

## Forum Review Article

# Redox Signaling Across Cell Membranes

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### Abstract

Generation of reactive oxygen species (ROS) by plasma membrane-localized NADPH oxidase (Nox 2) is a major mechanism of cell signaling associated with activation of the enzyme by a variety of agonists. With activation, the integral membrane flavocytochrome of Nox 2 transfers an electron from intracellular NADPH to extracellular  $O_2$ , generating superoxide anion ( $O_2^{\bullet-}$ ). The latter dismutates to  $H_2O_2$  which can diffuse through aquaporin channels in the plasma membrane to elicit an intracellular signaling response.  $O_2^{\bullet-}$  also can initiate intracellular signaling by penetration of the cell membrane through anion channels (Cl<sup>-</sup> channel-3, ClC-3). Endosomes containing Nox2 and ClC-3 (called signaling endosomes) are composed of internalized plasma membrane and generate  $O_2^{\bullet-}$  in the endosomal lumen to initiate signaling at intracellular sites. Thus, cellular signaling by Nox2 is dependent on the transmembrane flux of ROS. The role of this pathway has only recently been described and will require additional investigation to appreciate its physiological significance fully. *Antioxid. Redox Signal.* 11, 1349–1356.

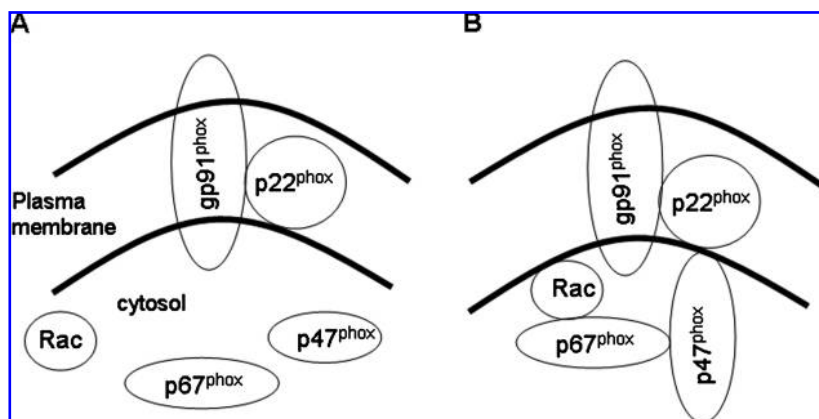
### Introduction

THE GENERATION of reactive oxygen species (ROS) in tissues was first proposed >50 years ago by Gerschman, Gilbert, and co-workers (20, 21) as a possible mechanism for the toxic effects of oxygen at increased partial pressure. Approximately 10 years later, McCord and Fridovich (42) isolated an enzyme (superoxide dismutase) from cells that degrades superoxide ( $O_2^{\bullet-}$ ) and postulated that it functions to protect against oxidant stress. Subsequently, cells were shown to produce  $O_2^{\bullet-}$  and other ROS when exposed to redox active agents, but also as a by-product of normal metabolism. For example,  $O_2^{\bullet-}$  is produced by “leakage” of electrons from the mitochondrial respiratory chain or from microsomal redox-active enzymes (6, 19). Early on, a “physiologic” role for ROS was discovered related to the killing of bacteria by phagocytic cells through a pathway involving superoxide anion plus hypochlorous acid generated by the myeloperoxidase reaction (3). Although it was appreciated that this reaction is beneficial to the host organism, it represents a toxic effect of ROS on the bacteria. These concepts resulted in emphasis on the injurious effects of ROS and their role in various pathologies. More recently, it has become clear that cells generate ROS at lower levels to serve a signaling function that is crucial

for cellular homeostasis. These oxygen-derived species function in signal transduction by reversible oxidative modification of proteins, leading to phosphorylation cascades and altered gene transcription (18). ROS-mediated signaling can promote cell proliferation and survival, but also can regulate programmed cell death or even necrosis, thereby playing a key role in the modulation of cell turnover (23, 40, 41, 49).

ROS are produced in the intact cell by both enzymatic and nonenzymatic reactions. The latter include the autooxidation of xenobiotics such as paraquat or of endogenous metabolites such as components of the mitochondrial respiratory chain. These autooxidation reactions are not regulated, and, although the ROS that are generated may interact (or interfere) with signaling pathways, this is unlikely to constitute a physiologically important system. Conversely, the enzymatic generation of ROS can be more finely controlled and can constitute a physiologic signaling pathway. These enzyme systems have specific subcellular localization and thus give rise to the concept of compartmentalization of both ROS production and the signaling response.

Compartmentalization was noted relatively early in the evolution of our knowledge of ROS biology, because the production of superoxide by phagocytic cells was found to occur by a plasma membrane-localized enzyme (NADPH



**FIG. 1. Activation of NADPH oxidase.** (A) The unassembled NADPH oxidase complex consists of cytoplasmic and intrinsic membrane components. (B) After an activating stimulus, the enzyme is assembled by translocation of the cytoplasmic components to the cell membrane.

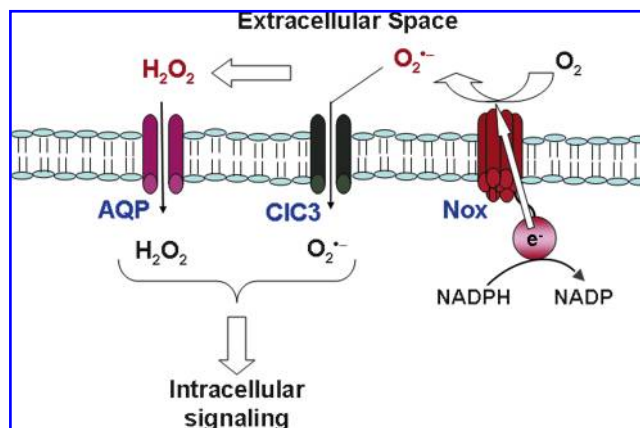
oxidase) into the well-circumscribed space of a phagolysosome. Compartmentalization is a likely explanation for many of the diverse effects of ROS. Antioxidant defenses are likewise compartmentalized, with specific enzymes for mitochondria (MnSOD), cytosol (CuZnSOD, GSH peroxidase), peroxisomes (catalase), and the extracellular space (ECSOD). By accepting the compartmentalization of ROS production, the question arises as to mechanisms for the possible transfer of ROS across membranes. In that regard, it is helpful to start with a discussion of the plasma membrane-associated NADPH oxidases.

#### Generation of ROS by NADPH Oxidase in Phagocytes

NADPH oxidase has long been recognized as an important generator of ROS in phagocytic cells such as polymorphonuclear leukocytes (PMNs) or macrophages in which it is localized to the plasma membrane (3, 4). This enzyme represents a multiprotein complex that is assembled after appropriate stimulation (16, 59). The assembled complex consists of both plasma membrane and cytosolic proteins (Fig. 1). The intrinsic membrane component is a heterodimeric flavocytochrome with gp91<sup>phox</sup> and p22<sup>phox</sup> subunits. These designations were chosen to indicate a protein (p) or glycoprotein (gp) of the phagocyte oxidase (phox), whereas the numeric designation indicates the nominal molecular mass on gel electrophoresis. The heterodimeric flavocytochrome is also called cytochrome *b*<sub>558</sub>, based on its spectroscopic properties. It is the catalytic subunit responsible for transferring an electron from NADPH to molecular oxygen, thereby forming O<sub>2</sub><sup>•-</sup>. Activity of cytochrome *b*<sub>558</sub> requires its interaction with cytosolic regulatory subunits. The primary regulatory proteins are p47<sup>phox</sup> and p67<sup>phox</sup>, which are phosphorylated during the activation process and translocate to the plasma membrane, where they associate with the flavocytochrome. The p47<sup>phox</sup> is important for organization of the complex, and p67<sup>phox</sup> activates the electron-transfer process. A small G protein, rac2 in PMNs, is crucial for initiation of the activation process resulting in phosphorylation and translocation of cytosolic components to the plasma membrane. Several additional proteins (p40<sup>phox</sup>, p29) also appear to play a role in activation of the complex, although their function is incompletely understood (16, 33, 59). It has been suggested that binding of p67<sup>phox</sup> to endosomal membranes through its interaction with p40<sup>phox</sup> is required for its delivery to the plasma membrane (43). The NADPH oxidase complex can be activated after exposure to

stimuli such as opsonized bacteria, various cytokines, or bacterial lipopolysaccharides.

The gp91<sup>phox</sup> is a membrane-spanning protein oriented so that electron transfer occurs from cytoplasmic NADPH to an acceptor oxygen on the extracellular side of the plasma membrane (Fig. 2). In the phagocyte, this results in the generation of O<sub>2</sub><sup>•-</sup> into the phagolysosome. Activation of the complex and O<sub>2</sub><sup>•-</sup> production by PMNs and other phagocytic cells is a major bactericidal mechanism, and absence of one or more proteins of the enzyme complex is manifested as chronic granulomatous disease, a syndrome characterized by increased susceptibility to chronic infections. In addition to bactericidal activity, the respiratory burst in phagocytes can result in activation of protein tyrosine kinases or inactivation of protein tyrosine phosphatases (14, 54). These observations provided support for the concept of NADPH oxidase involvement in ROS-mediated signaling.



**FIG. 2. Generation of reactive oxygen species by activated NADPH oxidase (Nox).** NOX2 transfers an electron from intracellular NADPH across the cell membrane to molecular oxygen to generate O<sub>2</sub><sup>•-</sup> in the extracellular space. Extracellular O<sub>2</sub><sup>•-</sup> can dismutate to H<sub>2</sub>O<sub>2</sub>, which traverses the cell membrane through aquaporin channels (AQP). Extracellular O<sub>2</sub><sup>•-</sup> also can penetrate cell membranes through a chloride channel-3 (CIC3). H<sub>2</sub>O<sub>2</sub> and O<sub>2</sub><sup>•-</sup> may interact with specific ligands to initiate intracellular signaling. Modified with permission from (14). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at [www.liebertonline.com/ars](http://www.liebertonline.com/ars)).

### The NADPH Oxidase Family

It is now recognized that the phagocytic NADPH oxidase is the prototype for a family of enzymes that are widely distributed and that differ primarily in the flavocytochrome component (16, 31, 59). These enzyme complexes have been designated the Nox proteins (for NADPH oxidase), and the classic phagocyte oxidase is now called Nox2. Five Nox proteins have been described, along with two additional enzymes called Duox (for dual oxidase). All of the Nox proteins appear to have the membrane-associated p22<sup>phox</sup> component (except perhaps Nox5) and are activated by proteins analogous to those described for Nox2. Some of these proteins may be expressed on internal (organellar) membranes and may show constitutive activity, but those details are still under investigation. Membrane oxidases identical to the phagocytic NADPH oxidase complex (Nox2) have been demonstrated in endothelial cells, fibroblasts, mesangial cells, smooth muscle cells, neurons, and probably are expressed in nearly all cell types (31, 59). The rate of superoxide generation by Nox2 in nonphagocytic cells is lower than the levels required for microbicidal function, but is sufficient for cell signaling.

### ROS-Mediated Signaling in Endothelium

The Nox pathways have been well studied in endothelial cells (1, 2, 22, 28, 58). These cells express Nox2 and also Nox4 and perhaps Nox1; as in the phagocytic cells, endothelial Nox2 is localized to the plasma membrane, although it may also be expressed on endocytic vesicles derived from plasma membrane. The location of Nox4 is less certain and may be expressed primarily by the nuclear and other organellar membranes. Endothelial Noxs have been shown to participate in cell signaling and the generation of O<sub>2</sub><sup>•-</sup> results in the activation of tyrosine kinases and protein phosphorylation. Analogous to its localization in PMN, activated Nox2 generates superoxide anion on the external side of the plasma membrane, as indicated by the cytochrome *c* reduction assay (8, 39). (Cytochrome *c* added to the medium would be expected to remain in the extracellular space, whereas most superoxide generated intracellularly would dismute before it could cross the plasma membrane; therefore, the reduction of cytochrome *c* added to the medium, and its inhibition by extracellular SOD, corroborates an extracellular site of O<sub>2</sub><sup>•-</sup> production). O<sub>2</sub><sup>•-</sup> production by activated Nox2 in bovine pulmonary artery cells was ~50% of the corresponding value for the respiratory burst in PMN, although superoxide production by rat pulmonary microvascular endothelium was only about one tenth as great (8, 39). Nox2 in endothelial cells can be activated by a variety of agents including hormones and agonists such as angiotensin II and thrombin, and inflammatory mediators such as TNF- $\alpha$  and IL-1 (22).

Endothelial cells *in situ* are exposed to a variety of mechanical forces, including intraluminal pressure and tangential shear stress. *In vitro* studies have demonstrated that endothelial cells undergo changes with shear stress, leading to what has been described as a flow-adapted state (10). It is assumed that endothelial cells *in situ* have been flow adapted by the physiologic exposure to shear. Endothelial cells *in situ* respond to abruptly decreased shear with ROS production (1, 15, 63), and endothelial cells *in vitro* respond similarly (8, 47). Increased shear stress also activates signaling through ROS generation associated with the activation of NOX2

(10, 26). Thus, the concept has evolved that endothelial cells respond to an alteration of shear either in a positive or negative direction from the "set" point.

Our laboratory has described the signaling pathways associated with acute loss of shear, an event that models "ischemia" due to vascular obstruction. The loss of shear is sensed by caveoli, either indirectly through cytoskeletal elements or directly through shear sensitivity of these organelles, resulting in a cascade manifested initially by closure (decreased open probability) of cell membrane-associated K<sub>ATP</sub> channels (8, 46). Channel "closure" results in partial depolarization of the endothelial cell plasma membrane, estimated in our studies with pulmonary microvascular endothelium as a change of approximately +20 mv (assuming a resting endothelial cell membrane potential of -60 mv) (8, 63). This signal is transduced by membrane-associated phosphatidylinositol-3 kinase (PI3K) and protein kinase B (Akt) to activate the small G protein (rac1 in endothelium) that orchestrates the assembly of the NADPH oxidase complex and the generation of O<sub>2</sub><sup>•-</sup> (63). Downstream signaling events include the activation of MAP kinases (Erk, Jnk) and transcription factors (NF- $\kappa$ B, AP1), eventuating in endothelial cell proliferation (46, 60). Our preliminary (unpublished) studies indicate increased angiogenic potential of flow-adapted endothelial cells subjected to acute loss of shear, which presumably could result in the formation of new vessels as compensation for the loss of blood flow. The physiologic "purpose" for the signaling response to increased shear has not been determined.

### Mediators of ROS-Dependent Transmembrane Signaling

In both the PMN and endothelial cell models, extracellularly generated O<sub>2</sub><sup>•-</sup> results in an intracellular signaling response. How is this response initiated, because the plasma membrane represents an obvious barrier to the free diffusion of O<sub>2</sub><sup>•-</sup>? Superoxide in solution dismutates to H<sub>2</sub>O<sub>2</sub> with a rate constant of ~10<sup>5</sup> M<sup>-1</sup>s<sup>-1</sup> at pH 7; extracellular superoxide dismutase (if present) can increase the rate of O<sub>2</sub><sup>•-</sup> dismutation by ~4 orders of magnitude. Thus, the lifetime and potential diffusion distance for O<sub>2</sub><sup>•-</sup> will be very short, estimated at 0.5  $\mu$ m (44), and it seems likely that a reaction product of superoxide (*i.e.*, H<sub>2</sub>O<sub>2</sub>) rather than the anion itself is responsible for initiating the signaling response (Fig. 2). H<sub>2</sub>O<sub>2</sub> is relatively stable in solution and readily crosses cell membranes, where it could initiate the signaling cascade through a variety of redox-mediated reactions such as the oxidation of protein sulfhydryl moieties (18). Although the plasma membrane has been assumed to be freely permeable to H<sub>2</sub>O<sub>2</sub>, current evidence suggests that, similar to H<sub>2</sub>O, the pathway for H<sub>2</sub>O<sub>2</sub> diffusion is through aquaporin channels (5). The selectivity of these channels for H<sub>2</sub>O<sub>2</sub> is not known but could possibly influence the signaling response. The intracellular concentration of H<sub>2</sub>O<sub>2</sub> is regulated through enzymatic scavenging by peroxidoreductases, glutathione peroxidases, and catalase.

Support for H<sub>2</sub>O<sub>2</sub> as the mediator of signal transduction has been gained from experiments in which scavengers of ROS were added to the extracellular milieu. Thus, the signaling cascade is relatively unaffected by the presence of superoxide dismutase, which converts O<sub>2</sub><sup>•-</sup> to H<sub>2</sub>O<sub>2</sub>, but is abrogated by the presence of catalase or other H<sub>2</sub>O<sub>2</sub> scavengers (46). Additional possibilities for the signal transducer include other

products formed from  $O_2^{\bullet-}$ , such as  $\bullet OH$  from its interaction with  $H_2O_2$ , peroxynitrite (ONOO $\bullet$ ) from its interaction with  $\bullet NO$ , or oxidized membrane components including lipid hydroperoxides or protein carbonyls. However, all of these more likely represent toxic by-products of superoxide generation rather than physiologic signaling molecules (27).

The hypothesis that  $H_2O_2$  represents the signal transducer for extracellularly generated  $O_2^{\bullet-}$  is compatible with results after the addition of exogenous  $H_2O_2$  to cells (56). The pitfalls (and strengths) of this approach have been reviewed, and the properties of  $H_2O_2$  that coincide with the theoretic requirements for a second-messenger function have been described (17, 27): (a)  $H_2O_2$  is produced enzymatically; (b) its concentration is effectively regulated through rapid removal by various enzymes with specificity for  $H_2O$ ; (c) a steep gradient of  $H_2O_2$  emanates from its site of production; and (d)  $H_2O_2$  is relatively specific in its biochemical sites of action. A major caveat is that past studies frequently have relied on supra-physiologic concentrations of exogenous  $H_2O_2$  for studies of cellular activation, but more recent studies using concentrations of  $H_2O_2$  within the physiologic range have confirmed many of the previous observations (17, 27, 56).

Although  $H_2O_2$  is now generally accepted as a signaling molecule and specifically as the second messenger related to NADPH oxidase activity, several studies have suggested that  $O_2^{\bullet-}$  also may operate in this context. Further,  $O_2^{\bullet-}$  and  $H_2O_2$  may have different and discrete signaling roles. For example,  $O_2^{\bullet-}$  added to intact HEPG2 cells results in cytochrome *c* release from mitochondria, an index of programmed cell death or apoptosis, an effect that was not seen with equivalent concentrations of  $H_2O_2$  (37). In these experiments, pretreatment with a SOD-mimetic abolished the effect of  $O_2^{\bullet-}$ , indicating that  $H_2O_2$  produced by dismutation was not responsible for the observed results. As another example, T-cell receptor-stimulated activation of the FAS ligand promoter was dependent on  $O_2^{\bullet-}$  but independent of  $H_2O_2$ ; the activation was blocked by an SOD-mimetic but was unaffected by enzymes that scavenge  $H_2O_2$  (13).

Finally, extracellular  $O_2^{\bullet-}$  (generated by activated alveolar macrophages) has been shown to evoke a large intracellular  $Ca^{2+}$  transient in endothelial cells associated with activation of inositol 3-phosphate (IP3) receptors (38). This latter response was inhibited by SOD and was not reproduced by the addition of  $H_2O_2$ . The differential signaling function of  $O_2^{\bullet-}$  and  $H_2O_2$  in higher organisms recaps similar observations in bacteria in which the SoxR transcription factor appears to be an  $O_2^{\bullet-}$ -specific sensor, whereas the Oxy-R transcription factor is sensitive to  $H_2O_2$  (9). This has been explained as a result of differential chemistry in which  $H_2O_2$  modification of proteins is primarily through oxidation of sulfhydryl groups, whereas  $O_2^{\bullet-}$  specifically reacts with iron-sulfur clusters related to high electrostatic attraction (9).

How can extracellular  $O_2^{\bullet-}$  result in intracellular signaling events? A possible mechanism was indicated by studies of  $O_2^{\bullet-}$  flux across cell membranes. It was shown many years ago by using extracellular cytochrome *c* as a trap that  $O_2^{\bullet-}$  generated inside erythrocyte ghosts could traverse the cell membrane through anion channels (36). More recent studies evaluated transmembrane flux of extracellular  $O_2^{\bullet-}$  generated by xanthine/xanthine oxidase or by the addition of  $KO_2$  (potassium superoxide) to endothelial cells (24). Cells that were loaded with the superoxide-sensitive dye hydroethidine showed a

burst of fluorescence after the addition of  $O_2^{\bullet-}$ , suggesting transmembrane flux of this radical. The burst of fluorescence was followed by a transient increase of cytosolic  $Ca^{2+}$  (24) that, based on previous observations, results from activation of IP3 receptors (38). Both oxidation of hydroethidine and release of intracellular  $Ca^{2+}$  induced by extracellular  $O_2^{\bullet-}$  were blocked by the presence of an anion channel blocker (DIDS) or the selective silencing of the chloride channel-3 (ClC-3) by treatment with siRNA (24, 38). The increase in intracellular  $Ca^{2+}$  induced by extracellular superoxide in endothelial cells resulted in the activation of mitochondrial superoxide anion production, thereby amplifying the initial signal, as indicated by increasing fluorescence of the hydroethidine indicator (24). The mechanism for  $Ca^{2+}$ -induced mitochondrial ROS production appears to be alteration (depolarization) of the mitochondrial membrane. This amplified signal (*i.e.*, ROS release by mitochondria) induced cellular apoptosis, which was inhibited when cell penetration by extracellular  $O_2^{\bullet-}$  was blocked (24). Thus, these results indicate that extracellular superoxide can penetrate the cell membrane through a chloride channel and thereby activate an intracellular signaling cascade (Fig. 2). Because of its short lifetime and limited diffusivity, the effects of extracellular  $O_2^{\bullet-}$  must be confined to the area immediately surrounding the channel, although the manifestations of the secondary signaling events can be more widespread. Previous publications indicating inhibition of physiologic effects after receptor activation by  $Cl^-$  channel blockers raise the possibility that an  $O_2^{\bullet-}$ -mediated increase of intracellular  $Ca^{2+}$  regulates granule exocytosis by PMNs (29) and possibly other secretory cells (7, 51, 52).

An alternative but unexplored explanation for the observed role of  $O_2^{\bullet-}$  in signaling is that the anion channel itself serves a second-messenger function, and its oxidation specifically by superoxide (but not  $H_2O_2$ ) leads to intracellular signaling, but this would not explain the initial oxidation of hydroethidine on addition of exogenous  $O_2^{\bullet-}$ . Further complicating the issue is the demonstration that ClC-3 is present in the PMN secretory granules and, through  $Cl^-$  flux, may play a role in activation of Nox2 (48). Clearly the relation between Nox2 activation,  $O_2^{\bullet-}$  generation, and ClC-3 will require further study.

Although  $H_2O_2$  permeation through aquaporin channels and  $O_2^{\bullet-}$  penetration through anion channels are potential agents for transmitting NOX-generated chemical signals across the cellular plasma membrane, another possibility for signal transduction associated with activation of NADPH oxidase is related to proton ( $H^+$ ) movements. Transfer of electrons from NADPH across the cell membrane to extracellular  $O_2$  to generate  $O_2^{\bullet-}$  results in a relative excess of  $H^+$  in the cell cytosol, and extrusion of these protons is necessary to maintain the balance of electrical charge in the cell (12). Recent studies have demonstrated the presence of cellular proton channels that show outward rectification, thereby permitting proton efflux (11). This field of investigation is still controversial, and an alternate explanation is that charge neutrality is maintained through the NADPH oxidase itself (25). The recent identification of a "proton channel" gene indicates that these channels do exist separate from the NADPH oxidase (53, 55). Thus, a so-far-undiscovered effect of the proton efflux pathway could be responsible for cell signaling by NADPH oxidase activity unrelated to a role for either  $H_2O_2$  or  $O_2^{\bullet-}$ . However, proton efflux could not explain the specificity of

individual ROS or the specific effects of ROS added to the extracellular milieu (in the absence of NOX activity).

In summary, the published data support the conclusion that transmembrane diffusion of  $\text{H}_2\text{O}_2$  and probably  $\text{O}_2^{\bullet-}$  is the mechanism for NOX-mediated signaling.

### ROS-Mediated Signaling in Endosomes

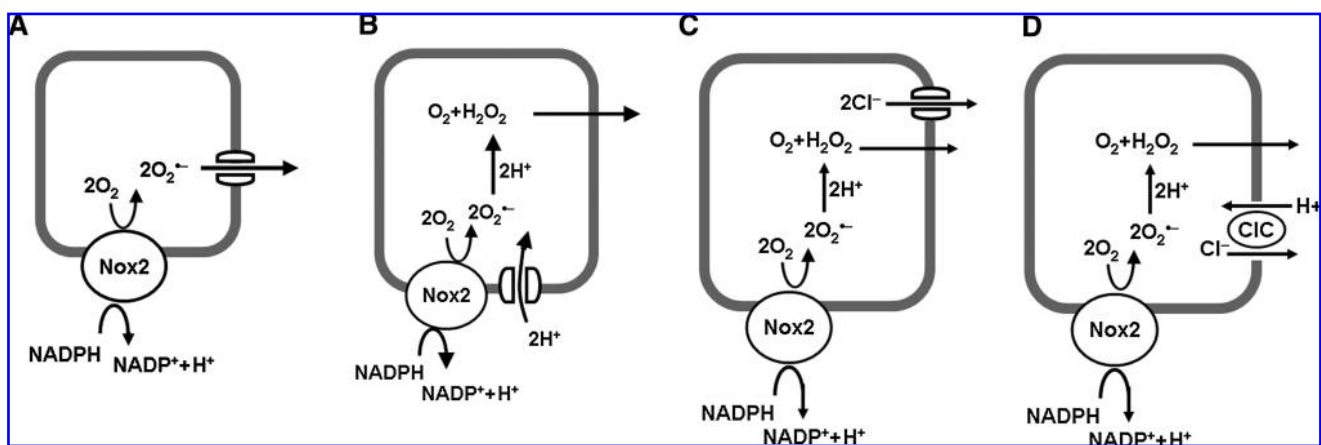
Recently, a subset of endosomes was identified that contain the Nox1 or Nox2 membrane-spanning proteins and is called "signaling endosomes" (34, 45). These organelles represent a novel pathway for NADPH oxidase-mediated intracellular signaling. The significance of the signaling endosome is that generation of ROS as second messengers is not limited to the cytoplasmic area near the plasma membrane, but rather can occur at intracellular sites, depending on endosomal localization (34, 35). Endosomes are formed by internalization of a patch of plasma membrane. Within endosomes, the cytoplasmic face of the membrane corresponds to the cytoplasmic face of the plasma membrane, whereas the internal (luminal) face and the endosomal contents correspond to the extracellular face of the plasma membrane and the extracellular space, respectively. This configuration reflects that for phagolysosomes of PMNs and other phagocytic cells. Signaling endosomes have been shown to generate  $\text{O}_2^{\bullet-}$  within the luminal space, transferring the electron from cytoplasmic NADPH to "extracellular"  $\text{O}_2$  (34, 45). Thus, the spatial relation between the membrane protein and electron transfer to  $\text{O}_2$  are maintained in the endosome as for the cell membrane (Fig. 3A). As in prior studies with the plasma membrane (5, 24), the endosomal membrane is permeable to  $\text{H}_2\text{O}_2$  and also to permeation by  $\text{O}_2^{\bullet-}$ , possibly through  $\text{Cl}^-$  channels (50). In a recent study, the efflux of ROS from endosomes of MCF-7 cells stimulated with interleukin-1 $\beta$  showed that 60% was  $\text{O}_2^{\bullet-}$ , and this efflux was abolished by  $\text{Cl}^-$  channel inhibitors (50). Further, these endosomes recruited SOD to their cytoplasmic surface, which would result in the rapid and local generation of  $\text{H}_2\text{O}_2$  from  $\text{O}_2^{\bullet-}$  traversing membrane channels. SOD-deficient cells showed defective signaling in response to interleukin 1, suggesting that rapid dismutation of  $\text{O}_2^{\bullet-}$  to

$\text{H}_2\text{O}_2$  is necessary for this signaling pathway. A possible role for  $\text{O}_2^{\bullet-}$  itself in endosome-mediated signaling has not yet been described.

As described earlier for the cell membrane, transfer of electrons from cytosol to the endosomal lumen to generate  $\text{O}_2^{\bullet-}$  requires charge compensation. This can be provided by diffusion of  $\text{O}_2^{\bullet-}$  through endosomal membrane channels (50), which transfers the electrons back to the cytosol (Fig. 3A); as mentioned earlier, this pathway may account for much but not all of the electron flux (50). For the remaining  $\text{O}_2^{\bullet-}$ , dismutation to  $\text{H}_2\text{O}_2$  occurs within the endosome, and transmembrane movement of  $\text{H}^+$  from the cytosol to lumen or of anion from lumen to cytosol is necessary to equilibrate the charges. One possibility is that proton channels on the endosomal membrane allow proton flux, as proposed for the plasma membrane (Fig. 3B). Another possibility is that  $\text{Cl}^-$  efflux from the endosomal lumen to the cytoplasm provides charges compensation; this could occur either through a  $\text{Cl}^-$  channel (Fig. 3C) or through an  $\text{H}^+/\text{Cl}^-$  exchanger (Fig. 3D). It has been proposed that CIC-3 functions as an  $\text{H}^+/\text{Cl}^-$  antiporter similar to that described for CIC-4 and CIC-5 (32, 45). Charge compensation through  $\text{Cl}^-$  efflux alone would result in alkalinization of the endosome, because of utilization of  $\text{H}^+$  in  $\text{O}_2^{\bullet-}$  dismutation. The precise effects of  $\text{H}^+/\text{Cl}^-$  exchange on endosomal pH would depend on the stoichiometry of the process, which has been reported as 1  $\text{H}^+$  for 2  $\text{Cl}^-$  in bacteria but is not known for mammalian endosomes (30). Inhibition of  $\text{Cl}^-$  translocation in endosomes could explain the effect of  $\text{Cl}^-$  channel inhibitors on Nox activity (because a failure of charge compensation would quickly inhibit activity of the oxidase) but would leave unexplained the mechanism for superoxide permeation through the membrane.

### Site-Specific Generation of Signaling Molecules

It has recently become clear that site-specific localization of Nox2-mediated ROS production can play an important role in physiologic function. Localization of Nox2 to the leading edge of migrating endothelial cells has been shown to be important for angiogenesis and wound healing (57, 61). The generation



**FIG. 3. Superoxide generation in a signaling endosome.** Activated NADPH oxidase (Nox 2) transfers an electron from cytoplasmic NADPH to  $\text{O}_2$  in the lumen of the endosome to generate  $\text{O}_2^{\bullet-}$ . The  $\text{O}_2^{\bullet-}$  can exit the endosome through a chloride channel (CIC) (A) or dismute to  $\text{H}_2\text{O}_2$  (B–D). Charge compensation theoretically can be provided by efflux of  $\text{O}_2^{\bullet-}$  (A), influx of  $\text{H}^+$  through a proton channel (B), efflux of  $\text{Cl}^-$  through a  $\text{Cl}^-$  channel (C), or exchange of luminal  $\text{Cl}^-$  for cytoplasmic  $\text{H}^+$  through an  $\text{H}^+/\text{Cl}^-$  antiporter (D). Modified with permission from (32).

of ROS on the extracellular side of lamellipodia could provide a signaling gradient for directed cell migration. Localization of ROS production to focal complexes of the leading edge may be regulated through binding of the p47 subunit of Nox2 to the orphan adaptor TRAF4 and subsequent binding to the focal contact scaffold Hic-5 (57, 61), whereas caveolae or lipid rafts may be the intramembranous sites for NADPH localization (62). These results suggest that the discrete localization of ROS production in the membrane can provide spatial discrimination to the signaling process.

### Summary

In summary, NADPH oxidases, in addition to their role in microbial killing, are now known to be involved in intracellular signaling. The best studied of this family of enzymes is Nox2 (gp91<sup>phox</sup>), which is present in phagocytes, endothelial cells, and probably most other cell types. This enzyme transfers an electron to molecular oxygen to generate superoxide on the extracellular face of the plasma membrane or into the lumen of a phagolysosome or signaling endosome. Nox localization in specialized membrane domains may provide spatial resolution to the signaling process. Signaling is accomplished by dismutation of O<sub>2</sub><sup>•-</sup> to H<sub>2</sub>O<sub>2</sub> extracellularly with subsequent plasma-membrane permeation through aquaporin channels. O<sub>2</sub><sup>•-</sup> also can permeate the plasma and endosomal membranes through anion (Cl<sup>-</sup>) channels, where it can interact with nearby proteins to initiate intracellular signaling. Still unresolved is the precise mechanism for localizing the extracellularly generated oxidants and for directing the signaling molecule to its downstream target. And finally, the major question: what is the biologic advantage for extracellularly generated signaling molecules, and how did the primitive cell co-opt this pathway for its survival advantage? Future study will undoubtedly shed additional light on the roles and mechanisms for cell membrane-associated signaling pathways.

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### Abbreviations

Akt, protein kinase B; ClC, chloride channel; IL-1, interleukin-1; IP<sub>3</sub>, inositol 3-phosphate; NOX, NADPH oxidase; PMN, polymorphonuclear leukocyte; PI3K, phosphatidylinositol-3 kinase; ROS, reactive oxygen species; SOD, superoxide dismutase.

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