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GENETIC DEFECTS OF PHAGOCYTE NADPH OXIDASE ACTIVITY AND ACTIVATION

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NADPH oxidase is the key enzyme of the free radical-generating oxidative matabolism of phagocytes. Work from our and other's laboratories has recently established that the oxidase is not a single molecular entity, but it is a multicomponent system including a NADPH-binding protein, a flavoprotein, a b-type cytochrome and other unidentified factors. A working model of the molecular nature and of the activation mechanism of phagocyte NADPH oxidase is here proposed. This model is suitable for the study and the classification of the molecular pathology of the oxidase system. The various genetic defects of the NADPH oxidase, that are the cause of chronic granulomatous disease, (CGD) are here presented and discussed.

Keywords: NADPH oxidase, Phagocytes, Chronic Granulomatous Disease, Superoxide.

Killing of microorganisms by professional phagocytes (neutrophils, eosinophils, monocytes and macrophages) involves the dramatic increase of oxygen consumption, with concomitant production of oxygen free radicals and hydrogen peroxide. If this metabolic activity, which is called respiratory burst, is genetically deficient, a severe predisposition to infections occur early in childhood.

The enzymatic basis of the respiratoy burst have been extensively studied in the past decades (1-5). A membrane-bound enzyme, NADPH oxidase, is responsible for the generatione of superoxide anion (O_2^-) by using electrons provided by NADPH, which, in turn, is generated by the hexose monophosphate pathway of glucose catabolism. From O_2^- then H_2O_2 and other free radicals may be generated both enzymatically (superoxide dismutase) and non enzymatically (iron-catalyzed Haber-Weiss reaction). Products of oxygen matabolism are utilized for bactericidal and tumoricidal function, or they are degraded by scavenger systems when they diffuse inside the cell. These scavenger systems (catalase, GSH peroxidase, vitamin E, ascorbate) are essential to mantain the integrity of the cell during the respiratory burst.

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An important feature of NADPH oxidase is that this system is usually dormant in phagocytes, but may be activated when the cells are stimulated by opsonized bacteria and other soluble or particulate compounds. Moreover, the extent of the activation may be up - and down - regulated by other factors such as bacterial toxins, hormones, cytokines, peptides, etc. (6-8).

Because of the complexity of the respiratory burst and of its regulation, a series of possible genetic defects may be the cause of its malfunction, namely: a) defects of the activation machinery, such a those related to opsonization, receptors, transduction systems, phagocytosis; b) defects of the glucose transport and metabolism, that cause impairment of NADPH production, such as severe glucose 6-phosphate dehydrogenase deficiency; c) absence or structural defect of the various constituents of the NADPH oxidase enzyme; d) defects of scavenger systems, such as those of the glutathione cycle, leading to self-toxicity of free radicals. This brief review is focused on genetic defects of the NADPH oxidase (point c).

Knowledge of the nature and molecular pathology of NADPH oxidase has been greatly facilitated by the existence of a syndrome, called chronic granulomatous disease (CGD), wich is due to the absence of this oxidase activity from the patient's phagocytes. CGD is characterized by severe and recurrent fungine and bacterial infections and other pathological modifications of the reticuloendothelial system (9,10). The disease was described in 1957 as fatal granulomatosis of childhood (11,12) and its primary metabolic lesion was discovered in 1967 (13). Subsequent investigations showed that this disease is both genetically and biochemically heterogeneous. The various molecular lesions that lead to CGD syndrome will be better understood after a description of the structure and activation mechanism of NADPH oxidase.

1. *Molecular structure and activation mechanism of the respiratory burst enzyme*

According to the most accepted hypothesis, the NADPH oxidase activity is due to a multicomponent system that would be assembled as an electron-transport chain capable of transferring electrons through the membrane from NADPH to molecular oxygen. The assembly of the various components could be promoted by phosphorylation of particular proteins, by changes of the lipid milieu of the membrane, or by other unidentified transduction pathways (4,5,14-16). Recent evidence indicates that some factors are translocated from the cytosolic compartment to the plasmamembrane, and the GPT-binding proteins may participate in the activation process (17-19).

During the past ten years several laboratories have attempted the extraction and purification of NADPH oxidase from neutrophils and macrophages, in order to characterize its structure in molecular terms (20-28). As a consequence of these studies, much information has been accumulated, but the data reported by the various groups were often controversial. The main reason for this is the extreme lability of the enzyme upon detergent extraction. Furthermore, the oxidase appears to be composed of several subunits that may be dissociated in different ways according to different purification procedures.

Table I reports most of the molecular constituents of the oxidase, resulting from the above outlined studies. However, it is worth noting that until now nobody has been able either to purify at homogeneity the "whole" oxidase system, or to reconstitute it by starting from separate components.

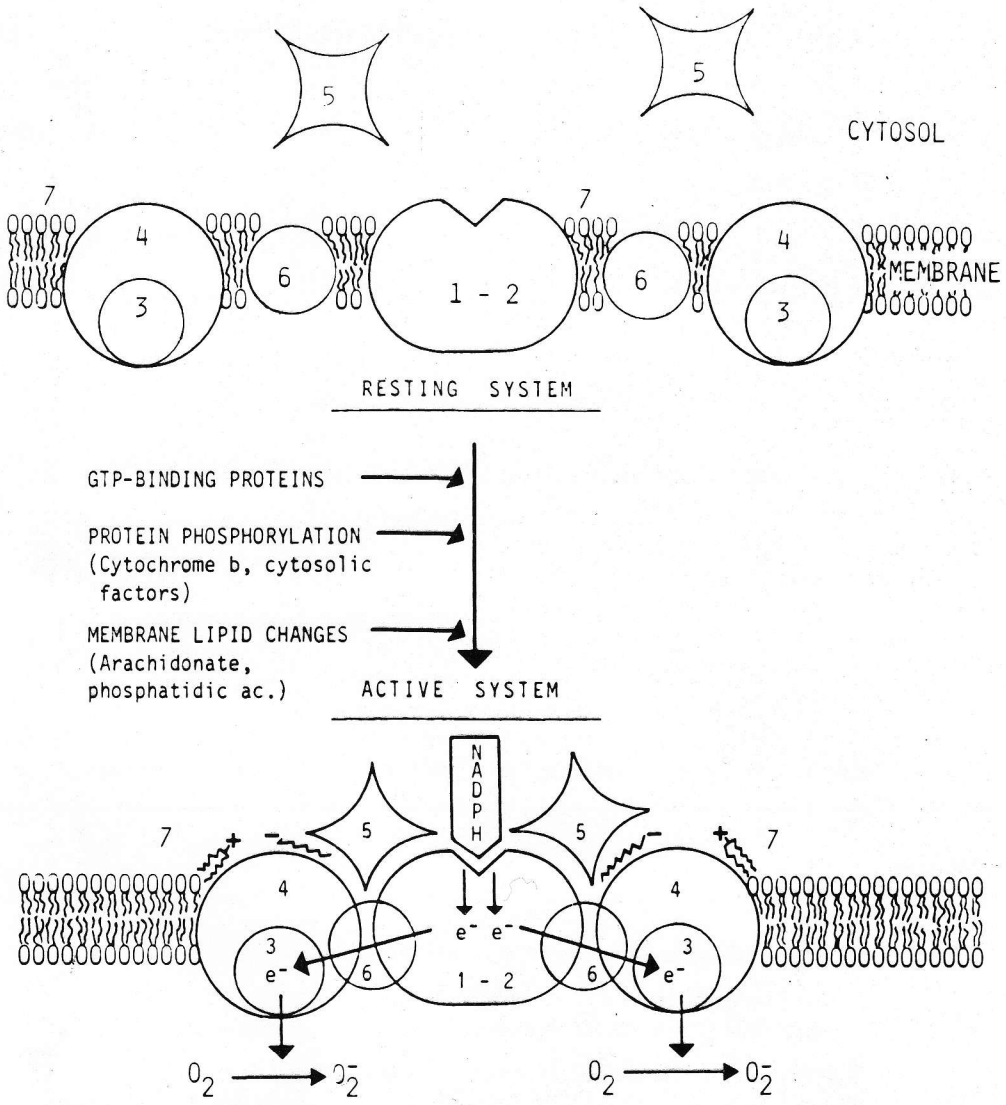

Figure 1. Hypothetical scheme of NADPH oxidase structure and activation. Numbers refer to the components of NADPH oxidase that have been described in table I. For explanation see text.

Table I. Molecular components of NADPH oxidase

	Mr (KDa, SDS-gel electrophoresis)	Reference
1. NADPH-binding protein	66	29-31
2. FAD-Flavoprotein	45	32
	51	27
	65-67	23, 25
Cytochrome b_{558} (α subunit)	20-23	33, 36
4. X-CGD protein (β subunit of cytochrome b)	68-92	33, 37
5. Cytosolic factors	47	38, 39
	65	38, 39
6. Other unidentified proteins	32	21, 25
	65	24
	14-16/18	40
7. Phospholipids	—	—

Table II. Molecular pathology of Chronic Granulomatous Disease

Type	Biochemical lesion	Inheritance	Relative frequency (%)
I.	Absence of both subunits of cytochrome b_{558}	X-linked	50-60
	Variant: Absence of cyt. b spectrum and deficiency of flavoprotein	X-linked	10-20
	Variant: absence of cyt. b spectrum with low-affinity NADPH oxidase	X-linked	rare
II.	Absence or malfunction of cytosolic factor	A.R.	20-30
III.	Absence of both subunits of cytochrome b_{558}	A.R.	rare
IV.	Unknown (cytochrome b malfunction?)	X-linked	very rare

Therefore, the sequential order and the organization of the electron transport chain is still hypothetical. A working model of the oxidase system and of its activation, which accounts for the present state of knowledge, is shown in figure 1.

Obviously, the first protein of the chain (n. 1 in table I and in fig. 1) should bear the NADPH binding site. This protein has been described by two independent groups as a band of about 66KDa that is present on the membrane of neutrophils and binds labeled NADPH analogues. In a study on X-linked CGD patients (31), NADPH binding protein was normal.

Various groups have identified a FAD-flavoprotein as part of the oxidase complex (n. 2 in table I and fig. 1). This flavoprotein has a Mr ranging from 45 to 67 KDa according to different groups. It can not be excluded that two or more different flavoproteins are involvend. The most consistent Mr is however 65-67 KDa, that is the same of the NADPH binding protein. This observation suggest that this flavoprotein has actually the NADPH binding site, although there is not direct demonstration yet. Kinetic studies showed that FAD is reduced by NADPH in anaerobiosis, and this reduction is faster when the enzyme has been activated (41). These data indicate that the activation process involves either the interaction of the flavoprotein with NADPH (assuming that it bears the nucleotide binding site), or the interaction with a proximal electron transport component.

The third factor of the complex is cytochrome b_{558}, which is also called cytochrome b_{-245}, on the basis of its characteristic and unusually low mid-point potential. Structural and functional properties of cytochrome b_{558} have been defined in better detail as compared with the flavoprotein. Evidence of its participation in O_2^- formation is overwhelming and has been reviewed elsewhere (5). Most methods of purification of this cytochrome resulted in the co-purification of two proteins, with Mr of 20-23 KDa (defined as small, or α, subunit) and of 68-92 KDa (defined as large, or β, subunit). However, a recent work from Kakinuma's group (36), clearly showed that the small subunit binds the heme and that the large subunit can be dissociated from it by using non-denaturing detergents. Therefore, the "true" cytochrome b_{558} is the 20-23 KDa protein (n. 3 in table I and fig. 1). The large subunits (n. 4 in table I and fig. 1) migrates on SDS/PAGE as a broad band, because it is heavily glycosylated. Removal of sugars changes its Mr to 55 KDa. Both proteins are phosphorylated during cell activation (42), suggesting that a protein kinase-mediated mechanism may be involved in the control of oxidase activity or in the assembly of the oxidase complex.

The genes for both subunits have been cloned and sequenced (43,44). The gene for the large subunit is located on the X-chromosome and is defective in the most frequent form of CGD (see section 2). Knowledge of its aminoacid sequence gave little insight into its function, also because no obvious homology with other known proteins was found. Evidence is accumulating that this glycoprotein is a membrane-anchoring protein for the small subunit (cytochrome b) and possibly for other proteins of the oxidase complex. In fact: a) Most procedures of isolation of the cytochrome b_{558} resulted in co-purification of the large subunit, indicating that they are strictly associated; b) we and others have found that the apparent molecular mass of the non-denatured cytochrome is about 200 kDa (45-47); c) mRNA for the small subunit is present in a variety of cell types, but the protein is expressed only in the cells that express also the large Mr protein and it s mRNA (44); d) A subgroup of X-linked and cytochrome b-negative CGD patients exhibit a deficiency of

flavoprotein (48-49). This observation suggests either the existence of two genetic deficiencies in the same patient, or, more probably, that the large subunit of the cytochrome b serves also for the assembly or the stabilization of a flavoprotein-cytochrome complex into the membrane; e) recent data from Segal's group (50) indicate that this protein is also required for the binding of regulatory cytosolic components that are translocated to the membrane during the activation process.

In our laboratory we have described a protein of 31.5 KDa which is associated with cytochrome b_{558} in membranes of guinea pig and pig neutrophils (5,21,45). This protein is markedly phosphorylated during phagocytosis and in phorbol-myristate acetate treated cells. We could not find a similar protein in human neutrophils and antibodies against the small subunit of the cytochrome b did not react with it (unpublished experiments carried out in collaboration with Dr. Garcia and Dr. A. W. Segal). Cloning of the gene and search for homologies with other proteins will hopefully clarify the nature of the 31.5 kDa phosphoprotein.

The oxidase model reported in fig. 1 shows putative flavoprotein surrounded by more than one cytochrome b/large subunit complex. This model accounts for our observation that in partially purified and active oxidase preparations the cytochrome b_{558} is in large molar excess with respect to FAD (22,45).

An important progress in the understanding of the nature and activation machanism of the oxidase has been provided by studies on cell-free systems of oxidase activation (51,52). It has been established that in these systems the participation of a series of cytosolic cofactors is required (n. 5 in table I and fig. 1). At least two of these cofactors have been defined in molecular terms and they are proteins of 47 KDa and 65 KDa. Also in this case, studies on CGD have contributed to demonstrate that both of these cofactors are required for the activation of the enzyme. In fact, there are particular variants of this disease, in whom the cytochrome b is normally present, but the activation mechanism is deficient. Studies in cell-free systems have shown that the cytosolic fraction of neutrophils from these patients can not sustain the activation of the oxidase and that the defect may be due either to deficiency of the 47 KDa protein or to the deficiency of the 65 KDa protein. By mixing the cytosolic fraction of two patients having different molecular defects, the activation capacity is restored.

The 47 KDa cytosolic cofactor is phosphorylated in activated cells and is translocated to the plasmamembrane. A defect of phosphorylation of this protein was found in CGD patients before it was established that it corresponds to the cytosolic cofactor which is necessary for the activation in cell-free system (53).

Other proteins (n. 6 in table I and fig. 1) could be part of the oxidase, even if no specific function has been assigned to them. Among these proteins, there is that one described by Doussière and Vignais (24) as the oxidase from bovine granulocytes. Its Mr is identical to the flavoprotein described by others, but neither FAD, no cytochrome b prosthetic groups could be found. Furthermore, in pig neutrophil membranes, a heterodimer of 14 and 16-18 KDa, which is immunoprecipitated by a monoclonal antibody that inhibits the NADPH oxidase activity, has been recently described in our laboratory (40). We are currently investigating the nature of this novel protein that should have important structural or regulatory function in the oxidase system.

Finally, the role of phospholipids has be mentioned. In fact, addition of phospholipids to the assay mixture markedly increase the O_2^- forming activity of

Triton X-100 solubilized enzyme (54) and we have found large amount the phospholipid in association with the oxidase in active state after solubilization and partial purification (21,45). Probably phospholipids give the enzyme the right conformation and spatial orientation, as known for several other membrane-bound enzymes. Another function of phospholipids could be to isolate the electron transport chain from external electron acceptors. We have shown that the oxidase is able to donate electrons only to molecular oxygen while other acceptors such as cytochrome c and dichlorophenolindophenol cannot withdraw electrons from the chain (55). This is a distinctive property of NADPH oxidase with respect to other oxidases that usually can reduce artificial electron acceptors.

In fig. 1 a simplified scheme of the activation mechanism is shown. This model of assembly of several components does not imply that these components are dissociated in the resting state and become associated during activation. Translocation of cytosolic cofactors is highly probable, but movement of flavoproteins and citochromes in the membrane plane is only speculative. It is also possible that activation induces functional coupling between various factors that are already physically associated. Several mechanism may be involved: a) Participation of GTP-binding proteins is indicated by inhibitory effect of pertussis toxin on the stimulus induced by many (but not all) stimulants, and by the observation that GTP analogues markedly potentiate the activation in cell-free systems; b) phosphorylation is a universal mechanism of regulation of protein function and the oxidase is not an exception. A number of evidences support the participation of protein kinase C in the activation of the oxidase, including the direct demonstration of the activation induced by this kinase in cell free systems (56). However, this direct activation accounts for only a small fraction of the activation occurring in intact cells; c) It is known that a number of lipid changes in the plasmamembrane are triggered by interactions of the stimulant with its receptors. It is probable that these changes contribute to the oxidase activation independently of their consequences on calcium fluxes and protein kinase activation. In fact, studies in cell-free systems have shown that arachidonic acid (whose formation is catalysed by phospholipase A2, wich is activated during the respiratory burst) and phospatidic acid (whose formation is catalysed both by phospholipase and by diacylglycerol kinase) may promote the activation of the oxidase in the absence of ATP (57). In summary, there is evidence that different transduction pathways and different final modifications of selected components of the system or of its lipid environment lead to critical conformational changes associated with the triggering of O_2 formation.

2. Genetic and biochemical lesions in CGD Phagocytes.

Structural and functional complexity of the NADPH oxidase system is reflected by the heterogeneity of CGD syndrome. The first structural defect of the oxidase that was described was the absence of cytochrome b (58) in male patients with classic X-linked patterns of transmission. Soon after, CGD patients with normal amounts of cytochrome b and with an autosomal recessive pattern of transmission were described. An important cooperative European study showed that most patients were affected by the cytochrome b-negative form of the disease (59). In recent years, several different forms have been described, including cytochrome b-negative variants with autosomal recessive inheritance and cytochrome b-positive variants

with X-linked inheritance. A provisional classification of the various types of CGD so far described is reported in table II. This classification follows, with little modification, that proposed by Curnutte (10).

It is worth noting that the identification of new variant forms of CGD has to be expected because of the use of new diagnostic tools such as western blot analysis of neutrophil proteins with antibodies directed against specific components and on northern blot analysis or in situ hybridization of the respective mRNAs. Further biochemical studies that are necessary to determine the CGD subtype include the measurement of Vmax and Km of NADPH oxidase, the level of cytosolic activation factors as measured in cell-free systems, and reconstitution of NADPH oxidase activity with the components that are presumably lacking in particular patients.

The first form of the classification is the classic X-linked CGD. Studies with cells from these patients were determined both to establish the role of the cytochrome in the oxidase activity, and to approach the cloning of the gene for the protein that is missing in this disease. The latter was accomplished by Orkin's group by "reverse genetic" before the gene product could be isolated (43). This protein was in fact initially designed as X-CGD protein and then identified as the large subunit of cytochrome b. The genetic lesion in most of the type I cases studied thus far is presumably a point mutation of the gene, which results in the absence of mRNA transcripts for the large subunit of cytochrome b_{558}. In a few patients interstitial gene deletions and also chromosomal deletions involving the Xp21 region have been described. The mRNA for the small subunit of cytochrome b is normally represented in CGD patients, but western blot analysis showed that the protein has an essential role in the stabilization of the small subunit and for its incorporation into the membrane.

A discrete subgroup of classic CGD patients also exhibits a partial deficiency of flavoprotein. It is possible that in these patients the molecular lesion involves also the incorporation of the flavoprotein into the oxidase complex. Until now, FAD-negative patients have not differentiated from FAD-positive patients by other analysis such as northern or western blots.

A further (rare) variant of the type I is characterized by the presence of some residual NADPH oxidase activity and the cells from these patients are able to produce trace amounts of superoxide. This pattern is associated with a milder clinical symptomatology and, very interestingly, to a positive response to in vivo and in vitro treatment with interferon-gamma (60). In these patients, phagocyte cytochrome b spectra, immunoreactive heavy chain protein and mRNA transcript for the heavy chain gene in granulocytes before and at selected times after in vivo treatmnet with interferon increased from an almost undetectable amount to a small fraction of the normal value. This in vivo effect was surprisingly prolonged, indicating that the lymphokine affected not only mature granulocytes but also progenitor cells (61,62).

The second CGD type is inherited with autosomal recessive inheritance and accounts for about one third of the cases. In this form the molecular lesion affects the cytosolic factors that are necessary for NADPH oxidase activation. Forms that affect either the 47 KDa factor and the 65 KDa factor have been reported. It is still unclear whether there is complete absence or malfunction of these factors. Also this group of patients respond to interferon-gamma treatment and they are actually the most responsive (62). This fact suggests that probably another interferon-gamma sensitive gene is involved in the genetic defect of this class of CGD.

Eight cases of CGD patients with pedigrees typical of autosomal recessive inheritance and with lack of cytochrome b spectrum have been reported and are now classified as type III CGD. The biochemical defect could involve the small subunit of the cytochrome, although at present we have no information about the chromosomal location of the gene for this protein. According to Curnutte (10), also in this variant, both subunits are undetectable on western blot analysis, indicating that both are necessary for their membrane anchoring.

Finally, type IV includes a few cases where an X-linked pattern of inheritance is associated with a biochemical lesion other than the absence of cytochrome. In fact, in this form the cytochrome b is expressed at apparently normal levels but, despite this fact, the membrane fraction from these patients is not activated in the cell-free activation system when incubated with normal cytosol. This finding is open to two different explanations, namely either there is a deficiency of an unidentified component that is coded by chromosome X, or alternatively, there is a subtle qualitative defect of cytochrome b which renders the protein nonfunctional. Further studies are necessary to clarify the biochemical basis of this defect.

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