

Luminex
complexity simplified.



**Flexible, Intuitive, and
Affordable Cytometry.**

LEARN MORE >

Guava® easyCyte™ Flow Cytometers.

 **The Journal of
Immunology**

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

This information is current as
of October 17, 2019.

Qingdan Chen, David W. Powell, Madhavi J. Rane, Saurabh
Singh, Waseem Butt, Jon B. Klein and Kenneth R. McLeish

J Immunol 2003; 170:5302-5308; ;
doi: 10.4049/jimmunol.170.10.5302
<http://www.jimmunol.org/content/170/10/5302>

References This article **cites 43 articles**, 30 of which you can access for free at:
<http://www.jimmunol.org/content/170/10/5302.full#ref-list-1>

Why *The JI*? [Submit online.](#)

- **Rapid Reviews! 30 days*** from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Fast Publication!** 4 weeks from acceptance to publication

**average*

Subscription Information about subscribing to *The Journal of Immunology* is online at:
<http://jimmunol.org/subscription>

Permissions Submit copyright permission requests at:
<http://www.aai.org/About/Publications/JI/copyright.html>

Email Alerts Receive free email-alerts when new articles cite this article. Sign up at:
<http://jimmunol.org/alerts>

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852
Copyright © 2003 by The American Association of
Immunologists All rights reserved.
Print ISSN: 0022-1767 Online ISSN: 1550-6606.



Akt Phosphorylates p47^{phox} and Mediates Respiratory Burst Activity in Human Neutrophils¹

Qingdan Chen,* David W. Powell,[†] Madhavi J. Rane,* Saurabh Singh,* Waseem Butt,* Jon B. Klein,*^{†‡} and Kenneth R. McLeish^{2*†‡}

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 ³²P 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, Ser³⁰⁴ and Ser³²⁸, 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 b₅₅₈ and Rap1A to form the active oxidase (1). The flavocytochrome b₅₅₈ 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 b₅₅₈ and p67^{phox} (2, 3).

Several kinases that phosphorylate components of the NADPH oxidase and regulated oxidase activity have been identified. Pro-

tein 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

Departments of *Medicine and [†]Biochemistry and Molecular Biology, University of Louisville, Louisville, KY 40202; and [‡]Veterans Affairs Medical Center, Louisville, KY 40206

Received for publication November 7, 2002. Accepted for publication March 20, 2003.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ This work was supported by grants from Department of Veterans Affairs (to K.R.M. and J.B.K.), the National Institutes of Health HL66358 and DK62086 (to J.B.K.), and the American Heart Association Ohio Valley Affiliate (to M.J.R.).

² Address correspondence and reprint requests to Dr. Kenneth R. McLeish, Molecular Signaling Group, Kidney Disease Program, Room 102, Baxter Building, 570 South Preston Street, University of Louisville, Louisville, KY 40202.

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

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}. Ser³⁰⁴ and Ser³²⁸ on p47^{phox} were mutated to alanine with the Clontech (Palo Alto, CA) site-directed mutagenesis kit. The mutation and selection primers for Ser³⁰⁴ were 5'-CCCGCAGGTCGGCCATCCGCAA-3' and 5'-GGAATTCGAACCTTGATCCGG-3', respectively. The mutation and selection primers for Ser³²⁸ were 5'-CTATCGCCGCAACGCGTCCGTTTC-3' and 5'-GGAATTCGAACCTTGATCCGG-3', respectively. The selection primer 5'-GAGTGAAGGAGTTCGAAGCTTG-3' was used for the double mutant. Mutations were verified by DNA sequencing.

Neutrophil isolation

Neutrophils were isolated from healthy donor blood using plasma-Percol 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 μ Ci [γ -³²P]ATP (167 TBq/mmol; ICN Biomedicals, Irving, CA) and 1 μ g recombinant p47^{phox} in 20 μ l of kinase buffer containing 20 mM HEPES, 10 mM MgCl₂, and 10 mM MnCl₂. 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% bromophenol blue) to give a total volume of 135 μ l. Following overnight hydration, IPG gels were isoelectrically focused at 8000 V_{max} and 50 μ A/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 μ g of Akt template DNA to be transcribed and translated was added to 40 μ l 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 μ Ci of [³⁵S]methionine (Amersham Pharmacia Biotech, Piscataway, NJ).

GST pull down of neutrophil lysate

Neutrophil lysates were prepared by suspending 2 \times 10⁷ cells in 200 μ l of lysis buffer containing 1% Nonidet P-40, 10% glycerol, 137 mM NaCl, 20 mM Tris-HCl, 1 μ g/ml aprotinin, 1 μ g/ml leupeptin, 4 mM PMSF, 20 mM NaF, 1 mM Na₃VO₄, and 1% Triton X-100. A total of 10 μ l of glutathione-Sepharose beads coupled to GST or GST-p47^{phox} was incubated with 100 μ l 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 ³⁵S-labeled proteins

A total of 10 μ l of Akt rabbit reticulocyte lysate was added to 40 μ l 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 MgCl₂. 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 MgCl₂. Sepharose was transferred to fresh tubes and washed twice with the equilibration buffer lacking Nonidet P-40. Pelleted Sepharose was boiled in 20 μ l 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 O₂⁻ by neutrophils was measured in duplicate samples by cytochrome *c* reduction at 550 nm in spectrophotometer, as previously described (31). A total of 5 \times 10⁶ neutrophils was incubated at room temperature for 1 h in the presence of 200 μ M of Akt inhibitory peptide and 200 μ M 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 \times 10⁻⁷ M fMLP.

Phagocytic activity and H₂O₂ production

Phagocytosis and H₂O₂ production by neutrophils were measured by a flow cytometric assay, as previously described (31). A total of 5 \times 10⁶ PMNs was incubated at room temperature for 1 h in the presence of 200 μ M of Akt inhibitory peptide, 200 μ M scrambled peptide, or Krebs buffer, followed by hypotonic shock. Neutrophils were loaded with 2',7'-dichlorofluorescein 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 μ M final concentration) was added to the bottom of the transwells (lower chambers). Transwells were incubated at 37°C with 5% CO₂ 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 \times 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 \pm 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. 1A 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. 1B 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. 1A, 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. 2A 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 release (Fig. 2B). To determine whether Akt plays a role

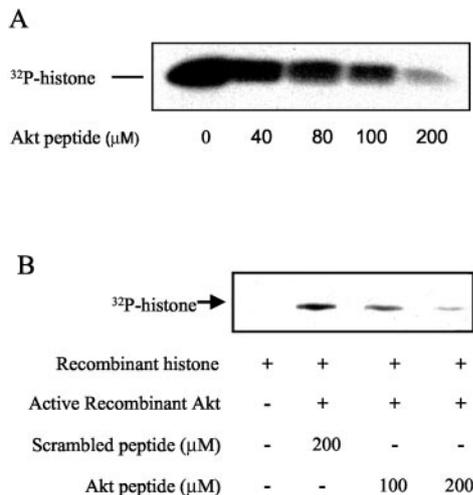


FIGURE 1. Inhibition of Akt activity by Akt inhibitory peptide. *A*, An in vitro kinase assay with active recombinant Akt and histone as substrate was performed in the presence or absence of various concentrations of Akt inhibitory peptide. The figure shows an autoradiograph of phosphorylated histone. 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.

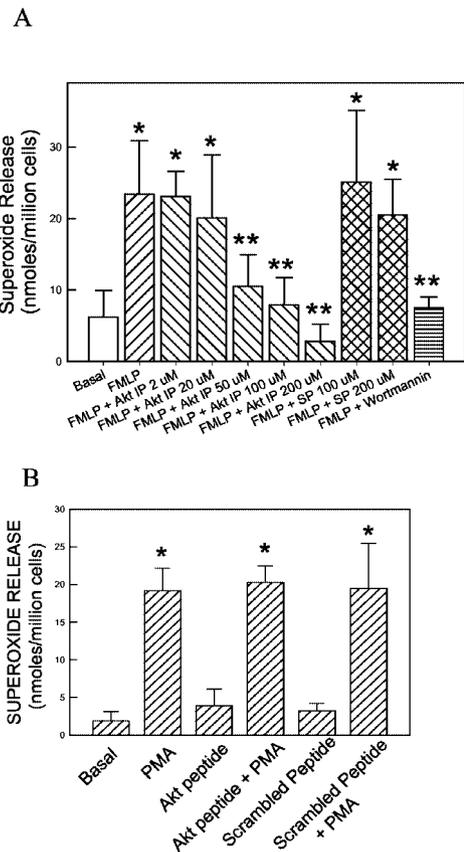


FIGURE 2. Role of Akt in fMLP-stimulated superoxide release. *A*, The release of superoxide from neutrophils pretreated with or without various concentrations of Akt inhibitory peptide (Akt-IP) ranging from 2 to 200 μM , scrambled peptide (SP) at 100 and 200 μM , or 100 nM wortmannin, followed by stimulation 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*/10⁶ 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. 3A) without significantly altering bacterial phagocytosis (Fig. 3B). 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

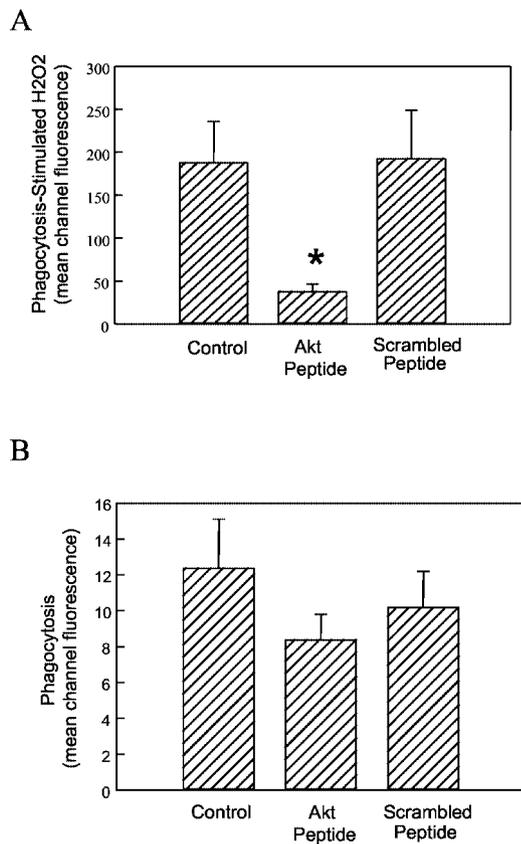


FIGURE 3. Role of Akt in phagocytosis-stimulated H₂O₂ production. Following pretreatment with 200 μ M Akt inhibitory peptide or 200 μ M scrambled peptide, neutrophils were loaded with 2',7'-dichlorofluorescein before stimulation by incubation with propidium iodide-labeled, opsonized, killed *Staphylococcus aureus*. All cells were subjected to hypotonic shock before addition of bacteria. Phagocytosis and H₂O₂ oxidation of dichlorofluorescein were measured by a flow cytometric assay. **A**, Shows the results of H₂O₂ production as mean \pm SEM of mean channel fluorescence for four separate experiments. Pretreatment with the Akt inhibitory peptide significantly reduced H₂O₂ 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.

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. 4A shows the results of a GST-p47^{phox} pull down of [³⁵S]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. 4B 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. 5A shows autophosphorylation of active recombinant Akt and Akt phosphorylation of p47^{phox} in the absence of peptides and in

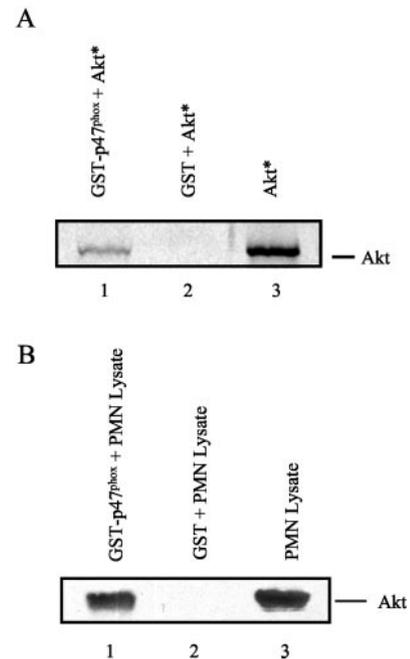


FIGURE 4. GST pull down demonstrates the Akt and p47^{phox} interact. **A**, Shows a pull down of [³⁵S]methionine-labeled Akt (Akt*) with GST-p47^{phox}-coupled glutathione-Sepharose or GST-coupled glutathione-Sepharose. Following 10% SDS-PAGE, autoradiography was performed. Lane 3, Loaded with labeled 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.

the presence of 200 μ M scrambled peptide. Addition of 200 μ M 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. 5B 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, Thr⁴⁵, Thr⁹³, Ser³⁰⁴, and Ser³²⁸, when low stringency was used. The consensus motif for Akt phosphorylation is reported as RXXRXXpS/T, in which X is any amino acid, R represents arginine, and pS/T phosphorylated serine or threonine (29). Phosphorylation of Ser³⁰⁴ and Ser³²⁸ 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

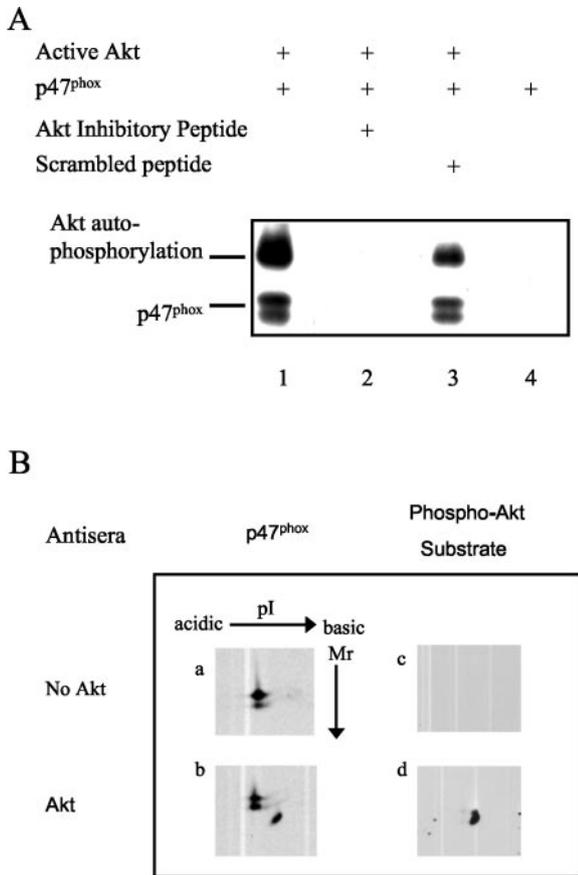


FIGURE 5. Akt phosphorylates p47^{phox}. *A*, Shows the results of an in vitro kinase assay in which active recombinant Akt was incubated with recombinant p47^{phox} and [³²P]ATP in the presence or absence of 200 μ M Akt inhibitory peptide or 200 μ M scrambled peptide. An autoradiogram of protein separated 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*).

Ser³⁰⁴ and Ser³²⁸ 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 Ser³⁰⁴ or Ser³²⁸ 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

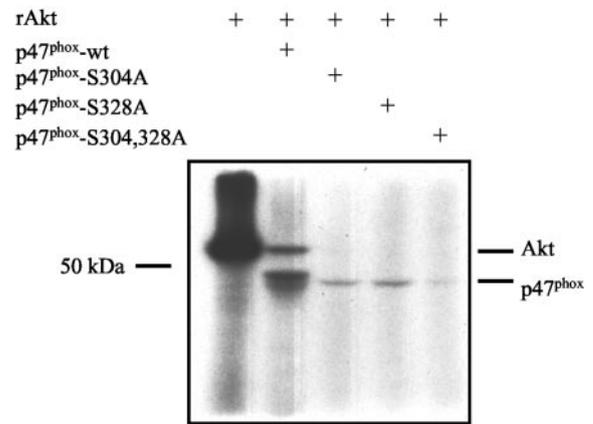


FIGURE 6. Akt phosphorylates Ser³⁰⁴ and Ser³²⁸ on p47^{phox}. The ability of active recombinant Akt to phosphorylate mutants of p47^{phox} in an in vitro kinase assay. Equivalent amounts of wild-type p47^{phox}, p47^{phox}-S304A, p47^{phox}-S328A, or p47^{phox}-S304,328A were incubated with 400 ng active recombinant Akt and [³²P]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 μ M 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 μ M, while 200 μ M 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

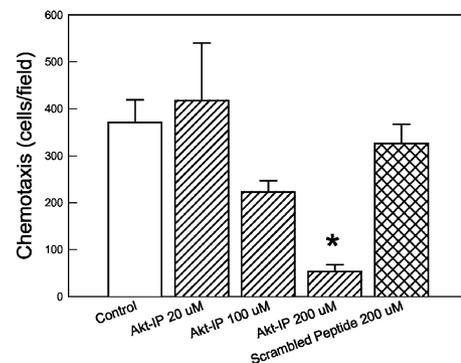


FIGURE 7. Role of Akt in neutrophil chemotaxis. Neutrophils were pretreated with hypotonic shock alone (control), or with 20, 100, or 200 μ M Akt inhibitory peptide (Akt-IP), or with 200 μ M of scrambled peptide before assaying migration across a polyethylene membrane stimulated by 3×10^{-7} M fMLP. Pretreatment with 100 μ M Akt inhibitory peptide numerically, but not significantly, reduced the number of migrating cells, while 200 μ M significantly reduced neutrophil chemotaxis (*, $p < 0.01$). The scrambled peptide had no significant effect on chemotaxis. Results are expressed as mean \pm 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-3K γ -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 pleckstrin 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 B₄, IL-8, FcR γ 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 B₄, 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%. Di-

dichenko 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, PKC ζ 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, Ser³⁰⁴ and Ser³²⁸, as potential sites for Akt phosphorylation. Ago et al. (34) reported that mutation of Ser^{303/304} and Ser³²⁸ 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 Ser³⁰⁴ and Ser³²⁸ 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.

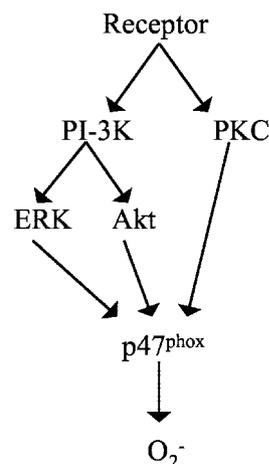


FIGURE 8. Model of PI-3K regulation of respiratory burst activity. Postulated pathways by which PI-3K regulates NADPH oxidase assembly and activity. See text for details.

Acknowledgments

We acknowledge the excellent technical assistance of Rachel Wu and Suzanne Eades.

References

- Babior, B. M. 1999. NADPH oxidase: an update. *Blood* 93:1464.
- Babior, B. M., J. D. Lambeth, and W. Nauseef. 2002. The neutrophil NADPH oxidase. *Arch. Biochem. Biophys.* 397:342.
- Huang, J., and M. E. Kleinberg. 1999. Activation of the phagocyte NADPH oxidase protein p47^{phox}: phosphorylation controls SH3 domain-dependent binding to p22^{phox}. *J. Biol. Chem.* 274:19731.
- Dewas, C., M. Fay, M. A. Gougerot-Pocidal, and J. El-Benna. 2000. The mitogen-activated protein kinase extracellular signal-regulated kinase 1/2 pathway is involved in formyl-methionyl-leucyl-phenylalanine-induced p47^{phox} phosphorylation in human neutrophils. *J. Immunol.* 165:5238.
- Dang, P. M., A. Fontayne, J. Hakim, J. El Benna, and A. Perianin. 2001. Protein kinase C ζ phosphorylates a subset of selective sites of the NADPH oxidase component p47^{phox} and participates in formyl peptide-mediated neutrophil respiratory burst. *J. Immunol.* 166:1206.
- El Benna, J., L. R. P. Faust, J. L. Johnson, and B. Babior. 1996. Phosphorylation of the respiratory burst oxidase subunit p47^{phox} as determined by two-dimensional phosphopeptide mapping. *J. Biol. Chem.* 271:6374.
- Inanami, O., J. L. Johnson, J. K. McAdara, J. E. Benna, L. R. Faust, P. E. Newburger, and B. M. Babior. 1998. Activation of the leukocyte NADPH oxidase by phorbol ester requires the phosphorylation of p47^{phox} on serine 303 or 304. *J. Biol. Chem.* 273:9539.
- El Benna, J., J. Han, J. W. Park, E. Schmid, R. J. Ulevitch, and B. M. Babior. 1996. Activation of p38 in stimulated human neutrophils: phosphorylation of the oxidase component p47^{phox} by p38 and ERK but not by JNK. *Arch. Biochem. Biophys.* 334:395.
- Lal, A. S., A. D. Clifton, J. Rouse, A. W. Segal, and P. Cohen. 1999. Activation of the neutrophil NADPH oxidase is inhibited by SB 203580, a specific inhibitor of SAPK2/p38. *Biochem. Biophys. Res. Commun.* 259:465.
- Coxon, P. Y., M. J. Rane, D. W. Powell, J. B. Klein, and K. R. McLeish. 2000. Differential mitogen-activated protein kinase stimulation by Fc γ receptor IIa and Fc γ receptor IIIb determines the activation phenotype of human neutrophils. *J. Immunol.* 164:6530.
- Rane, M. J., S. L. Carrithers, J. M. Arthur, J. B. Klein, and K. R. McLeish. 1997. Formyl peptide receptors are coupled to multiple mitogen-activated protein kinase cascades by distinct signal transduction pathways: role in activation of reduced nicotinamide adenine dinucleotide oxidase. *J. Immunol.* 159:5070.
- McLeish, K. R., J. B. Klein, P. Y. Coxon, K. Z. Head, and R. A. Ward. 1998. Bacterial phagocytosis activates extracellular signal-regulated kinase and p38 mitogen-activated protein kinase cascades in human neutrophils. *J. Leukocyte Biol.* 64:835.
- Zu, Y.-L., J. Qi, A. Gilchrist, G. A. Fernandez, D. Vazquez-Abad, D. L. Kreutzer, C.-K. Huang, and R. I. Sha'afi. 1998. p38 Mitogen-activated protein kinase activation is required for human neutrophil function triggered by TNF- α or FMLP stimulation. *J. Immunol.* 160:1982.
- Nick, J. A., N. J. Avdi, S. K. Young, C. Knall, P. Gerwins, G. L. Johnson, and G. S. Worthen. 1997. Common and distinct intracellular signaling pathways in human neutrophils utilized by platelet activating factor and FMLP. *J. Clin. Invest.* 99:975.
- Waite, K. A., D. Qualliotine-Mann, and L. C. McPhail. 1997. Phosphatidic acid-mediated phosphorylation of the NADPH oxidase component p47^{phox}: evidence that phosphatidic acid may activate a novel protein kinase. *J. Biol. Chem.* 272:15569.
- Park, H. S., S. M. Lee, J. H. Lee, Y. S. Kim, Y. S. Bae, and J. W. Park. 2001. Phosphorylation of the leukocyte NADPH oxidase subunit p47^{phox} by casein kinase 2: conformation-dependent phosphorylation and modulation of oxidase activity. *Biochem. J.* 358:783.
- Vlahos, C. J., W. F. Matter, R. F. Brown, A. E. Traynor-Kaplan, P. G. Heyworth, E. R. Prossnitz, R. D. Ye, P. Marder, J. A. Schelm, K. J. Rothfuss, et al. 1995. Investigation of neutrophil signal transduction using a specific inhibitor of phosphatidylinositol 3-kinase. *J. Immunol.* 154:2413.
- Arcaro, A., and M. P. Wymann. 1993. Wortmannin is a potent phosphatidylinositol 3-kinase inhibitor: the role of phosphatidylinositol 3,4,5-trisphosphate in neutrophil responses. *Biochem. J.* 296:297.
- Ding, J., C. J. Vlahos, R. Liu, R. F. Brown, and J. A. Badwey. 1995. Antagonists of phosphatidylinositol 3-kinase block activation of several novel protein kinases in neutrophils. *J. Biol. Chem.* 270:11684.
- Li, Z., H. Jiang, W. Xie, Z. Zhang, A. V. Smrcka, and D. Wu. 2000. Roles of PLC- β 2 and - β 3 and PI3K γ in chemoattractant-mediated signal transduction. *Science* 287:1046.
- Hirsch, E., V. L. Katanaev, C. Garlanda, O. Azzolino, L. Pirola, L. Silengo, S. Sozzani, A. Mantovani, F. Altruda, and M. P. Wymann. 2000. Central role for G protein-coupled phosphoinositide 3-kinase. *Science* 287:1049.
- Sasaki, T., J. Irie-Sasaki, R. G. Jones, A. J. Oliveira-dos-Santos, W. L. Stanford, B. Bolon, A. Wakeham, A. Ltie, I. Koziarzdzki, N. Joza, et al. 2000. Function of PI3K γ in thymocyte development, T cell activation, and neutrophil migration. *Science* 287:1040.
- Kettritz, R., M. Choi, W. Butt, M. J. Rane, S. Rolle, F. C. Luft, and J. B. Klein. 2002. Phosphatidylinositol 3-kinase controls antineutrophil cytoplasmic antibodies-induced respiratory burst in human neutrophils. *J. Am. Soc. Nephrol.* 13:1740.
- McLeish, K. R., C. Knall, R. A. Ward, P. Gerwins, P. Y. Coxon, J. B. Klein, and G. L. Johnson. 1998. Activation of mitogen-activated protein kinase cascades during priming of human neutrophils by TNF- α and GM-CSF. *J. Leukocyte Biol.* 64:537.
- Knall, C., S. Young, J. A. Nick, A. M. Buhl, G. S. Worthen, and G. L. Johnson. 1985. Interleukin-8 regulation of the Ras/Raf/mitogen-activated protein kinase pathway in human neutrophils. *J. Biol. Chem.* 271:2832.
- Tilton, B., M. Andjelkovic, S. A. Didichenko, B. A. Hemmings, and M. Thelen. 1997. G-protein-coupled receptors and Fc γ -receptors mediate activation of Akt/protein kinase B in human phagocytes. *J. Biol. Chem.* 272:28096.
- Klein, J. B., M. J. Rane, J. A. Scherzer, P. Y. Coxon, R. Kettritz, J. M. Mathiesen, A. Buridi, and K. R. McLeish. 2000. Granulocyte-macrophage colony-stimulating factor delays neutrophil constitutive apoptosis through phosphoinositide 3-kinase and extracellular signal-regulated kinase pathways. *J. Immunol.* 164:4286.
- Klein, J. B., A. Buridi, P. Y. Coxon, M. J. Rane, T. Manning, R. Kettritz, and K. R. McLeish. 2001. Role of extracellular signal-regulated kinase and phosphatidylinositol-3 kinase in chemoattractant and LPS delay of constitutive neutrophil apoptosis. *Cell. Signal.* 13:335.
- Obata, T., M. B. Yaffe, G. G. Leparo, E. T. Piro, H. Maegawa, A. Kashiwagi, and L. C. Cantley. 2000. Peptide and protein library screening defines optimal substrate motifs for AKT/PKB. *J. Biol. Chem.* 275:36108.
- Haslett, C., L. A. Guthrie, M. M. Kopaniak, R. B. Johnston, and P. M. Henson. 1985. Modulation of multiple neutrophil functions by preparative methods or trace concentrations of bacterial lipopolysaccharide. *Am. J. Pathol.* 119:101.
- Ward, R. A., and K. R. McLeish. 1995. Polymorphonuclear leukocyte oxidative burst is enhanced in patients with chronic renal insufficiency. *J. Am. Soc. Nephrol.* 5:1697.
- Zu, Y.-L., A. Gilchrist, Y. Ai, M. E. Labadia, R. I. Sha'afi, and C.-K. Huang. 1996. Activation of MAP kinase-activated protein kinase 2 in human neutrophils after phorbol ester or FMLP peptide stimulation. *Blood* 87:5287.
- Yaffe, M. B., G. G. Leparo, J. Lai, T. Obata, S. Volinia, and L. C. Cantley. 2001. A motif-based profile scanning approach for genome-wide prediction of signaling pathways. *Nat. Biotechnol.* 19:348.
- Ago, T., H. Nunoi, T. Ito, and H. Sumimoto. 1999. Mechanism for phosphorylation-induced activation of the phagocyte NADPH oxidase protein p47^{phox}: triple replacement of serines 303, 304, and 328 with aspartates disrupts the SH3 domain-mediated intramolecular interaction in p47^{phox}, thereby activating the oxidase. *J. Biol. Chem.* 274:33644.
- Knall, C., G. S. Worthen, and G. L. Johnson. 1997. Interleukin 8-stimulated phosphatidylinositol-3-kinase activity regulates the migration of human neutrophils independent of extracellular signal-regulated kinase and p38 mitogen-activated protein kinases. *Proc. Natl. Acad. Sci. USA* 94:3052.
- Sevant, G., O. D. Weiner, P. Herzmark, T. Balla, J. W. Sedat, and H. R. Bourne. 2000. Polarization of chemoattractant receptor signaling during neutrophil chemotaxis. *Science* 287:982.
- Hannigan, M., L. Zhan, Z. Li, Y. Ai, D. Wu, and C. K. Huang. 2002. Neutrophils lacking phosphoinositide 3-kinase γ show loss of directionality during N-formyl-Met-Leu-Phe-induced chemotaxis. *Proc. Natl. Acad. Sci. USA* 99:3603.
- Didichenko, S. A., B. Tilton, B. A. Hemmings, K. Ballmer-Hofer, and M. Thelen. 1996. Constitutive activation of protein kinase B and phosphorylation of p47^{phox} by a membrane-targeted phosphoinositide 3-kinase. *Curr. Biol.* 6:1271.
- Rane, M. J., P. Y. Coxon, D. W. Powell, R. Webster, J. B. Klein, W. Pierce, P. Ping, and K. R. McLeish. 2001. p38 kinase-dependent MAPKAPK-2 activation functions as 3-phosphoinositide-dependent kinase-2 for Akt in human neutrophils. *J. Biol. Chem.* 276:3517.
- Downey, G. P., J. R. Butler, H. Tapper, L. Fialkow, A. R. Saltiel, B. B. Rubin, and S. Grinstein. 1998. Importance of MEK in neutrophil microbicidal responsiveness. *J. Immunol.* 160:434.
- Labadia, M. E., Y.-L. Zu, and C.-K. Huang. 1996. Synthetic peptide containing a predominant protein kinase C site within p47^{phox} inhibits the NADPH oxidase in intact neutrophils. *J. Leukocyte Biol.* 49:116.
- Faust, L. R. P., J. E. Benna, B. M. Babior, and S. J. Chanock. 1998. The phosphorylation targets of p47^{phox}, a subunit of the respiratory burst oxidase: functions of the individual target serines as evaluated by site-directed mutagenesis. *J. Clin. Invest.* 96:1499.
- Johnson, J. L., J. W. Park, J. E. Benna, O. Inanami, and B. M. Babior. 1998. Activation of p47^{phox}, a cytosolic subunit of the leukocyte NADPH oxidase: phosphorylation of Ser-359 or Ser-370 precedes phosphorylation at other sites and is required for activity. *J. Biol. Chem.* 273:35147.