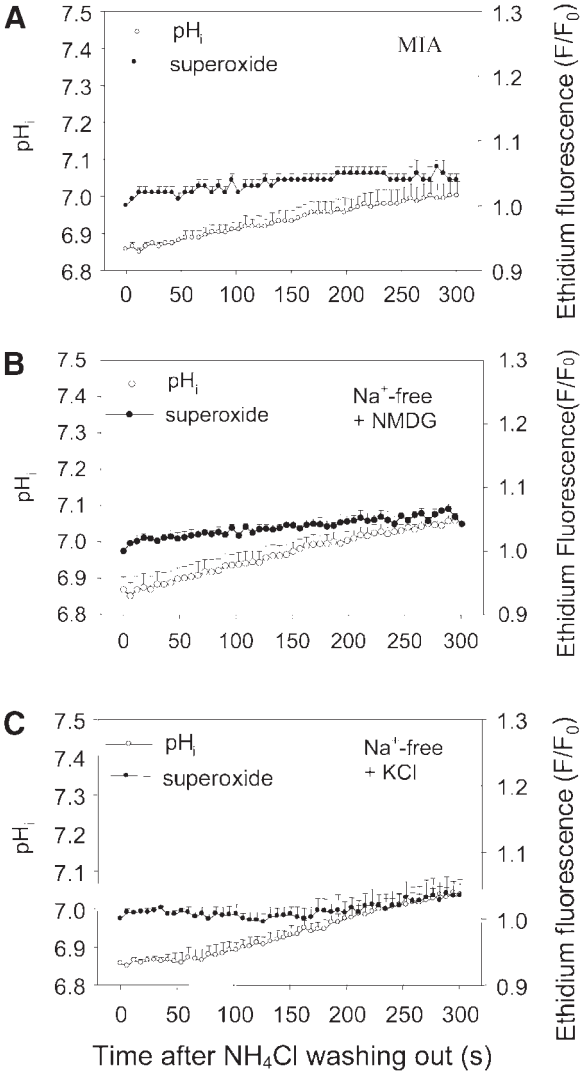

Fig. 2. Effects of inhibition of Na^+/H^+ exchange on pH_i and $O_2^-\cdot$ levels in NH_4Cl-prepulsed TALHs. *A*: effects of a Na^+/H^+ exchanger (NHE) inhibitor, methylisobutyl-amiloride (MIA; 100 μM). *B*: effects of replacement of Na^+ with *N*-methyl-D-glucamine (NMDG) in bath solution. *C*: effect of replacement of Na^+ by K^+ in bath solution ($n = 6$).

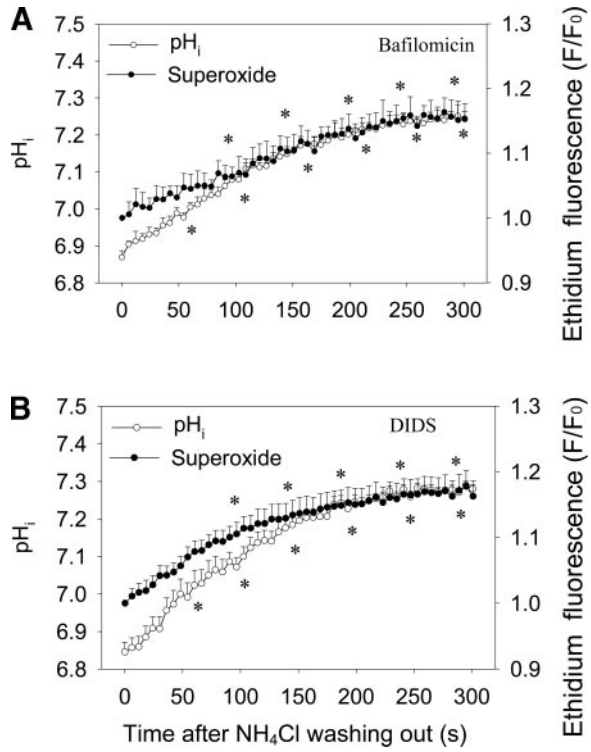

Fig. 3. Effects of inhibition of H^+-ATPase or Na/HCO_3 cotransporter on pH_i recovery and $O_2^-\cdot$ production in NH_4Cl-prepulsed TALHs. *A*: effects of bafilomycin, an inhibitor of H^+-ATPase. *B*: effects of DIDS, an inhibitor of Na/HCO_3 cotransporter. $^*P < 0.05$ compared with the values obtained at 0 s after washing out NH_4Cl ($n = 6$).

remained the same as that observed in the absence of Tiron. Then, a similar effect of DPI was observed. When TALHs were preincubated with this inhibitor of NAD(P)H oxidase, increase in intracellular $O_2^-\cdot$ was blocked, but the pattern and magnitude of pH_i increase were not altered (Fig. 4*B*).

Effects of pH_i on $O_2^-\cdot$ production by loading different concentrations of NH_4Cl in TALHs. To illustrate whether production of $O_2^-\cdot$ was related to pH_i value, different acidification levels of TALHs were achieved by loading different concentrations of NH_4Cl. However, when TALHs were loaded with NH_4Cl without washing out, there was no change in $O_2^-\cdot$ during these time periods. In the TALHs with different pH_i, the $O_2^-\cdot$ levels remained unchanged (Fig. 5).

Effects of H^+ influx and efflux on $O_2^-\cdot$ production in TALHs pretreated with H^+ ionophore. To further confirm the coupling of H^+ movements across cell membrane and $O_2^-\cdot$ production in TALHs and to define the role of H^+ influx or efflux in mediating activation of NAD(P)H oxidase, a H^+ ionophore, CCCP (10 μM), was used to allow H^+ to freely cross cell membrane in TALHs. In TALHs pretreated with CCCP, alterations of pH in the bath solution drove H^+ flow in or out of TALH cells. As shown in Fig. 6*A*, an outward flow of H^+ by adjusting pH to 8.2 in the bath solution increased both pH_i and intracellular $O_2^-\cdot$ in parallel. The H^+ outflow stopped 4–5 min later when pH_i reached a steady state. The pattern of $O_2^-\cdot$ increase was similar to the changes in pH_i. In contrast, influx of H^+ by reducing pH to 6.7 in the bath solution only caused a decrease in pH_i, but did not cause an increase in $O_2^-\cdot$ levels (Fig. 6*B*). In vehicle experiments, exchanging the bath solution

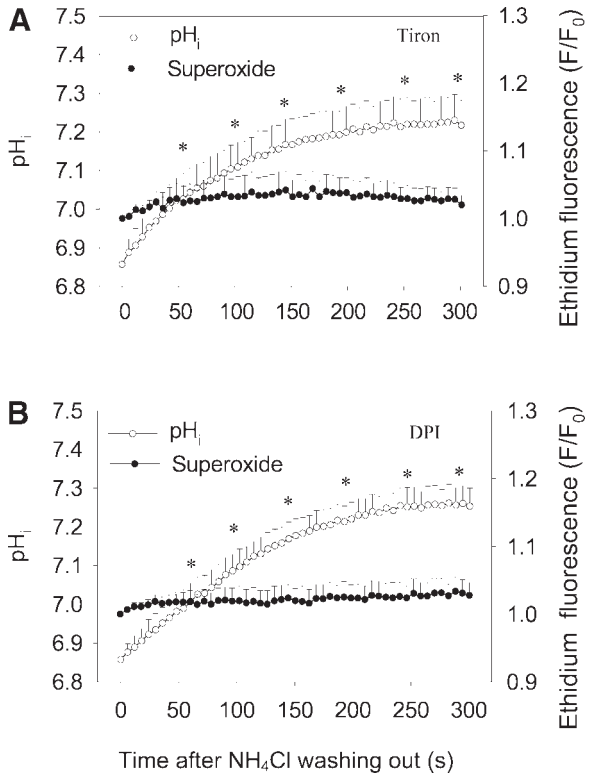

Fig. 4. Effects of $O_2^-\cdot$ dismutase (SOD) mimetic, Tiron, and inhibitor of NAD(P)H oxidase, DPI, on pH_i and $O_2^-\cdot$ levels in NH_4Cl-prepulsed TALHs. *A*: effects of Tiron (1 mM). *B*: effects of DPI. $^*P < 0.05$ compared with the values obtained at 0 s after washing out NH_4Cl ($n = 5$).

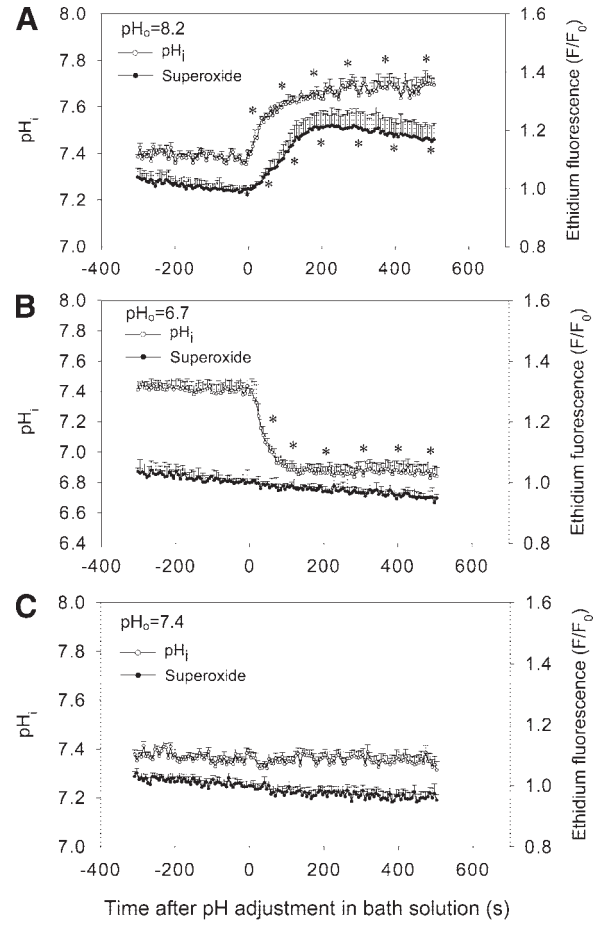

Fig. 6. Increased production of $O_2^-\cdot$ following outward movement of H^+ induced by raising pH in the bath solution in TALHs pretreated with a proton ionophore, CCCP. *A*: effects of efflux of H^+ by increasing pH in the bath solution (pH_o). *B*: effects of influx of H^+ by reducing pH in the bath solution. *C*: no change in pH in the bath solution. $^*P < 0.05$ compared with the values obtained at 0 s after changing the bath solution ($n = 6$).

without changing pH did not produce any H^+ flow and therefore neither pH_i nor $O_2^-\cdot$ level was altered (Fig. 6*C*).

Specific inhibition of $O_2^-\cdot$ increases associated with H^+ efflux in TALHs pretreated with H^+ ionophore. Experiments above demonstrated that only H^+ efflux in CCCP pretreated TALHs produced corresponding increase in intracellular $O_2^-\cdot$. To address whether this response was associated with activation of NAD(P)H oxidase, two inhibitors of this enzyme were used in the preparation. As shown in Fig. 7*A*, pretreatment of TALHs with DPI specifically blocked the increase in intracellular $O_2^-\cdot$ levels induced by adjusting pH to 8.2 in the bath solution, but it had no effect on the increase in pH_i. Another mechanistically different inhibitor of NAD(P)H oxidase, apocynin, similarly uncoupled the H^+ efflux-induced increase in $O_2^-\cdot$ from the changes in pH_i (Fig. 7*B*).

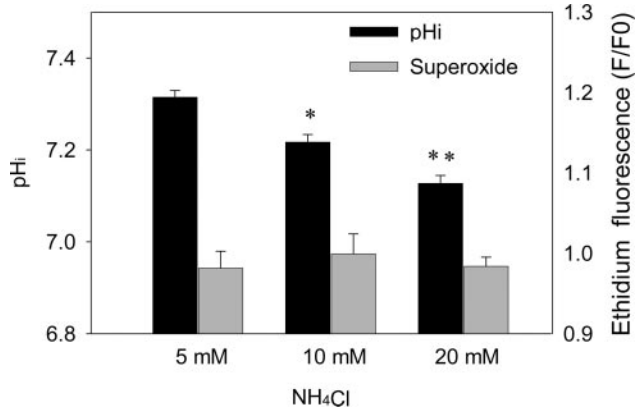

Fig. 5. Effects of different acidification on $O_2^-\cdot$ production by loading different concentrations of NH_4Cl in TALHs. $^*P < 0.05$ compared with the values obtained from TALHs treated with 5 mM of NH_4Cl. $^{**}P < 0.05$ compared with the values obtained from TALHs treated with 10 mM of NH_4Cl ($n = 6$).

Effect of inhibition of Na^+/H^+ exchange on renal redox status in vivo. To determine the physiological significance of H^+ outflow-activated NAD(P)H oxidase, we examined changes in redox status by analyzing renal interstitial H_2O_2 using microdialysis methods. In anesthetized rats, infusion of MIA, an inhibitor of NHE exchanger, into the renal outer medulla was used to inhibit Na^+/H^+ exchange activity in TALHs in this kidney region. As shown in Fig. 8, MIA significantly increased urinary sodium excretion, which sustained for more than 1 h even after termination of renal medullary interstitial infusion of MIA. In contrast, medullary interstitial concentration of H_2O_2 measured in the microdialysates was significantly decreased, which also sustained after stopping administration of MIA. By comparing the patterns of increase in $U_{Na}V$ and decrease in H_2O_2, the changes in $U_{Na}V$ occurred at a relatively earlier stage after the MIA infusion.

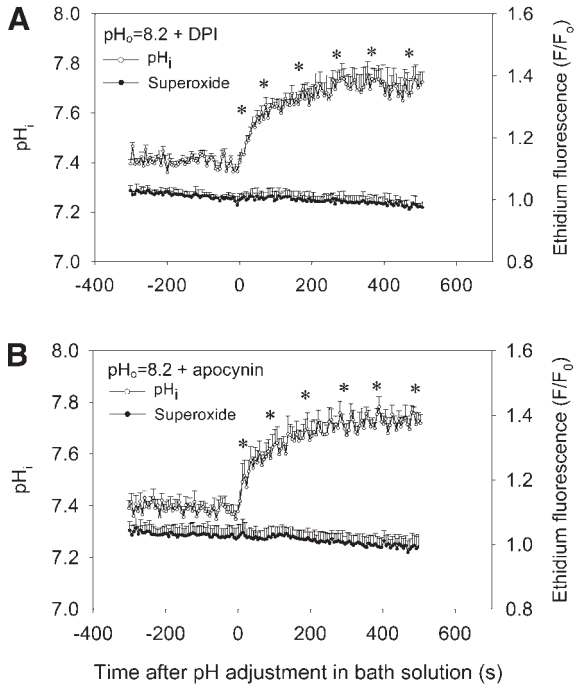

Fig. 7. Effects of inhibition of NAD(P)H oxidase on production of $O_2^-\cdot$ induced by H^+ efflux in TALHs pretreated with proton ionophore, CCCP. *A*: effects of DPI. *B*: effects of NAD(P)H oxidase inhibitor, apocynin (100 μM). *$P < 0.05$ compared with the values obtained at 0 s after changing the bath solution ($n = 5$).

DISCUSSION

The present study demonstrated that in isolated TALHs, intracellular levels of $O_2^-\cdot$ were increased in parallel to the elevation of pH_i induced by Na^+/H^+ exchange-dependent outflow of H^+. This response was substantially abolished by inhibition of Na^+/H^+ exchange and NAD(P)H oxidase. In H^+ ionophore-treated TALHs, H^+ efflux was also coupled to $O_2^-\cdot$ production, which was blocked by NAD(P)H oxidase inhibitors, whereas the influx of H^+ decreased pH_i, but did not increase $O_2^-\cdot$ production. Interstitial infusion of Na^+/H^+ exchange inhibitor remarkably decreased intrarenal superoxide production as measured by H_2O_2 concentration in dialysate from intramedullary microdialysis. These data suggested that outflow of H^+ mediated by NHE increases production of $O_2^-\cdot$ via NAD(P)H oxidase in TALHs.

Despite extensive studies in phagocytes, the relationship between H^+ outflow and $O_2^-\cdot$ production has not yet been elucidated in nonphagocytes. In the present study, we determined whether $O_2^-\cdot$ production in TALH cells was associated with outflow of H^+. The major goal of these experiments is to address the role of H^+ movements across cell membrane in the regulation of $O_2^-\cdot$ production. We first examined the effect of NHE-dependent H^+ outflow on intracellular $O_2^-\cdot$ production in TALH cells. NH_4Cl-prepulsed technique, a widely used method to produce NHE-dependent H^+ outflow in different cells (6, 7, 17, 19, 27), was used in the present study. Our data showed that outflow of H^+ was induced during the recovery period after removal of NH_4Cl from the bath solution, as indicated by the increase in pH_i. In parallel to the elevation of pH_i, intracellular $O_2^-\cdot$ levels were increased and it reached a plateau 3 min after washing out NH_4Cl. This result indicates that there is a coupling of H^+ outflow and $O_2^-\cdot$ production in these TALH cells.

To further confirm this coupling of H^+ outflow and $O_2^-\cdot$ production, we determined the effect of inhibition of Na^+/H^+ exchange on $O_2^-\cdot$ production as this NH_4Cl prepulse-induced H^+ outflow is mainly dependent on the activity of NHE. When NHE inhibitor, MIA, was used to pretreat TALH cells, H^+ outflow after washing out NH_4Cl was substantially blocked. At the same time, the increase in intracellular $O_2^-\cdot$ levels was almost completely blocked. This MIA-induced inhibition of both H^+ outflow and increase in intracellular $O_2^-\cdot$ reflects their dependence on Na^+/H^+ exchange activity in these cells, suggesting a linkage of the production of $O_2^-\cdot$ to NHE function.

Another approach used to inhibit Na^+/H^+ exchange was to remove Na^+ from the bath solution (6, 7, 17, 19, 27). It was found that removal of Na^+ from the bath solution remarkably inhibited H^+ outflow and the same time abolished the increase in $O_2^-\cdot$ production in TALH cells induced by NH_4Cl prepulse. This result further supports the view that NHE-mediated outflow of H^+ contributes to $O_2^-\cdot$ production in TALHs. These findings are consistent with previous reports that inhibition of NHE attenuated $O_2^-\cdot$ production in neutrophils (39) and macrophage (4). More interestingly, a recent study reported similar results that inhibition of NHE abolished the increase in $O_2^-\cdot$ levels induced by elevations of metabolic activity in dissected renal medullary TALHs that were overloaded with NaCl, D-glucose, or triiodo-thyronine (38). However, this study did not determine the role of H^+ movement across the cell membrane in $O_2^-\cdot$ production. Therefore, the present study elucidates a H^+ movement-dependent mechanism coupling tubular metabolic activity to the production of $O_2^-\cdot$ in TALHs.

Although NHE is the major pathway in the pH_i recovery after NH_4Cl prepulse, there are other acid/base transporters that may participate in the regulation of H^+ outflow under this condition. We therefore examined the roles of H^+-ATPase and Na/HCO_3 cotransporter in the pH_i recovery and intracellular $O_2^-\cdot$ production. Our data did not show that these two mechanisms were involved in the changes in pH_i and $O_2^-\cdot$ levels after NH_4Cl prepulses. It has been shown that H^+-ATPase participates in the pH_i recovery after NH_4Cl prepulse, but only contributes to a very small portion, ~5% of the total recovery under basal condition, although it plays more important role in the presence of its stimuli (17, 41). Thus the role of H^+-ATPase in the pH_i recovery and $O_2^-\cdot$ production is not important under our experimental condition. DIDS-sensitive Na/

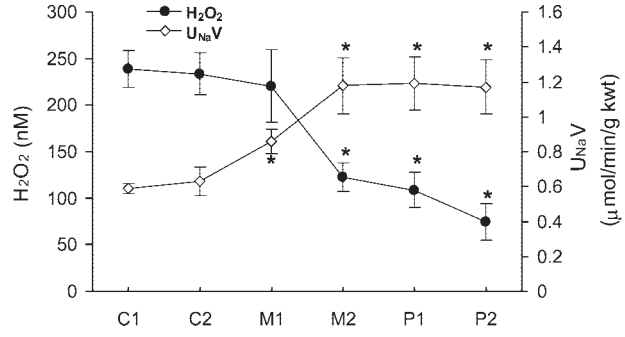

Fig. 8. Effects of infusion of MIA, a NHE inhibitor, on H_2O_2 levels in the renal medulla of rats. $U_{NA}V$, urinary sodium excretion rate; C, control; M, MIA; P, post-MIA. *$P < 0.05$ vs. control ($n = 5$).

HCO_3 cotransporter has also been reported to participate in the pH_i recovery after NH_4Cl prepulse by inducing base influx in the presence of HCO_3/CO_2 (2, 45). However, our results suggest that its role was negligible in the present experimental condition as its inhibitor did not have effect on either pH_i or $O_2^-\cdot$ levels.

It should be noted that remaining small increase in pH_i did not increase $O_2^-\cdot$ production by TALHs when NHE was inhibited (Fig. 2). There may be two reasons that result in this phenomenon. First, it is possible that the remaining increase in pH_i may be due to other mechanisms, rather than H^+ outward movements. It has been reported that pH recovery after NH_4Cl prepulse is dependent on several mechanisms, despite the fact that NHE is the primary one (6, 7, 17, 19, 27). Therefore, $O_2^-\cdot$ production may not be changed if that small increase in pH_i is not due to H^+ outward movement during NHE inhibition. Second, it is also possible that the sensitivity of DHE/Eth fluorescence monitoring for superoxide levels is not enough to detect changes induced by small pH_i changes due to H^+ outward movements.

Previous studies demonstrated that in macrophage-like cells H^+ efflux was coupled to the activation of NAD(P)H oxidase, enhancing production of $O_2^-\cdot$ (11, 13, 15, 28, 29). The next question we addressed was whether the $O_2^-\cdot$ production induced by H^+ outflow observed in the present study was NAD(P)H oxidase dependent. By using a SOD mimetic, Tiron, and the inhibitors of NAD(P)H oxidase, we observed the contribution of this enzyme to the $O_2^-\cdot$ production associated with H^+ outflow. Tiron was found to block the increase in $O_2^-\cdot$, but it had no effects on the increase in pH_i induced by removal of NH_4Cl, indicating that increase in intracellular $O_2^-\cdot$ levels was initiated by the outward movement of H^+. Inhibition of NAD(P)H oxidase by DPI also significantly attenuated increase in intracellular $O_2^-\cdot$ levels induced by H^+ outflow. These results confirmed that increase in intracellular $O_2^-\cdot$ levels in these TALH cells was derived from NAD(P)H oxidase. However, DPI did not block increase in pH_i induced by removal of NH_4Cl from the bath solution, suggesting that activation of NAD(P)H oxidase resulted from outflow of H^+. These results were consistent with the previous findings obtained from human eosinophils that NAD(P)H oxidase was activated when H^+ current was increased substantially, while DPI inhibited activation of NAD(P)H but not enhanced H^+ current. (11). Our data support the view that outward movement of H^+ is not just a compensation for the electrogenic activity of NAD(P)H oxidase.

To further determine the role of H^+ movement across the cell membrane in the activation of NAD(P)H oxidase, another strategy was used to induce H^+ influx or efflux in TAHL cells, which was based on the action of CCCP, a proton ionophore on H^+ permeability across cell membrane. When TALHs were pretreated with CCCP, outward movement of H^+ was induced by increasing pH in the bath solution. Under this condition, pH_i and intracellular $O_2^-\cdot$ levels were increased in parallel. In contrast, inward movement of H^+ induced by decreasing pH in the bath solution only reduced pH_i, but did not cause increase in the level of intracellular $O_2^-\cdot$. These findings further support the view that outward, but not inward, movement of H^+ increases $O_2^-\cdot$ production in these tubular cells. Similarly, we also examined whether the production of $O_2^-\cdot$ induced by H^+ efflux was derived from NAD(P)H oxidase in CCCP-treated TALHs. It was found that the inhibitors of this enzyme, either DPI or apocynin, completely blocked the increases in $O_2^-\cdot$ without altering the changes in pH_i. It is NAD(P)H oxidase that is activated to produce $O_2^-\cdot$ by H^+ outward movement. It should be pointed out that our H^+ influx experiments only provide information that inflow of H^+, unlike outflow of H^+, could not increase $O_2^-\cdot$ production. We cannot exclude that inflow of H^+ might inhibit $O_2^-\cdot$ production as DHE/Eth ratio measurement could not detect the decrease in $O_2^-\cdot$ levels. However, this limitation of our method should not weaken our conclusion that outward movements of H^+, rather than its inward movement, activate NAD(P)H oxidase in TALHs.

There are concerns whether activation of NAD(P)H oxidase is pH_i dependent and whether this activation of NAD(P)H oxidase is specific to any H^+ ion transporters in TALHs. It was found that in both NH_4Cl prepulse experiments and CCCP pretreat experiments, when pH_i recovery reached a steady state, $O_2^-\cdot$ production ceased, implying that pH_i value was not related to the activity of NAD(P)H oxidase, and it was pH_i changing process that activated the enzyme activity. In other words, the absolute pH_i values did not contribute to activation of this enzyme to produce superoxide. This view is also supported by the findings in our experiment that activities of NAD(P)H oxidase were not altered by different acidifications of TALHs when loading different concentrations of NH_4Cl. The activation of NAD(P)H oxidase may be associated with the electrogenic feature of this enzyme. It is proposed that H^+ outward movement produces a H^+ gradient or potential across cell membrane, which may directly activate the enzyme as superoxide-producing process through NAD(P)H oxidase, in fact, is an electron movement process via its transport system across cell membrane. Although there are reports that the electron flow for superoxide production mediated by NAD(P)H oxidase is a driving force for H^+ outward movements, the present study and other studies suggested that H^+ movements could also drive electron flow of NAD(P)H oxidase. If there was no H^+ outward movement, there would be no $O_2^-\cdot$ production. Our data showed that when increases in pH_i reached a plateau, meaning the H^+ outward movement was stopped, the production of $O_2^-\cdot$ also ceased. This electrogenic mechanism may be one of the major mechanisms mediating NAD(P)H oxidase activation.

In the present study, activation of NAD(P)H oxidase was associated with outward movements of H^+ that were induced by two different methods. Previous reports showed that in phagocyte-like cells the activation of NAD(P)H oxidase is coupled to H^+ efflux mediated by voltage-gated proton channel (23, 24, 26). It appears that any pathways, as long as they lead to H^+ moving out of the cells, would cause activation of NAD(P)H oxidase. The pathways mediating H^+ outflow in the regulation of NAD(P)H oxidase may be related to cell type or function. In our tubular preparation, NEH activity was shown to be importantly involved in the activation of NAD(P)H oxidase. The present study did not attempt to explore the mechanism by which H^+ outward movement activates NAD(P)H oxidase. How outflow of H^+ activates NAD(P)H oxidase remains to be determined in the future studies.

To address the physiological relevance of this H^+ outflow-induced production of $O_2^-\cdot$ to renal tubular activity, we performed in vivo experiments to determine whether blockade of H^+ outward movement by inhibition of NHE altered $O_2^-\cdot$

production and whether NHE-coupled $O_2^-\cdot$ production contributed to a high level of oxidant stress in the renal medulla (10, 48). In the present study, interstitial H_2O_2 concentration in renal medulla was used to reflect the oxidative status as H_2O_2 accumulation is closely related to $O_2^-\cdot$ (22) and highly NAD(P)H oxidase dependent (42), and it can be dialyzed for quantification with a high dialysis efficiency (96%) through the membrane of dialysis probe (10). It was demonstrated that intramedullary infusion of MIA, a NHE inhibitor, remarkably increased urinary Na^+ excretion and simultaneously decreased the levels of H_2O_2 in the renal medulla, as measured in renal medullary microdialysates. This is consistent with the results obtained from our in vitro experiments that inhibition of NHE decreased $O_2^-\cdot$ production in dissected TALHs. These results from our in vivo experiments provide evidence that H^+ extrusion via NHE importantly regulates renal medullary oxidative status, which is coupled to the alterations of tubular function. Although oxidative stress in the kidneys changes tubular sodium transport activity, the present study demonstrates that sodium reabsorption through NHE activity is one of the important mechanisms determining the oxidative status in the kidneys, particularly in the renal medulla.

In summary, the present study showed that $O_2^-\cdot$ production was increased by outflow of H^+ in isolated renal TALHs and this increase in $O_2^-\cdot$ production was substantially abolished by inhibition of outflow of H^+. Inhibition of NAD(P)H oxidase blocked $O_2^-\cdot$ production but had no effect on pH_i changes in these TALH cells. Intrarenal administration of NHE inhibitor reduced the levels of intrarenal ROS, as indicated by decreased H_2O_2 levels in medullary microdialysates. These results suggest that outward movements of H^+ across cell membrane enhance oxidative stress in TALHs by promoting the production of $O_2^-\cdot$ via NAD(P)H oxidase. This action of outward movements of H^+ may represent an important mechanism mediating the activation of NAD(P)H oxidase coupled to the metabolic activity in these tubular cells.

GRANTS

This study was supported by National Institutes of Health Grants HL-70726 and DK-54927.

REFERENCES

1. **Akgur FM, Brown MF, Zibari GB, McDonald JC, Epstein CJ, Ross CR, and Granger DN.** Role of superoxide in hemorrhagic shock-induced P-selectin expression. *Am J Physiol Heart Circ Physiol* 279: H791–H797, 2000.
2. **Amlal H, Wang Z, Burnham C, and Soleimani M.** Functional characterization of a cloned human kidney Na^+:HCO_3^- cotransporter. *J Biol Chem* 273: 16810–16815, 1998.
3. **Bastani B.** Immunocytochemical localization of the vacuolar H^+-ATPase pump in the kidney. *Histol Histopathol* 12: 769–779, 1997.
4. **Bidani A and Heming TA.** Effects of lidocaine on cytosolic pH regulation and stimulus-induced effector functions in alveolar macrophages. *Lung* 175: 349–361, 1997.
5. **Bindokas VP, Jordan J, Lee CC, and Miller RJ.** Superoxide production in rat hippocampal neurons: selective imaging with hydroethidine. *J Neurosci* 16: 1324–1336, 1996.
6. **Brokl OH, Martinez CL, Shuprisha A, Abbott DE, and Dantzler WH.** Regulation of intracellular pH in proximal tubules of avian long-looped mammalian-type nephrons. *Am J Physiol Regul Integr Comp Physiol* 274: R1526–R1535, 1998.
7. **Brown DA, Melvin JE, and Yule DI.** Critical role for NHE1 in intracellular pH regulation in pancreatic acinar cells. *Am J Physiol Gastrointest Liver Physiol* 285: G804–G812, 2003.
8. **Cai H and Harrison DG.** Endothelial dysfunction in cardiovascular diseases: the role of oxidant stress. *Circ Res* 87: 840–844, 2000.
9. **Carter WO, Narayanan PK, and Robinson JP.** Intracellular hydrogen peroxide and superoxide anion detection in endothelial cells. *J Leukoc Biol* 55: 253–258, 1994.
10. **Chen YF, Cowley AW Jr, and Zou AP.** Increased H_2O_2 counteracts the vasodilator and natriuretic effects of superoxide dismutation by tempol in renal medulla. *Am J Physiol Regul Integr Comp Physiol* 285: R827–R833, 2003.
11. **Cherny VV, Henderson LM, Xu W, Thomas LL, and DeCoursey TE.** Activation of NADPH oxidase-related proton and electron currents in human eosinophils by arachidonic acid. *J Physiol* 535: 783–794, 2001.
12. **Decoursey TE.** Voltage-gated proton channels and other proton transfer pathways. *Physiol Rev* 83: 475–579, 2003.
13. **DeCoursey TE, Cherny VV, DeCoursey AG, Xu W, and Thomas LL.** Interactions between NADPH oxidase-related proton and electron currents in human eosinophils. *J Physiol* 535: 767–781, 2001.
14. **DeCoursey TE, Cherny VV, Zhou W, and Thomas LL.** Simultaneous activation of NADPH oxidase-related proton and electron currents in human neutrophils. *Proc Natl Acad Sci USA* 97: 6885–6889, 2000.
15. **DeCoursey TE, Morgan D, and Cherny VV.** The voltage dependence of NADPH oxidase reveals why phagocytes need proton channels. *Nature* 422: 531–534, 2003.
16. **Deem S, Hedges RG, Kerr ME, and Swenson ER.** Acetazolamide reduces hypoxic pulmonary vasoconstriction in isolated perfused rabbit lungs. *Respir Physiol* 123: 109–119, 2000.
17. **Feifel E, Krall M, Geibel JP, and Pfaller W.** Differential activities of H^+ extrusion systems in MDCK cells due to extracellular osmolality and pH. *Am J Physiol Renal Physiol* 273: F499–F506, 1997.
18. **Fleming I.** Cytochrome P450 and vascular homeostasis. *Circ Res* 89: 753–762, 2001.
19. **Ford P, Rivarola V, Kierbel A, Chara O, Blot-Chabaud M, Farman N, Parisi M, and Capurro C.** Differential role of Na^+/H^+ exchange isoforms NHE-1 and NHE-2 in a rat cortical collecting duct cell line. *J Membr Biol* 190: 117–125, 2002.
20. **Greenstock CL and Miller RW.** The oxidation of tiron by superoxide anion. Kinetics of the reaction in aqueous solution in chloroplasts. *Biochim Biophys Acta* 396: 11–16, 1975.
21. **Griendling KK, Sorescu D, Lassegue B, and Ushio-Fukai M.** Modulation of protein kinase activity and gene expression by reactive oxygen species and their role in vascular physiology and pathophysiology. *Arterioscler Thromb Vasc Biol* 20: 2175–2183, 2000.
22. **Halliwell B and Gutteridge JM.** Role of free radicals and catalytic metal ions in human disease: an overview. *Methods Enzymol* 186: 1–85, 1990.
23. **Henderson LM.** NADPH oxidase subunit gp91phox: a proton pathway. *Protoplasma* 217: 37–42, 2001.
24. **Henderson LM, Banting G, and Chappell JB.** The arachidonate-activable, NADPH oxidase-associated H^+ channel. Evidence that gp91-phox functions as an essential part of the channel. *J Biol Chem* 270: 5909–5916, 1995.
25. **Henderson LM, Chappell JB, and Jones OT.** Superoxide generation by the electrogenic NADPH oxidase of human neutrophils is limited by the movement of a compensating charge. *Biochem J* 255: 285–290, 1988.
26. **Henderson LM, Thomas S, Banting G, and Chappell JB.** The arachidonate-activatable, NADPH oxidase-associated H^+ channel is contained within the multi-membrane-spanning N-terminal region of gp91-phox. *Biochem J* 325: 701–705, 1997.
27. **Inglis SK, Finlay L, Ramminger SJ, Richard K, Ward MR, Wilson SM, and Olver RE.** Regulation of intracellular pH in Calu-3 human airway cells. *J Physiol* 538: 527–539, 2002.
28. **Jankowski A and Grinstein S.** Modulation of the cytosolic and phagosomal pH by the NADPH oxidase. *Antioxid Redox Signal* 4: 61–68, 2002.
29. **Jankowski A, Scott CC, and Grinstein S.** Determinants of the phagosomal pH in neutrophils. *J Biol Chem* 277: 6059–6066, 2002.
30. **Jones RD, Hancock JT, and Morice AH.** NADPH oxidase: a universal oxygen sensor? *Free Radic Biol Med* 29: 416–424, 2000.
31. **Jones RD, Thompson JS, and Morice AH.** The NADPH oxidase inhibitors iodonium diphenyl and cadmium sulphate inhibit hypoxic pulmonary vasoconstriction in isolated rat pulmonary arteries. *Physiol Res* 49: 587–596, 2000.
32. **Kwon TH, Fulton C, Wang W, Kurtz I, Frokiaer J, Aalkjaer C, and Nielsen S.** Chronic metabolic acidosis upregulates rat kidney Na-HCO cotransporters NBCn1 and NBC3 but not NBC1. *Am J Physiol Renal Physiol* 282: F341–F351, 2002.

33. **Lassegue B and Clempus RE.** Vascular NAD(P)H oxidases: specific features, expression, and regulation. *Am J Physiol Regul Integr Comp Physiol* 285: R277–R297, 2003.
34. **Li N, Yi FX, Spurrier JL, Bobrowitz CA, and Zou AP.** Production of superoxide through NADH oxidase in thick ascending limb of Henle's loop in rat kidney. *Am J Physiol Renal Physiol* 282: F1111–F1119, 2002.
35. **Mohanty JG, Jaffe JS, Schulman ES, and Raible DG.** A highly sensitive fluorescent micro-assay of H_2O_2 release from activated human leukocytes using a dihydroxyphenoxazine derivative. *J Immunol Methods* 202: 133–141, 1997.
36. **Mohazzab KM, Kaminski PM, and Wolin MS.** NADH oxidoreductase is a major source of superoxide anion in bovine coronary artery endothelium. *Am J Physiol Heart Circ Physiol* 266: H2568–H2572, 1994.
37. **Mori T and Cowley AW Jr.** Angiotensin II-NAD(P)H oxidase-stimulated superoxide modifies tubulovascular nitric oxide cross-talk in renal outer medulla. *Hypertension* 42: 588–593, 2003.
38. **Mori T and Cowley AW Jr.** Renal oxidative stress in medullary thick ascending limbs produced by elevated NaCl and glucose. *Hypertension* 43: 341–346, 2004.
39. **Muraki S, Morris CD, Budde JM, Zhao ZQ, Guyton RA, and Vinten-Johansen J.** Blood cardioplegia supplementation with the sodium-hydrogen ion exchange inhibitor cariporide to attenuate infarct size and coronary artery endothelial dysfunction after severe regional ischemia in a canine model. *J Thorac Cardiovasc Surg* 125: 155–164, 2003.
40. **Napoli C, de Nigris F, and Palinski W.** Multiple role of reactive oxygen species in the arterial wall. *J Cell Biochem* 82: 674–682, 2001.
41. **Oliveira-Souza M, Musa-Aziz R, Malnic G, and de Mello Aires M.** Arginine vasopressin stimulates H^+-ATPase in MDCK cells via V1 (cell Ca^{2+}) and V2 (cAMP) receptors. *Am J Physiol Renal Physiol* 286: F402–F408, 2004.
42. **Qin WM, Lan WZ, and Yang X.** Involvement of NADPH oxidase in hydrogen peroxide accumulation by *Aspergillus niger* elicitor-induced *Taxus chinensis* cell cultures. *J Plant Physiol* 161: 355–361, 2004.
43. **Rink TJ, Tsien RY, and Pozzan T.** Cytoplasmic pH and free Mg^{2+} in lymphocytes. *J Cell Biol* 95: 189–196, 1982.
44. **Salvi A, Quillan JM, and Sadee W.** Monitoring intracellular pH changes in response to osmotic stress and membrane transport activity using 5-chloromethylfluorescein. *AAPS PharmSci* 4: E21, 2002.
45. **Usui T, Hara M, Satoh H, Moriyama N, Kagaya H, Amano S, Oshika T, Ishii Y, Ibaraki N, Hara C, Kunimi M, Noiri E, Tsukamoto K, Inatomi J, Kawakami H, Endou H, Igarashi T, Goto A, Fujita T, Araie M, and Seki G.** Molecular basis of ocular abnormalities associated with proximal renal tubular acidosis. *J Clin Invest* 108: 107–115, 2001.
46. **Yi FX, Zhang AY, Campbell WB, Zou AP, Van Breemen C, and Li PL.** Simultaneous in situ monitoring of intracellular Ca^{2+} and NO in endothelium of coronary arteries. *Am J Physiol Heart Circ Physiol* 283: H2725–H2732, 2002.
47. **Zhang DX, Zou AP, and Li PL.** Ceramide reduces endothelium-dependent vasodilation by increasing superoxide production in small bovine coronary arteries. *Circ Res* 88: 824–831, 2001.
48. **Zou AP, Li N, and Cowley AW Jr.** Production and actions of superoxide in the renal medulla. *Hypertension* 37: 547–553, 2001.