Membrane Redox Systems in Porcine Neutrophils

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Introduction

Neutrophils undergo a series of changes in their oxygen metabolism during phagocytosis or stimulation by a number of soluble compounds (3,15,24). These changes are referred to as the respiratory burst and are generally characterized by increases in oxygen consumption, superoxide and hydrogen peroxide production, and hexose monophosphate shunt activity. Activation of the respiratory burst is initiated by perturbation of the plasma membrane and does not require phagocytosis (21). The purpose of the burst appears to be (2,4,16) to provide the cell with a group of highly reactive products which the cell can use for the destruction of invading microorganisms. Many studies indicate that the respiratory burst results from (5,11,14) the activation of an enzyme in the plasma membrane which catalyzes the reduction of oxygen to superoxide using NADPH or NADH as the electron donor. The burst can be activated by a wide variety of agents. In the present study, concanavalin A, phorbol myristate acetate, and sodium fluoride were employed to stimulate the respiratory burst. In this study the aspect of the respiratory burst focused upon was the stimulation of superoxide production.

The existance of a redox enzyme which is transmembranous in orientation has been shown in the plasma membrane of both animal (7,9,13) and plant (8) cells. This enzyme transmits electrons from internal reducing compounds to the impermeable electron acceptor ferricyanide (22,17). NADH has been shown to be the electron donor (8,9) which is reducing external ferricyanide. Löw and Crane (18,19) have proposed that this enzyme may be involved in the control of cellular metabolic functions because it is affected by physiological levels of anabolic and catabolic hormones. This idea is further supported by the work of Sun and Crane (25) which shows this enzyme to be inhibited by several anticancer drugs.

The ability of the neutrophil to reduce oxygen to the superoxide anion has been well established. In this paper we examine both the superoxide production and the transmembranous dehydrogenase activities of porcine neutrophils. Superoxide dismutase sensitive cytochrome c reduction is often used as a detector of the superoxide anion (20). Ferricyanide reduction is used to measure the activity of the transmembranous dehydrogenases (7). By observing each systems response to three agents known to stimulate superoxide production, we attempted to determine if all membrane redox activity observed in porcine neutrophils is associated with the generation of superoxide or if there is some superoxide independent redox function at the surface of porcine neutrophils.

Materials and Methods

Horse heart ferricytochrome c (grade VI), superoxide dismutase Type 1: from bovine blood, 4-phorbol 12-myristate 13-acetate, concanavalin A (Type IV) and sodium fluoride were purchased from Sigma Chemical Co., St. Louis, Mo All other reagents were of analytical grade.

Porcine neutrophils were prepared from blood that was treated with 15 mM ethylenediaminetetraacetate (EDTA) to prevent coagulation. Neutrophils were

isolated using a modification of the procedure of Dioguardi et al. (12). Blood was centrifuged at 3000 g for 10 min., plasma was removed by aspiration and the neutrophil rich fraction was drawn off. This fraction was then treated with .83% NH_4Cl in a ratio of 1 part blood:8 parts NH_4Cl and centrifuged at 120 g for 2 min. The resulting pellet was washed with .83% NH_4Cl and centrifuged at 120 g for 20 min. A third wash was done to ensure elimination of contaminating erythrocytes and platelets, resulting in a cell suspension composed primarily of neutrophils. Neutrophils were suspended in whole cell buffer (pH 7.0 containing 137 mM NaCl, 2.7 mM KCl, 1.2 mM $CaCl_2$, 8.4 mM NaH_2PO_4, 7.3 mM sucrose, 0.5% dextrose and 2% fetal calf serum.

Neutrophil suspensions were treated with agents which stimulate superoxide generation and incubated at 37°C with constant stirring for 3 min. Cells were determined viable using Trypan blue exclusion. Incubation conditions varied with the stimulator. The assay buffer used for concanavalin A, and phorbol myristate acetate was whole cell buffer. The assay buffer for sodium fluoride was based on Dulbecco's phosphate saline (PBS) (10) and contained .117 mM NaCl and .31 mM $CaCl_2$. This low salt buffer was used to correct for the additional electrolytes from sodium fluoride and to prevent the precipitation of calcium fluoride. In assays with less than 20 mM sodium fluoride, NaCl was used to restore tonicity.

Cytochrome c and ferricyanide reduction were measured with an Aminco DW-2a spectrophotometer in the dual wavelength mode, subtracting absorbance changes at 541 nm from 550 nm for cytochrome c reduction and at 500 nm fom 420 nm for ferricyanide reduction. Extinction coefficients used for calculation of ferricyanide and cytochrome c reduction were 1 and 19 mM^{-1} cm^{-1} respectively.

The reaction mixture for ferricyanide reduction contained whole cell buffer, 0.3 mM potassium ferricyanide, the stimulator and superoxide dismutase concentrations as specified, in a total volume of 3.0 mls. The conditions for cytochrome c reduction were the same except 67 $\mu g/ml$ cytochrome c and 0.33 mM potassium cyanide were substituted for ferricyanide. The average concentration of neutrophils in the final reaction mixture was 0.00953 g cell wet weight.

Results

Porcine neutrophils were assayed in the absence of a stimulus in order to obtain a resting or control rate for cytochrome c and ferricyanide reduction. The effect of superoxide dismutase (SOD) on these resting rates is shown in Fig. 1 and is expressed as percent inhibition of the respective control rate. The maximum inhibition of the resting rate obtained in the presence of SOD are 25% and 35% for ferricyanide and cytochrome c respectively.

We examined the effects of three agents, which are known to stimulate superoxide production, by monitoring the rates of cytochrome c and ferricyanide reduction. When phorbol myristate acetate (PMA) was the stimulus employed (Fig. 2), there was up to 500% stimulation above the control rate of cytochrome c reduction. In addition, PMA shows a 150% stimulation above the ferricyanide control rate. The effect of SOD on these PMA(15 ng/ml)-stimulated rates of reduction is shown in Fig. 3. When the rates of reduction were followed in the presence of SOD there was 90% maximum inhibition of the PMA-stimulated rate of cytochrome c reduction. PMA-stimulated ferricyanide reduction showed a 60% maximum inhibition in the presence of SOD.

Concanavalin A (Con A) showed a 5-15% stimulation in the rate of ferricyanide reduction and a 10-135% stimulation in the rate of cytochrome c reduction (Fig. 4). At high concentrations of Con A (2.5 mg/ml) there is a further increase in
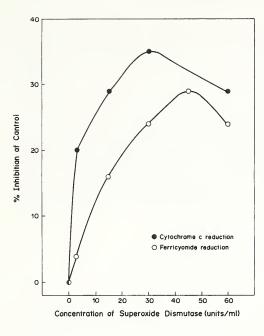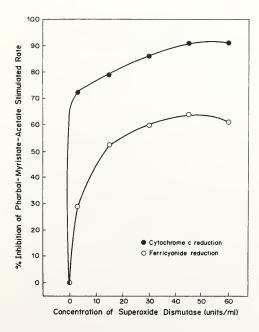
FIGURE 1. *Effect of superoxide dismutase on cytochrome c and ferricyanide reduction in porcine nuetrophils. Control rates of 16.19 and 218.2 nmol/min/g cells wet weight for cytochrome c and ferricyanide reduction respectively were obtained.*
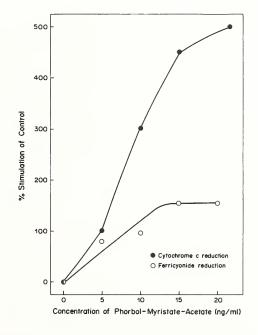
FIGURE 2. *Effect of phorbol myristate acetate on cytochrome c and ferricyanide reduction in porcine neutrophils. Control values of 3.93 and 242.67 nmol/min/g cells wet weight were obtained for cytochrome c and ferricyanide reduction respectively.*

FIGURE 3. *Effect of superoxide dismutase on phorbol myrisate acetate (15 ng/ml) stimulated cytochrome c and ferricyanide reduction in porcine neutrophils. Control values of 25.68 and 487.46 nmol/min/g cells wet weight for cytochrome c and ferricyanide reduction respectively were obtained. The phorbol myristate acetate (15 ng/ml) stimulated rates for cytochrome c and ferricyanide reduction were 143.46 and 1285.63 nmol/min/g cells wet weight respectively.*
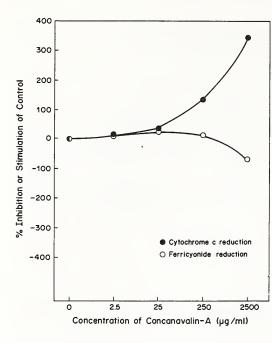
FIGURE 4. *Effect of concanavalin A on cytochrome c and ferricyanide reduction in porcine neutrophils. Control values of 16.38 and 136.05 nmol/min/g cells wet weight were obtained for cytochrome c and ferricyanide reduction respectively.*

FIGURE 5. *Effect of superoxide dismutase on concanavalin A (250 µg/ml) stimulated cytochrome c and ferricyanide reduction in porcine neutrophils. Control values of 1.15 and 355.19 nmol/min/g cells wet weight were observed for cytochrome c and ferricyanide reduction, respectively. The concanavalin A (250 µl/ml) stimulated rates of cytochrome c and ferricyanide reduction observed were 2.34 and 409.84 nmol/min/g cells wet weight respectively.*
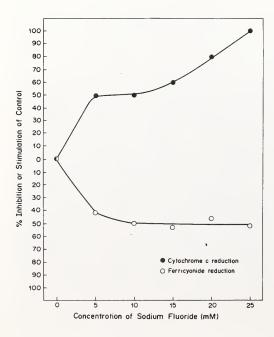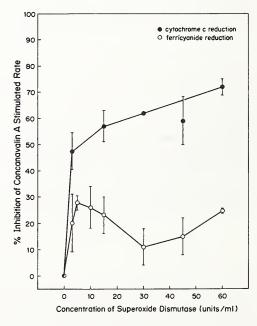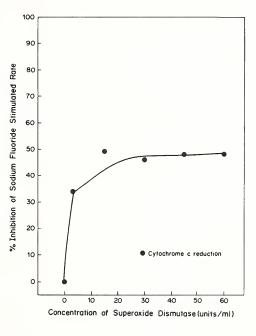

FIGURE 6. *Effect of sodium fluoride on cytochrome c and ferricyanide reduction in porcine neutrophils. Control values of 5.60 and 125.18 nmol/min/g cells wet weight were obtained for cytochrome c and ferricyanide reduction respectively.*

the cytochrome c rate, while the rate of ferricyanide reduction is inhibited. We examined the effect of SOD on the Con A (.25 mg/ml) stimulated rates of both ferricyanide and cytochrome c reduction as shown in Fig. 5. It was observed that a maximum of 75% of the Con A-stimulated cytochrome c rate is inhibited by SOD. The maximum inhibition of the ferricyanide rate achieved with SOD is 30% of the Con A-stimulated rate reduction.

The last agent used was sodium fluoride (NaF). At all concentrations of NaF employed in these experiments there was a stimulation in the rate of cytochrome c reduction and an inhibition in the rate of ferricyanide reduction, as shown in Fig. 6. The effect of SOD on the NaF (200 mM)-stimulated cytochrome c reduction is shown in Fig. 7. There was a maximum of 50% inhibition of this rate in the presence of SOD.

FIGURE 7. *Effect of superoxide dismutase on sodium fluoride (20 mM) stimulated cytochrome c reduction. Control value for cytochrome c reduction obtained was 28 nmol/min/g cells wet weight. The sodium fluoride (20 mM) stimulated rate of cytochrome c reduction was 47.45 nmol/min/g cells wet weight.*

Discussion

The results in this paper are in accord with earlier reports dealing wth the superoxide generating enzyme (3,15,16,24). The agents PMA, Con A, and NaF all increased the rate of cytochrome c reduction. A majority of this stimulated rate of cytochrome c reduction, 90% and 75% for PMA and Con A respectively, was found to be sensitive to SOD. These rates could therefore be attributed to the generation of superoxide. The results for NaF differ in that only 50% of the stimulated rate is SOD sensitive. This suggests that NaF may be stimulating some reduction of cytochrome c by a pathway which does not depend on superoxide. Our data indicates different modes of stimulation of neutrophil metabolism by various agents. The fact, that NaF activated system is different from that of Con A in human neutrophils, also suggests more than one mechanism exist for activation of the respiratory burst enzymes (21).

Evidence has shown that fluoride-mediated activation of the respiratory burst in human neutrophils requires the presence of Ca^{++} (10). Our assay buffer for NaF is based on Dulbecco's PBS which contains a considerable amount of Ca^{++} (0.31 mM). Higher NaF stimulated rate may be reached under a higher Ca^{++} concentration, however it can not be achieved due to a precipitation of CaF_2 in the incubation mixture.

The present work also looks at the transmembranous dehydrogenase that has been observed in several cell types (8.9). When ferricyanide reduction was examined, a different response than that observed with cytochrome c was noted for all stimuli employed. When NaF was the stimulus, opposite responses were observed for the two systems. When the rate of ferricyanide reduction was stimulated, as was the case with PMA and Con A, the SOD sensitivity was examined. It was observed that 30% and 60% of the stimulated rates for Con A and PMA respectively were inhibited in the presence of SOD, indicating that very little of the Con A-stimulated ferricyanide rate is the result of superoxide generation. It can also be seen that the PMA-stimulated rate of reduction can not be solely attributed to generation of the superoxide anion. The enzyme involved in the ferricyanide reduction does not seem to play a major role for superoxide production in the respiratory burst of neutrophils.

Evidence has been presented that the activated form of oxidase for the respiratory burst is on the plasma membrane of phagocytic cells (23). It has also been reported (1) that the main superoxide forming oxidase is embedded in the plasma membrane of human neutrophils with a portion extending into the cytoplasm and the rest buried in the lipid bilayer. Our data suggests there are at least two membrane redox systems in the plasma membrane of porcine neutrophils: 1) the redox system involved in the reduction of oxygen to superoxide, using NADPH or NADH as the electron donor, and 2) the transmembranous dehydrogenase which transmits electrons from internal reducing compounds to the impermeable electron acceptor ferricyanide.

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