Mechanisms of Activation of NADPH Oxidases

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SUMMARY: The members of the NOX family of enzymes are expressed in a variety of tissues and serve a number of functions. There is a high level of conservation of primary protein sequence, as well as functional features, although specialized responses are beginning to emerge. In this context, our data demonstrate that the NOX1 cytoplasmic domains interact efficiently with the cytoplasmic subunits of the phagocyte NADPH oxidase and identify the second cytoplasmic loop of NOX electron transporters as a crucial domain for enzyme function. Studies of cytosolic co-factors showed that the C-terminal cytoplasmic domain of NOX1 was absolutely required for activation with NOXO1 and NOXA1 and that this activity required interaction of the putative NADPH-binding region of this domain with NOXA1. Finally, we have provided the first example of how alternative splicing of a NOX co-factor may be involved in the regulation of NADPH oxidase function.

NOX family of NADPH oxidases: The NADPH oxidase (NOX) gene family has been recently defined based on homology with the phagocyte respiratory burst oxidase, a well known multi-component enzyme system that plays a critical role in antimicrobial host defenses (1). The essential components of the phagocyte oxidase (designated *phox*) include a membrane-bound flavo-heme catalytic subunit comprised of a gp91^{phox}-p22^{phox} heterodimer, as well as the cytosolic co-factors p47^{phox}, p67^{phox}, and the small GTPase Rac 1 or Rac 2. The gp91^{phox} protein contains two heme groups associated with transmembrane segments, and binding sites in its cytoplasmic domains for FAD, NADPH, and the cytosolic co-factors. Stimulus-dependent assembly of the complete oxidase complex leads to catalyzed electron transport from cytosolic NADPH to molecular oxygen, producing the free radical superoxide anion (O_2^-) (2). Superoxide then serves as an intermediate for the formation of other reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^·$).

The NOX gene family has 5 members, all homologous to gp91^{phox}, which is also designated NOX2. Although the members of the family exhibit structural similarity, they differ greatly in their range

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of tissue expression, co-factor and activation requirements, rate of superoxide formation, and apparent physiologic functions. For example, NOX1 is highly expressed in colon epithelium, NOX2 in phagocytes, and NOX4 in kidney. The preferred cytosolic co-factors for NOX1 are homologues of p47^{phox} and p67^{phox}, designated NOXO1 (O=organizer) and NOXA1 (A=activator), respectively (3). NOX5 has an N-terminal extension containing Ca^{2+}-binding EF-hand domains and is directly activated by calcium (4). EF-hand domains are also found in the NOX-related DUOX proteins, which in addition, have an extracellular peroxidase domain. The highest rates of superoxide formation are observed with the phagocyte oxidase, presumably due to the need to generate toxic concentrations of ROS for microbial killing, the main function of this NOX family member. A similar function has been postulated for NOX1 in colon cells. In contrast, other NOX family members appear to produce much lower superoxide fluxes, compatible with their putative roles in cell signaling.

NOX1/gp91^{phox} structure-function analyses: Sequence comparisons of NOX1 and gp91^{phox} indicate relatively high conservation of cytoplasmic domains that are known to serve in gp91^{phox} as binding sites for NADPH, FAD, and cytosolic oxidase proteins. Thus, we generated chimeric molecules comprised of the external and trans-membrane domains of gp91^{phox} and selected cytoplasmic domains of NOX1. The 7D5 antibody was used to sort high-expressing cells. Interestingly, this approach has demonstrated that the cytoplasmic domains of NOX1 are fully competent for superoxide formation. Indeed, a chimera that contains all of the NOX1 cytoplasmic domains exhibits rates of superoxide formation 5- to 6-fold those of intact gp91^{phox}. The key NOX1 element responsible for this super-activity is the third cytoplasmic loop, which is identical to the same region of gp91^{phox}, save for three residues. Point mutations of this region identified NOX1 Glu195 as a critical residue and defined the overall role of this domain in enzyme function. In contrast, substitution of the cytoplasmic domains of gp91^{phox} into the NOX1 backbone resulted in diminished superoxide production. These data demonstrate that the NOX1 cytoplasmic domains interact efficiently with the cytoplasmic subunits of the phagocyte NADPH oxidase and identify the second cytoplasmic loop of NOX electron transporters as a crucial domain for enzyme function.

NOX interactions with cytosolic co-factors: Continuing this line of investigation, we have found that NOX1 produces superoxide with both the p47^{phox}/p67^{phox} and NOXO1/NOXA1 systems, although activity is more robust with NOXO1 and NOXA1. In contrast, gp91^{phox} does not produce an appreciable amount of superoxide with the non-phagocytic co-factors. Using the domain-swapping chimeric approach described above, we have investigated the ability of the intracellular domains of NOX1 and gp91^{phox} to interact functionally with NOXO1 and NOXA1. Exchange of cytoplasmic domains between gp91^{phox} and NOX1 revealed that: i) while gp91^{phox} was essentially inactive with NOXO1 and NOXA1, en bloc replacement of the gp91^{phox} domains with those of NOX1 resulted in a highly active gp91^{phox}-based chimera, ii) the C-terminal cytoplasmic domain of NOX1 was absolutely required for activation with NOXO1 and NOXA1, and iii) this activity required interaction of the putative NADPH-binding region of this domain with NOXA1.

Variant forms of the NOXO1 co-factor: Although NOX1 was originally identified in the human CaCo2 colon cancer cell line, these cells display only low superoxide-generating activity, suggesting the possible absence or modification of the NOXA1 and NOXO1 cofactors. By RT-PCR analysis of CaCo2 mRNA, we have identified a number of NOXO1 transcripts. Some of these are incomplete versions of the native 14-exon NOXO1 gene, in which a variable number of internal exons are deleted. However, two other variants are of particular interest. The first, NOXO1-*trunc*, contains a C to T transversion resulting in the change of a glutamine residue at position 274 to a termination codon. Further downstream is a nucleotide deletion and upstream are two codon-neutral single nucleotide changes. The multiple changes in this mRNA suggest that it is derived either from a mutated NOXO1 allele in CaCo2 cells or from a duplicate gene. To investigate the biologic function of NOXO1-*trunc*, we established a K562 cell line that stably expresses both NOX1 and NOXA1. Both transfected wild-type and variant NOXO1 supported superoxide generation in these cells (although with differing kinetic patterns), indicating that enzymatic activity requires neither the putative PB1 domain nor the SH3 domains in NOXO1.

The second NOXO1 variant identified in CaCo2 cells is even more interesting. This variant NOXO1-*inhib* is missing exons that encode the region of the putative activation domain, but more significantly, due to an alternative upstream splice acceptor site in exon 14, it has an additional heptapeptide sequence inserted into the C-terminal SH3 domain (SH3^{+7}). Transfection studies showed, not surprisingly, that this variant is unable to support superoxide generation. Interestingly, co-transfection studies indicated that NOXO1-*inhib* acts in a dominant negative manner, inhibiting the activity of both NOXO1 and NOXO1-*trunc*. To address the question of what effect this additional peptide in the SH3 domain might have on NOXO1 activity in the whole molecule, we prepared chimeric proteins between the N-terminal region of both wild-type NOXO1 and NOXO1-*trunc* and the C-terminal end of the NOXO1-*inhib* molecule. This completely blocked the activity of both molecules, despite the presence of the activation domain and high expression in the K562 cells. Furthermore, these chimeric proteins still acted in a dominant-negative fashion. These studies provide insights into the structural basis of NOXO1 function and give the first indications of how alternative splicing of the NOXO1 gene may regulate activity of the NOX1/NOXA1/NOXO1 system.

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