Detection of NADPH diaphorase activity associated with human neutrophil NADPH-O_2 oxidoreductase activity

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At approximately equimolar concentrations (~70 μM), and in the presence of excess catalase and superoxide dismutase, DCIP, ferricytochrome *c* and ferricyanide abstracted 21, 6 and 61%, respectively, of the electron equivalents given up by NADPH to the NADPH-O_2 oxidoreductase complex derived from phorbol myristate acetate-stimulated human neutrophils. With a 10-fold increase in ferricyanide, all of the electron equivalents given up by NADPH to the oxidoreductase complex were shunted to ferricyanide concomitant with complete inhibition of NADPH-dependent O_2 consumption. These results substantiate the existence of intrinsic diaphorase activity associated with the superoxide generating NADPH-O_2 oxidoreductase of human neutrophils.

NADPH-O_2 oxidoreductase *Superoxide* *NADPH diaphorase neutrophil*

1. INTRODUCTION

Superoxide formation in human neutrophils is catalyzed by NADPH:O_2 oxidoreductase embedded in the plasmalemma of the cell [1–5]. Activation of the oxidoreductase occurs with stimulation of resting neutrophils and is a critical element in allowing the neutrophil to express toxic oxygen metabolites required in containing bacterial infections [6]. We previously demonstrated intrinsic NADPH-dependent DCIP and ferricytochrome *c* diaphorase activity associated with the oxidoreductase based upon direct spectral assays and noted its absence in two patients with the X-linked form of CGD [7,8], a rare genetic disorder in which the neutrophil lacks the capacity to express oxidoreductase activity [9]. Authors in [10] have recently identified a flavoprotein component in detergent-treated oxidoreductase-rich membrane fractions which exhibits NADPH-dependent DCIP reductase activity. However, the existence of intrinsic diaphorase activity associated with the oxidoreductase remains controversial. Authors in [11] found no apparent diaphorase activity in their oxidoreductase preparations derived from human neutrophils. Authors in [12] observed little DCIP and ferricytochrome *c* reductase activity in membrane fractions prepared from lysates of guinea pig neutrophils. The latter authors suggested that the apparent diaphorase activity is artifactual and due entirely to superoxide-mediated reduction of these two electron-accepting dyes. These results are in conflict with earlier work from our laboratory [7,8] with the human neutrophil oxidoreductase, and with findings of authors in [13] who employed similar assays and confirmed the presence of DCIP reductase activity in association with oxidoreductase activity derived from pig neutrophils.

We present here our findings on the efficacy of using DCIP, ferricytochrome *c* and potassium ferricyanide as electron acceptors of the NADPH–O_2 oxidoreductase complex derived from human neutrophils employing excess SOD and catalase in

Abbreviations: DCIP, 2,6-dichlorophenolindophenol; DTPA, diethylenetriaminepentaacetic acid; CGD, chronic granulomatous disease; PMA, phorbol myristate acetate; SOD, superoxide dismutase

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the final reaction mixtures, and contrast enzyme activities recovered in phorbol myristate acetate-stimulated cell lysates with similarly prepared lysates derived from resting cells.

2. MATERIALS AND METHODS

Ferricytochrome *c*, SOD, catalase, NADPH, DCIP, potassium ferricyanide, DTPA, and PMA were obtained from Sigma (St. Louis, MO). All other chemicals and reagents were of the best grade available.

Isolation of neutrophils from whole blood, activation of the oxidoreductase with PMA, and subsequent recovery of the 27000 × *g* particulate fraction was as in [8]. Spectroscopic measurements were made on a Shimadzu UV-250 double beam spectrophotometer at 25°C in thermostatted cuvettes.

Ferricytochrome *c* reductase activity was assayed at 550 nm using a millimolar absorptivity coefficient of 21.1 in a final reaction mixture of 63 μM ferricytochrome *c* (final concentration), 32 mM Tris–HCl (pH 7.6) containing 0.25 mM $CaCl_2$, 0.16 mM $MgCl_2$, 100 μM NADPH, 200 μM DTPA, 1.5×10^4 units catalase and 150 units SOD. Equal aliquots of the reaction mixture (total volume, 1.3 ml) were aliquoted between sample and reference cuvettes; enzyme activity was initiated upon addition of the 27000 × *g* particulate fraction derived from either PMA-stimulated or resting cell suspensions (see text) to the sample cuvette. Enzyme was pretreated with Triton X-100 to enhance detection of latent activity [8]. Blank assays were conducted with omission of NADPH from the final reaction mixture; the difference ± NADPH was then calculated as the true rate of enzyme activity. DCIP reductase and ferricyanide reductase activities were similarly assayed upon replacement of ferricytochrome *c* with either DCIP or ferricyanide (see text) and assaying at 600 and 405 nm using millimolar absorptivity coefficients of 16.1 and 1.04, respectively. NADPH oxidation was assayed in the presence and absence of ferricyanide as noted in the text except that enzyme activity was monitored at 340 nm using a millimolar absorptivity coefficient of 6.22. Superoxide generating activity was also monitored in the same fashion as ferricytochrome *c* reductase activity but with omission of SOD from the sample cuvette and addition of enzyme to both sample and reference cuvettes. Enzyme activities were expressed in milliunits (mU)·ml^{-1} enzyme suspension taking into account that oxidation of NADPH and reduction of DCIP corresponds to the loss of two, and gain of two, electron equivalents per mole, respectively. One mU corresponds to 1 nmol·min^{-1} expressed in electron equivalents transferred or given up.

O_2 consumption was measured at 25°C under the same conditions as the spectral assays using a Clark cell and Gilson (Model K-IC) oxygen analyzer as in [14].

3. RESULTS

With oxidoreductase-rich membrane fractions derived from PMA-stimulated neutrophils, but with the exclusion of SOD from the sample cuvettes, we observed an approximate 10-fold faster rate

Table 1

NADPH diaphorase-linked:O_2 oxidoreductase activity in 27000 × *g* membrane fraction derived from lysates of stimulated *vs* resting neutrophils

Activity assayed[a]	Resting	PMA-stimulated
NADPH oxidation	28 ± 8 (n = 2)	248 ± 10 (n = 2)
O_2^- generation	1 ± 2 (n = 3)	173 ± 10 (n = 3)
Cytochrome *c* reduction	4 ± 2 (n = 3)	16 ± 4 (n = 3)
DCIP reduction	12 ± 16 (n = 3)	54 ± 10 (n = 3)
Ferricyanide reduction	2 ± 5 (n = 3)	151 ± 12 (n = 5)

[a] All enzyme activities are expressed in mU·ml^{-1} ± 1 SD. The number of replicate assays are in parentheses. Enzyme assays were conducted on the same set of cells divided into two equal cell suspensions of which half were then activated with PMA (see section 2). The final concentration of ferricytochrome *c*, DCIP and ferricyanide employed in diaphorase assays was 63, 70 and 70 μM, respectively; SOD (150 units) and catalase (1.5×10^4 units) were also included in the diaphorase and NADPH oxidase assays; SOD was omitted from the sample cuvette in the O_2^- generating assays

of ferricytochrome *c* reduction, a 3-fold faster rate of DCIP reduction, and a 10% faster rate of ferricyanide reduction over that with SOD included in the sample cuvette. Hence, in the case of DCIP and ferricytochrome *c*, and to a much lesser extent with ferricyanide, superoxide-mediated reduction of these electron acceptors is significant. With catalase and SOD in the final reaction mixtures, we detected markedly more diaphorase activity in membrane fractions derived from activated cells than from resting cells concomitant with expression of NADPH–O_2 oxidoreductase activity (table 1). In terms of percent electron equivalents accepted by each dye with respect to electron equivalents given up by NADPH the relative rates were 6 ± 1.5 (± SD, n = 3), 22 ± 4 (n = 3) and 61 ± 5% (n = 3) for ferricytochrome *c* (final concentration, 63 μM), DCIP (70 μM) and ferricyanide (70 μM), respectively. In a separate set of experiments (see below) employing a 10-fold increase in ferricyanide concentration, all of the electron equivalents given up by NADPH were accounted for in the form of ferricyanide reductase activity. Because of the high absorptivity coefficients of cytochrome *c* and DCIP it was not feasible to use significantly higher concentrations of either of these latter two dyes because of technical difficulties in measuring residual transmitted light in the sample and reference cuvettes.

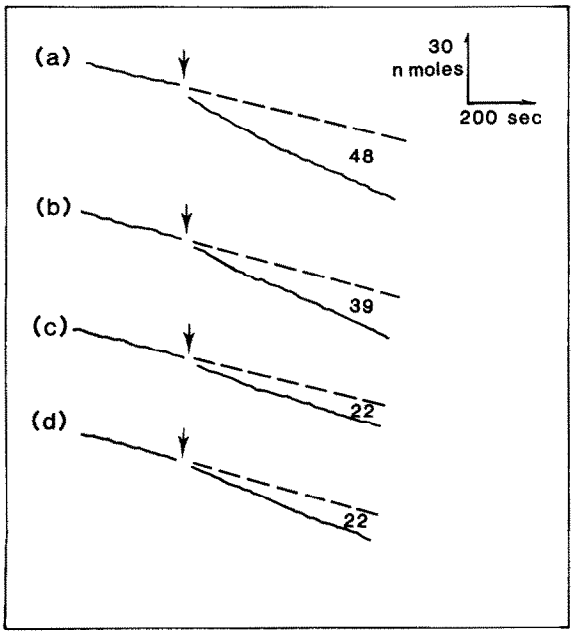

Fig.1. Effect of ferricyanide on net O_2 consumption of NADPH:O_2 oxidoreductase derived from PMA-stimulated neutrophils. (a) Uptake rate in the presence of NADPH, SOD, and catalase, (b) same as (a) + 0.07 mM ferricyanide in the final reaction mixture, (c) same as (a) + 0.7 mM ferricyanide in the final reaction mixture, (d) uptake rate in the presence of SOD and catalase minus NADPH. Arrows indicate the point of addition of enzyme suspension; dashed lines represent the background O_2 consumption rate prior to addition of the enzyme. Numbers indicate actual net O_2 consumption rate associated with enzyme addition. All assays were conducted at 25°C in a total volume of 1.5 ml of 32 mM Tris–HCl (pH 7.6), 0.25 mM $CaCl_2$, 0.16 mM $MgCl_2$, 150 units SOD, 200 μM DTPA and 1.5×10^4 units catalase. The final concentration of NADPH employed in experiments a–c was 100 μM.

In the presence of 0.07 mM ferricyanide, NADPH-dependent O_2 consumption was partially blocked; at 0.7 mM ferricyanide complete inhibition of NADPH-dependent O_2 consumption was evident (table 2). Tracings illustrating the effect of ferricyanide on O_2 consumption are shown in fig.1. It can be seen that there is also a significant rate of O_2 consumption which occurs in the absence of NADPH (fig.1d). This latter route of O_2 consumption is the subject of a separate study (in preparation). The data shown in table 2 were obtained by averaging the difference between O_2

Table 2

Effect of ferricyanide on net O_2 consumption of NADPH:O_2 oxidoreductase derived from PMA-stimulated neutrophils

Additions[a]	Net O_2 uptake ($mU \cdot ml^{-1}$)
None	24 ± 5 (n = 6)
+ 0.07 mM $K_3Fe(CN)_6$	16 ± 2 (n = 3)
+ 0.70 mM $K_3Fe(CN)_6$	0 (n = 4)

[a] All O_2 uptake experiments were conducted at 25°C in 32 mM Tris–HCl (pH 7.6) containing 0.25 mM Ca^{2+}, 0.16 mM Mg^{2+}, 200 μM DTPA, 1.5×10^4 units catalase, 150 units SOD and 100 μM NADPH (total volume, 1.5 ml) with enzyme added to initiate the reaction. $K_3Fe(CN)_6$ was also included in the final assay mixture at the concentrations shown. For further details see section 2. Values shown represent the mean ± SD with the number of replicate assays shown in parentheses

uptake rates measured in the presence and absence of NADPH. In a separate set of spectral assays conducted under the same assay conditions, NADPH oxidation rates were measured as 88 ± 4 mU·ml^{-1} (± SD, n = 5) in the absence of ferricyanide compared with 80 ± 6 mU·ml^{-1} (n = 6) in the presence of ferricyanide (0.7 mM); ferricyanide reductase activity measured at 405 nm was 75 ± 2 mU·ml^{-1} (n = 5).

4. DISCUSSION

A significant level of diaphorase activity measured in the presence of excess SOD and catalase is clearly expressed concomitant with the expression of the oxidoreductase in membrane fractions recovered from activated, but not resting, neutrophils. The diaphorase activity reported here cannot be explained in terms of superoxide-mediated reduction of the electron acceptors employed here since all of the assays were conducted with excess SOD included in the final reaction mixtures. These data, and the absence of diaphorase activity in two patients with CGD [7,8], indicate that diaphorase activity is intrinsic to the oxidoreductase. The markedly slower rates of ferricytochrome *c* and DCIP reduction compared with the oxidation rate of NADPH suggest that these two dyes are inefficient in oxidizing the reduced form of the oxidoreductase. These observations may explain difficulties in detecting DCIP and ferricytochrome *c* reductase activities associated with the oxidoreductase recovered from guinea pig lysates as in [12], and earlier difficulties in initial studies with the human neutrophil oxidoreductase reported in [11]. It is possible that species differences, and even different methods of preparing the oxidoreductase, may render the oxidoreductase more or less accessible to oxidation by DCIP or ferricytochrome *c*. Furthermore, it follows from our observations that the relatively low efficiency of DCIP and ferricytochrome *c* in abstracting electrons from the oxidoreductase complex makes these two dyes poor probes in demonstrating inhibition of O_2 consumption with inclusion of NADPH in the final reaction mixtures. For example, because of the inability of DCIP and ferricytochrome *c* to intercept more than 20 and 6% of the electron equivalents given up by NADPH to the oxidoreductase complex, respectively, under the conditions employed in our study, the maximum degree of inhibition would not be expected to exceed 20 and 6%, respectively. Since the coefficient of variation of O_2 uptake assays is in the order of 10 to 20% (cf. table 2), the small degree of inhibition in the net O_2 uptake rate expected with inclusion of either DCIP or ferricytochrome *c* in the reaction mixtures would be experimentally difficult to measure.

On the other hand, ferricyanide is an efficient electron acceptor of the oxidoreductase complex. This is evident by the comparable rates of ferricyanide reduction and oxidation of NADPH, and the capacity of ferricyanide to completely block O_2 consumption associated with oxidation of NADPH. Inhibition of O_2 consumption is evidence that ferricyanide successfully competes with O_2 for electrons given up by NADPH to the oxidoreductase complex. It cannot be argued that ferricyanide blocked O_2 consumption by inhibition of the oxidoreductase. The enzyme-catalyzed oxidation of NADPH measured in the presence of ferricyanide rules out this latter argument. Furthermore, the absence of net O_2 consumption cannot be attributed to cycling of superoxide back to O_2 with transfer of its electron to ferricyanide because excess SOD was employed in the O_2 uptake experiments. The amount of SOD included in the reaction mixtures (e.g., 150 units) was in great excess and more than adequate to scavenge all superoxide produced by the oxidoreductase. Of the three electron acceptors examined, we conclude that ferricyanide is the most efficient electron accepting dye in abstracting electron equivalents from the oxidoreductase. Work is currently underway in identifying in molecular terms the nature of the catalytic site responsible for this diaphorase type activity.

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