Blockade of Class IA Phosphoinositide 3-Kinase in Neutrophils Prevents NADPH Oxidase Activation- and Adhesion-dependent Inflammation*

Received for publication, November 2, 2006, and in revised form, December 14, 2006 Published, JBC Papers in Press, December 29, 2006, DOI 10.1074/jbc.M610248200

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We examined the role of class IA phosphoinositide 3-kinase (PI3K) in the regulation of activation of NADPH oxidase in PMNs and the mechanism of PMN-dependent lung inflammation and microvessel injury induced by the pro-inflammatory cytokine TNF-α. TNF-α stimulation of PMNs resulted in superoxide production that was dependent on CD11b/CD18-mediated PMN adhesion. Additionally, TNF-α induced the association of CD11b/CD18 with the NADPH oxidase subunit Nox2 (gp91phox) and phosphorylation of p47phox, indicating the CD11b/CD18 dependence of NADPH oxidase activation. Transduction of wild-type PMNs with Δp85 protein, a dominant-negative form of the class IA PI3K regulatory subunit, p85α, fused to HIV-TAT (TAT-Δp85) prevented (i) CD11b/CD18-dependent PMN adhesion, (ii) interaction of CD11b/CD18 with Nox2 and phosphorylation of p47phox, and (iii) PMN oxidant production. Furthermore, studies in mice showed that i.v. infusion of TAT-Δp85 significantly reduced the recruitment of PMNs in lungs and increased in lung microvascular permeability induced by TNF-α. We conclude that class IA PI3K serves as a nodal point regulating CD11b/CD18-integrin-dependent PMN adhesion and activation of NADPH oxidase, and leads to oxidant production at sites of PMN adhesion, and the resultant lung microvascular injury in mice.

PMNs3 play an important antimicrobial role in protecting the host from a range of infectious agents by the generation of reactive oxygen species (ROS). Superoxide anion (O₂⁻) is formed from molecular oxygen (O₂) through the enzymatic activity of the NADPH oxidase complex (1). O₂⁻ gives rise to H₂O₂, spon-

* This study was supported in part by National Institutes of Health Grants HL46350, HL77806, HL45638, and HL64573 (to A. B. M.), and AI52109 (to X. Z.) and by a research grant from the American Lung Association (to R. S. F.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "i n accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The abbreviations used are: PMN, polymorphonuclear leukocyte; NIF, neutrophil inhibitory factor; BAL, bronchoalveolar lavage; KcsA, capillary filtration coefficient; PKC, protein kinase C; PI3K, phosphoinositide 3-kinase; TNF, tumor necrosis factor; WT, wild type; mAb, monoclonal antibody; i.e., intravenous; ROS, reactive oxygen species; PBS, phosphate-buffered saline; GFP, green fluorescent protein; MPO, myeloperoxidase.

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exists in an unassembled state in resting cells, but upon stimulation, p47phox, p67phox, and Rac 2 translate to the plasma membrane where they co-operatively form a complex with cytochrome b558; however, the signaling mechanism regulating NAPDH oxidase assembly in adherent PMNs and consequences of adhesion-dependent PMN activation in mediating vascular inflammation remain incompletely understood.

In the present study, we transduced PMNs with Δp85 protein, a dominant negative form (Δ478–513 amino acids, p110 binding site) (23) of the class IA PI3K regulatory subunit, p85α, which was fused to HIV-TAT (TAT-Δp85) to facilitate cell membrane permeation of the protein. Our findings demonstrate that class IA PI3K activation is required for CD11b/CD18-dependent PMN adhesion, adhesion-dependent phosphorylation of p47phox, and ROS production by PMNs. In studies in mice, i.v. infusion of TAT-Δp85 protein prevented PMN-dependent lung microvascular injury and inflammation induced by TNF-α. Thus, selective inhibitors of class IA PI3K represent a novel therapeutic strategy for treating lung inflammation and injury resulting from the inappropriate sequestration and adhesion-dependent activation of PMNs in microvessels.

**EXPERIMENTAL PROCEDURES**

**Mice**—C57BL/6 mice (WT) were obtained from Jackson Laboratories (Bar Harbor, ME). Mac-1-deficient mice (Mac-1−/−) were obtained from Dr. C. Ballantyne, Baylor College of Medicine, Houston, TX (24). We generated NIF transgenic mice (NIF/+/+) as described (25). All mice were housed under specific pathogen-free conditions with access to food and water ad libitum in the Animal Care Facility and all studies were made in accordance with institutional guidelines.

**Isolation of Mouse PMNs**—Neutrophils were purified from mouse bone marrow and peripheral venous blood using a discontinuous Percoll gradient as described with modifications (25, 26). Purity of PMN preparations as assessed by examination of HEMA3 (Fisher)-stained cytospin (Shandon, Pittsburgh, PA) preparations (27) was 90–95% and viability assessed by Trypan blue exclusion was >95%.

**Purification of TAT-Δp85**—A detailed method for the generation of dominant negative TAT-Δp85 and TAT-GFP has been described (23, 28, 29). Briefly, a cDNA fragment encoding dominant negative p85 was amplified by PCR from the p85 cDNA with the deletion of 35 amino acids from residues 478–513 with the insertion of two amino acids in pGEX. The PCR product was digested with Agel/EcoRI and ligated into an Agel/EcoRI-digested pTAT vector using T4 ligase. Purification of TAT fusion proteins was performed using modification of a method from Myou et al. (28). Briefly, TAT-Δp85 was purified by sonication of high expressing BL21 Escherichia coli in 10 ml of buffer that was 20 mM HEPES, pH 8.0 and 100 mM NaCl. Cellular lysates were resolved by centrifugation, loaded onto 5-mL Ni-nitrilotriacetic acid column, washed, and eluted with 0.25–1.0 M imidazole in PBS. Imidazole was removed from the resultant protein solution using a PD-10 column. Each fusion protein preparation was flash-frozen at −80 °C.

**PMN Adhesion to Immobilized Fibrinogen**—For PMN adhesion to immobilized fibrinogen, 96-well microplates were coated with 10 μg/ml of fibrinogen overnight at 4 °C and washed three times with HBSS before use. Mouse PMNs loaded with calcein-AM (2 μg/ml) (Molecular Probes, Eugene, OR) for 30 min at 37 °C were added to fibrinogen-coated plates. The assay for PMN adhesion was performed as described by us (25).

**Microscopy of PMN Adhesion**—Freshly isolated PMNs (2 × 10⁶), added to 8-well Lab-Tek II chamber slide with fibrinogen-coated surface (10 μg/ml, for 60 min at 37 °C), were challenged with TNF-α at 37 °C for 1 h. The cells were then fixed with 3.3% paraformaldehyde (Electron Microscopy Sciences) and washed. Photomicrographs of PMNs in contact with surface-coated fibrinogen were acquired with a Zeiss LSM 510 confocal microscope.

**PMN Superoxide Generation**—PMNs without or with the treatment of TNF-α at 5 × 10⁶ cells/ml were seeded into a white, 96-well, flat-bottom tissue culture dish (E&K Scientific) coated with fibrinogen overnight. O₂⁻ generation by PMNs was measured as described, using isoluminol-enhanced chemiluminescence (30). Briefly, isoluminol was added to the cell suspension to a final concentration of 50 μM, and horseradish peroxidase was added to a final concentration of 40 units/ml. O₂⁻ production was determined after cells were stimulated with TNF-α for the indicated times, following preincubation with or without anti-CD11b mAb (8 μg/ml, clone M1/70, BD Biosciences, San Jose, CA), isotype-matched control antibodies (8 μg/ml, rat IgG2b, BD Biosciences) for anti-CD11b mAb, TAT-Δp85 (300 nM), TAT-GFP (300 nm), or LY294002 (50 μM, Calbiochem, La Jolla, CA) 15 min prior to TNF-α stimulation. The relative concentration of O₂⁻ in the culture medium was expressed as counts per second.

**Polyacrylamide Electrophoresis**—The BCA method (31) was used to measure total protein concentration. Equal amounts of total protein were loaded per lane (specific amounts are given in figure legends). Cells were lysed in the following buffer: 100 mM Tris-HCl, pH 7.5, 0.5% sodium deoxycholate, 1% Nonidet P-40, 0.1% SDS, 5 mM EDTA, 5 mM EGTA, 150 mM NaCl, 5 mM NaF, 1 mM Na3VO4 to which a protease inhibitor mixture (AEBSF, pepstatin A, E-64, bestatin, leupeptin, and aprotonin) was added before each experiment (Sigma). We used pre-cast NuPAGE Bis-Tris (4–12%) gels purchased from Invitrogen, per the manufacturer’s protocol.

**Immunoprecipitation and Immunoblotting**—Activation of Akt was assessed using a 1:500 dilution of an antibody that recognizes Akt phosphorylated on Ser473 (Cell Signaling, Beverly, MA). An antibody against non-phosphorylated Akt (Cell Signaling, Beverly, MA) was used to assess protein loading.

Association of CD11b/CD18 with the NADPH oxidase subunit Nox2 was studied using a soluble enriched membrane fraction. After homogenization of the lungs in PBS (containing 1% protease inhibitors), the samples were centrifuged for 15 min at 14,000 × g. The pellets were sonicated 2×12 s in lysis buffer containing 100 mM Tris-HCl, pH 7.5, 1% Nonidet P-40, 5 mM EDTA, 5 mM EGTA, 150 mM NaCl, to which a protease inhibitor mixture (Sigma) was added. After centrifugation again for 15 min at 14,000 × g, the supernatants of enriched membrane fraction were collected for immunoprecipitation. Equal amounts of protein (200 μg/ml total protein) in supernatants were precleared using purified IgG followed by protein
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A-Sepharose beads. The preincubated supernatants were recovered by centrifugation, and incubated overnight at 4 °C with primary Abs (3 μg/ml) (antibody against CD11b, purchased from Santa Cruz Biotechnology, Santa Cruz, CA), followed by incubations with protein A-Sepharose beads for 2 h. The beads were collected by centrifugation (10,000 × g, 5 min using an Eppendorf refrigerated microcentrifuge at 4 °C), washed extensively (5 × 10 min), solubilized and run on 4–20% SDS-PAGE. After transfer to nitrocellulose, the membrane was then incubated with 1:500 anti-Nox2 (BD Biosciences) or anti-CD11b antibody overnight at 4 °C. After washing, horseradish peroxidase-coupled secondary antibodies were then used to probe the membranes. The membranes were developed by manufacturer’s instructions.

For analysis of total p47phox phosphorylation, lungs and cells were homogenized in PBS containing 1% protease inhibitors, centrifuged at 14,000 × g, and the pellet was sonicated in lysis buffer, as described above. Total cell lysates were immunoprecipitated with p47phox antibody (from B. Babior and S. Catz, Scripps Research Institute, La Jolla, CA) and subjected to SDS-PAGE. Gels were stained with Pro-Q Diamond phosphoprotein gel stain (Molecular Probes, Eugene, OR) (32). The gels were scanned with Molecular Imager FX (Bio-Rad) and images of phosphoproteins were obtained. Non-phospho p47phox loading of gels was obtained by Western blotting of total cell lysates.

**TNF-α Challenge of Mice**—Mice were challenged with TNF-α (100 μg/kg, intraperitoneal). This dosage did not result in death within the 6-h experimental period after challenge. Control mice were injected intraperitoneal with an equal volume of PBS. Lungs were obtained at select time points after TNF-α challenge. One hour was used to assess the phosphorylation of Akt, p47phox in lungs, leukocyte oxidative burst, lung PMN recruitment, and lung vascular permeability and edema formation. To determine the role of PI3K in acute lung injury, mice were first injected intraperitoneal with LY294002 (100 mg/kg, 1 h). In another group, animals received i.v. TAT-Δp85 (10 mg/kg) or a control injection via tail vein. The animals receiving injection of TAT-Δp85 or control proteins (His-Δp85 or TAT-GFP) were assigned randomly to the experimental groups consisting of six mice each. In preliminary studies, we demonstrated that TAT protein vector (administered as TAT-GFP) had no effect on TNF-α-induced lung vascular permeability versus saline buffer control. The lungs obtained at different time points after TNF-α challenge were used to assess the changes as described above.

**ROS Production by BAL Leukocytes**—ROS in leukocytes obtained from mouse broncho-alveolar lavage (BAL) was measured as described (33). Briefly, leukocytes from BAL fluid were collected with or without intraperitoneal TNF-α challenge and incubated for 15 min with ROS-sensitive dye dichlorofluorescin-diacetate (DCFH-DA, 10 μM, Molecular Probes) at 37 °C in phosphate-buffered solution (PBS, Sigma Aldrich). After 1 × wash, the cells were suspended in PBS, and kept on ice until FACS analysis on the same day. The samples were analyzed by measuring fluorescence of 15,000–20,000 events from the gated PMN population using a Coulter EPICS Elite ESP (Coulter Corporation, Miami, FL) with the laser at 530 nm. The fluorescent product of oxidation (DCFH) was measured by flow cytometry using four decade logarithmic amplification.

**Lung PMN Sequestration**—Sequestration of PMNs in lung tissue was assessed by determining myeloperoxidase (MPO) activity in lungs and by morphometrically quantifying PMN infiltration as described (2, 27).

**Pulmonary Microvascular Permeability and Edema Formation**—Kw was measured to determine pulmonary microvascular permeability to liquid and the rate of edema formation was continuously monitored by determining lung wet weight changes as described (25, 27).

**Statistical Analysis**—Data are expressed as mean ± S.E. Statistical analysis was performed using the 2-way analysis of variance and Newman-Keuls test for multiple comparisons. Significance was set at p < 0.05.

**RESULTS**

Effectiveness of TAT-Δp85 in Preventing PI3K Activation Caused by TNF-α in PMNs—We have shown that the synthesized TAT-Δp85 fusion protein (useful for transducing human eosinophils (23)) specifically inhibits the activity of class IA PI3K. Experiments showed that all PMNs were transduced with TAT fusion protein after 2 min (data not shown). Western blot analysis showed that TAT-Δp85 was also successfully transduced into mouse PMNs. TAT-Δp85 expression was observed with little overlap with endogenous p85 after incubation of PMNs with 100–300 nM TAT-Δp85 (Fig. 1A, top panel). The functional efficacy of TAT-Δp85 expression was assessed by its ability to inhibit phosphorylation of Akt, downstream target of PI3K. As shown in Fig. 1A (middle panel), the TNF-α-induced phosphorylation of Akt was inhibited by TAT-Δp85 in
a concentration-dependent manner. The effectiveness of TAT-Δp85 (300 nM) was similar to the nonspecific PI3K inhibitor LY294002 (50 μM) used as a positive control (Fig. 1B). TAT-GFP (used as a control) had no effect on Akt phosphorylation (Fig. 1B).

Inhibition of Class IA PI3K Reduces PMN Adhesion to Immobilized Fibrinogen—We initially determined the role of the β2 integrin CD11b/CD18 in mediating PMN firm adhesion to purified fibrinogen, a CD11b/CD18 ligand, induced by TNF-α (Fig. 2, A and B). PI3K inhibition by 300 nM TAT-Δp85 or 50 μM Ly294002 prevented PMN adhesion to fibrinogen induced by TNF-α. Control peptide (TAT-GFP and TAT-p85) had no effect on PMN adhesion to fibrinogen induced by TNF-α. Control peptide (TAT-GFP and TAT-p85) had no effect on PMN adhesion to fibrinogen induced by TNF-α. Control peptide (TAT-GFP and TAT-p85) had no effect on PMN adhesion to fibrinogen induced by TNF-α. Control peptide (TAT-GFP and TAT-p85) had no effect on PMN adhesion to fibrinogen induced by TNF-α. Control peptide (TAT-GFP and TAT-p85) had no effect on PMN adhesion to fibrinogen induced by TNF-α. Control peptide (TAT-GFP and TAT-p85) had no effect on PMN adhesion to fibrinogen induced by TNF-α. Control peptide (TAT-GFP and TAT-p85) had no effect on PMN adhesion to fibrinogen induced by TNF-α. Control peptide (TAT-GFP and TAT-p85) had no effect on PMN adhesion to fibrinogen induced by TNF-α.

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class IA PI3K in mediating PMN O$_2^\bullet$ release induced by TNF-α. TNF-α caused time-dependent O$_2^\bullet$ release by PMNs adherent to plated fibrinogen (Fig. 3A). PI3K inhibition by 300 nM TAT-Δp85 or 50 μM Ly294002 blocked O$_2^\bullet$ release caused by TNF-α. By contrast, control peptide (TAT-GFP, TAT-p85, or His-Δp85) had no effect on O$_2^\bullet$ release induced by TNF-α. O$_2^\bullet$ release was also prevented by preincubation with anti-CD11b antibody. Combination of TAT-Δp85 and anti-CD11b did not further reduce O$_2^\bullet$ production of PMNs to plated fibrinogen. To further determine the role of CD11b/CD18 in PMN O$_2^\bullet$ generation, PMNs isolated from mice knock-out of Mac-1 were used for these studies. Compared with PMNs isolated from WT mice, decreased O$_2^\bullet$ release was observed in PMNs isolated from Mac-1 mice. Pretreatment Mac-1$^{-/-}$ PMNs with TAT-Δp85 resulted in no further reduction of O$_2^\bullet$ release (Fig. 3).

Because PMN activation and oxidant production are rapid signaling-dependent events (35), we also determined the early association of CD11b with Nox2. We addressed whether PI3K regulates the TNF-α-induced phosphorylation of p47$^{phox}$ in PMNs. TNF-α induced p47$^{phox}$ phosphorylation at 15–30 min, and introduction of TAT-Δp85 prevented phosphorylation of p47$^{phox}$ (Fig. 4C). Western blotting showed that the same amount of p47$^{phox}$ was immunoprecipitated from each sample.

Class IA PI3K Regulates Activation of NADPH Oxidase in Vivo—We examined the efficacy of TAT-Δ85 introduced with NIF. As shown in Fig. 4B, pretreatment of WT PMNs with TAT-Δp85 reduced the time points of PMN O$_2^\bullet$ release. As shown in Fig. 3B, TNF-α-induced O$_2^\bullet$ release by PMNs adherent to plated fibrinogen reached maximum within 15–30 min. Pretreatment of PMNs with TAT-Δp85 significantly reduced TNF-α-induced O$_2^\bullet$ release in a dose-dependent manner at early time points. Control peptide (TAT-GFP and TAT-p85) had no effect on TNF-α-induced O$_2^\bullet$ release (Fig. 3B). Because class IA PI3K plays a central role in PMN chemotaxis and oxidant production by chemotactants (36), we also studied the effects of TAT-Δp85 on fMLP-induced O$_2^\bullet$ release by PMN. TAT-Δp85 (100 nM and 300 nM) reduced fMLP (1 μM and 5 μM)-induced O$_2^\bullet$ release in a dose- and time-dependent manner at the early time points within 30 min (data not shown).

Inhibition of Class IA PI3K Reduces CD11b/CD18 Association with Nox2 and p47$^{phox}$ Activation—We examined whether NADPH oxidase subunits are co-localized with CD11b/CD18 on PMN membrane after activation by TNF-α. We found that CD11b/CD18 co-immunoprecipitated with Nox2 within 1 h of TNF-α challenge in PMNs. This response was attenuated by either pretreatment with anti-CD11b mAb or NIF (Fig. 4A).

We addressed the role of PI3K in signaling the interaction of CD11b/CD18 with NADPH oxidase. As shown in Fig. 4B, pretreatment of WT PMNs with TAT-Δp85 reduced the

FIGURE 3. Inhibition of class IA PI3K and CD11b/CD18 β$_2$ integrin reduces adhesion-dependent ROS production. A, PMNs (1 × 10$^5$) isolated from WT, Mac-1$^{-/-}$, and NIF$^{-/-}$ mice were preincubated with indicated reagents for 15 min and then added to plated fibrinogen for 1 to 2 h. Superoxide production was measured as described. n = 3. * denotes significant increase (p < 0.05) compared with unchallenged control. ** denotes significant decrease (p < 0.05) compared with TNF-α alone-treated group. † denotes significant decrease (p < 0.05) in PMNs isolated from genetically modified mice compared with PMNs isolated from WT mice post-TNF-α stimulation. Bars indicate mean ± S.E., R$_2$O production was determined in real-time based on isoluminol-ECL. Bars indicate mean ± S.E., R$_2$O generation was determined in real-time based on isoluminol-ECL. pmol-ECL.

A

B

Time (min)

Superoxide (CPS)

Maximum Superoxide (CPS)

1 hr

2 hr

Basal

TAT-Δp85

TAT-GFP

TNF-α

TAT-GFP+TNF-α

TAT-Δp85 (100 nM)+TNF-α

TAT-P85 (300 nM)+TNF-α

6120 JOURNAL OF BIOLOGICAL CHEMISTRY

VOLUME 282•NUMBER 9•MARCH 2, 2007

6120 JOURNAL OF BIOLOGICAL CHEMISTRY

VOLUME 282•NUMBER 9•MARCH 2, 2007
Because phosphorylation of p47phox is a key determinant of NADPH oxidase activation, we surmised that CD11b/CD18 activation was involved in the response. We observed a reduction in phosphorylation of p47phox in TNF-α-challenged lungs of Mac-1−/− mice; similar results were also obtained with WT mice pretreated with the anti-CD11b mAb prior to TNF-α challenge (Fig. 5C).

PI3K Regulates TNF-α-dependent ROS Production in BAL Leukocytes—ROS production was measured in BAL leukocytes obtained from TNF-α-challenged WT mice at 1 h compared with buffer-challenged control mice (Fig. 6A); the response persisted up to 6 h (data not shown). To address the in vivo relevance of class IA PI3K in TNF-α-induced oxidant production in leukocytes, mice were pretreated with the peptide inhibitor of PI3K activity described above. In positive control experiments, the PI3K inhibitors wortmannin or LY 294002 significantly reduced oxidant generation in leukocytes obtained from BAL of TNF-α-challenged mice (Fig. 6, A and B). Systemic pretreatment with TAT-Δp85 in a concentration-dependent manner reduced ROS production in the BAL leukocytes (Fig. 6C). Pretreatment of WT mice with anti-CD11b mAb also significantly reduced ROS production after TNF-α challenge (Fig. 6D). However, no additive reduction of ROS production was seen with combination of both TAT-Δp85 and anti-CD11b mAb (Fig. 6D). In contrast, TAT-GFP had no effect on oxidant production by the BAL leukocytes (Fig. 6E).

To address the in vivo role of CD11b/CD18 in TNF-α-induced leukocytes ROS production, studies were made in NIF+/+ and Mac-1−/− mice. ROS generation was impaired in leukocytes obtained from Mac-1−/− (Fig. 6G) and NIF+/+ (Fig. 6H) mice. Pretreatment of TAT-Δp85 had no inhibitory effect on ROS generation in these genetically modified leukocytes.

Class IA PI3K Mediates TNF-α-induced PMN Sequestration and Vascular Injury in Mouse Lungs—To address the in vivo role of PI3K in mediating the TNF-α-induced PMN recruit-
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A

LY294002

B

Wortmannin

C

TAT-Δp85 (5 mg/kg, iv) or TAT-Δp85 + CD11b Ab

D

TAT-Δp85 (10 mg/kg, iv) or TAT-Δp85 + CD11b Ab

E

TAT-GFP

F

Anti-CD11b Ab

G

Mac-1−/−

H

NIF+/−

Oxidative burst

FIGURE 6. Effect of class IA PI3K inhibition or CD11b/CD18 blockade on ROS generation from leukocytes isolated from BAL after TNF-α challenge. WT mice (A–F) or Mac-1−/− (G) and NIF+/− mice (H) were pretreated with or without PI3K chemical inhibitor, 100 μg/kg LY294002 (A) or 2 mg/kg wortmannin (B), peptide inhibitor TAT-Δp85 (5 and 10 mg/kg, i.v) or TAT-GFP (10 mg/kg, i.v.) (C, E, G, H), and anti-CD11b antibody (D, F) for 30 min and then challenged with TNF-α (100 μg/kg, intraperitoneal). Oxidant generation from leukocytes isolated from BAL 1 h after challenge was measured by flow cytometry after dichlorofluorescin staining. Data are representative of three independent experiments.

ment, PMN number in lungs was measured as described (27). Lung tissue PMN counts increased from 551.3 ± 52.1 cells/30 mm2 lung tissue to 3395.3 ± 73.8 cells/30 mm2 1 h after TNF-α challenge in WT mice. Systemic pretreatment with TAT-Δp85 reduced lung PMN sequestration after TNF-α challenge (Fig. 7). PMN sequestration was reduced by ~55% at 1 h by TAT-Δp85, whereas the control p85 protein had no effect (Fig. 7A). TNF-α-induced PMN sequestration was reduced ~40% by anti-CD11b mAb (Fig. 7A) whereas isotype-matched control antibody IgG2a did not affect PMN sequestration in lungs. No additive blockade of PMN sequestration was observed after combination of both anti-CD11b and TAT-Δp85. PMN sequestration was also significantly reduced in Mac-1−/− and in NIF+/− mice (Fig. 7A). Systemic pretreatment with TAT-Δp85 also reduced lung myeloperoxidase (MPO) activity by ~51% at 1 h after TNF-α challenge (Fig. 7B), whereas the control p85 protein had no effect.

Activation of PMN adherent to the microvessels plays a key role in lung inflammation and contributes to the mechanism of lung microvascular injury by the release of oxidants, proteases, chemokines, and other mediators (25, 37), because the above data show an important role for class IA PI3K in signaling PMN ROS generation and sequestration in lungs following TNF-α challenge, we addressed the possibility that TAT-Δp85 would also prevent lung vascular microvascular injury induced by TNF-α. Pulmonary capillary filtration coefficient (Kf,c), a measure of microvascular permeability, and lung edema formation were determined in WT lungs perfused with TNF-α. TNF-α challenge resulted in increased Kf,c (Fig. 8A). Kf,c value at 1 h after TNF-α was elevated ~6-fold over basal, indicating a marked increase in microvessel permeability. Systemic treatment with TAT-Δp85, but not TAT-GFP, prevented the TNF-α-induced increase in lung microvascular permeability and edema formation (Fig. 8A). In negative control experiments, the increases in Kf,c induced by TNF-α were reduced by ~50% in Mac-1−/− mice and ~75% in NIF+/− mice (Fig. 8A) and by pretreatment with either NIF or anti-CD11b mAb (Fig. 8A). No further reduction in Kf,c was observed after combination of both anti-CD11b mAb and TAT-Δp85 in WT mice. TAT-Δp85 did not prevent further the lung vascular permeability response seen in Mac-1−/− or NIF+/− mice after TNF-α. Fig. 8B shows the protective effect of TAT-Δp85 on lung edema formation. TAT-Δp85, but not TAT-GFP reduced lung wet weight increase after TNF-α challenge (Fig. 8B) and no further protective effect after combined treatment with anti-CD11b and TAT-Δp85. Fig. 8C shows the protective effect of anti-CD11b mAb on lung edema formation, whereas anti-CD11a mAb was not protective. TAT-Δp85 had no further inhibitory effect on lung wet weight increases seen in Mac-1−/− (Fig. 8D) or NIF+/− (Fig. 8E) mice.

DISCUSSION

The augmented oxidant generation by the adherent PMNs may be a crucial factor mediating lung inflammation and microvascular injury (25, 37, 38). Because oxidant generation is linked to β2 integrin-dependent PMN adhesion, we addressed the role of the PMN β2 integrin CD11b/CD18 (Mac-1) in activating PMN NADPH oxidase complex, and thereby in the mechanism of PMN-mediated lung inflammation and microvascular injury. We showed that (i) activation of the class IA PI3K isoform is an important signal mediating PMN adhesion...
and adhesion-dependent ROS production by PMNs, (ii) class IA PI3K isoform mediates this response by inducing CD11b/CD18 interaction with Nox2 subunit of NADPH oxidase, and (iii) selective inhibition of class IA PI3K prevents lung PMN sequestration and microvascular injury induced by TNF-α challenge of mice.

Previous studies have shown involvement of PI3K in signaling the activation of NADPH oxidase by G protein-coupled receptors (GPCRs) such as the fMLP receptor (39) and certain non-GPCRs such as TNF receptors (40). However, the isoforms of PI3K regulating NADPH oxidase activation in PMNs have not been clearly delineated, in part, because of the lack of isoform-specific inhibitors. Prior studies in PI3K-γ-deficient mice showed impaired fMLP-stimulated PMN respiratory burst activity (17, 41) indicating the important role of GPCR-coupled PI3Kγ in the activation of NADPH oxidase. Using dominant negative protein inhibitors of class IA and IB PI3K, a recent study has demonstrated the dominant role of class IA PI3K in fMLP-stimulated superoxide generation (36). In the present study, we addressed the role of class IA PI3K in mediating TNF-α-induced activation of PMN NADPH oxidase and its consequences in the mechanism of lung inflammation and injury.

Studies were made using the Δp85 protein, a dominant negative form of the class IA PI3K regulatory subunit, p85α, fused to HIV-TAT (TAT-Δp85) enabling the protein to readily permeate the plasmalemmal barrier of PMNs (32). We observed that PI3K inhibition by TAT-Δp85 significantly reduced CD11b/CD18-dependent PMN adhesion to fibrinogen and ROS generation induced by TNF-α challenge. TAT-Δp85 also abrogated the phosphorylation of p47**phox** and association of CD11b/CD18 with NADPH oxidase. In mouse studies, we showed that TAT-Δp85 i.v. infusion significantly reduced the recruitment of PMNs into lungs and the increase in lung microvascular permeability induced by TNF-α. These results demonstrate the critical role of class IA PI3K-activated NADPH oxidase and resultant ROS production in the mechanism of lung inflammation and microvascular injury.

Several possible mechanisms may explain the PI3K-dependent activation of NADPH oxidase. Inhibition of PI3K has been shown to reduce the phosphorylation of p47**phox** caused by fMLP (42). A number of kinases are dependent on the PI3K activity, including Akt, ERK, p38 MAPK, PKCδ, and of these Akt and PKCδ were shown to promote p47**phox** phosphorylation (39, 43). Phosphorylation of p47**phox** induced a conformational change in p47**phox** leading to its interaction with both flavocytochrome b558 and p67**phox** (18). Inhibition of p47**phox** phosphorylation markedly decreased the membrane translocation of p47**phox**, association of p47**phox** with Nox2 at the membrane, and activation of NADPH oxidase-induced oxidant generation (44). In addition, activation of the small GTPase Rac2, a prerequisite for
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NADPH oxidase assembly (35, 45), was shown to be dependent on PI3K activity (46–48).

The present study also advances our understanding of the mechanism of lung inflammation and injury induced by PMN ROS generation subsequent to CD11b/CD18-mediated PMN adhesion. We observed that PMNs isolated from BAL of WT mice induced significant ROS production after TNF-α challenge, whereas the response was attenuated in BAL leukocytes from NIF+/+ and Mac-1−/− mice. Pretreatment of mice with NIF or anti-CD11b mAB, but not with anti-CD11a mAB, prevented PMN ROS production after TNF-α challenge and PMN sequestration in lungs and the increase in microvascular permeability. Thus, the TNF-α-induced PMN ROS production as the consequence of CD11b/CD18-dependent adhesion is a crucial factor mediating lung inflammation in our model. These in vivo observations are consistent with the evidence showing an important role of CD11b/CD18 in mediating PMN oxidant production (37). Using anti-CD11b mAB or NIF infusion and NIF+/+ or Mac-1−/− mice, we observed that CD11b/CD18 contributed to ∼50% of lung PMN sequestration response following TNF-α challenge, a finding in accord with an important role for CD11b/CD18 in the mechanism of PMN sequestration in vivo (49). Thus, our results from both in vitro and in vivo studies demonstrate that CD11b/CD18-mediated PMN adhesion is a critical factor inducing NADPH oxidase activation, and thereby lung inflammation and injury.

In summary, we have demonstrated that class IA PI3K isoform regulates the association of β2 integrin CD11b/CD18 with NADPH oxidase subunits in PMNs and mediates the activation of NADPH oxidase and oxidant production. Therefore, class IA PI3K may serve as a nodal point signaling CD11b/CD18-dependent PMN adhesion and activation of PMN NADPH oxidase. Furthermore, class IA PI3K-mediated ROS production by PMNs appears to be a critical determinant of lung inflammation and microvascular injury. In this regard, strategies aimed at inhibiting the p85 subunit of the class IA PI3K may provide novel therapeutic strategy for the treatment of lung inflammation and injury resulting from inappropriate sequestration and activation of PMNs in lung microvessels.

Acknowledgments—We thank Dr. Bao-Shiang Lee and Sangeeth Krishnanthettier for making TAT-Δp85 protein, assistance of Dr. Karen Hagen in flow cytometry studies, Dr. Richard D. Ye for insightful discussions, and Dr. Kelly Price for the review of the article. We also thank Guilan Liu for technical assistance.
