Production of superoxide through NADH oxidase in thick ascending limb of Henle’s loop in rat kidney

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Li, Ningjun, Fu-Xian Yi, Jamie L. Spurrier, Carol A. Bobrowitz, and Ai-Ping Zou. 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. First published January 29, 2001; 10.1152/ajprenal.00218.2001.—We recently reported that NADH oxidase is one of the major enzymes responsible for superoxide (O2·-) production in the rat kidney. However, the functional significance of NADH oxidase-mediated O2·- production and the mechanisms regulating this enzyme activity are poorly understood. Using fluorescence microscopic imaging analysis, the present study demonstrated that thick ascending limbs of Henle’s loop (TALHs) exhibited red fluorescence when incubated with dihydroethidium (DHE), suggesting that O2·- is produced in this tubular segment. Compared with other nephron segments, TALHs from both renal cortex and medulla showed the highest fluorescence intensity. By incubating cortical TALHs (cTALHs) with the substrates of NADH oxidase, xanthine oxidase, nitric oxide synthase, arachidonic acid-metabolizing enzymes, and intramitochondrial oxidases, NADH oxidase was found to be one of the most important enzymes for O2·- production in this tubular segment. The NADH oxidase inhibitor diphenyleneiodonium (DPI; 100 μM) completely blocked NADH-induced O2·- production in cTALHs. Exposure of cTALHs to low PO2 (5–10 Torr) significantly increased O2·- production regardless of the absence or presence of NADH. Furthermore, angiotensin II (100 nM) increased NADH oxidase activity by 32%, which was completely blocked by DPI. These results suggest that NADH oxidase is a major enzyme responsible for O2·- production in the TALHs and that the production of O2·- via NADH oxidase may be regulated by renal tissue oxygenation and circulating hormones.

oxidative stress; reactive oxygen species; renal tubule; hypoxia; redox signaling

REACTIVE OXYGEN SPECIES (ROS) are conventionally considered as cytotoxic byproducts of cellular metabolism. Recently, evidence has been rapidly accumulating that ROS play an important role in the intracellular signaling, whereby ROS may participate in the regulation of cell function or activity under physiological conditions. In this regard, ROS have been reported to be involved in the growth, division, transformation, apoptosis, and senescence of a variety of mammalian cells, in addition to their physiological role in the respiratory burst of phagocytic cells. Many studies have indicated that ROS participate in the regulation of ion transport systems, protein phosphorylation, phospholipase activity, and gene expression (12, 13, 23).

More recently, ROS signaling has been indicated to contribute to the control of vascular tone or vasomotor response. It has been demonstrated that ROS serve as a vascular O2·-sensing factor to control the vascular reactivity in response to tissue metabolic activity (39). Moreover, ROS were found to play a role in the response of vessels to pressure, flow, and receptor agonists (39). With respect to their pathological actions, ROS have been reported to participate in the development of atherosclerosis, hypertension, and progressive renal disease (10, 17, 20, 40, 43).

Although several pathways may account for the production of O2·- or ROS in various cells and tissues, such as xanthine oxidase, mitochondrial respiratory chain enzymes, arachidonic acid-metabolizing enzymes, and nitric oxide synthase (NOS), NADH oxidase has been found to be the most important enzyme actively involved in the O2·-mediated signaling and physiological process (15). Many studies showed that NADH oxidase is an important pathway for O2·- production in vascular tissues (15, 16), and therefore activation of this enzyme in spontaneously hypertensive rats or during the elevation of plasma ANG II levels produced sustained hypertension (31, 43). These results have indicated that NADH oxidase may be a key enzyme to the production of O2·- in vascular cells under physiological or pathological conditions.

Despite intensive studies of NADH oxidase-mediated production and action of O2·- in the vasculature, the action of O2·- and the mechanisms regulating O2·- production mediated by NADH oxidase in the kidney are poorly understood. We recently demonstrated that NADH oxidase is a major enzyme responsible for O2·- production, using NADH or NADPH as a substrate in the rat kidney, suggesting the presence of this enzymatic pathway in the nephron system (46). The present study was designed to further determine the localization of NADH oxidase along the nephron and to address the regulatory mechanism of NADH oxidase activity. Because TALH is a nephron segment with great

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ion transport activity or high metabolic rate (11) and $O_2^-$ production has been related to oxygen consumption (2, 23), we chose this tubular segment to explore the mechanism responsible for the regulation of NADH oxidase activity associated with tubular oxygenation and circulating hormone, ANG II.

MATERIALS AND METHODS

**Microdissection of nephron segments in the rat kidney.** Microdissection was performed as we described previously (45). Briefly, male Sprague-Dawley rats weighing between 250 and 300 g were anesthetized with pentobarbital sodium (80 mg/kg body wt ip), and the aorta below the left renal artery was isolated and cannulated. After the aorta was ligated at a site between the origin of left and right renal arteries, the left kidney was flushed with 20 ml ice-cold dissection solution containing (in mM) 135 NaCl, 3 KCl, 1.5 CaCl$_2$, 1 MgSO$_4$, 2 KH$_2$PO$_4$, 5.5 glucose, 5 l-alanine, and 5 HEPES (pH 7.4). Then, the kidney was perfused with 10 ml digestion solution, which was prepared by adding 1 mg/ml collagenase (243 U/mg, Worthington) and 1 mg/ml bovine albumin to the dissection solution. After perfusion, the kidney was removed and cut into 1- to 2-mm-thick sections containing the entire corticomedullary axis. The sections were incubated at 37°C for 30 min in the same digestion solution with gentle shaking. During incubation, the samples were bubbled with 95% O$_2$-5% CO$_2$. The sections were then rinsed twice with collagenase-free dissection solution and transferred into petri dishes filled with ice-cold dissection solution containing 0.1 mg/ml trypsin inhibitor and 20 μg/ml aprotinin. A petri dish was mounted on the microscope stage and maintained at 4°C during dissection.

Microdissection was performed under a Leica MZ8 stereomicroscope equipped with darkfield illumination. The nephron segments including glomeruli, proximal convoluted tubule (PCT), proximal straight tubule (PST), cortical thick ascending limb of Henle’s loop (cTALH), medullary thick ascending limb of Henle’s loop (mTALH), thin limb of Henle’s loop, cortical collecting duct (CCD), and medullary collecting duct (MCD) were dissected. The time period for dissection was limited to 1 h.

**$O_2^-$ assay in microdissected renal segments.** $O_2^-$ concentrations within cells of glomeruli and tubular segments were monitored by measuring the changes in fluorescence resulting from the oxidation of dihydroethidium (DHE; Molecular Probes). DHE can enter the cell and be oxidized by $O_2^-$ to yield ethidium (Eth), which binds to DNA to produce bright red fluorescence. The increase in Eth-DNA fluorescence is suggestive of $O_2^-$ production within cells (3, 6).

With the use of a 96-well microtiter plate, dissected nephron segments were first incubated in the dissection solution at 37°C for 20 min and then with 20 μM DHE at room temperature. Twenty minutes after the loading of DHE into the cells, the plate was mounted on the stage of a fluorescence microscope (Nikon E-600) equipped with ×10 and ×20 objectives, and the fluorescence images were captured with a digital charge-coupled device camera (Roper Scientific RTE/CCD-1300-Y/HYS) controlled by Metamorph image-analysis software (Universal Imaging). The Eth-DNA fluorescence was monitored every 5 min for 30 min at 490-nm excitation and 610-nm emission. The images were saved in a departmental intranet system for off-line analysis, and the fluorescence intensity of 5–10 segments of renal tubules (3–6 mm in length) was integrated and normalized to the area of all counted tubules. The average fluorescent intensity at the maximal response time period (usually 25 min) was presented as units per square millimeter of tubule cross-sectional area.

**Analysis of different pathways for $O_2^-$ production.** It has been reported that the possible intracellular sources of $O_2^-$ include NADH oxidase, xanthine oxidase, arachidonic acid metabolism, NOS, and intramitochondrial enzyme systems (26, 30). To determine which pathway is responsible for $O_2^-$ production in TALHs, different enzyme substrates were incubated with cTALHs at 37°C for 20 min, and then 20 μM DPI was added to measure $O_2^-$ production as described above. NADH or NADPH (0.1 mM) was used to test the enzyme activities of xanthine oxidase, arachidonic acid-metabolizing enzymes, NOS, and mitochondrial enzymes, respectively (26, 38). Because mitochondria produce $O_2^-$ when the respiratory chain is blocked, antimycin was used to block the respiratory chain reaction.

**Regulation of $O_2^-$ production via NADH oxidase.** To further confirm that the activity of NADH oxidase contributes to the production of $O_2^-$ in cTALH, we examined the effects of NADH oxidase inhibitor or superoxide dismutase (SOD) blocker on Eth-DNA fluorescence intensity. Diphenyleneiodonium chloride (DPI; 100 μM, Aldrich) was used as an inhibitor of NADH oxidase to test whether the activity of this enzyme in TALH was blockable. Diethylthiocarbamate (DETC; 1 mM), a SOD inhibitor, was used to block SOD activity and consequently increase intracellular $O_2^-$ concentrations. These experiments would confirm that $O_2^-$ derived from NADH oxidase is SOD sensitive, which has usually been used to determine the specificity of $O_2^-$ assays (35).

In additional experimental groups of cTALHs, the mechanisms regulating $O_2^-$ production via NADH oxidase were explored. ANG II has been reported to stimulate $O_2^-$ production in renal proximal tubules, mesangial cells, and artery wall through AT$_1$ receptors (18, 20, 28, 33). These AT$_1$ receptors were found expressed in cTALHs (25). It remains unknown whether ANG II stimulates $O_2^-$ production in this tubular segment. The present study examined the effect of ANG II on $O_2^-$ production through NADH oxidase. ANG II (100 nM) was added to the bath solution and incubated with cTALHs for 30 min, and $O_2^-$ production was determined as described above. We also performed experiments to determine the effects of hypoxia on $O_2^-$ levels within cTALHs. In these experiments, cTALHs loaded with DHE were incubated in a low-PO$_2$ chamber (PO$_2$ = 5–10 Torr) as we described previously (47), and then the $O_2^-$ levels were measured. These experiments determined the possible association of tubular oxygenation with $O_2^-$ production.

**Expression of mRNA for NADH oxidase subunits in cTALHs.** RT-PCR was performed to determine mRNA expression of NADH oxidase in cTALHs. The mRNAs of 5 NADH oxidase subunits, including p22phox, p40phox, p47phox, p67phox, and Gp91phox (where phox indicates phagocyte oxidase), were PCR amplified and detected in this tubular segment. RNA extraction and RT-PCR were performed as we described previously (45). In brief, total RNA from microdissected segments (20 mm) was extracted using 450 μl TRIzol reagent (GIBCO BRL, Life Technologies). The resultant RNA was resuspended in 8 μl of RNase-free water. A first-strand cDNA synthesis kit (Pharmacia Biotech) was used to synthesize cDNA by RT from mRNA. According to the instruction of the manufacturer, 8 μl of total RNA, 0.2 μg random hexadeoxynucleotides, and 100 U Maloney murine leukemia virus RT were used. The reaction mixture was incubated at 37°C for 60 min and then heated to 65°C for 10
min to inactivate the activity of RT and to denature cDNA hybrids.

PCR reactions were performed in a total volume of 50 μl using a PCR Supermix kit (GIBCO BRL) containing 22 mM Tris-HCl (pH 8.4), 55 mM KCl, 1.65 mM MgCl2, 200 μM dNTPs, 5 μl RT reaction mixture, 22 U recombinant Taq DNA polymerase, and 400 pmol of the specific primer pairs for NADH oxidase subunits or GAPDH as we described previously (45, 47). The reactions were cycled 30 times from 94°C for 1 min to 52°C (for subunits p22phox, p47phox, and Gp91phox) or 55°C (for subunits p40phox and p67phox) for 1 min, and then 72°C for 1.5 min. Samples were incubated at 72°C for an additional 5 min after the last cycle was completed. NADH oxidase subunits p22phox, p40phox, p47phox, p67phox, p91phox, and GAPDH primers spanned fragments of 134, 611, 394, 599, 459, and 555 bp from their respective cDNAs. Negative control PCRAs with a substitution of dissection solution or total RNA of TALHs without RT reaction were performed in parallel.

The structure of the primers was as follows: p22: sense 5'-CCG GGA AAG AAA AAG and antisense 5'-GCC GAC GAC AGT AAG; p40: sense 5'-CCG CCG CTA TCG CCA GGT TTA and antisense 5'-CCT CCT CCA CCG CAA TGT CCT; p47: sense 5'-GCC TGA TGA CTA GAA ACT and antisense 5'-GCC TTC ACC CTC AGA CAG; p67: sense 5'-CCC AAA ACC CCA GAA ATC and antisense 5'-CCC ACC GTA TGC TCA CAC; Gp91: sense 5'-ATG AGG TGG TGA TGT TAG TGG and antisense 5'-AGT TGG AGA TGG TTT GTT TAC; and GAPDH: sense 5'-GGC GCC CAA AAT AAC and antisense 5'-GGG ACA GGA ATT and antisense 5'-GGG ACC GTG TCA CAC. The base sequences of these primers were sequenced by Genemed Synthesis (San Francisco, CA). The plasmid DNA was digested with EcoRI and sequenced as we described previously (45). Briefly, RT-PCR products of NADH oxidase subunits from TALHs were ligated into pCR II TOPO vector (Invitrogen, San Diego, CA), and the subsequent plasmid DNA was purified using an ion-exchange column (Qiagen, Chatsworth, CA). The plasmid DNA was digested with EcoRI to confirm the positive clones, and the inserts in these positive clones were sequenced by Genemed Synthesis (San Francisco, CA).

Immunohistochemistry for NADH oxidase within the kidney. Immunohistochemistry was performed as described previously (25). In brief, the rat kidneys were removed and fixed in 10% neutral formalin and embedded in paraffin. Then, kidney sections were prepared, incubated with xylene, and hydrated through several washes in ethanol and distilled water to remove the paraffin. Endogenous peroxidase activity was blocked by incubation of the sections with 3% H2O2 in methyl alcohol for 30 min and several rinses with distilled water. DAKO target antigen retrieval solutions were used to retrieve antigen by incubation of the sections in a water bath at 95–100°C for 30 min, and endogenous biotin was blocked by a DAKO biotin-blocking system. After a nonspecific staining block, a primary monoclonal antibody against Gp91 subunit of NADH oxidase (Transduction Laboratory, Lexington, KY) was used for immunostaining with a dilution of 1:100 in DAKO antibody diluents with background-reducing components. The kidney sections were incubated with the primary antibody overnight at room temperature in a humidified chamber, and a kidney section was incubated with vehicle (DAKO antibody diluent) as a negative control. Secondary antibody biotinylated cocktails were incubated with the sections for 60 min and then with streptavidin peroxidase conjugates (60 min; DAKO, Carpinteria, CA). The immunoreactive bands were detected with a diaminobenzidine-chromogen solution. Then, the sections were rinsed, nuclear stained, dehydrated, cleared, and mounted with permount media.

Statistical analysis. Data are presented as means ± SE. The significance of differences within and between multiple groups was evaluated using one- or two-way ANOVA followed by a post hoc test (Duncan’s multiple range test; SigmaStat, San Rafael, CA). A P value <0.05 was considered statistically significant.

RESULTS

O2·− production in the glomeruli and different tubular segments. As shown in Fig. 1A, the O2·− levels were detected in the glomeruli and all microdissected tubules, as measured by red fluorescence of Eth-DNA complex. Cortical and medullary TALHs (cTALHs and

Fig. 1. O2·− production in the glomeruli and different renal tubular segments. Microdissected glomeruli and tubular segments were incubated with dihydroethidium (DHE). The formation and binding of ethidium (Eth) from DHE produced red fluorescence, which represents O2·− production. A: typical fluorescence microscopic images showing maximal O2·−-induced Eth-DNA red fluorescence response recorded at 25 min after incubation of NADH with different renal segments. PCT, proximal convoluted tubule; PST, proximal straight tubule; cTAL, cortical thick ascending limb of Henle’s loop; CCD, cortical collecting duct; mTAL, medullary thick ascending limb of Henle’s loop; MCD, medullary collecting duct; Thin, thin limb of Henle’s loop; Glom, glomerulus. B: summary of the intensity of Eth-DNA fluorescence detected in the glomeruli and tubular segments. *P < 0.05 compared with the value obtained within PCT.
mTALHs) exhibited the brightest fluorescence compared with other tubular segments, indicating that the $O_2^\cdot$ levels were highest in the TALHs. These results were repeatedly observed ($n = 6$ rats) and are summarized in Fig. 1B. The $O_2^\cdot$ levels detected by DHE oxidation were highest in the cTALH and mTALH among all dissected renal segments, including the glomeruli. Compared with PCT or PST, cTALH, mTALH, CCD, and glomeruli all showed significantly higher levels of $O_2^\cdot$.

Different pathways responsible for $O_2^\cdot$ production. Figure 2A presents typical fluorescence microscopic images showing $O_2^\cdot$-induced Eth-DNA red fluorescence within cTALHs through different enzymatic pathways. These TALHs were incubated with different enzyme substrates, which were used for enzymes to produce $O_2^\cdot$, and then incubated with DHE. It was shown that with incubation of cTALHs with NADH oxidase substrate, NADH produced the brightest Eth fluorescence. Figure 2B summarizes the results of these measurements ($n = 7$). Incubation of cTALHs with NADH produced the most intensive Eth fluorescence compared with the incubation of other substrates such as xanthine, L-arginine, and AA.

Inhibition of NADH oxidase by DPI and blockade of $O_2^\cdot$ dismutation by DETC in cTALHs. Figure 3 summarizes the results showing the effects of DPI, the NADH oxidase inhibitor, and DETC, the SOD blocker, on $O_2^\cdot$ production. DPI at a concentration of 100 μM produced a 51% decrease in basal $O_2^\cdot$ levels when incubated with cTALHs for 30 min. NADH was found to increase $O_2^\cdot$ levels by 52% compared with control. In the presence of DPI, however, addition of NADH into the incubation mixtures did not significantly increase $O_2^\cdot$ levels ($n = 6$). DETC increased basal $O_2^\cdot$ levels by 52% and enhanced NADH-induced $O_2^\cdot$ production by 35% ($n = 7$).

Identification of mRNA of NADH oxidase subunits in cTALHs. Figure 4 illustrates a representative photograph of Eth bromide-stained RT-PCR products of five subunits of NADH oxidase and GAPDH in cTALHs. Four of these subunits were detected in total RNA extracted from cTALHs ($n = 5$). The sizes of the RT-PCR products of p40phox, p47phox, p22phox, and Gp91phox were 611, 394, 134 and 459 bp, respectively, which were identical to the predicted sizes based on

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**Fig. 2.** Different pathways responsible for $O_2^\cdot$ production in cortical thick ascending limbs of Henle’s loop (cTALHs). cTALHs were incubated with substrates of different enzymes and DHE. A: typical fluorescence microscopic images showing $O_2^\cdot$ levels within cTALHs incubated with different enzyme substrates for 25 min. Ctrl, control; AA, arachidonic acid; L-Arg, L-arginine; B: results measured when cTALHs were incubated with different substrates. $\Delta$Fluorescence, the increment of Eth-DNA fluorescence intensity measured during incubation of various substrates vs. that measured under control condition; mito-, mitochondria; Xan, xanthine. *$P < 0.05$ vs. vehicle.

**Fig. 3.** Effects of superoxide dismutase (SOD) or NADH oxidase inhibition on $O_2^\cdot$ production in cTALHs. Summarized data depicting the effects of diphenyleneiodonium (DPI) and diethyldithiocarbamate (DETC) on basal $O_2^\cdot$ levels and NADH-induced $O_2^\cdot$ production in cTALHs. *$P < 0.05$ vs. control (C). **$P < 0.05$ vs. NADH. ***$P < 0.05$ vs. DETC.

**Fig. 4.** RT-PCR detection of NADH oxidase subunits in the total RNA extracted from cTALHs. p22, p47, p67, p40 and Gp91 represent subunit p22phox, p47phox, p67phox, p40phox, and Gp91phox, respectively. (–), PCR without reverse transcription; DS, PCR with dissection solution as negative control; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
their cDNA sequences deposited in GenBank. Direct addition of total RNA from cTALHs into PCR reaction without reverse transcription or dissection solution used for PCR did not produce any signal (lane RT and lane DS), suggesting that PCR products are derived from cDNA synthesized by RT. Subunit p67phox with a predicted size of 599 bp was not detected.

Immunohistochemistry of NADH oxidase subunit Gp91phox within the rat kidney. Identification of NADH oxidase mRNA described above provided strong evidence showing the mRNA expression of this enzyme in the TALHs. However, the presence of mRNA does not necessarily indicate the expression of enzyme proteins. Therefore, we performed immunohistochemical examination to identify the protein expression of this enzyme in the TALHs. Using a monoclonal antibody against a major membrane subunit of NADH oxidase, Gp91phox, a high level of expression of this NADH oxidase subunit (brown staining) was found in the renal outer medulla with the high abundance on the cell membrane of the TALHs (n = 4). Lesser brown staining was found in the renal cortex, and no signal could be detected in the renal papilla (inner medulla; Fig. 5).

Effects of hypoxia on O$_2^-$ production in cTALHs. DHE-loaded cTALHs were incubated in a low-P$_O_2$ chamber, whereby P$_O_2$ in the incubation solution decreased to 5–10 Torr (~1.5% O$_2$), as measured by a polarographic microelectrode. Figure 6 presents the effects of hypoxia on O$_2^-$ levels within cTALHs. Low P$_O_2$ increased O$_2^-$ levels within cTALHs by 35 and 60%, respectively, in the absence and presence of NADH in the incubation solution (n = 5).

Effects of ANG II on O$_2^-$ production in the absence or presence of DPI. As shown in Fig. 7, incubation of TALHs with ANG II (100 nM) significantly stimulated O$_2^-$ production. When TALHs were pretreated with the...
NADH oxidase inhibitor DPI, the effects of ANG II on \( \text{O}_2^\cdot \) production were completely abolished. In the presence of both DPI and ANG II, \( \text{O}_2^\cdot \) levels were even lower compared with control values.

DISCUSSION

The present study detected \( \text{O}_2^\cdot \) in microdissected renal tubules and explored the regulatory mechanisms of intracellular \( \text{O}_2^\cdot \) concentrations in TALH cells by fluorescence microscopic imaging analysis of DHE oxidation. It has been reported that DHE as a nonfluorescent dye can easily enter into cells and be oxidized to form a fluorescent product, Eth. The formation of Eth by oxidation of DHE within cells is directly proportional to the levels of \( \text{O}_2^\cdot \), but not to that of \( \text{O}_2 \), \( \text{H}_2\text{O}_2 \), and other free radicals (37, 38). It is generally accepted that the fluorescence intensity produced by DHE oxidation reflects the \( \text{O}_2^\cdot \) concentrations in solutions or cell cytoplasm and that the determination of \( \text{O}_2^\cdot \) levels based on the oxidation of DHE is specific. In addition, Eth formed by oxidation of DHE can bind to DNA of cell nuclei, which enhances fluorescence intensity by >40-fold (38). Therefore, this method is the most sensitive assay suitable for the measurement of \( \text{O}_2^\cdot \) levels within cells and has been extensively used to detect intracellular \( \text{O}_2^\cdot \) concentrations or to determine the enzyme activity responsible for \( \text{O}_2^\cdot \) production in a variety of mammalian cells (37, 38, 46).

Using this assay of intracellular \( \text{O}_2^\cdot \), we found that microdissected TALHs exhibited the highest \( \text{O}_2^\cdot \) levels compared with other renal segments such as glomeruli, proximal tubules, and collecting duct. The high levels of \( \text{O}_2^\cdot \) in the TALHs may be associated with its functional and metabolic activity (11). Therefore, we chose cTALH as a prototype tubular segment to explore the mechanisms regulating intracellular \( \text{O}_2^\cdot \) concentrations under physiological conditions. First, we determined the enzymatic pathways responsible for \( \text{O}_2^\cdot \) production in this tubular segment. It has been demonstrated that different enzymatic pathways are involved in \( \text{O}_2^\cdot \) generation in nonphagocytic cells under physiological or pathological conditions, which include NADH oxidase, xanthine oxidase, cyclooxygenase/lipoxygenase, mitochondrial respiratory enzyme chain system, and NOS (17, 26, 30). By incubation of cTALHs with the substrates of all these enzymes as described in previous studies (37, 38, 46), we found that incubation with NADH produced the most intense Eth-DNA fluorescence in these TALH cells, suggesting that NADH oxidase-mediated \( \text{O}_2^\cdot \) production may be a primary source of intracellular \( \text{O}_2^\cdot \) in this tubular segment. Because NADH has much higher physiological concentrations (at mM range) in mammalian cells compared with the substrates at micromolar range for other possible enzymes producing \( \text{O}_2^\cdot \) listed above (22), NADH-derived \( \text{O}_2^\cdot \) may predominately contribute to the high levels of \( \text{O}_2^\cdot \) in this tubular segment under physiological conditions.

In previous studies, NADH oxidase has been shown to be the major oxidase in vascular tissue and in cardiac cells compared with the production of ROS from xanthine oxidase, arachidonic acid, and mitochondrial oxidases (5, 16, 23, 27, 29, 31). Although early work demonstrated xanthine oxidase as a prime source of endothelium-dependent \( \text{O}_2^\cdot \) production, recent studies have indicated that NADH oxidase activities in smooth muscle cells and fibroblasts account for the majority of \( \text{O}_2^\cdot \) produced in the normal vessel wall, suggesting that NADH oxidase may be the critical determinant of the redox state of blood vessels and the myocardium (16). In the kidney, NADH oxidase has been reported to mediate the production of \( \text{O}_2^\cdot \) in response to ANG II in mesangial cells (20). More recently, we have reported that NADH oxidase is a major enzyme responsible for \( \text{O}_2^\cdot \) production in the rat kidney homogenate (46). Taken together, these results suggest that NADH oxidase may play a critical role in producing ROS in the kidney and thereby in the regulation of renal function.

In the present study, increased intracellular \( \text{O}_2^\cdot \) concentrations in cTALHs were detected when these tubular segments were incubated with NADH or NADPH, indicating that NADH oxidases produce \( \text{O}_2^\cdot \) intracellularly. This orientation of \( \text{O}_2^\cdot \) production or release in cTALH cells was consistent with that in vascular smooth muscle cells, in which the production of \( \text{O}_2^\cdot \) and \( \text{H}_2\text{O}_2 \) is mainly intracellular (14, 42). In neutrophils, however, NADH oxidases span cell membranes and transfer electrons from intracellular NAD(P)H to extracellular \( \text{O}_2 \) to form \( \text{O}_2^\cdot \) outside of the cells, thereby increasing extracellular \( \text{O}_2^\cdot \) concentrations to produce a respiratory burst (16). The reason for different orientations of \( \text{O}_2^\cdot \) production or release through NADH oxidases in different cells remains unknown. It is possible that the cell type-specific assembling models of the enzyme subunits or different regulatory mechanisms determine the orientation of \( \text{O}_2^\cdot \) production and release.

To further confirm that an increase in intracellular \( \text{O}_2^\cdot \) in response to NADH is derived from NADH oxidases, we examined the effects of DPI, an inhibitor of flavin-containing oxidases, on NADH-induced \( \text{O}_2^\cdot \) production in cTALHs. Previous studies have indicated that inhibition of \( \text{O}_2^\cdot \) production by DPI is one of the common pharmacological characteristics of NADH ox-
idase, despite the differences in the orientations, kinetics, and efficiency of O$_2^\cdot$ production by this enzyme and its homologues in different tissues or cells (16). It was found that DPI significantly decreased intracellular O$_2^\cdot$ levels in cTALHs under control condition, suggesting that NADH oxidase can produce O$_2^\cdot$ with endogenous NADH or NADPH as a substrate. When NADH was added into the incubation mixtures with cTALHs, increased production of intracellular O$_2^\cdot$ by NADH in these tubules was completely blocked by DPI, whereby O$_2^\cdot$ was maintained at a level observed in cTALHs treated with DPI alone. These results provide further evidence supporting the view that NADH oxidase is importantly responsible for O$_2^\cdot$ production in the TALHs in the presence of NADH.

It has been demonstrated that the activity of O$_2^\cdot$ dismutases (SODs) is a key determinant of intracellular O$_2^\cdot$ concentrations. These enzymes function to accelerate the removal of O$_2^\cdot$ from 8 $\times$ 10$^{-4}$ mol$^{-1}$·l$^{-1}$·s$^{-1}$ to 2 $\times$ 10$^{-6}$ mol$^{-1}$·l$^{-1}$·s$^{-1}$ (39). Previous studies have shown that only picomolar O$_2^\cdot$ can be detected in vascular tissues in the presence of SODs, which is much lower compared with micromolar O$_2^\cdot$ in the absence of SOD (39). We demonstrated that incubation of TALHs with DETC, a SOD inhibitor, remarkably increased the Eth-DNA red fluorescence in the absence and presence of NADH. This result suggests that SODs also importantly contribute to the regulation of O$_2^\cdot$ concentrations in TALHs by removal of excess O$_2^\cdot$.

NADH or NAPDH oxidase in mammalian professional phagocytes is well characterized among the O$_2^\cdot$-producing systems (1, 16, 34). The enzyme complex comprises five components: p40phox , p47phox , p67phox , p22phox , and Gp91phox (1). The catalytic core of the phagocyte NADH oxidase is the membrane-integrated flavocytochrome b558, comprising the two subunits p22phox and Gp91phox, the latter of which contains a complete electron-transferring apparatus from NAD(P)H to molecular oxygen with binding sites for heme, FAD, and NAD(P)H. In the resting cells, three of these five components including p40phox, p47phox, and p67phox exist in the cytosol as a complex. When the resting phagocytes are exposed to any of a wide variety of stimuli, the cytosolic component p47phox becomes heavily phosphorylated, and the entire cytosolic complex migrates to the membrane, where it binds to cytochrome b-558 to assemble into an active oxidase (34). Recently, NADH oxidase activity has been demonstrated in nonphagocytic tissues or cells, such as vascular smooth muscle cells (36), endothelium (24), carotid body (8), lung (13, 41), joint tissues (19), and kidney (9, 21, 34). However, the expression of NADH oxidase components was found to be different from that in phagocytic cells (15, 24). For instance, Jones et al. (21) have reported that Gp91phox mRNA could not be detected in human glomerular mesangial cells, and others have shown that there was no expression of p40phox in vascular tissues (24, 39). On the basis of these results, it has been proposed that tissue-specific expression of different NADH oxidase components may confer the differences in the regulation of the enzyme activity in those tissues or cells (24). Using RT-PCR, we can only detect four of five NADH oxidase components in cTALHs. p67phox was not expressed in this tubular segment. It remains to be determined why p67phox is not expressed in these TALHs and of what relevance is the lack of this subunit to enzyme activity. By immunohistochemical examinations, we demonstrated that a subunit of NADH oxidase, Gp91phox, predominately expressed in the renal outer medulla, especially on the cell membrane of the TALHs in this kidney region. This suggests that the high abundance of NADH oxidase protein in the TALHs may be responsible for increased production of O$_2^\cdot$ in this tubular segment.

To further determine the effects of tissue or cell oxygenation on the production of O$_2^\cdot$ via NADH oxidase in the TALHs, we examined the response of NADH oxidase to hypoxia in this tubular segment. When these TALHs were exposed to hypoxia, O$_2^\cdot$ production within this tubular segment using NADH as substrate was significantly increased. These results confirm that this O$_2^\cdot$-producing enzyme system is coupled to cell oxygenation. It appears that decreased cell oxygenation could activate NADH oxidase to produce O$_2^\cdot$. Given that O$_2^\cdot$ increases adenosine production (7) or directly contracts vessels (32), increased O$_2^\cdot$ production in response to a decrease in cell oxygenation in the TALHs may be importantly involved in the tubuloglomerular feedback response. It is possible that O$_2^\cdot$ production in the TALHs in response to hypoxia mediates or enhances vasoconstriction of afferent arterioles in the tubuloglomerular feedback response through its direct vasoconstrictor action or stimulation of adenosine (7, 32).

The present study also determined whether the production of O$_2^\cdot$ via NADH oxidase is regulated by ANG II. ANG II was chosen because it was reported to participate importantly in the regulation of ion transport activity in the TALHs (11). Furthermore, there is a large body of evidence indicating that ANG II is a potent stimulator of oxidative stress in vascular tissue or cells and mesangial cells (20, 28, 33). It has been demonstrated that ANG II stimulates O$_2^\cdot$ production by activation of a membrane-bound NADH oxidase (18, 44). In the present study, ANG II was found to enhance NADH-induced O$_2^\cdot$ production in cTALHs, which was blocked by DPI. This suggests that the activity of NADH oxidase may be regulated by ANG II. When the role of O$_2^\cdot$ as a signaling molecule in various cells (13) is considered, it is possible that this free radical plays a critical role in mediating the effects of ANG II on ion transport activity of the TALHs.

In summary, the present study demonstrated that NADH oxidase was the major enzyme responsible for O$_2^\cdot$ production in TALHs and that the production of O$_2^\cdot$ via NADH oxidase was enhanced by cell hypoxia or ANG II. These results indicate that tubular cell oxygenation and circulating hormones may importantly regulate NADH oxidase activity in the TALHs.
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