Mechanism of Production of Toxic Oxygen Radicals by Granulocytes and Macrophages and their Function in the Inflammatory Process

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SUMMARY

The paper deals with 1) the freatures of the respiratory burst (increase of the respiration with production of O_2 metabolites, O_2^-, H_2O_2, $OH^\cdot$) of the inflammatory cells; 2) the factors responsible for its activation; 3) the methods for its measurement; 4) the molecular events which take place at the level of the plasma membrane following the interaction between the stimuli and the cell surface (the Ca^{++} changes, the modification of membrane potential, the activation of phospholipid turnover) and the hypothesis of the activation of the pretein kinase C; 5) the nature of the NADPH oxidase whose activation is responsible for the respiratory burst and the production of O_2 metabolites; 6) the defensive, toxic, proinflammatory and modulatory effects due to the reactivity of the oxygen metabolites.

Introduction

Granulocytes and macrophages, that is the professional phagocytes, respond to a variety of stimuli with aggregation, activation of movement, secretion, endocytosis, activation of the respiration, production of mediators. Through these responses these cells exert a key role in the defense against invading organisms and tumor cells. The most impressive response, which will be the object of this presentation, is the activation of the respiration with a concomitant production of an enormous amount of intermediate products of O_2 reduction such as superoxide anion (O_2^-), hydroxyl radical ($OH^\cdot$), hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$)[1, 3, 25, 39]. This activation of the respiration is called "respiratory burst" and is due to the activation of a membrane bound NADPH oxidase, which is dormant in resting state, induced by perturbation of the plasma membrane during phagocytosis or following interaction between the cell surface and a number of environmental stimuli. The intermediate products of O_2 reduction play a relevant role in the host defenses, in the evolution of the inflammatory process and in a series of cytotoxic effects responsible for tissue damage.

Feature of the Respiratory Burst

1. The term respiratory burst refers to the increase in the non-mitochondrial O_2 consumption, with a concomitant production of O_2^-, H_2O_2 and other oxygen radicals, and in the oxidation of glucose through the hexose monophosphate shunt (HMPS), which occurs when phagocytes are exposed to appropriate stimuli. The linking between the O_2 consumption and the HMPS has been discussed in a previous review[39]. Briefly, the O_2 consumption is due to the activation of the oxidation of NADPH, and the increase in HMPS to the decrease of the ratio NADPH/$NADP^+$ and to the degradation of H_2O_2 by glutathione peroxidase and glutathione reductase. In other words the oxidation of glucose through the HMPS has two functions: the production of NADPH for the oxidase and the degradation of H_2O_2.

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Table 1. The Most Commun Agents that Stimulate the Oxidative Metabolism of Phagocyte

Phagocytosable particles	Phorbol-esters
Adhesion to surface	N-Formyl-Peptides
Surfactants	Ca^{++} ionophores
Anti-leucocyte antibody	Cytochalasin D, E
Phospholipase C	Pyrogen
Immunocomplexes (Fc)	Platelet activating factor
Complement fragments	Leukotrienes
Fatty acids	Concanavalin A

2. The respiratory burst is induced (Table 1) by phagocytosable particles (bacteria, viruses, aggregate materials, cell debris etc.) and by a number of soluble factors. It is worth pointing out that the activation of the respiration is not a postengulfment event, because it starts when a particle interacts with the cell plasma membrane, is associated with the act of engulfment and ceases when the phagocytic act is completed[40]. The respiratory burst is induced also when phagocytes adhere to non-phagocytosable substrates, for example surfaces covered with immunocomplexes.

Some of the stimulatory agents interact with the phagocytes in a non-specific way (for example detergents, fatty acids), other ones bind to specific receptors (for example Concanavalin A, immunocomplexes, N-formyl-peptides). Most of the stimulatory agents such as immunocomplexes, chemotactic peptides, lectins, endotoxins, leukotrienes etc. are of great biological importance, since they frequently come in contact with the phagocytic cells at the inflammatory sites or in the blood stream. Many of the stimuli induce more than one response such as chemotaxis, secretion, aggregation, respiratory burst etc. Although these different responses are induced at different concentration of the stimulant (for example the optimal chemotactic response to N-formylated peptide takes place at 10^{-10} M and the respiratory one at 10^{-8}–10^{-7} M), it seems that the activation of the respiration is a frequent condition of the phagocytes engaged in different functions as during the chemotactic movement, adhesion to endothelial cells or to collagen fibers, phagocytosis, aggregation inside the blood vessels induced by complement factors etc. The regulation of the different responses, the reciprocal influences and the molecular signals involved are quite complex and, at present, practically unknown. Some recent results indicate that this matter is of great importance. For instance, the secretion of lysosomal granule content modifies the number of receptors for chemotactic peptides and, as a consequence, the magnitude of the response to these agents[15]. The intermediates of O_2 reduction produced during the burst modifies the structural and biological properties of chemotactic peptides[37] and leukotrienes[22] that become inactive. Thus the secretory activity and the respiratory burst can modify the cell response to environmental stimuli.

3. The respiratory burst is a very impressive phenomenon, with regard to the intensity and the rapidity of its occurrence. The stimulation of O_2 consumption and O_2^- production is already evident few seconds after the addition of bacteria or of soluble stimulants, like chemotactic peptides, phorbol-esters or other ones.

The respiratory burst can be estimate[25] by measuring the increment of O_2 consumption (with a Clark oxygen electrode, or with a Warburg respirameter); the O_2^- production (as superoxide dismutase sensitive cytochrome c reduction = O_2^- + cyt. c → O_2 + red. cyt. c); the H_2O_2 production (by using scopoletin or homovanillic acid as compound to be oxidized in presente of exogenous horse radishperoxidase); the chemiluminescence; the $^{14}CO_2$ production from 1 – C^{14} glucose; the nitroblutetrazolium reduction to formazan (NBT test).

4. The respiratory burst occurs in all the so called professional phagocytes (granulocytes, macrophages). The intensity of the activation of the respiration varies depending on cell type, cell sources, animal species and, for macrophages, on the functional state (resident, elicited, activated). The neutrophils and eosinophils of mammals are more responsive than monocytes. Among the macrophages the most responsive are the so called "immunologically activated" [29, 41].

Mechanism of the stimulation of the respiration

The triggering of the respiratory response of phagocytes involves a series of molecular events which take place at the level of the plasma membrane: recognition, transduction and activation of the target.

1. The Recognition

Consists in the interaction between the stimulant and the cell surface. In many cases the interaction involves a specific receptor (for Fc, C5a, lectins, chemotactic peptides etc.). In other cases, receptors are not involved but the interaction is somehow specific because the stimuli act in a quite precise manner. The best example of this type of interaction not involving receptors is the triggering by phospholipase C[33], that removes the phosphorylated basis from membrane phospholipids, and by phorbol esters (PMA), that activates a Ca^{++} and phospholipid dependent protein kinase C[24].

The interaction at the cell surface is one of the steps that regulate the intensity, the rapidity and the time course of the respiratory response. Relevant are the relationship between the response and the number of receptors or binding sites on the cell surface, their kinetic properties and modulation, the rate of formation and the fate of ligand receptors (or binding sites) complexes. Some of these aspects have been recently clarified.

a) It has been recently found in our and other laboratories that the continuous binding of the stimulants (at least of those interacting with specific receptors) is necessary for maintaining the respiration of neutrophils in an activated state. In fact, the removal of Con A by α-methylmannoside[36], of arachidonic acid by albumin[11], and of f-Met-Leu-Phe by oxidative inactivation[37] or by antibodies[47] cause the cessation of the activated respiration within few seconds.

b) The magnitude of the respiratory response is proportional to the dose of the stimulant or to the number of particles to be ingested. However, the dose being equal, the magnitude of the response (in terms of maximal velocity of oxygen consumption) is regulated, at least in the case of stimulants involving receptors, also by the rate of the presentation of the stimulants. Infact, it has been recently shown that when the presentation of chemotactic peptides is made over a period of minutes, the respiratory response is greatly depressed and, sometimes, absent[13]. Apart from the mechanism responsible for this desensitivation, that has been discussed elsewhere, this finding is relevant for the events occurring in vivo, where it is likely that the contact between the cell surface and the stimulatory agents (for example chemotactic peptides, complement factors etc.) takes place not instantaneously but slowly.

c) By comparing the time course of the binding of f-Met-Leu-Phe and the respiratory response in human neutrophils we have shown that while the amount of the peptide bound to its receptors progressively increases, the velocity of the respiration reaches its maximum value very shortly and then progressively decreases[37]. The lack of summation of the effect of the stimulus-receptor complexes as they form indicate that the efficacy of the complexes is short-lasting. It remains to be verified if this short-lasting effect is a characteristic of the binding involving receptors or also of each type of interaction between stimuli and the cell surface.

d) The last point to be mentioned is that the maximum value of the stimulation of the respiration is reached when only about 20% of the receptor are occupied by f-Met-Leu-Phe[13].

2. *The Transduction*

Includes the molecular and functional modifications of the plasma membrane[13] triggered by the recognition events and generating the appropriate signals for the biological response, in this case the respiratory one. A number of biochemical changes at the level of plasma membrane have been described. These include change of ion fluxes, activation of phospholipase A_2 and C, changes of phospholipid turnover, of protein phosphorylation, of transmembrane potential etc. (Table 2).

Great uncertainty exists about the role of each molecular change in the various responses. It is likely that some are necessary for one type of response and not for others. It is also likely that some of these modifications are concomitant events not related with the functional responses of the cells. For example we know that some stimuli induce a release of Ca^{++} from cell membranes[28] but we do not know whether this event triggers the respiratory burst. Infact, a comparison of the effect of different concentrations of the chemotactic peptide f-Met-Leu-Phe on the release of Ca^{++} and the stimulation of the respiration shows that Ca^{++} release takes place at concentrations of the stimulant that do not trigger the respiration (unpublished results). Furthermore, it is known that some stimuli, for example phorbol esters, induce a respiratory burst in a Ca^{++} independent manner[48].

Another membrane event whose relevance remains to be clarified is the change of transmembrane potential[50, 55]. It is known that a membrane depolarization takes place very shortly (seconds) after the interaction between the stimulus and the plasma membrane of leukocytes. The fact that this depolarization does not occur in leukocytes of patients affected by chronic granulomatous disease (CGD)[55], cells that do not present a respiratory response, seems to indicate a strict correlation between the changes of membrane potential and the respiratory burst. However, a depolarization per se is not a trigger for the respiratory response as indicated by results, obtained in our laboratory, which show that the depolarization induced in human neutrophils by gramicidin, high extracellular K^+, ouabain and valinomycin, do not cause a stimulation of the respiration (Table 3).

Another problem is the meaning of the increase of turnover of phospholipids, in particular of phosphatidylinositol, which occurs with some stimuli (chemotactic peptide) and not with other (phorbol esters)[46]. Many years ago we have shown that the degradation of phospholipids of leukocytes membrane by exogenous phospholipase C induced a typical respiratory burst[33]. This old observation become today very relevant since it seems to be the direct

Table 2. Biochemical Changes Possibly Involved in or Controlling the Coupling Stimulus-repiratory Response in Phagocytes

Ca^{++} mobilization	Changes of phospholipid turnover
↑ permeability	↑ phospholipase activity
↑ release from stores redistribution	↑ phosphatydil-inositol turnover
Changes of monovalent ions fluxes (Na^+, K^+)	↑ diacylglycerol formation
Changes in trasmembrane potential	↑ phosphatydic acid formation
H^+ extrusion	Activation of protein Kinase C
Changes of cyclic nucleotide turnover	Changes of protein phosphorylation
↑ AMPc	Activation of arachidonic acid cascade
	↓ Phospholipidi methylation

Table 3. Correlation between the Changes of Transmembrane Potential and the Respiratory Burst in human Neutrophils

Stimulant	Depolarization	Respiratory Burst
N-formyl-peptides	yes	yes
Phagocytosis	yes	yes
Phorbol-myristate-acetate (PMA)	yes	yes
Gramicidin	yes	no
High-K^+-medium	yes	no
Ouabain	yes	no
Valinomycin (in K^+)	yes	no

demonstration that the formation of diacylglycerol in the plasma membrane is the key event in the transduction of the signal to the target. Thus, the sequence of events could be the following: interaction of the stimulus with receptor or binding sites of plasma membrane, activation of phospholipase C (via Ca^{++} changes), formation of diacylglycerol, activation of proteinkinase C, phosphorylation of protein(s). Although many data support the validity of this scheme, at least with regards to the formation of diacylglycerol, much work remains to be done for a final demonstration of its validity. It is worth to pointing out that this scheme can be valid also in the case of the burst induced by phorbol esters which is independent both on Ca^{++} and phosphatidylinositol turnover changes. It has been shown, infact, that phorbol esters can substitute diacylglycerol in the activation of proteinkinase C[24].

3. *The Activation of the Target (NADPH Oxidase)*

The recognition of the enzymatic system involved in the activated respiration of phagocytes has been a very controversial problem for the last twenty years[1, 3, 25, 39, 40]. It is now universally accepted that the enzyme responsible for the respiratory burst is an oxidase that oxidizes NADPH with formation of O_2^-. The oxidase is embedded in the plasma membrane, with the active center directed towards the cytosol. In the course of phagocytosis only the enzyme of the portion of the membrane that is invaginated and forms the phagosome, is activated[5]. This segmentary activation allows the formation of toxic oxygen compounds (O_2^-, H_2O_2, $OH^\cdot$) in a strategic position for killing ingested organisms. When the stimulus is a soluble factor it is very likely that the activation involves the oxidase distributed in all the plasmamembrane.

The nature of this enzyme is under investigation in many laboratories. It is widely accepted that the oxidase is a sort of respiratory system composed of a flavoprotein[2, 26], a newly discovered cytochrome b with very negative (–245 mV) redox potential[44, 45] and possible ubiquinone[10]. Apart from cytochrome b, we believe that the presence of the other factors, including flavins, must be better demonstrated.

Attempts are in progress in our laboratory to isolate and purify the oxidase system. By treating the membrane of activated guinea pig granulocytes with detergents we have extracted a molecular complex of about 1.000.000 daltons containing proteins, phospholipids, flavin and cit b_{-245} and a very high NADPH oxidase activity[6, 7]. Further purification of the complex has shown that the oxidase activity copurifies with a protein of 31.000 daltons. The mechanism of electron transport from NADPH to oxygen remains to be elucidated.

Function of the Respiratory Burst

The effect and the function of the respiratory burst are linked to the production, fate and reactivity of the free radicals and of other intermediates of O_2 reduction. The first product of the oxidase is the free radical O_2^-, which rapidly dismutates to H_2O_2 ($2\,O_2^- + 2\,H^+ \rightarrow H_2O_2 + O_2$). The reaction between O_2^- and H_2O_2 (Haler-Weiss reaction) generates the strong oxidant hydroxyl radical ($H_2O_2 + O_2^- \rightarrow OH^\cdot + OH^- + O_2$). The rate constant of this reaction is very small and it certainly could not occur at the low concentration of O_2^- and H_2O_2. Since several transition metal ions, expecially iron, are present in a significant amount in living systems, it is likely that the Haber-Weiss reaction is catalyzed by iron ($Fe^{3+} + O_2^- \rightarrow Fe^{2+} + O_2$; $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^\cdot + OH^-$)[3, 19]. Lactoferrin, a neutrophil granule protein, may catalyze this reaction. Other free radicals may derive from metal catalyzed reaction of O_2^- with peroxide or other organic hydroperoxide to form a variety of oxidizing radicals (alkoxy and acyloxy radical in addition to $OH^\cdot$) which form a family of $OH^\cdot$-like oxidizing species[25].

Another possible reactive product is singlet oxygen ($^1O_2$) which can be formed by the metal-catalyzed Haber-Weiss reaction or as a product of H_2O_2 and myeloperoxidase[25].

The myeloperoxidase-H_2O_2-halide system generates other toxic molecules including hypoalous acids (HOX), such as hypochlorous acid (OHCl), long lived oxidants N-chloromine (RNHCl) and aldehydes[25, 43, 52].

O_2^- may act as an oxidant or as a reducent, but the main effect is linked to the strong oxidants $OH^\cdot$, OHCl and RNHCl, and to the action of Myeloperoxidase-H_2O_2-halide system. The interaction of all these oxidants with many molecular targets including protein, lipids, polisaccarides, nucleic acids, cofactors such as glutathione, tocopherol, ascorbate, β-carotene; biological factor such as chemotactic factors, α-antitrypsin, leukotrienes (that are inactivated by oxidation), cause a series of functional and harmful changes of cells and extracellular structures. It is worth pointing out that the inflammatory cells are equipped with efficient mechanisms for degradation or inactivation of free radicals and of H_2O_2. These mechanism include scavengers (sugar, unsatured fatty acids, sulfur containing aminoacids, glutathione, vitamine E, metals etc.) and enzymes (superoxide dismutase, catalase, peroxidases).

The effects of the respiratory burst can be distinguished in beneficial and harmful, and within these two categories, the main action are four: defensive, toxic, proinflammatory and modulatory.

Defensive action: this is the principal function of the burst because in addition to other factors[1] it provides a potent battery of weapons for killing ingested organism and tumor cells. The oxygen-dependent mechanisms of killing are two (Table 4): mediated by myeloperoxidase and direct. The first takes place inside the phagosome (where H_2O_2 is formed by dismutation of O_2^- and myeloperoxidase is discharged from the azurophilic granules) or outside (following the discharge of H_2O_2 and myeloperoxidase outside the cell). The killing is likely to be due to the formation of hypochlorous acid, of chloramine and of aldehydes[25, 43, 52, 54] and to the interaction between these toxic compounds and molecular targets of the engulfed organisms. This mechanism can also be

operative against tumor cells, independently on phagocytosis, but triggered by the contact with activated macrophages, mediated by antibodies (ADCC) or by arming factors.

The myeloperoxidase-independent killing is due to direct interaction between the free radicals or other oxidants with molecular components of the victim (bacteria, virus, protozoa, tumor cells), and can take place both after phagocytosis inside the phagosome and following the contact between the phagocyte and the invader.

Table 4. Microbicidas and Cytocydal Activity of Intermediates of O_2 Reduction

Mechanisms: 1. *Myeloperoxidase dependent*
($MPO\text{-}H_2O_2$-halide)
↓
OHCl; RNHCl; aldehydes
↓
Oxidation and inactivation of molecules of the victim

2. *MPO-independent*
direct oxidant effect

- Killing of infective agents inside the phagosome
- Killing of infective agents in extracellular environment (inflammatory sites)
- Killing of target cells without phagocytosis
  a) Antibody mediated cytotoxicity (antibody causes the hooking of the victim to granulocytes, macrophages, lymphocytes)
  b) Armed or activated macrophages

Table 5. Relationship between the Respiratory Burst and Inflammation

	Death of phagocyte itself	→ Tissue damage
O_2^-, H_2O_2, $OH^\cdot$, $^1O_2$	*Release of cell constituent*	→ Amplification of inflammation
OHCl, RNHCl	*Defensive mechanism (killing)*	→ Recovery
Other oxidizing radicals	*Amplification of Modulation*	
	↑ Vascular permeability	
	↑ Adhesion of leukocytes to endothelium – Endothelium damage	
	↑ Histamine release	
	↑ Platelet aggregation	
	↑ Platelet secretion	
	↑ Generation of chemotactic factor	
	Inactivation of chemotactic factors	
	Inactivation of proteases	
	Inactivation of anti-protease	
	Inactivation of leukotrienes	
	Transformation of prostanglandins	
	Modification of collagens	
	Depolarization of ialuronic acid	
	Antinflammatory effect of superoxide dismutase.	

Toxic action. On the basis of our knowledge of free radical pathology, it is evident that the free radicals of O_2 and H_2O_2, and the other oxidants generated produce chemical modification of virtually all components of the cell and of extracellular medium, followed by cell degeneration, cell death and tissue destruction. The matter has been reviewed recently[14, 16, 18]. Among the effect of oxygen radical generated by the burst also cytogenetic changes have been shown[53].

Effect on the inflammatory process

Evidences are continuously growing that the intermediates of O_2 reduction produced during the respiratory burst exert a amplificatory and modulatory function on the inflammatory process. The matter is quite complex and a detailed analysis has been the object of many reviews[14, 16] and meetings. A number of actions of the intermediates of

Table 6. Actions of Intermediates of O_2 Reduction on the Development and on the Effects of the Inflammatory Process

1. Proinflammatory
   Increase of vascular permeability
   Increase of adhesion of leukocytes to endothelium
   Generation of chemotactic factor
   Activation of histamine secretion
   Activation of prostaglandin formation
   Activation of platelet secretion and aggregation
2. Modulatory
   Inactivation of enzymes secreted (collagenase, elastase, etc.)
   Inactivation of α-1 proteinase inhibitor
   Inactivation of chemotactic peptides
   Inactivation of leukotrienes
   Transformation of prostaglandins
3. Defensive
   Killing of invading organism
   Killing of tumor cells
4. Toxic
   Extracellular
   Depolimerization of collagen
   Structural modification of collagen
   Decomposition of proteoglicans
   Protein denaturation
   Lipid peroxidation
   Cellular
   Cell damage with loss of function; mutations; degenerations; death (granulocytes, macrophages, erithrocytes, fibroblasts, endothelial, platelets, parenchimal)

O_2^- reduction or of their derivatives on inflammatory events and on cells participating in the inflammatory process have been described (Table 5). Examples are: increase in vascular permeability[12], secretion of serotonin by and aggregation of platelets[20], activation of histamine secretion by mast cells[30], stimulation of collagen synthesis[23], stimulation of arachidonic acid cascade[21], generation of chemotactic factor in the plasma[34], direct and non enzymatic formation of chemotactic peptide by arachidonic acid[51], increase in adhesive interaction between endothelia and white cells[12], inactivation of chemotactic factors[9], regulation of protease and antiprotease activities at the inflammatory sites through the inactivation of α 1-proteinase inhibitor[8], inactivation of leukotrienes by $OH^{\cdot}$[22], transformation of prostaglandins[32], depolimerization of ialuronic acid[27], modification of collagen[17], endothelial injury[42]. Relevant is also the antiinflammatory effect of SOD[4, 27, 31].

In an attempt of simplifying this matter the relationships between the burst and the inflammatory process has been classified in four type: proinflammatory, modulatory, defensive and toxic. Table 6 reports this classification.

In our opinion the problem of the function of the respiratory burst in the inflammation is not completely understood. Apart from some precise experimental findings the matter needs a more accurate revisitation. Many results wait for confirmation, many conclusions are indirect, many practical applications seem rather rash, many exaggerations (perhaps for commercial purposes) have been done.

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