Vol. 104, No. 1, 1982 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 15, 1982 Pages 200-206

INHIBITORY EFFECT OF CIBACRON BLUE F3GA ON THE O_2^- GENERATING ENZYME OF GUINEA PIG POLYMORPHONUCLEAR LEUKOCYTES

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Received November 23, 1981

SUMMARY: Cibacron Blue F3GA(CB-F3GA), which interacts with nucleotide-requiring enzymes, was used to determine the cellular location of the NADPH-binding site of the O_2^- generating enzyme in polymorphonuclear leukocytes(PMN). A fraction of plasma membranes, in which the O_2^- generating enzyme is located, was prepared from myristate(MA)-activated PMN by Percoll density gradient centrifugation. CB-F3GA was found to be a competitive inhibitor of the membrane-bound NADPH dependent O_2^- generating enzyme; the Ki value of CB-F3GA was about 0.8 μM. The dye did not inhibit O_2^- generation and release from PMN activated with MA or phorbol myristate acetate(PMA). These results suggest that the NADPH binding site of the O_2^- generating enzyme is located on the inner surface of the plasma membrane. The low Ki value of this enzyme for the dye suggests that the NADPH binding site has a dinucleotide fold.

Cyanide-insensitive respiration of polymorphonuclear leukocytes(PMN) has been evaluated by addition of bacteria and soluble stimulants(1-3). An NADPH oxidase that generates O_2^- and H_2O_2 by coupled oxidation of NADPH is responsible for the respiratory burst of phagocytosing PMN, because this activity is dormant in resting cells and appears only when the cells are exposed to phagocytic particles or stimulants(4,5). Some investigators have suggested that the O_2^- generating enzyme is located in the plasma membrane(6,7). Consistent with this suggestion, in previous experiments we found by Percoll density gradient centrifugation(8), that the O_2^- generating enzyme was present in plasma membranes isolated from guinea pig PMN treated with a metabolic stimulant. In 1977, Briggs et al.(9) demonstrated an increase in H_2O_2 generation by phagocytosing human PMN in the presence of extracellular NAD(P)H. This finding suggests that the NAD(P)H binding site is located on the outer

Abbreviations: PMN, polymorphonuclear leukocytes; CB-F3GA, Cibacron Blue F3GA; MA, myristate; PMA, phorbol myristate acetate.

0006-291X/82/010200-07$01.00/0

surface of the PMN plasma membrane, because pyridine nucleotides do not usually penetrate the cell membrane readily. On the other hand, Green et al. postulated that the NADPH binding site of this oxidase is located on the inner surface of the plasma membrane(10). However, this postulation was based on data on NADPH oxidase activity measured by following oxygen consumption in the presence of Mn^{2+}, which is well known to enhance non-enzymatic NAD(P)H oxidation in the peroxidase reaction(11).

Stellwagen's group(12,13) and Wilson(14) showed that Cibacron Blue F3GA (CB-F3GA) interacts with nucleotide-requiring enzymes and binds strongly to some of these enzymes that have a dinucleotide fold, presumably owing to the structural similarity between the dye and nucleotide. In this paper, we used CB-F3GA to determine the cellular location and nature of the NADPH binding site of the O_2^- generating enzyme, because this dye is highly specific for the NAD(P)H binding site. The results obtained provide direct evidence that the NADPH binding site of the O_2^- generating enzyme is located on the inner surface of the PMN plasma membrane. In addition, from kinetic studies on the O_2^- generating enzyme with CB-F3GA we obtained information on the nature of the interaction between the O_2^- generating enzyme and NADPH.

MATERIALS AND METHODS

Materials Cibacron Blue F3GA(CB-F3GA) was purchased from Pierce Biochem. Co., Illinois. The concentration of CB-F3GA was measured spectrophotometrically at 610 nm using an extinction coefficient of 13.6 $mM^{-1}cm^{-1}$, as reported previously(13). Cytochrome c(Type IV, from horse heart), catalase and NADPH were purchased from Boehringer Co., Mannheim. Superoxide dismutase, phorbol myristate acetate, bovine serum albumin were from Sigma Chem. Co., St. Louis, sodium myristate was from Nakarai Chem. Co., Tokyo, and Percoll was from Pharmacia Fine Chem. Co., London. Acetylated cytochrome c was prepared as reported previously(15). Other chemicals used were of analytical grade.
Cell preparation Guinea pig PMN were isolated from the peritoneal cavity after injection of sterilized caseinate in 0.9% NaCl as reported previouly (16). The cells, 98% of which were PMN, were suspended in N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid buffer saline(HBS) containing 5 mM KCl.
Preparation of the plasma membrane fraction from myristate-activated PMN The plasma membrane fraction was prepared from myristate(MA)-activated PMN as reported previously(8). A PMN suspension(2.5×10^7 cells/ml HBS) containing 5 mM glucose was incubated at 37°C for 10 min and then the cells were activated with MA(110 nmol/10^7 cells) for 30 sec. After incubation with MA, the cell suspension was loaded on a layer of silicone oil(density = 1.02) in a siliconized glass tube and promptly centrifuged at 1,000 x g for 3 min. All subsequent procedures were carried out at 0 to 4°C. The precipitated cells were suspended in 0.34 M sucrose containing 10 mM Tris-HCl buffer, pH 7.4, and homogenized in a homogenizer with a Teflon pestle operating at

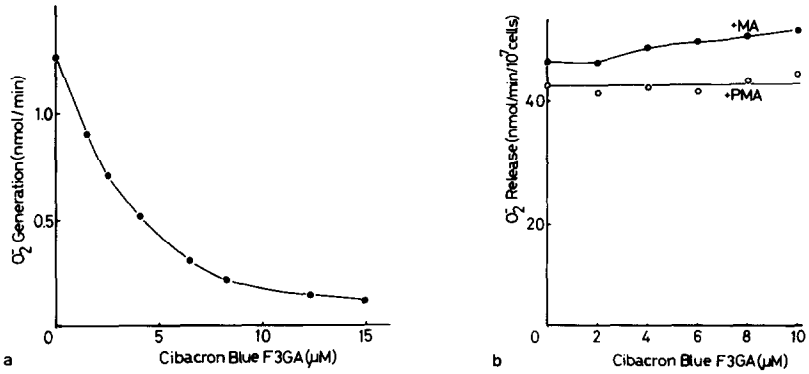

Figure 1-a. Effect of CB-F3GA on O_2^- generation by plasma membrane-bound NADPH oxidase. Plasma membranes were isolated from MA-activated PMN by Percoll density gradient centrifugation. The assay mixture contained 41 μg/ml protein of plasma membranes, 15 μM acetylated cytochrome c, 5 μg/ml catalase, 0.1 mM NADPH, 0.17 M sucrose and various concentrations of CB-F3GA in 65 mM sodium potassium phosphate buffer, pH 7.0. The reaction was started by addition of NADPH.

Figure 1-b. Effect of CB-F3GA on the O_2^- generation by activated cells. The assay mixture contained 4 x 10^6 cells, 15 μM cytochrome c, 5 μg/ml catalase, 5 mM glucose and various amounts of CB-F3GA in 1.6 ml of N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid buffer saline. PMN were activated by myristate(MA)(110 nmol/10^7 cells) or phorbol myristate acetate(PMA)(0.1 μg/1.6 ml).

1,000 rpm(total of 80 strokes) in an ice bath. The homogenate was centrifuged at 450 x g for 10 min to remove cell debris and the resulting supernatant was fractionated on a 0 to 50% Percoll density gradient. Peak fractions of plasma membranes were pooled and centrifuged at 100,000 x g for 2 h. The resulting pellet was washed with 0.34 M sucrose containing 10 mM Tris-HCl buffer and suspended in the same sucrose buffer.

Assay of O_2^- generation The rate of O_2^- generation by cellular or subcellular fractions of PMN was measured at 37°C by recording the reduction of unmodified or acetylated cytochrome c as described previously(17). Reduction of cytochrome c was measured continuously in a cell mixer(18) by recording increase in absorbance at 550-540 nm. For assay of O_2^- release from intact cells, the assay mixture contained 15 μM cytochrome c, 5 μg/ml of catalase, 5 mM glucose, and 4 x 10^6 cells in 1.6 ml of HBS. For assay of O_2^- generation by subcellular fractions, the assay medium contained 15 μM acetylated cytochrome c, 5 μg/ml of catalase, an aliqout of the plasma membrane fraction, 0.17 M sucrose and various amounts of NADPH in 65 mM sodium potassium phosphate buffer, pH 7.0. The reaction was started by addition of NADPH. CB-F3GA was added at various concentrations to the assay medium.

Protein Protein was determined by the method of Lowry et al.(19) with bovine serum albumin as standard. Interference by Tris or other components of reaction mixtures was avoided by using samples precipitated with 5% trichloroacetic acid.

RESULTS

Effects of CB-F3GA on O_2^- generation by the subcellular fraction and intact cells: Figure 1-a shows the effect of CB-F3GA on the O_2^- generating enzyme of plasma membranes purified from MA-activated PMN. CB-F3GA was markedly

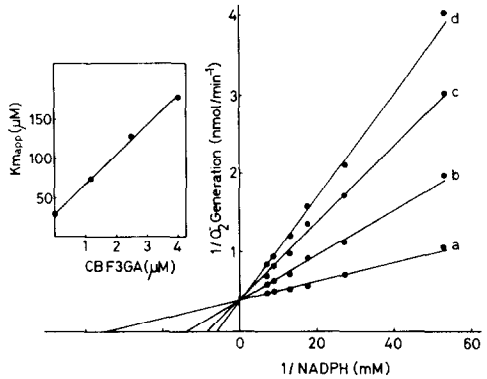

Figure 2. Inhibition by CB-F3GA of O_2^- generation by plasma membrane-bound NADPH oxidase. The assay mixture was as for Fig. 1, except that 69 μg/ml protein of plasma membranes was used. The concentrations of CB-F3GA used were as follows: a, 0; b, 1.2 μM; c, 2.5 μM; d, 4.0 μM. The inset is a replot of the apparent Km at various CB-F3GA concentrations.

inhibitory at a low concentration: 10 μM CB-F3GA reduced the O_2^- generating activity to about one-tenth of the control value.

To determine the cellular location of the NADPH binding site of the O_2^- generating enzyme, we tested the effect of CB-F3GA on O_2^- generation by intact cells activated with MA and PMA. As shown in Fig. 1-b, CB-F3GA slightly stimulated, rather than inhibited, O_2^- generation by MA-activated PMN. Moreover it did not inhibit O_2^- generation on addition of PMA at a concentration sufficient to inhibit O_2^- generation by the subcellular fraction.

Kinetic studies on CB-F3GA: Next we examined the kinetics of inhibition by CB-F3GA using various NADPH concentrations with four constant concentrations of dye: 0, 1.2, 2.5, 4.0 μM. Lineweaver-Burk plots(20) of the results are shown in Fig. 2. The apparent Km value for NADPH was 29 μM in the absence of CB-F3GA, and increased in the presence of dye, but the apparent Vmax for O_2^- generating activity was not affected by the presence of the dye. Replots of the apparent Km in the inset of Fig. 2 show that the Ki value of CB-F3GA was about 0.8 μM, which is very low compared with the Km value for NADPH. These results show that CB-F3GA is a competitive inhibitor of the O_2^- generating enzyme. The low Ki value suggests that the NADPH binding site of the O_2^- generating enzyme has a dinucleotide fold, as mentioned in the Discussion.

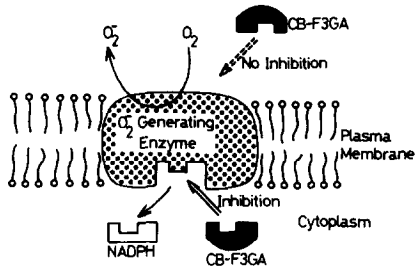

Figure 3. Model of the NADPH binding site of the O_2^- generating enzyme on the PMN plasma membrane. CB-F3GA, which does not penetrate the plasma membranes, cannot inhibit O_2^- generation by intact PMN, but it competitively inhibited O_2^- generation by the NADPH oxidase, probably because it bound to the NADPH binding site on inside-out vesicles of plasma membrane. Therefore, the NADPH binding site of this enzyme is probably located on the inner surface of the plasma membrane.

DISCUSSION

There is some evidence that the O_2^- generating enzyme is located in the plasma membranes of PMN(6,7). Recently, we obtained very high O_2^- generating activity concentrated in a single peak by Percoll density gradient centrifugation of the post-nuclear supernatant of MA-activated PMN(8). The O_2^- generating activity of MA-activated PMN was consistently associated with 5'-nucleotidase activity as a membrane marker enzyme, and was not associated with lysosomal marker enzymes such as myeloperoxidase and lysozyme. In the present experiment, we found that CB-F3GA competitively inhibited the NADPH-dependent O_2^- generating enzyme located in the plasma membrane. However, CB-F3GA did not inhibit O_2^- generation from MA- and PMA-activated PMN. (The dye slightly stimulated O_2^- generation by MA-activated PMN, for some unknown reason.) The present work provides direct evidence that the NADPH binding site of this oxidase is located on the inner surface of the plasma membrane. Recently, Kakinuma and Kaneda(21) suggested that phagocytosing PMN release O_2^- to the opposite site of cytoplasm, from the data obtained by experiments using cellular and subcellular fractions under aerobic and anaerobic conditions. These are summarized in Fig. 3. Green et al.(10) proposed a similar scheme to ours, but they used an assay medium containing 1 mM Mn^{2+}, which accelerates non-enzymatic oxidation of NAD(P)H(11). Futhermore, they used a high concentration of NADP(H)(1 mM) that was 30-40 times higher than the Km value of

this oxidase for NADPH(22,23). Other researchers(24, 25) also suggested the cellular localization of the oxidase, but provided only indirect evidence to support their suggestions.

Wilson(14) generalized the effects of CB-F3GA on nucleotide-requiring enzymes on the basis of his data and those of Stellwagen's group(12,13). He found that the Ki values of nucleotide-requiring enzymes for this dye are ~0.1 - 1.0 μM for enzymes possessing a dinucleotide fold and over 10 μM for enzymes that interact relatively weakly with the dye. CB-F3GA was found to be a competitive inhibitor of the O_2^- generating enzyme of the plasma membrane of PMN(Fig. 1-a and 2), and the Ki value of this enzyme was about 0.8 μM. This small Ki value indicates that this dye binds tightly to the O_2^- generating enzyme, and thus suggests that the NADPH binding site of this enzyme has a dinucleotide fold.

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