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Opening the black box: Lessons from cell-free systems on the phagocyte NADPH-oxidase

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Abstract

The NADPH-oxidase complex of phagocytic cells plays a key role in the defense against invading pathogens, through the release of superoxide anion, precursor of other reactive oxygen species (ROS). NADPH-oxidase deficiency is called Chronic Granulomatous Disease (CGD), in which patients suffer from recurrent infections and from the formation of granulomas in various organs. Research on NADPH-oxidase has much benefited from the discovery of cell-free systems, i.e. reconstitution assays from broken resting (unstimulated) phagocytes, in which activation of the oxidase is elicited *in vitro*. Cell-free systems were developed in parallel to studies of molecular defects of patients with CGD, both approaches leading to the identification of the major proteins implicated in enzyme activation. Variations around the cell-free system allowed molecular dissection of the mechanism of NADPH-oxidase activation and provided insights into its relationship to phagocytosis.

Keywords: NADPH-oxidase; Phox; Cell-free system; Phagocytosis; Cytochrome b_{558}

1. Introduction: Early history of the cell-free system

A broken cell system was used for studies of the regulation of adenylate cyclase by a cholera toxin sensitive G protein [1]. At the same time, the observation that phospholipase A_2 (PLA_2) inhibitors inhibited the NADPH-oxidase in intact phagocytes suggested that a PLA_2 product could be an activator. The two PLA_2 products, arachidonate and lysophosphatidylcholine, were therefore tested and arachidonate was shown to induce NADPH-dependent superoxide (O_2^-) generation by a nuclei-free cell homogenate, derived from guinea pig macrophages [2]. Three other groups discovered this effect of arachidonate almost simultaneously on horse and human neutrophils [3–5]. In these studies, a high speed supernatant (cytosol) of a cell homogenate, subjected to centrifugation, was mixed with a high speed pellet (membrane) in the presence of arachidonate or other long-chain unsaturated fatty acids, and O_2^- production was assayed by cytochrome *c* reduction after addition of the substrate, NADPH. Superoxide dismutase, that transformed O_2^- to H_2O_2, was then used to forestall cytochrome *c* reduction, as proof that cytochrome *c* was indeed reduced by O_2^- anions generated by the fatty acid-stimulated cell homogenate.

The cell-free system was such a valuable tool that, in the following few years, it was adapted, modified and extended to neutrophils from different species by virtually all the groups working on the NADPH-oxidase. Variations included the replacement of fatty acids by sodium dodecyl sulfate (SDS)

Abbreviations: AIR, autoinhibitory region; CGD, chronic granulomatous disease; GEF, Guanine nucleotide exchange factor; LiDS, lithium dodecyl sulfate; PA, phosphatidic acid; PAK, p21-activated kinase; PG, phosphatidyl glycerol; PI-3-kinase, phosphatidylinositol-3-kinase; PKC, protein kinase C; PLA2, phospholipase A2; phox, phagocyte oxidase; PRR, proline rich region; Rho-GDI, Rho-GDP-dissociation inhibitor; ROS, reactive oxygen species; SDS, sodium dodecyl sulfate; TPR, tetratricopeptide repeat.

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[6], followed by lithium dodecyl sulfate (LiDS) [7], phosphatidic acid (PA) [8], and short-chain saturated fatty acids [9]. These amphiphilic molecules have polar heads and hydrophobic chains. Addition of GTP-γ-S [10] enhanced the activity in most systems but was poorly stimulating in guinea pig macrophage homogenates [7]. The optimum pH of enzyme activation ranged from 6.5 to 7.0 in the first model, and from 7.5 to 8 in others. Some cell-free systems required Mg^{2+} whereas others did not, and supplementation by FAD usually enhanced NADPH-oxidase activation but was not found to be an absolute requirement in all situations. In the macrophage-derived cell-free system, supplementation with Mg^{2+} compensated partially for the absence of FAD. Later work demonstrated that the extent of the dependence of cell-free NADPH-oxidase activation on exogenous FAD is related to the degree of loss of non-covalently bound FAD from flavocytochrome b_{558}, which was found to be the only catalytic component of the system. For a more detailed discussion of the roles of FAD and Mg^{2+}, see [11]. With macrophages, the non-mitochondrial origin of the ROS forming activity was ascertained by the lack of inhibitory effect of NaN_3. Some groups supplemented the system with ATP; it is possible that an enhancing effect of ATP supplementation could be explained by it acting as a source of GTP in conjunction with a nucleoside diphosphate kinase (NDPK) present in phagocyte membranes [12,13]. Some groups included a preactivation step, before the addition of NADPH and cytochrome *c*, and thus separated the assembly and the "catalytic" phases in the activation of the enzyme [14,15]. SDS was poorly effective in a system derived from bovine neutrophils (personal observation) and porcine neutrophils, that did respond to long-chain unsaturated fatty acids and some saturated fatty acids, respectively [9,14]. Early reports describing differences between NADPH-oxidase activation *in vitro* by fatty acids and anionic amphiphiles, which are not fatty acids, such as SDS, appear to have been due to methodological problems, probably related to the use of total cytosol and a variety of membrane preparations before the introduction of the semi-purified cell-free systems. There is no reason to believe that the two categories of activators work by different mechanisms and, more likely, the contrary is true.

2. Cell-free systems as purification tools

2.1. The cytosolic factors

What the cell-free systems first evidenced was that, in order to activate a membrane-bound component, a thermolabile cytosolic fraction (called cytosolic factor) was required [2–6,14,16]. Cell-free systems were used to monitor the activity of cytosolic fractions isolated during various purification trials. At this stage, the number of cytosolic factors started to multiply at the same time as their activity was vanishing. The amphiphilic reagents used (arachidonate or SDS) exhibit typical bell-shaped dose-effect curves, with lower activities at both low and high concentrations of amphiphile. The optimal amount of activator was shifted by the presence of salt in the elution buffers used in column fractionation experiments based on salt gradients, or by variations in the protein concentration of the fractions [17]. Cytosol fractionation experiments by ammonium sulfate precipitation, anion exchange chromatography, and gel filtration suggested that there were at least two cytosolic components. The presence of two cytosolic factors was also evidenced by complementation studies using autosomal recessive forms of CGD [18]. Next, using a complementation method [19], it became possible to purify these cytosolic factors: a 63 kDa protein from porcine and bovine neutrophils [20,21], and a 47 kDa protein [22]. Shortly before, an antibody raised against a cytosolic fraction partially purified by affinity chromatography on GTP-agarose, recognized two proteins absent in distinct forms of CGD [23]. Its use led to the cloning of the cDNA of the human cytosolic proteins, p47phox and p67phox (phox = phagocyte oxidase) [24–26]. Human p67phox corresponds to the porcine and bovine 63 kDa proteins. p47phox had previously been identified as a phosphoprotein absent in some CGD cases and purified following its phosphorylation [27].

2.2. The small G protein, a cytosolic factor at the border between membrane and cytosol

Involvement of a GTP binding protein was deduced from the enhancing effect of GTP on NADPH-oxidase activation in most cell-free systems. GTP binding proteins are able to associate with the membrane, due to their post-translational modification by a hydrophobic isoprenyl chain at their C-terminus. In bovine neutrophils, the membrane fraction used for reconstitution of the activity contained enough G protein and required only p47phox and p67phox to be activated [22,28]. A candidate GTP binding protein was Rap1A, which was shown to co-immunoprecipitate with the membrane component of the NADPH-oxidase complex [29]. A C-terminally truncated (soluble) Rap1A protein was shown to enhance the activity of the cytosol [30]. However, the membrane component was found to be fully active after elimination of Rap1A during the purification process [31,32]. Another candidate, the small GTP binding protein Rac, was identified simultaneously by two groups. Purification to homogeneity of sigma1, an activating factor from the cytosol of guinea pig macrophages yielded a complex of Rac1 with its regulatory protein, Rho-GDP-Dissociation Inhibitor (Rho-GDI) [33]. Similarly, the Rac2/Rho-GDI complex was isolated from the cytosol of human neutrophils as a GTP binding fraction [34]. Rac was found in the cytosol in soluble form by virtue of it forming a complex with Rho-GDI, which masks its hydrophobic tail. Although the function of Rho-GDI is to maintain Rac in a GDP-bound form, supposed to be inactive, the Rac/Rho-GDI complex was able to stimulate the NADPH-oxidase [35,36]. The complex could also be replaced by recombinant Rac1 protein, that had been preloaded with GTP [37]. Another argument in favor of Rac being the GTP binding protein involved in NADPH-oxidase activation was derived from a study showing that Rac2 antisense oligonucleotides inhibited NADPH-oxidase activation in B lymphocytes (a cultivatable cell-line that possesses a NADPH-oxidase, although less active