NADPH-binding component of the superoxide-generating oxidase in unstimulated neutrophils and the neutrophils from the patients with chronic granulomatous disease

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The NADPH-binding component of the neutrophil superoxide-generating oxidase was studied in the particulate oxidase fractions obtained from the neutrophils of normal and chronic-granulomatous-disease (CGD) patients. The molecular mass of the NADPH-binding component of the stimulated human neutrophils, which was labelled with the 2',3'-dialdehyde derivative of NADPH and sodium cyanoboro[³H]hydride, was 66 kDa. The 66 kDa component was also labelled in monocytes, but not in red blood cells, platelets and lymphocytes. The particulate oxidase fractions obtained from the patients with CGD had a diminished amount of FAD, whether they contained cytochrome *b*₅₅₈ or not. The fractions labelled with the NADPH analogue showed that CGD patients had the NADPH-binding component in the neutrophils. The molecular mass of the component was identical with that of the normal neutrophils. The patients are thought to have an intact NADPH-binding domain of the oxidase in the neutrophils in spite of a diminished amount of FAD in the particulate fractions. The component of the oxidase in the resting neutrophils was also labelled with the analogue. The molecular mass of the component in the resting neutrophils was identical with that of the stimulated neutrophils, and the component was not phosphorylated during the activation process. These results indicate that the NADPH-binding component of the oxidase, which is specific to phagocytes, is present in the resting neutrophils and that the component does not change with respect to molecular mass during the activation process.

INTRODUCTION

Normal human neutrophils contain a membrane-bound NADPH-dependent superoxide-generating oxidase system that is important in the microbicidal activity (Badwey & Karnovsky, 1980; Babior, 1984*a,b*). The oxidase has been postulated to be a multicomponent electron-transport chain consisting of a flavoprotein and a cytochrome *b*₅₅₈. Although the activity of the oxidase is dormant in unstimulated neutrophils, appropriate stimuli initiate a sharp increase in the production of superoxide anions. The activation of the oxidase is a highly specific process controlled by a complex mechanism(s) that is yet to be elucidated.

Chronic granulomatous disease (CGD) is a disorder in which the patients suffer from severe recurrent infections with bacteria and fungi. In patients with this disease, the superoxide-generating activity is completely defective in neutrophils and monocytes (Klebanoff & Clark, 1978; Tauber *et al.*, 1983). Cytochrome *b*₅₅₈ was not detectable in the neutrophils from most patients with the X-chromosome-linked form of CGD (Segal *et al.*, 1983). Cytochrome *b*₅₅₈ was present, but failed to be reduced after stimulation in the neutrophils from the patients with the autosomal recessive form of the disease (Segal & Jones, 1980; Gabig & Lefker, 1984). A flavoprotein defect in CGD has been proposed on the basis of the observation that the FAD content was greatly diminished in the particulate oxidase fraction of the neutrophils from CGD patients. The disease may become manifest

not only when components of the enzyme system are missing or structurally altered, but also when defects occur in the activation mechanism.

We have recently reported that the NADPH-binding component of this enzyme can be labelled with the 2',3'-dialdehyde derivative of NADPH (Umei *et al.*, 1986). The molecular mass of the labelled component was essentially identical with that of the molecule that two groups of investigators have prepared as the oxidase (Markert *et al.*, 1985; Doussiere & Vignais, 1985). The affinity-labelling technique has enabled us to identify the component of the superoxide-generating oxidase in unstimulated neutrophils and the neutrophils from the patients with CGD that is, in both cases, unable to produce superoxide anions. We show here that the NADPH-binding component of the oxidase is intact in the neutrophils from the patients with CGD and that the component which is specific to phagocytes neither changes in molecular mass nor is phosphorylated during the activation process.

EXPERIMENTAL

Materials

Ferricytochrome *c* (type 3), superoxide dismutase (bovine erythrocyte), PMA, standard molecular markers and isocitrate dehydrogenase (pig heart) were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Sodium deoxycholate (Difco Laboratories, Detroit, MI,

Abbreviations used: CGD, chronic granulomatous disease; PMA, phorbol 12-myristate 13-acetate; NADPH 2',3'-dialdehyde or 'NADPH analogue', 2',3'-dialdehyde derivative of NADPH.

U.S.A.), NADPH (Oriental Yeast Co., Tokyo, Japan), sodium periodate (Nakarai Chemicals, Kyoto, Japan), polyethyleneimine sheets (Merck, Darmstadt, Germany), FAD and FMN (Wakamoto Chemicals, Tokyo, Japan), DEAE-Sepharose 6B (Pharmacia, Uppsala, Sweden), Enlightning (New England Nuclear, Boston, MA, U.S.A.), sodium cyanoboro[^3H]hydride (sp. radioactivity 11 Ci/mmol) (Amersham, Tokyo, Japan) and $\text{H}_3^{32}\text{PO}_4$ (Nihon-Genshiryoku Institute, Tokyo, Japan) were obtained from the suppliers cited. All other reagents were commercially available and of analytical-reagent grade.

Preparation of the particulate oxidase fraction from blood cells

Neutrophils were prepared from fresh acid-citrate/dextrose-treated blood by dextran sedimentation, by hypo-osmotic haemolysis of red blood cells and by the Conray-Ficoll method (Böyum, 1968). Neutrophils ($10^8/\text{ml}$ in phosphate-buffered saline) were stimulated by incubation at 37°C with PMA ($1\text{ }\mu\text{g}/\text{ml}$) in the presence of $1\text{ mM-Na}_2\text{S}_2\text{O}_8$ for 20 min. Stimulated and unstimulated neutrophils from normal volunteers and stimulated neutrophils from CGD patients were sonically disrupted and fractionated by differential isopycnic sedimentation by the method of Gabig *et al.* (1982). The particulate fraction was enriched in the NADPH-dependent superoxide-generating oxidase activity. Red-blood-cell, platelet, lymphocyte and monocyte membranes were prepared as described previously (Evans, 1978).

Assay of superoxide generation by the particulate oxidase fraction

NADPH-dependent superoxide generation was measured by a continuous spectrophotometric assay of superoxide dismutase-inhibitable ferricytochrome *c* reduction at 25°C with the use of a dual-wavelength spectrophotometer (Hitachi, model 557) (Umei *et al.*, 1986). The reaction mixture contained $50\text{ mM-phosphate buffer}$, $\text{pH } 8.0$, $50\text{ }\mu\text{M-ferricytochrome } c$, 0.5 mM-NADPH and 0.02% sodium deoxycholate. Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Determination of cytochrome b_{558} and FAD

The particulate oxidase fraction was analysed for cytochrome b_{558} content by reduced-minus-oxidized spectroscopy, an ϵ_{558} value of $21.6\text{ mM}^{-1}\cdot\text{cm}^{-1}$ (Cross *et al.*, 1982a) being used. Non-covalently bound FAD in the oxidase fraction was assayed by the method of Faeder & Siegel (1973).

Preparation of the 2',3'-dialdehyde derivative of NADPH

The NADP^+ 2',3'-dialdehyde prepared by periodate oxidation was enzymically converted into NADPH 2',3'-dialdehyde with the use of isocitrate dehydrogenase (Umei *et al.*, 1986). The NADPH analogue was purified with a DEAE-Sepharose column and its purity was checked by t.l.c. on a polyethyleneimine sheet.

Inactivation of human NADPH oxidase by NADPH 2',3'-dialdehyde

Inactivation of the NADPH oxidase from human neutrophils by NADPH 2',3'-dialdehyde was examined

as previously described (Umei *et al.*, 1986). The oxidase fraction of human neutrophils was incubated at 10°C with $1\text{ mM-NADPH } 2',3'\text{-dialdehyde}$.

Labelling of the NADPH-binding component with NADPH 2',3'-dialdehyde and sodium cyanoboro[^3H]hydride

Labelling of the NADPH-binding component was performed as previously described (Umei *et al.*, 1986). The particulate oxidase fraction, incubated with $0.1\text{ mM-NADPH } 2',3'\text{-dialdehyde}$ in the presence or absence of 100 mM-NADPH , was treated with $0.15\text{ mM-sodium cyanoboro[}^3\text{H]hydride}$ ($170\text{ }\mu\text{Ci}$). The labelled proteins precipitated by trichloroacetic acid were analysed by SDS/polyacrylamide-gel electrophoresis as described by Laemmli (1970). The gels, stained with Coomassie Brilliant Blue, were treated with Enlightning, dried, and exposed to Fuji RX X-ray film at -70°C . The molecular mass of each band was determined on 7.5% (w/v)-polyacrylamide separating gels using standard molecular-mass markers (egg albumin, 45 kDa ; bovine serum albumin, 66 kDa ; phosphorylase *b*, 97.4 kDa ; β -galactosidase, 116 kDa ; and myosin, 205 kDa).

^{32}P labelling of neutrophils and autoradiography

^{32}P labelling of neutrophils was achieved by incubation of the cells with carrier-free $\text{H}_3^{32}\text{PO}_4$ ($1\text{ mCi}/\text{ml}$) for 60 min at 37°C (Irita *et al.*, 1984). The neutrophils, loaded with [^{32}P]P $_i$, were exposed to PMA ($1\text{ }\mu\text{g}/\text{ml}$) (stimulated) or dimethyl sulphoxide alone (unstimulated) for 15 min and the particulate oxidase fractions of the neutrophils were prepared. The proteins were separated by SDS/polyacrylamide-gel electrophoresis. The gels were stained, dried, and exposed to the X-ray film at -70°C .

RESULTS

Content of FAD and cytochrome b_{558} in the particulate fractions

The stimulated neutrophils obtained from CGD patients and normal unstimulated neutrophils failed to produce superoxide anions. The subcellular particulate oxidase fractions were prepared from the stimulated neutrophils of normal and CGD patients and from the normal unstimulated neutrophils. The superoxide generation by the fraction from normal stimulated neutrophils was $172 \pm 41\text{ nmol}/\text{min}$ per mg of protein ($n = 5$), whereas those of the unstimulated neutrophils and CGD patients were not measurable. The contents of FAD and cytochrome b_{558} in the particulate fractions are given in Table 1. The particulate oxidase fractions obtained from normal stimulated and unstimulated neutrophils contained essentially an equimolar amount of FAD and cytochrome b_{558} . The contents of FAD and cytochrome *b* in the fraction of the normal unstimulated neutrophils were about two-thirds that of the normal stimulated neutrophils. The fraction from an X-chromosome-linked-CGD patient (CGD1) had about 40% of the normal amount of FAD and no detectable cytochrome b_{558} . Four other CGD patients with the X-chromosome-linked cytochrome *b*-negative form had 20–40% of the normal amount of FAD in the particulate oxidase fraction. The fraction from an autosomal-CGD patient (CGD2) had about 65% of the normal amount of FAD and a normal

Table 1. FAD and cytochrome b_{558} content of the particulate oxidase fractions

Data represent mean $\pm$ S.E.M. The particulate oxidase fractions were prepared from normal and two CGD patients [CGD1 (male) and CGD2 (female)].

Particulate oxidase fraction	n	Content (nmol/mg of protein)	
		FAD	Cytochrome b_{558}
Normal, stimulated	5	0.28 $\pm$ 0.02	0.28 $\pm$ 0.03
Normal, unstimulated	5	0.18 $\pm$ 0.01	0.18 $\pm$ 0.01
CGD1	3	0.11 $\pm$ 0.01	< 0.01
CGD2	3	0.18 $\pm$ 0.01	0.29 $\pm$ 0.02

Fig. 1. Dithionite-reduced-minus-oxidized absorption spectra

The spectra of particulate oxidase fractions of the male and female CGD patient (CGD1 and CGD2) were recorded with a Hitachi model-340 spectrophotometer. The spectrum of the fraction from the female patient (—, 0.48 mg/ml) was identical with that of normal neutrophils characterized with α -peak at 558 nm, β -peak at 529 nm and γ -peak at 426 nm. The peak at 474 nm was due to contamination by myeloperoxidase. No peaks due to cytochrome b_{558} were recorded in the spectrum of the oxidase fraction from the male patient (····, 0.46 mg/ml), although there was a little contamination by myeloperoxidase.

amount of cytochrome b_{558} . Fig. 1 shows the dithionite-reduced-minus-oxidized absorption spectra of the fractions from the X-chromosome-linked-CGD patient (CGD1) and the autosomal-CGD patient (CGD2). The

spectrum for CGD2 is characterized by the α -peak at 558 nm, the β -peak at 529 nm and the γ -peak at 426 nm, which are identical with those of normal cytochrome b_{558} specifically present in neutrophils. The spectrum for CGD1 shows the absence of cytochrome b_{558} . Thus the oxidase fractions obtained from CGD patients in our study contained a diminished amount of FAD, whether they had cytochrome b_{558} or not.

Inactivation of human NADPH oxidase by NADPH 2',3'-dialdehyde

The human oxidase preparation with 1 mM-NADPH 2',3'-dialdehyde also lost 62% of the superoxide-generating activity (compared with the control) after incubation for 30 min at 10 °C as for pig neutrophils. The inactivation of the activity was diminished 75% by NADPH. Therefore it seems likely that the NADPH-binding component of human neutrophils is labelled by NADPH 2',3'-dialdehyde.

Labelling of the NADPH-binding component of the oxidase in CGD-patient neutrophils

The particulate oxidase fraction was labelled with the NADPH analogue and sodium cyanoboro[^3H]hydride and then analysed by SDS/polyacrylamide-gel electrophoresis. Fluorography of the gels revealed that the component labelled with the NADPH analogue was present in all oxidase fractions of normal subjects and both X-chromosome-linked- and autosomal-CGD patients and had the same molecular mass, 66 kDa (Fig. 2). The component was not labelled in the presence of excess NADPH, indicating that the NADPH analogue

Fig. 2. Fluorography of the particulate oxidase fractions from normal and CGD patients

The particulate oxidase fractions of normal (lanes 1 and 2) and CGD patients (lanes 3–10) were labelled with the NADPH analogue and sodium cyanoboro[^3H]hydride in the presence (lanes 2, 4 and 6) or absence (lanes 1,3,5 and 7–10) of NADPH. The fluorography of the labelled proteins analysed by SDS/polyacrylamide-gel electrophoresis is shown. CGD patients examined were an autosomal cytochrome b -positive female CGD2 (lanes 3 and 4), an autosomal cytochrome b -positive male CGD6 (lane 10) and X-chromosome-linked cytochrome b -negative [CGD1 (lanes 5 and 6), CGD3 (lane 7), CGD4 (lane 8) and CGD5 (lane 9)]. The 66 kDa molecule was also not labelled in CGD3, CGD4, CGD5, and CGD6 when treated with the NADPH analogue in the presence of NADPH (result not shown). The molecular mass (left) is given in kDa.

bound specifically to the component. A 45 kDa polypeptide that was recently suggested to be a component of the oxidase with pig neutrophils by affinity labelling with diphenylene iodonium (Cross & Jones, 1986) was not labelled by NADPH 2',3'-dialdehyde, even in normal neutrophils. The patterns of protein bands of the fractions from the CGD patients with the X-chromosome-linked and autosomal forms of the disease were essentially the same as that of the normal neutrophils, and consistent changes in the protein bands were not observed (Fig. 3). This is in accord with the results of two-dimensional gel electrophoresis of the neutrophil proteins from normal subjects and CGD patients (Ishii *et al.*, 1986).

Labelling of the NADPH-binding component of the oxidase in unstimulated neutrophils and blood cells

The particulate fraction of unstimulated neutrophils was also labelled with the NADPH analogue and sodium cyanoboro[^3H]hydride and then analysed by SDS/polyacrylamide-gel electrophoresis. Fluorography of the gels revealed that the NADPH-binding component was also labelled by the NADPH analogue in the unstimulated neutrophils and not labelled in the presence of excess NADPH (Fig. 4). The molecular mass of the labelled

Fig. 3. SDS/polyacrylamide-gel electrophoresis of the particulate oxidase fraction from normal and CGD patients

The particulate oxidase fractions of normal (lane 1) and CGD patients (lanes 2–7) were analysed by SDS/polyacrylamide gel electrophoresis. Proteins were stained with Coomassie Brilliant Blue. CGD patients examined were X-chromosome-linked cytochrome *b*-negative CGD1 (lane 2), CGD3 (lane 4), CGD4 (lane 5), CGD5 (lane 6), an autosomal cytochrome *b*-positive female CGD2 (lane 3), and an autosomal cytochrome *b*-positive male CGD6 (lane 7). For electrophoresis, 150 μg of protein was placed in each well. Molecular masses (left) are given in kDa.

Fig. 4. Fluorography of the particulate oxidase fractions from normal stimulated and unstimulated neutrophils

The particulate oxidase fractions of normal stimulated (lanes 1 and 2) and unstimulated (lanes 3 and 4) leucocytes were treated with the NADPH analogue and sodium cyanoboro[^3H]hydride in the presence (lanes 2 and 4) or absence (lanes 1 and 3) of NADPH. The fluorography of the labelled proteins analysed by SDS/polyacrylamide-gel electrophoresis is shown. Molecular mass (left) is given in kDa.

component of the unstimulated neutrophils was 66 kDa, which is identical with that of the component in stimulated neutrophils. These results indicate that the NADPH-binding domain of the component in the unstimulated neutrophils exists, and does not change its molecular mass, during the activation process. Incubation of the membranes of red blood cells, platelets and lymphocytes with the NADPH analogue and sodium cyanoboro[^3H]hydride did not show any radioactive band corresponding to about 66 kDa, but incubation of monocyte membranes did reveal a radioactive band in the 66 kDa region (Fig. 5). We propose that the NADPH-binding protein of 66 kDa is present only in phagocytes (neutrophils and monocytes).

Phosphorylation of the particulate fraction during the activation process

Because the involvement of protein kinase C in the activation of the NADPH oxidase has been suggested (Fujita *et al.*, 1984; Wolfson *et al.*, 1985), we examined the phosphorylation of the proteins in the particulate

Fig. 5. Fluorography of blood-cell membranes labelled with the NADPH analogue and sodium cyanoboro[^3H]hydride

The membranes of monocytes (lane 1), lymphocytes (lane 2), red blood cells (lane 3) and platelets (lane 4) were treated with the NADPH analogue and sodium cyanoboro[^3H]hydride. The fluorography of the labelled proteins analysed by SDS/polyacrylamide-gel electrophoresis is shown. Molecular mass (left) is shown in kDa.

Fig. 6. Autoradiography of the stimulated and unstimulated particulate oxidase fractions of neutrophils

Normal neutrophils loaded with radioactive P_i were exposed to PMA (stimulated, lane 2) or only dimethyl sulphoxide (unstimulated, lane 1) and the particulate oxidase fractions were prepared from the cells. The proteins were separated by SDS/polyacrylamide-gel electrophoresis, stained and autoradiographed. Molecular mass (left) is given in kDa.

oxidase fraction of the PMA-stimulated neutrophils. The particulate oxidase fractions were prepared from the stimulated and unstimulated neutrophils loaded with ^{32}P and analysed by SDS/polyacrylamide-gel electrophoresis. The gels were stained, dried, and subjected to autoradiography (Fig. 6). Several labelled proteins were found, but the 66 kDa protein did not alter in radioactivity after the exposure to PMA. These results indicate that the 66 kDa protein is not phosphorylated during the activation process.

DISCUSSION

The NADPH oxidase that produces superoxide anions has been postulated to be a system consisting of a flavoprotein and a cytochrome b_{558} , on the basis of studies of the neutrophils in CGD patients. Most CGD patients have either the X-chromosome-linked cytochrome b -negative form or the autosomal cytochrome b -positive form of the disease, though other variations exist (Weening *et al.*, 1985). The patients in the present study belong to either group. FAD in the oxidase fraction was greatly decreased in all cases, a finding in agreement with previous reports (Cross *et al.*, 1982b;

Borregaard & Tauber, 1984; Gabig & Lefker, 1984; Weening *et al.*, 1985). FAD is a cofactor for the dehydrogenase portion of many oxidases, and the decrease of FAD in the oxidase fraction in CGD patients suggest a possible defect of the NADPH-dehydrogenating flavoprotein. The presence of the NADPH-binding component with essentially the same molecular mass as that of normal neutrophils, however, was demonstrated in CGD patients by affinity labelling with the NADPH analogue. The decrease in FAD may not necessarily be related to a deficiency of the dehydrogenase component. It is possible that the flavoprotein is altered in CGD without its NADPH-binding domain and its mobility in SDS/polyacrylamide-gel electrophoresis being affected. Some groups of investigators have purified the cytochrome b_{558} . The molecular mass of the purified cytochrome b_{558} is 127 kDa (Lutter *et al.*, 1985) or 68–78 kDa (Harper *et al.*, 1984). The NADPH-binding domain is not likely to be located in the cytochrome b_{558} protein itself, not only because of the difference in the molecular mass between them, but also because of the presence of the domain in the X-chromosome-linked cytochrome b -negative patients.

Because red blood cells, platelets and lymphocytes had

no NADPH-binding component of 66 kDa, but neutrophils and monocytes, which have cytochrome b_{558} , did, the 66 kDa NADPH-binding protein would seem to be specific to phagocytes. Two groups of investigators have purified a superoxide-generating oxidase with a molecular mass of 65 kDa (Markert *et al.*, 1985; Doussiere & Vignais, 1985). A similarity in molecular mass suggests that their preparation corresponds to the component that is labelled with the NADPH analogue. If the superoxide-generating oxidase is a single molecule of 66 kDa, the alteration of the molecule in CGD may be restricted to a portion other than the NADPH-binding domain, so that the oxidase can accept electrons from NADPH but fails to donate them to oxygen.

Although the NADPH oxidase is dormant in unstimulated neutrophils, appropriate stimuli change the oxidase from an inactive form to an active form that produces superoxide anions. The mechanism(s) by which the oxidase is activated is a highly specific process. Recently, activation of the oxidase *in vitro* has been accomplished with arachidonic acid (Bromberg & Pick, 1984) and SDS (Bromberg & Pick, 1985), but the mechanism by which an inactive form of the oxidase changes to an active form has not yet been elucidated. In our study the NADPH-binding component is not phosphorylated during the activation process, although the phosphorylation of the NADPH oxidase by protein kinase C has been suggested (Cox *et al.*, 1985; Papini *et al.*, 1985; Segal *et al.*, 1985).

Gabig & Lefker (1985) have proposed that the activation of the oxidase results in the coupling between ubiquinone-10 and cytochrome b_{558} , because ubiquinone-10 was reduced by NADPH in unstimulated neutrophils. The participation of ubiquinone-10, however, is still controversial (Cross *et al.*, 1983; Lutter *et al.*, 1984). The study involving unstimulated neutrophils and the NADPH analogue indicates that the NADPH-binding domain labelled with the analogue has the same molecular mass as that of normal stimulated neutrophils. These results indicate the component does not alter its molecular mass during the activation process and that the activation appears to induce coupling between the NADPH-binding component and the succeeding component which accepts electrons from it (probably cytochrome b_{558}).

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