Oxygen availability limits renal NADPH-dependent superoxide production

Yifan Chen, Pritmohinder S. Gill, and William J. Welch
Division of Nephrology and Hypertension, Georgetown University, Washington, District of Columbia

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Chen, Yifan, Pritmohinder S. Gill, and William J. Welch. Oxygen availability limits renal NADPH-dependent superoxide production. Am J Physiol Renal Physiol 289: F749–F753, 2005.—Renal oxygenation are exacerbated by hypertension (26) and diabetes (16). We showed that PO\textsubscript{2} is reduced by ~10 to 33 mmHg in the outer and to 19 mmHg in the inner cortex in spontaneous hypertensive rats (SHR) compared with normotensive Wistar-Kyoto (WKY) rats (26). However, it remains undetermined whether the low oxygenation is sufficient to regulate renal O\textsubscript{2}•\textsuperscript{-} production.

NADPH oxidase is the primary source of O\textsubscript{2}•\textsuperscript{-} in the kidney under physiological conditions (9). The induction of oxidative stress or overproduction of O\textsubscript{2}•\textsuperscript{-} often depends on increased NADPH oxidase expression or activity. Renal NADPH oxidase is upregulated with a high-salt diet (9), ANG II infusion (26), diabetes (5, 8), and ischemia-reperfusion injury (7). In many of these models, the role of concomitant hypoxia on the O\textsubscript{2}•\textsuperscript{-} production is beginning to emerge. Several in vitro models have shown that NADPH-dependent O\textsubscript{2}•\textsuperscript{-} production decreases in coronary (13) or pulmonary arteries (15) or cardiomyocytes (14) when exposed to hypoxia (PO\textsubscript{2}: 8–10 mmHg). Reoxygenation (160 mmHg) or reperfusion increases O\textsubscript{2}•\textsuperscript{-} production compared with the prehypoxic period or to ischemic tissue. However, regulation of renal NADPH oxidase activity by O\textsubscript{2}• remains unclear, as one report showed that NADPH-dependent O\textsubscript{2}•\textsuperscript{-} production is enhanced in rat cortical thick ascending limbs of Henle’s loop exposed to hypoxia (PO\textsubscript{2}: 5–10 mmHg) (11). Because the oxygen environment in the kidney is unique, with a marked hypoxic medulla in normal animals, O\textsubscript{2}• may limit O\textsubscript{2}•\textsuperscript{-} formation in this region of the normal kidney. Thus we determined whether NADPH-dependent O\textsubscript{2}•\textsuperscript{-} production is altered during changes in the physiological range of PO\textsubscript{2} in the kidney.

We tested the hypothesis that the low PO\textsubscript{2} commonly found in the kidney limits NADPH-dependent O\textsubscript{2}•\textsuperscript{-} production. Specifically, we studied real-time, in vitro NADPH-dependent O\textsubscript{2}•\textsuperscript{-} production in kidney tissue and cultured cells in response to various O\textsubscript{2} concentrations.

METHODS

Preparation of the Kidney Homogenate

The protocol describing the use of animals in this study was approved by the Georgetown University Animal Care and Use Committee. Male adult rats (225–265 g) were anesthetized, and kidney tissue was prepared as described previously (9). The right external jugular veins were cannulated and the abdominal cavity was opened through a midline incision. Kidneys were removed and perfused with an ice-cold, pH 7.4, PBS via the renal artery to wash out blood. The kidney was cleaned of connective tissues and immediately frozen in liquid nitrogen. The left ventricle was prepared similarly. All the tissues were kept at ~80°C. After being thawed, the samples were placed on ice in test tubes containing PBS and homogenized. Thereafter, the homogenate was centrifuged at 3,000 rpm for 20 min. The
supernatant was used to study NADPH-dependent $O_2^\bullet^-$ production. Pilot studies showed that $O_2^\bullet^-$ levels were similar in fresh and frozen tissue and thawed from tissue.

Preparation of the Cultured Cells

The cells used in this study were: rat proximal tubule cells (PT; kindly provided by Dr. U. Hopfer, Case Western Reserve University, Cleveland, OH), mouse inner medulla collecting duct (IMCD; provided by Dr. B. A. Stanton, Dartmouth Medical School, Hanover, NH), rat vascular smooth muscle cells (VSM; A-10 cell line, ATCC, Manassas, VA), and rat cardiomyocytes (CM; Cell Applications, San Diego, CA). The cells were grown and maintained at 37°C in humidified atmosphere of 5% $CO_2$, in media containing 10% fetal bovine serum and other supplements as per instructions. The cells were grown to 80% confluence and washed with PBS. Cells were dislodged with scrapping in PBS. Each measurement used 1 x 10⁶ cells.

Measurement of Superoxide Production

Lucigenin (final concentration 5 µM)-enhanced chemiluminescence was used to determine $O_2^\bullet^-$ generation after adding excess NADPH (100 mM), the substrate for NADPH oxidase. This method is specifically designed to measure NADPH oxidase capacity. A tube chemiluminescence apparatus was custom-constructed to include a light-tight box and a photodetector (Electron Tubes, Rockaway, NJ). This configuration permits simultaneous gassing and photon counting of the sample, allowing for rapid switching between two different concentrations of $O_2$ while a constant airflow rate is maintained. The chemiluminescence signals were sampled at 100 Hz and recorded cumulatively every second after background adjustment. For the tissue samples, the final values, expressed as relative light units (RLU), were corrected for protein concentration determined by protein assay. The integration of peak values recorded continuously for ≥20 s was calculated for the final comparison.

In this preparation, we showed that tempol, a superoxide dismutase (SOD) mimic or SOD, inhibited 90% of the NADPH-dependent signal (9, 25). The signal was also reduced by 70% by apocynin (100 mM). However, because apocynin acts to prevent assembly of NADPH oxidase subunits, its effectiveness in this in vitro preparation is unclear. The addition of rotenone (100 µM, a mitochondrial complex inhibitor; 95 ± 4%) did not affect the NADPH-dependent signals in the kidney or culture cell preparations within the tested range of $Po_2$. The involvement of mitochondrial complex was further evaluated using succinate (100 µM). The succinate-dependent signal from the kidney homogenate was less than 8% of the NADPH-dependent signal. There was no detectable succinate-dependent signal in the cultured cell preparations. The addition of Nα-nitro-L-arginine methyl ester (100 mol) or tetrahydrobipterin (10 mM) did not alter the basal $O_2^\bullet^-$ production, suggesting the $O_2^\bullet^-$ signal is not linked to nitric oxide synthase. $O_2^\bullet^-$ was not formed by the addition of NADH or xanthine. Thus the lucigenin-enhanced, NADPH-dependent $O_2^\bullet^-$ signals were almost exclusively generated by NADPH oxidase in the cells and kidney tissue.

Measurement of $O_2$ and Experimental Protocol

Oxygen tension was measured using a combination needle $O_2$ electrode with internal reference (model 768–20R, Diamond General Development, Ann Arbor, MI). Within the experimental range of $Po_2$ increased linearly with increasing concentrations of gassing $O_2$ ($r^2 = 0.998, P < 0.0001$). The airflow was set at a slow rate to minimize bubbling. When concentrations of $O_2$ were changed, equilibration was achieved within 90 s.

All the samples were gassed for 3 min with 0, 1, 2, 3, or 4% $O_2$ (2, 7, 14, 20, 29, or 76 mmHg $Po_2$) before and after baseline gassing at 10% $O_2$ measurement. We were unable to completely eliminate residual $O_2$ in these preparations, as gassing with 0 $O_2$ resulted in $Po_2$ of ~2 mmHg. Subsequently, NADPH, NADH, or xanthine (100 µM) was applied, and the response was allowed to reach a steady state.

Chemicals

Lucigenin (bis-N-methylacridinium nitrate), NADH, NADPH, succinate, rotenone, and apocynin were purchased from Sigma (St. Louis, MO). NADH oxidase (from bacillus) was obtained from Calbiochem (San Diego, CA). All the chemicals were dissolved in PBS.

Experimental Series

Series 1. $O_2^\bullet^-$ production was assessed in normotensive and hypertensive rats. RLU output was measured in homogenates of kidney and left ventricle after the addition of oxidase substrates, NADPH, NADH, or xanthine. The homogenates were gassed with 10% $O_2$.

Series 2. NADPH-dependent $O_2^\bullet^-$ production in kidney homogenates from normotensive and hypertensive rats was measured by continuous RLU during exposure of kidney homogenates to 0, 1, 2, 3, or 4% $O_2$, before and after exposure to 10% $O_2$.

Series 3. NADPH-dependent $O_2^\bullet^-$ production in renal cells was assessed by RLU during exposure to 2% $O_2$ before and after exposure to 10% $O_2$.

Series 4. $O_2$ sensitivity of NADH-stimulated $O_2^\bullet^-$ generated by purified NADH oxidase was measured during exposure to 0, 1, 2, 3, or 4% $O_2$ followed by 10% $O_2$.

Data Processing and Statistical Analysis

Data are reported as means ± SE. The chemiluminescence signals at various $Po_2$ were calculated as relative changes to their levels at $Po_2 = 76$ mmHg (gassing with 10% $O_2$) for further comparison. Nonlinear regression or t-test was used to compare the changes in fluorescent intensity in response to changing $Po_2$. The results were considered significant at $P ≤ 0.05$.

RESULTS

Series 1

The addition of NADPH to homogenates of rat kidney tissue generated high levels of $O_2^\bullet^-$, whereas the addition of NADH or xanthine had minimal effects (Fig. 1). Although we previously performed experiments to identify the source of $O_2^\bullet^-$, these data provide the best evidence NADPH oxidase is the exclusive source in this preparation. NADPH-dependent $O_2^\bullet^-$ production was also found in the rat left ventricle. The NADPH-dependent $O_2^\bullet^-$ production was higher in hypertensive kidneys ($P < 0.05$), but not in hypertensive left ventricles. These data confirm that the major oxidative enzyme in these kidney and heart preparations is NADPH oxidase, which is increased in hypertensive kidneys.

Series 2

NADPH-initiated $O_2^\bullet^-$ production was reduced when equilibrated with lower $Po_2$ levels. A representative experiment is shown in Fig. 2. RLU was increased within seconds of switching the $O_2$ of the reaction mixture from 2 to 10%. This response was reversible. Regression analysis of data from exposure to 0, 1, 2, 3, 4, and 10% $O_2$ showed that the relative renal NADPH-dependent $O_2^\bullet^-$ production fell exponentially with $O_2$ levels below 4% (Fig. 3). Although the basal activity levels were higher in hypertensive kidneys, there was no difference between the responses in tissue from normotensive and hypertensive rats ($n = 3$ per group). The $K_m$ values were $15.4 ± 1.3$
and 13.9 ± 2.9 mmHg Po2 for the normotensive and hypertensive, respectively (not significant).

Series 3

Similar dependency of the NADPH-dependent O2•− production on O2 levels was observed in cultured cells. As shown in Fig. 4, the NADPH-dependent O2•− production decreased in the PT (−56.8 ± 0.2%), IMCD (−57.9 ± 2.6%), VSM (−42.1 ± 4.5%), and CM (−56.8 ± 1.1%) when these cells were exposed to 2% O2 compared with 10% O2 (P < 0.01, n = 3 each cell type).

Series 4

We further examined the in vitro Po2 effects using purified NADH oxidase in place of tissue or cells (3). NADH-initiated O2•− production by NADH oxidase decreased exponentially with decreasing Po2 especially below ~30 mmHg (Fig. 5). The Km was 11.4 ± 0.6 mmHg Po2, similar to our results with renal tissue and cells.

DISCUSSION

We show that levels of O2 below 4% reduce NADPH-dependent O2•− production by kidney tissue homogenates, cells, and purified oxidase compared with 10% O2. These data suggest that the NADPH-dependent O2•− production is regulated by O2 in areas of the kidney where Po2 is low. Medullary oxygen levels of 10–20 mmHg Po2 (2–4% O2) (2, 10, 26) are sufficiently low to limit excess O2•− formation by endogenous NADPH oxidase under normal conditions. O2•− production in the cortex may also be limited, especially during pathophysiological conditions such as hypertension or diabetes, when renal cortical Po2 has been reported as low as 30 mmHg, equivalent to ~4% O2 (16, 26).

Oxygen is required for O2•− production catalyzed by NADPH oxidase: NADPH + 2O2 = NAD(P)+ + 2O2•− + H+ (6). Therefore, O2 becomes rate limiting when its availability fails to match the need for electron acceptor in this process. In vitro analyses in this study demonstrate these limits. The in vivo requirement for O2 is at least as high as in vitro. In vivo NADPH oxidase concentration is higher than in the diluted tissue homogenate tested in this study. Oxygen is used for O2•− production as well as other cellular reactions including mitochondria respiratory activity. These competitive processes may intensify demands for O2. Thus the principles demonstrated by our in vitro preparation remain relevant and low O2 should reduce O2•− production in a dose-dependent manner under in vivo conditions.
NADPH oxidase as characterized in neutrophils is a complex enzyme consisting of five subunits: cytosolic p47phox, p67phox, p40phox, and membrane bound α- and β-subunits, p22phox and gp91phox (2). Tissue NADPH oxidases are not as well characterized, and although similar, homologs to the flavoprotein gp91phox and to p47phox and p67phox have been identified. Various studies suggest that p22phox and the flavoproteins are essential for NADPH oxidase function.

NADPH oxidase activity in increased in hypoxia/reperfusion injury in lungs and heart compared with hypoxic conditions (4, 13–15, 17, 24, 27). However, when measured during the hypoxic (PO2 = 8–10 mmHg) phase of these studies, NADH-stimulated O2•− production was reduced in coronary or pulmonary arteries and abolished in CM compared with basal (PO2 = 160 mmHg) (13–15). These results are consistent with our observation that NADPH oxidase activity is limited by low O2.

The reduced O2•− production during hypoxia is not associated with a decrease in NADPH oxidase expression. In cultured endothelial cells exposed to 8 h of hypoxia, O2•− production was reduced, but expression for p22phox was unchanged (20). Studies in the kidney confirm that NADPH oxidase activity is limited by hypoxia. Oxidative stress, assessed by glutathione oxidation after 24 h, was not altered in kidneys of hypoxic mice despite upregulation of the β-subunit NOX-4 (22). Therefore, O2•− production is limited by the availability of O2 not the amount of enzyme.

However, O2•− production by NADPH oxidase may increase during hypoxia. Acute exposure of cultured smooth muscle cells from small bovine pulmonary arteries to moderate hypoxia (PO2 = 40 mmHg) increased O2•− production, which was blocked by the NADPH oxidase inhibitor diphenyliodonium (DPI) but not by the mitochondrial inhibitor myxothiazol (12). Also, anoxia generates a burst of ROS in cultured endothelial cells from porcine aorta that is blocked by DPI and by mitoQ, an inhibitor to mitochondrial ROS (18). Incubation of rat thick ascending limb of Henle’s loop with 1.5% O2 increased NADH-induced O2•− production (11). These observations of hypoxia-induced O2•− production may have several causes. First, we find that NADPH oxidase production is little affected until the O2 level is below 4% or ~30 mmHg PO2. This may explain why increases in O2•− associated with PO2 values above 30 mmHg may not be limited by hypoxia. Second, some metabolites such as lactate may potentiate the oxidase activity especially during prolonged exposure to hypoxia (14). Third, there may be differences in tissue responses, suggesting that the level of hypoxia and responses to hypoxia may differ in pulmonary, coronary, and renal vessels. However, we detected a rather uniform effect in renal, vascular, and cardiac cells when measured in vitro.

Our results confirm that NADPH oxidase is the dominant source for O2•− in the kidney in normal and hypertensive rats (9, 26) and that NADPH-dependent O2•− production is increased in hypertensive kidneys. However, an effect of hypertension on NADPH-dependent O2•− production was not seen in the heart. This observation needs further investigation. The absence of NADH- or xanthine-stimulated O2•− in both the kidney and heart confirms that the dominant oxidase in these tissues is NADPH oxidase. Some studies have shown that the NADH pathway is a greater source than the NADPH pathway for O2•− generation in the kidney (11) and cardiovascular system (13, 14). Both the NADH (11) and NADPH (9) oxidases have been identified in the kidney. They have similar membrane and cytosolic components and are able to use both NADH and NADPH as substrates, depending on the availability of cofactors. The addition of FAD to the purified NADPH oxidase used in this study limits this enzyme activity to NADH, as FAD prevents the oxidation of NADPH. Therefore, in vitro estimation of O2•− production depends critically on the availability of necessary cofactors, which was carefully controlled in these studies.

The availability of substrate may also be a rate-limiting process in vivo. Our measurements were made in the presence of saturating levels of substrates. The primary in vivo source for NADPH is from glucose metabolism through the pentose capacitation pathway. Glucose 6-phosphate is converted into ribulose 5-phosphate and CO2, leading to the synthesis of NADPH. The NADPH supply is low during oxidative stress (1, 19) or hypoxic conditions (23). Therefore, when oxidative expression is increased especially in hypertension and diabetes, NADPH availability could also limit O2•− production (5, 21).

Although the total NADPH oxidase activity was higher in hypertensive rats, the sensitivity of the enzyme was similar in tissue from normotensive and hypertensive rats. This greater basal production of O2•− is due to the increased expression of NADPH oxidase mRNA and protein in hypertensive kidneys (6, 9). However, the sensitivity differences to the level of O2•− was reduced, but expression for p22phox was unchanged.

![Fig. 4. NADPH-dependent O2•− production in cultured cells. Open bars, 10% O2; filled bars, 2% O2. PT, rat proximal tubule; IMCD, mouse inner medulla collecting duct; VSM, rat vascular smooth muscle; CM, rat cardiomyocyte. *P < 0.01 compared with 10% O2.](http://ajprenal.physiology.org/)

![Fig. 5. Regression analysis of NADH-dependent O2•− production by NADPH oxidase as a function of PO2 (r² = 0.90).](http://ajprenal.physiology.org/)
may be a defense mechanism to avoid even greater oxidative stress. As we showed previously, the reduced renal PO2 in hypertension may be due to the reduced efficiency of O2 usage relative to sodium transport (26). Thus the reduced PO2 would limit O2 production by NADPH oxidase and reduce the damage caused by oxidative stress in the kidney.

Because we show the same sensitivity of NADPH oxidase to PO2 in kidneys from normotensive and hypertensive rats, this suggests that the different level of O2 concentration at baseline under normal oxygen (Fig. 1) could be due to enhanced NADPH oxidase expression and perhaps also to substrate or cofactor availability. However, a more plausible explanation may be related to the reduced O2 availability in hypertensive rat kidneys. We showed that outer and inner cortical PO2 were reduced to 32 and 22 mmHg, respectively, in hypertensive kidneys (26). Under these conditions, NADPH oxidase activity may be suppressed sufficiently to limit the damage caused by oxidative stress in the kidney. However, when extracted and exposed to similar O2 levels, the enzyme is equally active.

Superoxide production by NADPH oxidase in harvested renal tissue and cultured cells is reduced at O2 levels below 4% (≤30 mmHg). This is similar to the activity profile of purified NADPH oxidase. Whereas the reduced PO2 levels observed in the renal medulla may not prohibit oxidative stress associated with hypertension or diabetes, it may nevertheless limit O2 production and its consequences. Therefore, low O2 availability may be protective against oxidative stress in the renal medulla of normal rats. In addition, during hypertension or diabetes when PO2 levels throughout the kidney are reduced, a protective role of low O2 may extend into the cortex and ultimately reduce the tissue damage and other consequences of oxidative stress.

GRAINS
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REFERENCES