KINETICS OF NICOTINAMIDE ADENINE DINUCLEOTIDES IN OLEATE-STIMULATED POLYMORPHONUCLEAR LEUKOCYTES

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1. Introduction

During phagocytosis by polymorphonuclear (PMN) leukocytes, a number of metabolic changes take place. The most dramatic of these are the stimulation of a cyanide-insensitive oxygen metabolism and the increase of glucose oxidation via the hexose monophosphate shunt (HMS) pathway. Stimulation of the HMS is presumed to result from a production of NADP from NADPH as a consequence of the activation of either a NADPH oxidase [1], or a NADH oxidase [2]. Although the substrate specificity of the oxidase involved remains controversial, recent reports favor the involvement of a NADPH oxidase [3–5].

During phagocytosis of particles such as bacteria or polystyrene spherules, the cellular NADP/NADPH ratio is increased, whereas the NAD/NADH ratio does not change [6–8]. Our results confirm the increase of NADP for PMN leukocytes treated with sodium oleate which was shown to be a potent non-phagocytic stimulator of the cyanide-resistant oxidative metabolism. We also report that a portion of the cellular NAD(H) is converted into NADP(H).

2. Materials and methods

PMN leukocytes were isolated from heparinized fresh horse blood by a slight modification of the Böyum technique [9] and finally suspended in Hanks medium (pH 7.4). Differential counting revealed >95% PMN leukocytes in all preparations. Oxygen consumption was measured amperometrically in a microvessel [10] equipped with a Yellow Springs Instrument Clark electrode. Release of hydrogen peroxide from stimulated cells was measured fluorometrically by means of the peroxidase-catalyzed oxidation of reduced scopoletin [11]. Superoxide production was quantitated by following spectrophotometrically the reduction of 25 μM cytochrome *c* [12]. All experiments were done in Hanks solution at 37°C and the maximal rates were deduced from the slope of the apparently linear portion recorded during the first 3 min.

The extent of lipid peroxidation in cell suspensions was assessed by the thiobarbituric acid assay which measures the formation of malondialdehyde [13]. The concentration of the coenzymes, NAD, NADH, NADP and NADPH in PMN leukocytes was determined by the method of enzymatic cycling as in [14]. Internal standards of the coenzymes were added to the cell suspensions and simultaneously analyzed.

Sodium oleate (Hopkin and Williams, London), solubilized in 0.9% NaCl, was added to initiate the oxidative processes. The coenzymes, alcohol dehydrogenase and glucose-6-phosphate dehydrogenase were purchased from Boehringer, Mannheim. All other chemicals were of the highest grade available.

3. Results and discussion

Certain fatty acids induce the production of superoxide anions by PMN leukocytes [15]. The results in fig.1 show that a micellar solution of the salt of an unsaturated fatty acid, sodium oleate, can induce an increase in oxygen uptake in resting horse leukocytes accompanied with generation of superoxide and hydrogen peroxide. These metabolic responses of PMN are dependent on the concentration of oleate, insensitive to cyanide (1 mM) or azide (1 mM), and require intact, viable cells. The potency of oleate as stimulator is illustrated by the fact that oleate-activated PMN consume oxygen ~4-times more rapidly

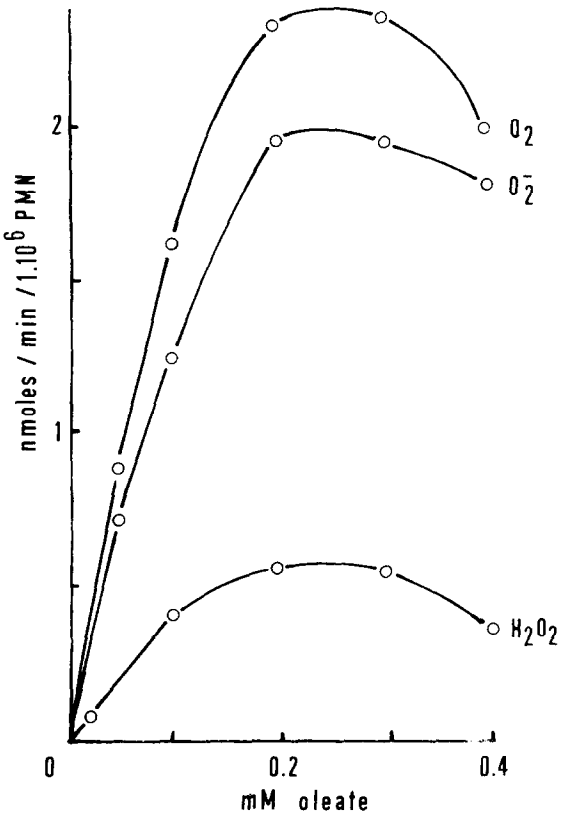

Fig.1. Effect of oleate concentration on O_2-uptake and release of H_2O_2 and O_2^- from PMN leukocytes. PMN were suspended in Hanks medium (1×10^7/ml for the oxygen and superoxide assay, 2×10^6/ml for the peroxide assay) and oleate was added after 10 min preincubation at 37°C. Measurements of O_2, O_2^- and H_2O_2 were performed as in section 2 and are mean result of 3 assays.

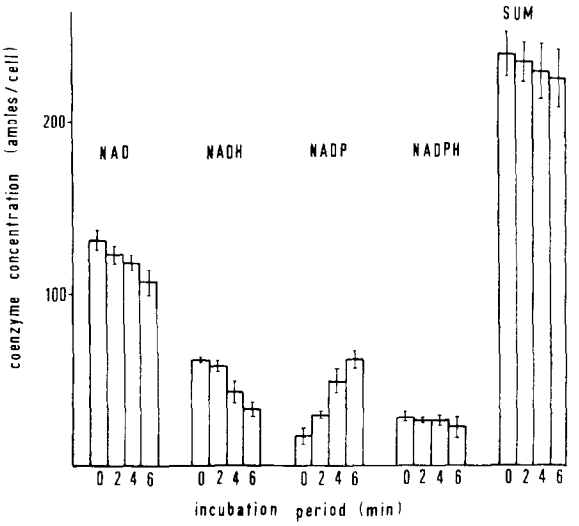

Fig.2. Intracellular coenzyme concentration of PMN leukocytes as a function of incubation time with 0.2 mM sodium oleate. Cells (5×10^7/ml) were preincubated for 10 min at 37°C. After incubation with oleate for the indicated time, the coenzymes were extracted with 0.1 N HCl or 0.1 N NaOH. Extracts were heated for 2 min at 90°C, neutralized and assayed for coenzyme content by the enzymatic cycling method. Data are expressed as mean ± SEM of 5 separate extracts.

than cells which are phagocytizing latex particles (0.68 nmol . min^{-1} . 10^6 PMN^{-1}).

When the metabolism of the cells is initiated at 37°C, a change of the coenzymes levels was noticed during the first minutes of incubation, which confirms [6], whereafter no further significant variations occur. Accordingly, all experiments were carried out using a 10 min preincubation at 37°C. The concentrations of the different nicotinamide adenine dinucleotides in horse PMN leukocytes at rest and during treatment with sodium oleate are shown in fig.2. The results reveal a striking influence of the oxidative stimulator. After a few minutes a marked increase of NADP is observed, while the concentration of NADPH has only slightly dropped. A concomitant decrease in both NAD and NADH levels is noticed. Since the total amount of the coenzymes does not change significantly, apparently no net synthesis or breakdown occurs in the short incubation time employed. It seems likely that parallel to activation of the oxidative system, oleate treatment of PMN triggers a mechanism by which cellular NAD(H) is converted into NADP(H).

To exclude the possibility that the oleate-induced oxygen burst and the concomitant alterations in cellular nicotinamide adenine dinucleotide levels are due to lipid peroxidation [16], the production of malondialdehyde was monitored. The results (table 1) indicate that no significant lipid peroxidation occurs during the incubation period employed. Initiation of intracellular lipid peroxidation by addition of Fe(III)-ADP [17] to PMN leukocytes in the presence of oleate was minimal and yielded no quantifiable increase of the oxygen consumption.

In particle-phagocytizing PMN a 2-fold increase of the NADP/NADPH ratios has been described while the NAD and NADH levels are unaffected [6,7]. The extent of change in the NADP/NADPH ratio is expected to depend upon the relative activities of the NADP-linked dehydrogenases of the HMS and to that of the

Table 1
Effect of various compounds on the oxygen consumption, malondialdehyde production and intracellular coenzymes of oleate-activated PMN leukocytes

Conditions	O_2-consumption (nmol . min^{-1} . 10^6 PMN^{-1})	Malondialdehyde (ΔA_{532}/10 min)	Coenzymes (amol/cell)			
			NAD	NADH	NADP	NADPH
Activated PMN	2.7 ± 0.2	0.032	91	50	68	14
+ Fe^{3+}-ADP (50 μM)	2.6 ± 0.3	0.050	87	56	73	16
+ NaN_3 (1 mM)	2.5 ± 0.2	–	96	54	61	14
+ Cytochalasin B (10 μg/ml)	3.0 ± 0.2	–	76	42	82	13
+ PCMBSA[a] (1 mM)	1.2 ± 0.4	–	116	62	36	20
Resting PMN (control)	0.4 ± 0.1	0.029	125	79	16	21

[a] PCMBSA, *p*-chloromercuribenzene sulfonic acid

PMN leukocytes in Hanks medium were stimulated after a 10 min preincubation at 37°C by the addition of 0.2 mM sodium oleate. Oxygen consumption (expressed as the mean ± SEM of 4 expt) and malondialdehyde production were measured as in section 2. Extractions of NAD, NADH, NADP and NADPH were made after 5 min at 37°C and assays were performed as in fig.2. The mean results from 3 expt are shown

NADPH oxidase. The concentration of NADP has been demonstrated to be the rate limiting factor for shunt activity [18]. Following stimulation with oleate a >6-fold increase of the cellular NADP/NADPH ratio is noticed within a few minutes. Whole-cell contents of the different forms of the coenzymes were measured. Since intracellular concentration gradients may exist and since coenzymes may reside substantially in a bound state with enzymes, it is possible that an even more pronounced change of the nicotinamide nucleotide ratios takes place in the pool which serves both the stimulated NADPH oxidase and the NADP-linked dehydrogenases of the shunt. Combining the data on oxygen consumption (fig.1) with those on cellular NADPH content in stimulated cells (fig.2) and assuming that all NADPH is available as substrate for the oxidase, a turnover of ~100/min can be calculated for the coenzyme. Under these conditions the NADPH level can become rate limiting for the oxidative processes. The results show that the cellular NADPH content remains at ≲40 μM (assuming cell vol. 3000 μm^3). This value corresponds rougly with the app. K_m for the NADPH-oxidase [4,5]. Increasing the NADP(H) pool by a NAD kinase-catalyzed phosphorylation of NAD would enable the cell to carry out an elevated HMS activity and allow the generation of greater quantities of reactive oxygen species. A NAD kinase has been described in human neutrophils [19]. Although its physiological function has to be further established, NAD kinase may play a regulatory role in coenzyme metabolism.

The effects of some effectors on the oleate-induced oxygen burst and on the concomitant changes in cellular coenzyme levels are shown in table 1. Addition of 1 mM azide has no effect, whereas preincubation with cytochalasin B results in a slight potentiation of both phenomena, thereby confirming the observations made for other non-phagocytic stimulants on oxygen metabolism of cytochalasin-treated granulocytes [20]. The inhibitory effect of the non-penetrating sulfhydryl reagent *p*-chloromercuribenzene sulfonic acid on the oxygen burst correlates well with a decreased alteration of the coenzyme pattern. Altogether these results indicate that at the least in oleate-stimulated granulocytes the phosphorylation of NAD is closely associated with the remarkable alterations in oxidative metabolism referred to as the respiratory burst.

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