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

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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.—The present study was designed to test the hypothesis that the production of superoxide (O₂⁻) 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 O₂⁻ levels and pH (pHᵢ) in renal medullary thick ascending limb of Henle (TALH) cells were simultaneously measured. It was found that intracellular O₂⁻ levels in these cells were increased in parallel to the elevation of pHᵢ by outflow of H⁺ induced via NH₄Cl loading followed by rapid removal. This increase in intracellular O₂⁻ 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 pHᵢ and intracellular O₂⁻ levels, but the influx of H⁺ did not increase intracellular O₂⁻ levels. The H⁺ efflux-induced increase in intracellular O₂⁻ levels was completely blocked by DPI and another NAD(P)H oxidase inhibitor, apocynin (100 μM). In in vivo experiments, renal medullary infusion of MIA (100 μM) was found to significantly decrease the concentrations of H₂O₂ 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 O₂⁻ 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

It had been well documented that NAD(P)H oxidase reduces O₂ to form O₂⁻ using NAD(P)H as an electron donor. In phagocytes or phagocyte-like cells, proton efflux is coupled with the production of O₂⁻ by NAD(P)H oxidase and that inhibition of proton efflux leads to inhibition of O₂⁻ 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 (pHᵢ) 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 O₂⁻ 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 Zn²⁺ and Cd²⁺ significantly inhibited the O₂⁻ production via NAD(P)H oxidase and blocked hypoxic pulmonary vasoconstriction (12, 30, 31) and that inhibition of carbonic anhydrase changed pHᵢ 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 O₂⁻ was the primary source of intracellular O₂⁻ in thick ascending limb of Henle (TALH) and it importantly contributed to the tissue O₂⁻ levels in the kidneys (34, 48). This NAD(P)H oxidase-derived O₂⁻ 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.
The present study was designed to further test the hypothesis that the production of O$_2^·$ 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$_2^·$ 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$_2^·$ 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 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$_2^·$ 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 and O$_2^·$ in TALHs.

**Simultaneous measurement of pH$_i$ and O$_2^·$ levels in TALHs.** The levels of pH$_i$ and O$_2^·$ 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$_2$HPO$_4$, 0.4 KH$_2$PO$_4$, 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), dihydroethidine (DHE; for O$_2^·$ 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$_2^·$ 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$_2^·$ production within cells (5, 9). The fluorescence excitation profile of BCFED 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$_2^·$. 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 Metaffluor 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$_4$Cl for 3 min and then washed by NH$_4$Cl free Hanks’ solution. This NH$_4$Cl loading and removal will rapidly eliminate NH$_3$ and result in retention of H$^+$ within the cells. After removal of NH$_4$Cl, a time-dependent outflow of H$^+$ occurs, which is primarily dependent on Na$^+/H^+$ exchanger (NHE) activity. This NH$_4$Cl 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$^+-$/Na$^+$ exchanger (32) and Na/HCO$_3$ cotransporter (32) in H$^+$ outflow and O$_2^·$ production after NH$_4$Cl 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$_2^·$ production and H$^+$ movements across cell membrane of TALHs was examined by a parallel monitoring of pH$_i$ and O$_2^·$. To confirm the Eth fluorescence was O$_2^·$ specific, a superoxide scavenger, Tiron (20, 47) (1 mM, Sigma), was used to block the changes in Eth fluorescence. To determine whether O$_2^·$ production was derived from NAD(P)H oxidase, different inhibitors of NAD(P)H oxidase, DPI (100 μM, Sigma) and apocynin (100 μM, EMBD Bioscience, La Jolla, CA), were utilized.

**Effects of Na$^+/H^+$ exchange-depending H$^+$ movements on renal redox status 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 h$^{-1}$.100 g body wt$^{-1}$ throughout the experiment to replace fluid loss and maintain a constant hematocrit.
In control TALHs without loading NH4Cl, a parallel recording of both pH and O2⁻ was also performed. There were no significant changes observed in the fluorescent intensity of BCECF and Eth in these control TALHs (Fig. 1B), which served as time control, indicating that alterations of pH and O2⁻ were not due to time-dependent autofluorescence in NH4Cl-prepulsed tubules.

Production of intracellular O2⁻ dependent on Na⁺/H⁺ exchange-mediated H⁺ outflow in NH4Cl-prepulsed TALHs. In the presence of a Na⁺/H⁺ exchanger inhibitor, MIA (100 μM), increase in pH was blocked during the recovery period after removal of NH4Cl, which was accompanied by abolishment of

increased intracellular O2⁻ production coupled with Na⁺/H⁺ exchange-dependent outflow of H⁺ in NH4Cl-prepulsed TALHs. During the recovery period after washing out NH4Cl from the batch solution, an outward flow of H⁺ in TALHs occurred, as indicated by increase in pH, that was monitored by fluorescence intensity of BCECF. In parallel,
intracellular O2⁻ levels were elevated, as indicated by enhanced Eth fluorescence (a ratio of Eth/DHE fluorescence at 610/430 nm). Intracellular O2⁻ levels were presented as the ratio of Eth/DHE fluorescence compared with the 0 time point when NH4Cl was washed out. After preloading and washing out 20 mM of NH4Cl, pH 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 is due to H⁺ outflow mainly dependent on Na⁺/H⁺ exchanger activity (6, 7, 17, 19, 27). In parallel to the increase in pH, intracellular O2⁻ levels increased, as shown by increase in Eth/DHE ratio from 1 to 1.18 ± 0.025 (units) in TALHs. This increase in O2⁻ production was terminated when pH increase reached a plateau (Fig. 1A). Although increases in pH and O2⁻ were parallel, statistically the increase was earlier in pH than that in O2⁻ after washing out NH4Cl. By comparing with 0 time point after washing out NH4Cl, increases in pH reached statistical significance at 60 s, while O2⁻ levels at 90 s.

RESULTS

Increased intracellular O2⁻ production coupled with Na⁺/H⁺ exchange-dependent outflow of H⁺ in NH4Cl-prepulsed TALHs. During the recovery period after washing out NH4Cl from the batch solution, an outward flow of H⁺ in TALHs occurred, as indicated by increase in pH that was monitored by fluorescence intensity of BCECF. In parallel,
increase in the production of intracellular $O_2^·$ (Fig. 2A). Similarly, removal of Na$^+$ from the bath solution by substituting NaCl for N-methyl-n-glucamine (Fig. 2B) or KCl (Fig. 2C) inhibited Na$^+$/H$^+$ exchange (17, 19, 25, 27) and blocked both increases in pH$^-$ and intracellular $O_2^·$. In the presences of inhibitor of H$^+$-ATPas or Na/HCO$_3$ cotransporter, the patterns of increases in pH$^-$ and intracellular $O_2^·$ levels caused by NH$_4$Cl prepulse were the same as that in control TALHs (Fig. 3 vs. Fig. 1A).

Specific inhibition of $O_2^·$ increases in NH$_4$Cl-prepulsed TALHs. To determine whether the Eth fluorescence increase is specific to $O_2^·$ production, we examined the effect of chemical mimetic of SOD, Tiron, and a selective inhibitor of NAD(P)H oxidase, DPI, on $O_2^·$ levels during $H^+$ extrusion after washing out NH$_4$Cl. First, incubation of TALHs with Tiron (1 mM) inhibitor of H$^+$ extrusion increased in pHi and intracellular $O_2^·$ vs. Fig. 1

Specific inhibition of $O_2^·$ increases in NH$_4$Cl-prepulsed TALHs. To determine whether the Eth fluorescence increase is specific to $O_2^·$ production, we examined the effect of chemical mimetic of SOD, Tiron, and a selective inhibitor of NAD(P)H oxidase, DPI, on $O_2^·$ levels during $H^+$ extrusion after washing out NH$_4$Cl. First, incubation of TALHs with Tiron (1 mM) specifically blocked the increase in intracellular $O_2^·$ levels, but it had no effect on the elevation of pHi induced by removal of NH$_4$Cl (Fig. 4A). The pattern and magnitude of increase in pH$^-$ 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 $O_2^·$ was blocked, but the pattern and magnitude of pH$^-$ increase were not altered (Fig. 4B).

Effects of pH$_i$ on $O_2^·$ production by loading different concentrations of NH$_4$Cl in TALHs. To illustrate whether production of $O_2^·$ was related to pH$_i$ value, different acidification levels of TALHs were achieved by loading different concentrations of NH$_4$Cl. However, when TALHs were loaded with NH$_4$Cl without washing out, there was no change in $O_2^·$ during these time periods. In the TALHs with different pH$_i$, the $O_2^·$ levels remained unchanged (Fig. 5).

Effects of $H^+$ influx and efflux on $O_2^·$ production in TALHs pretreated with $H^+$ ionophore. To further confirm the coupling of $H^+$ movements across cell membrane and $O_2^·$ 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 $\mu$M), 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. 6A, an outward flow of $H^+$ by adjusting pH to 8.2 in the bath solution increased both pH$_i$ and intracellular $O_2^·$ in parallel. The $H^+$ outflow stopped 4–5 min later when pH$_i$ reached a steady state. The pattern of $O_2^·$ 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 $O_2^·$ levels (Fig. 6B). In vehicle experiments, exchanging the bath solution...
without changing pH did not produce any H⁺ flow and therefore neither pHi nor O₂⁻/H₂O₂ level was altered (Fig. 6C).

Specific inhibition of O₂⁻/H₂O₂ 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 O₂⁻/H₂O₂. 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. 7A, pretreatment of TALHs with DPI specifically blocked the increase in intracellular O₂⁻/H₂O₂ levels induced by adjusting pH to 8.2 in the bath solution, but it had no effect on the increase in pHi. Another mechanistically different inhibitor of NAD(P)H oxidase, apocynin, similarly uncoupled the H⁺ efflux-induced increase in O₂⁻/H₂O₂ from the changes in pHi (Fig. 7B).

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 H₂O₂ 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 H₂O₂ measured in the microdialysates was significantly decreased, which also sustained after stopping administration of MIA. By comparing the patterns of increase in UNaV and decrease in H₂O₂, the changes in UNaV occurred at a relatively earlier stage after the MIA infusion.
that there is a coupling of H⁺ outflow and O₂⁻ production in these TALH cells.

To further confirm this coupling of H⁺ outflow and O₂⁻ production, we determined the effect of inhibition of Na⁺/H⁺ exchange on O₂⁻ production as this NH₄Cl 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 NH₄Cl was substantially blocked. At the same time, the increase in intracellular O₂⁻ levels was almost completely blocked. This MIA-induced inhibition of both H⁺ outflow and increase in intracellular O₂⁻ reflects their dependence on Na⁺/H⁺ exchange activity in these cells, suggesting a linkage of the production of O₂⁻ 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 O₂⁻ production in TALH cells induced by NH₄Cl prepulse. This result further supports the view that NHE-mediated outflow of H⁺ contributes to O₂⁻ production in TALHs. These findings are consistent with previous reports that inhibition of NHE attenuated O₂⁻ production in neutrophils (39) and macrophage (4). More interestingly, a recent study reported similar results that inhibition of NHE abolished the increase in O₂⁻ levels induced by elevations of metabolic activity in dissected renal medullary TALHs that were overloaded with NaCl, α-glucose, or triiodothyronine (38). However, this study did not determine the role of H⁺ movement across the cell membrane in O₂⁻ production. Therefore, the present study elucidates a H⁺ movement-dependent mechanism coupling tubular metabolic activity to the production of O₂⁻ in TALHs.

Although NHE is the major pathway in the pHi recovery after NH₄Cl 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/HCO₃ cotransporter in the pHi recovery and intracellular O₂⁻ production. Our data did not show that these two mechanisms were involved in the changes in pHi and O₂⁻ levels after NH₄Cl prepsules. It has been shown that H⁺-ATPase participates in the pHi recovery after NH₄Cl 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 pHi recovery and O₂⁻ production is not important under our experimental condition. DIDS-sensitive Na/
HCO$_3$ cotransporter has also been reported to participate in the pH$_i$ recovery after NH$_4$Cl 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$_2^·$ levels.

It should be noted that remaining small increase in pH$_i$ did not increase O$_2^·$ 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$_4$Cl prepulse is dependent on several mechanisms, despite the fact that NHE is the primary one (6, 7, 17, 19, 27). Therefore, O$_2^·$ 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$^+$ influx was coupled to the activation of NAD(P)H oxidase, enhancing production of O$_2^·$ (11, 13, 15, 28, 29). The next question we addressed was whether the O$_2^·$ production induced by H$^+$ influx 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$_2^·$ production associated with H$^+$ influx. Tiron was found to block the increase in O$_2^·$, but it had no effects on the increase in pH$_i$ induced by removal of NH$_4$Cl, indicating that increase in intracellular O$_2^·$ levels was initiated by the outward movement of H$^+$. Inhibition of NAD(P)H oxidase by DPI also significantly attenuated increase in intracellular O$_2^·$ levels induced by H$^+$ influx. These results confirmed that increase in intracellular O$_2^·$ 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$_4$Cl 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 TALH 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$_2^·$ 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$_2^·$. These findings further support the view that outward, but not inward, movement of H$^+$ increases O$_2^·$ production in these tubular cells. Similarly, we also examined whether the production of O$_2^·$ 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$_2^·$ without altering the changes in pH$_i$. It is NAD(P)H oxidase that is activated to produce O$_2^·$ 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$_2^·$ production. We cannot exclude that inflow of H$^+$ might inhibit O$_2^·$ production as DHE/Eth ratio measurement could not detect the decrease in O$_2^·$ 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$_4$Cl prepulse experiments and CCCP pretreat experiments, when pH$_i$ recovery reached a steady state, O$_2^·$ 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$_4$Cl. 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$_2^·$ production. Our data showed that when increases in pH$_i$ reached a plateau, meaning the H$^+$ outward movement was stopped, the production of O$_2^·$ 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$_2^·$ to renal tubular activity, we performed in vivo experiments to determine whether blockade of H$^+$ outward movement by inhibition of NHE altered O$_2^·$.
production and whether NHE-coupled \( \text{O}_2^- \) production contributed to a high level of oxidant stress in the renal medulla (10, 48). In the present study, interstitial \( \text{H}_2\text{O}_2 \) concentration in renal medulla was used to reflect the oxidative status as \( \text{H}_2\text{O}_2 \) accumulation is closely related to \( \text{O}_2^- \) (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 outward movements of \( \text{H}^+ \) across cell membrane enhance oxidative stress in TALHs by promoting the production of \( \text{O}_2^- \) via NAD(P)H oxidase. This action of outward movements of \( \text{H}^+ \) may represent an important mechanism mediating the activation of NAD(P)H oxidase coupled to the metabolic activity in these tubular cells.

In summary, the present study showed that \( \text{O}_2^- \) production was increased by outflow of \( \text{H}^+ \) in isolated renal TALHs and this increase in \( \text{O}_2^- \) production was substantially abolished by inhibition of outflow of \( \text{H}^+ \). Inhibition of NAD(P)H oxidase blocked \( \text{O}_2^- \) production but had no effect on \( \text{pH} \) changes in these TALH cells. Intrarenal administration of NHE inhibitor reduced the levels of intrarenal ROS, as indicated by decreased \( \text{H}_2\text{O}_2 \) levels in medullary microdialysates. These results suggest that outward movements of \( \text{H}^+ \) across cell membrane enhance oxidative stress in TALHs by promoting the production of \( \text{O}_2^- \) via NAD(P)H oxidase. This action of outward movements of \( \text{H}^+ \) may represent an important mechanism mediating the activation of NAD(P)H oxidase coupled to the metabolic activity in these tubular cells.

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