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Akt Phosphorylates p47^{phox} and Mediates Respiratory Burst Activity in Human Neutrophils[1]

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Respiratory burst activity and phosphorylation of an NADPH oxidase component, p47^{phox}, during neutrophil stimulation are mediated by phosphatidylinositol 3-kinase (PI-3K) activation. Products of PI-3K activate several kinases, including the serine/threonine kinase Akt. The present study examined the ability of Akt to regulate neutrophil respiratory burst activity and to interact with and phosphorylate p47^{phox}. Inhibition of Akt activity in human neutrophils by an inhibitory peptide significantly attenuated fMLP-stimulated, but not PMA-stimulated, superoxide release. Akt inhibitory peptide also inhibited hydrogen peroxide generation stimulated by bacterial phagocytosis. A direct interaction between p47^{phox} and Akt was shown by the ability of GST-p47^{phox} to precipitate recombinant Akt and to precipitate Akt from neutrophil lysates. Active recombinant Akt phosphorylated recombinant p47^{phox} in vitro, as shown by ^{32}P incorporation, by a mobility shift change detected by two-dimensional gel electrophoresis, and by immunoblotting with phospho-Akt substrate Ab. Mutation analysis indicated that 2 aa residues, Ser^{304} and Ser^{328}, were phosphorylated by Akt. Inhibition of Akt activity also inhibited fMLP-stimulated neutrophil chemotaxis. We propose that Akt mediates PI-3K-dependent p47^{phox} phosphorylation, which contributes to respiratory burst activity in human neutrophils. *The Journal of Immunology*, **2003, 170: 5302–5308.**

Neutrophils are a critical cellular component of the innate immune response to invading microorganisms. The ability to generate toxic oxygen radicals by a multicomponent enzyme complex, termed the NADPH oxidase, is necessary for microbicidal activity. In resting neutrophils, the NADPH oxidase complex consists of unassembled cytosolic and membrane components. Following activation, the cytosolic components, p40^{phox}, p47^{phox}, p67^{phox}, and Rac-2, translocate to plasma or phagosome membranes, where they associate with flavocytochrome b_{558} and Rap1A to form the active oxidase (1). The flavocytochrome b_{558} serves as the electron transporter that generates superoxide from oxygen and NADPH (2). p47^{phox} is postulated to act as an adaptor protein that assembles the components of the functional enzyme. Stimulation of neutrophils by receptors for chemoattractants, chemokines, complement components, and Ig and by phorbol diesters results in extensive phosphorylation of p47^{phox}. Phosphorylation of p47^{phox} produces a conformational change leading to exposure of SH3 motifs, proline-rich regions, and a PX domain that mediate interaction with both flavocytochrome b_{558} and p67^{phox} (2, 3).

Several kinases that phosphorylate components of the NADPH oxidase and regulated oxidase activity have been identified. Protein kinase C (PKC)[3] phosphorylates a number of serines on p47^{phox}, and activation of PKC by phorbol diesters is a potent stimulus for oxidase activity (4–7). Two mitogen-activated protein kinases (MAPK), extracellular signal-regulated kinase (ERK) and p38 MAPK, phosphorylate p47^{phox} in human neutrophils (4, 6, 8). Inhibitors of each of these kinases have been reported to attenuate neutrophil respiratory burst activity stimulated by specific agonists (4, 5, 9–14). Other kinases that are reported to phosphorylate p47^{phox} include a phosphatidic acid-activated kinase and casein kinase-2 (15, 16).

Neutrophil stimulation by chemoattractants and FcR, but not phorbol diesters, results in activation of phosphatidylinositol 3-kinase (PI-3K), leading to the generation of phosphatidylinositol 3,4,5-trisphosphate and phosphatidylinositol 3,4-bisphosphate (17). Pharmacologic inhibition of PI-3K activity or genetic depletion of PI-3K blocks neutrophil respiratory burst activity stimulated by fMLP, C5a, and IL-8, while phorbol diester-stimulated activity is unaffected (17–22). Inhibition of PI-3K also blocks respiratory burst activity in human neutrophils stimulated by anti-neutrophil cytoplasmic Abs (23). Ding et al. (19) reported that inhibition of PI-3K activity reduced fMLP-, but not phorbol diester-induced p47^{phox} phosphorylation by 50%.

The mechanism by which PI-3K regulates p47^{phox} phosphorylation and respiratory burst activity has not been determined. Increased ERK and p38 MAPK activity following stimulation of human neutrophils by fMLP, immune complexes, and bacterial phagocytosis is dependent on PI-3K activity (10–12, 24, 25). Another PI-3K-dependent kinase activated in human neutrophils by chemoattractants, chemokines, cytokines, and immune complexes is Akt (26–28). Although Akt plays an important role in constitutive neutrophil apoptosis (27, 28), regulation of respiratory burst

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[3] Abbreviations used in this paper: PKC, protein kinase C; ERK, extracellular signal-regulated kinase; IPG, immobilized pH gradient; KRPB, Krebs-Ringer phosphate buffer; MAPK, mitogen-activated protein kinase; PI-3K, phosphatidylinositol 3-kinase.

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activity or phosphorylation of p47^{phox} by Akt has not been examined previously. The present study was initiated to determine whether Akt plays a role in respiratory burst activity. We report that inhibition of Akt activity by an inhibitory peptide significantly attenuates respiratory burst activity stimulated by fMLP or bacterial phagocytosis. Additionally, we show that Akt directly interacts with and phosphorylates p47^{phox}.

Materials and Methods

Materials

An Akt inhibitory peptide (ARKRERTYSFGHHA), based on the report of Obata et al. (29), and a scrambled peptide (HAKEAYGHARRPRA) were synthesized by the Peptide Synthesis Facility, University of Kentucky (Lexington, KY).

Expression of recombinant proteins

Escherichia coli BL21(Lys) (Invitrogen, San Diego, CA) was transformed with plasmid pGEX-2T-p47^{phox} (provided by M. Yaffe, Massachusetts Institute of Technology, Boston, MA) or pGEX-2T. Purified GST and GST-p47^{phox} fusion protein were stored bound to glutathione-Sepharose in PBS containing 0.5 mM DTT and 0.2 mM PMSF at 4°C. Full-length p47^{phox} cDNA was subcloned from pGEX-2T-p47^{phox} into the *Bam*HI/*Eco*RI site of the *E. coli* expression vector pRSETA (Invitrogen), which was used for bacterial expression and purification of recombinant p47^{phox}. Ser^{304} and Ser^{328} on p47^{phox} were mutated to alanine with the Clontech (Palo Alto, CA) site-directed mutagenesis kit. The mutation and selection primers for Ser^{304} were 5′-CCCGCAGGTCGGCCATCCGCAA-3′ and 5′-GGAATT CGAACCTTGATCCGG-3′, respectively. The mutation and selection primers for Ser^{328} were 5′-CTATCGCCGCAACGCCGTCCGTTTTC-3′ and 5′-GGAATTCGAACCTTGATCCGG-3′, respectively. The selection primer 5′-GAGTGGAAGGAGTTCGAAGCTTG-3′ was used for the double mutant. Mutations were verified by DNA sequencing.

Neutrophil isolation

Neutrophils were isolated from healthy donor blood using plasma-Percoll gradients, as described by Haslett et al. (30). Trypan blue staining revealed that at least 97% of cells were neutrophils with greater than 95% viability. After isolation, neutrophils were suspended in Krebs-Ringer phosphate buffer (KRPB), pH 7.2, at the desired concentration. The Human Studies Committee of the University of Louisville approved use of human donors.

Akt in vitro kinase assay

Active recombinant Akt (400 ng) (Upstate Biotechnology, Lake Placid, NY) was incubated with 10 μCi [γ-^{32}P]ATP (167 TBq/mmol; ICN Biomedicals, Irving, CA) and 1 μg recombinant p47^{phox} in 20 μl of kinase buffer containing 20 mM HEPES,10 mM $MgCl_2$, and 10 mM $MnCl_2$. Reactions were incubated at room temperature for 2 h and terminated by addition of Laemmli SDS sample dilution buffer. Proteins were separated by 10% SDS-PAGE, and phosphorylation was visualized by autoradiography.

Two-dimensional gel electrophoresis

Recombinant p47^{phox} was incubated in the presence or absence of active recombinant Akt, as described above. Proteins were separated by isoelectric focusing with immobilized pH gradient (IPG) strips (7 cm, pH 7–10; Bio-Rad, Hercules, CA), followed by 10% SDS-PAGE. IPG strips were hydrated with the kinase reaction mixture and rehydration buffer (7 M urea, 2 M thiourea, 2% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, 0.01 M DTT, 2% ampholytes (pH 3–10), 0.01% bromphenol blue) to give a total volume of 135 μl. Following overnight hydration, IPG gels were isoelectrically focused at 8000 V_{max} and 50 μA/gel for 20,000 V hours. Following focusing, IPG gels were incubated for 10 min with buffer containing 6 M urea, 2% DTT, and 30% glycerol, and then incubated for 10 min in buffer containing 6 M urea, 2.5% iodoacetamide, and 30% glycerol. IPG gels were then applied to 10% SDS-PAGE, and proteins were electrophoretically transferred to nitrocellulose and immunoblotted with anti-p47^{phox} Ab (Upstate Biotechnology) or with phospho-Akt substrate Ab (Cell Signaling Technology, Boston, MA).

In vitro protein expression

A total of 1 μg of Akt template DNA to be transcribed and translated was added to 40 μl of coupled transcription translation rabbit reticulocyte lysate (Promega, Madison, WI). Transcription/translation was performed for 90 min at 30°C in the presence of 20 μCi of [^{35}S]methionine (Amersham Pharmacia Biotech, Piscataway, NJ).

GST pull down of neutrophil lysate

Neutrophil lysates were prepared by suspending 2×10^7 cells in 200 μl of lysis buffer containing 1% Nonidet P-40, 10% glycerol, 137 mM NaCl, 20 mM Tris-HCl, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 4 mM PMSF, 20 mM NaF, 1 mM Na_3VO_4, and 1% Triton X-100. A total of 10 μl of glutathione-Sepharose beads coupled to GST or GST-p47^{phox} was incubated with 100 μl of neutrophil lysate at 4°C overnight. Following incubation, beads were washed four times with lysis buffer, and bound proteins were resolved by SDS-PAGE and detected by immunoblotting with anti-Akt pleckstrin homology domain specific Ab (Upstate Biotechnology).

GST pull down of ^{35}S-labeled proteins

A total of 10 μl of Akt rabbit reticulocyte lysate was added to 40 μl p47^{phox}-GST-Sepharose or GST-Sepharose equilibrated in 25 mM Tris-HCl, pH 7.9, 1 mM DTT, 150 mM NaCl, 0.01% Nonidet P-40, and 25 mM $MgCl_2$. Binding was conducted at 4°C overnight. The bound complexes were washed twice by centrifugation with 1 ml of 25 mM Tris-HCl (pH 7.9), 150 mM NaCl, 1 mM DTT, 0.05% Nonidet P-40, and 25 mM $MgCl_2$. Sepharose was transferred to fresh tubes and washed twice with the equilibration buffer lacking Nonidet P-40. Pelleted Sepharose was boiled in 20 μl Laemmli buffer, and eluted proteins were separated by SDS-PAGE. The gel was fixed with 50% methanol, 40% water, and 10% acetic acid for 30 min; rinsed with 7% methanol, 7% acetic acid, and 1% glycerol; and dried at 80°C for 40 min with a gel dryer. Radiolabeled proteins were visualized by autoradiography.

Superoxide production

Release of O_2^- by neutrophils was measured in duplicate samples by cytochrome *c* reduction at 550 nm in spectrophotometer, as previously described (31). A total of 5×10^6 neutrophils was incubated at room temperature for 1 h in the presence of 200 μM of Akt inhibitory peptide and 200 μM scrambled peptide, or for 10 min in KRPB with 100 nM wortmannin. Cells were subjected to hypotonic shock by addition of distilled water for 5 s to release endocytosed peptides, as previously reported by Zu et al. (32). Cells were then incubated with cytochrome *c* in KRPB buffer and stimulated with 3×10^{-7} M fMLP.

Phagocytic activity and H_2O_2 production

Phagocytosis and H_2O_2 production by neutrophils were measured by a flow cytometric assay, as previously described (31). A total of 5×10^6 PMNs was incubated at room temperature for 1 h in the presence of 200 μM of Akt inhibitory peptide, 200 μM scrambled peptide, or Krebs buffer, followed by hypotonic shock. Neutrophils were loaded with 2′,7′-dichloroflourescin diacetate (Molecular Probes, Eugene, OR) and then incubated with heat-fixed, propidium iodide-labeled *S. aureus* that was opsonized with human plasma for 10 min. Hydrogen peroxide production was measured by the hydrolysis of dichlorofluorescein to its fluorescent analog by flow cytometry. The flow cytometer was calibrated before the analysis of each set of samples with Standard-Brite beads (Corixa, Seattle, WA).

Chemotaxis

Neutrophils were washed and resuspended in KRPB. Cells were added to polypropylene microtubes (upper chambers), and fMLP (0.3 μM final concentration) was added to the bottom of the transwells (lower chambers). Transwells were incubated at 37°C with 5% CO_2 in the tissue culture incubator for 30 min. Following incubation, the polyester membranes were fixed and stained with H&E and dried at room temperature overnight. Membranes were cut and fixed on glass slides, keeping the bottom surface upright, and viewed by light microscopy using ×100 magnification. Cells within the scale that had passed through the pores and were at the focal plane of the pores were counted. Results are expressed as the mean + SEM number of cells migrating across a 6.5-mm-diameter circle of the membrane.

Statistical analysis

Statistical analysis by one-way or two-way ANOVA was performed using GraphPad Instat (GraphPad Software, San Diego, CA). Differences between groups were determined using Bonferroni's posttest, and significance was defined as $p < 0.05$.

Results

To determine whether Akt contributes to neutrophil respiratory burst activity, a peptide that acts as an Akt substrate, reported previously by Obata et al. (29), was introduced into neutrophils by

hypotonic shock. To establish the ability of this peptide to inhibit Akt phosphorylation of substrate, a dose inhibition of in vitro Akt activity was performed. Fig. 1*A* shows the concentration-dependent inhibition of Akt phosphorylation of histone by Akt inhibitory peptide, which was partially inhibitory at 80 and 100 μM and was maximally inhibitory at 200 μM. Fig. 1*B* compares the ability of Akt inhibitory peptide and a scrambled peptide to inhibit Akt phosphorylation of histone in an in vitro kinase assay. As shown in Fig. 1*A*, 100 μM Akt inhibitory peptide partially inhibited phosphorylation of recombinant histone, while 200 μM markedly reduced Akt activity, compared with 200 μM of a scrambled peptide. Confocal microscopy was used to confirm the introduction of peptide into neutrophils using hypotonic shock and to establish peptide distribution. Fluorescein-labeled peptide at 100 or 200 μM was incubated with neutrophils, which were then subjected to hypotonic shock. Confocal microscopy showed diffuse cytosolic staining of all cells, with more intense staining of cells incubated with 200 μM (data not shown).

To determine the role of Akt in respiratory burst activity, neutrophils were loaded with various concentrations of Akt inhibitory peptide or scrambled peptide or pretreated with 100 nM of the PI-3K inhibitor wortmannin. All cells were exposed to hypotonic shock, whether or not peptide was introduced. Fig. 2*A* shows the effect of these treatments on fMLP-stimulated superoxide release. Akt inhibitory peptide induced a dose-dependent reduction in superoxide release, which was significant at concentrations of 50 μM or greater. Akt inhibitory peptide at 200 μM completely blocked fMLP-stimulated superoxide release. Pretreatment with wortmannin also blocked superoxide release, while the scrambled peptide had no effect at either 100 or 200 μM. Separate studies determined that introduction of 200 μM Akt inhibitory peptide or 200 μM scrambled peptide had no effect on phorbol diester (PMA)-induced superoxide relase (Fig. 2*B*). To determine whether Akt plays a role

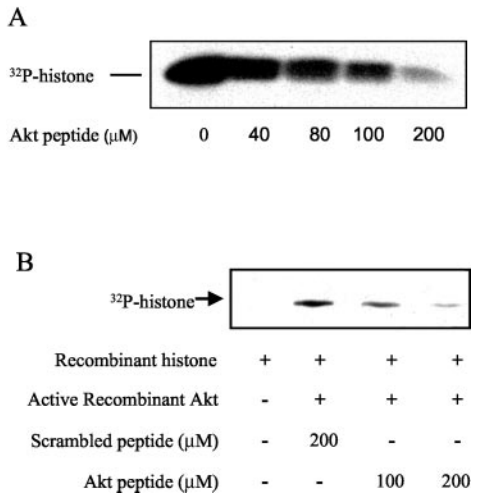

FIGURE 1. Inhibition of Akt activity by Akt inhibitory peptide. *A*, An in vitro kinase assay with active recombinant Akt and histone as substrate was performed in the presence or absence of various concentrations of Akt inhibitory peptide. The figure shows an autoradiograph of phosphorylated histone. A concentration-dependent reduction in histone phosphorylation is shown, with 200 μM of inhibitory peptide maximally inhibiting Akt activity. *B*, Comparison of the ability of Akt inhibitory peptide and scrambled peptide to alter Akt phosphorylation of histone. Akt inhibitory peptide at 100 and 200 μM and scrambled peptide at 200 μM were added to an in vitro kinase assay containing active recombinant Akt and histone as substrate. As shown in *A*, 200 μM Akt inhibitory peptide markedly reduced Akt phosphorylation of histone, compared with the addition of scrambled peptide.

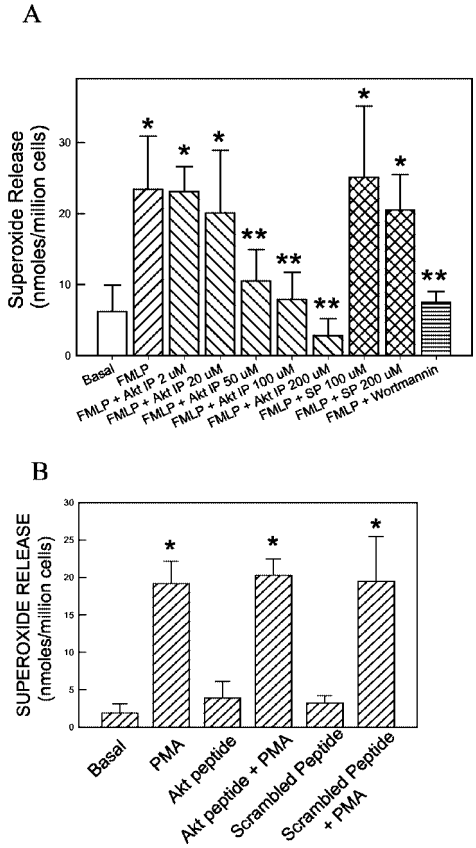

FIGURE 2. Role of Akt in fMLP-stimulated superoxide release. *A*, The release of superoxide from neutrophils pretreated with or without various concentrations of Akt inhibitory peptide (Akt-IP) ranging from 2 to 200 μM, scrambled peptide (SP) at 100 and 200 μM, or 100 nM wortmannin, followed by stimulation for 5 min with 3×10^{-7} M fMLP was determined. All cells were subjected to hypotonic shock before addition of fMLP. Results are expressed as mean $\pm$ SEM in nmol of reduced cytochrome $c/10^6$ cells for at least five separate experiments. fMLP alone, in cells pretreated with Akt inhibitory peptide at 2 or 20 μM, or in cells pretreated with scrambled peptide stimulated a significant increase in superoxide release (*, $p < 0.001$). Wortmannin and Akt inhibitory peptide at 50, 100, and 200 μM significantly reduced fMLP-stimulated superoxide release (**, $p < 0.001$). Pretreating cells with 100 or 200 μM Akt inhibitory peptide reduced superoxide release to or below basal levels. *B*, The release of superoxide from neutrophils pretreated with or without 200 μM Akt inhibitory peptide or 200 μM scrambled peptide before incubation with 100 nM PMA for 20 min. The results are expressed as mean $\pm$ SEM for three separate experiments. PMA stimulated a significant increase (*, $p < 0.05$) in superoxide release in the presence or absence of peptides, and neither peptide significantly reduced PMA-stimulated superoxide release.

in respiratory burst activity stimulated by other physiologically relevant stimuli, the effect of Akt inhibitory peptide or scrambled peptide on hydrogen peroxide production induced by bacterial phagocytosis was examined. Akt inhibitory peptide significantly reduced hydrogen peroxide production (Fig. 3*A*) without significantly altering bacterial phagocytosis (Fig. 3*B*). The scrambled peptide did not alter either phagocytosis or hydrogen peroxide production. Thus, inhibition of Akt activity attenuates respiratory burst activity stimulated by either chemotactic peptides or phagocytosis.

Based on these data and previous reports that PI-3K inhibition blocks phosphorylation of $p47^{phox}$ (19), we postulated that PI-3K-dependent phosphorylation of $p47^{phox}$ was mediated by Akt. To test this hypothesis, we determined whether there was a direct

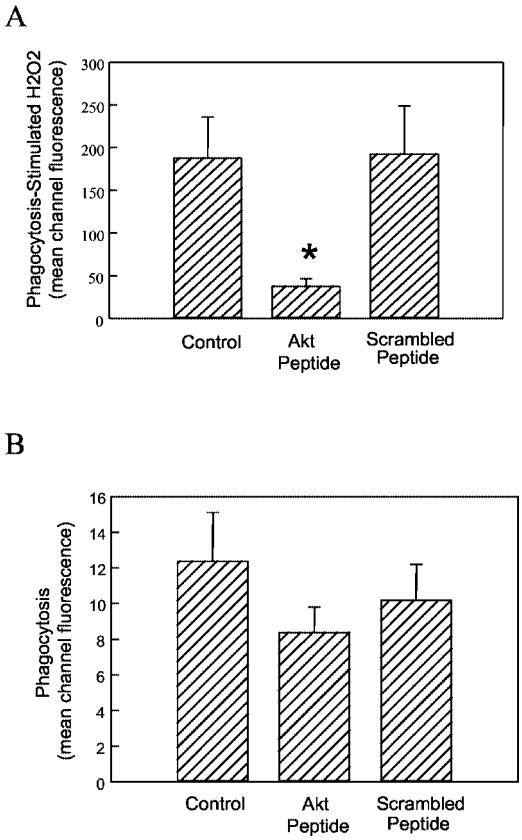

FIGURE 3. Role of Akt in phagocytosis-stimulated H_2O_2 production. Following pretreatment with 200 μM Akt inhibitory peptide or 200 μM scrambled peptide, neutrophils were loaded with 2′,7′-dichlorofluorescein before stimulation by incubation with propidium iodide-labeled, opsonized, killed *Staphylococcus aureus*. All cells were subjected to hypotonic shock before addition of bacteria. Phagocytosis and H_2O_2 oxidation of dichlorofluorescein were measured by a flow cytometric assay. *A*, Shows the results of H_2O_2 production as mean ± SEM of mean channel fluorescence for four separate experiments. Pretreatment with the Akt inhibitory peptide significantly reduced H_2O_2 production, and compared control and scrambled peptide, $p < 0.05$. *B*, Shows the results of phagocytosis for the same four experiments, measured as fluorescence intensity of propidium iodide-labeled bacteria. Neither Akt inhibitory peptide nor scrambled peptide significantly altered phagocytosis.

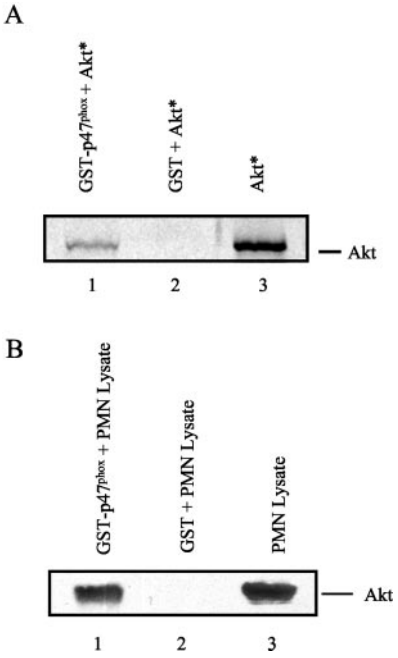

FIGURE 4. GST pull down demonstrates the Akt and $p47^{phox}$ interact. *A*, Shows a pull down of [^{35}S]methionine-labeled Akt (Akt*) with GST-$p47^{phox}$-coupled glutathione-Sepharose or GST-coupled glutathione-Sepharose. Following 10% SDS-PAGE, autoradiography was performed. *Lane 3*, Loaded with labeled Akt alone. *Lane 1*, Shows that GST-$p47^{phox}$ precipitated Akt, while GST-coupled Sepharose alone failed to precipitate Akt (*lane 2*). *B*, Shows a pull down of neutrophil lysate with GST-$p47^{phox}$-coupled glutathione-Sepharose or GST-coupled glutathione-Sepharose. Following precipitation, proteins were separated by 10% SDS-PAGE, transferred to nitrocellulose electrophoretically, and immunoblotted for Akt. The immunoblot shows that GST-$p47^{phox}$ precipitates Akt from neutrophil lysates, while GST-Sepharose alone did not precipitate Akt.

interaction between $p47^{phox}$ and Akt and whether Akt phosphorylated $p47^{phox}$. To determine whether there was a direct interaction between $p47^{phox}$ and Akt, GST pull down assays using GST-$p47^{phox}$-coupled glutathione-Sepharose were performed. Fig. 4*A* shows the results of a GST-$p47^{phox}$ pull down of [^{35}S]methionine-labeled Akt produced by rabbit reticulocyte lysate transcription and translation. Akt was precipitated by GST-$p47^{phox}$, but not by GST-coupled glutathione-Sepharose alone. To determine whether Akt interacted with $p47^{phox}$ in neutrophils, a GST pull down of neutrophil lysate was performed, followed by immunoblotting of the precipitate for Akt. Fig. 4*B* shows that GST-$p47^{phox}$-coupled glutathione-Sepharose precipitated Akt from neutrophil lysate, while GST-coupled glutathione-Sepharose did not. These data suggest that Akt is able to directly interact with $p47^{phox}$.

To determine whether Akt is capable of phosphorylating $p47^{phox}$, an in vitro kinase assay was performed in which active recombinant Akt was added to recombinant $p47^{phox}$ in the presence and absence of Akt inhibitory peptide or scrambled peptide. Fig. 5*A* shows autophosphorylation of active recombinant Akt and Akt phosphorylation of $p47^{phox}$ in the absence of peptides and in the presence of 200 μM scrambled peptide. Addition of 200 μM Akt inhibitory peptide blocked both autophosphorylation and Akt phosphorylation of $p47^{phox}$. The ability of Akt to phosphorylate $p47^{phox}$ was confirmed using two-dimensional gel separation of recombinant $p47^{phox}$ following an in vitro kinase assay with active recombinant Akt. Phosphorylation of proteins results in addition of negative charge, leading to a shift in position of the protein toward the basic pole of an isoelectric focusing gel. Fig. 5*B* shows an immunoblot of these two-dimensional gels with Abs directed against $p47^{phox}$ or Abs that recognize phosphorylated Akt substrates. In the absence of active Akt, immunoblotting for $p47^{phox}$ showed two separate proteins at the same pI (*a*), while immunoblotting for phospho-Akt substrate was negative (*c*). Addition of active recombinant Akt resulted in the appearance of a negatively charged form of $p47^{phox}$ (*b*), consistent with phosphorylation of $p47^{phox}$. This new protein spot was also recognized by the phospho-Akt substrate Ab (*d*). Taken together, these data support the hypothesis that Akt phosphorylates $p47^{phox}$.

Scansite, a Web-interfaced program using motif-based profile scanning (http://scansite.mit.edu) (33), found four potential sites of Akt phosphorylation, Thr^{45}, Thr^{93}, Ser^{304}, and Ser^{328}, when low stringency was used. The consensus motif for Akt phosphorylation is reported as RXRXXpS/T, in which X is any amino acid, R represents arginine, and pS/T phosphorylated serine or threonine (29). Phosphorylation of Ser^{304} and Ser^{328} on $p47^{phox}$ was reported previously to be necessary for assembly and activation of the NADPH oxidase (34). The amino acid sequences of these sites are PPRRSS (304) and AYRRNS (328). Based on the identification of

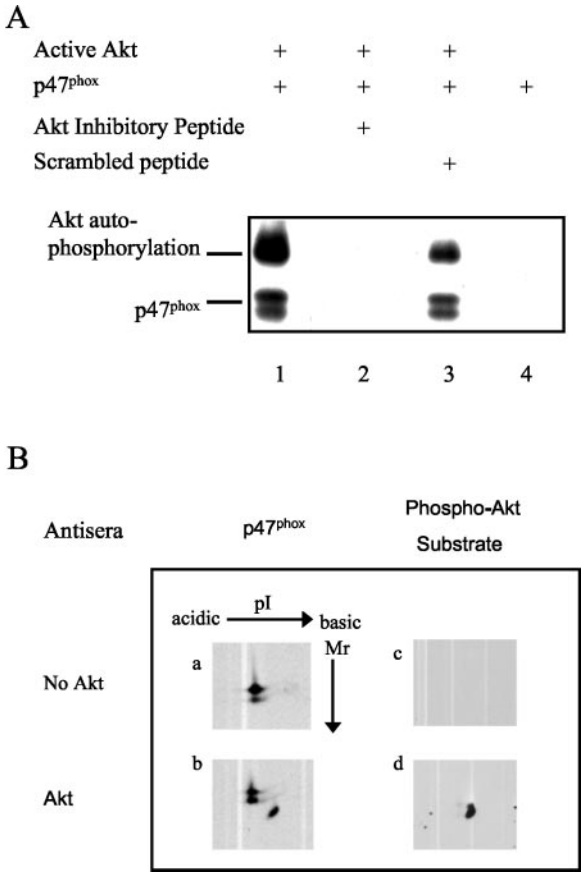

FIGURE 5. Akt phosphorylates p47^{phox}. *A*, Shows the results of an in vitro kinase assay in which active recombinant Akt was incubated with recombinant p47^{phox} and [^{32}P]ATP in the presence or absence of 200 μM Akt inhibitory peptide or 200 μM scrambled peptide. An autoradiogram of protein separated by 10% SDS-PAGE is shown. Akt underwent autophosphorylation, and p47^{phox} was phosphorylated in the presence of active Akt alone or Akt and scrambled peptide. Akt inhibitory peptide blocked both p47^{phox} phosphorylation and Akt autophosphorylation. *B*, Shows the immunoblots of two-dimensional gels using antisera toward p47^{phox} or phosphorylated Akt substrate. Recombinant p47^{phox} was incubated in the presence or absence of active recombinant Akt and ATP for 30 min at 30°C, then the products were separated by two-dimensional gel electrophoresis and transferred to nitrocellulose. In the absence of Akt (*a*), two isoforms of p47^{phox} at the same pI were identified by immunoblot analysis with anti-p47^{phox} Ab. Immunoblot analysis for phosphorylated Akt substrate (*c*) failed to recognize any protein. Following incubation with active recombinant Akt, immunoblot analysis revealed the appearance of a new, negatively charged form of p47^{phox} (*c*). This same protein spot was recognized by anti-Akt phospho-substrate Ab (*d*).

Ser^{304} and Ser^{328} as potential Akt phosphorylation sites and their importance in p47^{phox} function, we mutated these sites to alanine and examined the ability of Akt to phosphorylate wild-type and mutant p47^{phox}. Fig. 6 shows Akt phosphorylation of p47^{phox} was reduced when either Ser^{304} or Ser^{328} was mutated to alanine, and Akt-induced phosphorylation was almost completely absent when both sites were mutated to alanine. These data suggest that Akt is capable of phosphorylating two serine residues that have been shown previously to be necessary for p47^{phox} assembly and activation of the NADPH oxidase (34).

Previous reports indicate that PI-3K activity is critical to neutrophil chemotaxis (20–22, 35). The pleckstrin homology domain of Akt is selectively recruited to the leading edge of chemotaxing neutrophils (36, 37). To determine whether Akt activity plays a

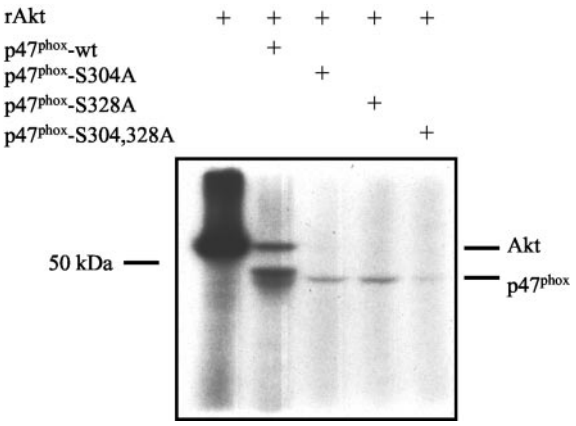

FIGURE 6. Akt phosphorylates Ser^{304} and Ser^{328} on p47^{phox}. The ability of active recombinant Akt to phosphorylate mutants of p47^{phox} in an in vitro kinase assay. Equivalent amounts of wild-type p47^{phox}, p47^{phox}-S304A, p47^{phox}-S328A, or p47^{phox}-S304,328A were incubated with 400 ng active recombinant Akt and [^{32}P]ATP for 2 h before termination by addition of Laemmli SDS sample dilution buffer and separation by 10% SDS-PAGE. Phosphorylation was detected by autoradiography. *Lane 1*, Demonstrates autophosphorylation of Akt that is reduced in the presence of wild-type p47^{phox} (p47^{phox}-wt, *lane 2*) and completely absent in the presence of the mutants. Phosphorylation of p47^{phox}-S304A (*lane 3*) and p47^{phox}-S328A (*lane 4*) was reduced compared with p47^{phox}-wt (*lane 2*), and phosphorylation of p47^{phox}-S304,328A (*lane 5*) was barely detectable.

role in chemotaxis, neutrophils were pretreated with various concentrations of Akt inhibitory peptide or 200 μM of scrambled peptide before measurement of fMLP-stimulated migration across a polyester membrane. Fig. 7 shows a concentration-dependent inhibition of chemotaxis by Akt inhibitory peptide. Chemotaxis was numerically, but not significantly, reduced at 100 μM, while 200 μM significantly inhibited chemotaxis. The scrambled peptide had no significant effect. These data indicate that Akt activation participates in neutrophil chemotaxis, as well as respiratory burst activity.

Discussion

A number of studies indicate that PI-3K plays a critical role in neutrophil respiratory burst activity. Pharmacologic inhibition of

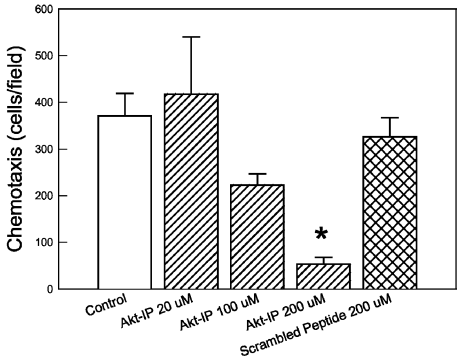

FIGURE 7. Role of Akt in neutrophil chemotaxis. Neutrophils were pretreated with hypotonic shock alone (control), or with 20, 100, or 200 μM Akt inhibitory peptide (Akt-IP), or with 200 μM of scrambled peptide before assaying migration across a polyethylene membrane stimulated by 3×10^{-7} M fMLP. Pretreatment with 100 μM Akt inhibitory peptide numerically, but not significantly, reduced the number of migrating cells, while 200 μM significantly reduced neutrophil chemotaxis (*, $p < 0.01$). The scrambled peptide had no significant effect on chemotaxis. Results are expressed as mean ± SEM in number of cells crossing the member per 6.5-mm-diameter field for four separate experiments.

PI-3K with wortmannin or LY294002 inhibits respiratory burst activity stimulated by chemoattractant receptors (11, 17–19, 24), bacterial phagocytosis (12), and anti-neutrophil cytoplasmic Abs (23). In contrast, stimulation of PKC by phorbol diesters does not activate PI-3K, and inhibition of PI-3K does not alter phorbol diester-stimulated respiratory burst activity (17, 19). PI-3Kγ-deficient mice demonstrate impaired fMLP-stimulated neutrophil respiratory burst activity (20–22), while phorbol diester stimulation of superoxide release is not affected (22). Finally, introduction of membrane-targeted PI-3K into the GM-1 monoblastic cell line resulted in NADPH oxidase assembly (38). Thus, PI-3K and PKC are members of two independent pathways leading to respiratory burst activity in neutrophils.

The mechanism(s) by which PI-3K regulates respiratory burst activity has not previously been identified. Activation of a number of neutrophil kinases is dependent on PI-3K activity, including Akt (26, 39), ERK (10–12, 25), p38 MAPK (10, 11), and several unidentified kinases (19). Inhibition of both ERK and p38 MAPK attenuates neutrophil respiratory burst activity (10–14, 24, 40), suggesting that either of these kinases could be components of the PI-3K-dependent pathway. The results of our study suggest that Akt also mediates PI-3K-dependent NADPH oxidase activation in human neutrophils. Inhibition of Akt activity by an inhibitory peptide significantly attenuated fMLP-stimulated, but not PMA-stimulated, respiratory burst activity. This role of Akt is not specific for G protein-coupled chemoattractant receptors, as the inhibitory peptide also attenuated respiratory burst activity stimulated during bacterial phagocytosis.

Inhibition of PI-3K activity by genetic deletion or pharmacologic inhibition was shown previously to inhibit neutrophil chemotaxis (20–22, 35). Sevant et al. (36) and Hannigan et al. (37) demonstrated that green fluorescent protein-labeled Akt pleckstin homology domain localizes to the leading edge of chemotaxing neutrophils, suggesting that PI-3K activation is occurring primarily at that location. These observations suggested that Akt activity may play a role in neutrophil chemotaxis. In the present study, inhibition of Akt activity by introduction of an inhibitory peptide markedly inhibited chemoattractant-stimulated chemotaxis. Thus, our data support a role for Akt in neutrophil chemotaxis, as well as respiratory burst activity.

Akt is a serine/threonine kinase activated by growth factors, cytokines, insulin, and G protein-coupled receptors in a PI-3K-dependent manner. Akt plays a role in a number of cellular processes, including glucose metabolism, cell proliferation, apoptosis, and gene transcription. Akt was shown previously to be activated in human neutrophils by fMLP, leukotriene B_4, IL-8, FcRγ cross-linking, LPS, GM-CSF, and anti-neutrophil cytoplasmic Abs (23, 26–28, 39). We reported previously that Akt activity was necessary for delay of constitutive neutrophil apoptosis by GM-CSF, IL-8, leukotriene B_4, and LPS (27, 28). The absence of respiratory burst activity in the presence of optimal concentrations of Akt inhibitory peptide suggests that Akt activity is necessary for NADPH oxidase activity. The ability of GM-CSF and LSP to stimulate Akt activity without stimulating respiratory burst activity suggests that Akt activation alone is insufficient to induce NADPH oxidase activity. Thus, activation of Akt appears to be necessary, but not sufficient, for respiratory burst activity.

The present study establishes Akt as a PI-3K-dependent kinase that directly interacts with and phosphorylates $p47^{phox}$. Previous reports indicate that $p47^{phox}$ phosphorylation during receptor-mediated neutrophil activation is dependent on PI-3K activity. Ding et al. (19) reported that pretreatment of guinea pig neutrophils with wortmannin or LY294002 inhibited fMLP-stimulated, but not phorbol diester-stimulated, $p47^{phox}$ phosphorylation by ~50%. Didichenko et al. (38) reported that expression of membrane-targeted PI-3K in a monoblastic cell line resulted in wortmannin-sensitive phosphorylation of $p47^{phox}$.

Phosphorylation of $p47^{phox}$ is necessary for its translocation to the plasma membrane and for its ability to function as an adaptor protein for assembly of NADPH oxidase components (3, 7, 34, 41–43). At least nine different serines on the C-terminal portion of $p47^{phox}$ are phosphorylated during activation, and phosphorylation of serines at positions 303, 304, 328, 359, and 370 have been reported to play a role in NADPH oxidase activation (6, 34, 43). A number of kinases are reported to phosphorylate $p47^{phox}$, including certain PKC isoforms, ERK, p38 MAPK, casein kinase 2, p21-associated kinase, and a phosphatidic acid-dependent kinase (4–6, 15, 16). Of these, PKCζ and ERK have been shown to mediate fMLP-stimulated phosphorylation of $p47^{phox}$, while inhibition of p38 MAPK had no effect on $p47^{phox}$ phosphorylation (4, 5). Dewas et al. (4) reported that inhibition of PKC and ERK1/2 resulted in an additive inhibition of $p47^{phox}$ phosphorylation. Using motif-based profile scanning (33), we identified two C-terminal serine residues, Ser^{304} and Ser^{328}, as potential sites for Akt phosphorylation. Ago et al. (34) reported that mutation of $Ser^{303/304}$ and Ser^{328} to aspartic acid or glutamic acid resulted in $p47^{phox}$ activation of NADPH oxidase activity under cell-free conditions. Additionally, substitution of alanine for these three serines led to defective superoxide production. Our results indicate that both Ser^{304} and Ser^{328} are phosphorylated by Akt. Taken together, these results suggest that PI-3K-mediated Akt activation is required for receptor-stimulated neutrophil respiratory burst activity, and Akt phosphorylates a component of the NADPH oxidase, $p47^{phox}$, on serine residues known to control oxidase activity.

Based on our results and previous studies, we propose a model of signal transduction pathways leading to respiratory burst activity shown in Fig. 8. Multiple independent pathways are stimulated by various plasma membrane receptors. One of these pathways leads to PKC activation and partial phosphorylation of $p47^{phox}$. Another pathway results in PI-3K-mediated activation of Akt and ERK1/2. Both Akt and ERK1/2 mediate partial phosphorylation of $p47^{phox}$. The combined phosphorylation of $p47^{phox}$ by ERK, Akt, and PKC results in translocation of $p47^{phox}$ to the plasma membrane and induction of the adaptor function, leading to assembly of the NADPH oxidase.

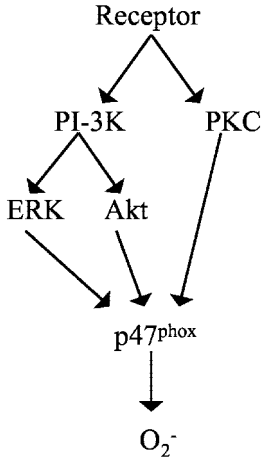

FIGURE 8. Model of PI-3K regulation of respiratory burst activity. Postulated pathways by which PI-3K regulates NADPH oxidase assembly and activity. See text for details.

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