Regulation of phosphatidylinositol 3–kinase by polyisoprenyl phosphates in neutrophil-mediated tissue injury

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Neutrophils play a central role in host defense, inflammation, and tissue injury. Recent findings indicate a novel role for polyisoprenyl phosphates (PIPPs) as natural down-regulatory signals in neutrophils. The relationship between PIPPs and neutrophil early activating signals, such as phosphoinositides, has not been previously determined. Here, we establish presqualene diphosphate (PSDP) as an endogenous PIPP regulator of phosphatidylinositol 3–kinase (PI3K). In human neutrophils, leukotriene B4 (LTB4) triggered rapid decreases in PSDP and reciprocal increases in PI3K activity. In addition, PSDP was identified by gas chromatography/mass spectrometry in p110γ–PI3K immunoprecipitates obtained 30 s after LTB4, indicating a physical interaction between PSDP and PI3K in activated neutrophils. Moreover, PSDP (0.4–800 pmol) directly inhibited recombinant human p110γ–PI3K activity. During an experimental model of lung injury and inflammation, a reciprocal relationship was also present in vivo for lung PSDP and PI3K activity. To investigate its therapeutic potential, we developed a new PSDP structural mimic that blocked human neutrophil activation and mouse lung PI3K activity and inflammation. Together, our findings indicate that PSDP is an endogenous PI3K inhibitor, and suggest that in inflammatory diseases characterized by excessive neutrophil activation, PIPPs can serve as structural templates in a novel antineutrophil therapeutic strategy to limit tissue injury.
activity as a potent counterregulatory mediator that prevents ROS generation (6, 7). In sharp contrast, PSMP is >100-fold less active than PSDP for inhibition (6, 7). Thus, incoming positive signals for PMN (e.g., LTB₄) initiate rapid degradation and inactivation of an inhibitory lipid signal (i.e., PSDP) coincident with cell responses (e.g., ROS generation). PSDP levels quickly return to baseline in a time frame that parallels cellular deactivation. Intracellular targets for PSDP to control PMN activity remain to be elucidated. Select PSDP structural mimetics are also active in vivo, dampening mouse responses to zymosan A–induced peritonitis (8).

In addition to PSDP remodeling, LTB₄ also initiates phosphatidylinositol 3–kinase (PI3K) activation in PMN to promote NADPH oxidase assembly and ROS production (9, 10). Phosphoinositide signaling initiated by PI3K is a critical early event in PMN responses, such as phagocytosis (11) and chemotaxis (12), and contributes to ALI pathogenesis (13). Because LTB₄ initiates PMN PI3K activation and PIPP remodeling, we hypothesized that these signaling events were related in the regulation of PMN responses. Here, we report that PI3K activity and PSDP remodeling are linked during PMN activation and deactivation with direct inhibition of PI3K by PSDP to limit PMN responses and lessen the severity of experimental lung inflammation.

RESULTS AND DISCUSSION

PSDP and PI3K regulate LTB₄–triggered O₂⁻ release by human PMN

To determine if PSDP and PI3K regulate LTB₄-stimulated responses, we exposed freshly isolated human PMN to a new structural PSDP mimetic (Fig. 1 A) or a potent and specific inhibitor of PI3K activity (LY294002) before LTB₄. A marked increase in the rate of O₂⁻ generation was observed within seconds after LTB₄ addition that was transient, slowing considerably by 60 s (Fig. 1 B). The presence of either PSDP mimetic (100 nM) or PI3K inhibitor (3 μM) markedly blocked O₂⁻ generation. Exposure to LTB₄ (10 min) induced 1.6 ± 0.3 nmol O₂⁻/10⁶ PMN (as compared with vehicle 0.5 ± 0.1 nmol O₂⁻/10⁶ PMN; P < 0.01). Both the PSDP mimetic and PI3K inhibitor led to >50% inhibition of LTB₄–triggered O₂⁻ generation (0.8 ± 0.3 nmol/10⁶ PMN and 0.7 ± 0.2 nmol/10⁶ PMN, respectively; P < 0.05) (Fig. 1 C). The PSDP mimetic concentration in these experiments (i.e., 100 nM) was 1,000-fold lower than its critical micellar concentration (CMC) (~100 μM; Fig. S1, at http://www.jem.org/cgi/content/full/jem.20052143/DC1). To verify that the observed inhibition was not secondary to micelle formation and sequestration of the lipid agonist LTB₄, there was pretreatment with the mimetic, a wash, and stimulation with LTB₄. Although this extra step reduced total ROS generation in response to LTB₄, the PSDP mimetic still led to a >50% decrease (71.2 ± 27.7% inhibition, n = 4). Together, these results indicate that human PMN activation by LTB₄ is highly dependent on PI3K activity and can be inhibited by a new PSDP structural mimetic.

Figure 1. Superoxide anion generation by human PMN is regulated by PI3K and PSDP. (A) Structure of PSDP and a new amido PSDP structural mimetic (CS ChemDraw software). (B) Freshly isolated human PMN were exposed to the PSDP mimetic (100 nM) ( ● ), a PI3K inhibitor (3 μM) ( ● ● ), or vehicle ( ○ ) before LTB₄ (100 nM) and O₂⁻ generation was determined. Results are representative for n = 3. (C) Total O₂⁻ generation was also determined for LTB₄–activated PMN (10 min) in the presence or absence of the test compounds (mean ± SEM; n = 4 from separate PMN donors; *, P < 0.01 as compared with vehicle; **, P < 0.05 as compared with LTB₄).

Relationship between PI3K activity and PSDP during PMN activation

To determine if PSDP remodeling and PI3K activation were related, we first examined their kinetics in human PMN after exposure to LTB₄. Because G protein–coupled receptors can activate class IA and IB PI3Ks (10), we measured PIP₃ formation in vitro by members of these PI3K classes. After LTB₄, p110γ–PI3K activity in PMN rapidly increased (within 5 s), reached a maximum rate of activity by 20 s, and then declined to basal levels within 30 s (Fig. 2 A, representative for n = 3). LTβ₄ also rapidly stimulated p85–based PI3K activity in PMN, but at lower levels than p110γ–PI3K. By 5 s, significant decrements were also evident in total PSDP levels (7.9 ± 1.2%; P < 0.01) (see supplemental
Materials and methods, available at http://www.jem.org/cgi/content/full/jem.20052143/DC1) that progressed to 27.1 ± 4.8% reductions (P = 0.001) within 30 s (Fig. 2 B). These results indicate that LTB4-triggered activation of PMN PI3K and PSDP remodeling was concurrent and temporally overlapped with initiation of ROS generation. Although both of these signaling events were rapid in onset, the kinetics for PI3K activation and deactivation differed from the time course for total PSDP remodeling during the initial 30-s interval. To determine if PSDP directly interacted with PI3K as its activity decreased, PMN were exposed to LTB4 for 30 s and p110γ–PI3K was immunoprecipitated from cellular materials. Lipid extracts were performed and analyzed by gas chromatography/mass spectrometry (GC/MS) (6). Selective ion monitoring at m/z 137 (2 isoprenoid units) revealed a unique peak at 18.1 min in LTB4-exposed PMN extracts. MS spectral diagnostic ions (Fig. 2 C), namely m/z 567 [M+ − (H2O)], 488 [M+ − (H2PO4)], 410 [M+ − (HP2O7)], 341 [M+ − (HP2O7)− 69], 205 [M+ − (HP2O7)− 69 − [CH2C(CH3)CHCH2] × 2], 137 [C6H17], 97 [H2PO4], 81 [CH2C(CH3)CH(CH2)2 − H+] and 69 [base peak; (CH3)2CCHCH2], were consistent with authentic PSDP in the PI3K immunoprecipitated material.

Direct inhibition of p110γ–PI3K by PSDP
Because PI3K activity and PSDP remodeling were both early signaling events in PMN with interaction between PSDP and p110γ–PI3K, next we questioned if PSDP could directly regulate PI3K activity. Recombinant human (rh) p110γ–PI3K activity was determined by PIP3 formation in vitro in the presence of PSDP, PSMP, or the PI3K inhibitor LY294002. PIP3 formation was significantly decreased by PSDP (800 pmol) with 94.7 ± 5.3% inhibition (P < 0.001) and a PI3K inhibitor (500 pmol) with 46.7 ± 6.7% inhibition (P = 0.01) (Fig. 3 A). PSDP inhibited p110γ–PI3K in a concentration-dependent fashion (Fig. 3 B). In sharp contrast, PSMP (8–800 pmol) did not significantly impact p110γ–PI3K activity. The IC50 for PSDP (38 pmol) had a stoichiometry with PI3K of 9:1. These results indicate that PSDP is a potent direct inhibitor of p110γ–PI3K with a structure–activity relationship that suggests an important role for the diphosphate structure in PSDP’s action on p110γ–PI3K activity. The LTB4-mediated PMN remodeling of PSDP corresponds to an ~50 pmol change in PSDP/106 PMN, a decrease that is within the concentration range for regulation of p110γ–PI3K activity (Fig. 3 B). After cell activation, the percent change in total PSDP (i.e., 28%) is similar to the change in phosphatidylinositol (17%) that occurs in activated PMN membranes (14). Collectively, our new findings indicate that receptor-mediated agonists for PMN remodel PSDP in time-frames and amounts consistent with functional impact on PI3K activity and cellular responses.

PSDP remodeling in vivo during tissue injury and inflammation
Because PI3K activity occupies a central role in regulating PMN activation during lung injury and inflammation (13), next we determined PSDP remodeling in vivo in mouse lungs during an experimental model of mild ALI secondary
to aspiration of gastric acid (15), which is a common clinical event (3). To simulate acid aspiration, hydrochloric acid (HCl) (0.1 N, pH = 1.5) was selectively instilled into the animals’ left lungs (15). Lung PMN infiltration was maximal 12 h after HCl injury (14.2 ± 1.8 × 10⁴ PMN/mg lung; P < 0.01) (Fig. 4 A). Expression of class IA and IB PI3Ks in mouse lungs were both increased at 2 and 12 h after HCl (Fig. 4 B). Lungs were removed and lipid extracts were prepared for PSDP determination. Of interest, despite increased PMN numbers, PSDP levels were significantly lower in the left lungs of HCl-injured mice (4.6 ± 0.3 μg PSDP vs. 9.0 ± 1.6 μg PSDP in control lungs; P < 0.02). These results indicate that experimental lung injury led to decrements in PSDP concomitant with increased PMN, suggesting an inverse relationship in vivo between lung PSDP and inflammation.

**PSDP mimetic blocks PMN infiltration and PI3K activity**

To determine if PSDP can block pulmonary inflammation and PI3K in vivo, we administered a PSDP structural mimetic (0.8 μg/mouse, i.v.) or vehicle 15 min before HCl instillation into the left main-stem bronchus. PSDP markedly reduced lung PMN 12 h after injury (Fig. 5 A). Tissue morphometry on LY-6G–stained histological sections (for identification of mouse PMN) revealed significant inhibition with the PSDP mimetic (46.8 ± 7.1% LY-6G staining [HCl] vs. 18.8 ± 5.8% LY-6G staining [HCl plus PSDP mimetic]; P < 0.05) (Fig. 5 B). In view of the prominent class IA PI3K lung expression that increased markedly after ALI (Fig. 4 B), we next determined class IA PI3K activity after acid injury in the presence or absence of the PSDP mimic. HCl injury induced significant increases in lung PI3K activity in p85 immunoprecipitates (0.59 ± 0.17 PIP₃/mg lung with HCl vs. 0.05 ± 0.02 pmol PIP₃/mg lung with PBS; P < 0.05). Administration of the PSDP mimetic blocked the HCl–induced increase in class IA PI3K activity to near basal levels (0.05 ± 0.01 pmol PIP₃/mg lung; P < 0.05) (Fig. 5 C). Thus, PSDP can regulate PMN activation, tissue accumulation, and total PI3K activity in vivo during experimental acid-initiated ALI.

During acute inflammation, PI3Ks orchestrate several cellular responses for host defense, including PMN ROS generation (12). Befitting its central role in cell activation, several mechanisms are in place to restrain PI3K activity (16–18). Previous reports have suggested a link between decreased PIPP formation and increased PI3K (19, 20). Results presented here are the first to demonstrate direct inhibition of PI3K by a PIPP and inverse relationships between PI3K
activity and PSDP levels both in vitro and in vivo. In addition, PSDP bound to p110γ–PI3K in activated PMN and potently inhibited rhp110γ–PI3K in vitro and a PSDP structural mimic blocked PI3K activity in vivo. Together, these new findings support a signaling relationship between PI3K and PIPPs in the regulation of leukocyte functions during inflammation.

Pivotal regulatory properties have been ascribed to isoprenoids. For example, polyisoprenyl glycolipids form antigen complexes with CD1 to activate T cells (21), and cholesterol is critical to PMN cell membrane organization and polarization in response to chemotactic stimuli (22). Although PIPPs are appreciated as cholesterol biosynthetic intermediates, PSDP is also present in cells, such as human PMN, that cannot use it for cholesterol biosynthesis because they lack squalene cyclase and other mixed function oxidase activities (23). There are now several lines of evidence to support a role for PSDP as a counterregulatory signal in PMN functional responses. PSDP is also present in cells, such as human PMN, that cannot use it for cholesterol biosynthesis because they lack squalene cyclase and other mixed function oxidase activities (23). There are now several lines of evidence to support a role for PSDP as a counterregulatory signal in PMN functional responses. PSDP is also present in cells, such as human PMN, that cannot use it for cholesterol biosynthesis because they lack squalene cyclase and other mixed function oxidase activities (23).

Figure 5. PSDP reduced acid-initiated ALI. (A) PSDP structural mimetic (0.8 μg/mouse) or vehicle was administered (i.v.) 15 min before HCl injury. 12 h after HCl instillation, histological specimens were prepared and mouse PMN were identified by LY-6G immunostaining (arrows). Bar, 100 μm. (B) Tissue morphometry was performed to determine the percentage of LY-6G staining cells in mouse lungs (n = 4 measurements in each group; *, P < 0.05 as compared with HCl-injured left lung). (C) Class IA (p85-based) PI3K activity was determined in lung lysates after ALI. Data are mean ± SEM. n = 3; *, P < 0.05 as compared with control; **, P < 0.05 as compared with HCl-injured lung.
decreased in acid-injured lungs and a novel PSDP mimetic blocked PI3K, PMN ROS generation, and PMN accumulation in the lung. Because the PSDP mimetic was administered intravenously, regulation of cells other than PMN may have also contributed to the marked inhibition of leukocyte trafficking after acid injury.

In conclusion, the ability of PSDP and a new PSDP mimetic to directly inhibit PMN early intracellular activating signals, such as PI3K, and to lessen the inflammation associated with experimental ALI provides insight into new mechanisms for in vivo protection from excess PMN-driven inflammation and tissue injury. Together, our findings suggest that PIPP signaling pathways, and specifically PSDP, can serve as natural templates for the design of new therapeutic strategies in inflammatory diseases.

MATERIALS AND METHODS

Materials. PSDP and PSMP were isolated from human PMN or prepared by total organic synthesis (8). The bisphosphonate PSDP structural mimetic, tetraethyl presulfolane carboxamido-methylene-diphosphonate was prepared from presulfolane carboxylic acid. All synthetic compounds were characterized by NMR spectroscopy.

Human PMN incubations. Peripheral blood was obtained by venipuncture from healthy volunteers who denied taking any medications for at least 2 wk and had given written informed consent to a protocol approved by Brigham and Women’s Hospital’s Human Research Committee. PMN were isolated from whole blood as described previously (6). Freshly isolated PMN (1–5 × 10^6 PMN/ml HBSS plus 1.6 mM CaCl_2) were incubated (5 min, 37°C) in the presence of 3 μM LY294002, 100 nM PSDP mimetic, or vehicle (0.1% ethanol), then exposed to LTB_4 (100 nM) in the presence of 7 μg/ml cytochrome c. This concentration of LTB_4 was chosen because it initiates PMN NADPH oxidase assembly (7) and is similar to amounts measured in vivo at sites of acute inflammation (30). In some incubations, PMN in HBSS without calcium were exposed to PSDP mimetic or vehicle, pelleted by centrifugation (700 g, 3 min), and resuspended in HBSS plus 1.6 mM CaCl_2 without PSDP before the addition of agonist. Superoxide anion generation was determined (37°C) as superoxide dismutase-inhibitable cytochrome c reduction by monitoring (550 nm) at 5-s intervals in a continuously flowing water-bath–jacketed cassette or after timed incubations. For PSDP identification, PMN (50–100 × 10^6 cells/ml) were activated (LTB_4, 100 nM, 30 s) before disruption by N_2 cavitation (350 psi, 20 min, 4°C). Remaining intact cells and nuclei were pelleted (500 g, 10 min, 4°C) and supernatants were used for immunoprecipitation with anti-p110γ−μ−phosphatidylinositol and 1 μM ATP and allowed to react for 90 min at room temperature. Incubations were stopped with 50 μL of Kinase-Glo reagent and incubated an additional 10 min at room temperature.

Luminescence was measured with a FLx800 microplate luminometer (Bio-Tek Instruments, Inc.).

Experimental model of ALL. All animal protocols were approved by the Harvard Medical Area Institutional Review Board. Acid (0.1 N HCl, pH 1.5, 50 μL) was instilled intratracheally into the left lung of anesthetized mice (FVB, male, 10–12 wk; Charles River Laboratories) (15). A PSDP mimetic (0.8 μg in 100 μL 0.9% saline) or vehicle (1% ethanol) was administered by tail vein 15 min before HCl instillation. After 12 h, lungs were removed, prepared for MPO (15) or PI3K assay, or were fixed in IHC zinc buffer and paraffin embedded for immunostaining with LY-6G (1:50 dilution). Area and number of positively staining cells was measured with National Institutes of Health Image software and percentage of positive cells/area calculated.

Statistical analysis. Results are expressed as the mean ± SEM. Statistical significance of differences was assessed by Student’s t test and one-way analysis of variance. P < 0.05 was set as the level of significance.

Online supplemental material. Fig. S1 shows the CMC determination for the PSDP mimetic and related compounds. Further information on materials and experimental protocols are supplied as the supplemental Materials and methods. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20052143/DC1.

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SUPPLEMENTAL MATERIALS AND METHODS

LTB₄ was obtained from Cayman Chemical; LY294002 was obtained from Cell Signaling Technology; dolichol monophosphate was obtained from American Radiolabeled Chemical, Inc.; o-dianisidine dihydrochloride, cytochrome c, and ATP were obtained from Sigma Chemical Co.; human p110γ PI3K was obtained from Alexis Biochemicals; l-α-phosphatidylinositol was obtained from Avanti Polar Lipids, Inc.; and anti-p110γ and anti-p85–PI3K antibodies were obtained from Upstate Biotechnology. IHC zinc buffer and mouse anti–LY-6G antibody were obtained from BD Biosciences.

PSDP determination.
The amount of PSDP was determined by densitometry (Scion Image software) after materials were saponified (10% KOH in methanol, 30 min, 37°C), extracted, and separated by TLC. Dolichol monophosphate (2 μg) was used as an internal control to correct for extraction losses.

P85 and p110γ-PI3K gene expression.
Total RNA was extracted from snap-frozen lungs and semi-quantitative gene expression was determined using specific primers for murine p85 (sense primer 5′-ACCCCAGTTTTGTGCTTG-3′, antisense primer 5′-CCTGCCCAACATT-TAGTCCA-3′), p110γ PI3K (sense primer 5′-TTCTCGTGTGTCACCACATGT-3′, antisense primer 5′-CCTGGGACATCT-CAGTGAT-3′), and β-actin (internal control). After electrophoresis, densitometry was performed using Scion Image software.

CMC measurement.
CMC was determined by light scattering (Levy, B.D., N.A. Petasis, and C.N. Serhan. 1997. Nature. 389:985–990). In brief, PSDP mimetic, the related PIPPs PSMP and farnesyl diphosphate (FDP), or l-α-phosphatidylinositol (PI) (10⁻⁹–10⁻³ M) were added to HBSS containing 1.6 mM CaCl₂. After vortexing (30 s, room temperature) and sonication (30 s × 3, full power), materials were kept at room temperature for 30 min. For each compound, the relationship between absorbance at 762 nm (Abs 762 nm) and concentration was determined and the breakpoint in Abs 762 nm was taken as an estimate of the CMC (see Fig. S1).