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Effect of Arginine Modification of Cytosolic Component $p47^{phox}$ by Phenylglyoxal on the Activation of Respiratory Burst Oxidase in Human Neutrophils

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Abstract: The NADPH oxidase of phagocytes catalyzes the reduction of oxygen to O_2^- at the expense of NADPH. The enzyme is dormant in resting neutrophils and becomes activated on stimulation. During activation, $p47^{phox}$ (phagocyte oxidase factor), a cytosolic oxidase subunit, becomes extensively phosphorylated on a number of serines located between S303-S379. Although the biochemical role of phosphorylation is speculative, it has been suggested that phosphorylation could neutralize the strongly cationic C-terminal which may result in the change of conformation of $p47^{phox}$ and subsequent translocation of this protein and other cytosolic components to the membrane. In order to mimic the effect of phosphorylation in terms of neutralizing the positive charges, recombinant $p47^{phox}$ was treated with phenylglyoxal, which removes positive charges of arginine residues. Modification of recombinant $p47^{phox}$ resulted in the activation of oxidase in a cell-free translocation system as well as a conformational change in recombinant $p47^{phox}$, which may be responsible for the activation of the enzyme.
Key words: conformational change, NADPH oxidase, $p47^{phox}$, phenylglyoxal, phosphorylation.

NADPH oxidase (also called respiratory burst oxidase), a complex enzyme found in phagocytes and B-lymphocytes, catalyzes the reduction of oxygen to O_2^- using NADPH as the electron donor and plays an important role in host defense against microbial infection (Babior, 1978, 1992). The oxidase is dormant in resting neutrophils but acquires catalytic activity when cells are exposed to appropriate stimuli. The catalytic activity of the oxidase is located in the plasma membrane, but it is known that in resting cells the oxidase components are distributed between the plasma membrane and cytosol (Bromberg and Pick, 1984, 1985; Heyneman and Vercauteren, 1984; Curnutte, 1985; McPhail *et al.*, 1985). When the cells are activated, the cytosolic components migrate to the plasma membrane, where they associate with the membrane-bound components to assemble the catalytically active oxidase (Heyworth *et al.*, 1991; Park *et al.*, 1992).

Among the cytosolic oxidase components of NADPH oxidase, $p47^{phox}$ is a basic protein that becomes extensively phosphorylated when the oxidase is activated. The phosphorylation targets are a group of serines in the highly basic carboxyl-terminal quarter of the polypeptide (Hayakawa *et al.*, 1986; El Benna *et al.*, 1994a, 1994b). The relationship between the phosphorylation of $p47^{phox}$ and the initiation of O_2^- production by neutrophils exposed to activating agents such as phorbol-12-myristate 13-acetate suggested that phosphorylation of this protein is an essential step in the mechanism of the activation of the oxidase.

The phosphorylation of $p47^{phox}$ appears to play an important role in the translocation of the cytosolic components from the cytosol to the membrane (Heyworth *et al.*, 1991; Park and Ahn, 1995), but the biochemical basis for the transfer of $p47^{phox}$ to the membrane remains a matter of speculation. Recently, it has been proposed that in intact neutrophils, the phosphorylation of $p47^{phox}$ during activation is necessary to neutralize the strongly positive charge in the arginine-rich carboxyl-terminal domain of the protein (Nauseef *et al.*, 1993).

To verify the proposed role of phosphorylation as a charge neutralization, we investigated the possibility that modifications of purified recombinant $p47^{phox}$ by phenylglyoxal could reduce the strongly cationic charge, which may mimic the effect of charge neutralization

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by phosphorylation. It is known that the arginyl-directed reagent phenylglyoxal reacts with the guanidino group of the arginine residues under mild conditions (Takahashi, 1968).

Materials and Methods

Materials

Chemicals, enzymes, and molecular biology reagents were obtained from the following sources: pGEX-1λT vector, dextran, Ficoll-Hypaque from Pharmacia Uppsala, Sweden); bovine erythrocyte superoxide dismutase (SOD), NADPH, ferricytochrome c (type VI), GTPγS, glutathione (GSH), GSH-agarose, Nonidet P-40, and carboxymethyl (CM)-Sepharose, phenylmethylsulfonyl fluoride (PMSF), phenylglyoxal, and thrombin from Sigma (St. Louis, USA); N-[2-^{3}H]ethylmaleimide ([^{3}H]-NEM; specific activity, 50.08 Ci/mmol, 1.0 mCi/ml) from Dupont New England Nuclear (Boston, USA); and Bio-Rad protein assay kit, electrophoresis and immunoblotting reagents from Bio-Rad (Hercules, USA).

Preparation of neutrophil fractions

Neutrophil cytosol and membrane were prepared as described previously (Park and Babior, 1992). Briefly, neutrophils were obtained from normal subjects by dextran sedimentation and Ficoll-Hypaque fractionation of freshly drawn citrate-coagulated blood. The neutrophils were suspended at a concentration of 10^8 cells/ml in a modified relaxation buffer (100 mM KCl/3 mM NaCl/3.5 mM $MgCl_2$/10 mM PIPES buffer, pH 7.3), and plasma membrane and cytosol were prepared by nitrogen cavitation and centrifugation through Percoll by the method of Borregaard (Borregaard *et al.*, 1983). Both cytosol and membrane were divided into aliquots and stored at −70℃ until use.

Preparation of recombinant p47^{phox} protein

The pGEX-1λT plasmid construct containing full length p47^{phox} cDNA was transformed into *Escherichia coli* JM101 and grown at 37℃ overnight in 100 ml of YT/ampicillin broth. The overnight culture was diluted into 1 liter of fresh YT/ampicillin broth and grown an additional hour at 37℃ and then was amended with isopropyl β-D-thiogalactoside (0.1 mM, final concentration) and grown for an additional 3 h at 37℃. All cultures were agitated vigorously during growth. At the conclusion of incubation, the bacteria were recovered by centrifugation at 5,000×*g* for 10 min at 4℃. The pellet was suspended in 10 ml of ice-cold MTPBS (150 mM NaCl/16 mM Na_2HPO_4/4 mM NaH_2PO_4, pH 7.3) containing 0.2 mM PMSF/0.5 mM EDTA/0.5% (v/v) Nonidet P-40, and disrupted by sonication (three 10-sec pulses) at 0℃. The sonicate was clarified by centrifugation at 10,000×*g* for 20 min at 4℃. The fusion proteins were isolated from the clarified sonicate by purification over GSH-agarose as described previously (Park *et al.*, 1994). The fusion protein was cleaved by treatment with thrombin (10 U/ml) for 2 h at room temperature in elution buffer containing 150 mM NaCl and 2.5 mM $CaCl_2$. The protein mixture was further purified on a CM-Sepharose column (0.9×10 cm) equilibrated with 5 mM phosphate buffer, pH 7.0, containing 0.1 mM PMSF and eluted with a 40-ml gradient of 0~0.4 M NaCl in the same buffer. The concentration of proteins was determined with the Bio-Rad assay kit using bovine serum albumin as a standard.

Reaction with phenylglyoxal

Purified recombinant p47^{phox} (50 μg) or cytosol (5×10^7 cell eq) was incubated at room temperature for 3 h in a final volume of 1 ml in relaxation buffer containing 10 mM phenylglyoxal. After reaction, reaction mixtures were desalted on a PD-10 column using ice-cold relaxation buffer and concentrated by an Amicon Centriprep-10. Cell-free NADPH oxidase assay O_2 production by arachidonate-activated NADPH oxidase was measured as the superoxide dismutase-inhibitable reduction of ferricytochrome c by NADPH (Park *et al.*, 1992). For the measurements, assay mixtures contained 0.1 mM cytochrome c, 90 μM SDS, 0.16 mM NADPH, 1.6×10^6 cell eq of membrane, 1×10^6 cell eq of cytosol, recombinant p47^{phox}, and relaxation buffer to a final volume of 0.75 ml. All components except NADPH were mixed in the cuvette and equilibrated at 25℃ for 1 min. Reactions were then started by adding the NADPH in 25 μl relaxation buffer. Cytochrome c reduction was followed at 550 nm and corrected for background observed in the presence of 45 μg of superoxide dismutase.

In vitro translocation

In vitro translocation was carried out essentially as described previously (Park, 1996). Translocation mixtures contained 1.25×10^8 cell eq. membrane, 10 μM GTPγS, 5×10^7 cell eq. of cytosol, either 10 μg of phenylglyoxal-treated or untreated p47^{phox}, relaxation buffer, and 90 μM SDS in a total volume of 2 ml. After incubation for 5 min at 25℃, the translocation mixtures were carefully layered onto a discontinuous gradient composed of 1 ml of 15% (w/v) sucrose layered over 0.5 ml of 50% (w/v) sucrose, both in relaxation buffer, and centrifuged at 105,000×*g* for 30 min at 25℃. After centrifugation, a 0.75 ml portion was carefully removed from the bottom of the gradient for use as "pellet". The O_2 -forming activity of the pellet was determined as described in an earlier section, ex-

cept that the reaction mixture contained 115 μl of cytochrome c/$MgCl_2$ solution (final concentrations, 0.1 and 6.25 mM, respectively), 20 μl of pellet, relaxation buffer, and 25 μl of 5 mM NADPH in a total volume of 0.75 ml.

Electrophoresis and immunoblotting

Protein samples were subjected to SDS-PAGE on 8% polyacrylamide gels using the Laemmli buffer system (Laemmli, 1970). The separated proteins were electrophoretically transferred onto a nitrocellulose sheet (Towbin *et al.*, 1979), which was blocked with dried milk, then probed with mixed partially purified rabbit polyclonal antibody raised against synthetic peptide from $p47^{phox}$ and used at dilution of 1:10000; it was finally detected with a 1:2000 dilution of alkaline phosphatase-labeled goat anti-rabbit Ig antibody (Sigma, St. Louis, USA) using 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium as substrate (Bio-Rad, Richmond, USA).

Labeling of $p47^{phox}$

Preparations of 2 μg each of $p47^{phox}$ or phenylglyoxal-treated $p47^{phox}$ were treated at room temperature with 2 μl (2 μCi) of [^{3}H]-NEM in a total volume of 0.1 ml. After incubation for the indicated time, the sample was placed on ice for 5 min and the labeled protein precipitated by adding 50% (w/v) trichloroacetic acid to a final concentration of 10%. Transfer RNA (250 μg) was also added as a carrier. After a further 10 min on ice, the proteins were pelleted by centrifugation at 10,000×*g* for 10 min at 4℃. The precipitate was suspended in 100 μl of H_2O, mixed with water-compatible scintillation fluid (Ready Safe, Beckman) and assayed for radioactivity in a liquid scintillation counter.

Results and Discussion

The recombinant human $p47^{phox}$ was isolated by purification of a glutathione S-transferase (GST) fusion protein from an *E. coli* expression system and subsequently cleaved with thrombin. A variety of conditions for thrombin cleavage were tested; Adding 10 U/ml of thrombin and incubating for 2 h at room temperature provided the best result as judged by electrophoresis through an 8% SDS-PAGE gel and immunoblotting with antipeptide antibody against $p47^{phox}$. The principal band on the immunoblot of the $p47^{phox}$ fusion protein, which migrated at 69 kDa, was completely converted to 47 kDa by thrombin cleavage (Fig. 1). Although it is known that the best way to purify the protein of interest from the thrombin cleavage product

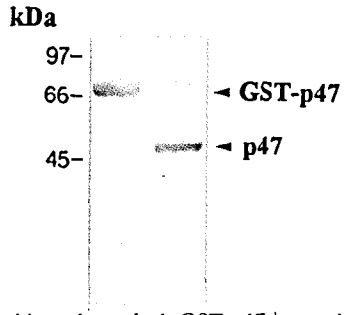

Fig. 1. Immunoblot of purified GST-$p47^{phox}$ and thrombin-cleaved GST-p47phox with anti-$p47^{phox}$ antibody. The samples are: lane 1, GST-$p47^{phox}$ (2 μg); lane 2, thrombin-cleaved GST-$p47^{phox}$ (2 μg). Immunoblotting was performed as described under Materials and Methods.

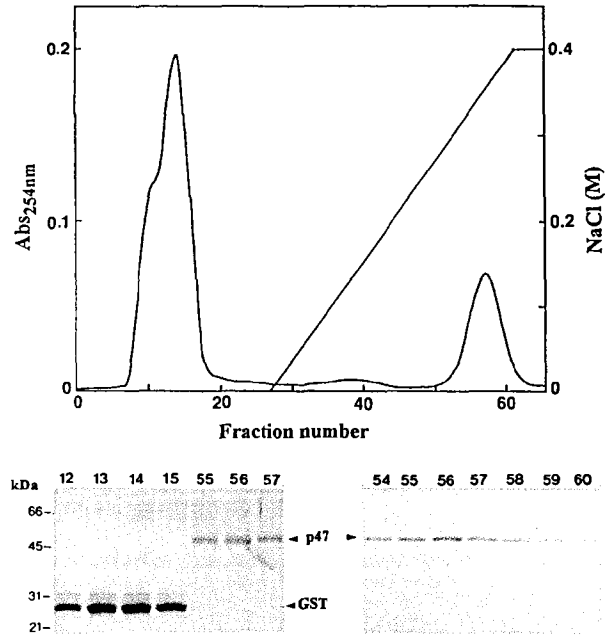

Fig. 2. CM-Sepharose chromatography of thrombin-cleaved GST-$p47^{phox}$. Chromatography was carried out as described under Material and Methods. Top, Chromatogram. Protein was measured as absorbance at 254 nm. Bottom, In the Western blot (right) and Coomassie staining (left), each lane contained 30 μl of the column fraction indicated at the head of the lane.

is re-chromatography with a GSH-agarose column (Smith and Johnson, 1988), this method was not applicable to $p47^{phox}$ due to the nonspecific binding of $p47^{phox}$ to GSH-agarose beads. Because $p47^{phox}$ is a strongly basic protein the thrombin-cleaved $p47^{phox}$ preparations were chromatographed on a CM-Sepharose and eluted with a 0~0.4 M NaCl gradient in a CM-Sepharose buffer. As shown in Fig. 2, GST eluted in the flow-through fractions of this column and $p47^{phox}$ eluted at ~0.3 M NaCl. Immunoblotting confirms that this protein cross-reacted with antipeptide antibody against $p47^{phox}$.

The activity of recombinant $p47^{phox}$ was determined

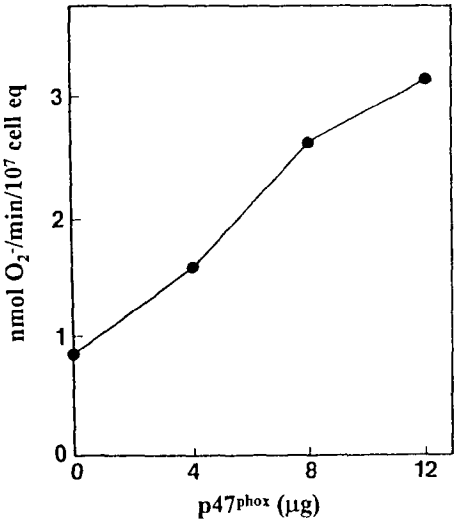

Fig. 3. *Effect of recombinant* p47^{phox} on O_2^- production by a SDS-mediated cell-free oxidase activating system that uses the sub-optimal amount of cytosol (1×10^6 cell eq.). Values are reported as means of two independent experiments.

by a SDS-induced cell-free oxidase activation system with a suboptimal amount of cytosol (1×10^6 cell eq.), which is 10% of the cytosol used for a standard cell-free assay (Park *et al.*, 1992). The results showed that the O_2^--forming activity was increased by the recombinant p47^{phox}, the extent of the increase depending on the amount of p47^{phox} added to the assay mixture (Fig. 3).

Although p47^{phox} is known as an essential component of the NADPH oxidase activation, the molecular function of this cytosolic subunit has not been elucidated clearly. *In vivo* (Heyworth *et al.*, 1991) and *in vitro* (Park *et al.*, 1992) results indicate that the only known function of p47^{phox} so far is as a mediator of translocation of whole cytosolic components of NADPH oxidase. It is a consensus opinion that p47^{phox} can migrate on its own from the cytosol to the membrane during oxidase activation. Other cytosolic factors such as p67^{phox} and ras related G-protein rac2 can only migrate to the membrane in the presence of p47^{phox} (Heyworth *et al.*, 1991; Park *et al.*, 1992). Recent evidence shows direct interaction between the carboxyl-terminal regions of cytochrome b_{558} and p47^{phox} during activation of the oxidase (Nakanishi *et al.*, 1992; DeLeo *et al.*, 1995). This interaction is sufficient to carry essential cytosolic components to the membrane for the activation of oxidase. Therefore, it is reasonable to assume that any modifications of p47^{phox} which mimic the phosphorylation may activate the NADPH oxidase. Although it has not been studied yet, the site-specific mutagenesis of phosphorylable Ser (S304, S305, S315, S320, S328, S345, S348, S359, S370, and S379) (Faust *et al.*, 1995) to Asp or Glu, which provides negative charges, may

291 QRQIKRGAPPRR**S**SIRNAHS

IHQR**S**RKRL**S**QDAYRRN**S**VR

FLQQRRRQARPGPQ**S**PG**S**PL

EEERQTQR**S**KPQPAVPPRP**S**

ADLILNRC**S**ESTKRKLASAV 390

Fig. 4. Amino acid sequence of C-terminal quarter of p47^{phox}. Phosphorylable Ser residues are indicated by underlining.

Table 1. Effect of phenylglyoxal-modified p47^{phox} on the O_2 forming activity of pellet after translocation with cytosol and membranes. The results shown here represent the mean± S.D. of three separate experiments. PG, phenylglyoxal

Composition of translocation mixture	Activity of pellet (nmol O_2 min/mg pellet protein)
Cytosol, membranes	0
Cytosol, membranes, SDS (90 μM)	182± 14
Cytosol, membrane, p47^{phox}	0
Cytosol, membrane, PG-treated p47^{phox}	44± 6

mimic the effect of phosphorylation. Alternatively, if the consequence of phosphorylation is the neutralization of strongly positive charges in the carboxyl-terminal Arg residues, elimination or reduction of positive charges of Arg may have a similar consequence. The amino acid sequence shows that 20 of the carboxyl-terminal quarter (Q291-V390) of p47^{phox} are Arg, as shown in Fig. 4.

In general, chemical modification of a protein leads to partial or complete loss of activity. When we treated cytosol with phenylglyoxal, and this treated cytosol was used in a SDS-induced cell-free activation system, complete loss of activity was observed. This result may suggest that Arg modification affects the capacity of cytosolic factors to activate oxidase. To avoid the inactivation of oxidase factors, recombinant p47^{phox} was treated with phenylglyoxal and the unreacted phenylglyoxal was removed by PD-10 column. In the SDS-induced cell-free oxidase activation system with a suboptimal amount of cytosol (Fig. 3) the modified p47^{phox} was as active as untreated p47^{phox} in the concentration-dependent enhancement of activity (data not shown). The modified p47^{phox} was added to an *in vitro* translocation system without adding SDS. To evaluate transfer of cytosolic components to plasma membranes and resulting activation of NADPH oxidase we recovered the membrane pellet from the translocation mixture through a discontinuous sucrose gradient. When phenylglyoxal-treated p47^{phox} in the presence of cytosol and mem-

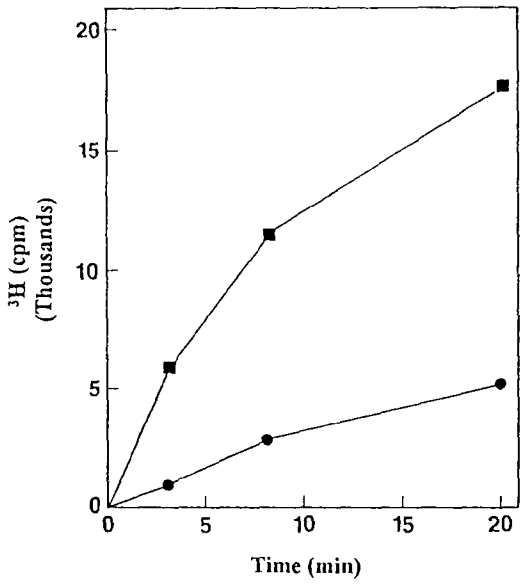

Fig. 5. Effect of treatment with phenylglyoxal on the labeling of recombinant p47^{phox} by [^{3}H]-NEM. Rates of labeling of untreated (■) or phenylglyoxal-treated recombinant p47^{phox} (■) by [^{3}H]-NEM as measured by liquid scintillation counting. The results are representative of three experiments.

brane were subjected to *in vitro* translocation, the rate of O_2^- generation of the recovered pellet was 44± 6 nmol O_2^-/min/mg pellet protein (mean± S.D., n=3). However, membranes and cytosol with untreated p47^{phox} but without adding SDS exhibited no activity (Table 1). Activity obtained by phenylglyoxal modification of p47^{phox} is 24% of SDS-induced cell-free activation, while physiological cell-free activation mediated by phosphorylation with protein kinase C provided 10~ 20% of the activity of the SDS-induced system (El Benna *et al.*, 1995). Therefore, activity obtained by phenylglyoxal-treated p47^{phox} may be comparable to that obtained by *in vitro* phosphorylation.

We recently have developed a method to covalently label p47^{phox} to assess changes in its conformation during activation (Park and Babior, unpublished results). The chemical reactivity, toward a thiol reagent, of a protein containing cysteine residues is very dependent upon the accessibility of the thiol groups. p47^{phox} has an NEM-reactive cysteine residue at res idue 378 which lies only 13 residues from the C-terminus of the molecule. A decrease in the accessibility of sulfhydryl reagents were observed upon treatment with an anionic amphiphile, such as arachidonate, and upon phosphorylation; both conditions result in the activation of oxidase. The purpose of the conformational change is most likely to create binding sites for membrane component cytochrome b_{558} which is an essential step for activation. This implies that modification of p47^{phox} by phenylglyoxal may result in a similar conformational change. As shown in Fig. 5, modification of p47^{phox} resulted in a considerable reduction in the rate of alkylation of the protein by [^{3}H] NEM, which is a result similar to that of phosphorylation and arachidonate treatment of p47^{phox}. However, further studies are required to determine whether the same conformational changes are induced by Arg modification, amphiphile treatment and phosphorylation of p47^{phox}.

It has been proposed that the membrane binding site of p47^{phox} is concealed by an interaction involving the protein's highly cationic carboxyl-terminal tail, whose positive charges could associate with a concentration of negative charge elsewhere on the surface of the molecule in the resting cell (Faust *et al.*, 1995). Phosphorylation could reverse this positive charge, releasing the tail and exposing the membrane binding site to allow assembly of the active oxidase. Modification of Arg residues in the carboxyl-terminal of p47^{phox} by phenylglyoxal somehow may bring about a similar consequence by reduction of positive charges.

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