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Phosphorylation of NADPH-cytochrome *c* reductase in guinea pig peritoneal macrophages stimulated with phorbol myristate acetate

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The plasma membrane-bound NADPH-cytochrome *c* reductase of guinea pig macrophages (Mϕ) was found to be phosphorylated when [^{32}P]phosphate-labeled cells were stimulated with 12-phorbol 13-myristate acetate (PMA). The time course of phosphorylation was parallel to that of O_2^--generating activity elicited. These results suggest that the reductase participates as a flavoprotein in activation of the respiratory burst NADPH oxidase, when phosphorylated.

NADPH oxidase; NADPH-cytochrome *c* reductase; Phosphorylation; (Macrophage)

1. INTRODUCTION

NADPH-cytochrome *c* reductase exists in the plasma membrane of polymorphonuclear leukocytes [1–5]. Sakane et al. [6] found that the highly purified reductase generated O_2^- when coupled with cytochrome *b*-559 in the presence of phosphatidylcholine [6]. These properties of the reductase suggest that it may be a putative flavoprotein of the respiratory burst NADPH oxidase, and also that the association of the reductase with cytochrome *b*-559 which was induced by appropriate stimuli may result in formation of the NADPH oxidase.

Many studies demonstrated a possible role of a certain protein kinase in the triggering mechanism for the respiratory burst, and the phosphorylation of a number of the cellular proteins on stimulation of monocytes and neutrophils [7–17]. We found that the membrane-bound NADPH-cytochrome *c* reductase of guinea pig macrophages (Mϕ) was phosphorylated on stimulation of the cells with PMA. The phosphorylation of the reductase is reported in this paper, with discussion of its possible relationship with the respiratory burst.

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Abbreviations: BSA, bovine serum albumin; DFP, diisopropyl fluorophosphate; HIFCS, heat-inactivated fetal calf serum; P-450 reductase, liver NADPH-cytochrome P-450 reductase; Mϕ, macrophages; MEM, minimum essential medium; PBS, 10 mM phosphate-buffered saline, pH 7.4; PMA, phorbol 12-myristate 13-acetate; PS, phosphatidylserine; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; SOD, superoxide dismutase

2. MATERIALS AND METHODS

2.1. *Materials*

Guinea pig liver NADPH-cytochrome P-450 reductase (P-450 reductase), highly purified [3], was coupled with Affi-gel 15 (Bio-Rad Labs). The gel thus prepared was used for specific purification of rabbit antibody to P-450 reductase; the antibody bound on the gel was eluted with 0.23 M glycine-HCl buffer, pH 2.6. Guinea pig anti-ovalbumin antibody was also specifically purified as described in [18]. The F(ab')$_2$s of these antibodies were prepared by pepsin digestion [18,19] and conjugated to CNBr-activated Sepharose CL-4B (Pharmacia) [20]. Protein kinase C was partially purified from guinea pig brain by the first step of the procedure described by Inagaki et al. [21]. The specific activity of the enzyme preparation was 4 U/mg protein when protein kinase C activity was assayed according to [21]. One unit of the enzyme was defined as in [22].

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2.2. *Isolation of the NADPH-cytochrome c reductase labeled with [^{35}S]Met and phosphorylated with [^{32}P]phosphate*

Guinea pig Mϕ were isolated from peritoneal cells of animals injected with thioglycollate 4 days before harvest [23]. The cells were incubated with minimum essential medium (MEM) supplemented with 10% heat-inactivated fetal calf serum (HIFCS) in 10-cm Falcon plastic dishes. After incubation for 2 h at 37°C in a CO_2-air incubator, the adherent cell monolayers were used as Mϕ for experiments. Mϕ NADPH-cytochrome *c* reductase was labeled with [^{35}S]Met (ICN Biochemicals) by incubating the cells with Met-free RPMI 1640 supplemented with 100 μCi [^{35}S]Met (spec. act. >800 Ci/mmol) and 10% HIFCS previously dialyzed against the same medium, for 4 h at 37°C in a CO_2/air incubator [24]. The labeled cells were solubilized with 10 mM phosphate-buffered saline, pH 7.4 (PBS), containing 1% Nonidet P-40 (Nakarai), 1% BSA (Sigma) and 2 mM DFP (Kishida), and the ^{35}S-labeled reductase was immunoprecipitated with 25 μl anti-P-450 reductase F(ab′)$_2$-Sepharose after preadsorption with 40 μl anti-ovalbumin F(ab′)$_2$-Sepharose, since the reductase is cross-reactive with anti-P450 reductase antibody [3]. The reductase thus isolated was analyzed by SDS-PAGE [25] followed by fluorography [26].

To determine the in vivo phosphorylation of the reductase, Mϕ monolayers in Falcon dishes (2.5×10^5 cells/cm^2) were incubated with phosphate-free MEM supplemented with 180 μCi [^{32}P]phosphate (spec. act. ~285 Ci/mg P; ICN) and 5% HIFCS previously dialyzed against the same medium, for 20 h at 37°C in a CO_2/air incubator. Monolayers were then stimulated with 0.3 μg/ml of PMA (Sigma) dissolved in the same medium for up to 1 h at 37°C. After termination of the reaction by removal of the reaction medium, the cells were solubilized with extraction buffer [1% Nonidet P-40, 25 mM Hepes, 10 mM ATP, 30 mM NaF, 2 mM DFP, 10 μg/ml aprotinin (Sigma), 1 μM leupeptin (Sigma), 2 mM EDTA, 1% BSA, 100 mM NaCl, 5 mM $Na_2P_4O_7$, pH 7.4]. The reductase was isolated from the cell lysate, and phosphorylation of the enzyme was determined by SDS-PAGE followed by autoradiography, as in the case of the ^{35}S-labeled reductase.

2.3. *Determination of O_2^- generation by Mϕ*

The Mϕ monolayers prepared in a Falcon 24-well plate (5×10^5 cells/well) as in the case of in vivo phosphorylation of the reductase were stimulated with 0.3 μg/ml of PMA dissolved in Hanks' balanced salt solution containing 80 μM cytochrome *c* (Sigma) in the presence or absence of 50 μg/ml superoxide dismutase (SOD, Sigma). The amounts of O_2^- generated were estimated by measuring the SOD-inhibitable reduction of cytochrome *c* [27].

3. RESULTS AND DISCUSSION

[^{35}S]Met-labeled Mϕ were solubilized and the NADPH-cytochrome *c* reductase was isolated by immunoprecipitation, as described in section 2. The fluorograph obtained showed an 80 kDa band corresponding to the molecular mass of the reductase [4,5], and no other cellular protein was detectable, indicating that the method used is useful for selective and rapid isolation of the reductase (not shown). Whether phosphorylation of the reductase occurs upon stimulation of Mϕ with PMA was examined by the use of the same method. The reductase was phosphorylated time-dependently upon stimulation; 15 min stimulation resulted in maximum phosphorylation, and thereafter, dephosphorylation proceeded (fig.1B). A control experiment using anti-ovalbumin F(ab′)$_2$-Sepharose gave no band. The stimulation with PMA does not seem to vary the cellular content of the reductase, as judged from protein staining of the gel (fig.1A).

When the reductase, which was isolated from unstimulated Mϕ by immunoprecipitation, was incubated with partially purified kinase C, it was phosphorylated with [γ-^{32}P]ATP (ICN) in the presence of Ca^{2+} and phosphatidylserine (PS, Sigma) (fig.2B, lane 3). This in vitro phosphorylation was enhanced with 1-oleoyl-2-acetylglycerol (Sigma) (fig.2B, lane 4), and completely inhibited with 30 μM H-7 (Seikagaku Kogyo) (fig.2B, lane 5). These results indicate that the reductase is able to be a substrate for protein kinase C.

When the time course of in vivo phosphorylation of the reductase induced by PMA was

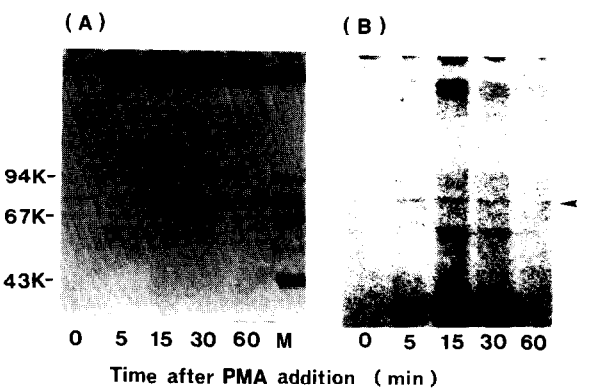

Fig.1. In vivo phosphorylation of the reductase. The reductase was immunoprecipitated from the lysate of [^{32}P]phosphate-labeled Mϕ (1×10^7 cells) that had been stimulated with 0.3 μg/ml of PMA for the periods indicated. After elution from the precipitate by boiling with SDS-PAGE sample buffer containing 5% 2-mercaptoethanol [24], the reductase was electrophoresed on 7.5% polyacrylamide gel. Gel stained with Coomassie blue and autoradiograph of the same gel are shown in (A) and (B), respectively. Arrowhead on the right indicates position of the reductase. Bars (left) indicate positions of protein standards (M); phosphorylase *b* (94 kDa), BSA (67 kDa) and ovalbumin (43 kDa).

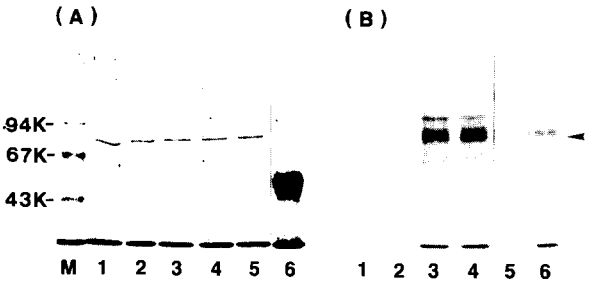

Fig.2. In vitro phosphorylation of the immunoprecipitated reductase by protein kinase C. The immunoprecipitated reductase from the lysate of unstimulated Mϕ (1×10^7 cells eq.) was phosphorylated by 30 μg partially purified protein kinase C in 0.2 ml of 25 mM Tris-HCl (pH 7.4) containing 5 mM $MgCl_2$, 10 μM [γ-^{32}P]ATP (5 μCi), and other additions described below, for 5 min at 30°C. Additions to reaction mixtures – lanes: (1) no further additions; (2–6) 1 mM Ca^{2+}; (3–6) 4 μg PS; (4–6) 1 μg oleoylacetylglycerol; (5) 30 μM H-7. Lane 6 shows a control experiment using anti-ovalbumin F(ab')$_2$-Sepharose in place of anti-P-450 reductase F(ab')$_2$-Sepharose. Phosphorylation of the reductase was analyzed as described in fig.1. Gel stained with Coomassie blue and autoradiograph of the same gel shown in (A) and (B), respectively. Arrowhead on the right indicates position of the reductase. Bars (left) indicate positions of standards (M).

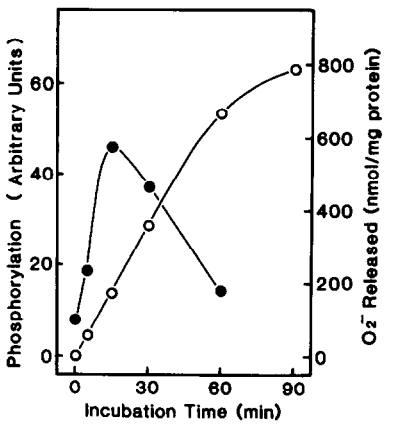

Fig.3. Time courses of O_2^- generation by Mϕ monolayers and in vivo phosphorylation of the reductase. Mϕ (5×10^5 cells) were stimulated with 0.3 μg/ml of PMA for the periods indicated. The amounts of O_2^- generated were determined by measuring the SOD-inhibitable cytochrome *c* reduction (○). [^{32}P]Phosphate-labeled Mϕ (1×10^7 cells) were similarly stimulated with PMA, and ^{32}P incorporation into the reductase (●) was quantified by measuring the darkness of the 80 kDa band on the autoradiograph by densitometry. Level of ^{32}P incorporation is represented in arbitrary units. Each point represents the mean of duplicate determinations (SD < 10% mean). Protein was determined by the method of Lowry et al. [29].

estimated by scanning of the 80 kDa band of the autoradiograph, it was found to be parallel with that of eliciting of O_2^--generating activity (fig.3). In addition, the dephosphorylation observed after 15 min seems to accompany the decrease in O_2^- generation. Thus, it suggests that the amount of phosphorylated reductase determines the NADPH oxidase activity. A preliminary experiment showed that at 15 min where the amount of O_2^- generated was maximum, several percent of the reductase was phosphorylated (to be published).

The results so far obtained suggest that the reductase is a putative flavoprotein of the respiratory burst NADPH oxidase, and constitutes the NADPH oxidase with cytochrome *b*-559, when phosphorylated by a certain protein kinase, probably kinase C which is activated in response to many different extracellular signals [28]. If this is the case, phosphorylation may modify the reductase molecule, resulting in facilitation of the formation of the NADPH oxidase complex with cytochrome *b*-559 in the plasma membrane. To clarify the relationship of phosphorylation of the reductase with the respiratory burst, further investigation is now in progress.

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