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The role of NADPH oxidase in carotid body arterial chemoreceptors

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Abstract

O₂-sensing in the carotid body occurs in neuroectoderm-derived type I glomus cells where hypoxia elicits a complex chemotransduction cascade involving membrane depolarization, Ca²⁺ entry and the release of excitatory neurotransmitters. Efforts to understand the exquisite O₂-sensitivity of these cells currently focus on the coupling between local P_{O₂} and the open-closed state of K⁺-channels. Amongst multiple competing hypotheses is the notion that K⁺-channel activity is mediated by a phagocytic-like multisubunit enzyme, NADPH oxidase, which produces reactive oxygen species (ROS) in proportion to the prevailing P_{O₂}. In O₂-sensitive cells of lung neuroepithelial bodies (NEB), multiple studies confirm that ROS levels decrease in hypoxia, and that E_M and K⁺-channel activity are indeed controlled by ROS produced by NADPH oxidase. However, recent studies in our laboratories suggest that ROS generated by a non-phagocyte isoform of the oxidase are important contributors to chemotransduction, but that their role in type I cells differs fundamentally from the mechanism utilized by NEB chemoreceptors. Data indicate that in response to hypoxia, NADPH oxidase activity is increased in type I cells, and further, that increased ROS levels generated in response to low-O₂ facilitate cell repolarization via specific subsets of K⁺-channels.

1. Introduction

The survival of multicellular aerobic organisms is dependent on a continuous supply of molecular oxygen. O₂ is consumed in enzymatically catalyzed electron transfer reactions which occur in cell membranes, at multiple sites throughout the cell, and within particular organelles, such as mitochondria. Much of the O₂ used in these reactions is completely reduced via the transfer of 4 electrons to form 2 molecules of H₂O. However, important amounts of O₂ can also be partially reduced, by single electron reduction to form the highly reactive metabolite, superoxide anion (O₂⁻). O₂⁻ is converted to hydrogen peroxide (H₂O₂) by an isozyme of superoxide dismutase (SOD) either in the cytosolic compartment (ZnCuSOD), or in mitochondria (MnSOD). In the presence of transition metal ions, O₂⁻ can also undergo

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conversion to the highly reactive hydroxyl radical (OH^\cdot) (Thannickal et al., 2000). These partially reduced metabolites of O_2 have been designated “reactive oxygen species” (ROS) because they are variously more reactive than molecular O_2 . High levels of ROS have traditionally been regarded as toxic (oxidative stress), and their numerous potentially damaging effects collectively constitute the basis for one of the most popular theories of aging. However, recent investigations have also demonstrated that low concentrations of ROS, produced locally and acting on nearby effectors, are important signaling molecules (Thannickal et al., 2000; Sauer et al., 2001; Griendling et al., 2000; Lambeth, 2002; Finkel, 2000). Thus, an emerging body of data indicate that specific extracellular stimuli including cytokines, neurotransmitters and hypoxia initiate signaling cascades involving the production of ROS from mitochondria and non-phagocyte forms of NADPH oxidase (Chandel et al., 2000b; Thannickal et al., 2000). Like cyclic nucleotides (cAMP, cGMP), inositol phosphates (IP_N) and nitric oxide (NO), ROS affect specific targets within local cellular compartments (Pani et al., 2001), frequently involving select cysteine and methionine residues which are highly labile for oxidation/reduction (Thannickal et al., 2000; Xu et al., 2002). Changes in the redox state at these sites has been demonstrated to critically alter important effector molecules such as ion channels (K^+ -channels (Hoshi and Heinemann, 2001; Tang et al., 2001)), protein kinases (Thannickal et al., 2000) and transcription factors (HIF-1 α , NF- κ B, AP-1 (Thannickal et al., 2000; Görlach et al., 2001; Zhu et al., 2002; Chandel et al., 2000b)).

1.1 ROS formation in mitochondria

Mitochondrial cytochrome oxidase catalyzes the 4-electron reduction of O_2 in the final step of an elaborate electron transport chain which captures metabolic energy in the terminal phosphate bond of ATP. In addition, 1–3% of the O_2 consumed by mitochondria is partially reduced to O_2^\cdot by enzyme-complex I, the ubisemiquinone site of complex III, and other electron transfer proteins (Chandel et al., 2000b; Thannickal et al., 2000). Extended periods of hypoxia (1–2 hr) may depress the V_{max} of cytochrome oxidase, and produce a shift in the redox state within mitochondria (i.e., increased levels of NADH), thereby promoting the formation of O_2^\cdot (Chandel et al., 2000b). Although its negative charge prevents this anion from crossing mitochondrial membranes, it is rapidly converted by MnSOD to highly membrane permeant H_2O_2 (Sauer et al., 2001), which appears to mediate apoptotic signaling and cell proliferation (Irani, 2000). Mitochondria have also been implicated as cellular O_2 sensors in erythropoietin (Epo)-producing Hep3B cells and pulmonary artery smooth muscle cells. Mitochondrial ROS production evoked by hypoxia has been correlated with modulation of K^+ -channels and vasoconstriction (hypoxic pulmonary vasoconstriction, HPV) (Michelakis et al., 2002; Chandel and Schumacker, 2000b; Waypa et al., 2001). However, the mechanism by which mitochondrial ROS are coupled to contractile elements has not been elucidated, and the role of cell redox in HPV is a point of considerable controversy (Sylvester, 2001).

1.2 ROS formation by NADPH oxidase

For many years it has been known that large quantities of ROS are produced by phagocyte cells as part of an extracellular killing mechanism activated in response to invading microorganisms. ROS-generating phagocyte NADPH oxidase is a complex enzyme comprised of two trans-membrane and four cytosolic subunits (Figure 1). The large 91 kD glycoprotein (gp91^{phox}; phox: phagocyte oxidase) and a 22 kD protein (p22^{phox}) form a membrane bound cytochrome b558 (Sauer et al., 2001; Diebold et al., 2001; Babior, 1999). Immunologic stimulation initiates a protein kinase C (PKC)-dependent process in which the cytosolic subunits p67^{phox}, p40^{phox}, p47^{phox} and a small GTPase (Rac-1 or Rac-2), unite at the membrane to form the active enzyme. An electron is then transferred from NADPH (produced in the hexose monophosphate shunt pathway [HMS] of glucose metabolism) to O_2 , thus forming O_2^\cdot plus NADP^+ and H^+ (Babior, 1999; Diebold et al., 2001). Despite advances in demonstrating the importance of ROS in cell signaling, the role of the phagocyte oxidase is

perplexing because it is inactive in resting cells, and produces O_2^- primarily on the extracellular side of the plasma membrane (Diebold et al., 2001), whose location is thus entirely inconsistent with the action of an intracellular messenger. However, recent studies of non-phagocyte cells have shown that NADPH oxidase may also be constitutively active, and produce ROS intracellularly at low levels (Brar et al., 2002b; Brar et al., 2002a; Li et al., 2002; Szöcs et al., 2002; Görlach et al., 2001). For example, vascular endothelial cells contain an NADPH oxidase in which cytochrome b558 and the cytosolic subunits are preassembled on cytoskeletal elements in the perinuclear region, where they are engaged in constitutive production of intracellular ROS (Li et al., 2002). Moreover, recent studies in the lung have documented the involvement of ROS generated by NADPH oxidase in chemosensory cells of neuroepithelial bodies (NEB) which respond to hypoxia by inhibition of a voltage-dependent K^+ -current. Furthermore, K^+ -channels in these cells are activated by low concentrations of H_2O_2 , and hypoxia-evoked depression of channel activity is occluded in the presence of oxidase inhibitors, suggesting that ROS production is a key factor which couples local P_{O_2} to cell activity (Wang et al., 1996). Importantly, the depolarizing response is absent in null-mutant mice lacking gp91^{phox} (Wang et al., 1996; O'Kelly et al., 2000; Fu et al., 2000a)

Homologs of gp91^{phox} (Figure 1) have been cloned and sequenced from a variety of cell types, suggesting that there are multiple forms of the enzyme which are adapted to function in specific signaling pathways (Cheng et al., 2001; Lambeth, 2002). Some of these alternative forms of NADPH oxidase (Nox1–5, plus Duox1 and Duox2) have likewise been proposed as O_2 -sensors which produce ROS in proportion to local levels of P_{O_2} (Acker, 1994; Porwol et al., 2001). ROS generated by Nox homologs may also mediate cell proliferation and expression of transcription factors in multiple cell types (e.g., airway smooth muscle, melanoma cells, vascular endothelial cells (Brar et al., 2002b; Brar et al., 2002a; Irani, 2000)) in response to a variety of physical (e.g., shear stress) and chemical (e.g., cytokines, neurotransmitters) stimuli (Griendling et al., 2000; Irani, 2000). Chemosensory type I cells in the carotid body, like NEB cells in the lung, are O_2 -sensors which respond to acute hypoxia (AH) by depolarization, Ca^{2+} entry, the release neuroactive agents, and activation of sensory nerves. ROS have been proposed as intracellular mediators of chemotransduction events in type I cells, coupling moment-to-moment cellular activity to the prevailing P_{O_2} (Acker, 1994), but efforts to determine the precise mode(s) of involvement of Nox in ROS production have resulted in conflicting views (Obeso et al., 1999).

2. Oxygen Sensing in the Carotid Body and the Potential Involvement of ROS

Exposure of the carotid body to AH elicits increased neural activity in the carotid sinus nerve (CSN), and reflex cardio-pulmonary adjustments which mitigate the adverse effects of hypoxia. Increased carotid body activity occurs at low to moderate arterial P_{O_2} levels, in contrast to the severe hypoxia required to elicit metabolic and functional adjustments in non- O_2 sensing tissues (Fidone et al., 1997; Fidone et al., 1995; Gonzalez et al., 1994). Neuroectodermal type I cells are responsible for this exquisite sensitivity, and contemporary efforts to understand their unique hypoxic chemotransduction mechanisms have focused primarily on the relationship between P_{O_2} and the activity of K^+ -channels (Gonzalez et al., 1994; Peers et al., 1995; Lopez-Lopez et al., 1989; Lopez-Barneo, 1994; Lopez-Barneo et al., 2001). In the rat, studies of type I cells have demonstrated that hypoxia inhibits two types of K^+ -channel: 1) a large-conductance, voltage- and calcium-dependent channel (maxiK (Peers, 1990; Wyatt et al., 1995)), and 2) a voltage-independent TASK-type channel which mediates a background leak current (Buckler, 1997; Buckler et al., 2000; Buckler et al., 2006). Available data indicate that TASK-like channels play a major role in setting the E_m , and they strongly suggest that closure of these channels initiates cell depolarization (Donnelly, 1997; Buckler, 1999). The properties of maxiK, however, are not consistent with the initiation of cell activity evoked by hypoxia because their current-voltage characteristics suggest that they do not open until the membrane

potential is more positive than -20 mV. Because of these properties, and the fact that specific maxiK blockers fail to activate type I cells, some authors conclude that maxiK channels modulate cell activity and facilitate repolarization of the membrane (Prabhakar, 2000; Buckler, 1999; Donnelly, 1997). In this view, hypoxic inhibition of the maxiK current would contribute to cell activity by delaying recovery of the cell to the resting state.

There is general agreement that the K^+ -channels which are also expressed in non- O_2 sensing tissues are not inherently sensitive to molecular oxygen and must somehow be coupled to responsive cellular components modulated by the prevailing P_{O_2} in type I cells (Patel et al., 2001). Foremost amongst the differing points of view regarding O_2 -sensing are that either an O_2 -binding heme-protein in the plasma membrane (Lopez-Lopez et al., 1992), or specialized O_2 -sensitive cytoplasmic components (i.e., mitochondria or Nox (Acker, 1994; Wilson et al., 1994; Buckler et al., 2006)), modulate the activity of K^+ -channels. Importantly, a recent review by Prabhakar pointed out that these different mechanisms are not necessarily mutually exclusive, and that chemoreceptor cells may integrate signals from multiple O_2 -sensors, which collectively determine K^+ -channel activity (Prabhakar, 2000). This concept is particularly attractive because it also embodies the notion that hypoxia targets multiple cellular components, and that the response depends on both the severity and duration of the stimulus.

3. Diverse Views of Nox Function in Carotid Body

The Nox hypothesis of O_2 -sensing (figure 2) in type I cells suggests that a phagocyte Nox2 or other isoform couples P_{O_2} to K^+ -channel activity by generating ROS in proportion to available O_2 (Acker, 1994). Accordingly, H_2O_2 derived from O_2^- by the action of SOD would facilitate K^+ -channel activity either by direct action on channel protein or via a shift in the cell redox balance. Indeed, application of low concentrations of H_2O_2 to the *in vitro* rat carotid body/CSN preparation has been shown to depress chemoreceptor nerve activity (Acker et al., 1992), and a non-specific inhibitor of the oxidase, diphenyleneiodonium (DPI), alters nerve activity evoked by hypoxia (Cross et al., 1990). Furthermore, certain subunits common to the phagocyte and non-phagocyte forms of the enzyme, including p22^{phox}, gp91^{phox} (or a closely related Nox isoform), p47^{phox}, and p67^{phox}, have been localized to type I cells by immunocytochemical staining (Kummer et al., 1995). The spectral absorption studies of Acker and his colleagues have enhanced the Nox hypothesis by showing that hypoxia reduces an isoform of cytochrome b558 (Lahiri et al., 1999). Also, a specific Nox inhibitor, 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF), was shown to first excite carotid sinus nerve activity, and then block the chemoreceptor response to hypoxia (Lahiri et al., 1999).

Opposing these findings are multiple observations which question the validity of the NADPH oxidase hypothesis of chemotransduction. First, studies in the rat have shown that the heme-binding molecule, carbon monoxide (CO), activates K^+ -channels, and blocks the inhibitory effects of hypoxia, suggesting that a heme-protein physically associated with channel protein mediates the effects of hypoxia on channel activity (Lopez-Lopez et al., 1992). Second, although early studies demonstrated that maxiK and TASK-like channels fail to respond to hypoxia following excision of membrane patches from type I cells (Wyatt et al., 1995; Buckler, 1999), more recent studies have obtained opposite results indicating that cytosolic factors (i.e., subunits of Nox) are not required for low- O_2 inhibition of the K^+ -current (Riesco-Fagundo et al., 2001). Third, studies by Gonzalez and his colleagues showed that inhibitors of Nox (neopterin and arsine oxide) did not promote catecholamine (CA) release from the normoxic carotid body, nor block hypoxia-evoked CA release, indicating that O_2 -sensing in type I cells is not dependent on Nox function (Obeso et al., 1999). Fourth, although physiological hypoxia increases NAD(P)H levels in type I cell mitochondria (Duchen et al., 1992), it does not change the primary factor controlling the cytosolic redox state, namely, the reduced-to-oxidized glutathione (GSH/GSSH) ratio; moreover, pharmacological manipulation of the redox

environment is not correlated with chemoreceptor excitation (Gonzalez et al., 2004; Gonzalez et al., 2002). Finally, experiments using genetically modified mice lacking the gp91^{phox} subunit (targeted gene deletion: knockout, KO) demonstrated normal inhibition of I_K by hypoxia in type I cells, as well as normal hypoxia-evoked Ca²⁺-responses and CSN activity (He et al., 2002; Roy et al., 2000). On the other hand, mice lacking p47^{phox} displayed an enhanced electrophysiological chemoreceptor response to hypoxia (Sanders et al., 2002). Thus, inhibiting Nox function via p47^{phox} gene KO resulted in an outcome (enhanced CSN response) which is diametrically opposed to findings obtained with the Nox inhibitor AEBSF (absence of CSN response), albeit in different species. On the other hand, the finding that chemosensitivity is elevated in the absence of the cytosolic subunit, p47^{phox}, but remains unaltered following KO of gp91^{phox} indicates that type I cells express a non-phagocyte isoform of Nox.

4. Chemoreceptor Function in Normal and Nox-Gene-Deleted Cells

These controversial issues led us to focus further studies on whether the enhanced chemoreceptor discharge following p47^{phox} gene-deletion reflected an underlying negative modulation of type I cells by Nox-generated ROS. In patch-clamp experiments greater than 70% of the voltage-dependent current in mouse type I cells was sensitive to iberiotoxin, consistent with the presence of large conductance maxiK channels (He et al., 2005). Moreover, we found that application of H₂O₂ decreased hypoxic inhibition of voltage-dependent K⁺-currents in normal mouse type I cells (figure 3), a result that agreed with previous studies of rat NEB cells and their analog, the H146 cell-line (O'Kelly et al., 2000). In accord with Nox-mediated negative modulation, hypoxia-evoked depression of the maxiK-like current was greater in the p47^{phox} KO model, and the Nox inhibitor, AEBSF, enhanced hypoxia-induced current depression in normal, but not KO type I cells (fig. 3). Studies of Ca²⁺-levels in type I cells further showed that basal and hypoxia-evoked [Ca²⁺]_i was higher in Nox gene-deleted cells, and that AEBSF enhanced Ca²⁺-responses in normal, but not KO type I cells (He et al., 2005) (figure 4). In new experiments we have found that AEBSF enhances hypoxia-evoked CSN activity in the rat carotid body (figure 5), a result which conflicts with earlier studies (Lahiri et al., 1999). Collectively, these recent findings strongly suggest that ROS generated by Nox enhance maxiK-like currents, and promote cell repolarization. The data further confirm that hypoxia-induced depression of the voltage-sensitive K⁺-current occurs independently of Nox function.

A hypothetical scheme has emerged in which ROS levels increase in hypoxia, in a mechanism which opposes the excitatory effects of low-O₂. Testing this idea required direct evaluation of the effects of hypoxia on ROS generation in type I cells. Experimental conditions for measuring ROS were established using mouse polymorphonuclear (PMN) cells loaded with the dye, dihydroethidium (DHE), which is oxidized by O₂⁻ to fluorescent ethidium. Normal mouse PMN displayed a rapid increase in fluorescence when stimulated with a cocktail consisting of the chemotactic peptide *N*-formyl-Met-Leu-Phe (fMLP; 1 μM), arachidonic acid (AA; 10 μM), and cytochalasin B (CB; 5 μg/ml). This response was blocked by the Nox inhibitor diphenyleneiodonium (DPI, 10 μM) (He et al., 2005) (figure 6). The mitochondrial uncoupler, azide (5 μM), also elicited a substantial increase in cell fluorescence. PMN from p47^{phox} KO mice did not respond to the cocktail, but did fluoresce like normal cells when treated with azide. The metabolic uncoupler also elicited a large increase in fluorescence in normal and p47^{phox} KO type I cells, but when stimulated by hypoxia, only normal type I cells generated an AEBSF-sensitive response, indicating increased production of O₂⁻ originating from Nox (He et al., 2005) (figure 7). We have since reported similar results in rat carotid body lending further support to the hypothesis that low levels of O₂ activate a unique form of Nox in type I cells (He et al., 2006). Because hypoxia has been shown to lower ROS levels in the H146 cell-line, in accord with a primary role for Nox in O₂-sensing (O'Kelly et al., 2000), the data strongly

indicate that hypoxia triggers opposite effects with respect to Nox activation in carotid body versus airway chemoreceptors.

The finding that ROS generation increases as O₂ levels decrease may appear as overtly counterintuitive to fundamental concepts of enzyme-substrate interactions. However, the affinity of the type I cell Nox isoform for O₂ is unknown, and the reported mean K_m for O₂ of phagocyte Nox is P_{O₂}=12.7 Torr (Cross et al., 1986), about half of P_{O₂} used to stimulate ROS production (He et al., 2005). Thus in physiological hypoxia, O₂ availability may not limit Nox activity. In fact, the phagocyte Nox is adapted to function in naturally hypoxic conditions, including wounds. On the other hand, a potentially relevant factor for Nox activation is the cellular concentration of enzyme cofactor, NADPH. In a previous study we demonstrated that hypoxia increases ouabain-sensitive glucose uptake in carotid body type I cells (Obeso et al., 1993). This finding is consonant with the hypothesis that hypoxia-evoked cell depolarization activates Na⁺/K⁺-ATPase, thereby triggering metabolic flux of glucose through the HMS. The resulting increase of cofactor could thus be responsible for activation of Nox after the initial hypoxia-induced depolarization. In preliminary experiments, we have found support for this scheme by showing that inhibitors of the HMS (epiandrosterone, 6-aminonicotinamide) block hypoxia-induced ROS production in rat type I cells, and enhance hypoxia-evoked CSN activity.

5. Summary: Adaptation of Nox Isoforms in Airway versus Arterial Chemoreceptor Cells

Nox isoforms are present in airway (NEB) and arterial (carotid body) chemoreceptors where they subserve diverse signaling functions (Kummer et al., 1995; Youngson et al., 1997). In NEB, Nox appears to be a primary O₂-sensor which produces ROS in proportion to local P_{O₂}. K⁺-currents are activated by H₂O₂, and pharmacological inhibition of Nox partially blocks low-O₂ depression of currents (O'Kelly et al., 2000; O'Kelly et al., 2001; Fu et al., 2000b). Gene-deletion of the phagocyte gp91^{phox} subunit (Nox2), inhibits responses to low-O₂ (Fu et al., 2000a). Finally, detailed studies of the NEB analog cell-line (H146) suggest that Nox-mediated O₂-sensing may be only one of multiple mechanisms detecting O₂ in airway chemoreceptor cells (O'Kelly et al., 2001).

In arterial chemoreceptor type I cells, the available data indicate that primary O₂-sensing occurs via a membrane-delimited (but also see Buckler, 1999; Wyatt et al., 1995) detector which responds to a decrease in P_{O₂} by closure of K⁺-channels (Riesco-Fagundo et al., 2001). Cell depolarization activates voltage-dependent Ca²⁺-channels and exocytotic release of neurotransmitters. Unlike NEB cells, functional Nox is not necessary for the full expression of O₂-sensing in type I cells. Nox gene-deletion (p47^{phox}) in fact enhances chemoreceptor sensitivity, consistent with a modulatory role for ROS. This conclusion is supported by studies of voltage-sensitive K⁺-currents, cell Ca²⁺-responses, and measurement of ROS production in type I cells. Data from the normal versus p47^{phox} KO mice, and rats, uniformly indicate that hypoxia activates Nox-dependent ROS production, and that ROS increase voltage-dependent K⁺-currents, thereby promoting cell repolarization. Importantly, the primary O₂-sensing machinery in type I cells appears to be insensitive to the cytosolic redox state. Moreover, hypoxia does not alter the cytosolic GSH/GSSH redox potential in carotid body, suggesting that changes in the redox status are confined to microdomains closely associated with target molecules (Gonzalez et al., 2002; Gonzalez et al., 2004). Consistent with this conclusion is a recent report showing that co-expression of TASK-1 K⁺-channels and Nox4 in HEK293 cells results in association in the plasma membrane (Lee et al., 2006).

6. Perspectives and Future Directions

The Nox model of O₂-sensing as originally formulated by Acker and his colleagues (Acker et al., 1989; Cross et al., 1990) stimulated numerous studies which elucidated novel mechanisms involving ROS in cellular and systemic responses to hypoxia (Acker, 2005). Compelling evidence for Nox-mediated O₂-sensing has been obtained in NEB, and ROS have been implicated in regulating ion channel activity and gene expression in multiple cells and tissues (Acker, 2005). In the carotid body available evidence suggests that Nox fills a unique supporting role as a modulator of type I cell activity. However, several issues remain which require further investigation for complete understanding of Nox involvement in arterial chemoreception. Of paramount importance is the identification of the Nox isoform expressed in type I cells, and detailed examination of the role of NADPH cofactor in regulating ROS production. To this end, it will be important to examine the relationship between glucose uptake and cofactor production in the HMS. It is encouraging that recent studies of pulmonary versus coronary vascular smooth muscle have shown that inherently different levels of ROS production by Nox4 are correlated with cellular concentrations of NADPH (Gupte et al., 2005).

Although our data strongly support a role for ROS in the enhancement of maxiK currents in type I cells, virtually nothing is known about the influence of ROS on TASK-like channel activity in carotid body. This is particularly important in view of the fact that these O₂-sensitive channels appear to control type I cell E_M via a background leak current (Buckler, 1999; Buckler et al., 2000). Importantly, a study of TASK-1 channels co-expressed in HEK293 cells along with Nox4 showed that background K⁺-current was correlated with Nox activity (Lee et al., 2006). Thus, hypoxia-initiated ROS production may promote type I cell recovery via both maxiK and TASK-like channels. Although not fully understood, recent findings indicate that hypoxia inhibits TASK-1 and TASK-3 in type I cells via changes in mitochondrial energy metabolism, perhaps involving cytosolic concentrations of ATP (Buckler et al., 2006).

A final important consideration is the role of Nox in adaptation of the carotid body during chronic exposure to hypoxia. It has been repeatedly demonstrated that chronic hypoxia (CH) elicits remarkable structural changes and chemosensory hypersensitivity in mammalian carotid body (Dinger et al., 2003). Moreover, systemic hypoxia is known to increase levels of ROS in tissues, consistent with increased O₂⁻ production in mitochondria (Chandel et al., 2000a) and/or via enhanced Nox activity. The finding that type I cell activity in acute hypoxia is routinely modulated by ROS suggests the possibility that Nox acts as an important regulatory mechanism which could limit CH-induced hyperexcitability. In contrast, a different paradigm of prolonged exposure, namely, chronic intermittent hypoxia (CIH), has been shown to elicit chemoreceptor hypersensitivity involving cellular mechanisms which differ fundamentally from CH (Peng et al., 2004). Interestingly, Prabhakar and his colleagues have demonstrated that ROS scavengers prevent the CIH-induced increase in chemosensitivity (Peng et al., 2003). Future studies may determine that diverse mechanisms involving Nox are critical for resetting chemosensitivity in carotid body following CH versus CIH.

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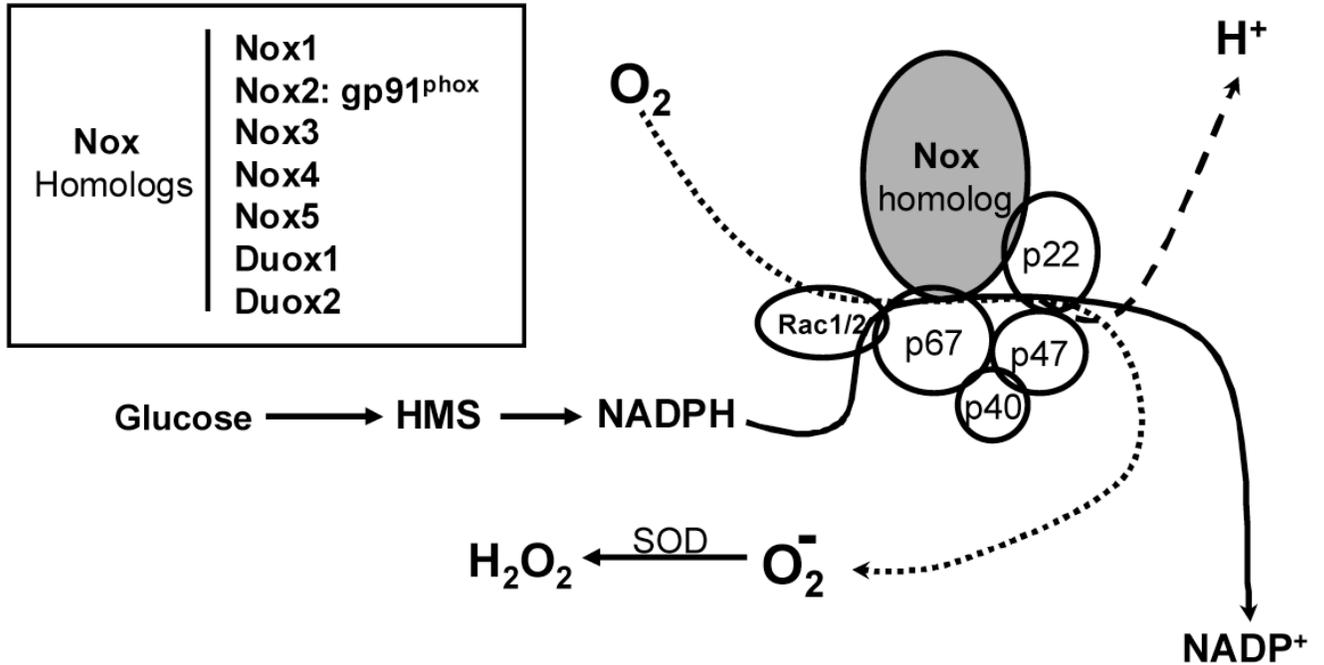


Figure 1.

Composition and function of NADPH oxidase (Nox). A heme-containing Nox-homolog plus p22^{phox} form a cytochrome b558 which in the phagocytic enzyme is embedded in plasma membrane. Cytosolic components [p67^{phox}, p47^{phox}, p40^{phox}, plus a GTPase (Rac 1 or 2)] translocate in a protein kinase-dependent process to form the functional enzyme. The cofactor, NADPH, is formed in the hexose monophosphate shunt (HMS) pathway of glucose metabolism. Superoxide anion, O₂⁻, is converted to hydrogen peroxide (H₂O₂) by superoxide dismutase (SOD). Inset indicates various superoxide-generating Nox homologs (Nox 1-5) which may be associated with membranes, organelles or cytoskeletal components. Duox1 and Duox2 function as hydrogen ion transporters.

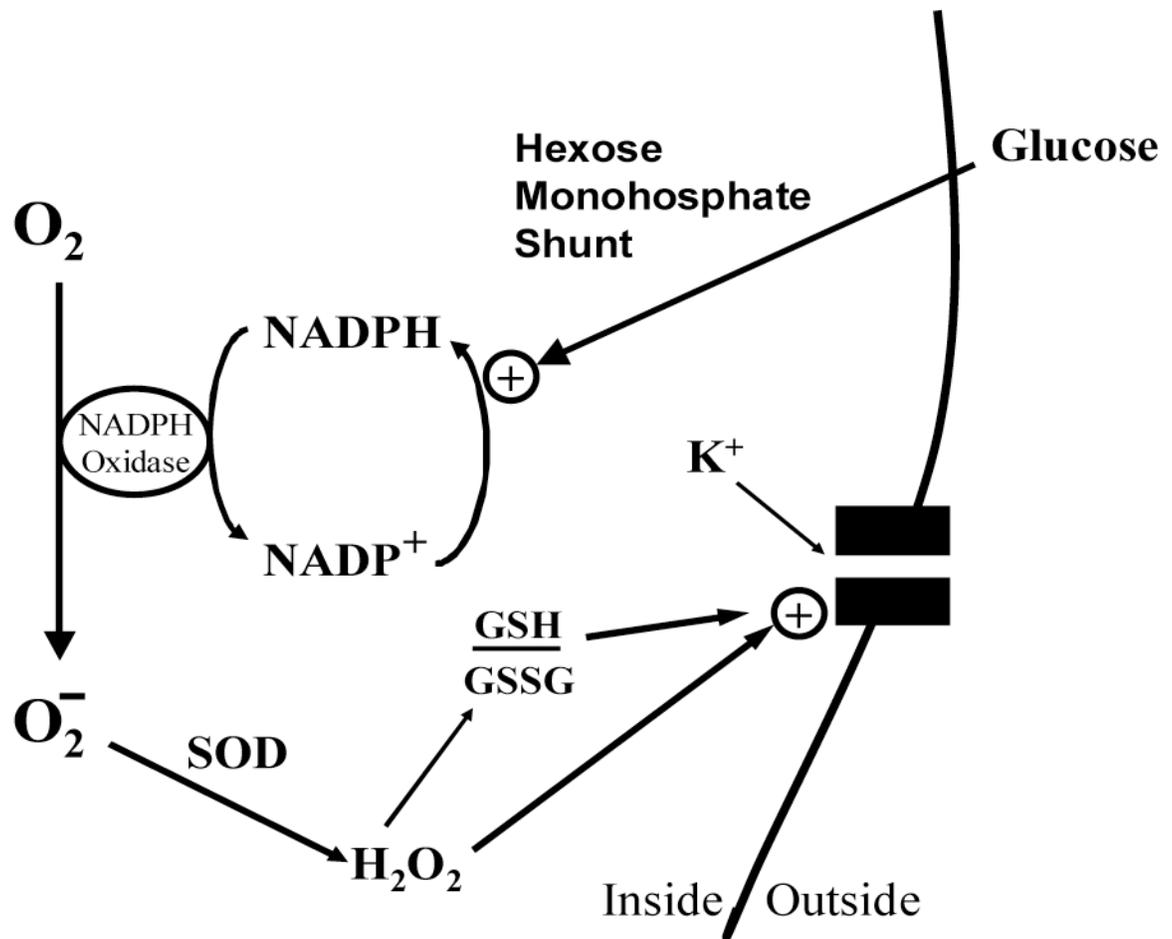


Figure 2. Nox function in O₂-sensitive chemoreceptor cells. Diagram depicts activation of K⁺-channel by H₂O₂, and/or shift in ratio of reduced (GSH) versus oxidized (GSSG) glutathione (i.e., cellular redox potential). The rate of superoxide generation may depend on the concentrations of molecular O₂, and/or NADPH cofactor produced by glucose flux through the hexose monophosphate shunt. SOD: superoxide dismutase.

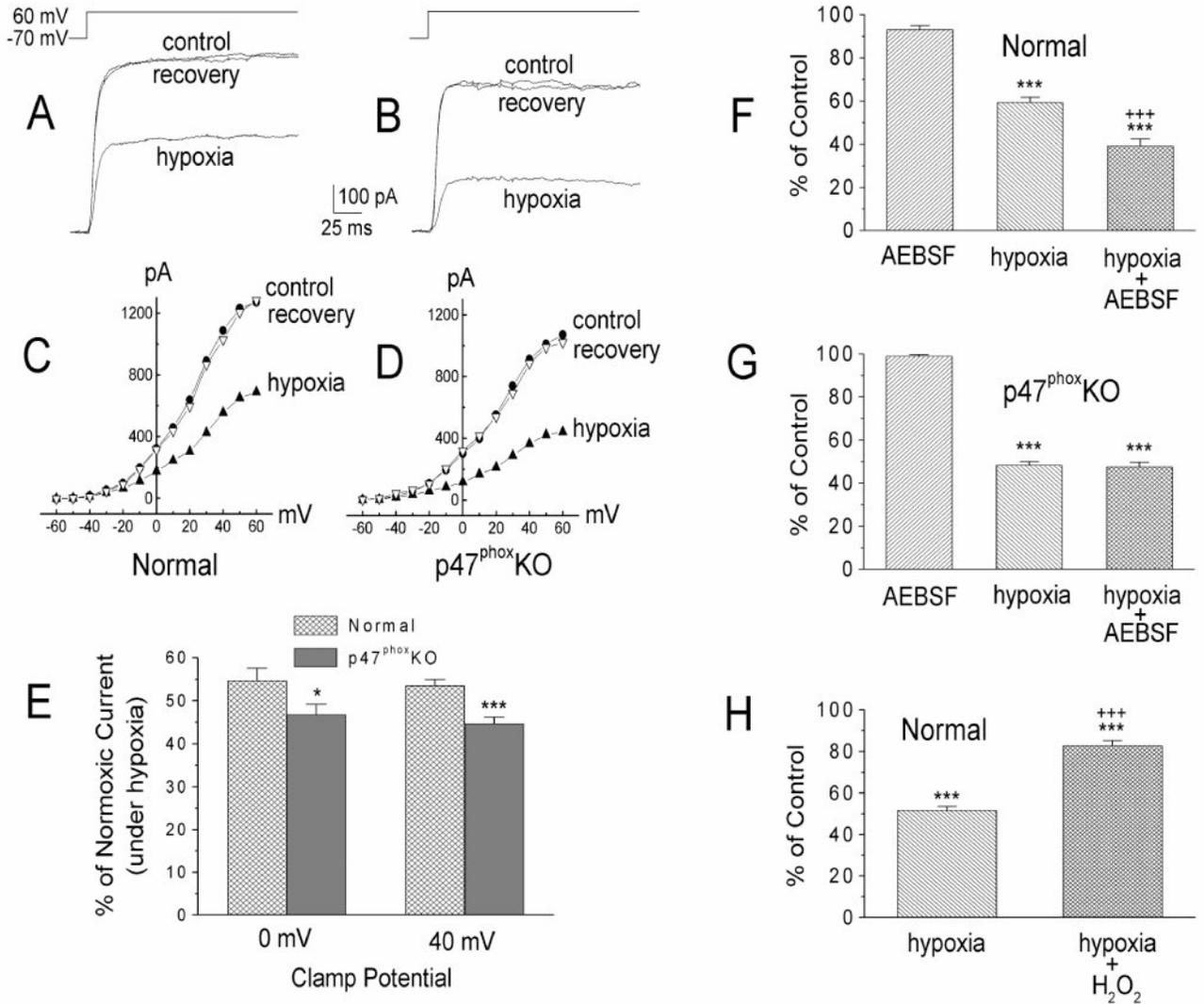


Figure 3.

A and *C*: sample records (*A*) and current-voltage curves (*C*) obtained in a normal type I cell in normoxia, hypoxia, and again in normoxia (recovery). *B* and *D*: sample records (*B*) and current-voltage curves (*D*) obtained in a cell from a p47^{phox}KO mouse. *E*: mean hypoxic depression of the normoxic voltage-dependent K⁺ current (I_K) at membrane potentials (E_m) of 0 and +40 mV obtained in 31 normal and 32 p47^{phox} KO cells. * $P < 0.05$; *** $P < 0.001$ vs. normal cells. *F* and *G*: effects of AEBSF (3 mM) in normal ($n = 10$; *F*) and p47^{phox} KO type I cells ($n = 12$; *G*). *** $P < 0.001$ vs. AEBSF. +++ $P < 0.001$ vs. hypoxia. *H*: H₂O₂ (100 μM) decreased hypoxic inhibition of I_K in normal cells. Values are means ± SE; $n = 12$ cells. *** $P < 0.001$ vs. normoxia (100%). +++ $P < 0.001$ vs. hypoxia. From (He et al., 2005) with permission.

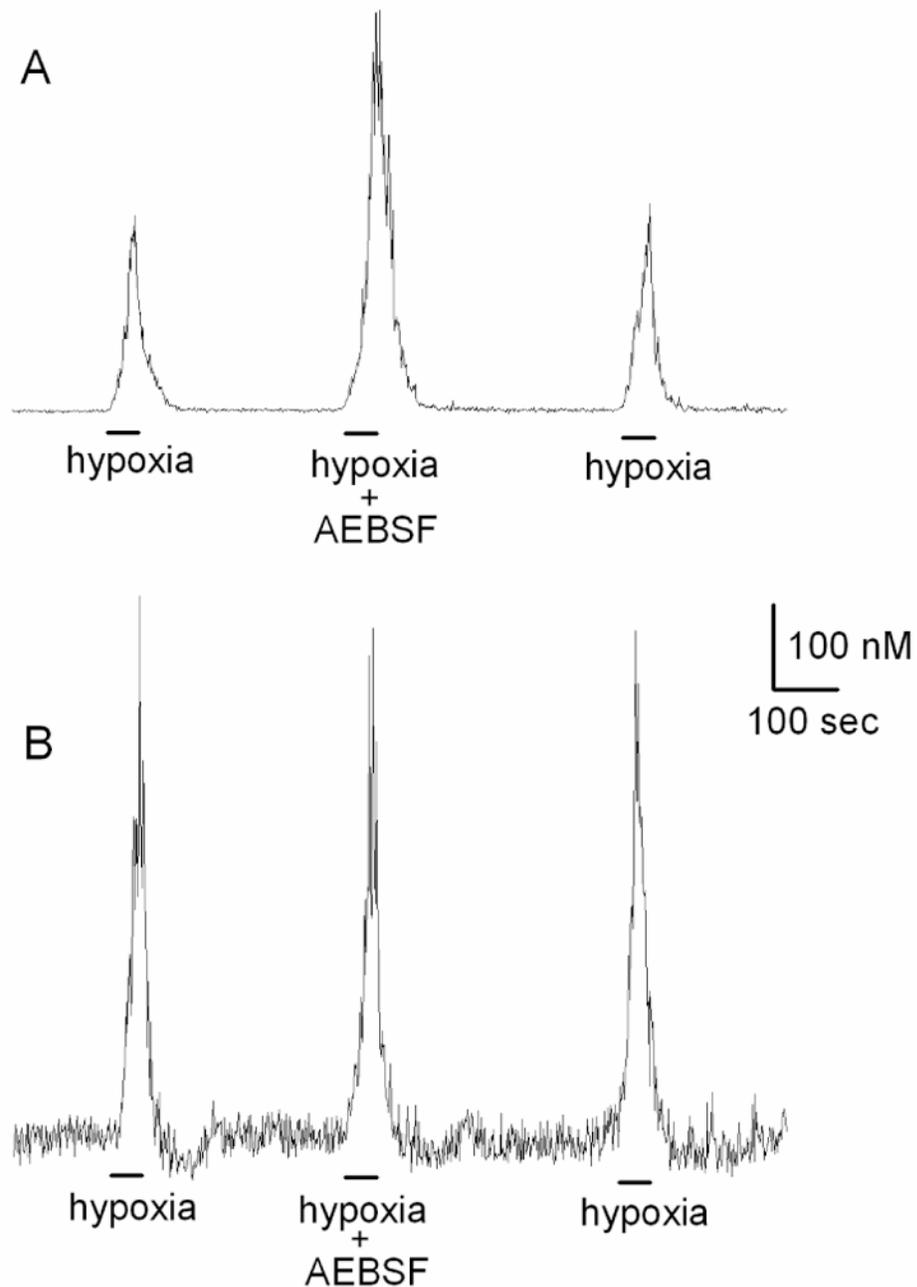


Figure 4. Hypoxia-evoked Ca²⁺-responses in Fura-2-loaded mouse type I cells. A: Response in normal cell is potentiated in presence of a specific Nox inhibitor, 4-(2-Aminoethyl)-benzenesulfonyl fluoride (AEBSF; 3 mM). B: In a cell from a p47^{phox} KO mouse, hypoxia evokes a larger response; however, AEBSF has no effect, consistent with the absence of functioning Nox.

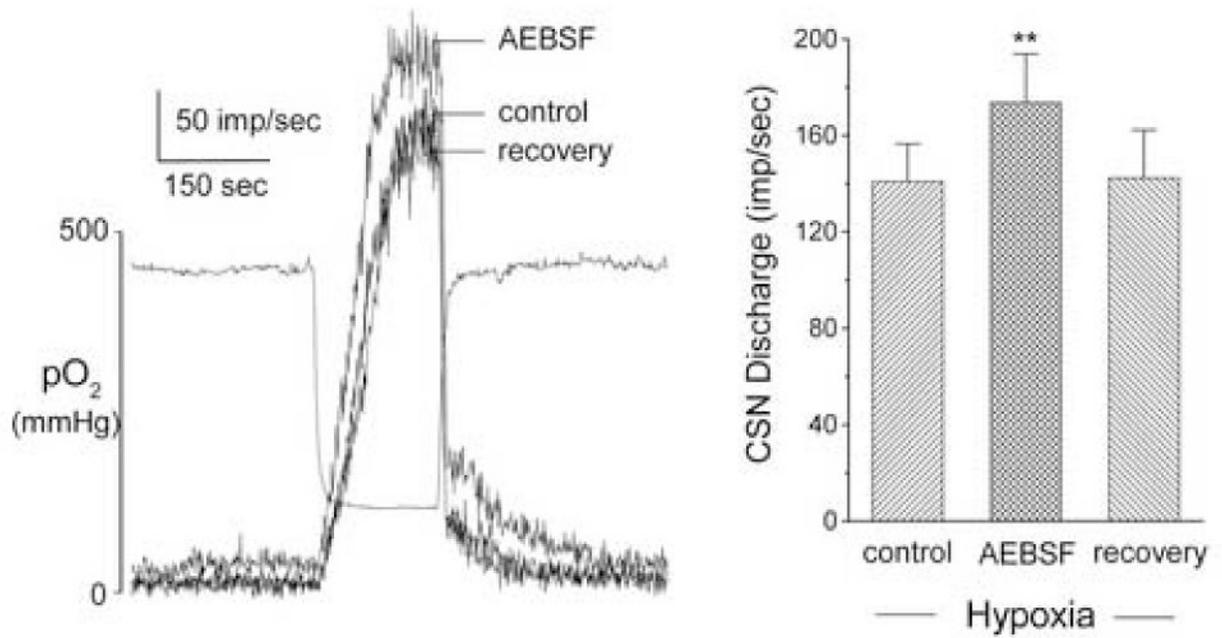


Figure 5. Effect of specific Nox inhibitor, AEBSF (3 mM) on rat carotid sinus nerve (CSN) activity evoked by acute hypoxia. *In vitro* superfused preparation. Left panel shows 3 superimposed traces of integrated nerve activity. Separate record indicates bath P_{O₂}. Left panel summarizes data from 4 preparations, and demonstrates significant ($p < 0.01$) enhancement of hypoxia-evoked response in presence of AEBSF.

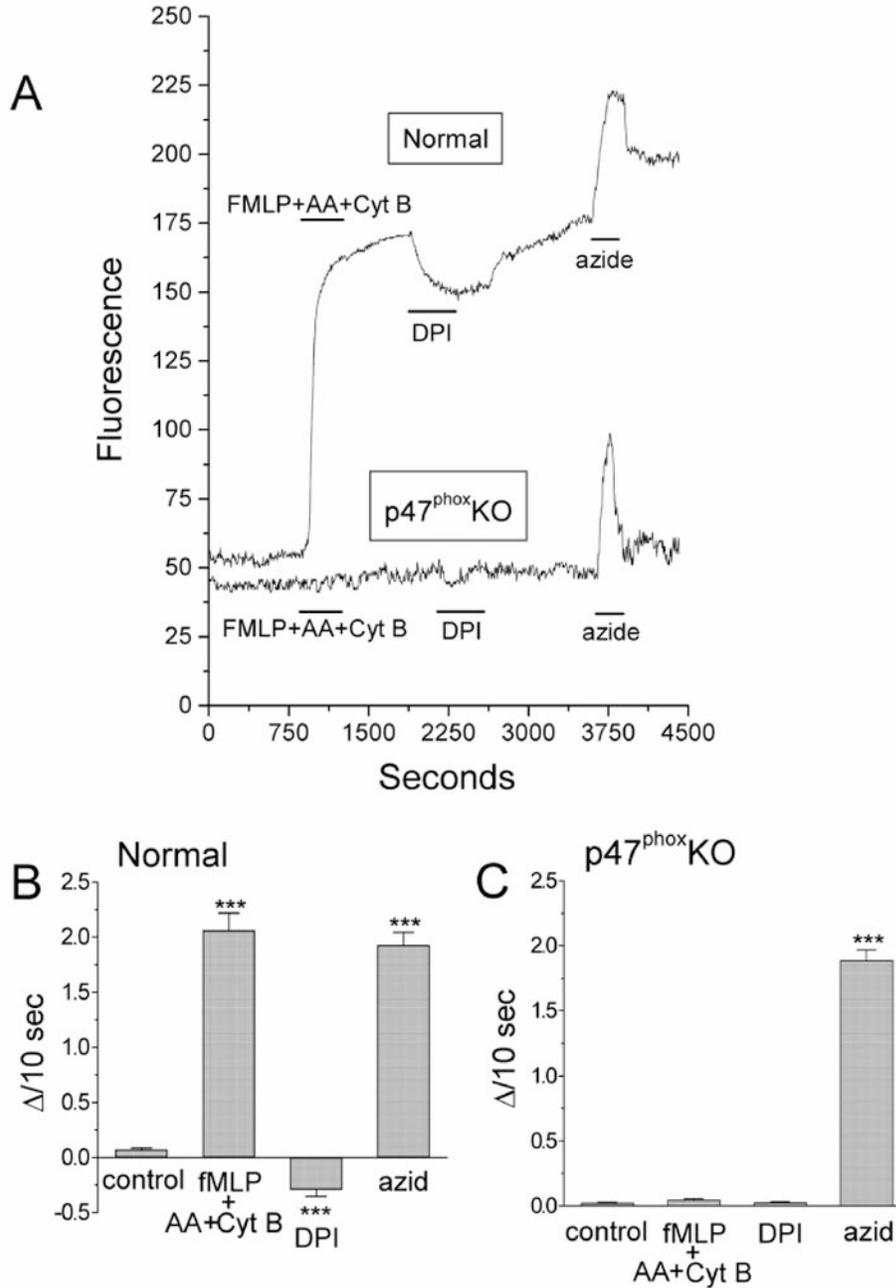


Figure 6. *A:* a cocktail of neutrophil-stimulating agents [*N*-formyl-Met-Leu-Phe (fMLP peptide; 1 μM), arachidonic acid (AA; 10 μM), and cytochalasin B (CytB; 5 μg/ml)] produced a sharp increase in dihydroethidium (DHE) fluorescence in a single attached polymorphonuclear neutrophil (PMN) from a normal mouse and no response in a p47^{phox} knockout (KO) PMN. Diphenyleiiodonium (DPI; 10 μM) decreased the fluorescence signal in the normal mouse and had no effect on the p47^{phox} KO PMN. Azide (5 μM) caused a similar increase in fluorescence in the normal and p47^{phox} KO PMN. *B* and *C*: summaries of DHE fluorescence for 98 normal (*B*) and 185 p47^{phox} KO PMN (*C*). Data are expressed as slopes of fluorescence

intensity change ($\Delta I/10$ s) during the initial 100 s of the experimental conditions designated in A. *** $P < 0.001$ vs. drug-free conditions. From (He et al., 2005) with permission.

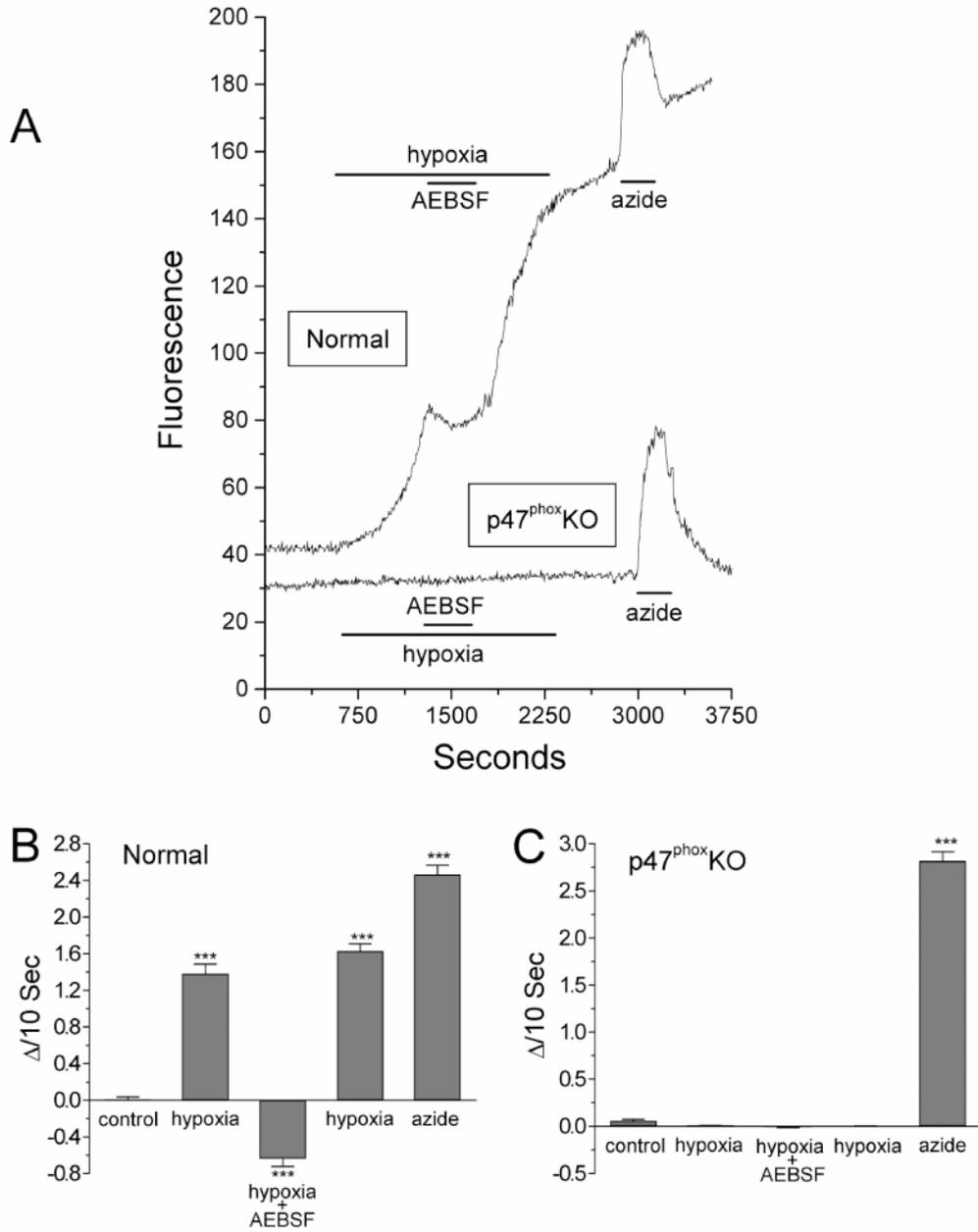


Figure 7.

A: effect of hypoxia on DHE fluorescence in carotid body type I cells isolated from normal and p47^{phox} KO mice. In normoxia, fluorescence gradually increases and is markedly elevated by hypoxia in the normal but not in the p47^{phox} KO cell. 4-(2-Aminoethyl)-benzenesulfonyl fluoride (AEBSF; 3 mM) depresses hypoxic increase of reactive oxygen species (ROS) production in the normal type I cell. Azide (5 μ M) increased the fluorescence in the normal and p47^{phox} KO cells. B and C: summaries of DHE fluorescence for 32 normal (B) and 44 p47^{phox} KO type I cells (C) harvested from 4 normal and 4 KO animals. Slopes ($\Delta I/10$ s) were quantified as described in Fig. 6. *** $P < 0.001$ vs. control, normoxic conditions. From (He et al., 2005) with permission.