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HYPERPHOSPHORYLATED p47-PHOX LOST THE ABILITY TO ACTIVATE NADPH OXIDASE IN GUINEA PIG NEUTROPHILS

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p47-phox is one of the cytosolic activation factors of NADPH oxidase in neutrophils and known to translocate to plasma membranes and function by protein kinase C-phosphorylation. In cytosol fraction, prepared from calyculin A-treated neutrophils, the activity of cytosolic factor to activate NADPH oxidase was more reduced than that from PMA-treated cells. But, p47-phox did not translocate to the membranes, even if p47-phox was hyperphosphorylated in the calyculin A-treated neutrophils. Such hyperphosphorylated p47-phox seemed to lose the activity to constitute NADPH oxidase complex.

Superoxide anion(O_2^-) production in neutrophils is stimulated during phagocytosis and by treatment with a variety of stimuli, such as certain chemoattractants, activators of protein kinase C[1]. Active oxygen metabolites, such as superoxide anion, hydrogen peroxide and hydroxyl radicals, are contributory towards the microbicidal action. It is generally accepted the O_2^- production is catalyzed by an activated form of the membrane-associated NADPH oxidase which is dormant in the resting state. It is also known that cytochrome b_{558}, p47-phox, p67-phox and rac are essential constituents of the activated form[2]. We previously described that phosphorylation of the p47-phox by protein kinase C is essential for activation of O_2^- producing complexes, so called NADPH oxidase in intact cells[3,4].

On the other hand, calyculin A, which is one of the inhibitors of protein phosphatase, induced apparent phosphorylation of p47-phox without causing O_2^- production[5].

In this report, we describe that the hyperphosphorylated p47-phox lose the ability to translocate to the membrane and to activate NADPH oxidase.

Materials and Methods

Materials

Anti-mouse p47-phox antibody was generated to 24 COOH-terminal peptides, PRPSSDLILHRCTESTKRKLTSAV against it. All other chemicals were reagent grade from standard commercial sources.

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Subcellular fractionation of guinea pig neutrophils

Neutrophils were obtained from the peritoneal cavities of female guinea pigs of the Hartley strain as reported previously[6]. Neutrophils (1 x 10^7 cells/ml) were preincubated at 37 °C for 5 min. After preincubation, 1 nM PMA or 100 nM calyculin A was added, and the mixture was incubated at 37 °C for 10 min. The reaction was stopped by chilling, and the mixture was centrifuged at 4 °C and 120g for 5 min to sediment intact neutrophils. The cytosol and membrane fraction was preparated as reported previously[5].

Measurements of O_2^- production in neutrophils

O_2^- production by neutrophils was measured on the basis of superoxide dismutase (SOD)-inhibitable reduction of ferricytochrome c (final concentration, 100 μM)[7].

Measurement of NADPH oxidase activity in membrane fraction and the activity of cytosolic activation factor(s) for NADPH oxidase activation

NADPH oxidase activity in the membrane fraction and the activity of cytosolic activation factor(s) in the cytosol fraction was measured as reported previously[8].

Preparation of phosphorylated cytosol

Protein kinase C was partially purified from guinea pig brain cytosol. The protein kinase C was added in the presence or absence of 1 μM staurosporine to the cytosol prepared from resting neutrophils, and the mixture was incubated for 5 min at 30 °C in the presence of 0.5 mM $CaCl_2$, 10 mM $MgCl_2$, 100 μg/ml phosphatidylserine, 0.8 μg/ml 1,2-diolein, and 25 μM ATP. The reaction was stopped by addition of 3.5 mM EGTA and chilling in an ice water bath.

Immunoblotting

The membrane fraction was subjected to SDS-PAGE prior to electrophoretic transfer to nitrocellulose blotting membrane (Schleicher & Schuell, Germany), according to a general methods[9].

Immunoprecipitation

Neutrophils were labeled with carrier-free ^{32}Pi as described previously[6]. The labeled neutrophils (10^7 cells/ml) were preincubated at 37 °C for 5 min. After the preincubation, PMA or calyculin A were added, and the mixture was incubated at 37 °C for 10 min. The cell pellet was lysed with NP-40 lysis buffer (150 mM NaCl, 1.0% NP-40, 50 mM Tris (pH 8.0) containing 200 μM PMSF, 2 μM leupeptin, 2 μM pepstatin, 100 nM calyculin A, 1 μM staurosporine and 100 μM orthovanadate). Immunoprecipitataion was performed according to a general methods[9].

Results

Activity of NADPH oxidase and localization of cytosolic factor

Neutrophils stimulated with 1 nM PMA released O_2^- at 15.0 nmol/5 min/10^6 cells, whereas calyculin A, a protein phosphatase inhibitor, hardly induced O_2^-(3.5 nmol/5 min/10^6 cells). PMA- and calyculin A-treated neutrophils were disrupted and membrane and cytosol fraction were prepared. Then we measured the activity of NADPH oxidase in the membrane fraction and that of cytosolic factor for NADPH oxidase activation remaining in the cytosol (Fig. 1). The NADPH oxidase activity of the membranes and the activity of cytosolic factor, which were prepared from PMA stimulated neutrophils, were 19.6±1.3 nmol/min/mg membrane protein, and 75.9±2.4

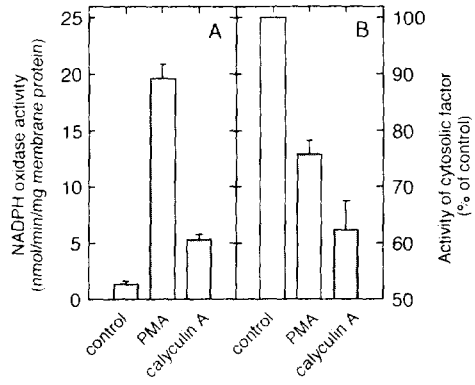

Fig. 1. Activity of the NADPH oxidase in membranes and localization of cytosolic factors. Neutrophils were treated with 1 nM PMA or 100 nM calyculin A for 10 min, then the membrane and cytosol fraction were prepared. A) The NADPH oxidase activity in the membrane fraction, B) the activity of the cytosolic factor for NADPH oxidase activation in the cytosol fraction were measured. The values are the mean±SE of at least three experiments.

% of control, respectively. On the other hand, the NADPH oxidase activity and the activity of cytosolic factor, which were prepared from calyculin A-treated neutrophils, were 5.3±0.5 nmol/min/mg membrane protein and 62.3±5.2 % of control, respectively. The activity of cytosolic factor prepared from calyculin A-treated neutrophils was more decreased than that from PMA-treated neutrophils. These results may indicate that the cytosolic factor translocate to the membranes without activation of NADPH oxidase in the calyculin A-treated neutrophils.

Translocation of the p47-phox

The translocation of the p47-phox to membranes in PMA- and calyculin A-treated cells was measured using anti-mouse p47-phox antibody (Fig. 2). The p47-phox protein was detected in the membrane fraction prepared from PMA-treated cells, but not in the membranes prepared from calyculin A-treated cells. These findings suggest that the activity of cytosolic factor is reduced in calyculin A-treated cells, although the p47-phox does not translocate to the membranes.

Cell-free NADPH oxidase activation by phosphorylated p47-phox

Calyculin A is a well known protein phosphatase inhibitor, and induces phosphorylation of numerous proteins including p47-phox in intact cells[5]. It is plausible that when the p47-phox is once phosphorylated, the ability to activate oxidase will be diminished.

Calyculin A hyperphosphorylate numerous proteins, so it is difficult to detect phosphorylated p47-phox, even in 2-dimensional gel electropherograms. Thus, we

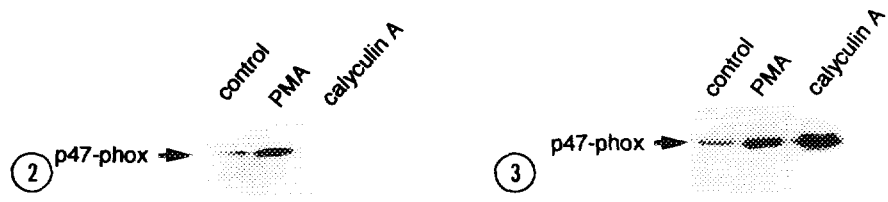

Fig. 2. Immunoblotting of the membrane fractions. The membranes prepared from PMA or calyculin A-treated cells were subsequently analyzed by SDS-PAGE and immunoblotting as described under Materials and Methods.

Fig. 3. Phosphorylation of p47-phox. ^{32}P-labeled neutrophils were treated with 1 nM PMA and 100 nM calyculin A for 10 min, then lysed with NP-40 lysis buffer. The lysate was immunoprecipitated by anti-mouse p47-phox as described in Materials and Methods.

performed immunoprecipitation to visualize p47-phox. Fig. 3 shows that calyculin A markedly induced phosphorylation of p47-phox. We further examined the above mentioned possibility using the cytosol artificially hyperphosphorylated by protein kinase C (see Material and Methods). The activity of the cytosolic factor to activate NADPH oxidase was lost in phosphorylated cytosol (Table 1). These results indicated that the p47-phox was inactivated by the hyperphosphorylation.

Effect of calyculin A on PMA-induced O_2^- production

Fig. 4 shows that effect of calyculin A on PMA-induced O_2^- production. Calyculin A enhanced O_2^- production in neutrophils in response to suboptimal amounts of PMA. At high concentration of PMA, however, calyculin A showed inhibition rather than enhancement. These results also suggest that hyperphosphorylated p47-phox lose the ability to activate NADPH oxidase in intact cells.

Table 1

The ability of phosphorylated cytosol to activate NADPH oxidase

Cytosol	NADPH oxidase activity (nmol/min/mg cytosol protein)
phosphorylated (non-treatment)	5.97 ± 1.00
non-phosphorylated (staurosporine)	14.2 ± 0.29

The activity of cytosolic factor of non-treated cytosol (phosphorylated) and staurosporine treated cytosol (non-phosphorylated) was measured as reported previously[6] . The values are the mean±SE of at least three experiments.

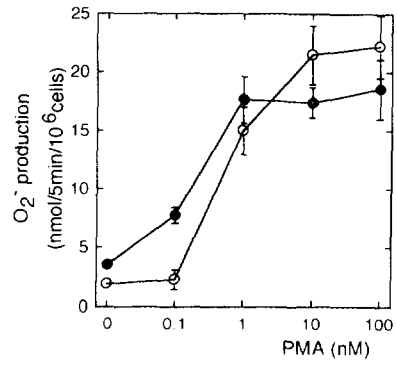

Fig. 4. Effect of calyculin A on PMA-induced O_2^- production. Neutrophils were preincubated for 5 min at 37 °C with (●) or without (○) 100 nM calyculin A. Then, indicated concentrations of PMA were added, and the mixture was further incubated at 37 °C for 5 min.

Discussion

We have reported that phosphorylation of p47-phox is increased in parallel with stimulation of O_2^- production in guinea pig neutrophils[1]. And the p47-phox is translocated concomitantly with its phosphorylation. Thus, such phosphorylation and translocation appear to be necessary for activation of NADPH oxidase[10].

In this study, we reported that calyculin A induced phosphorylation of p47-phox as PMA does. The translocation of the phosphorylated p47-phox, however, was not caused by calyculin A, in spite of reduction of the activity of cytosolic factor from the cytosol. In intact cells, several workers also reported that pretreatment of the cells with okadaic acid or calyculin A reduced the magnitude of respiratory burst in response to PMA(Fig. 4, [11-13]). The activity of p47-phox phosphorylated by protein kinase C was also reduced in cell-free system(Table 1). Thus, this inhibitory effect in intact cells of the protein phosphatase inhibitors was assumed to be due to an increase in phosphorylation of p47-phox.

Though, we and many researchers reported that phosphorylation of p47-phox is prerequisite for NADPH oxidase activation. Calyculin A induced hyperphosphorylation of p47-phox and it lost the ability to constitute activated NADPH oxidase. Since calyculin A is protein phosphatase inhibitor, some protein kinases would be activated apparently. It is reasonable to assume that in calyculin A treatment, NADPH oxidase activity is reduced by some conformational changes caused by various kinases other than protein kinase C. Consequently, hyperphosphorylated p47-phox inhibits oxidase activation. This inhibitory effect by the hyperphosphorylation may be one of the regulatory mechanisms against overproduction of superoxide. It is currently unknown what kinase causes hyperphosphorylation of p47-phox in calyculin A-treated cell. This study is now in progress.

Acknowledgments

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