Role of components of the phagocytic NADPH oxidase in oxygen sensing

K. A. Sanders, K. M. Sundar, L. He, B. Dinger, S. Fidone, and J. R. Hoidal. Role of components of the phagocytic NADPH oxidase in oxygen sensing. J Appl Physiol 93: 1357–1366, 2002. First published July 5, 2002; 10.1152/japplphysiol.00564.2001.—It has been hypothesized that O₂ sensing in type I cells of the carotid body and erythropoietin (EPO)-producing cells of the kidney involves protein components identical to the NADPH oxidase system responsible for the respiratory burst of phagocytes. In the present study, we evaluated O₂ sensing in mice with null mutant genotypes for two components of the phagocytic oxidase. Whole body plethysmography was used to study unanesthetized, unrestrained mice. When exposed to an acute hypoxic stimulus, gp91phox-null mutant and wild-type mice increased their minute ventilation by similar amounts. In contrast, p47phox-null mutant mice demonstrated increases in minute ventilation in response to hypoxia that exceeded that of their wild-type counterparts: 109.6 ± 13.29% vs. 72.54 ± 7.65% (n = 8 and 7, respectively, P = 0.206). Consequently, the hypoxia-evoked CSN discharge (stimulus-basal) was ~58% larger in p47phox-null mutant mice. Quantities of EPO mRNA in kidney were similar in gp91phox- and p47phox-null mutant mice and their respective wild-type controls exposed to hypobaric hypoxia for 72 h. These findings confirm the previous observation that absence of the gp91phox component of the phagocytic NADPH oxidase does not alter the O₂-sensing mechanism of the carotid body. However, absence of the p47phox component significantly potentiates ventilatory and chemoreceptor responses to hypoxia. O₂ sensing in EPO-producing cells of the kidney appears to be independent of the gp91phox and p47phox components of the phagocytic NADPH oxidase.

carotid body; erythropoietin; hypoxia

IN MAMMALS, HYPOXIA INITIATES three adaptive adjustments that mitigate the adverse effects of decreased ambient O₂ by augmenting O₂ transport to tissues. These homeostatic mechanisms include carotid body-stimulated increases in ventilation, renal production of erythropoietin (EPO) with resulting augmentation of red blood cell mass, and contraction of pulmonary artery smooth muscle cells, which enhances ventilation-perfusion matching. Cells in these tissues exhibit an unusual sensitivity to hypoxia, wherein they respond to PO₂ levels that do not alter normal oxidative metabolism or threaten cell survival (14). Numerous efforts to understand the microphysiology of these systems have failed to identify the molecular O₂ sensor (or sensors) utilized in each response. Potential signaling molecules include members of the NADPH oxidase family of enzymes (34), which are responsible for generating the well-known respiratory burst in phagocytic cells. The phagocytic form of the oxidase (phox) comprises a heme-containing flavocytochrome (cytochrome bo₅₅a) that consists of two membrane-bound components, gp91phox and p22phox, in addition to the cytosolic proteins p47phox, p67phox, and p40phox, which bind to the flavocytochrome to form the active enzyme complex. Activation also requires the participation of two low-molecular-weight guanine nucleotide-binding proteins: the cytoplasmic Rac2 and the cytochrome-associated Rap1A (5). According to the hypothesis of Acker (1), O₂ sensing involves the generation of reactive oxygen species (ROS) in proportion to local tissue PO₂ by a membrane-bound NADPH oxidase, thereby altering the intracellular redox balance. Thus, as the intracellular environment becomes reduced, mechanisms intended to alleviate tissue hypoxia are initiated. O₂-sensing mechanisms used by the type I cells of the carotid body (2), EPO-producing cells of the kidney (7), smooth muscle cells of the pulmonary arteries (53), and cells of the neuroepithelial bodies (NEB) (55) [presumed airway chemoreceptors (15)] have been proposed to function in this fashion.

Recent studies utilizing gp91phox-null mutant mice suggest that the phagocytic NADPH oxidase may function as an O₂ sensor in a tissue-dependent fashion. O₂ sensing is unaltered in pulmonary arterial tissue (4) and carotid body type I cells (23, 42) from gp91phox-null mutant mice. In contrast, O₂-sensitive K⁺ currents in NEB cells from gp91phox-null mutant mice are not inhibited by hypoxia or the flavoenzyme inhibitor diphenyleneiodonium (DPI) (15). NEB are thought to be

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particularly relevant to O$_2$ sensing in the newborn (17), and, consistent with this notion, neonatal gp91$^{\text{phox}}$-null mutant mice have a lower hypoxic ventilatory response than wild-type mice (25). Renal EPO production in response to hypoxia has not been examined in gp91$^{\text{phox}}$-null mutant mice. Thus the role of a phagocyte-like NADPH oxidase in O$_2$ sensing by critical peripheral sensors remains unclear. Furthermore, the possible involvement of NADPH oxidase components other than gp91$^{\text{phox}}$ in O$_2$ sensing has not been evaluated.

We have conducted experiments to further examine the hypothesis that the NADPH oxidase of phagocytes is the O$_2$ sensor responsible for carotid body-mediated increases in ventilation and renal EPO gene expression in response to hypoxia. Awake and unrestrained gp91$^{\text{phox}}$-null mutant and wild-type mice had identical ventilatory responses to acute hypoxia. In contrast, p47$^{\text{phox}}$-null mutant mice demonstrated increased normoxic (basal) carotid sinus nerve (CSN) activity, as well as increased hypoxic ventilation and hypoxia-evoked CSN activity, compared with wild-type mice. Wild-type and p47$^{\text{phox}}$- and gp91$^{\text{phox}}$-null mutant mice demonstrated identical EPO mRNA production in kidney after 72 h of hypobaric hypoxia. These findings suggest that the O$_2$ sensor of the carotid body may utilize the p47$^{\text{phox}}$ component of the phagocytic oxidase. However, neither gp91$^{\text{phox}}$ nor p47$^{\text{phox}}$ of the phagocytic NADPH oxidase is a necessary component of the O$_2$-sensing mechanism mediating renal EPO gene expression.

METHODS

Animals. All procedures involving animals were approved by the University of Utah and Department of Veterans Affairs Salt Lake City Health Care System Institutional Animal Care and Use Committees. The gp91$^{\text{phox}}$-null mutant mice were a gift from Mary C. Dinauer (University of Indiana) (41), and the p47$^{\text{phox}}$-null mutant mice were provided by Steve Holland (National Institutes of Health) (24). Wild-type mice were purchased from the Jackson Laboratory (Bar Harbor, ME). These mice were similar in genetic background (C57BL/6J) to both strains of null-mutant mice to control for varying degrees of hypoxic ventilatory response among different strains of mice (47). Wild-type mice were age and sex matched with comparison null mutant mice.

Hypoxic ventilatory response. Whole body plethysmography was utilized to measure the ventilatory response to hypoxia (Buxco, Troy, NY) (40). Compressed gas with a composition reflecting normoxia (21% O$_2$-balance N$_2$) or hypoxia (10% O$_2$-balance N$_2$) was applied to the plethysmograph according to the desired O$_2$ concentration in each experiment (Airgas-Intermountain, Denver, CO). The plethysmograph was operated in the flow mode, while gas was applied passively to the open pneumotachograph and reference chamber port. A bias airflow was utilized within the main chamber to prevent excessive increases in CO$_2$ concentration due to rebreathing. The mouse was placed in the main chamber for 30 min before the initiation of data collection to allow it to become familiar with its environment. Every 5 min during this first 0.5 h, mice were briefly exposed to increased flow rates of normoxic gas. This enabled the animal to become accustomed to the sound of gas at the higher flow rates used to flush the plethysmograph. After the 30-min acclimatization period, the animal was exposed to a flow rate of 8.0 l/min of normoxic gas for 35 s to flush the plethysmograph. The flow rate was then decreased to 3.0 l/min for the next 5 min, during which time data were collected. Preliminary experiments demonstrated that these flow rates were appropriate to achieve and maintain the desired O$_2$ concentration within the plethysmograph without altering the quality of data. The plethysmograph was then exposed to the hypoxic gas mixture for 5 min. Flow rates identical to those used during exposure to normoxia were again used during flushing of the plethysmograph and during data acquisition. Breathing frequency, tidal volume, and minute ventilation (V$_E$) were measured at 10-s intervals and recorded using Buxco software. Gas effluent from the main chamber (bias flow) was monitored with an in-line O$_2$ concentration analyzer (Hudson RCI, Temecula, CA) equipped with a class R-13 O$_2$ sensor (Teledyne Analytical Instruments, City of Industry, CA) to confirm that the animal was being exposed to the desired concentration of gas. All values for V$_E$ over the middle 3 min of the recording were averaged. From each averaged set of data, mean, SDs were determined, and data points that fell outside this range were eliminated. This was done to exclude transient marked increments in recorded values that were likely due to artifacts during recording. A new average was then calculated and considered to be representative of the V$_E$ that occurred over this period of time. Normoxic V$_E$ was used as a baseline from which to assess change in V$_E$ due to hypoxia. For each animal, the average V$_E$ during hypoxic gas breathing was divided by the average V$_E$ during normoxic gas breathing to obtain a percent increase in ventilation due to hypoxia. This enabled each animal to serve as its own control.

Electrophysiological recording of CSN activity. Under pentobarbital sodium anesthesia and with the aid of a dissecting microscope, the carotid bifurcations containing the carotid bodies were located and removed from wild-type and p47$^{\text{phox}}$-null mutant mice and placed in a Lucite chamber containing 100% O$_2$-equilibrated modified Tyrode solution at 0–4°C. Each carotid body, along with its attached nerve, was carefully removed from the artery and cleaned of surrounding connective tissue. The preparation was then placed in a conventional superfusion chamber, where the carotid body was continuously superfused (up to 4 h) with modified Tyrode solution maintained at 37°C and equilibrated with a selected gas mixture. Bath PO$_2$ was continuously measured with an O$_2$ electrode (model 760, Diamond General). The CSN was positioned in the tip (~75 μm ID) of a glass suction electrode for monopolar recording of chemoreceptor activity (22). The bath was grounded with a Ag-AgCl$_2$ wire. Neuronal activity was led to an alternating current-coupled preamplifier, filtered, and transferred to a window discriminator and a frequency-to-voltage converter. Signals were processed by an analog-to-digital/digital-to-analog converter for display of frequency histograms on a personal computer monitor. Basal (resting) CSN activity was established in solutions equilibrated with 100% O$_2$, which resulted in a bath PO$_2$ of ~450 Torr. The superfused preparations were stimulated in solutions equilibrated with air (~120 Torr PO$_2$). In a previous report, our laboratory demonstrated that this level of PO$_2$ is a moderate stimulus that elicits ~50% of maximal low-O$_2$-evoked CSN activity (23). Basal and stimulus-evoked CSN activities were analyzed over 100-s periods during superfusion at 450 and 120 Torr PO$_2$, respectively.

Renal EPO gene expression due to hypoxia. The gp91$^{\text{phox}}$- and p47$^{\text{phox}}$-null mutant and the respective wild-type mice were maintained in hypobarcic hypoxia (0.5 atm) or normobaric normoxia for 72 h. In Salt Lake City, UT (647 Torr
barometric pressure), this degree of hypobaria corresponds to a dry gas inspired PO2 of 63 Torr. Similar conditions of hypobaria have been found to induce maximal EPO production in mice (44). To evaluate for increased sensitivity to hypoxia, the p47phox-null mutant and wild-type mice were also exposed to an intermediate hypobaric (0.75 atm) stimulus corresponding to a dry gas inspired PO2 of 94.5 Torr for 48 h.

Once daily, the chamber was opened to allow assessment and care of the animals. Care provided to the mice under normobaric conditions did not differ from that under hypobaric conditions. At the conclusion of the experiment, mice were anesthetized with tribromethanol (10 mg ip; Aldrich Chemical, Milwaukee, WI). The abdominal cavity was opened, and the kidneys were removed. Animals were then euthanized by exsanguination. Specimens were maintained on ice until they were frozen at −80°C. Thawed kidney samples were homogenized in Tri-Reagent (Molecular Research, Cincinnati, OH) using a PowerGen 700 homogenizer (Fisher Scientific, Hampton, NH). Total RNA was prepared according to the manufacturer's protocol. Total RNA (0.6 mg) was used to synthesize cDNA with Moloney's murine leukemia virus RT and random hexamer primers; 10% of the RT product was electrophoresed on a 1.0% Trevigel 500 (Trevigen, Gaithersburg, MD) containing ethidium bromide (1.0 mg/ml). Gels were illuminated by ultraviolet light and photographed using Polaroid type 55 film, and the negatives were analyzed by densitometry. The densitometric value of the RT-PCR product using EPO primers was divided by that of the β-actin primers to correct for differences in RNA loading and subsequent RT reaction between samples. These ratios were expressed as relative densitometric units (RDU).

Statistical analysis. Values are means ± SE. A two-tailed, two-sample, equal variance Student’s t-test was used to compare results between wild-type and null mutant mice in all studies.

RESULTS

Hypoxic ventilatory response. We initially evaluated the hypoxic ventilatory responses of wild-type and null mutant mice. When exposed to the acute hypoxic stimulus (10% inspiratory O2 fraction), wild-type and gp91phox-null mutant mice increased their \( \dot{V}E \) by similar amounts: 21.0 ± 9.0 and 17.0 ± 13%, respectively (\( n = 6, P = 0.79 \); Fig. 1). Likewise, the wild-type mice that were used as controls for the p47phox-null mutant mice had similar increases in \( \dot{V}E \) in response to hypoxia: 20.0 ± 13.0% (\( n = 11 \); Fig. 1). In contrast, p47phox-null mutant mice demonstrated a marked increase in \( \dot{V}E \) in response to the acute hypoxic stimulus: 98.0 ± 18.0% (\( n = 11 \); Fig. 1). The percent change in \( \dot{V}E \) due to hypoxia was significantly greater in the p47phox-null mutant mice than in the respective wild-type mice (\( P = 0.003 \)). These findings demonstrate that there is no difference in hypoxic ventilatory response between mice that lack the gene encoding the gp91phox protein and those with the wild-type genotype. However, mice lacking the gene encoding the p47phox protein demonstrate an accentuated acute hypoxic ventilatory response.

Resting and hypoxia-evoked CSN activity. Figure 2A presents typical integrated CSN activity recorded in vitro in wild-type and p47phox-null mutant mouse preparations, along with superimposed records of bath PO2. In the p47phox-null mutant preparation, resting (basal) neural activity measured at ~450 Torr PO2 is slightly higher, and, moreover, an equal reduction of PO2 to ~120 Torr for 100 s elicits a substantially greater increase in the CSN discharge. In multiple experiments summarized in Fig. 2B, the mean basal CSN activity was 17.82 ± 1.92 (\( n = 7 \)) and 24.58 ± 1.6 impulses/s (\( n = 8 \)) in wild-type and p47phox-null mutant mouse preparations, respectively (\( P = 0.017 \)). Figure 2B also shows that hypoxia-evoked chemoreceptor activity was increased after gene deletion: average CSN activity was 72.54 ± 7.65 (\( n = 7 \)) and 109.61 ± 13.29 impulses/s (\( n = 8 \)) in wild-type and p47phox-null mutant mouse preparations, respectively (\( P = 0.026 \)). Likewise, the differences in CSN discharge between basal and hypoxic states were greater in p47phox-null mutant than in wild-type mice: 86.29 ± 11.48 (\( n = 7 \)) vs. 54.71 ± 6.57 impulses/s (\( n = 8, P = 0.039 \); Fig. 2C). These findings confirm the results of the experiments assessing hypoxic ventilation in the unanesthetized, unrestrained mice. Compared with wild-type mice, CSN activity is marginally increased in the p47phox-null mutant mice under conditions of normoxia but markedly increased by hypoxia, a finding that correlates with the elevated hypoxic ventilatory response in these animals.

Renal EPO gene expression due to hypoxia. To evaluate the role of gp91phox or p47phox in a different O2-sensing system, EPO gene expression in response to hypoxia was assessed in null mutant and wild-type mice. EPO gene expression was low or undetectable in
null mutant and the respective wild-type mice, respectively \((n = 6, P = 0.35; \text{Fig. 3B})\). An intermediate hypoxic stimulus \((0.75 \text{ atm for 48 h})\) did not induce EPO gene expression in \(p47^{\text{phox}}\)-null mutant or wild-type mice \((n = 3, \text{data not shown})\). Thus the absence of the \(gp91^{\text{phox}}\) protein does not alter renal \(O_2\) sensing and resulting EPO gene expression. Likewise, in contrast to the carotid body data, the findings demonstrate that loss of the \(p47^{\text{phox}}\) protein does not result in an increased expression of the EPO gene in response to a hypoxic stimulus.

**DISCUSSION**

There is considerable controversy regarding the identity of the \(O_2\) sensor utilized by physiological systems that regulate global tissue oxygenation. Mitochondria have been implicated in the \(O_2\)-sensing mechanisms relevant to the carotid body \((11, 12)\) and pulmonary vasculature \((29, 52)\). Functional and immunohistochemical studies have implicated the phagocytic NADPH oxidase in \(O_2\) sensing. This latter oxidase is the focus of the present investigation. Previous findings in mice lacking the gene encoding \(gp91^{\text{phox}}\) suggest that the phagocytic oxidase is not relevant to \(O_2\) sensing in the carotid body \((23, 42)\) or the pulmonary vasculature \((4)\). Our results demonstrate that absence of the \(p47^{\text{phox}}\) component of the phagocytic oxidase alters \(O_2\) sensing by the carotid body, but not hypoxia-induced renal EPO gene expression.

Chemoreception in the carotid body occurs within \(O_2\)-sensitive type I (glomus) cells, which release neurotransmitters in response to hypoxia \((14)\). When superfused with a low \(P_{O_2}\), the rat carotid body exhibits an optical absorbance spectrum that resembles the reduced spectrum of NADPH oxidase of neutrophils \((3)\). DPI, an inhibitor of NADPH oxidase \((21)\), attenuates the reduced absorbance spectrum in response to hypoxia. A \(b\)-type cytochrome capable of \(H_2O_2\) formation has been detected in rat carotid body tissue \((8)\). \(H_2O_2\) formation, as well as hypoxia-induced increases in CSN discharges, was inhibited by DPI. From these findings, it was postulated that an oxidase such as the NADPH oxidase of neutrophils acts as the \(O_2\) sensor in the carotid body. Further support for this argument was provided by the observation that antibodies raised against the neutrophil NADPH oxidase components \(p20^{\text{phox}}, gp91^{\text{phox}}, p47^{\text{phox}}, \) and \(p67^{\text{phox}}\) immunostain type I cells of guinea pig, rat, and human carotid bodies \((27)\).

Much of the evidence implicating the phagocytic NADPH oxidase as an \(O_2\) sensor has relied on the assumption that DPI functions as a specific inhibitor of the oxidase \((3, 35)\). Recent findings of Obeso and colleagues \((36)\) refute this concept. Under normoxic conditions, in vitro preparations of intact rat and rabbit carotid bodies demonstrated the dose-dependent release of radiolabeled catecholamines in response to the application of DPI. In contrast, normoxic application of the NADPH oxidase inhibitors phenylarsine oxide \((30)\) and neopterin \((26)\) did not stimulate the release of

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**Fig. 2.** A: effect of hypoxia on carotid sinus nerve (CSN) activity in wild-type (Normal) and \(p47^{\text{phox}}\)-null mutant (\(p47 \text{ KO}\)) mice. Superimposed record shows bath \(P_{O_2}\). Resting (basal) neural activity measured at \(-450 \text{ Torr} P_{O_2}\) is slightly higher, and an equal reduction of \(P_{O_2}\) to \(-120 \text{ Torr}\) for 100 s elicits a substantially greater increase in CSN discharge in \(p47^{\text{phox}}\)-null mutant preparation. Imp, impulses. B: chemoreceptor activity averaged during 100 s at 450 and 120 Torr \(P_{O_2}\) in wild-type \((n = 7)\) and \(p47^{\text{phox}}\)-null mutant mice \((n = 8)\). \(*P = 0.017\) for differences at 450 Torr. \(\ast P = 0.026\) for differences at 120 Torr. C: hypoxia-evoked CSN discharge (stimulus-basal) in wild-type \((n = 7)\) and \(p47^{\text{phox}}\)-null mutant mice \((n = 8)\). \(\ast P = 0.039\).

Kidneys from wild-type and \(gp91^{\text{phox}}\) - and \(p47^{\text{phox}}\)-null mutant mice under normoxic conditions (Fig. 3A). After 72 h of hypobaric hypoxia, kidneys from control and null mutant mice demonstrated increases in EPO mRNA. Results of RT-PCR using \(\beta\)-actin primers verify similar RNA loading and quality of RT reactions between samples. In experiments comparing \(gp91^{\text{phox}}\) -null mutant with wild-type mice, EPO mRNA levels were \(0.64 \pm 0.09\) and \(0.79 \pm 0.12\) RDU, respectively \((n = 9, P = 0.32; \text{Fig. 3B})\). EPO mRNA levels were \(0.97 \pm 0.03\) and \(0.93 \pm 0.03\) RDU in hypoxic \(p47^{\text{phox}}\) null mutant and the respective wild-type mice, respectively \((n = 6, P = 0.35; \text{Fig. 3B})\). An intermediate hypoxic stimulus \((0.75 \text{ atm for 48 h})\) did not induce EPO gene expression in \(p47^{\text{phox}}\)-null mutant or wild-type mice \((n = 3, \text{data not shown})\). Thus the absence of the \(gp91^{\text{phox}}\) protein does not alter renal \(O_2\) sensing and resulting EPO gene expression. Likewise, in contrast to the carotid body data, the findings demonstrate that loss of the \(p47^{\text{phox}}\) protein does not result in an increased expression of the EPO gene in response to a hypoxic stimulus.
catecholamines. None of these three inhibitors prevented catecholamine release in response to hypoxia. In fact, the simultaneous exposure of the carotid bodies to DPI and hypoxia produced an additive response. DPI is a nonspecific inhibitor of flavoenzymes (32, 51, 57) and, therefore, might also inhibit enzymes sharing structural properties with the phagocytic NADPH oxidase. Phenylarsine oxide binds to vicinal and neighboring thiol groups in the gp91phox component of the neutrophil NADPH oxidase (10, 30) and diminishes the affinity of the oxidase for O2 (9). The mechanism by which neopterin inhibits the NADPH oxidase of neutrophils has not been characterized. Whether phenylarsine oxide and neopterin might inhibit other oxidases is unknown. Thus one explanation for the findings of Obeso et al. is the presence of a unique NAD(P)H oxidase within the type I cells of the carotid body that is structurally similar, yet distinct, from that of phagocytes. This NAD(P)H oxidase might be sensitive to inhibition by DPI and insensitive to inhibition by phenylarsine oxide and neopterin.

Several homologs of gp91phox have been recently characterized as to cDNA sequence, pattern of tissue expression, and possible function. Nox1 (for NADPH oxidase) (28) is expressed in human colon, uterus, prostate, and rat vascular smooth muscle tissue (46). Nox4 mRNA is expressed in murine renal proximal convoluted tubule cells (16), and the corresponding protein is detected in human renal distal tubule cells (45). An NAD(P)H oxidoreductase containing cytochrome b5 and b5 reductase domains cloned from Hep 3B cells has also been described and proposed as an O2 sensor (58). Similar to gp91phox, these homologs possess binding sites for heme, flavin, and NAD(P)H, features that parallel the function of phagocytic NADPH oxidases. Their existence raises the possibility that a protein structurally related to gp91phox could support a similar function in a nonphagocytic NAD(P)H oxidase in the carotid body.

The disparate findings between gp91phox- and p47phox-null mutant mice in the hypoxic ventilatory and CSN studies could be consistent with the participation of a nonphagocytic NAD(P)H oxidase in sensing O2 by type I cells in the carotid body. Rather than being identical to the phagocytic oxidase, this NAD(P)H oxidase might utilize some components of the phagocytic oxidase (p47phox), but not others (gp91phox). According to the model proposed by Acker (1), O2 sensors produce ROS relative to the surrounding Po2. Thus, as Po2 is lowered, decreased ROS production alters the redox state of type I cells, resulting in K+ channel closure and depolarization. Deletion of any component of this oxidase might result in a diminished production of ROS under conditions of normoxia. This incomplete oxidase would produce even less ROS during hypoxia, with resulting enhanced K+ channel closure and type I cell depolarization at a given Po2. Alternatively, ROS production may play a modulatory or regulatory role in type I cells, whereby the redox state influences O2 chemotransduction via indirect or nonspecific mechanisms.

Activation of the phagocytic NADPH oxidase is accompanied by phosphorylation of multiple serines within p47phox (13) by a protein kinase C (PKC)-mediated process (48). There is evidence that a similar activation process occurs in O2 sensing. Cells of the small cell lung carcinoma line H-146 are thought to represent immortal cells from NEB and possess a specific component of the whole cell K+ current that is sensitive to hypoxia and influences membrane potential (38, 39). A PKC-activating phorbol ester suppressed the ability of hypoxia to inhibit K+ currents
(37), suggesting that PKC modulates the affinity of NAD(P)H oxidase for O₂ by phosphorylation of the p47^phox component. Likewise, NEB cells have been shown to increase H₂O₂ levels after PKC activation (49). Absence of p47^phox might result in the opposite effect: diminished affinity of the NAD(P)H oxidase for O₂. Such a theory is consistent with what we observed in the p47^phox-null mutant mice. CSN activity of these animals was elevated at baseline, with a subsequent marked increase in nerve activity and ventilation noted during hypoxia. These findings may represent a dysfunctional carotid body O₂ sensor that is producing diminished ROS at baseline. With hypoxia, the production of ROS decreases more precipitously, manifesting as increased CSN discharges and ventilation.

One limitation of this study is the use of mice obtained from an outside colony as controls (“wild-type”) for the null mutant mice. Interstrain variations in ventilatory responses to hypoxia have been demonstrated (47). Although all mice used in these experiments were derived from the same background strain (C57BL/6J), it is possible that unrecognized traits comigrating with the deleted p47^phox gene could contribute to differences observed between wild-type and p47^phox-null mutant mice. However, given the substantial increase in hypoxic ventilation and CSN activity observed in the p47^phox-null mutant mice compared with the wild-type mice, deletion of the p47^phox gene would seem the most likely cause of the observed phenotype.

Deletion of genes encoding the gp91^phox and p47^phox components of the phagocytic NADPH oxidase had no impact on EPO gene expression in response to a hypobaric hypoxic stimulus. Previous studies suggested that the phagocytic NADPH oxidase might function as an O₂ sensor in renal tissue. Antibodies raised against p22^phox stain renal parenchymal cells possessing EPO mRNA, suggesting at least a proximate relationship between this NADPH oxidase component and EPO production within the kidney (6). When exposed to low PO₂, cells from a human hepatoma line (Hep 3B) produce EPO mRNA and protein by a mechanism sensitive to the application of carbon monoxide as well as heme synthesis inhibitors. These findings imply the involvement of a heme protein in this mechanism of O₂ sensing (19). EPO gene expression in response to hypoxia has been reported to be inhibited by DPI (18); however, this has not been a consistent finding (20).

We have found that, under conditions of hypobaric hypoxia, gp91^phox, and p47^phox-null mutant mice and their respective wild-type mice express the EPO gene to the same extent. In response to an intermediate hypoxic stimulus (0.75 atm for 48 h), the wild-type and p47^phox-null mutant mice demonstrated a lack of EPO gene expression. Thus, in contrast to the acute hypoxic ventilatory response, absence of the p47^phox protein does not impart the EPO-producing cells of the kidney with an increased responsiveness to hypoxia. This may reflect differences in O₂-sensing mechanisms mediating type I cell depolarization vs. EPO gene expression in response to hypoxia. EPO production is regulated at the level of gene transcription by the heterodimeric complex hypoxia inducible factor-1 (7, 50), as are vascular endothelial growth factor (31, 33) and aldolase (43). Previous work using B cell lines derived from patients suffering from the genetic absence of gp91^phox or p22^phox demonstrated normal hypoxia-induced gene expression of vascular endothelial growth factor and aldolase (54), findings that are in agreement with normal levels of EPO gene expression in response to a hypoxic challenge in gp91^phox- and p47^phox-deficient animals. Thus neither gp91^phox nor p47^phox of the phagocytic NADPH oxidase are relevant to the O₂-sensing mechanism mediating EPO gene expression within the kidney.

In contrast, the loss of K⁺ current sensitivity to hypoxia and DPI in NEB cells from gp91^phox-null mutant mice (15) suggests that an NAD(P)H oxidase and ROS may be involved in O₂ sensing via regulation of membrane potential. The increased hypoxia-evoked CSN activity demonstrated in p47^phox-null mutant mice is likewise consistent with altered regulation of membrane properties in response to changes in ambient PO₂. However, further detailed studies of type I cell membrane currents in these animals are required to elucidate the role of the p47^phox protein and ROS production in carotid body chemotransduction.

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