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Attenuation of $p47^{phox}$ and $p67^{phox}$ Membrane Translocation as the Inhibitory Mechanism of *S*-Nitrosothiol on the Respiratory Burst Oxidase in Human Neutrophils

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The effect of the S-nitrosothiol (RSNO) on the activation of NADPH oxidase in human neutrophils was studied using an *in vitro* translocation system in which an anionic amphiphil, such as sodium dodecyl sulfate or arachidonate, plays a role as an activator. When membranes pretreated with RSNO and a cytosol fraction from resting neutrophils were combined to reconstitute the NADPH oxidase, both translocation of the cytosolic NADPH oxidase components such as $p47^{phox}$ and $p67^{phox}$ to the plasma membrane fraction and subsequent superoxide generation was inhibited. However, RSNO had no effect on O_2^- production when added after enzyme activation. A similar inhibition of translocation of recombinant $p47^{phox}$ was observed with RSNO-treated membrane. When the RSNO-treated membrane fraction was exposed to 2-mercaptoethanol the inhibition was reversed. The data suggest that RSNO inhibits translocation of $p47^{phox}$ or $p47^{phox}$ containing cytosolic complex via a direct effect on the membrane component of the NADPH oxidase.

The NADPH oxidase (also called respiratory burst oxidase), a complex enzyme so far found exclusively 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 (1, 2). The oxidase is dormant in resting neutrophils but acquires catalytic activity when cells are exposed to appropriate stimuli. In a cell-free system, catalytic activity of the NADPH oxidase in membranes from unstimulated neutrophils can be induced by anionic amphiphiles, such as sodium dodecyl sulfate (SDS) or arachidonate, in the presence of a cytosolic fraction (3–5). The catalytic activity is located in the plasma membrane, but it is known that in resting cells the oxidase components are distributed between the plasma membrane and the cytosol (3–7) and that when activation takes place either in intact neutrophil cells (8) or in a cell-free system (9, 10) the cytosolic components such as $p47^{phox}$ (the 47-kDa cytosolic phagocyte oxidase factor) and $p67^{phox}$, which exist as a $\sim$240 kDa complex (10), migrate to the membrane to associate with the membrane cytochrom b_{558} to assemble a functioning oxidase. Therefore, it is widely accepted that translocation of cytosolic components to the membrane is essential for the activation of oxidase.

The pathways involving the activation have been extensively studied; however, the regulation and the deactivation mechanism of NADPH oxidase is less understood. Recent reports suggest that nitric oxide (NO) may inhibit the generation of superoxide anion by neutrophils (11, 12). Since NO reacts with reduced thiol to form S-nitrosothiol (RSNO), the focus on biological effects on NO has expanded to include RSNO. RSNO is a biologically active and significantly more stable than NO itself, therefore, this adduct has been proposed as a biologically active intermediate of NO (13–15).

Although circumstantial evidence for the mechanism of NO inhibition to NADPH oxidase was suggested (11), its target in oxidase inhibition has not been elucidated clearly. In order to assess whether the inhibitory effect of RSNO is on the assembly of oxidase *versus* the activity of assembled oxidase, we employed a previously described *in vitro* translocation system (10, 16) consisting of cytosol or recombinant $p47^{phox}$ along with isolated plasma membrane treated with RSNO.

In this communication, we provide evidence that RSNO affects the translocation of cytosolic

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complex to the membrane by impairment of interaction between $p47^{phox}$ and the membrane component of NADPH oxidase, presumably cytochrome b_{558}.

MATERIALS AND METHODS

Materials. Bovine erythrocyte superoxide dismutase (SOD), NADPH, ferricytochrome c, GTPγS, phorbol-12-myristate 13-acetate (PMA), N-acetyl-DL-penicillamine, captopril, glutathione (GSH)-agarose, and CM-Sepharose, phenylmethylsulfonyl fluoride (PMSF), and thrombin were obtained from Sigma. Anti-$p47^{phox}$ and anti-$p67^{phox}$ antibodies were kindly provided by Dr. Bernard M. Babior (The Scripps Research Institute, USA). S-Nitrosothiols were prepared as described previously (14).

Preparation of neutrophil fractions. Neutrophil cytosol and membrane were prepared as described previously (6). 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 (17). Both cytosol and membrane were divided into aliquots and stored at −70°C until use. Activated membranes were obtained from neutrophils that had been treated with 1μg/ml PMA for 7 min at 37°C.

Preparation of recombinant $p47^{phox}$ protein. The transformed *Escherichia coli* containing the pGEX-1λT plasmid, with insert of $p47^{phox}$ cDNA, were grown, and fusion protein was purified by affinity chromatography on GSH-agarose (18). The fusion protein was cleaved by treatment with thrombin (10 U/ml) in elution buffer containing 150 mM NaCl and 2.5 mM $CaCl_2$ for 2 hr at room temperature. The protein mixture was further purified on a CM-Sepharose column equilibrated with the 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.

In vitro translocation. Thawed cytosol was centrifuged at 105,000 × g for 1 hr at 25°C to remove aggregated proteins. Membranes were treated with RSNO before use. For this purpose, 1.25×10^8 cell eq of membranes were incubated for 5 min with indicated concentrations of RSNO in relaxation buffer (final volume, 2 ml). The RSNO-treated membranes were then reisolated by centrifugation at 105,000 × g for 5 min over a discontinuous sucrose gradient composed of 1 ml of 15% (w/v) and 0.5 ml 50% sucrose in relaxation buffer. Translocation mixtures contained RSNO-treated membrane (1.25×10^8 cell eq), 10 μM GTPγS, 5×10^7 cell eq of centrifuged cytosol, relaxation buffer, and 90 μM SDS in a total volume of 2 ml. After incubation for 5 min at 25°C, the translocation mixtures were carefully layered onto a discontinuous gradient composed of 1 ml of 20% (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°C. 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 in a reaction mixture containing 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. Absorbance of cytochrome c was read at 550 nm and corrected for background observed in the presence of 45μg of superoxide dismutase.

Electrophoresis and immunoblotting. Protein samples were subjected to SDS-PAGE on 8% polyacrylamide gels using the Laemmli buffer system (19). The separated proteins were electrophoretically transferred onto a nitrocellulose sheet (20), which was blocked with dried milk, then probed with mixed partially purified rabbit polyclonal antibody raised against synthetic peptide from $p47^{phox}$ and $p67^{phox}$ used at dilutions of 1:1000 and 1:2000, respectively, and finally detected with a 1:2000 dilution of alkaline phosphatase-labeled goat anti-rabbit Ig antibody (Sigma) using 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium as susbtrate (Bio-Rad). Immunoblotting results are representative of three separate experiments.

RESULTS AND DISCUSSION

We investigated whether RSNO acts on the membrane or on the cytosolic components of the oxidase to affect *in vitro* translocation system which reconstitutes NADPH oxidase by SDS. To evaluate transfer of cytosolic components, we recovered the membrane pellet from translocation mixtures by centrifugation through a discontinuous sucrose gradient following SDS-PAGE and immunoblotting. Membrane and cytosol fractions were exposed separately to RSNO, and unreacted RSNO was eliminated either by ultracentrifugation for membranes or by running the cytosols through a Sephadex G-25 column. When treated-cytosol and control membrane were subjected to *in vitro* translocation, the rate of O_2^- production of recovered pellet was similar in untreated and RSNO-treated cytosol (data not shown). However, membranes treated with various concentrations of S-nitrosoglutathione (GSNO) before addition of cytosol inhibited translocation of $p47^{phox}$ and $p67^{phox}$, and subsequent O_2^- forming activity, in a concentration-dependent manner, as shown in Fig. 1. Since GSNO with a same concentration range had no effect on trapping of O_2^- by cytochrome c in the xanthine/xanthine oxidase system (data not shown), the possibility that RSNO

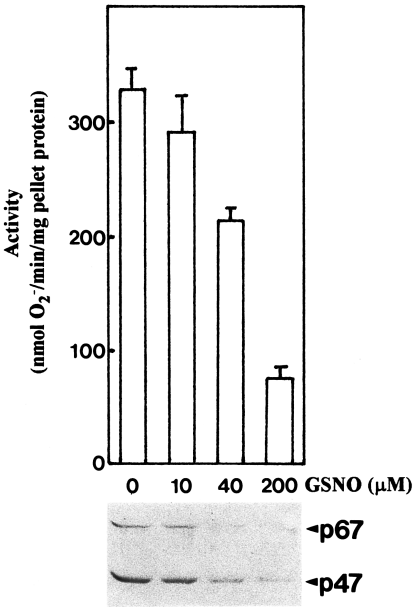

FIG. 1. Inhibition of translocation of $p47^{phox}$ and $p67^{phox}$ and O_2^- forming activity by the preincubation of membranes with the indicated concentrations of GSNO. Translocation mixtures contained cytosol, membranes pretreated with GSNO, 10 μM GTPγS, and 90 μM SDS. *Top*, O_2^- production by pellet. Values are mean ±S.D. of three experiments. *Bottom*, Western blot of pellets from translocation experiments. Western blotting was carried out as described in the text using 1.6×10^6 cell eq of pellet ($\sim$ 20 μg of protein) in each track.

acts as a radical scavenger has been ruled out. Translocation of $p47^{phox}$ and $p67^{phox}$ was varied in parallel with changes in activity. The treatment of membrane with 200 μM GSNO reduced SDS-induced superoxide production from 327±18 to 76±8 nmol O_2^-/min per mg of pellet protein (mean ± S.D., n = 3).

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 *rac* can only migrate to the membrane in the presence of $p47^{phox}$ in the intact neutrophils (8, 21). Recent evidence shows direct interaction between COOH-terminal regions of cytochrome b_{558} and $p47^{phox}$ during activation of the system (22, 23). Cytochrome b_{558}, which consists of two subunits ($gp91^{phox}$ and $p22^{phox}$), is thought to function as the terminal electron donor to O_2. The NADPH oxidase activity of the cell-free system could be fully reconstituted with recombinant cytochrome b_{558} and cytosolic components (24). Furthermore, it has been shown that $p47^{phox}$ was not translocated to the membrane from neutrophils of patients with cytochrome b_{558}-deficient chronic granulomatous disease (CGD) (10, 25). The translocation experiment was carried out with the same treatment conditions as described above, except cytosol is replaced with purified recombinant $p47^{phox}$. Inhibition of translocation of $p47^{phox}$ with GSNO-treated membrane was comparable to that with whole cytosol, which confirms that RSNO-modified membrane impaired interaction between membrane component of NADPH oxidase, probably cytochrome b_{558}, and $p47^{phox}$ (Fig. 2).

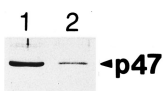

FIG. 2. Inhibition of translocation of recombinant $p47^{phox}$ by GSNO-treated membranes. Western blot of pellets from translocation mixtures contained recombinant $p47^{phox}$, 10 μM GTPγS, 90 μM SDS, and either untreated membranes (lane 1) or GSNO (200 μM)-treated membranes (lane 2).

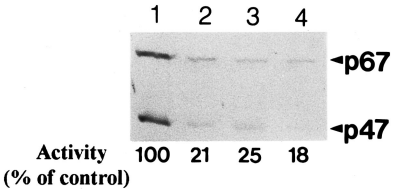

FIG. 3. Ability of various RSNO to inhibit SDS-induced $p47^{phox}$ and $p67^{phox}$ translocation and O_2-forming activity. Membranes were pretreated with 200 μM of RSNO and reisolated for the translocation experiments. Western blot of pellets from translocation mixtures contained cytosol, 10 μM GTPγS, 90 μM SDS, and either untreated membranes (lane 1), GSNO- (lane 2), S-nitrosocaptopril- (lane 3) or SNAP-treated membranes (lane 4). Activities are expressed as the percentage of the control rate in which the value obtained with untreated membrane was taken as 100%. The results are representative of three separate experiments.

The ability of RSNO with various structures to inhibit translocation was tested. As shown in Fig. 3, similar inhibition of translocation and superoxide-forming activity of pellet was observed with relatively stable RSNO such as GSNO and S-nitrosocaptopril as well as unstable RSNO such as S-nitroso-N-acetyl-DL-penicillamine (SNAP). It has been shown that RSNO, a nitrosating agent at neutral pH, acts by transferring its nitroso group directly to the sulfhydryl groups in the protein (26). A previous report shows that the ability of RSNO to provide the NO moiety to sulfhydryl group of protein was not dependent upon its stability (14).

Reversibility of the inhibitory effect of RSNO-treated membrane with the treatment of 5 mM 2-mercaptoethanol for 5 min at room temperature indicated involvement of a sulfhydryl group in the membrane component of NADPH oxidase (Fig. 4). In addition, this result confirms that RSNO did not nonspecifically affect the plasma membrane or irreversibly damage the NADPH oxidase. Previously, Bellavite *et al.* (27) suggested that p-chloromercuribenzoic acid inhibits NADPH oxidase by reaction with a thiol closely associated with the heme group of cytochrome b_{558}.

When active membrane pellets obtained from either the SDS-induced translocation or the fractionation of PMA-stimulated intact neutrophil cells were incubated with 10–200 μM of GSNO, the O_2^- forming activity of the preassembled oxidase complex was not significantly altered by GSNO (92–98% *versus* control activity). Furthermore, the $p47^{phox}$ and $p67^{phox}$ of activated pellet reisolated after GSNO treatment by a brief centrifugation was not changed significantly, as shown in Fig. 5. These findings suggest that once the oxidase is assembled, RSNO cannot exert its inhibitory action anymore. Therefore, it may assume that RSNO inhibits the activation of the broken cell oxidase rather than the activity of the assembled system. This result also suggests that RSNO did not act via a superoxide radical scavenging mechanism. Because RSNO has no effect on the assembled oxidase it is plausible to assume that RSNO may be involved in the regulatory mechanism of activation rather than deactivation of oxidase. It is possible that RSNO protects adjacent cells from neutrophil-mediated injury.

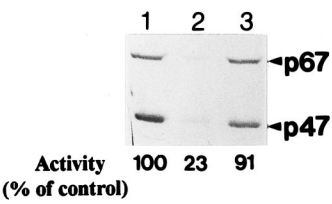

FIG. 4. Reversed inhibitory effect of GSNO on the translocation of $p47^{phox}$ and $p67^{phox}$ and the O_2^- generation by the treatment of GSNO-pretreated membranes with 2-mercaptoethanol. Western blot of pellets from translocation mixtures contained either untreated membrane (lane 1), GSNO (200 μM)-pretreated membranes (lane 2), or GSNO (200 μM)-pretreated membrane treated with 5 μM 2-mercaptoethanol for 5 min at room temperature (lane 3). Activities are expressed as the percentage of the control rate. The results are representative of three separate experiments.

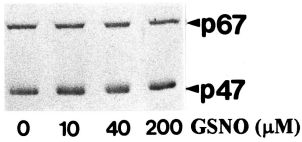

FIG. 5. Effects of GSNO on the preassembled NADPH oxidase. Western blot of reisolated pellets by centrifugation from active membrane pellets treated with the indicated concentrations of GSNO for 5 min at room temperature.

Taken together, we suggest that RSNO inhibits NADPH oxidase activity by reacting with the sulfhydryl group of cytochrome b_{558} and altering the conformation of cytochrome b_{558}, which is needed to provide binding site for $p47^{phox}$, that hampers the binding of $p47^{phox}$ or $p47^{phox}$ containing cytosolic complex.

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