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Role of cytochrome *b*-559 in arachidonic acid activation of resting human neutrophils

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Whether or not cytochrome *b*-559 is a necessary component of NADPH oxidase activity in neutrophils is still controversial. In highly purified plasma membranes isolated from resting neutrophils and lacking cytochrome *b*, addition of arachidonic acid induced an NADPH oxidase activity. This activity was similar to that of plasma membranes isolated from phorbol myristate acetate (PMA)-stimulated cells which possessed cytochrome *b*. Addition of arachidonic acid to the latter plasma membranes did not alter the oxidase activity. It can be concluded that plasma membranes isolated from resting neutrophils have, in the presence of arachidonic acid, an NADPH oxidase activity similar to that of PMA-stimulated cells, except that it is independent of cytochrome *b*-559.

Introduction

Human polymorphonuclear neutrophils (PMN) stimulated with a variety of particles or soluble compounds undergo an oxidative burst that generates large amounts of superoxide anion [1–3]. Superoxide anion (O_2^-) results from the monovalent reduction of oxygen through an NADPH-dependent enzymatic activity, the so-called NADPH oxidase. NADPH oxidase activity is not detected in membranes isolated from resting PMN, whereas it is clearly present in those isolated from stimulated cells [1–3]. Among the mechanisms which may activate the oxidase, a stimulus-induced fusion of sterically distinct components has been proposed [4,5]. In particular, several lines of evidence suggest that a low-potential *b* cytochrome, designated cytochrome *b*-559 and located in the granules of resting PMN, translocates to the plasma membrane upon stimulation [6,7]. However, the role of cytochrome *b*-559 in oxidase activity as well as that of its translocation remain a matter of controversy [8]. It has recently been reported that the addition of arachidonic acid to plasma membranes isolated from resting PMN enables them to produce O_2^- in the presence of cytosol and NADPH [9–14]. Whether or not cytochrome *b*-559 is necessary for this oxidase activation is not clear [15]. Using a technique [16] to isolate highly purified plasma membranes, we measured NADPH oxidase activity as well as cytochrome *b*-559 in plasma membranes isolated from resting and phorbol myristate acetate

Abbreviations: PMN, polymorphonuclear neutrophils; PMA, phorbol myristate acetate; FB1, floating band 1, plasma membranes; S, supernatant, cytosol; P, pellet, granules; PBS-sucrose, 2 mM phosphate-buffered saline (pH 7.4) (34 mosM) containing 0.34 M sucrose.

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(PMA)-stimulated PMN. NADPH oxidase was measured in the presence or absence of arachidonic acid. Results showed that plasma membranes from resting PMN possess neither oxidase activity nor cytochrome *b*-559. However, an oxidase activity develops when arachidonic acid is added to these isolated plasma membranes. The kinetic parameters of this arachidonic acid-activated oxidase are similar to those of the oxidase found in plasma membranes isolated from PMA-stimulated PMN. Furthermore, the addition of arachidonic acid to plasma membranes isolated from PMA-stimulated PMN does not alter the kinetic parameters of its oxidase activity.

Materials and Methods

Reagents. Dextran T 500, Ficoll-Hypaque were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). PMA, diisopropylfluorophosphate, superoxide dismutase, arachidonic acid and NADPH (type 1) were obtained from Sigma (St. Louis, MO). Ethanol and paraffin oil were from Merck (Darmstadt, F.R.G.). ^{57}Co-labeled vitamin B-12 (100–300 μCi/mg) were purchased from Amersham. The arachidonic acid stock solution (7.66 mM) was prepared by dissolving 25 mg in 25 ml absolute ethanol and then bringing the volume to 10 ml with H_2O. PMA was dissolved in dimethyl sulfoxide at 1 mg/ml and stored at −80 °C.

Isolation of PMN. Human PMN were obtained from venous blood (15 IU of lithium heparinate/ml of blood). They were isolated by Dextran T 500 sedimentation followed by Ficoll-Hypaque density centrifugation and hypotonic lysis of erythrocytes [7]. Leukocytes were washed with ice-cold Ca^{2+}- and Mg^{2+}-free Krebs-Ringer's phosphate buffer (pH 7.4) and resuspended in the same buffer at a concentration of $(1.0–2.0) \cdot 10^8$ PMN per ml. The cell suspension contained more than 97% PMN. Cell viability was higher than 98% as assessed by the trypan blue exclusion test.

PMA-stimulation of PMN. 0.1 μg of PMA was added to 5 ml of PMN suspension equilibrated at 37 °C. After 3 min incubation at 37 °C in a shaking water-bath (90 strokes per min), the reaction was stopped by adding 15 ml of ice-cold 20 mM phosphate-buffered saline (pH 7.4, 340 mosM). Resting PMN kept over an ice-cold bath were treated with 15 ml of ice-cold buffered saline. PMA-stimulated and resting PMN were then treated similarly, with all the steps conducted below 4 °C. The PMN were pelleted by centrifugation for 6 min at $200 \times g$. The supernatant was discarded and the cell pellet was resuspended in phosphate-buffered saline containing 0.34 M sucrose (PBS-sucrose). To inactivate serine proteinases, cell suspensions were incubated with 1 mM diisopropyl fluorophosphate for 15 min [17]. The PMN were then washed twice in PBS-sucrose and resuspended in the same PBS-sucrose buffer, except that it contained 2 mM $MgCl_2$.

Disruption of PMN and plasma membrane isolation. Resting and PMA-stimulated PMN were disrupted in paraffin oil as described [16]. Briefly, PMN $(50 \cdot 10^6$/ml) were mixed with paraffin oil and mechanically (33 kg/cm^2 pressure) disrupted with a Lox-press (AB Biox. Stockholm, Sweden). The resulting homogenate was collected into 1 ml of ice-cold PBS-sucrose. After centrifugation, the paraffin oil separated from the aqueous medium. Two floating bands were observed, one above the oil (FB1) and the other (FB2) at the interface of the oil and aqueous phases. FB2 was discarded because, as previously reported, this fraction contains markers of both the granules and the plasma membranes [16]. A pellet below the aqueous phase, which comprised nuclei and unbroken cells, was discarded. The aqueous phase, following centrifugation at $21\,000 \times g$ for 20 min, yielded a supernatant (S) and a pellet (P). As previously reported for resting PMN and shown here for PMA-stimulated cells, FB1, S and P were composed mainly of plasma membranes, cytosol and granules, respectively. FB1 was washed and solubilized with 0.1% Triton X-100 and 0.1% deoxycholate as previously described [16]. The following markers were used to show that subcellular organelles isolated from PMN were plasma membranes, cytosol and granules for FB1, S and P, respectively. Lactate dehydrogenase, measured according to Beutler [18], was used to monitor percent disruption and fate of the cytosol. The granule enzyme, myeloperoxidase, and vitamin B-12 binding protein were assayed as described [19,20] in order to determine the fate of primary and specific granules, respectively. Alkaline phosphatase was assayed to follow

the fate of plasma membranes [21]. Protein was determined by the Bio-Rad method using bovine serum albumin as a standard [22]. In order to compare recovery of subcellular components from resting and stimulated PMN disrupted in paraffin oil, the content of the above markers was measured in resting PMN disrupted by sonication in the presence of 0.2% Triton X-100 and 0.2% deoxycholate. Addition of these detergents to sonicated PMN either increased or did not alter the amount of protein and markers recovered.

NADPH oxidase activity. NADPH-dependent oxidase activity was measured by two techniques: superoxide dismutase-inhibitable reduction of cytochrome *C* and luminol-enhanced chemiluminescence. Cytochrome *C* reduction was monitored at 25°C with a Uvikon 810 dual beam spectrophotometer (Kontron Roche, Switzerland). The reference and sample cuvettes contained in a final volume of 1 ml: 0.1 M phoshate buffer (pH 7), 80 μM cytochrome *C*, 1 mM $MgCl_2$, 5–10 μg protein of FB1 and 0.25–0.75 mM NADPH. In addition, the reference cuvette contained 20 μg/ml of superoxide dismutase. NADPH was added and the difference in absorbance at 550 nm (sample minus reference) was followed for several minutes. Initial rate of O_2^- production was calculated using 21.1 mM as the absorption coefficient of cytochrome *C*. NADPH oxidase was also measured by luminol-enhanced chemiluminescence [23] using a Picolite model 6500 luminometer (Packard Corp). Activity was monitored at 37°C under magnetic stirring on program 2 (i.e., 10 s counting time, 18 counts sequence and zero background). The total chemiluminescence (3 min reaction) was recorded because of the stability of photon emission during this period of time. Each assay vial contained in a total volume of 150 μl: 0.1 M phosphate buffer (pH 7.5), 1 mM DTPAC, 34 mM sucrose or 5–10 μg protein of FB1, 1 μM luminol and NADPH at concentrations ranging from 0.25 to 0.75 mM. The reaction was started by injecting 65.6 μM AA or its solvent in the controls.

Cytochrome b-559. Cytochrome *b*-559 was measured in total PMN homogenate, FB1 and P fractions of resting and PMA-stimulated PMN. The technique used has been described in detail [24]. This technique routinely uses, before dithionite addition, bubbling of CO in aerated homogenate (i.e., in which cytochrome *b*-559 is in its oxidized form and does not bind CO, whereas hemoglobin is in a reduced functional form and firmly binds CO). Unbound CO is evacuated after a further aeration and dithionite is added. As previously reported [24], in preparations devoid of hemoglobin, the peak at 558–559 nm (dithionite reduced minus oxidized) did not change, whether or not CO was bubbled in the preparation. Moreover, in membrane preparations from PMA-stimulated PMN that are not contaminated with any hemoglobin, measured values of cytochrome *b*-559 were similar, whether or not CO was bubbled. In total homogenate, addition of detergents (Triton X-100 and deoxycholate), as routinely performed in membrane preparations, did not alter the results of cytochrome *b*-559 measurements.

Statistics. Results are given as means ± S.D. V_{max} and apparent K_m were calculated using a Lineweaver-Burk plot and the least-square method to establish regression lines.

Results

Plasma membrane isolation and its cytochrome b-559 content

Optimal conditions for disruption of PMA-stimulated PMN were found to be similar to those previously reported for resting PMN [16]. Table I shows the distribution of the organelle markers found in FB1 (plasma membrane), S (cytosol) and P (granules) fractions after disruption of resting and PMA-stimulated cells. PMN plasma membranes of stimulated cells were contaminated by less than 3% of granule markers (myeloperoxidase and vitamin B-12 binding protein). No contamination of granule markers or cytochrome *b*-559 was detected in FB1 isolated from resting PMN (Fig. 1). Based on the sensitivity of our technique, this means that the plasma membranes of resting PMN contain less than 3% of the total cell cytochrome. In resting PMN, cytochrome *b* was recovered in fraction P. It was 58 ± 7.4% of the total PMN cytochrome *b*. This low recovery is in part linked to the fact that the FB2 fraction as well as the pellet were discarded. The absence of cytochrome *b*-559 in the FB1 fractions suggests that the resting PMN were not activated during membrane preparation. FB1 of PMA-stimulated PMN contained

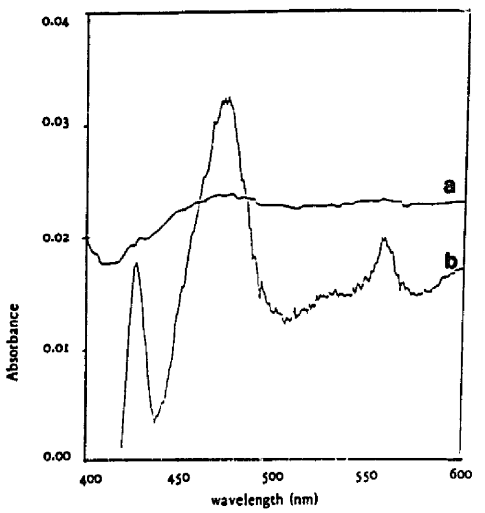

Fig. 1. Reduced-minus oxidized spectra of cytochrome *b*-559. (a) FB1 (membranes); (b) P fraction (granules) from $30 \cdot 10^6$ resting PMN per ml. Cytochrome *b* was determined as described under materials and Methods.

$26 \pm 14\%$ of total cytochrome, whereas the content of cytochrome in granules decreased to $16 \pm 6\%$.

NADPH-dependent O_2^--forming activity of FB1

In FB1 isolated from resting PMN, no O_2^--forming activity was found either by the superoxide dismutase-inhibitable reduction of cytochrome *C* or by the luminol-enhanced chemiluminescence methods. This absence of O_2^--forming activity shows that, under our experimental conditions of FB1 preparation, the PMN were not activated. By contrast, a NADPH-dependent O_2^--forming activity was found with both techniques when FB1 were isolated from PMA-stimulated cells. The kinetic parameters of the enzymatic activity were the following: V_{max} was 200 nmol O_2^- per min per mg of protein; the apparent K_m for NADPH was $35 \cdot 10^{-6}$ M. The NADPH oxidase activity was not expressed when denaturated protein (boiled for 5 min) was substituted for the FB1 fraction or when NADPH was omitted from the reaction mixture.

TABLE I

RECOVERY OF PROTEIN AND MARKERS IN SUBCELLULAR FRACTIONS OF HUMAN PMN

Values obtained after Lox-press disruption in paraffin oil of resting (R) or PMA-stimulated (S) PMN. Subcellular fractions were obtained as explained in Materials and Methods. The recovery values do not take into account the pellet and the FB2 fractions that were discarded in most experiments (see Materials and Methods). These fractions contain about 30% of the total protein and about 30–35% of cytochrome *b*-559 in resting or stimulated PMN. Values above are means ± S.D. of six different experiments, and units are indicated in parentheses. u.d., undetectable.

	Total PMN	FB1 (membranes)		S (cytosol)		P (granules)		Recovery (%)	
		R	S	R	S	R	S	R	S
Protein (mg/10^9 PMN)	124 ±22	2.24 ±0.4	2.54 ±0.3	49.6 ±2.1	50.3 ±1.8	13.2 ±1.2	11.7 ±2.0	52.5	51.5
Alkaline phosphatase (units/10^6 PMN)	86 ±12	42.3 ±2.3	46.1 ±2.5	2.24 0.17	2.38 0.36	6.9 2.24	6.6 0.55	59.8	64.0
Lactate dehydrogenase (units/10^6 PMN)	75 ±18.9	0.5 ±0.1	0.5 ±0.1	55 ±2.5	53 ±3.0	0.75 ±0.2	0.93 ±0.3	75	72
Myeloperoxidase (units/10^6 PMN)	137 ±26.7	1 ±0.2	3.8 ±0.2	3.0 ±0.1	3.8 ±0.2	89 ±4.2	78 ±3.0	67	62.5
Vitamin B-12 binding activity (mg B-12 found in 10^9 PMN)	490 ±160	5 ±0,2	15 ±0,5	42 ±5	35 ±6	276 ±7,8	85.2 ±3.0	65	27.5
Cytochrome *b*-559 (pmol/10^6 PMN)	8.1 ±1.3	u.d.	2.1 ±1.1	u.d.	u.d.	4.7 ±0.6	1.3 ±0.5	58	42

TABLE II

EFFECT OF ARACHIDONIC ACID ON NADPH-DEPENDENT O_2 ACTIVITY MEASURED ON MEMBRANES FROM RESTING PMN

Chemiluminescence and superoxide dismutase-inhibitable cytochrome *C* reduction of the membrane fraction from resting PMN, determined in the presence of NADPH and arachidonic acid (see Materials and Methods). V_{max} and K_m were calculated from a Lineweaver-Burk plot.

Method	V_{max}	k_{in} (μM)
Chemiluminescence	$1.6 \cdot 10^6$ cpm/min per mg protein	38.4 ± 1.6
Spectrophotometer	300 nmol O_2^-/min per mg protein	37 ± 2.1

Effect of arachidonic acid on NADPH-dependent O_2^--forming activity

Addition of arachidonic acid to FB1 from resting PMN allowed generation of O_2^- in amounts similar to those generated by FB1 isolated from PMA-stimulated PMN. Moreover, kinetic parameters (V_{max} and apparent K_m for NADPH) in the O_2^- generation of FB1 from resting PMN were similar to those of FB1 from PMA-stimulated cells (Table II). It is noteworthy that in these membranes cytochrome *b*-559 was not detectable. In contrast to its effect on FB1 isolated from resting cells, arachidonic acid did not modify the O_2^- production by FB1 from PMA-stimulated cells. Furthermore, the V_{max} and the apparent K_m for NADPH were not modified by the addition of arachidonic acid to FB1. The spectrophotometric and the chemiluminescence assays, used to measure NADPH oxidase activity in the FB1 fraction, are very sensitive to the KCN or azide levels currently used to demonstrate that the PMN NADPH oxidase is cyanide-insensitive. In order to circumvent this difficulty, we measured the cyanide (1 mM)-insensitive O_2 uptake by two FB1 fractions prepared from resting PMN and activated with arachidonic acid. Results were similar to those found with FB1 fraction prepared from PMA-stimulated PMN (not shown).

Discussion

The NADPH-oxidase system of human PMN is a subject of extensive research and debate concerning its components. Most investigators believe that two species, an unusual, low-potential *b*-type cytochrome, *b*-559, and a flavoprotein, take part in the electron transfer system. Cytochrome *b*-559 is located in the granules of the resting PMN and translocates to the plasma membranes upon stimulation [6,7]. Recently, the activation of the human neutrophil NADPH oxidase has been shown to occur in plasma membrane preparation from PMN which had not been stimulated before fractionation [9–14]. Here, we show that the activation of the membrane NADPH oxidase by arachidonic acid is not dependent on spectrophotometrically detectable cytochrome *b*. In particular, the apparent K_m and V_{max} of the activated NADPH oxidase are similar whether cytochrome *b*-559 is present, as in membranes isolated from PMA-stimulated PMN, or not detectable, as in the membrane isolated from resting, in vitro arachidonic acid-activated PMN. This latter result, showing a similar enzymatic activity in cell activation by PMA or isolated plasma membrane activated by arachidonic acid, strongly suggests that all components of the NADPH oxidase system are present in the plasma membrane of resting PMN. This is further supported by the fact that the enzymatic activity in the intact, PMA-stimulated PMN is not altered further by additional treatment of the plasma membrane with arachidonic acid. Activation of intact PMN under our experimental conditions was performed using 20 ng/ml (0.1 μg for 5 ml). Under these conditions of suboptimal PMN activation, the O_2^- production is about 30 nmol O_2^- per min per mg of protein. Because plasma membrane proteins account for about 3–4% of total proteins, the theoretical production of O_2^- by isolated plasma proteins should be about 3-times that found in our membrane preparation isolated from PMA-stimulated cells or from resting, subsequently arachidonic acid-activated cells. This relatively low recovery of NADPH oxidase activity might be due to our conditions of membrane preparation. These could select a membrane fraction with NADPH activity lower than that of discarded membrane fractions, such as FB2 and pellet, or other fractions in which NADPH oxidase activity was not measured, such as P [13]. Further measurements of NADPH oxidase activity in these fractions are

needed. However, the NADPH oxidase activity observed in the membrane fraction is similar or even higher than those reported by others under conditions involving the presence of cytosol [11–14].

Our results were obtained with a technique [16] recently shown to isolate highly purified membranes from resting PMN [16]. We show (Table I) that the technique is also efficient for isolation of plasma membranes from PMA-stimulated cells. Cytochrome *b*-559 has been reported to be located in the granule and plasma membrane fractions of resting PMN [6]. The differential spectrum (dithionite-treated minus oxidized) of cytochrome *b*-559 shows three absorption bands, α, β and γ at 557–559, 529–531 and 427–429 mm, respectively. The α band is specific for the cytochrome, but its height was much lower than that of the γ band. In the plasma membrane (FB1) of resting PMN, neither the α nor the γ band could be seen. Dilution of total PMN homogenate assured that down to 3% of total, the cytochrome could be clearly seen by its γ band (results not shown). This indicates that the plasma membrane of the resting PMN contains less than 3% of its total cytochrome *b*. Upon PMA stimulation, cytochrome *b* is translocated from the granules to the plasma membrane, in parallel with the degranulation of the specific granules.

In a cell-free system, as described by a number of authors [9–14,25], the NADPH oxidase of human neutrophil membrane can be activated by arachidonic acid without previous activation before fractionation. Under our experimental conditions, cytochrome *b* was not spectrophotometrically detected in the membrane of resting PMN. Therefore, the activation by arachidonic acid of resting PMN membranes did not require the presence of cytochrome *b*, in agreement with results on bovine PMN [26]. Furthermore, Kakinuma et al. [27] and Pick et al. [28] did not find any heme protein in the oxidase of pig PMN. In variance with Curnutte et al. [11] and Clark et al. [13], we find that the activation of NADPH oxidase by arachidonic acid does not require the presence of a cytosolic factor. Curnutte et al. found that the required cytosolic factors has two peaks of activity (200 and 40 kDa), whereas Clark et al. found a single peak of 10 kDa. In another report [12], the cytsolic factor was found not to be protein kinase C, since H-7 inhibition of protein kinase C had no effect on the induced oxidase activity. Our technique, using lactate dehydrogenase activity as a marker of cytosolic factor contamination into membranes, allows us to exclude such contamination. This method of plasma membrane preparation differs from that used by others [11–14]. It thus cannot be excluded that during paraffin oil disruption, a selective adherence of a required cytosolic factor for NADPH oxidase activity occurs. In fact, addition of cytosol to the membrane fraction (FB1) did not increase arachidonic acid-induced NADPH oxidase activity (results not shown). Another difference is that in the present study, FB1 was treated with Triton X-100 and deoxycholate to allow complete separation of FB1 proteins from paraffin oil. It cannot be excluded that such treatment may have effects similar to those resulting from addition of cytosol. Further efforts are now being devoted to understanding the reason(s) why our membrane preparation possesses an arachidonic acid-inducible NADPH oxidase activity, independent of a cytosolic factor.

In conclusion, the in vitro activation of NADPH oxidase by arachidonic acid in plasma membranes lacking cytochrome *b*-559 suggests that human intact PMN possess a superoxide-anion-forming activity which is cytochrome *b*-independent and whose mechanism of activation needs to be studied further.

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