Polymorphonuclear Leukocyte Activation: Effects of Phospholipase C (36498)

SANDRA S. KAPLAN, STUART C. FINCH, AND ROBERT E. BASFORD
(Introduced by Dane R. Boggs)

Departments of Pathology and Biochemistry, University of Pittsburgh Medical School, Pittsburgh, Pennsylvania 15213; and Department of Medicine, Yale University Medical School, New Haven, Connecticut 06510

The polymorphonuclear (PMN) leukocyte reacts to phagocytosis or treatment with surface-active agents with a large increase in the flow of glucose through the hexose monophosphate shunt (HMPS) (1–4). This reaction is stimulated by an increased availability of NADP through a group of reactions involving NADPH oxidase (5, 6) and glutathione reductase (7), both of which show increased activity very shortly following particle ingestion. The inciting phenomenon, whereby the activity of these enzymes becomes increased preceding increased HMPS activity is unknown. However, its requirement for an intact cell (8, 9) and recent work correlating structural alterations of the cell membrane with metabolic stimulation (10) suggest that the initial stimulus may come from changes in the cell membrane itself. The morphologic changes involve the plasma membrane and the membranes of subcellular particles (11). The metabolic alterations include increased incorporation of ^{14}C-acetate and $^{32}P_i$ into lipids (12) and increased synthesis of phospholipid (13).

Phosphoglycerides are a prominent constituent of the PMN leukocyte membrane. Their degradation and repair following phagocytosis may generate NADP for early activation of the HMPS and may serve to upset the existing equilibrium involving NADPH, its oxidase and glutathione reductase. Therefore, changes in leukocyte HMPS activity, acetate incorporation into lipids and NADP/NADPH ratios were studied in leukocytes treated with phospholipase C (plC) and the metabolic changes were compared to the base line levels of similarly incubated untreated cells.

Materials and Methods. PMN leukocytes were obtained from human donors with a moderate leukocytosis associated with infection or surgery and were prepared as previously described (14). The cells were suspended in Krebs–Ringer phosphate (KRP) buffer (pH 7.4); containing 1 m*M* $CaCl_2$, 5.5 m*M* glucose and 10% autologous plasma. Leukocyte counts ranged from 1 to 3 $\times$ 10^7 cells/ml, of which 80–90% were PMN leukocytes. All studies were performed using 3 ml of this cell suspension in 25 ml Erlenmeyer flasks which were incubated at 37° in a Dubnoff shaker. Phospholipase C (Sigma) was used at a concentration of 0.4 units/ml. Leukocyte counts were repeated after 15 and 60 min of incubation. For HMPS evaluation, 0.2 μCi 1-^{14}C-glucose was added to cells with and without plC. Incubations were continued for 1 hr in stoppered flasks also containing 0.2 ml Hyamine in a center well (Kontes) suspended from the stopper. The reaction was terminated by the injection through the stopper of 0.2 ml 6 *N* H_2SO_4, and after shaking at 37° for 30 additional minutes, the center wells were transferred to counting vials to which 10 ml of Bray's solution were added. Samples were counted in a Packard Tri-Carb scintillation spectrometer, model 3003. In all studies, reactions were performed in duplicate and blanks omitting cells were run each time. Several separate experiments were done with 1 m*M* KCN or with 10^{-3} *M* EDTA added to the cell suspension. Results are expressed as the mean counts per minute per 10^7 leukocytes, with significance evaluated by means of the paired *t* test.

Acetate incorporation into lipids was deter-

mined using 2 μCi 1-^{14}C-sodium acetate/flask with incubation at 37° for 15 min. The contents of each flask were transferred to separate ice-cold conical centrifuge tubes and spun at 5° for 15 min at 300*g*. The supernatant fluid was removed and the cell button was suspended in 20 vol of chloroform–methanol, 2:1. Lipids were extracted according to the method of Folch, Lees and Stanley (15). An aliquot of each extract then was placed in a counting vial and evaporated to dryness under nitrogen. These samples were counted, and results were expressed as described for glucose oxidation. A second aliquot was used for quantitation of lipid according to the method of Amenta (16).

Estimation of NADP and NADPH were carried out according to the method of Nesselbaum and Green (17). After incubation with and without plC for 10 min, the cell suspensions were transferred to separate ice-cold conical centrifuge tubes and spun for 10 min at 300*g*. The cells were resuspended in 1 ml fresh KRP buffer and the nucleotides were extracted by the addition either of 1 ml 0.1 *N* HCl for NADP or 1 ml 0.1 *N* NaOH containing 0.5 m*M* cysteine for NADPH. After the addition of acid or base the cells were mixed on a vortex mixer and placed in a water bath at 100° for 30 sec. The suspensions then were neutralized, brought to 5 ml with glycyl–glycine buffer and centrifuged at 20,000*g* for 20 min. An aliquot of the supernatant was used for the assay. Absorbance was followed manually on a Beckman DU spectrophotometer. Nucleotide concentrations were determined from the ΔOD/5 min by referring to a standard curve consisting of 3 points which was run for each set of unknowns.

Results. The effects of plC on HMPS activity are shown in Table I. There was a significant stimulation of the mean HMPS activity to 368% of control. This stimulation was not abolished by 1 m*M* KCN but was totally lost in the presence of 10^{-3} *M* EDTA. This is consistent with the known dependence of plC on calcium (18). Leukocyte counts performed at intervals during the course of the experiment revealed no change in control flasks or in plC flasks after 15 min, but showed a 6% decrease after 1 hr with plC. The HMPS activity per 10^7 leukocytes was based on the leukocyte count done after 1 hr of incubation.

Acetate incorporation into total lipids was enhanced in leukocytes incubated with plC (Table I). The mean increase in 9 separate experiments was to 132% of control. However, there was no significant difference in total lipid in μg/10^7 PMN leukocytes; values for control and plC cells were 174 and 167, respectively ($p > .4$).

NADP/NADPH ratio of cells incubated with and without plC is shown in Table I. There was a significant increase in this ratio ($p < .01$) after 10 min incubation with plC. The mean nucleotide concentration expressed as nanomoles $\times 10^{-2}$ per 10^7 PMN leukocytes was 8.2 and 37.7 for NADP and NAD PH without plC. This quantity of recovered nucleotide agrees well with the data of Silber, Gabrio and Huennekens (19). In the presence of plC the values for NADP and NAD PH were 14.3 and 33.0. Experiments in which known amounts of NADPH were added to the cells prior to extraction resulted in apparent complete recovery. The experimentally determined NADPH concentration was 105 and 97% of the calculated value in control and plC-treated cells.

Discussion. The effect of plC on some aspects of PMN leukocyte metabolism appears to be qualitatively similar to those changes observed following ingestion by the cell of particles (phagocytosis) (1, 12, 20). The metabolic effects of plC are associated with extensive hydrolysis of phospholipids (4). These effects are abolished by the omission or binding of calcium and such binding resulted in abolition of the HMPS stimulation. It would seem reasonable then, that this lipid hydrolysis may be associated with increased lipid synthesis by the cell for which there is some evidence with the increased incorporation of ^{14}C-acetate into lipids in plC-treated cells. Lipid hydrolysis due to plC, in equilibrium with lipid synthesis, could explain the failure to show a net increase in lipid while acetate incorporation into lipid was aug-

TABLE I. The Effect of Phospholipase C on Hexose Monophosphate Shunt Activity, Acetate Incorporation into Lipid and the NADP/NADPH Ratio.

	− plC	+ plC	p value[a]
HMPS activity (cpm/10^7 PMN/60 min incubation)	244 ± 41[b]	774 ± 112[b]	< .001
1-^{14}C-Acetate incor. into lipid, (cpm/10^7 PMN/ 15 min incubation)	458 ± 44	597 ± 63	< .001
NADP/NADPH (ratio after 10 min incubation)	0.22 ± 0.05	0.45 ± 0.08	< .01

[a] p is the double probability value for differences between paired data with and without plC.
[b] Standard error of the mean.

mented. It has been shown that PMN leukocytes lack acetyl-CoA carboxylase and thus are unable to synthesize fatty acids *de novo* (21). They are able to elongate fatty acid chains, however, and this may be the mechanism of increased acetate incorporation. The elongation of fatty acid chains requires NAD PH and produces NADP and may therefore serve as the initial force which alters the nucleotide equilibrium resulting in modestly increased HMPS activity and increased activity of HMPS-perpetuating enzymes such as NADPH oxidase and glutathione reductase. The increase of the NADP/NADPH ratio which has been observed in the PMN leukocyte following phagocytosis has been considered evidence for stimulated NADPH oxidase activity (20). Recent work, however, demonstrating early activation of glutathione reductase (7) which also serves to generate NADP from NADPH, provides an alternate explanation to NADPH oxidase stimulation for the early mechanisms of NADPH formation for HMPS stimulation. Furthermore, the link between particle attachment or entry, or the effect of surfactants and the increase in NADP levels by either NADPH oxidase or glutathione reductase remains unclear.

There is mounting evidence for significant alteration of the cell membrane during phagocytosis. The data presented above, linking increased acetate incorporation into lipids, an increased NADP/NADPH ratio and increased HMPS activity in cells treated with a lipolytic agent (plC) implicates the cell membrane as the source of NADP for cell activation. This evidence, however, is not conclusive, and the associations could be coincidental. Further work is in progress to attempt to define the nature of these associations.

Summary. Human polymorphonuclear leukocytes were incubated with and without phospholipase C (plC) to investigate the possible interrelationship of cell metabolism to membrane alterations. Cells treated with plC showed increased hexose monophosphate shunt (HMPS) activity, increased incorporation of acetate-1-^{14}C into lipids, and an increase in the NADP/NADPH ratio. These findings suggest a possible mechanism for the early activation of the HMPS during phagocytosis through hydrolysis and repair of the cell membrane.

This work was supported by Public Health Research Grant (CA HE 11106-06), Public Health Training Grant (HE 5316 09) and Health Research and Services Foundation, Pittsburgh, PA (M 22).

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Received Sept. 7, 1971. P.S.E.B.M., 1972, Vol. 140.