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Review Article

THE SUPEROXIDE-FORMING ENZYMATIC SYSTEM OF PHAGOCYTES

PAOLO BELLAVITE
Istituto di Patologia Generale, Universita' di Verona, Strada Le Grazie, 37134 Verona, Italy

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Abstract—The formation of oxygen-derived free radicals by the phagocytes (neutrophils, eosinophils, monocytes and macrophages) is catalysed by a membrane-bound NADPH oxidase which is dormant in resting cells and becomes activated during phagocytosis or following interaction of the cells with suitable soluble stimulants. This enzyme is under investigation in many laboratories but its molecular structure remains to be clarified. Possible components such as flavoproteins, cytochrome b_{558}, and quinones have been proposed on the basis of enzyme purification studies, effects of inhibitors, kinetic properties and analysis of genetic defects of the oxidase. An extensive discussion of the evidence for the participation of these constituents is reported. On the basis of the available information on the structure and the catalytic properties of the NADPH oxidase, a series of possible models of the electron-transport chain from NADPH to O_2 is presented. Finally, the triggering mechanism of the respiratory burst is discussed, with particular reference to the stimulus-response coupling and the final modification(s) of the oxidase (phosphorylation, assembly, change of lipid environment, etc.) which are involved in its activation.

Keywords—Superoxide formation, NADPH oxidase, Phagocyte metabolism, Cytochrome b_{558}, Flavoprotein, Respiratory burst, Transduction systems, Chronic granulomatous disease

INTRODUCTION

The generation of oxygen-derived free radicals such as superoxide anion (O_2^-), hydroxyl radical ($OH\cdot$) and singlet oxygen ($^1O_2$) and of hydrogen peroxide (H_2O_2) represents one of the main systems by which phagocytes kill invading organisms and tumor cells and may cause other harmful effects in inflammatory processes.[1–6] These intermediates of oxygen reduction are formed by phagocytes (neutrophils, eosinophils, monocytes, and macrophages) during the engulfment of particulate matter or when the cells are stimulated by a variety of soluble compounds. The peculiar metabolic pathway of activated phagocytes is called "respiratory burst" because it was first described as a sudden increase of oxygen consumption occurring a few seconds after the interaction of the cell with the stimulatory agent.[7–12] Early studies indicated that this oxygen metabolic pathway is insensitive to cyanide and therefore is independent of mitochondrial respiration.[10–12] Following these observations, increasing efforts were addressed to the investigation of the phagocyte's respiratory burst.

In spite of the great body of information accumulated in the past 20 years, the nature of the free-radical generating system has yet to be clarified. While there is substantial agreement on the properties of the respiratory burst in intact cells and on its products and its function in host defense and inflammation, there is a series of newly generated questions concerning the structure of the enzyme(s) involved and the mechanism of its activation. Here the main body of knowledge regarding phagocyte metabolism will be summarized, consolidating detailed reviews which have been published on this topic.[13–19] Then, the most recent and often controversial reports on the nature of the enzymatic system which is responsible for the respiratory burst will be presented.

Born April 9, 1952 at Verona (Italy), Paolo Bellavite studied in Classic Liceo secondary schools, then took a medical degree and a specialization as hematologist at the University of Trieste. At present he is employed as Associate Professor of General Pathology at the University of Verona. His research area is the function, metabolism and pathology of leukocytes. He is member of the European Society for Clinical Investigation.

I. THE RESPIRATORY BURST OF PHAGOCYTIC CELLS

Oxidative metabolism was first studied in intact phagocytes. The increased oxygen consumption and free radical generation can be stimulated by a large series of phagocytozable and soluble substances capable of reacting with specific receptors present on the cell surface or by inducing other kinds of membrane modification. A representative list of the compounds and of the experimental conditions which activate the respiratory burst is reported in Table 1.

The increased respiration of phagocytozing cells is accompanied by other metabolic modifications including: (1) increase of glucose uptake[20] and catabolism through hexose monophosphate pathway (HMP),[21] (2) decrease of the NADPH:NAD$^+$ ratio[22] and GSH:GSSG ratio,[23] (3) production and release of hydrogen peroxide,[24] of superoxide anion,[25] of hydroxyl radical,[26] of singlet oxygen,[27] and (4) emission of photons of light (chemiluminescence).[28] Coincident with the activation of these systems, the intracellular pH undergoes rapid acidification, followed by gradual alkalinization, due to extrusion of H^+ through a receptor-activated Na^+/H^+ antiport.[29-32] All these biochemical events are strictly interrelated and take place almost simultaneously.[33] A scheme of these interrelationships is proposed in Figure 1.

Virtually all the extra-oxygen consumption due to functional activation by phagocytosis is converted first to O_2^-, then to H_2O_2 through the dismutation reaction:

$$O_2^- + O_2^- + 2H^+ \longrightarrow O_2 + H_2O_2 \quad (1.1)$$

It has been calculated that only a minor portion of O_2 that undergoes reduction can be recovered as O_2 or H_2O_2 in the extracellular environment, because phagocytes utilize the products in phagocytic vacuoles or destroy them through their powerful scavenger systems represented by superoxide dismutase, catalase, glutathione peroxidase, ascorbate and tocopherol.[34-40]

The extent of these phenomena and the stoichiometries of $O_2:O_2^-:H_2^-$ vary according to the experimental conditions, the cell type, and the stimulant employed. Challenge of neutrophils with maximum doses of opsonized zymosan or of phorol-myristate-acetate (PMA, one of the most powerful soluble stimulatory agents) triggers an oxygen consumption (and a corresponding free-radical production) in the order of 5–10 nmol/min/10^6 cells.[18] It is a common experience of investigators working with these cells to observe 10–20 million neutrophils in the Clark-oxygraph, which consume all the oxygen of the solution in a few minutes. Considerable differences in the extent of the oxidative metabolism among the various mononuclear phagocytes have been reported.[31,41] Blood monocytes are more active than differentiated macrophages.[42] Particular types of tissue macrophages, such as liver Kupffer cells, appear to be almost totally impaired in their oxidative response.[43] Release of O_2^- and H_2O_2 by pulmonary alveolar macrophages is very low, due to the high efficiency of their scavenger systems.[44] Under appropriate stimuli such as inflammation, endotoxins, or γ-interferon, resident macrophages may maturate to cell types which are more active in bactericidal and cytotoxic functions. One of the characteristic modifications of "activated" or "inflammatory" macrophages is a higher production of oxygen-derived free radicals.[45-50] Interestingly, a phagocytosis-associated respiratory burst has been described even in protozoa such as the amoebae.[51,52]

The time-course of the respiratory burst varies according to the stimulant used. Some stimulants, such as phagocytozable particles, induce a progressive increase of respiration that accompanies the engulfing act and ceases when phagocytosis is completed;[53] chemotactic peptides trigger an almost instantaneous increase of O_2 consumption that often shows biphasic kinetics;[54,55] phorbol esters cause a progressive and irreversible activation;[56] activation by sodium fluoride shows a very long lag time.[57] The metabolic activation is reversible upon removal of the activator, which can be accomplished by washing the cells,[57] by displacing the ligand from receptor with competitive substances,[58,59] or by oxidative inactivation of the activator itself.[54] Once deactivated, the oxidative metabolism can be reactivated by a second stimulus,[54,60] but under particular conditions a "desensitization" of the system may occur.[61-63] Desensitization is stimulus-specific and is probably due to either down-regulation or uncoupling of the receptorial system. Different from deactivation is the termination of the respiratory burst taking place as a consequence of auto-inactivation of the enzymatic system that generates free radicals. Such an inactivation is due to toxic effects of hydrogen peroxide and myeloperoxidase, which are released and accumulate during the activation phase.[64-66]

Considerable advances in the knowledge of the res-

Table 1. Stimulants of the Phagocyte Oxidative Metabolism

Bacteria	Opsonized zymosan
Latex particles	*N*-formyl-peptides
Oil droplets	Pyrogen
IgG-coated surfaces	Ca^{2+} ionophores
Immune complexes	Cytochalasins D and E
Complement fragments C5a, C567	Phospholipase C
Fatty acids	Concanavalin A
Tumor necrosis factor	Leukotriene B_4
Phorbol myristate acetate	Anti-leukocyte antibodies
Sodium fluoride	Low-Na^+ incubation buffer
Diacylglycerol	Platelet activating factor

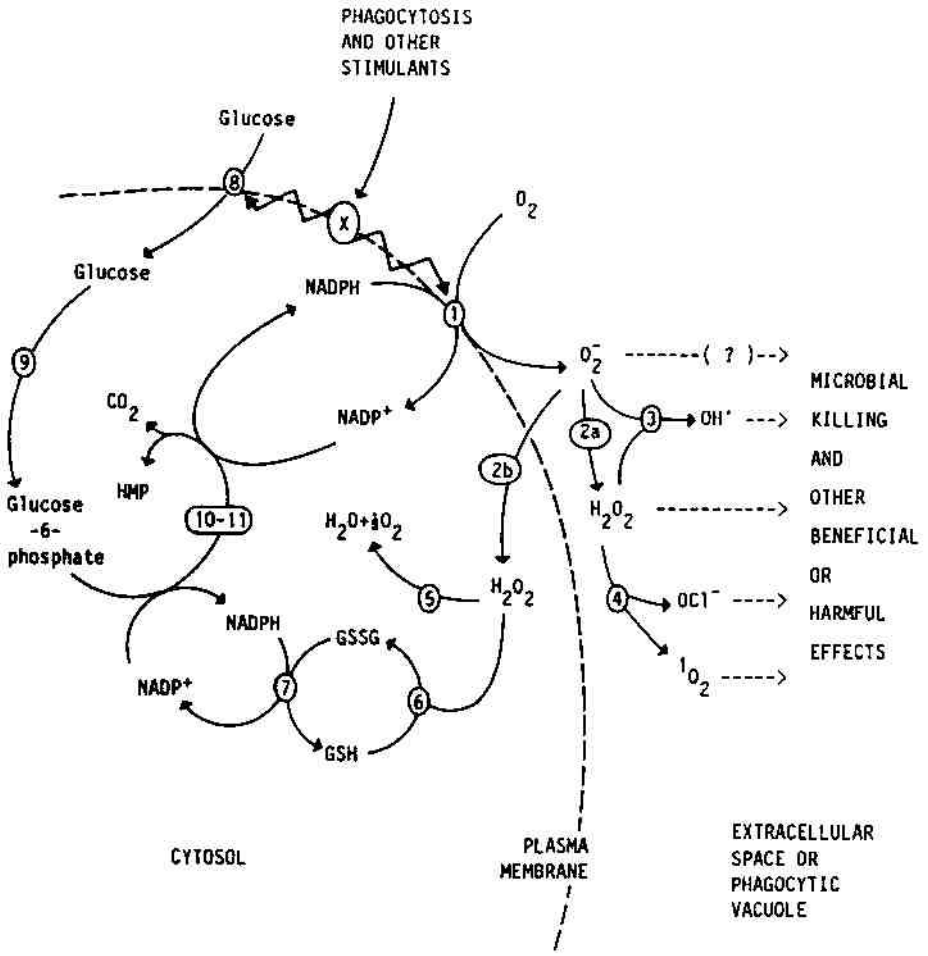

Fig. 1. Metabolic pathways of activated phagocytes. Perturbation of plasmamembrane (X) by various stimulants (see text) leads to activation of the primary oxidase (1) and of various correlated enzymatic and nonenzymatic systems. (2a) spontaneous dismutation; (2b) superoxide dismutase; (3) Haber-Weiss reaction; (4) myeloperoxidase; (5) catalase; (6) glutathione peroxidase; (7) glutathione reductase; (8) glucose transporter; (9) hexokinase; (10) glucose-6-phosphate dehydrogenase; (11) 6-phosphogluconate dehydrogenase; HMP = hexose monophosphate pathway.

piratory burst and of its mechanisms have been provided by studies on congenital and acquired defects of phagocytic metabolism (Table 2). The prototype of the congenital deficiencies is chronic granulomatous disease (CGD), a syndrome with many variants having in common the complete lack of superoxide and hydrogen peroxide generation by all types of phagocytes in the homozygous form.[67-72] For the biochemist and the genetecist, CGD is a formidable nature-experiment which is utilized for the investigation of the components of the respiratory burst enzyme. Unfortunately, patients affected by this disease (and also be severe glucose-6-phosphate dehydrogenase deficiency) suffer serious infections and often die in their early youth. Acquired cellular defects are milder in their expression and do not create severe clinical problems unless they are multiple or concomitant with leukopenia or with other immunodeficiencies. Table 2 also reports a list of drugs that are known to inhibit the respiratory burst. Some of them have a precise cellular target, while the effect of others has not been explained yet. It should be pointed out that many compounds that have been claimed to be inhibitory were tested only in vitro and at concentrations far above those commonly used in therapy.

II. THE ENZYMATIC BASIS OF THE RESPIRATORY BURST

The discovery of the peculiar oxygen metabolism of phagocytes opened a large debate on its biochemical basis. A variety of enzymes, such as NADH oxidase,[101-103] aminoacid oxidase,[104] myeloperoxidase,[105] NADPH oxidase,[53,106,107] and NADH-NBT reductase,[108-110] have been proposed as the respiratory burst enzyme.

Table 2. Defects of Phagocyte Oxidative Metabolism

Type of Defect	Reference Number
Hereditary diseases	
Chronic granulomatous disease	67–72
Glucose-6-phosphate dehydrogenase deficiency	73
Glutathione peroxidase deficiency	74
Mol-glycoprotein deficiency	75
Acquired defects	
Burns	76,77
Viral infections	78,79
Malnutrition	80
Iron deficiency	81
Liver diseases	82–84
Type IV hyperlipoproteinemia	85
Anaesthetic agents	86–88
Nonsteroidal antiinflammatory drugs	89–93
Corticosteroids	94
Antimicrobial agents	95–98
Busulphan, methotrexate	99,100

Most of these indications were not confirmed by subsequent investigations, and NADH oxidase and NADPH oxidase remained the sole candidate enzymes. The debate over the true substrate (NADH versus NADPH) was quite lengthy and various arguments in support of each theory were reported.[13,16,17,111–113] Awaiting a definite clarification, from time to time the term NAD(P)H oxidase was also used.[114–117]

In recent years, unequivocal evidence established that the physiological substrate of the free radical generating oxidase is NADPH, although in the test tube the enzyme is also capable of oxidizing NADH. The main reasons for such a conclusion are as follows.

A. Coupling of the oxidase with HMP activity

The stimulation of HMP is supported by an increase of the $NADP^+$:NADPH ratio, i.e. by oxidation of NADPH. Assuming that the respiratory burst was due to NADH oxidase, the linkage between NADH oxidation and HMP activity would be provided by ancillary reactions, which oxidize NADPH to $NADP^+$. Theoretically, these reactions could be catalyzed by NADPH/NAD^+ transhydrogenase or by NADPH-dependent lactate dehydrogenase. It has been shown that the activity of these systems in phagocytes is too low to account for a $NADP^+$ production sufficient to sustain all the glucose oxidation through the HMP.[16] Therefore, the HMP appears to be directly linked to oxygen consumption through generation of $NADP^+$ by NADPH oxidase. This is in agreement with the observation that during phagocytosis the intracellular concentration of NADPH decreases and that of $NADP^+$ increases, while the NADH:NAD^+ ratio does not significantly change.[22,118] A significant contribution to NADPH consumption in phagocytozing cells is provided by the gluthatione cycle (see Fig. 1), which is an important peroxide-scavenging system. However, the HMP is activated even in conditions where the glutathione cycle is not operative, as in guinea pig neutrophils.[119–121]

B. Identification of the oxidase in subcellular fractions

The superoxide-forming activity is detectable in subcellular organelles (usually 27,000 × *g* pellet of postnuclear supernatants, or membranes purified through sucrose or Percoll gradients) prepared from phagocytozing cells. Both NADH and NADPH oxidase activities are triggered by phagocytosis or by soluble stimulants. The Km for the substrate of the NADPH oxidizing activity is close to the NADPH concentration in the cell, whereas the Km of the NADH-oxidizing activity is much higher, suggesting that the preferred physiological substrate is NADPH.[16,113,117,118,122]

C. Adequacy of NADPH oxidase to account for the respiratory burst

The activated oxygen consumption and the O_2^- production of intact neutrophils and macrophages is accounted for by the oxygen-consumption and O_2^- production catalyzed by NADPH oxidase. This concordance was demonstrated by a simple experiment where the respiratory burst of PMA-stimulated macrophages was monitored (Fig. 2).[123,124] After stimulation the O_2^- production increased until a linear rate was attained (trace 1). At this point the cells were lysed with deoxycholate and the O_2^- production ceased, but addition of NADPH restored the activity at a rate similar to that sustained by whole cells. A similar result was obtained by adding $NADP^+$ plus glucose-6-phosphate, which together with glucose-6-phosphate dehydrogenase provided by the cell lysate form a NADPH generating system (trace 2). NADH was less effective than NADPH (trace 3), and NADPH oxidase activity was undetectable in unstimulated cells (trace 4). This experiment (and similar results published by others)[125] demonstrated that both the NADPH-dependent O_2^- generating system and the NADPH-generating system of phagocytes are competent for supporting the respiratory burst.

D. Studies of genetic defects

The primary molecular lesion in phagocytes of patients with CGD lies in the NADPH oxidase system,[126] while the deficiency of other enzymes in this disease is controversial.[127–129] The absence of NADPH-dependent O_2^- formation in CGD could be due to (1) lack of the oxidase or of one of is components, (2)