^{31}P NMR SPECTROSCOPY OF PHORBOL–MYRISTATE–ACETATE STIMULATED POLYMORPHONUCLEAR HUMAN LEUKOCYTES

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1. Introduction

High resolution nuclear magnetic resonance (NMR) of cellular suspensions and tissues has been applied in the study of various metabolic activities under physiologic conditions. In particular ^{31}P NMR has been used to monitor intracellular pH and directly measure various phosphorous metabolites as a function of selected cellular activities [1–3]. The data herein reported are the first employing NMR to study the polymorphonuclear (PMN) leukocyte, a circulating blood phagocyte which serves in the initial cellular defense against invading microorganisms. The PMN leukocyte manifests a complex respiratory burst when activated by phagocytosable particles or by the soluble agonist, phorbol-12-myristate-13-acetate (PMA) [4]. The respiratory burst of the PMN leukocyte is characterized as cyanide-insensitive to distinguish mitochondrial respiration from the activation of a flavin-dependent NADPH-oxidase, which reduces molecular oxygen to superoxide (O_2^-), and accounts for the principal enzymatic activity of the respiratory burst [5]. Reduction products of the consumed oxygen are utilized in various microbicidal mechanisms. ^{31}P NMR spectroscopy was employed to measure directly the cellular ATP pool, the pyridine nucleotide (NAD(H)) concentrations, and monitor pH fluctuation as a consequence of PMA and zymosan activation of the human PMN leukocyte respiratory burst. This first report of NMR spectroscopy applied to leukocytes demonstrates the ease and utility of direct measurement for study of the metabolism of various phosphorous compounds.

2. Materials and methods

Human venous blood was obtained from normal donors and PMN leukocytes were harvested by dextran sedimentation, hypotonic lysis, and centrifugation over Ficoll-Paque (Pharmacia Fine Chemicals, Piscataway NJ) in >99% final purity as in [6]. Cells ($3–6.7 \times 10^8$ cells/ml in Hanks balanced salt solution without phenol red (Microbiological Associates, Walkersville MD) with 10% D_2O for internal lock signal were placed in 10 mm NMR tubes. ^{1}H noise-decoupled ^{31}P NMR spectra at 109.3 MHz were obtained at 4°C with a Bruker 270 spectrometer. Typical acquisition parameters included 30° pulse angle, 0.6 s repetition rate, and 1000 transients. Under these conditions, none of the phosphate metabolite resonances were detectably saturated in these cells. Cells were stimulated with zymosan opsonized in autologous serum (6) or PMA (Sigma Chemical Co., St Louis MO) at a maximal stimulatory (non-cytotoxic) concentration of 1 μg/ml for 10 min at 37°C. Spectra of activated cells were subsequently obtained at 4°C.

^{31}P chemical shifts are referenced with respect to external H_3PO_4 (in some cases internal phosphocreatine was used). Determination of solution pH was based on the chemical shift of the P_i resonance [1,2]. Quantitation of phosphate metabolites was done with internal standards of known concentrations of phosphocreatine and P_i added to resting cells. The data for resting cells are the average (and standard deviation) determined from spectral duplicates of 3 leukocyte preparations. PMA activation data are derived from 2 different PMN leukocyte preparations and zymosan activation data are from a single leukocyte donor. In

each instance, duplicate spectral analyses were performed with each cell preparation. The ATP-β resonance (~18 ppm) was used to measure ATP concentration; ADP concentration was calculated from the difference in area of the ATP-γ,ADP-β resonance (~4.8 ppm) and the ATP-β peak. NAD(H) was estimated by integration of a shoulder, or in some cases a defined small peak at 10.5 ppm. The excess area (above that at 4.8 ppm) under the ATP-α,ADP-α resonance at 9.8 ppm is labeled 'X' and is indicative of a pyrophosphate compound. Sugar phosphates were determined from the area under −5 to −3 ppm.

3. Results and discussion

3.1. *Definition of NMR signal*

NMR spectra were obtained from both resting and PMA stimulated PMN leukocytes (fig.1). Five prominent signals were analyzed and found to correspond to a pool of sugar phosphates (SP) (~−4.75 ppm), inorganic phosphate (P_i) (~−2.6 ppm), adenosine triphosphate (ATP) (γ-P at 4.8 ppm, β-P at 18.7 ppm, α-P at 9.8 ppm), adenosine diphosphate (ADP), and pyridine dinucleotide diphosphate (NAD{H}) (10.5 ppm, visible as a shoulder in A and as a well-defined peak in B).

The ATP chemical shifts (γ-P, 4.81 ppm; α-P, 9.80 ppm; β-ATP, 18.69 ppm) in resting cells, suggest that all ATP is complexed to Mg^{2+} and not free [1]. The resonance peak of sugar phosphate and monophosphate nucleotide pool appears to contain little AMP and IMP, with relatively more glucose 6-phosphate, although detailed identification in this region will depend on spectra of $HClO_4$ extracts [7,8].

3.2. *pH change with stimulation*

A fall in pH was noted after 10 min leukocyte stimulation from the initial value of 7.1. Determined from the shift of the P_1 signal, the pH of PMA-stimulated PMN leukocytes was 5.9–6.2 and 6.4 for zymosan-activated cells. It is noteworthy that in fig.1A, the initial P_i signal is broad and somewhat split. This may represent either poorly differentiated compartmentalized pools or an equilibration of P_i inside the cell with the external medium.

Early work with animal granulocytes demonstrated the selective incorporation of ^{32}P into phosphatidic acid, phosphatidylinositol, and phosphatidylserine upon phagocytic stimulation [9]. In our studies

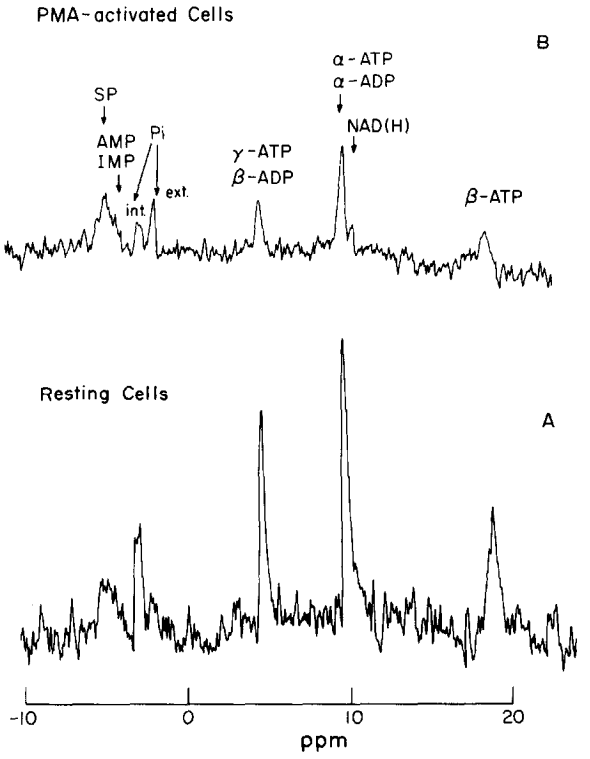

Fig.1. (A) Cells 3 × 10^8/ml resting PMN leukocytes at 4°C; acquisition parameters are 4000 Hz sweep width, 8192 data points, 30° flip angle, 896 transcents, 10 Hz line-broadening; (B) Cells 3 × 10^8/ml activated with PMA for 10 min at 37°C then cooled to 4°C for spectral acquisitions; similar parameters to (A) with the exception that 3344 transcents were accumulated.

employing NMR spectroscopy, the P_i signal was not lost by incorporation into complex phospholipids or phosphoproteins upon stimulation, but was found predominantly in the extra-cellular medium. After spectrum accumulation of stimulated PMN leukocytes, the cells were centrifuged, and resuspended in Hanks' solution containing <1 mM phosphate (a negligible quantity by this assay). Resuspended cells and the original supernatant were then again examined by NMR spectroscopy. Of the P_i signal, 50–75% was found in the extracellular supernatant. The significance or mechanism of secretion has not been determined.

Studies employing phagocytozed particles which were stained with pH-sensitive dyes demonstrated that the pH within the phagocytic vacuole was 6.5–≤5.0 for similar periods of activation [10,11]. While PMA has been shown to induce vacuolization in PMN leukocytes [12], NMR spectroscopy was unable to dif-

Table 1
Concentrations (nmol/10^8 cells) of phosphate compounds detected by ^{31}P NMR spectroscopy of PMN leukocytes in resting, PMA- and zymosan-activated states

	Resting	+PMA[a]	+Zymosan[a]
SP	81 ± 20[c]	77 ± 23	134 ± 30
ATP	149 ± 11	78 ± 12	113 ± 15
ADP	22 ± 12	22 ± 13	30 ± 15
X[b]	34 ± 6	50 ± 32	68 ± 30
NAD(H)/NADP(H)	48 ± 30	34 ± 13	47 ± 26

[a] Activation of cells for 10 min at 37°C, prior to accumulating the ^{31}P spectra at 4°C
[b] An unidentified component of the peak at 9.80 ppm, probably containing a pyrophosphate linkage
[c] Average ± standard deviation

ferentiate whether pH changes were uniform within intracellular compartments. The large pH shift (1.0 or 0.6 units) documented represents an average extracellular level with possibly wide intracellular compartment variation.

3.3. *Assessment of ATP–ADP levels*

Integration and standardization with internal phosphocreatine and P_i of the ATP-β phosphorous peak (fig.1) demonstrates 149 ± 11 nmol ATP/10^8 resting cells. The ADP levels did not vary significantly upon PMA stimulation, while a significant decrease in ATP levels was seen (table 1). This fall in ATP concentration is consistent with previous studies of human PMN leukocytes phagocytosing zymosan particles [13] and with our single experiment employing zymosan as the stimulus. The observation that mitochondrial respiration does not maintain resting ATP levels subsequent to maximal stimulation is thus observed with both zymosan and PMA activation. Studies employing extraction of ATP and assay with the luciferase system, reported ATP values comparable to those found in these direct NMR determinations [13,14]. The role of cAMP in the activation mechanism of the respiratory burst [15,16] or of ATP in modulating that activity are unsettled. That ATP may function as an inhibitor of the respiratory oxidase has been suggested for both the guinea pig [17] and human [18] enzymes; however we favor enzymatic autoinactivation [5] as the principal regulator of the activated oxidase. Excess area under the 9.8 ppm resonance (compared to the 4.8 ppm signal) was assigned to an unidentified pyrophosphate diester. Such discrepancies have also been seen in perfused heart spectra [1].

3.4. *Pyridine nucleotide concentrations*

By integration of the shoulder at 10.5 ppm, NAD(H) levels are estimated to be 34–48 nmol/10^8 cells (table 1). This value though error-prone because it overlaps with the ATP-α,ADP-α peak remained constant during PMA and zymosan stimulation which reflects that both the oxidized and reduced forms are pooled in this determination. Determinations in [19,20] of NADP(H) and NAD(H) levels in human PMN leukocytes are 14–16 nmol/10^8 cells, a figure 1/2–1/3 that reported here. The discrepancies may reflect differences of efficiency between methods relying upon extraction procedures with chemical assay and direct NMR observation.

These findings demonstrate the feasibility of applying ^{31}P NMR spectroscopy to the study of human PMN leukocytes. The technical ease and accuracy of determining the concentration of various phosphate-containing compounds, their sensitivity to pH shifts, and the ability to separate intra- and extracellular components of the ^{31}P NMR signal, further illustrate the broad applicability of such methodology. From these studies, the significance of the appearance of extracellular phosphate and a pH gradient established by PMA stimulated PMN leukocytes are the subject of continued study.

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