INHIBITION OF THE RESPIRATORY BURST AND OF PHAGOCYTOSIS BY NORDIHYDROGUAIARETIC ACID IN NEUTROPHILS

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1. Introduction

Leucocytes have an impressive capacity to respond to a variety of stimuli with excitation of random and oriented movements, with secretion of intracellular stored compounds, with an increase in O_2 consumption and in production of O_2^- and H_2O_2. The secretion and the excitation of oxidative metabolism (respiratory burst) are also induced by phagocytosis [1–4].

During the last decade information has accumulated on a series of molecular events that take place at the level of the plasma membrane following the interaction with the stimulating agents, this includes changes in transmembrane potential due to modification of K^+ and Na^+ permeability [5–7], modifications of calcium fluxes and disposal [8–13], activation of arachidonic acid cascades with generation of prostaglandins, tromboxanes, leucotrienes and hydroxy fatty acids [13–21], modification of cyclic nucleotide turnover [22]. However, the precise nature of the mechanism triggering the manifold responses and the interrelation between the various responses remain to be clarified.

The concept emerging is that oxygenated metabolites of arachidonate produced by lipoxygenase, such as monohydroxyeicosatetraenoic acids (HETES), have a role in many leucocyte functions. This view is based on a series of experimental results showing that HETES elicits a chemotactic response in neutrophils [23] and that depletion of arachidonic acid oxygenate products in leucocytes causes an inhibition of random migration and chemotaxis [24], of exocytosis [25,26], of aggregation [27], of calcium influx induced by chemotactic peptides [28], and of the respiratory response to chemotactic peptide and digitonin [26,29].

This report presents the effects of nordihydroguaiaretic acid (NDGA), an inhibitor of lipoxygenase [30]: (i) on the respiratory burst induced by PMA and by Zymosan; (ii) on phagocytosis of Zymosan; (iii) on the activity of the stimulated NADPH oxidase, the enzyme responsible for the respiratory burst, in guinea pig and human polymorphonuclear leucocytes.

2. Materials and methods

2.1. *Collection of cells*

Guinea pig polymorphonuclear leucocytes (PMN) were prepared from peritoneal exudates and human neutrophils from healthy donors as in [31]. Cells freed from erythrocytes by hypotonic lysis were suspended in Krebs-Ringer phosphate buffer (pH 7.4) (KRP) containing 5 mM glucose and 0.5 mM $CaCl_2$. The viability of the cells was determined with the trypan blue exclusion test.

2.2. *Metabolic studies*

The respiration of the cells was measured with a Clark oxygen electrode as in [31]. The NADPH oxidase activity was measured as oxygen consumption with a Clark oxygen electrode by using cell-free particles, obtained by centrifuging at 100 000 × *g* homogenates of leucocytes stimulated by PMA as in [32]. The assay system was as follows: 0.075 M Na,K-phosphate buffer (pH 7.0), 0.170 M sucrose, 2 μM NaN_3, 0.15 μM NADPH.

2.3. *Phagocytosis*

Phagocytosis was tested by optical examination of samples of leucocyte suspensions challenged with opsonized Zymosan particles (0.5 mg STZ: 10^7 PMN), withdrawn from the plastic chambers during the mea-

surement of leucocyte respiration (3 min after addition of STZ) and stained with May-Grümwald Giemsa.

2.4. *Materials*

Nordihydroguaiaretic acid (NDGA), NADPH, phorbol 12-myristate 13-acetate (PMA), Zymosan A were from Sigma.

3. Results

Fig.1,2 show the effect of NDGA on the respiratory burst of guinea pig PMN. It can be seen that:

(i) The drug causes a marked inhibition of the rate of extra-respiration induced both by PMA and by STZ;

(ii) A preincubation of NDGA with the cells is not necessary for the inhibition of the burst. In fact the inhibitory effect is achieved when NDGA is added before the stimulatory agent and after the activation of the respiration;

(iii) The magnitude of the effect of NDGA is proportional to the drug concentration.

In different experiments the ID_{50} ranged from 50–100 μM when the stimulatory agent was PMA, and from 20–40 μM with STZ. Similar results have been obtained in human blood granulocytes.

After these findings we have performed a series of experiments in order to clarify the mechanism of the inhibitory action.

(i) The inhibition is not due to a cytotoxicity of the drug. In fact by using the trypan blue exclusion test on samples of cells withdrawn from the electrode chamber during the measurement of the respiration, we have shown that the percentage of the cells taking up the dye (<5%) is not modified by NDGA at the concentrations that exert an inhibitory effect on the burst.

(ii) The effect of NDGA is reversible. In fact the washing of PMN after the treatment with 100–200 μM NDGA (5 min centrifugation of PMN at 1000 × *g* and resuspension in KRP) completely restores the capacity to show a respiratory response both to PMA and STZ.

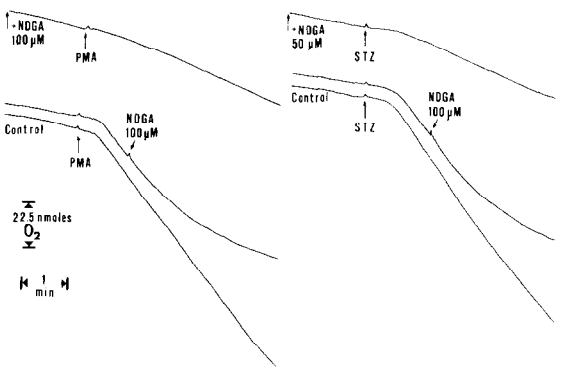

Fig.1. Polarographic traces of oxygen consumption by guinea pig PMN (1×10^7) stimulated by PMA (40 μg) and STZ (1 mg). Effect of NDGA: incubation medium (KRP) 2 ml; temp. 37°C.

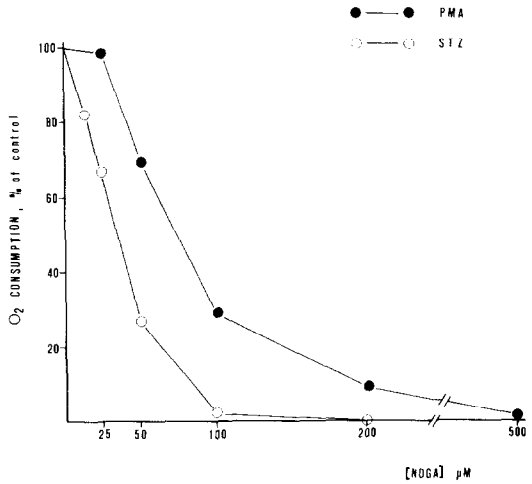

Fig.2. Effect of different concentrations of NDGA on the respiratory burst induced by PMA (20 μg/ml) and by STZ (0.5 μg/ml) guinea pig PMN.

The fact that NDGA is also inhibitory when added after the stimulatory agents prompted us to investigate the effect on phagocytosis and on the activity of the enzyme responsible for the burst. Table 1 shows that NDGA inhibits phagocytosis and that this inhibition parallels that of the associated respiratory burst. The washing of PMN treated with NDGA completely restores the phagocytic activity.

Furthermore NDGA has an inhibitory effect on NADPH oxidase activity of cell-free particles obtained from homogenates of leucocytes stimulated by PMA. In different experiments the ID_{50} ranges from 150–300 μM. These values are higher than those found for the effects on the respiratory burst and on phagocytosis of intact cells. The results of the experiment using different concentrations of NADPH show that NDGA exerts a inhibitory action by a non-competitive mechanism (fig.3).

Table 1
Effect of NDGA on phagocytosis of STZ and on associated respiratory burst in guinea pig PMN[a]

	Phagocytosing[b] PMN %	Phagocytic index[c]	% Inhibition	% Inhibition of respiratory burst
Control	90	1.94	–	–
NDGA 12.5 μM	67	1.41	−27.3	−18
NDGA 25 μM	52	1.01	−47.9	−33
NDGA 50 μM	17	0.46	−76.3	−73
NDGA 100 μM	3	0.04	−97.7	−98

[a] Means of 3 expt; [b] PMN containing STZ particles; [c] Mean number of STZ particles/cell

4. Discussion

By analogy with the interpretation advanced with regard to manifold functions of leucocytes such as aggregation [27], exocytosis [25,26], movement [23,24], hexose transport [33], calcium fluxes [28], the inhibitory effects of NDGA on phagocytosis and on the respiratory burst could be explained as being due to the inhibition of the lipoxygenase pathway of arachidonic acid metabolism. If this explanation is correct, the oxygenation products of the arachidonic acid cascade play a role also in the regulation of the mechanisms triggering the respiratory response of leucocytes. On the basis of the results reported here these hydroxy acids (HETES) would be involved in the recognition of the stimuli and/or in the coupling between the stimuli and the responses, that is the endocytic act, the activation and the activity of the NADPH oxidase, the enzyme responsible for the respiratory burst.

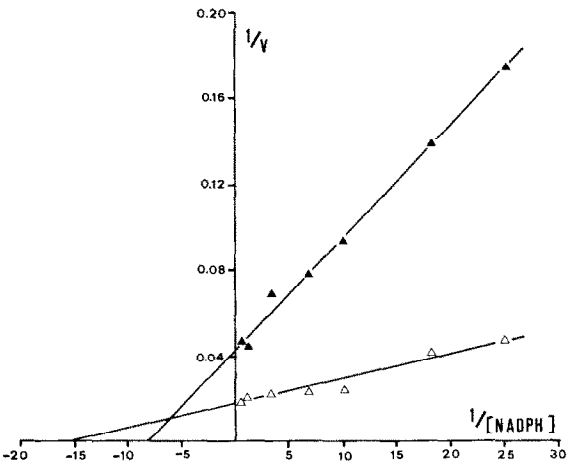

Fig.3. Inhibition of NADPH oxidase by NDGA: Lineweaver-Burk plot of NADPH oxidase activity of 100 000 × g particles obtained from PMA activated guinea pig PMN. (△) no NDGA; (▲) 300 μM NDGA; abscissa (mM^{-1}); ordinate (nmol O_2 consumed . min^{-1} . mg $protein^{-1}$).

However, a careful analysis of the results casts doubts on this interpretation and indicates that mechanisms different from the deprivation of lipoxygenase products could be responsible for the effect of NDGA.

(i) A preincubation of leucocytes with the drug, which is necessary for the decrease in the concentration of HETES in leucocytes [24], is not a perequisite for the inhibitory activity. In fact in our experiments this drug is active also when added a short time before the stimulant and when the respiratory burst has been activated.

(ii) The inhibitory activity is reversible by a simple lavage of leucocytes treated with active doses of NDGA. This finding indicates that the perequisite for the inhibitory activities is the continuous presence of the drug inside the cell or bound to the plasma membrane.

(iii) The concentrations of NDGA required for the inhibition of the respiratory burst by PMA and also of phagocytosis are higher than those capable of reducing the content of HETES in neutrophils [24].

(iv) NDGA causes a direct and non-competitive inhibition of the activated NADPH oxidase.

Whether NDGA acts independently on the effect on lipoxygenase and on a modification of the content of HETES in the membranes of leucocytes, what is the mechanism of the inhibitory activity of this compound on the burst and on phagocytosis? The scarcity of experimental information and the complexity of

the problem do not allow an answer to this question. Among the elements of this complexity is, for example, a possible correlation between the inhibitory effect of NDGA on methyl-transferase [28] and that on phagocytosis and on the burst.

Considering current mechanisms thought responsible for the burst, two levels of activity of NDGA should be taken into account to understand its action and to establish further investigations:

(1) The mechanism of activation of NADPH oxidase;
(2) The activity of the oxidase once activated.

The results presented here seem to indicate that NDGA acts at both of the above levels. It is likely however, that the main action is on the mechanism of NADPH activation. When leucocytes are challenged with phagocytosable particles, the presence of NDGA impedes the movements of the plasmamembrane, that consist in the phagocytic act, and the associated molecular rearrangements, that represent the triggering of the activation of the membrane bound respiratory enzyme. When PMA is the stimulatory agent, a similar mechanism would operate. In this case NDGA prevents the molecular perturbations of the plasma membrane, that follow the interaction with the soluble agents, and hence the activation of the oxidase.

We do not know whether there is a common mechanism for the inhibitory effect on phagocytosis and on the burst by PMA. A unifying hypothesis could be the occurrence of a modification of a lypoxygenase-independent calcium influx or disposal, that is of phenomena involved in the triggering and in the regulation of a variety of leucocyte functions [10].

More complex is the explanation of the inhibition of the respiratory burst that occurs when NDGA is added after the stimulation by PMA or by Zymosan. In this case two mechanisms can be postulated:

(1) Since the stimulation of the respiration induced by soluble factors is a reversible phenomenon [34] and that caused by phagocytosable matter is linked to the endocytic act [35], the addition of NDGA interrupts the movements of the membrane and the molecular rearrangements, whose occurrence must be continuous in order to maintain the oxidase in an activated state, or to cause a continuous renewal of the activation of the oxidase.
(2) A direct effect of NDGA on the activated NADPH oxidase.

However the fact that the inhibition of NADPH oxidase in vitro requires a concentration of the drug higher than that required for the inhibition of phagocytosis and of the burst by PMA, casts some doubts on the relevance of this second mechanism in intact cells.

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