Phosphorylation of the subunits of cytochrome b_{-245} upon triggering of the respiratory burst of human neutrophils and macrophages

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Cytochrome b_{-245}, the only clearly identified component of the microbicidal oxidase system of phagocytes, is a heterodimer consisting of a 23 kDa (α) and a 76–92 kDa (β) subunit. This study was conducted to examine whether, in common with a number of proteins, the subunits of the cytochrome were phosphorylated upon activation of the oxidase. Both subunits were phosphorylated after activation of neutrophils or macrophages with phorbol myristate acetate or a phagocytic stimulus, although the time course of this process did not parallel that of the oxidase. Phosphorylation of these proteins was normal in cells from two patients with autosomal recessive chronic granulomatous disease, in whom phosphorylation of a 47 kDa protein is defective.

INTRODUCTION

The microbicidal oxidase is important for the efficient killing of ingested microbes by phagocytic cells. It consists of an electron-transport chain containing cytochrome b_{-245} and probably also a flavoprotein (Cross et al., 1982b), which is activated by interaction with a stimulus. The cytochrome is a heterodimer composed of 23 kDa (α) and 76–92 kDa (β) subunits (Segal, 1987).

Chronic granulomatous disease (CGD) is a syndrome characterized by a severe predisposition to infection consequent upon a complete absence of oxidase activity from the phagocytic cells of these patients (Holmes et al., 1967). The cytochrome is missing from the cells of the subgroup with an X-chromosome-linked pattern of inheritance (Segal et al., 1983; Segal, 1987), and it has been demonstrated that the gene that is most commonly abnormal in this condition codes for the β subunit (Royer-Pokora et al., 1986; Teahan et al., 1987). In patients with an autosomal recessive pattern of inheritance the cytochrome is present, but fails to receive electrons when the cell is stimulated (Segal & Jones, 1980), indicating abnormal activation or the absence of a proximal electron donor. Activation of neutrophils by a variety of stimuli is associated with the phosphorylation of a number of proteins (Schneider et al., 1981; Andrews & Babior, 1983). Autosomal recessive CGD is associated with a selective failure to phosphorylate a 47 kDa protein (Segal et al., 1985; Heyworth & Segal, 1986), the functional significance of which remains to be established.

This study was conducted to determine whether one or both subunits of the cytochrome were phosphorylated in association with activation of the oxidase, and if so, whether this process was also abnormal in autosomal recessive CGD.

EXPERIMENTAL

Preparation and culture of cells

Neutrophils were purified from 50–100 ml of freshly drawn blood containing heparin (5 i.u./ml), as previously described (Segal et al., 1980). The cell pellet was washed with 0.9% (w/v) NaCl and then with a phosphate-free medium (PFM) (150 mM-NaCl/5 mM-KCl/10 mM-glucose/0.25 mM-CaCl₂/1 mM-MgCl₂/6 mM-Hepes, pH 7.4), by resuspension and centrifugation at 200 g for 5 min, at room temperature. Neutrophils were finally resuspended in PFM.

Monocytes were obtained as previously described (Garcia et al., 1986) and cultured in 80 cm² tissue-culture flasks (Nunc; Gibco, Paisley, Renfrewshire, Scotland, U.K.). Culture medium was RPMI containing 10% (v/v) fetal-calf serum (Gibco) and 10% (v/v) conditioned medium from phytohaemagglutinin-stimulated lymphocyte cultures. The adherent moncyte-derived macrophages were maintained in a CO_2/air (1 : 19) incubator at 37 °C for 3 days and then washed with PFM.

Patients with CGD

One subject with classical X-chromosome-linked CGD [patient no. 4 in Segal et al. (1983)] and two patients with autosomal recessive CGD were studied. The autosomal recessive patients were a male and female aged 30 and 17 years respectively. Their neutrophils demonstrated markedly depressed superoxide generation after stimulation with PMA. Their cells contained normal concentrations of cytochrome b_{-245} (110 ± 20 pmol/10^7 cells) but, in common with other subjects with this condition, failed to phosphorylate the 47 kDa protein (Segal et al., 1985; Heyworth & Segal, 1986).

Abbreviations used: PMNs, polymorphonuclear leucocytes; CGD, chronic granulomatous disease; PFM, phosphate-free medium; PMA, phorbol myristate acetate; iPr₂P-F, di-isopropyl fluorophosphate; PAGE, polyacrylamide-gel electrophoresis.

Labelling with [^{32}P]P$_i$ and stimulation of cells

Neutrophil suspensions [(3–10) × 10^7/ml] or adherent macrophages (8 × 10^6 cells/80 cm^2 flask) were incubated at 37 °C for 60 min in PFM containing [^{32}P]P$_i$ (20–70 MBq/ml; Amersham International, Amersham, Bucks., U.K.).

Stimulation of neutrophils was performed by adding PMA (200 ng/ml) or IgG-coated latex particles (Segal *et al.*, 1980) in a particle/cell ratio of 30:1, in the thermostatically controlled (37 °C) chamber of an oxygen electrode, with continuous stirring. Samples were removed at different times, pipetted into ice-cold PFM containing 50 mM-NaF and washed with the same medium. iPr$_2$P-F (1 mM) was added and the cells stored at −20 °C until extraction and purification of the cytochrome *b*.

Stimulation of macrophages was carried out by the addition of PMA (500 ng/ml). After the times indicated, the flasks were cooled down on ice and EDTA was added to a final concentration of 2 mM. The cells were mechanically detached, washed with PFM, iPr$_2$P-F (1 mM) was added, and they were stored at −20 °C until extraction and purification.

Measurement of oxygen metabolism

Oxygen consumption by neutrophils was determined in the thermostatically controlled (37 °C) chamber of an oxygen electrode (Rank Brothers, Bottisham, Cambridge, U.K.). Superoxide-anion generation by macrophages was determined at 37 °C as described previously (Garcia *et al.*, 1986), in parallel with the phosphorylation reaction.

Purification of cytochrome *b*$_{-245}$

The cytochrome was partially purified from frozen phosphorylated cell pellets by differential detergent extraction (first sodium cholate, then Triton N-101) and ion-exchange chromatography (Harper *et al.*, 1984; Segal, 1987), with the addition of NaF (10 mM) to all solutions. In the case of macrophages, 1 ml of non-radioactive Triton N-101 extract from normal PMNs containing 1.7 nmol of cytochrome haem was added as carrier before the ion-exchange-chromotography purification step.

Electrophoresis and autoradiography

An equal volume of electrophoresis sample buffer [4 % (w/v) SDS/10 % (v/v) 2-mercaptoethanol/20 % (v/v) glycerol/0.1 % (w/v) Bromothymol Blue/100 mM-Tris/HCl, pH 6.8] was added to portions of the fractions eluted from heparin–agarose columns corresponding to the peak of cytochrome haem, quantified as previously described (Cross *et al.*, 1982a). After 3–5 h at room temperature, the treated samples were electrophoresed on SDS/12 %-(w/v)-polyacrylamide slab gels (Laemmli, 1970). The gels were stained with Coomassie Blue R, destained, dried under vacuum and autoradiographed using pre-flashed Hyperfilm-MP (Amersham International). Autoradiographs were scanned with a Joyce–Loebl Chromoscan 3 instrument.

RESULTS

Both the alpha and beta subunits of the cytochrome *b*$_{-245}$ became phosphorylated after activation of the oxidase system of neutrophils with PMA (Fig. 1). The
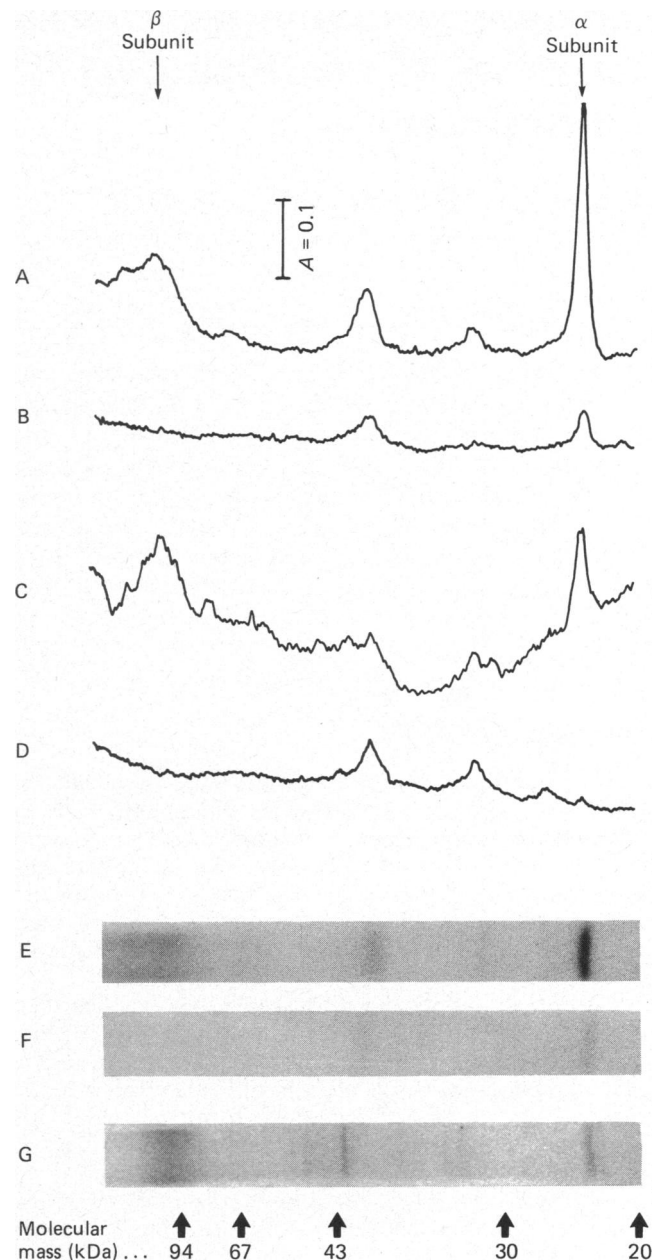

Fig. 1. Phosphorylation of subunits of cytochrome *b*$_{-245}$ from PMNs stimulated with PMA

PMNs were isolated from a normal subject and from patients with autosomal recessive or X-chromosome-linked CGD, labelled with [^{32}P]P$_i$ and stimulated or not with PMA, as described in the Experimental section. Cytochrome *b*$_{-245}$ was subsequently purified and subjected to PAGE (129 pmol of cytochrome haem/lane). The gels were dried, autoradiographed, and the films scanned. Scans and autoradiographs are shown from: A and E, stimulated normal PMNs; B and F, unstimulated normal PMNs; C, stimulated autosomal recessive CGD PMNs; D, stimulated X-chromosome-linked CGD PMNs. G shows the Coomassie Blue-stained pattern of protein separation on SDS/PAGE after a typical purification of cytochrome *b*$_{-245}$.

molecular mass of the β subunit was observed to be 93–113 kDa on the 12 % gels used, i.e. above the range of
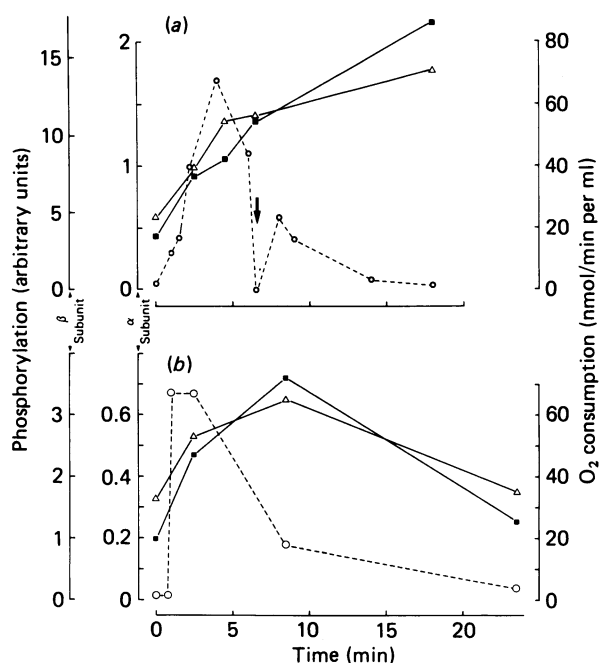
Fig. 2. Time course of oxygen consumption and phosphorylation of cytochrome subunits after stimulation with PMA (a) or phagocytosis of IgG-coated latex particles (b) by normal PMNs

(a) Normal PMNs were labelled with $[^{32}P]P_i$ and stimulated with PMA for the times indicated. The cytochrome b was purified and subjected to SDS/PAGE. The autoradiographs of the gels were scanned, and the radioactivity incorporated into the α ($\triangle$) and β ($\blacksquare$) subunits of the cytochrome was quantified by measuring the area under the respective peaks (arbitrary units). The rate of oxygen consumption ($\bigcirc$), determined simultaneously, is also shown. Re-oxygenation of the reaction mixture is indicated by an arrow. (b) Cytochrome b was purified from normal ^{32}P-labelled PMNs after incubation with opsonized latex particles for the times indicated. Phosphorylation of the α ($\triangle$) and β ($\blacksquare$) subunits of the cytochrome was evaluated as described above and compared with the rate of oxygen consumption ($\bigcirc$).

Fig. 3. Time course of phosphorylation of cytochrome subunits ($\triangle$, α; $\blacksquare$, β) and superoxide generation ($\bigcirc$) by macrophages after stimulation with PMA

Monocyte-derived macrophages after 3 days in culture were labelled with $[^{32}P]P_i$, stimulated for the times indicated, harvested, and the cytochrome purified. Phosphorylation of the cytochrome b subunits was determined as described in Fig. 2, and superoxide formation was determined as described in the Experimental section.

76–92 kDa reported previously for 10% gels (Segal, 1987). This anomalous variability in the mobility of the large subunit of the cytochrome in relation to the mobility of molecular-mass markers, as a function of the polyacrylamide concentration of the gels, is in agreement with the findings of Parkos et al. (1987).

The identity of the subunits was confirmed by their absence from cells of a patient with X-chromosome-linked CGD. Two other proteins, of 39 kDa and 32 kDa, which co-purified with the cytochrome, were phosphorylated in stimulated neutrophils from both normal individuals and the CGD patients. PMA-induced phosphorylation of both subunits of the cytochrome was normal in two CGD patients with the autosomal recessive pattern of inheritance, the results from one of which is shown in Fig. 1. The time course of this phosphorylation did not parallel that of PMA-stimulated oxygen consumption (Fig. 2a). Phosphorylation was still increasing when oxygen consumption had greatly slowed. Phosphorylation of both subunits was also observed after stimulation with phagocytic stimuli (Fig. 2b), where it
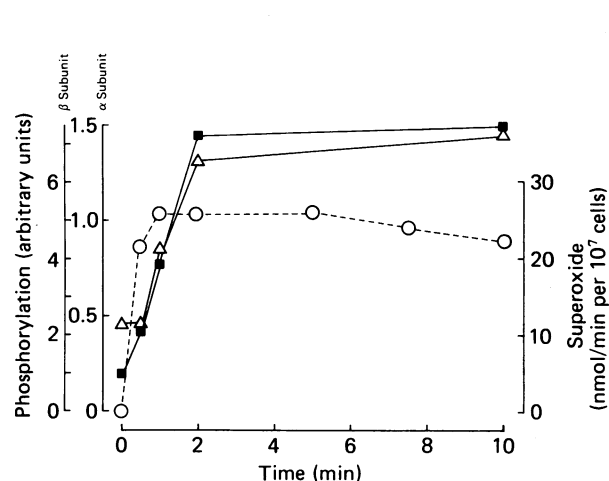
also demonstrated a different time course from that of the rate of oxygen consumption.

Phosphorylation of both cytochrome subunits was observed after stimulation of monocyte-derived macrophages with PMA, with a similar time course to that seen in peripheral-blood neutrophils.

DISCUSSION

The electron-transport chain that comprises the microbicidal oxidase of phagocytic cells is inactive until these cells are triggered by a phagocytic particle in vivo or a variety of artificial stimuli in vitro. This stimulation is associated with the phosphorylation of a number of proteins, including both subunits of cytochrome b_{-245}, as shown here.

The extensive phosphorylation of other cellular components made it necessary to partially purify the cytochrome in order to visualize its two subunits, the identity of which was confirmed by their absence from cells of patients with X-chromosome-linked CGD.

The normal pattern of phosphorylation of these proteins in patients with autosomal recessive CGD indicates that the failure of cells from these subjects to phosphorylate a 47 kDa protein (Segal et al., 1985; Heyworth & Segal, 1986) is a selective defect. Phosphorylation of the 47 kDa protein by normal and X-chromosome-linked CGD PMNs, but not by autosomal recessive PMNs, was observed in the experiments reported here. The 47 kDa phosphoprotein is found in the cholate extracts from stimulated cells, before solubilization of the cytochrome b with Triton N-101 from the cholate-extracted material (results not shown).

The phosphorylation of the cytochrome is likely to play a part in the regulation of electron transport down the chain. It could induce a conformational change in the cytochrome that facilitates electron transfer or integration with other components of the chain, or it could be responsible for the termination of electron flow.

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