

Pollen NAD(P)H Oxidases and Their Contribution to Allergic Inflammation

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The Latin definition of pollen is *fine powder*. Analogous to sperms of the animal kingdom, pollens are plant male germ cells containing haploid chromosomes. Released from the anther of the flower, pollens reach the plant female reproductive organ (stigma) by means of wind, water, or insects in a process termed *pollination*. Pollens not only carry genetic information from anther to stigma but also have a broad impact on human health. Inhaling pollens can induce symptoms of seasonal asthma and allergic rhinitis in sensitized individuals [1,2]. Because of their microscopic size, some pollen grains and subpollen particles can penetrate lower airways and induce allergic airway inflammation [3,4]. Pollens contain a large number of antigenic proteins, such as the pectate lyase Amb a 1 in ragweed; defensin-like Art v 1 in mugwort; Pla 1 in plantain; Che a 1 in goosefoot; and nonspecific lipid transfer proteins Par j 1 and Par j 2 in pellitory [5]. These antigenic proteins are processed in antigen-presenting cells and presented to T-helper

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cells, thereby triggering an adaptive immune response culminating in allergic inflammation.

In addition to these antigens, pollens contain myriad other proteins, some with enzymatic activities. The plasma membrane of plant cells contains NADPH oxidases that are similar to the inducible NADPH oxidase complex in the plasma membrane of mammalian phagocytes [6–9]. These plant oxidases play a critical role in vital physiologic functions, including defense against pathogens [10–12], growth and development [8,13,14], and expansion of cells in the root hairs [15]. The authors discovered the presence of NADPH oxidase activity in pollen grains of various plants, including grasses, weeds, and trees [16]. This article provides an overview of mammalian and plant NADPH oxidase and generation of reactive oxygen species. The role of NADPH oxidase-induced oxidative stress in allergic inflammation is also described.

Historical perspectives

Oxygen (O_2) constitutes 21% of the atmospheric air. O_2 is an essential element of survival for all animals and plants except anaerobic organisms. Aerobic energy production uses an O_2 -dependent electron transport chain present in eukaryotic mitochondria. The advantage of the aerobic respiration can be ascertained from the fact that aerobic glycolysis produces 36 ATP molecules compared with only 2 ATP molecules produced by anaerobic glycolysis. However, this enormous energy source has a caveat: O_2 is a toxic and mutagenic. Aerobic organisms survive in the presence of O_2 only because they have developed mechanisms that eliminate toxic O_2 products and repair damaged biomolecules such as DNA.

Phagocytic host defense is a component of the innate immune system and a first line of defense against invading microorganisms. Phagocytes generate reactive oxygen species (ROS) that kill the microorganisms. However, this response is nonspecific and bystander host cells are also damaged, analogous to collateral damage in military terms. In 1883, Metchnikoff [17] performed pioneering work in the field of phagocytic host defense by showing that when transparent starfish larvae were pierced with a thorn, cells surrounded the sharp thorn. Fifty years later, Balldridge and Gerard [18] showed that when neutrophils were exposed to bacteria, a transient but sharp increase in O_2 uptake by the cells occurred. They termed this phenomenon *respiratory burst*, because they attributed the increase to oxidative phosphorylation by the mitochondria. However, in 1959, Sbarra and Karnovsky [19] reported that mitochondrial poison cyanide had no effect on respiratory burst, ruling out the long-held notion that mitochondria were responsible for increased oxygen demand when the neutrophils were engaged in killing bacteria. These reports, in addition to the discovery that common human bacterial pathogens are not killed in an anaerobic environment despite their normal phagocytosis [20], fueled the

search for a molecular component in the cell that was responsible for oxygen uptake and host defense against bacteria. Later researchers discovered that activated neutrophils generated hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl). Myeloperoxidase enzyme present in neutrophils catalyzed the reaction between H_2O_2 and chloride ions to generate HOCl [21], commercially available as Clorox (The Clorox Company, Oakland, California).

The last piece in the puzzle was solved with the observation of an inherited syndrome in which patients were predisposed to frequent infections by an otherwise nonpathogenic bacterium, such as *Serratia marcescens*. This syndrome was initially believed to be a neoplastic condition because of formation of multiple granulomas infiltrated by macrophages and lymphocytes in these patients. This disease was termed *chronic granulomatous disease* (CGD) because of its pathologic manifestation of granulomas in multiple organs. Further studies showed that neutrophils from patients who had CGD were incapable of killing bacteria and could not generate H_2O_2 [22]. Around the same time, investigators discovered that biologic systems could convert O_2 into superoxide anions (O_2^-) [23–25], and Babior and colleagues [26] showed that O_2^- is responsible for bacterial killing, indicating the role of O_2^- in the pathogenesis of CGD. Different subunits of NADPH oxidase were characterized and molecular defects of these subunits were identified in patients who had CGD. Most patients who have CGD inherit the disease in an X-linked recessive manner with defects in the membrane-bound NADPH oxidase subunit, gp91^{phox} (phox for *phagocytic oxidase*). Mutations in other NADPH oxidase subunits, such as p47, p67, and p22, were identified in an autosomal-recessive form of CGD.

Mammalian NADPH oxidase

NADPH oxidase is an enzyme present in phagocytic cells such as macrophages, neutrophils, and eosinophils [27–29]. Eukaryotic NADPH oxidase is a complex multimolecular enzyme that transports electrons across the plasma membrane to convert O_2 into O_2^- . NADPH oxidase is composed of cytosolic and membrane subunits (Fig. 1). The cytosolic components are p47^{phox}, p40^{phox}, p67^{phox}, and the small G-proteins Rac2 and Cdc42 [30–32]. The membrane-bound components gp91^{phox} and p22^{phox} form a heterodimeric structure known as cytochrome b₅₅₈ (cyt b558) [33]. On cell stimulation, the p47^{phox}, p40^{phox}, and p67^{phox} translocate and form a complex with membrane-bound cyt b558 in 1:1:1:1 stoichiometry [34]. Small GTPase proteins, such as Rac2 and Cdc42, also participate in activating the enzyme complex [35]. Fig. 1 provides a conceptual model of NADPH oxidase components and its activation-induced assembly in the membrane. A brief overview of each NADPH oxidase component is presented here.



Fig. 1. Schematic illustration of NADPH oxidase in action. NADPH oxidase components are shown in the context of intact phagosome. Cytosolic and membrane subunits are in various stages of assembly to form activated NADPH oxidase on the membrane in response to bacterial stimulus. gp91^{phox} and p22^{phox} are shown as pink and blue helices anchored to the membrane. Rac2, GDP dissociation inhibitor (GDI), p40^{phox}, p47^{phox}, and p67^{phox} are coded by color and shape. Ingested bacterium is shown in purple. (Reproduced from Bokoch GM, Knaus UG. NADPH oxidase: not just for leukocytes anymore! Trends Biochem Sci 2003;28(9):502–8; with permission.)

Cytosolic subunits of NADPH oxidase

p47^{phox}

p47^{phox} is 390-amino-acid peptide containing phox homology (PX), SH3, autoinhibitory, and proline-rich region domains (Fig. 2). The PX domain is named after its presence in p47^{phox} and p40^{phox} [36]. This domain is present in the *N*-terminus of p47^{phox} and facilitates its anchoring to phosphatidylinositol-3,4 bisphosphate [PI(3)P2] and adjacent phosphatidylinositol-3 phosphate present in the cell membrane [37]. A tandem repeat of two SH3 domains occurs, the first of which binds to a proline-rich sequence in the *C*-terminus of p22^{phox}, and the second binds to a proline-rich region of p67^{phox} (see Fig. 2; Fig. 3). An autoinhibitory domain present in the *C*-terminal region of p47^{phox} exhibits intramolecular interaction with the SH3 domain, preventing it from interacting with p22^{phox} of cyt b558 (see Fig. 3) [38]. This masking effect ensures the inadvertent activation of NADPH oxidase and production of oxygen radicals.

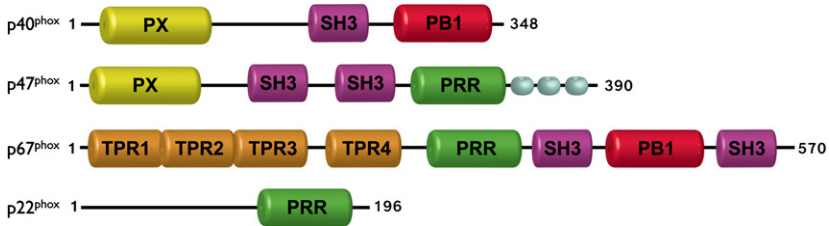


Fig. 2. Structural domains of NADPH oxidase subunits. The domain structures of $p40^{\text{phox}}$, $p47^{\text{phox}}$, $p67^{\text{phox}}$, and $p22^{\text{phox}}$ are shown in illustrative cartoon. Different domains in phox subunits are color coded. Phosphorylation sites in $p47^{\text{phox}}$ are shown as light blue spheres. The length of each subunit is approximated to total amino acids present in the proteins. PB1, phagocytic oxidase and Bem 1; PRR, proline-rich region; PX, Phox homolog; SH3, Src homology 3 domain; TPR, tetratricopeptide repeat.

Activation of the cell leads to phosphorylation of serines in the autoinhibitory domain, thereby disrupting its association with the SH3 domain and making it accessible for interaction with $p22^{\text{phox}}$ (Fig. 4) [38]. The proline-rich region of the $p47^{\text{phox}}$ domain binds to the SH3 domain in the

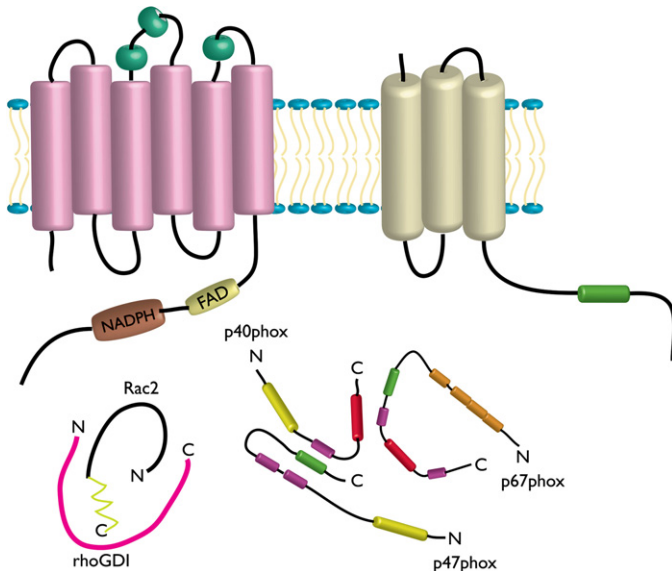


Fig. 3. NADPH oxidase components in resting state. Cytoplasmic and membrane bound subunits are shown in resting state. The pink- and cream-colored barrels indicate predicted transmembrane helices of $gp91^{\text{phox}}$ and $p22^{\text{phox}}$, respectively. Glycosylation sites in $gp91^{\text{phox}}$ are shown as green spheres. The FAD and NADPH binding sites in $gp91^{\text{phox}}$ are shown as light yellow and brown tubules. Rac2 bound to RhoGDI (guanine nucleotide dissociation inhibitor) is shown as black and pink thick lines. The thin green line on the C-terminus of Rac2 represents geranyl-geranyl moiety, which facilitates its association with membrane. $p40^{\text{phox}}$, $p47^{\text{phox}}$, and $p67^{\text{phox}}$ are displayed according to the structural model shown in Fig. 2. Interaction between $p40^{\text{phox}}$ and $p47^{\text{phox}}$ through the SH3 and PRR domains are shown.

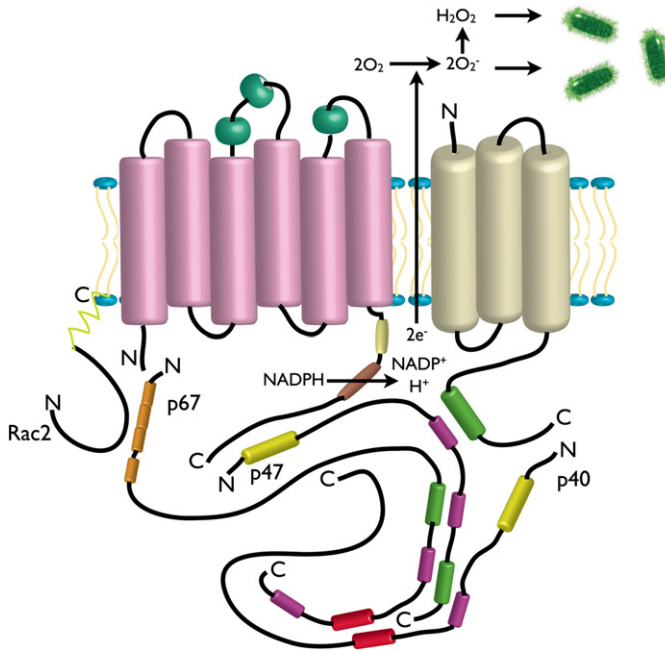


Fig. 4. Activation-induced NADPH oxidase subunit assembly in membrane. In the active state, RhoGDI dissociates from Rac2, and the latter then anchors to the membrane. P47^{phox} associates with the C-terminal end of gp91^{phox} and with p22^{phox} through its SH3 domain. p67^{phox} interacts with Rac2 through its TPR domains on the N-terminus, and with p47^{phox} through SH3 and PRR domain interactions. p40^{phox} weakly associates with p47^{phox} through SH3 and PRR interaction and with p67^{phox} through PB1 interaction. This assembly leads to activation of the NADPH oxidase complex, which converts NADPH to NADP⁺, and resulting electrons are transported across the membrane, converting molecular oxygen to superoxide. Superoxides are eventually converted to H₂O₂ by superoxide dismutase and together these ROS mediate the killing of pathogens.

C-terminus of p67^{phox} in a tail-to-tail interaction (Fig. 4). Phosphorylation of p47^{phox} is a prerequisite for its membrane translocation and interaction with p22^{phox}. Mutations in p47^{phox}, including the PX domain, have been associated with CGD [39,40]. However, in a cell-free system, the presence of p47^{phox} is not required when p67^{phox} and Rac2 are in excess [41]. Therefore, p47^{phox} mainly functions as an adaptor protein that binds to p67 and p40 and translocates them to the cell membrane.

p67^{phox}

p67^{phox} is a 526-amino-acid protein with tetratricopeptide repeat (TPR), activation, proline-rich region, SH3, and PB1 domains (see Fig. 2). Four TPR domains in the N-terminal of p67^{phox} interact with Rac in a guanosine triphosphate (GTP)-dependent manner (see Fig. 4) [42]. Once phosphorylated, the activation domain binds to gp91^{phox} and regulates electron flow

from NADPH to cyt b558 [43]. The presence of amino acids 199–210 is an absolute requirement for O_2^- production in a cell-free system [44]. The proline-rich region domain of p67^{phox} binds to a second SH3 domain of p47^{phox}. Two SH3 domains are found in p67^{phox}, one of which binds to the proline-rich region of p47^{phox}. The PB1 domain is named after its presence in phagocytic oxidase and Bem 1 proteins [45]. PB1 is essentially a protein–protein interaction motif that associates with PB1 in p40^{phox} to form a heterodimer (see Fig. 4). Translocation of p67^{phox}, p40^{phox}, and Rac2 from cytosol to membrane-bound cyt b558 involves p67^{phox} phosphorylation (see Figs. 3 and 4). p67^{phox} mutations are rare in CGD and, when present, result in decreased oxidase production [46].

p40^{phox}

p40^{phox} is a 339-amino-acid protein and the last subunit of the NADPH oxidase to be identified by copurification with p47^{phox} and p67^{phox}. It is composed of PX, SH3, and PB1 domains (see Fig. 2). The PX domain binds to PI(3)P2 and facilitates oxidase assembly in cell membrane. The SH3 domain of p40^{phox} interacts weakly with proline-rich region in p47^{phox} [47]. p40^{phox} contains a PB1 domain similar to p67^{phox} that mediates interaction between these proteins (see Fig. 4). The overall role of p40^{phox} remains under debate, although it has been reported to both up-regulate and down-regulate activity of NADPH oxidase [48,49]. At least in the cell-free system, p40^{phox} is not required for oxidase activity [50].

G-proteins

Rac and Cdc42 are G-proteins that belong to the Rho subfamily of proteins, which regulates a wide spectrum of functions such as intracellular signaling, cytoskeletal organization, generation of ROS, and cell growth. Three isoforms of Rac have been identified, with Rac1 ubiquitously expressed and Rac2 largely restricted to hemopoietic cells [51]. The predominant isoform in human neutrophils is Rac2. The association between NADPH oxidase and G-proteins was initially suspected because guanine nucleotides could stimulate NADPH oxidase [52]. In a resting state, Rac2 is bound to guanine-nucleotide dissociation inhibitor (GDI), inhibiting its interaction with the NADPH oxidase subunits p67 and cyt b558 (see Fig. 3). Appropriate stimuli induce dissociation of Rac2 from GDI and promote exchange of guanosine diphosphate (GDP) for GTP with the help of guanine-nucleotide exchange factors (GEF) [53]. Structural studies have shown the presence of switch I and II regions and an insert domain on Rac2. Interaction between Rac2 and cyt b558 is mediated through an insert region and is GTP-dependent. In the GTP-bound state, Rac2, through its switch I or effector region, can associate with the TPR domain of p67^{phox} (see Fig. 4).

The association of Rac2 and cytosolic subunits of NADPH oxidase with cyt b558 creates machinery for electron transfer from NADPH to O_2 involving two steps. In the first step, electrons are transferred from NADPH to the flavin adenine dinucleotide (FAD) moiety of cytochrome, and in the second phase, electrons are passed from FAD to heme in cyt b558 and then to molecular oxygen to form O_2^- [54]. A mutation in human Rac2 was identified that inhibits its association with GTP, resulting in decreased chemotaxis, O_2^- production, and azurophilic secretion [55].

Membrane-associated NADPH oxidase subunits

Cytochrome b₅₅₈

Cyt b558 is a flavohemoprotein composed of two subunits, gp91^{phox} and p22^{phox}. Cyt b558 is named because of its absorption peak at 558 nm. It is also known as Cyt b-245 because of its midpoint potential of -245 mV. Although the molecular weight of the large subunit gp91^{phox} is actually 65 kd, the extensive glycosylation process creates a diffuse band of approximately 91 kd on sodium dodecylsulfate (SDS) gels. Hydrophobic domains in the *N*-terminal region of gp91^{phox} form the membrane-spanning domains, whereas the hydrophilic *C*-terminal domain interacts with the p47^{phox} and heme moieties (see Figs. 3 and 4). Flavohemoprotein derives its name from the presence of FAD, which receives electrons from NADPH and two heme prosthetic groups that mediate further electron transfer to O_2 . The presence of FAD is mandatory for O_2^- generation, because removing FAD results in the loss of oxidase activity, and returning it restores the activity. Thus, gp91^{phox} contains all the components required for electron transfer, a concept supported by the report that gp91^{phox} functions similar to the H^+ channel and permits electron transport across the cell membrane [56]. Mutations of gp91^{phox} are associated with an X-linked CGD [57]. A phenotype similar to CGD is observed in gp91^{phox} knockout mice [58].

The p22^{phox} is a 21-kd protein that associates with gp91^{phox} in 1:1 stoichiometry (see Fig. 2). The heterodimer formation enhances the maturation and structural stability of gp91^{phox} [59]. The presence of three transmembrane α -helices in the *N*-terminal region of p22^{phox} forms the membrane insertion region, whereas the cytosolic *C*-terminal contains a proline-rich region that can interact with the SH3 domain of p47^{phox} (see Figs. 3 and 4). Mutations in p22^{phox} are associated with autosomal-recessive CGD [60].

Plant NAD(P)H oxidase

Plants are confined to a physical location because of their sessile growth and life. Therefore, developing defense mechanisms against broad range of biotic and abiotic stresses is critical. Pathogenic bacteria, viruses, and fungi are among the most dangerous threats to plant survival. Coevolution of

plants with various pathogens has enabled them to develop many different defense strategies through continuous exchange of molecular information between the groups. Some of the common defense reactions are cell-wall reinforcement and the production of antimicrobial agents, hydrolytic enzymes, and phytoalexins. The presence of an external threat is perceived through recognition of signal molecules known as *elicitors* released by microorganisms or plant cells in response to pathogens. Whether plants acquire resistance and clear the pathogen or become susceptible to the pathogen and develop disease depends largely on the speed and extent of intracellular signaling and the accumulation of defense products.

The defense responses are mainly regulated at transcription level by defense-related genes, resulting in delayed response time. However, occasionally the defense reactions primarily involve activation of preexisting components rather than *de novo* synthesis using the biosynthetic machinery of the cell. One of the most striking examples of this immediate response is the rapid, transient, and large release of ROS, termed *oxidative burst*, which is analogous to respiratory burst in mammalian cells. The function of ROS is not just limited to defense response; subsequent studies identified its vital role in a broad range of physiologic processes in plants, such as removal of aleurone layer, stomatal closure, allelopathy, root elongation, and gravitropism [15,61–64]. Current understanding of the mechanisms of respiratory burst largely derived from studies of effects of elicitors on suspension-culture plant cells. Doke [65] showed for the first time that potato tuber discs exposed to an avirulent strain of *Phytophthora infestans* generated an NAD(P)H-dependent rapid burst of ROS production. Furthermore, using a cytochrome C assay and nitroblue tetrazolium reduction assay, these investigators identified O_2^- as the major ROS released from the cells. Infection with a virulent strain of the same pathogen failed to induce ROS. Later, Jabs and colleagues [66] identified that diphenylene iodonium (DPI), an inhibitor of mammalian NAD(P)H oxidase, abrogated this ROS generation. Cell fractionation studies indicated the presence of NADPH-dependent ROS-inducing activities in plasma-membrane, similar to the presence of cell-membrane components of human NAD(P)H oxidase [67]. Collectively, these studies provided presumptive evidence that NAD(P)H oxidase exists in plants.

Respiratory burst oxidase homologues

Groom and colleagues [68] identified the first plant NAD(P)H oxidase gene in *Oryza sativa* and termed it *respiratory burst oxidase homologue* (*Rboh*) because of its homology to human *gp91^{phox}*. Several *Rboh* genes were later identified in many other plant species, including *Arabidopsis*, tomato, tobacco, and potato [11,69–71]. *A. thaliana* *Rboh* (*Atrboh*) genes are very well characterized and are described here as representative of similar *Rboh* genes in other plant species. Currently, 10 *Atrboh* genes have been

identified that code for highly similar proteins [69]. All Rboh proteins contain six transmembrane domains followed by FAD and NAD(P)H domains near their C-terminal end. The N-terminal regions boast two EF hands with a structural similarity to mammalian NOX5 and DUOX1 proteins. The EF hands can bind Ca^{2+} , allowing direct regulation of oxidase activity and O_2^- production through Ca^{2+} signaling [12]. This mechanism contrasts with that for mammalian gp91^{phox}, which lacks the EF hands and therefore depends on association with cytoplasmic components of NAD(P)H oxidase to generate O_2^- . Unlike mammalian gp91^{phox}, the Rboh crystal structure is not solved; however, a hypothetical model of its organization in plant cell membrane is shown in Fig. 5. Another common feature between mammalian and plant NAD(P)H oxidase exists in the form of regulatory proteins. Several Rho-like proteins (ROPs, also known as RAC) have been identified in different species of plants that differentially regulate activity of NAD(P)H oxidase during defense signaling and other physiologic processes [72–74].

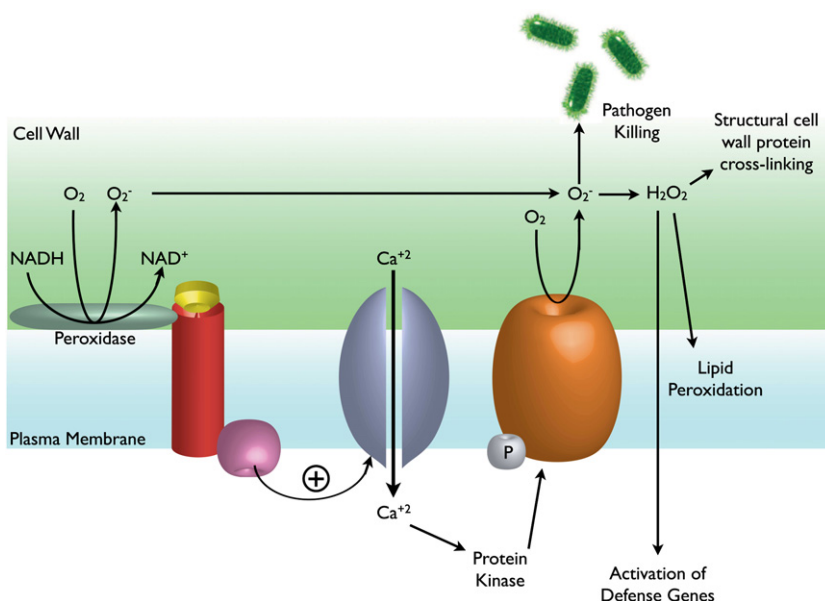


Fig. 5. A speculative model of plant NAD(P)H oxidase. Binding of elicitor (yellow) to its receptor (red barrel) in the membrane may stimulate membrane-associated peroxidases (green disk) through unknown mechanisms, which result in O_2^- synthesis. Alternatively, elicitor receptors coupled to G-proteins (pink sphere), and increased intracellular Ca^{2+} caused by Ca^{2+} channel opening (gray channel) and activation of protein kinase, may activate membrane-bound NAD(P)H oxidase (orange) through phosphorylation (gray sphere with P), resulting in conversion of O_2 to O_2^- . The O_2^- can be converted to H_2O_2 and these ROSs can induce pathogen killing, structural cell-wall protein cross-linking, and expression of various genes to constitute a full-blown defense response. (Modified from Mehdy MC. Active oxygen species in plant defense against pathogens. *Plant Physiol* 1994;105:467–72; with permission.)

Polyclonal antibodies raised against human p22^{phox}, p47^{phox}, and p67^{phox} have detected cross-reacting plant proteins of similar molecular sizes [75,76]. However, plant genes encoding any of these components have never been reported.

Oxidative stress and allergic inflammation

All aerobic organisms use O₂ in one form or another to generate energy requirements for various metabolic processes and to maintain cellular homeostasis. However, the high energy-generating aerobic metabolism comes at a cost. ROS are generated as byproducts of the oxidative metabolism, and cells neutralize them through various antioxidant defense mechanisms. When the delicate balance between ROS and antioxidants tips toward ROS, cellular components are damaged, resulting in various diseases involving practically every organ and system. ROS have been implicated in allergic disorders such as allergic rhinitis and atopic dermatitis [77–79].

In the respiratory system, NAD(P)H oxidase-generated ROS play an important role in physiologic processes such as airway and vasculature remodeling and O₂ sensing [80] at the same time excessive ROS play a key role in pathogenesis of allergic asthma and acute respiratory distress syndrome [81,82]. Exhaled H₂O₂ and nitric oxide (NO) are increased in patients who have allergic asthma compared with controls [83,84]. Also, the level of ROS in the airways is inversely correlated to the predicted forced expiratory volume in 1 second in patients who have asthma [85]. High levels of ROS are also encountered in the exacerbation of allergic asthma [86]. The direct effects of ROS on airway smooth muscle and mucin production are considered the mechanisms through which ROS induce and exacerbate allergic asthma [87,88]. ROS can inhibit β -adrenergic function in the airway smooth muscle cells and make them prone to acetylcholine-induced airway contraction [89,90].

Inflammatory cells such as macrophages, neutrophils, and eosinophils are considered the primary source of O₂⁻ anions in the airways. Airway macrophages in patients who have asthma produce more O₂⁻ than those of control subjects [91]. Even the monocytes and eosinophils collected from the blood of patients who have asthma produce more ROS compared with those of control subjects [91]. Environmental pollutants such as ozone, diesel exhaust, and cigarette smoke also increase O₂⁻ production in the lungs and worsen the symptoms of allergic asthma [92–94]. These studies unraveled the important role of ROS in allergic airway inflammation, and considered inflammatory cell-generated and environmental ROS to be the major source. Many patients have sensitivity to plant pollens and develop allergic symptoms on exposure to the specific plant pollens. Plant tissues possess NADPH oxidase and generate ROS when activated. Intrigued by this observation, the authors sought to determine whether pollens contain NADPH oxidase and, if so, what role pollen NADPH oxidase plays in allergic airway inflammation.

Pollen NADPH oxidase in allergic inflammation

Pollens are complex biologic entities that deliver sperms to the ovule in flowering plants. For reproductive function, matured pollen must germinate a polarized pollen tube that grows toward the ovule. The process of pollen tube growth is highly structured and regulated by Rac homologs and Ca^{2+} signaling [95,96]. Because NAD(P)H oxidase is present in various plant tissues, and homologs of Rac2 are present in pollens and pollen tubes [97], the authors hypothesized that NAD(P)H oxidase components were present in plant pollens and discovered that many plant pollen extracts contain NADH/NAD(P)H-dependent oxidase activity [16]. The authors showed that ragweed and other pollen extracts reduce nitroblue tetrazolium chloride (NBT) to formazan in the presence of NAD(P)H (Fig. 6A). Ragweed preparations that lacked NAD(P)H oxidase, such as Amb a 1 and heat-treated Ragweed extract (RWE^H), did not reduce NBT (see Fig. 6A). The authors

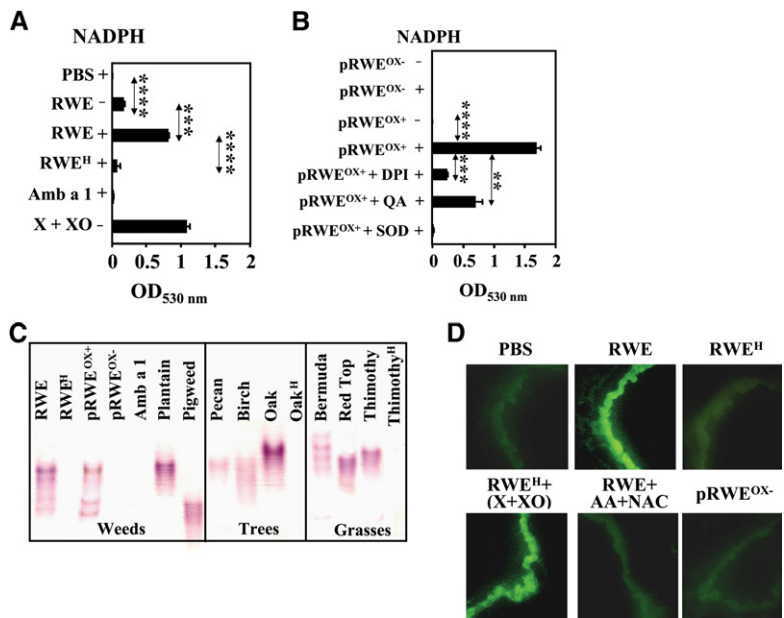


Fig. 6. Identification of pollen NAD(P)H oxidases. Plant pollen extracts possess NAD(P)H oxidase activity. (A) Reduction of NBT to formazan by allergenic extracts using NBT assay. RWE, RWE^H, and Amb a 1 were tested. X+XO was used as positive control. (B) pRWE^{OX+}-induced NBT reduction is inhibited by NAD(P)H oxidase inhibitors, DPI and quinacrine, and by the superoxide scavenger, SOD. (C) NBT reduction by allergenic extracts in situ after nondenaturing polyacrylamide gel electrophoresis. Oak^H, RWE^H, and Timothy^H are heat-inactivated extracts. (D) Change in ROS levels in lung epithelium of mice after ex vivo challenge as determined through DCF fluorescence. (Reproduced from Boldogh I, Bacsí A, Choudhury BK, et al. ROS generated by pollen NAD(P)H oxidase provide a signal that augments antigen-induced allergic airway inflammation. J Clin Invest 2005;115(8):2169-79; with permission.)

fractionated RWE using size-exclusion chromatography and identified fractions that possessed or lacked NAD(P)H oxidase activity, termed pRWE^{OX+} and pRWE^{OX-}. As hypothesized, pRWE^{OX+}, but not pRWE^{OX-}, reduced NBT in the presence of NAD(P)H (Fig. 6B). Adding NAD(P)H oxidase inhibitors DPI and quinacrine and the superoxide scavenger superoxide dismutase blocked this activity, suggesting the presence of NAD(P)H oxidase in the extracts. Furthermore, RWE also reduced redox-sensitive 2'-7' dihydro-dichlorofluorescein diacetate (H₂ DCF-DA) into fluorescent DCF. These experiments suggested the generation of O₂⁻ anions, which was confirmed through cytochrome C assay. In situ NBT gel assay on different pollen extract proteins after nondenaturing polyacrylamide gel electrophoresis showed well-defined protein bands only in the presence of NAD(P)H (Fig. 6C).

After the authors confirmed the presence of NAD(P)H oxidase in ragweed and other pollen extracts, they determined its role in allergic inflammation. They showed that RWE induces ROS in airway epithelial cells grown at the air-liquid interphase and in the murine airways (Fig. 6D). RWE^H and pRWE^{OX-} resulted in minimal induction of ROS in either cells or the airways (see Fig. 6D). Using a murine model of allergic airway inflammation, the authors showed that RWE-induced inflammatory cell and eosinophil recruitment and mucin production were dependent on NAD(P)H oxidase activity of RWE (Fig. 7A-C), and observed a similar phenomenon in a murine model of allergic conjunctivitis. They proposed a two-signal model for the development of allergic inflammation, wherein signal 1 constitutes pollen NAD(P)H oxidase induced ROS and signal 2 is induced by antigen recognition through adaptive immune response. Both signals are required for robust allergic inflammation, and compromising either signal substantially reduces allergic process. Thus, pollen NAD(P)H oxidase-induced ROS plays a major role in allergic inflammatory processes in the airways and conjunctiva.

Summary

ROS are inevitable byproducts of various physiologic processes in the body. Maintenance of homeostatic balance between production of ROS and the mechanisms that scavenge ROS is a cornerstone of preventing pathogenesis of many diseases. Oxidative stress is the end result of numerous molecular pathways and mechanisms in which the endogenous antioxidant defenses yield to free radicals. Apart from known sources of ROS, pollen NAD(P)H oxidase constitutes an important enzyme contributing to asthma pathogenesis. Similar to mammalian cells, plants have developed sophisticated mechanisms for self-defense, including NAD(P)H oxidase-induced ROS. NAD(P)H oxidase has been identified in various plant species that is homologous to human gp91^{phox}. The NAD(P)H oxidase present in many plant tissues, including pollens, regulate various physiologic processes.

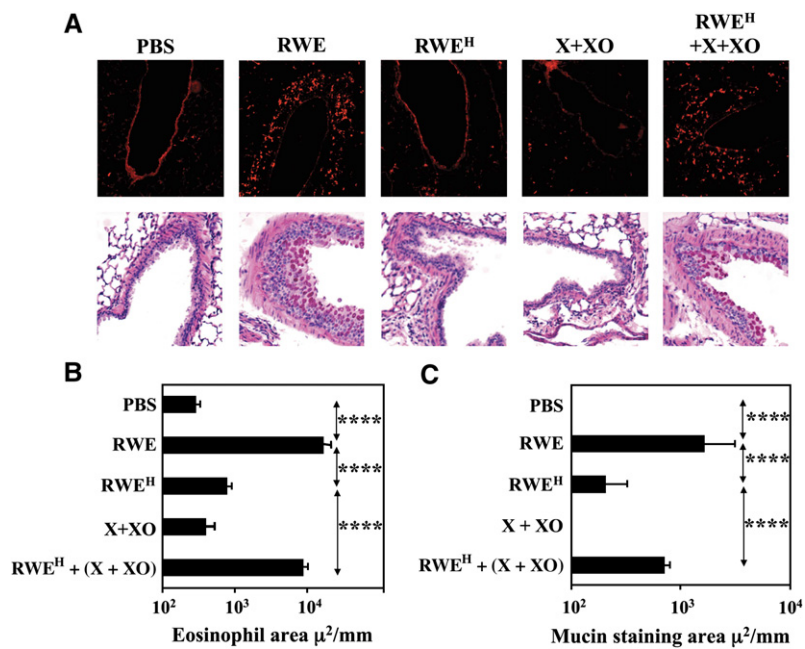


Fig. 7. ROS induced by NAD(P)H oxidase are required for allergic lung inflammation and mucin production. (A) Removal of NAD(P)H oxidase activity from RWE decreases accumulation of eosinophils in peribronchial location and mucin-containing cells in airway epithelium. Immunohistochemical staining of lung cryosections showing accumulation of eosinophils in peribronchial regions of lungs (upper panel). Periodic acid Schiff (PAS)-staining for mucin in representative lung sections (lower panel). Magenta-colored epithelial cells are positive for mucin. Magnification, 100X. (B) Total area of eosinophils in peribronchial area was quantified by morphometric analyses using Metamorph software. (C) Total area of mucin-containing cells in peribronchial area was quantified through morphometric analyses. Results are means $\pm$ s.e.m. (n = 7–9 mice per group). *** P < .001; **** P < .0001. (Reproduced from Boldogh I, Bacci A, Choudhury BK, et al. ROS generated by pollen NAD(P)H oxidase provide a signal that augments antigen-induced allergic airway inflammation. J Clin Invest 2005;115(8):2169–79; with permission.)

The same NAD(P)H oxidase that provides an important defense mechanism for plants is detrimental to the cells lining the airways in humans when they generate ROS and perpetuate allergic airway inflammation. The authors identified an important mechanism through which plant pollens induce allergic inflammation in the lungs and conjunctiva, which is just a beginning in the new direction to understanding the molecular mechanisms of allergic inflammation. Much work needs to be done before the entire mechanistic pathway can be identified that is activated during exposure of airway cells to ROS. Although these studies would be very challenging because of the short half-life of the ROS, this area is worth exploring considering its ubiquitous presence in many human diseases.

Important questions and future directions

Currently, only one homolog of the human NAD(P)H oxidase is identified in plants. Plants likely possess other components similar to mammalian NAD(P)H oxidase, and the discovery of these will help experts understand in detail the process of ROS generation in plants. The signaling pathway in cells from the moment of their exposure to ROS to the end result (allergic inflammation) is still largely unknown. Identification of effects of ROS on genome-wide changes in the cells and its expression pattern will provide crucial links to current understanding. The effect of plant NAD(P)H oxidase on allergic airway inflammation in humans is not known. Therefore, studies should be conducted to answer these questions and devise therapeutic strategies to control pollen-induced allergic inflammation. Development of a newer generation of antioxidants that has a long biologic half-life at the local site would be of immense importance for treating allergic inflammation.

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