Comparison of the Oxidative Burst Signaling Pathways of Plants and Human Neutrophils.

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I. Introduction

In both the plant and animal kingdoms, host defense appears to occur in 3 stages: pathogen recognition, pathogen destruction, and the strengthening of physical barriers to retard spread of surviving pathogens/parasites. [1,2] Although the mechanisms of pathogen destruction appear quite diverse between the two kingdoms, these mechanisms still follow the same basic set of strategies. Thus, both kingdoms attempt to poison the pathogen, [1-3] lyse the pathogen's cell membranes, [2-4] starve the pathogen by inducing their own infected cells to commit suicide, [5,6] or oxidize the pathogen.[7,8] The latter killing strategy, i.e. the release of superoxide and hydrogen peroxide in the direct vicinity of the invading organism, has attracted recent attention among plant biochemists, both with regard to its possible functions and mechanisms of formation.[9,10] As we have engaged in our own investigation of this pathway, we have been struck by both the similarities and differences between the plant and animal oxidative bursts. In this publication we compare the pathways of the two kingdoms and point out where they are similar and where they are different.

II. Background on the Oxidative Burst in Neutrophils

Mammals have many types of blood cells whose specialized functions collectively lead to recognition and destruction of invading pathogens. After transport through the circulatory system to a site of infection, the granulocytes (e.g. neutrophils), lymphocytes, and monocytes, etc. extravasate in response to cytokines and chemotactic peptides. The activated immune cells then migrate into the inflamed tissue where they attack pathogens and infected host cells that are marked by surface bound antibodies/

complement [11] or exposed complexes of MHC-I with foreign peptides.[12] Killing of the pathogens then arises from release of oxidants, lysis of the cell membranes, discharge of toxic compounds stored in intracellular granules, induction of apoptosis in infected cells, and phagocytosis and digestion of the foreign invaders. Among these cytotoxic mechanisms perhaps the fastest is the production of superoxide (O_2^-), which is rapidly dismutated to hydrogen peroxide (H_2O_2) and other more toxic oxidants.[13,14] Without this oxidative threat, a condition identified in humans as chronic granulomatous disease arises, where affected individuals are extremely susceptible to infectious organisms.[15,16]

III. Recognition of the Pathogen by Plant and Animal Cells

In neutrophils, many different ligands can act at cell surface receptors to trigger the oxidative burst. Examples include antigen-antibody complexes that bind to F_c receptors,[17] clustered complement fragments (e.g. C3b) that recognize complement receptors, [18] chemotactic peptides such as N-formyl-Met-Leu-Phe (fMLP) that dock at specific peptide receptors,[19] and various cytokines that associate with their individual cytokine receptors (Fig. 1). The latter stimulants, which include tumor necrosis factor,[20] and granulocyte/macrophage colony-stimulating factor (GM-CSF) [21] are generally released by other cells of the immune and circulatory systems that have already detected the pathogen's presence.

Less is known about induction of the oxidative burst in plants. Crude extracts from many fungi and bacteria trigger the rapid production of H_2O_2 in cultured plant cells, but few purified elicitors have been characterized. One potent elicitor that has been isolated is a cell wall breakdown product termed polygalacturonic acid (PGA). An oligomer of PGA with an approximate size of 10-16 sugar residues has been found to specifically stimulate the oxidative burst in soybean cells [8]. Preliminary studies indicate the presence of a plasma membrane receptor that binds the elicitor,[22] transmits the defense signal across the plasma membrane, and then endocytoses into the cell interior from where it is eventually deposited in the vacuole.[23] Since polymers of galacturonic acid are found in components of the plant cell wall, partial degradation of the cell wall by an invading microbe should create these PGA elicitors in close proximity to the plasma membrane, where the PGA can then bind to its receptor and trigger an oxidative burst.

In addition to ligand-mediated activation of the oxidative burst, both the neutrophil and soybean produce an oxidative burst upon dilution into media of lower osmotic strength.[24,25] For example, dilution of neutrophils from 300 mOsM to 100 mOsM initiates

biosynthesis of $O_2^{\cdot-}$ in the absence of any stimulating ligand. Similarly, dilution of a soybean cell suspension by as little as 25% with distilled water induces a typical oxidative burst [25]. In both kingdoms, this osmotic induction is rapidly reversed upon restoring normal tonicity, suggesting the response is still controlled by osmotic or mechanical regulatory mechanisms of a functioning cell. Furthermore, cross-talk and cross-desensitization are observed between ligand-stimulated and dilution-induced pathways. While the function of the osmotically-triggered burst in neutrophils is uncertain, we speculate that the same pathway in plants has evolved to respond to osmotic stress, cell expansion, and other mechanical signals. Thus, when the cell wall is subjected to excessive distortion or strain, release of H_2O_2 may promote de novo polymerization of cell wall components[26] or reinforcement of existing structures. Either process might protect against collapse of the cell wall under pressure or facilitate new cell wall deposition around an expanding or growing cell.

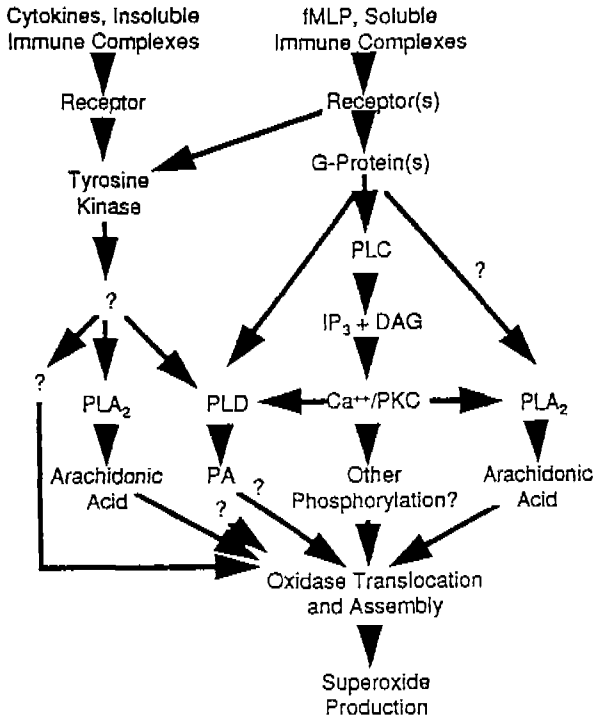

Fig. 1 Possible organization of signaling steps in generating the neutrophil oxidative burst. Abbreviations are: fMLP, N-formyl-Met-Leu-Phe; PLC, phospholipase C; IP_3, inositol trisphosphate; DAG, diacylglycerol; PKC, protein kinase C; PLA_2, phospholipase A_2; PLD, phospholipase D; PA, phosphatidic acid.

Once the oxidative burst is stimulated in both the animal and plant kingdoms, the physiology of the process appears similar. Following addition of an activating ligand, a lag period of 0.5 to 2 min is experienced before $O_2^{-\cdot}$ or H_2O_2 can be detected.[8-27] Thereafter, H_2O_2 is released at a rate of $\sim 10^{-14}$ mole/cell/min, resulting in accumulation of H_2O_2 in excess of 1 mM in the direct vicinity of the stimulated cell [27,8]. Following a productive period of upwards of 15 min, the generation of oxidants gradually subsides to near zero in both kingdoms. At this point, both plant and animal cells are refractory to further stimulation, at least for a very extended period.

IV. Second Messengers Along the Oxidative Burst Pathway

1. Heterotrimeric G Proteins.

The probable involvement of heterotrimeric G proteins in the neutrophil oxidative burst was implicated several years ago by the inhibitory effects of purtussis toxin on several ligand activated bursts.[28,29] However, for a few ligands, this inhibition was only partial,[30] implying the existence of pertussis toxin-insensitive pathways in triggering $O_2^{-\cdot}$ production. Cholera toxin was also found to specifically ADP-ribosylate G_{i2} and G_{i3} upon cell stimulation by fMLP,[31] and indeed, the predominant protein found associated with the purified fMLP receptor was G_{i2}.[32] Furthermore, activators of G proteins (e.g. GTP—S and AlF_4^-) have been demonstrated to stimulate $O_2^{-\cdot}$ production,[33] and the burst in permeabilized neutrophils has been shown to be dependent on the presence of GTP.[34] Finally, several of the relevant neutrophil receptors have been cloned, and they each reveal the classical G protein-coupled receptor hydropathy plots with expected conserved sequences.[19-35] Taken together, the involvement of G proteins in at least one pathway leading to the neutrophil oxidative burst seems assured (Fig. 1).

G-proteins have also been found to be involved in the oxidative burst of cultured soybean cells. Antibodies against a peptide of a highly conserved region in G-proteins specifically recognizes a protein of the anticipated 45kDa molecular weight.[36] Furthermore, the soybean G protein is ADP-ribosylated *in vivo* by an endogenous ADP ribosyl transferase, much like animal G proteins can be ADP-ribosylated by bacterial toxins. Most importantly, the oxidative burst in soybeans is dramatically enhanced by delivery of F_{ab} fragments of the anti-G protein antibody into the cultured cells. In contrast, intracellular delivery of the heat denatured F_{ab} has no effect. Taken together, these data also argue for involvement of a heterotrimeric G protein in the plant burst pathway.

An independent line of evidence for G protein participation in the soybean oxidative burst has come from studies with mastoparan, a 14 amino acid peptide from wasp venom. This peptide, which has been traditionally exploited as an exogenous stimulant of G proteins in animal cells, was found to independently induce a mild oxidative burst in plants. The activation was both saturable and concentration dependent. More importantly, Mas-17, an inactive analog of mastoparan that differs at two amino acids, causes no stimulation of H_2O_2 production.

2. Phospholipase C

At least one down-stream effector of G proteins in the neutrophil is phospholipase C.[37] Administration of GTP—S or fluoride is reported to induce phospholipase C activity, whereas inhibition of the fMLP-stimulated G protein by pertussis toxin is observed to abolish the enzyme's activity [28]. Direct measurements of the products of phospholipase C catalysis in activated neutrophils also confirm the involvement of this signal transducer. Thus, fMLP-stimulated neutrophils catalyze a rapid turnover of phosphoinositides, leading to a transient rise in cytoplasmic inositol trisphosphate (IP_3) and a concomitant elevation of diacylglycerol (DAG). The increase in IP_3 within 10s of stimulation has been further shown to be sufficient to facilitate release of Ca^{++} into the cytoplasm.[38] Finally, the phospholipase inhibitor U-73122AB, has been demonstrated to both inhibit $O_2^{\cdot-}$ production and block the rise in IP_3, DAG , and cytoplasmic Ca^{++}[39]. Thus, the classical phophotidylinositol signaling pathway may play a prominent role in inauguration of the neutrophil oxidative burst (Fig. 1).

Phospholipase C activation has also been found to participate in signaling the oxidative burst in plants. Stimulation of H_2O_2 production by either polygalacturonic acid or mastoparan (but not the inactive analog, Mas 17) was found to trigger a transient rise in IP_3 and a decrease in phosphatidylinositol 4,5-bisphosphate and phosphatidylinositol monophosphate.[40] The increase in IP_3 reached a maximum by 1 min post-stimulation and directly preceded the appearance of H_2O_2 in the same cell suspension. Since neomycin sulfate, a phospholipase C inhibitor, blocked IP_3 production and significantly reduced H_2O_2 release (by 60%) over the same concentration range, the involvement of the phosphatidylinositol pathway in eliciting an H_2O_2 burst in soybeans seems secure. Importantly, the reciprocal observation, that phosphatidylinositol turnover can be quantitatively eliminated without total abrogation of H_2O_2 generation also suggests that a phospholipase C-independent pathway for stimulating the burst in the plant kingdom may exist. Other observations (e.g. the dilution-induced oxidative burst does not proceed through an IP_3 intermediate) support this contention.

3. Phosphorylation

Phosphorylation probably contributes to several steps of the oxidative burst signaling cascade in neutrophils. Undoubtedly the best characterized among the responsible kinases is protein kinase C [41]. Thus, phorbol esters, artificial mimics of diacylglycerol (the natural activator of protein kinase C), appear to autologously initiate the pathway somewhere downstream of G proteins. Furthermore, inhibitors of protein kinase C, though undoubtedly active against other kinases also, significantly reduce the level of $O_2^{\cdot-}$ biosynthesis by stimulated cells.[42-43] While the specific protein kinase C isozyme that participates in triggering the burst in neutrophils remains to be identified, these and other in vitro studies [44] suggest at least one branch of the signaling pathway involves this well known kinase. Importantly, many of these same experiments reveal that other serine/threonine kinases, including calmodulin-dependent protein kinases [45] and as yet unidentified neutrophil kinases,[46] may also contribute essential steps to the signaling pathway (Fig. 1).

Recent studies of neutrophil activation also demonstrate the involvement of a separate tyrosine kinase-dependent signaling cascade.[47] Thus, fMLP not only initiates the phospholipase C-dependent pathway, but it also promotes the *in vivo* tyrosine phosphorylation of members of the MAP kinase family.[48] Since these kinases frequently occupy sites of convergence of feeder branches of common signaling routes, the MAP kinases may also represent a site of merger of the phospholipase C and tyrosine kinase dependent pathways. Regardless, the profound effects of tyrosine kinase inhibitors such as genistein on the neutrophil oxidative burst combined with the observation that phospholipase C independent routes to neutrophil activation exist together argue for a separate tyrosine kinase dependent arm of the pathway (Fig. 1).

A final line of evidence for the role of phosphorylation in the neutrophil oxidative burst is the finding that protein phosphatase inhibitors can autologously initiate the response. For example, simple addition of high concentrations of okadaic acid to resting granulocytes can promote generation of $O_2^{\cdot-}$, suggesting the phosphorylation reactions that initiate the response are contitutively operative. Curiously, when combined with natural inducers of the oxidative burst, the effects of phosphatase inhibitors can be very different. Thus, okadaic acid prolongs $O_2^{\cdot-}$ production initiated by fMLP, but inhibits oxidant biosynthesis by phorbol esters.[49]

In plants much less is known about the kinases and phosphatases that regulate assembly of the oxidase machinery. While the degree of phosphorylation of several proteins changes upon stimulation of the burst pathway in soybeans,[50] there is no indication whether these changes arise from kinase activation or phosphatase inhibition.

However, one clue that both processes may be important comes from the observations that okadaic acid or calyculin A (phosphatase inhibitors) can independently initiate the burst in plants (as they can in animals), and that a variety of protein kinase inhibitors can block the same response.[51]

V. Nature of the Oxidase Complexes

In neutrophils it is known that four proteins assemble on the membrane to form the active oxidase complex that transfers electrons from cytosolic NADPH to extracellular oxygen to generate $O_2^{\cdot-}$ (Fig. 2).[52] One of these components is the integral membrane flavocytochrome B_{558}[53] It is made up of two subunits, the 22 kDa subunit, p22-phox, and the 91 kDa glycosylated subunit, gp91-phox. Cytochrome B_{558} contains the NADPH binding site,[54] the FAD binding site,[55] and two hemes. It also serves as the membrane docking site for the other three proteins in the complex which are cytosolic in unstimulated neutrophils.[56]

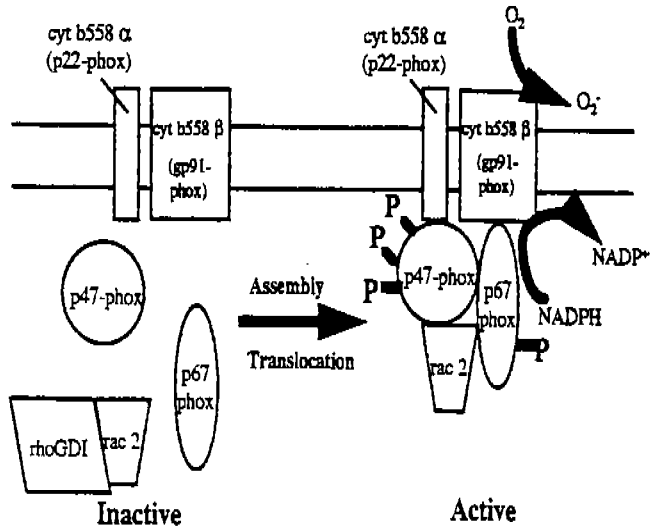

Fig. 2 Conversion of inactive oxidase subunits into an active complex on the neutrophil plasma membrane. The cyt b558 subunits are constitutively present in the membrane. Upon activation, rho GDI dissociates fro m rac2, allowing its activation by GTP in the neutrophil cytosol. Concurrently, p47-phox and p67-phox are each multiply phosphorylated, probably promoting their association via SH3 domains into an active oxidase complex with cyt b558 on the membrane.

The cytosolic components of the neutrophil oxidase are p47-phox,[57] p67-phox,[58] and

the small molecular weight GTPase rac2. [59] To date p47-phox and p67-phox have no known homologues in any other system of the body. Both p47-phox and p67-phox are multiply phosphorylated upon stimulation of neutrophils.[60-61] Thus, p47-phox exists in eight different phosphorylation states, the most heavily phosphorylated of which is generated once it has translocated to the membrane 60]. In the resting state, rac2 is bound to a guanine nucleotide dissociation inhibitor, rhoGDI. Upon stimulation of the neutrophil, rac2 separates from rhoGDI and translocates to the membrane.[62] It is not known how these cytosolic components activate the electron transport machinery of cytochrome B_{558}

In plants, the exact nature of the oxidase complex is not known. However, there is now evidence that at least two of the components of the oxidase complex in neutrophils, p47-phox and p67-phox, are also present in soybeans and other plant species. Antibodies to human neutrophil p47-phox and p67-phox stain specific single bands at the expected molecular weights of 47 kDa and 67 kDa when blotted against protein extracts from soybeans and other plant species.[63]

VI. Conclusion

There are many similar signaling elements in the oxidative burst pathways of plants and animals. They both bind pathogen generated ligands via specific receptors. They both employ heterotrimeric G-proteins, phospholipase C's, and protein kinases and phosphatases. There is also evidence that at least some of the components of the oxidase complex are homologous.

However, many differences between the pathways of the two kingdoms aHowere , many differences between the pathways of the two kingdoms also exist. Phospholipase A_2 is involved in the oxidative burst in neutrophils [64] but not utilized for that purpose in plants.[65] Phospholipase A may be involved in plant defense signaling, but it likely leads to the production of phytoalexins and not H_2O_2 [65]. Also H^+ extrusion is not a required step in the plant oxidative burst [66] as it is in neutrophils.[67] And finally, the involvement of tyrosine phosphorylation and phospholipase D activity in the plant oxidative burst has not been demonstrated or disproven.

Acknowledgments

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