CO-LOCALIZATION OF SUPEROXIDE GENERATION AND NADP FORMATION IN PLASMA MEMBRANE FRACTIONS FROM HUMAN NEUTROPHILS

PAMELA S. SHIRLEY, DAVID A. BASS, CYNTHIA J. LEES, J. WALLACE PARCE, B. MOSELEY WAITE and LAWRENCE R. DECHATELET

Department of Biochemistry and Department of Medicine
Bowman Gray School of Medicine of Wake Forest University
Winston-Salem, North Carolina 27103

Abstract—In order to resolve discrepancies in the literature concerning the subcellular localization of NADPH oxidase, we disrupted human neutrophils by nitrogen cavitation and fractionated the subcellular organelles on a discontinuous sucrose density gradient. The lightest fraction was 20- to 40-fold enriched for plasma membranes as determined by the marker enzymes alkaline phosphatase and phosphodiesterase I as well as by the ratio of lipid phosphorus to protein. There was a significant decrease in the specific activities of the granule markers myeloperoxidase, lysozyme, and β-glucuronidase. An intermediate fraction was enriched in membrane markers but not to the extent the lightest fraction was enriched. This fraction contained more granular contamination, as shown by the marker enzymes. In contrast, the densest bands of the gradient were enriched for granule markers with little contamination by plasma membrane. Superoxide generation and NADP formation were primarily associated with the two membrane-enriched fractions from polymorphonuclear leukocytes stimulated with phorbol myristate acetate. The NADP formation associated with a dense granule fraction observed previously in our laboratory was probably due to a cyanide-stimulated oxidation of NADPH by myeloperoxidase.

INTRODUCTION

During the process of phagocytosis, human neutrophils undergo marked alterations in oxidative metabolism collectively referred to as the respiratory burst (1, 2). These changes are generally conceded to be due to activation of a

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reduced pyridine nucleotide oxidase which preferentially oxidizes NADPH to NADP with production of the superoxide anion (O$_2^-$); subsequent dismutation of superoxide results in the production of hydrogen peroxide (1, 2). We have assayed for this oxidase activity by measuring the formation of the oxidized nucleotide (NADP) using either sensitive isotopic (3) or fluorometric (4) procedures. Others have assayed for the enzyme by measuring the production of superoxide, employing the superoxide dismutase-inhibitable reduction of cytochrome $c$ (5-8). Results with these assays have generally been comparable. In particular, the "oxidase" is inactive in resting cells but is activated by appropriate stimulation with either a phagocytic stimulus (3-5, 8) or with a soluble stimulus such as phorbol myristate acetate (PMA) (9). Further, cells obtained from patients with chronic granulomatous disease (CGD) do not exhibit a respiratory burst upon phagocytosis (10). Fractions obtained from such cells fail to show an activation of the oxidase regardless of whether NADP formation (11, 12) or superoxide generation (1, 6) is employed as the assay. For these reasons, it has been assumed that both assays were measuring precisely the same activity.

A difficulty arose, however, with regard to the subcellular localization of the enzyme. We fractionated cells on a sucrose density gradient and observed oxidase activity (determined by NADP formation) in a dense granule of the cell which sedimented at least as far into the gradient as the azurophil granule (4, 13). In contrast, Dewald et al. (14) performed almost identical experiments but reported that the oxidase activity (determined by superoxide generation) resided almost entirely in a light fraction which corresponded to the plasma membrane of the cell. This discrepancy in subcellular localization has not been adequately resolved.

In the present communication we describe a modified procedure which yields relatively pure plasma membrane preparations and demonstrate that when performed using similar conditions, NADPH oxidation is not separable from superoxide generation in terms of subcellular localization.

**MATERIALS AND METHODS**

*Isolation of Cells.* Leukocytes were isolated from the heparinized blood of normal human volunteers by sedimentation with plasma gel as previously described (15). Contaminating erythrocytes were removed by a brief (20-sec) hypotonic lysis with deionized water and isotonicity restored by the rapid addition of an appropriate amount of hypertonic sodium chloride. This procedure routinely yielded a preparation containing 80-90% granulocytes with greater than 95% viability as determined by trypan blue exclusion. In most experiments, cells were isolated from two units of blood obtained from separate normal donors; following isolation, the cells were pooled for membrane purification. In another experiment cells were obtained from a patient with CGD.