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PR-39, a proline-rich antibacterial peptide that inhibits phagocyte NADPH oxidase activity by binding to Src homology 3 domains of p47^{phox}

(neutrophil/superoxide anion/cytochrome b_{558}/p22^{phox})

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ABSTRACT **Reactive oxygen intermediates generated by the phagocyte NADPH oxidase are critically important components of host defense. However, these highly toxic oxidants can cause significant tissue injury during inflammation; thus, it is essential that their generation and inactivation are tightly regulated. We show here that an endogenous proline-arginine (PR)-rich antibacterial peptide, PR-39, inhibits NADPH oxidase activity by blocking assembly of this enzyme through interactions with Src homology 3 domains of a cytosolic component. This neutrophil-derived peptide inhibited oxygen-dependent microbicidal activity of neutrophils in whole cells and in a cell-free assay of NADPH oxidase. Both oxidase inhibitory and direct antimicrobial activities were defined within the amino-terminal 26 residues of PR-39. Oxidase inhibition was attributed to binding of PR-39 to the p47^{phox} cytosolic oxidase component. Its effects involve both a polybasic amino-terminal segment and a proline-rich core region of PR-39 that binds to the p47^{phox} Src homology 3 domains and, thereby, inhibits interaction with the small subunit of cytochrome b_{558}, p22^{phox}. These findings suggest that PR-39, which has been shown to be involved in tissue repair processes, is a multifunctional peptide that can regulate NADPH oxidase production of superoxide anion ($O_2^{\cdot-}$), thus limiting excessive tissue damage during inflammation.**

Neutrophils represent a first line of defense against infections; they are the first white blood cells to arrive at sites of infection and are well-equipped to sequester and eliminate pathogens. These cells possess multiple antimicrobial defense mechanisms, including both oxidative and nonoxidative microbial killing processes (1, 2). Nonoxidative neutrophil defense mechanisms include several antibacterial peptides, which are distributed widely in many species (3–7). A proline-arginine (PR)-rich antibacterial peptide, PR-39, kills bacteria by a non-pore-forming mechanism, presumably by interfering with DNA or protein synthesis (8). This peptide, which was isolated first from the porcine small intestine (9) and identified recently in porcine and human neutrophils (10, §), also has been isolated from wound fluid, where it has been shown to induce syndecan expression by mesenchymal cells (11). Because syndecans are proposed mediators of wound repair, PR-39 may have a larger role in inflammatory processes and tissue repair in addition to its antibacterial properties.

Phagocyte oxidative defense mechanisms are initiated by a plasma membrane-bound enzyme complex called reduced nicotinamide-adenine dinucleotide phosphate (NADPH) oxidase (12). This multicomponent enzyme catalyzes the reduction of molecular oxygen to superoxide anion ($O_2^{\cdot-}$) using NADPH as an electron donor. Although $O_2^{\cdot-}$ and other reactive oxygen intermediates are important components of host defense, these highly toxic oxidants cause significant tissue injury in inflammatory diseases and ischemia-reperfusion injury (13–17). Thus, their generation and inactivation must be tightly regulated. At least five proteins compose the NADPH oxidase complex: a membrane flavocytochrome b_{558}, which is composed of two subunits (gp91^{phox} and p22^{phox}) and three cytosolic components (p47^{phox}, p67^{phox}, and a GTP-binding protein named p21^{Rac}) (18, 19). Although mechanisms for activation and assembly of NADPH oxidase have not been elucidated fully, it is clear that multiple protein–protein interactions among its components are regulated by a number of signaling intermediates (20).

Our motivation in studying whether PR-39 influences oxidative killing mechanisms of neutrophils was stimulated by observations showing that assembly of phagocyte NADPH oxidase requires protein–protein interactions between Src homology 3 (SH3) domains in cytosolic components and proline-rich regions in other components (21–25). If PR-39 could mimic one of the proline-rich targets and bind to the SH3 domain(s), it might influence the assembly and activity of NADPH oxidase. Here, we report findings on PR-39 that link oxygen-independent and oxygen-dependent antimicrobial functions of neutrophils in a regulatory scheme.

MATERIALS AND METHODS

Peptide Design, Synthesis, and Antibacterial Assays. Predictions of peptide characteristics, relative to hydrophilicity and hydrophobicity were accomplished using a computer program (PEPTIDE COMPANION, Peptides International). Peptides were synthesized by the Biotechnology Core Facility at Kansas State University by the solid-phase method using t-Boc chemistry with a peptide synthesizer (Applied Biosystems; model 431). Peptide purification and characterization were conducted as described (10, 26). Briefly, the peptides were purified on a reversed-phase HPLC system (Beckman) with a C_{18} column (0.46 × 25 cm; Vydac, Hesperia, CA; model 218 TP), analyzed by fast-atom mass spectrometry (AUTOSPEC-Q; VG Analytical, Manchester, U.K.), and visualized by acid-urea PAGE (AU-PAGE). Antibacterial activity of the peptides was tested by overlaying the AU-PAGE gel with agarose containing bacteria (*Escherichia coli* and *Salmonella* spp.) and a lawn-spot antibacterial assay as described (10).

Superoxide Anion Production Assays. Whole-cell $O_2^{\cdot-}$ production by porcine peripheral blood neutrophils was determined by the superoxide dismutase-inhibitable reduction of

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Abbreviations: AU-PAGE, acid-urea PAGE; GST, glutathione *S*-transferase; PMA, phorbol 12-myristate 13-acetate; PR, proline arginine; PVDF, poly(vinylidene difluoride); SH3, Src homology 3.

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ferricytochrome *c* as described (27). Cell-free O_2^- production was measured essentially as described (28) using 96-well plates and a Molecular Devices Thermomax microplate reader. Reactions (100 μl) contained 10^6 cell equivalents of human neutrophil cytosol and 5×10^5 cell equivalents of deoxycholate-solubilized membranes prepared from human peripheral blood neutrophils. Reaction mixtures contained 50 mM potassium phosphate (pH 7), 0.2 mM acetylated cytochrome *c*, 4 mM $MgCl_2$, 1 mM EGTA, 10 μM FAD, 1 μM guanosine 5′-[γ-thio]triphosphate, and 200 μM NADPH. The reactions were initiated by addition of 40 μM arachidonic acid. Control reactions contained 5 μg of superoxide dismutase. Superoxide anion generation was calculated based on superoxide dismutase-inhibitable changes in cytochrome *c* absorbance observed at 551 nm. The reactions were followed for 20 min after addition of arachidonic acid, with absorbance readings taken at 1-min intervals. Maximum rates of superoxide generation were calculated from a linear least squares fit of 10 consecutive 1-min data points. Determinations were based on reactions performed in duplicate.

Binding Assays. Neutrophils were disrupted by sonication, and unbroken cells, debris, and granules were removed by centrifugation (13,000 × *g* for 60 min). Cytosol fractions were prepared by centrifugation (100,000 × *g* for 45 min) of the supernatants to remove residual membranes. Cytosolic proteins (15 μg in reducing buffer) were separated on an SDS/7.5% polyacrylamide gel, transferred to poly(vinylidene difluoride) (PVDF) membranes, and probed with biotinylated PR-39 (0.3 μM) for 2 hr. Binding between PR-39 and cytosolic proteins was visualized by a chemiluminescent streptavidin-alkaline phosphatase system using an Epson scanner (Torrance, CA) and the National Institutes of Health IMAGE program. PR-39 was labeled with biotin hydrazide at the carboxyl terminus (Pierce) and purified by gel filtration chromatography.

Competitive ligand-blot assays were performed by incubating recombinant $p47^{phox}$ (0.02 μM) without or with various concentrations (0.006, 0.06, and 0.6 μM) of PR-39 for 1 hr at 37°C and then with biotinylated PR-39 (0.06 μM) for an additional hr at 37°C. The solutions were dialyzed against ddH_2O for 1 hr at 4°C before blotting to nitrocellulose (5 μl) and detection with a streptavidin-alkaline phosphatase detection system as described above.

Methods for production of the glutathione *S*-transferase (GST)-$p47^{phox}$-SH3 (residues 151–284) fusion protein have been described (21). For solution binding assays, lysates of recombinant $p22^{phox}$ protein (residues 127–195) from baculovirus-infected cells (2.5×10^4 cell equivalents per assay) were mixed with peptides and incubated for 1 hr at 20°C with 100 μl of GST-$p47^{phox}$ bound to glutathione-Sepharose beads

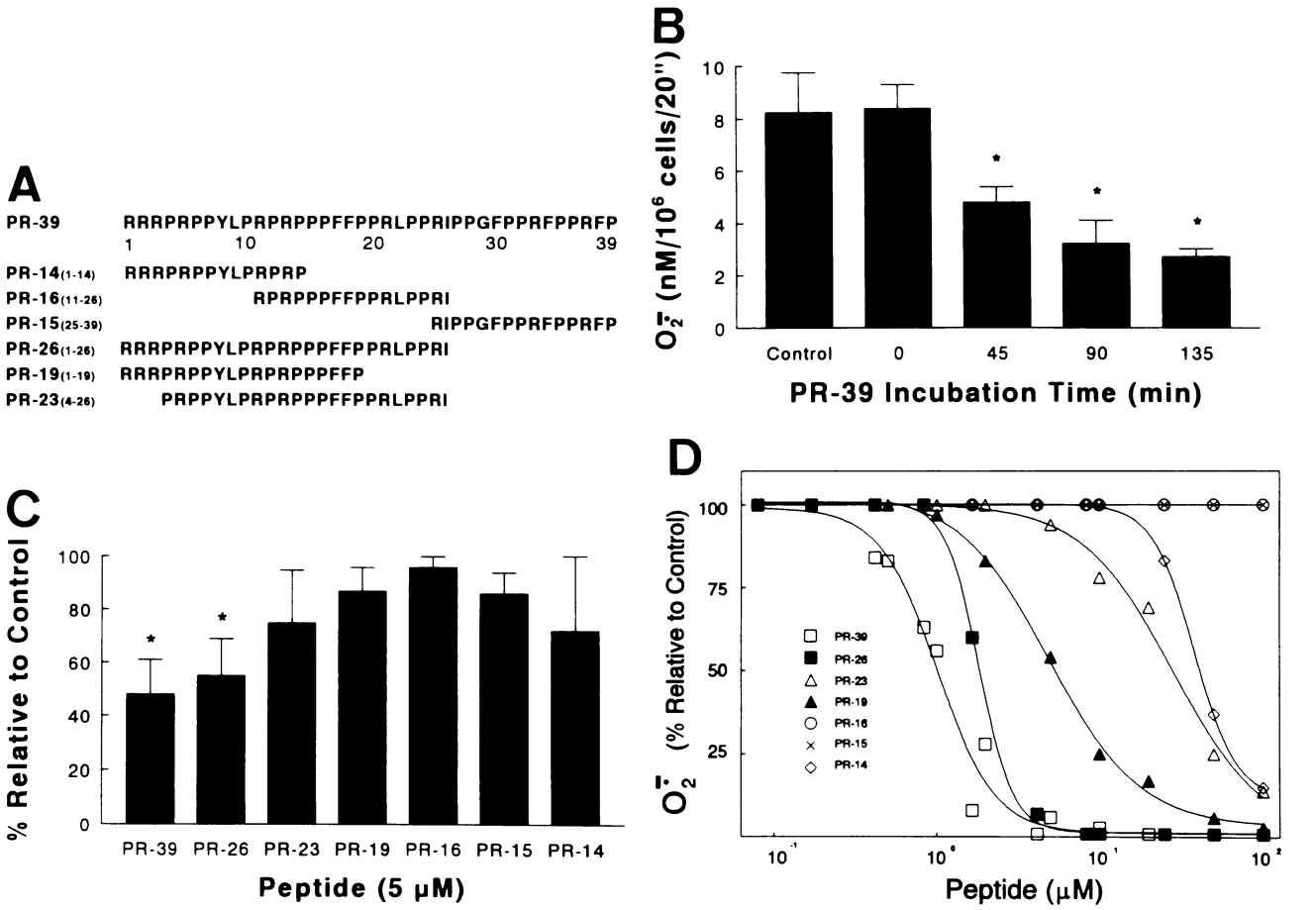

FIG. 1. Inhibition of neutrophil superoxide anion generation by PR-39. (*A*) Amino acid sequences of PR-39 and derivatives. The single-letter amino acid code is used. (*B*) Inhibition of whole-cell NADPH oxidase activity requires preincubation with PR-39. Neutrophils (1×10^6) were incubated without (Control) or with PR-39 (5 μM) for the indicated times before stimulation with phorbol 12-myristate 13-acetate (PMA). Values are mean ± SEM, $n = 5$. *, Different from control, $P < 0.05$. (*C*) Whole-cell NADPH oxidase inhibition by PR-39 and its derivatives. Neutrophils were incubated with PR-39 or derivatives for 150 min before stimulation with PMA. Results are reported as % relative to control (no peptides); however, the statistical analysis was based on nanomoles of superoxide anion produced. Values are means, $n = 3$. *, Different from control, $P < 0.05$. This experiment was conducted twice with similar results. (*D*) Cell-free NADPH oxidase inhibition by PR-39 and its derivatives. Data are averages of duplicate reactions and are representative of three to four independent experiments. Control (100%) activities ranged from 0.9–2.4 nmol of O_2^-/min/5×10^5 cell equivalents of membrane.

(15% suspension). Bound proteins were washed three times in 15 volumes of ice-cold 100 mM KCl/3 mM NaCl/3.5 mM $MgCl_2$/0.15 mM phenylmethanesulfonyl fluoride/10 mM Pipes (pH 7.5), eluted with 1% SDS, and analyzed by SDS/PAGE followed by immunoblotting with mouse anti-$p22^{phox}$ antibody (mAb 449, gift from A. Verhoeven, Central Laboratories of the Netherlands Red Cross).

RESULTS AND DISCUSSION

Using hydropathy analysis of PR-39, we designed and synthesized several peptides to explore its functional domains (Fig. 1*A*). Reverse-phase HPLC, mass spectrometry, and AU-PAGE confirmed peptide purity (>95%) and indicated that all synthetic peptides possessed the designed sequences. Bactericidal activity of these peptides was tested by overlaying the AU-PAGE gel with agarose containing bacteria. Consistent with our previous report (26), only PR-39 and PR-26 were found to have antibacterial activity against *E. coli* and *Salmonella* spp. In a lawn-spot antibacterial assay, PR-39, PR-26, PR-23, PR-19, PR-16, PR-15, and PR-14, and combinations of PR-14, PR-16, and PR-15 were tested. Again, only PR-39 and PR-26 had antibacterial activity; all of the other segments and their mixtures showed no antibacterial activity (data not shown). These results confirm that PR-26, the NH_2-terminal 26-residue segment, contains the antibacterial domain of PR-39.

When PR-39 was incubated with neutrophils for at least 45 min before stimulation with PMA, a significant reduction in $O_2^{\cdot-}$ generation was observed (Fig. 1*B*). This effect was even more apparent with longer pre-incubation periods, approaching as much as 70% oxidase inhibition. PR-26 also significantly reduced $O_2^{\cdot-}$ generation (Fig. 1*C*). However, the shorter peptides, PR-23, PR-19, PR-16, PR-15, and PR-14, did not significantly reduce $O_2^{\cdot-}$ generation by intact neutrophils. PR-39 (20 μM) added to neutrophils at the same time as PMA activation did not affect generation of $O_2^{\cdot-}$, while longer incubation periods with this peptide did not affect neutrophil viability, as judged by trypan blue dye exclusion.

To investigate the inhibition of neutrophil $O_2^{\cdot-}$ generation by PR-39 and its fragments in more detail, we used a cell-free assay system of $O_2^{\cdot-}$ generation. PR-39 and PR-26 were very potent inhibitors of $O_2^{\cdot-}$ generation in the cell-free assay; IC_{50} values were approximately 1 and 2 μM, respectively (Fig. 1*D*). At concentrations greater than 5 μM, both proline-rich peptides completely inhibited the generation of $O_2^{\cdot-}$. PR-16 and PR-15 did not affect $O_2^{\cdot-}$ generation; however, PR-14 did reduce $O_2^{\cdot-}$ generation at concentrations greater than 25 μM.

Because PR-26 contains the antibacterial activity of PR-39 and because this peptide also has oxidase inhibitory activity, we reasoned that truncated peptides of PR-26 might delineate important regions involved in both activities. PR-23 (residues 4–26; Fig. 1*A*) was designed to evaluate the importance of the first three arginine residues of PR-26, and PR-19 (residues 1–19) was designed to assess the contribution of the C terminus to the activity of PR-26. Unlike their parent peptide, neither of these truncated versions of PR-26 exhibited antibacterial activity. However, both peptides had some oxidase inhibitory activity; IC_{50} values of PR-19 and PR-23 were approximately 5 μM and 25 μM, respectively (Fig. 1*D*). These findings suggest that the first three arginine residues of PR-26 and the C-terminal segment (residues 20–26) are essential for antibacterial activity and that both peptides contain the oxidase inhibitory domain, although the arginine cluster contributes greatly to both activities.

The findings that PR-39 and PR-26 inhibited both cell-free and whole-cell $O_2^{\cdot-}$ production by neutrophils and that inhibition required preincubation of cells with peptides for at least 45 min before PMA stimulation suggested that these peptides act through some intracellular target, such as the NADPH oxidase components, themselves. To determine if PR-39 bound to specific neutrophil cytosol components, we separated human and porcine cytosolic proteins by SDS/PAGE, transferred the proteins to a PVDF membrane, and probed the membrane with biotinylated PR-39. Using this ligand-blot binding assay, we found that PR-39 bound to a 47-kDa protein in both human and porcine cytosol preparations (Fig. 2*A*). We conducted competitive binding assays to determine if PR-39 bound to recombinant $p47^{phox}$. These solution binding experiments with pure recombinant $p47^{phox}$ and PR-39 showed that increasing concentrations of nonbiotinylated PR-39 inhibited specific binding of biotinylated PR-39 to recombinant $p47^{phox}$ (Fig. 2*B*). Binding was inhibited 90% at equimolar concentrations of labeled and unlabeled peptide (0.06 μM) and was completely blocked by a 10-fold excess of unlabeled peptide. This finding shows that PR-39 binds to $p47^{phox}$ and that binding

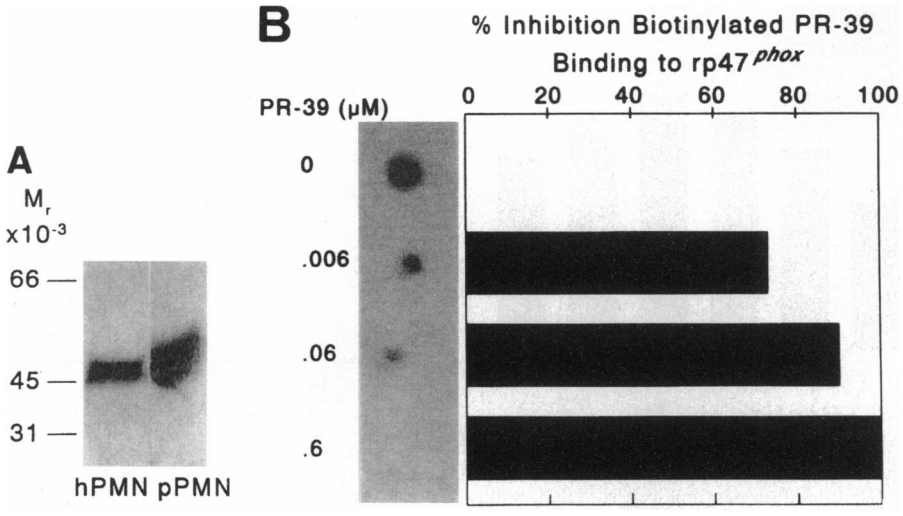

FIG. 2. Binding of PR-39 to the $p47^{phox}$ component of NADPH oxidase. (*A*) Biotinylated PR-39 binds to a 47-kDa neutrophil cytosol protein. Cytosolic protein (50 μg) from human and porcine polymorphonuclear (hPMN and pPMN) leukocytes was subjected to SDS-PAGE, transferred to a PVDF membrane, and then probed with biotinylated PR-39. Molecular masses of standards are shown on the left in kilodaltons. (*B*) Biotinylated PR-39 binds to recombinant $p47^{phox}$. Competitive binding analysis was conducted by incubating various concentrations of PR-39 (*Left*) with recombinant $p47^{phox}$ and then with biotinylated PR-39. After dialysis, solutions were dot-blotted onto PVDF membranes and probed for bound biotinylated PR-39.

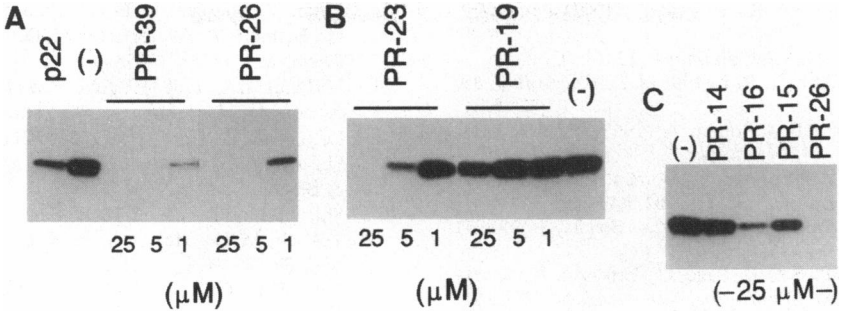

FIG. 3. Inhibition of binding of p47^{phox} SH3 domains to p22^{phox} by PR-39 or its derivatives. (*A*) PR-39 or PR-26 were mixed with recombinant p22^{phox} and incubated for 60 min with GST-p47^{phox}-SH3 fusion proteins (0.5 μg) bound to glutathione-Sepharose beads and analyzed as described. After SDS/PAGE, Sepharose-bound proteins were analyzed by immunoblotting with anti-p22^{phox} antibody. Controls included blotting of recombinant p22^{phox} lysate (5×10^3 cell equivalents) alone (p22) and bound complexes detected without competing PR-peptides (-). Concentrations of competing peptides defined above each blot are indicated below. (*B*) Inhibition by PR-23 or PR-19, conducted as in *A*. (*C*) Comparison of inhibitory activities of smaller peptides at 25 μM.

is specific, and implies that PR-39 decreases O_2^- generation by interfering with this cytosolic component of the NADPH oxidase complex.

At least two proline-rich sequences, the C-terminal region (residues 358–371) of p47^{phox} and the cytoplasmic region (residues 149–162) of p22^{phox}, are thought to bind to SH3 domains of p67^{phox} and p47^{phox}, respectively, and are essential for the activation of NADPH oxidase *in vivo* (21–25). Our data obtained by probing neutrophil cytosolic proteins with biotinylated PR-39 suggested that PR-39 did not bind to p67^{phox} (Fig. 2*A*). Because PR-39 bound directly to p47^{phox}, we reasoned that PR-39 could interfere with assembly of NADPH oxidase by blocking its interaction with p22^{phox}. To examine this hypothesis, we conducted solution binding assays between GST-p47^{phox}-SH3 domain fusion protein and a recombinant p22^{phox} cytoplasmic domain (residues 127–195) in the presence or absence of PR-39 or its derived fragments. PR-39 and PR-26 effectively blocked the interaction between GST-p47^{phox} and recombinant p22^{phox} at concentrations close to their IC_{50} values observed in the cell-free oxidase assay (Fig. 3*A*). Using this binding assay, analysis of the inhibition by PR-23, PR-19, PR-16, PR-15, and PR-14 revealed the importance of the C-terminal region of PR-26 in SH3 binding (Fig. 3 *B* and *C*). These data not only supported our hypothesis that PR-39 blocks the interaction between p47^{phox} and p22^{phox}, but also indicated that the main structural motif involved in the interaction between PR-39 and the SH3 domains of p47^{phox} was in the central segment (PR-16) of PR-39. This 16-residue segment contains structural elements compatible with consensus features of both classes of SH3 peptide ligands (29, 30). Two sequences within PR-16, RPPPFFP and PPRLPPRI, conform to consensus sequences for either Class I (+) and Class II (-) binding orientations, $X_1pX_2PpX_3P$ and $X_{3'}PpX_{2'}PpX_{1'}$, respectively (upper-case letters denote critical contact residues; P denotes proline; and X_1 or $X_{1'}$ favor arginine residues). Since PR-23 inhibits better than PR-19, a Class II binding orientation is proposed. However, because PR-19 had greater inhibitory activity in the cell-free assay but was less potent in blocking the interaction of p47^{phox} SH3 domains with p22^{phox} when compared with PR-23, the polybasic motif of the amino terminus of PR-26 also represents a separate important component of oxidase inhibition. This observation is supported by studies showing that several other cationic peptides effectively inhibit NADPH oxidase, including peptides derived from p47^{phox} (31, 32). Thus, we have defined critical regions at both ends of PR-26 based on dramatic losses of function seen with the deleted forms, PR-19 and PR-23. The polybasic motif at the amino termini of PR-39 and PR-26, in addition to their overall amphipathic character, may have a role in promoting internalization of these inhibitory peptides, since similar structural properties are thought to enable internalization of various synthetic peptides designed for intracellular targets (33–37). PR-39 uptake may involve an active endocytic process or direct membrane lipid interactions, since this peptide binds and induces conductance changes in pure lipid bilayers (38).

To our knowledge, PR-39 is the first naturally occurring down-regulator of phagocyte NADPH oxidase identified that interferes with assembly of this enzyme by binding to p47^{phox}. Little is known about the mechanisms governing respiratory burst kinetics, although models based on a continuous cycling of cytosolic components are consistent with the direct inhibitory effects of PR-39 on oxidase component interactions. The paradoxical finding of a neutrophil peptide possessing both antibacterial and oxidase inhibitory activities is intriguing, since a switch from oxygen-dependent to oxygen-independent bactericidal mechanisms by an accumulation of this peptide within inflammatory sites could serve several important functions. PR-39, which has a well-documented role as an antibacterial peptide, might have several roles in tissue repair by directly inhibiting NADPH oxidase activity and limiting related proinflammatory responses, while also affecting gene expression patterns that promote wound healing (11). Furthermore, since reactive oxygen intermediates have also been shown to function as second messengers that can regulate gene expression (39), PR-39 may indirectly influence gene expression patterns related to oxidative stress. These opposing activities of PR-39 illustrate a fine balance required in host defense mechanisms: antibacterial activity is necessary to control microbial pathogens, and oxidase inhibitory activity is important for restricting tissue damage caused by excessive oxygen radicals generated by NADPH oxidase. In the case of PR-39, one peptide possesses both activities. These findings suggest a mechanism for interaction between oxidative and nonoxidative antimicrobial systems of neutrophils and may serve as a basis for design of drugs effective against production of oxidants in chronic or acute inflammatory disease states.

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