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Original Contribution

Origin of the phagocytic respiratory burst and its role in gut epithelial phagocytosis in a basal chordate

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ABSTRACT

The vertebrate phagocytic respiratory burst (PRB) is a highly specific and efficient mechanism for reactive oxygen species (ROS) production. This mechanism is mediated by NADPH oxidase 2 (NOX2) and used by vertebrate phagocytic leukocytes to destroy internalized microbes. Here we demonstrate the presence of the PRB in a basal chordate, the amphioxus *Branchiostoma belcheri tsingtauense* (bbt). We show that using the antioxidant NAC to scavenge the production of ROS significantly decreased the survival rates of infected amphioxus, indicating that ROS are indispensable for efficient antibacterial responses. Amphioxus NOX enzymes and cytosolic factors were found to colocalize in the epithelial cells of the gill, intestine, and hepatic cecum and could be upregulated after exposure to microbial pathogens. The ROS production in epithelial cell lysates could be reconstructed by supplementing recombinant cytosolic factors, including bbt-p47phox, bbt-p67phox, bbt-p47phox, and bbt-Rac; the restored ROS production could be inhibited by anti-bbt-NOX2 and anti-bbt-p67phox antibodies. We also reveal that the gut epithelial lining cells of the amphioxus are competent at bacterial phagocytosis, and there is evidence that the PRB machinery could participate in the initiation of this phagocytic process. In conclusion, we report the presence of the classical PRB machinery in nonvertebrates and provide the first evidence for the possible role of PRB in epithelial cell immunity and phagocytosis.

The phagocytic respiratory burst (PRB) is a sophisticated mechanism for reactive oxygen species (ROS) production that has evolved in vertebrates and been used by migratory phagocytes to destroy phagocytosed microbes [1]. NADPH oxidase 2 (NOX2; also known as phox (phagocytic oxidase) or gp91phox) is the transmembrane catalytic subunit for the PRB, which is coupled with another transmembrane subunit, p22phox, to gain optimal activity and exportation to plasma membrane. In the resting state, NOX2–p22phox remain inactive by being separated from their cytosolic components—p47phox, p67phox, p40phox, and the GTPase Rac; at the initiation of phagocytosis, cytosolic regulators are relocated to the phagosomal or plasma membrane and bind NOX2–p22phox to trigger intense ROS production that may cause up to 50-fold oxygen consumption by phagocytes (Fig. 1A) [2–4]. The remarkable advantage of the PRB is the mobility endowed by migratory phagocytes and the high microbicidal activity and specificity achieved by maximizing local ROS concentration and minimizing global oxygen consumption and collateral damage.

In addition to NOX2, there are six other NOX family members in mammals, including NOXs 1, 3, 4, and 5 and DUOX1 and DUOX2. Though many of their physiological functions remain elusive, it is generally accepted that NOXs are broadly implicated in host defense, signaling, and biosynthesis [3,5–9]. NOX1 participates in colon epithelial immunity and cellular signaling in the vascular, nervous, and respiratory systems [10–13]. NOX3 participates in gravity perception of the inner ear and defects in NOX3 may cause hearing loss [14,15]. NOX4 is implicated in signal transduction in renal and vascular epithelia [16,17]. NOX5 participates in

Abbreviations: bbt, *Branchiostoma belcheri tsingtauense*; CYBA, cytochrome *b* light chain; DHE, dihydroethidium; DPI, diphenyleneiodonium; DUOX, dual oxidases; NAC, *N*-acetyl-l-cysteine; NBT, nitroblue tetrazolium salt; NCF, neutrophil cytosolic factor; NOX2, NADPH oxidase-2; NOXA1, NADPH oxidase activator 1; NOXO1, NADPH oxidase organizer 1; PRB, phagocytic respiratory burst; Rac, Ras-related C3 botulinum toxin substrate; ROS, reactive oxygen species; SH3, Src homology 3 domain; TEM, transmission electron microscopy; TLR, Toll-like receptor; TNF, tumor necrosis factor

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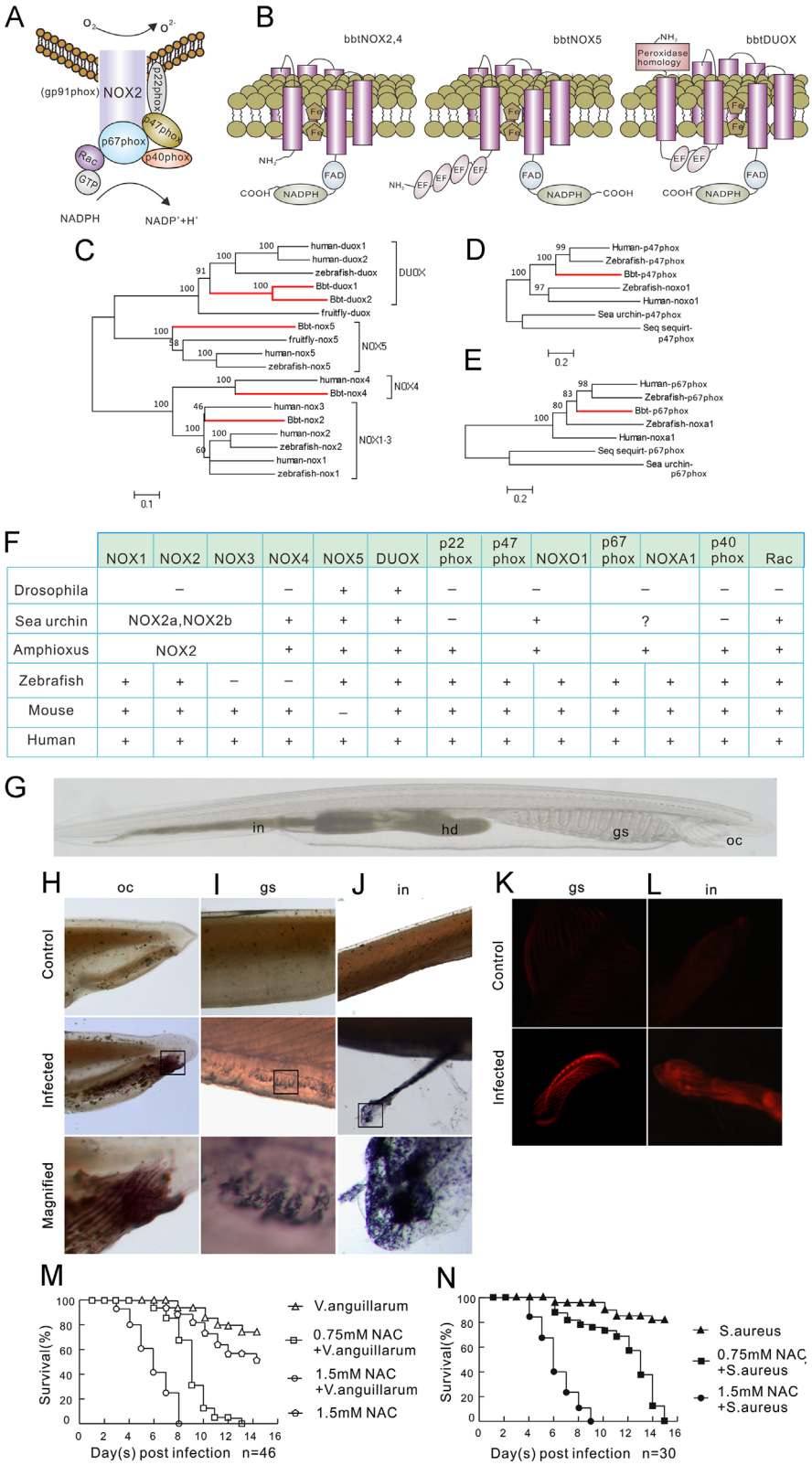

	NOX1	NOX2	NOX3	NOX4	NOX5	DUOX	p22 phox	p47 phox	NOXO1	p67 phox	NOXA1	p40 phox	Rac
Drosophila	–			–	+	+	–	–		–		–	–
Sea urchin	NOX2a,NOX2b			+	+	+	–	+		?		–	+
Amphioxus	NOX2			+	+	+	+	+		+		+	+
Zebrafish	+	+	–	–	+	+	+	+	+	+	+	+	+
Mouse	+	+	+	+	–	+	+	+	+	+	+	+	+
Human	+	+	+	+	+	+	+	+	+	+	+	+	+

Fig. 1. (A) The scheme of the supercomplex of the PRB machinery. NOX2 and p22phox are transmembrane components. Rac, p47phox, p67phox, and p40phox are cytosolic subunits that are relocated to bind with NOX–p22phox on activation. (B) The scheme of the architecture of amphioxus NOX enzymes. Bbt-NOX2 and bbt-NOX4 consist of six transmembrane domains, two heme (Fe) groups, one FAD-binding-8 domain, and a long NADPH-binding-6 cytoplasmic C-terminal. Bbt-NOX5 shares a backbone with bbt-NOX2 but has an N-terminal extension containing two to four EF-hand Ca^{2+}-binding sites. Bbt-DUOX shares its structure with bbt-NOX5, except that it has an additional N-terminal transmembrane domain and an extracellular peroxidase domain. The FAD-binding-8 domain of bbt-NOX2 shares almost 70% amino acid identity with its vertebrate homologs. (C–E) Phylogenetic analyses of amphioxus NOX2, p47phox, and p67phox protein sequences using the neighbor-joining method with 1000 bootstrap tests. Numbers on nodes indicate the bootstrap confidence values. (F) Comparison of the PRB gene repertoire in six species. "+", "−", and "?" indicate the gene status of presence, absence, and questionable, respectively. (G) Anatomy of a juvenile amphioxus. oc, oral cirri; gs, gill slit; hd, hepatic diverticulum; in, intestine. (H–J) NBT staining of the amphioxus exposed to *S. aureus* demonstrates formazan deposits on oc, gs, in, respectively. The controls are NBT staining of the amphioxus exposed to no bacteria. (K, L) Visualization of the ROS production in gs and in with the red fluorescent dye dihydroethidium. The amphioxus exposed to *S. aureus* shows stronger red fluorescent signals than the uninfected amphioxus (control). (M, N) Survival rates of amphioxus after infection by live *S. aureus* and *V. anguillarum* in the presence and absence of NAC. The survival rate experiments were repeated twice and showed similar results.