BBA 43181

Mechanism of the respiratory stimulation in saponine-treated leucocytes

The KCN-insensitive oxidation of NADPH

Polymorphonuclear leucocytes treated with surface-active agents exhibit changes in oxygen uptake and in carbohydrate oxidative metabolism comparable to those seen during phagocytosis[1–3]. However, while in phagocytosing leucocytes the glycolytic activity tends to increase, in saponine-treated cells it has been shown to be markedly lowered[3], which rules out the hypothesis[1,2] that an increased production of lactic acid represents the trigger mechanism for the oxidative changes.

Previous work in this laboratory has shown that the increased activity of the hexose monophosphate pathway in phagocytosing polymorphonuclear leucocytes is the result of a direct oxidation of NADPH by increased activity of intragranular NADPH oxidase[4–9]. In the present study the mechanism of the stimulation of oxygen uptake and hexose monophosphate pathway activity in surfactant-treated polymorphonuclear leucocytes has been investigated.

Polymorphonuclear leucocytes collected from guinea-pig peritoneal exudate[7], washed once and suspended in calcium-free Krebs–Ringer phosphate, were treated with saponine (Merck 7695) at a dose of 100 μg/ml of a cell suspension (containing about $1 \cdot 10^7$–$1.5 \cdot 10^7$ polymorphonuclear leucocytes per ml) for 7 min at 37° in shaken plastic tubes. Control tubes contained leucocytes without saponine. The cells were washed in 0.25 M cold sucrose and resuspended and homogenized in 0.34 M sucrose with a motor-driven Teflon pestle in a glass tube at 0°. After removal of the nuclear fraction and residual cells at low centrifugal force, each homogenate was divided into 2 aliquots, one (G + S) directly used for metabolic assay, the other centrifuged at 20000 × g for 30 min to obtain the supernatant (S) and granule (G) fractions. The latter was washed once and brought to the original volume with 0.34 M sucrose.

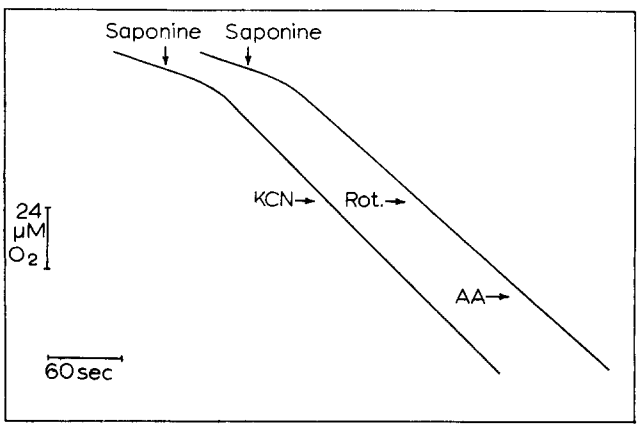

Fig. 1. Recording of the oxygen uptake of polymorphonuclear leucocytes in the presence of saponine. Composition of the system: $2 \cdot 10^7$ cells in 1.8 ml of calcium-free Krebs–Ringer phosphate containing 2.8 mM glucose; saponine, 200 μg; KCN, 2 μmoles; rotenone (Rot.), 5 mμmoles; antimycin A (AA), 2 μg. Temperature 37°.

The oxygen uptake of cell suspensions or homogenates was measured with a Clark oxygen electrode (Yellow Springs Instrument Co., Ohio) at 37°.

The results reported in Fig. 1 show that the saponine-stimulated respiration is unaffected by cyanide, rotenone or antimycin A. The stimulation appears within 20–30 sec after the addition of the surfactant.

The experiments on the oxidation of reduced nicotinamide–adenine dinucleotides by the subcellular fractions in a system containing cyanide show (Fig. 2A) that the oxygen uptake is strongly stimulated by NADPH in the G + S preparation from saponine-treated polymorphonuclear leucocytes, whereas little effect is observed in the same fraction from control leucocytes. In the presence of NADH the oxygen uptake of the same fraction of homogenate from saponine-treated cells is much lower although it appears to be clearly stimulated with respect to the control preparation.

Practically all the oxidase activities contained in the leucocyte homogenate (G + S) are recovered in the granule fraction (G) as shown in the experiments reported in Fig. 2B, where the oxygen uptake was measured by using the granule fractions separated at 20000 × *g*. The supernatants contained no oxidase activities for either NADH or NADPH.

It is evident that the most relevant change induced by the treatment of polymorphonuclear leucocytes with saponine appears to be the enormous increase in the

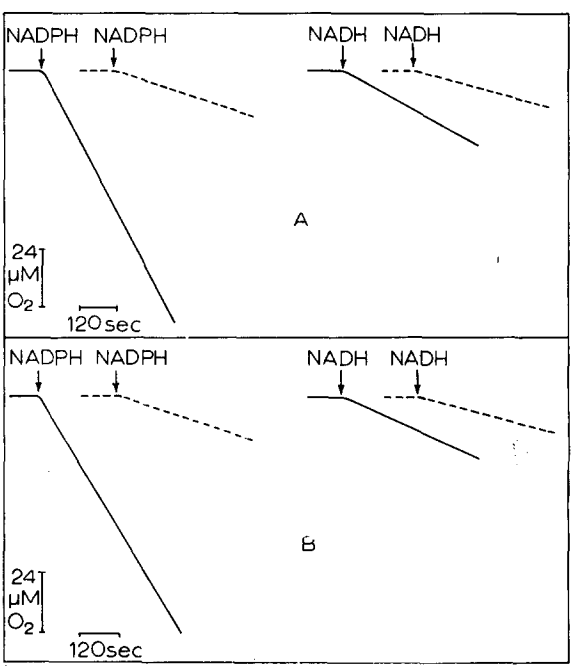

Fig. 2. Recording of the oxygen uptake of fractions of homogenates of polymorphonuclear leucocytes. The system was as follows: 140 mM sucrose; 36 mM phosphate buffer (pH 7); 19 mM KCl; 1.5 mM $MgCl_2$; 0.7 mM NADPH; 1 mM KCN; in (A) granules + supernatant, 2 mg of protein; in (B) granules corresponding to the quantity present in (A). Total vol. 1.8 ml. Temp. 37°. – – – –, preparations from control polymorphonuclear leucocytes; ———, preparations from saponine-treated polymorphonuclear leucocytes.

granule bound NADPH oxidase activity. The KCN-insensitive NADPH oxidase of granules from saponine-treated polymorphonuclear leucocytes is also unaffected by rotenone or antimycin A.

The behaviour of the oxidase activities in the granules of saponine-treated polymorphonuclear leucocytes appears quite similar to that observed in the preparations from phagocytosing cells[4,5,9]. Previous work has shown that in saponine-treated cells the lactic acid production is diminished[3], which rules out the hypothesis[1,2] of a stimulation of a soluble NADH oxidase as has been claimed to be brought about by an increased H^+ concentration when glycolysis is stimulated in phagocytosing polymorphonuclear leucocytes[10]. The present findings throw light on the processes underlying the increased oxygen uptake and hexose monophosphate pathway activity induced by the surface active agent in polymorphonuclear leucocytes as a result of direct oxidation of NADPH by the increased granule-bound KCN-insensitive oxidase activity. Such activation requires cellular integrity, since, as shown in Fig. 3, saponine strongly stimulates the oxygen uptake of intact polymorphonuclear leucocytes, but is without effect on the homogenate or granule fraction. This situation is similar to the stimulation elicited in intact cells but not in homogenates by NaF or particles[11].

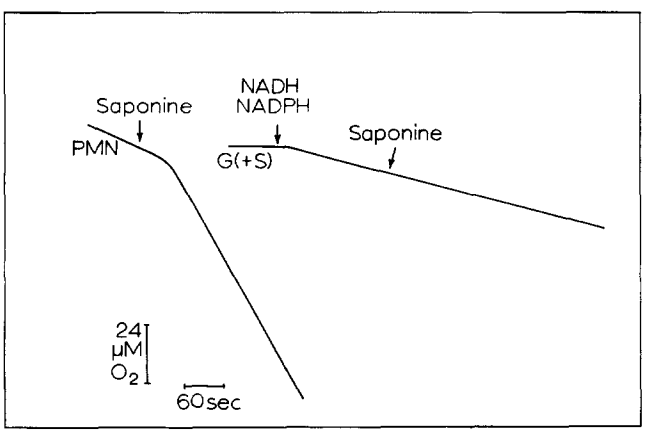

Fig. 3. Effect of saponine on the oxygen uptake of intact leucocytes (PMN), and of granules or granules *plus* supernatant G (+S) from normal leucocytes, in the systems, respectively described in Figs. 1 and 2. Saponine: 200 μg in the system containing intact leucocytes and 20, 100, 200, 400, 800 μg in the system containing subcellular fractions.

It therefore seems that the activation of the granule-bound oxidases does not result from a direct effect of saponine on the granules, in spite of the fact that it directly produces granule swelling[9]. It must be the alteration of the cell membrane produced by the surfactant, as well as that produced by contact with the opsonized particles during phagocytosis, which triggers a mechanism of activation of the granule-bound oxidases. Electron-microscopic examination has shown that, even after prolonged treatment of leucocytes with saponine (30 min), no degranulation appears, at variance with the phagocytosis which is followed by lysis of the specific granules. Although many investigations have been performed in recent years on membrane alterations produced by surfactants in a number of cellular or artificial systems[12–17], little is known about the effects of surface-active agents, as well as of phagocytosis,

on the leucocyte membrane and about its modifications concerned with the metabolic concomitants.

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Received September 29th, 1967

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BBA 43180

The redox potential of fungal laccase

Fungal laccase (*p*-diphenol:oxygen oxidoreductase, EC 1.10.3.2) has been found to contain four copper atoms per molecule[1]. Two of these are in a diamagnetic state, presumably Cu^{+}, while the other two exist as Cu^{2+} in the resting enzyme[2,3] and are reduced by substrate during catalysis[4]. However, only one cupric ion has the unique bonding properties which account for the high absorbance at 610 mμ and the narrow hyperfine structure splitting in the EPR spectrum[5]. We wish to report here that this cupric ion is further distinguished by having an exceptionally high redox potential.

In a previous communication it was shown that the enzyme undergoes a reversible loss of blue color at pH values greater than 6 (see ref. 6). EPR experiments confirmed that the reduction of one Cu^{2+} occurred concomitantly with loss of absorbance at 610 mμ. Such a pH-dependent reaction could be due to a reducing group on the enzyme or to reduction by solvent. Failure to detect sulfhydryl groups on the enzyme led us to a more serious consideration of the latter possibility. Assuming that one copper of laccase is coupled to the half-reaction

$$O_2 + 4H^+ + 4e^- = 2H_2O \qquad E_0 = 1.23\ \text{V (see ref. 7)}$$

and using the pK of 7.4 determined previously[6] for the pH-dependent reduction, it can be computed that the redox potential must be about 0.78 V.