CMLS, Cell. Mol. Life Sci. 59 (2002) 729–741
1420-682X/02/050729-13 $ 1.50 + 0.20/0

CMLS Cellular and Molecular Life Sciences

Polyisoprenyl phosphates: natural antiinflammatory lipid signals

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Abstract. Lipoxins (LX) and aspirin-triggered 15-epimer LX are leukocyte-derived eicosanoids generated during host defense that serve as down-regulatory signals. The specific intracellular events that govern cellular responses to inhibitory extracellular signals are of wide interest in order to understand pivotal intracellular events in diseases characterized by enhanced inflammatory responses, such as asthma, rheumatoid arthritis and atherosclerosis. We recently uncovered a novel role for polyisoprenyl phosphates, in particular presqualene diphosphate (PSDP), as natural down-regulatory signals in human neutrophils that directly inhibit phospholipase D and superoxide anion generation. Activation of LXA_4 receptors (ALXR) reverses proinflammatory receptor-initiated decrements in PSDP and inhibits cellular responses. These findings represent evidence for a novel paradigm for lipid-protein interactions in the control of cellular responses, namely receptor-initiated degradation of repressor lipids that is subject to regulation by aspirin treatment via the actions of aspirin-triggered 15-epimer LX at the ALXR, and identify new templates for antiinflammatory drugs by design.

Key words. Eicosanoids; lipid mediators; signal transduction; aspirin; inflammation; leukocytes.

Introduction

During host defense a wide array of compounds are generated by host tissues that tightly regulate cellular events that are essentially homeostatic in function. Their summation leads to acute inflammatory events that can progress to either chronic inflammation, resolution or healing [1]. Among the well-appreciated mediators of inflammation, novel lipid mediators are of special interest, in that agents that enhance the rate of resolution or healing can be defined as endogenous antiinflammatory mediators. Here, we discuss our recent evidence for endogenous antiinflammatory lipid signals that can serve in the hostís antiinflammatory cascade.

Leukotrienes (LT) and lipoxins are local effectors that control leukocyte-mediated events and are of interest in inflammation, reperfusion injury and tissue repair [2]. LTB_4 stimulates leukocyte extravasation and activation, and the peptido-LT (LTC_4 and LTD_4) are potent vaso- and bronchoconstrictors [3]. In sharp contrast to these potentially proinflammatory LT-mediated signals, LX display leukocyte selective actions that can be characterized as 'stop signals' operative in acute inflammation [4]. The timing and balance between LT and LX biosynthesis likely influence the intensity and duration of cellular responses during inflammation and other complex host responses, since they are derived from the common precursor, arachidonic acid.

The LXA_4 receptor (ALXR) was the first lipoxygenase-derived eicosanoid receptor to be cloned and identified as a member of the family of G-protein-linked receptors with seven transmembrane-spanning domains [5]. Recently, a similar cloning strategy resulted in the identification of an LTB_4 receptor (BLTR), a distinct G-protein-linked receptor closely related to the ALXR [6]. Despite structural similarities, activation of BLTR leads to neutrophil locomotion, superoxide anion generation and granule mobilization, in contrast to activation of ALXR by LXA_4, which inhibits these responses. It is of wide in-

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terest to elucidate key intracellular events that translate opposing extracellular signals, such as LXA_4 and LTB_4, into controlled, integrated cellular responses.

Polyisoprenyl phosphates in human neutrophils

Are there specific intracellular counter regulatory lipid signals in human neutrophils?

Receptor-ligand interactions initiate the elaboration of both positive and negative signals [2]. These events are exemplified by formyl-methionyl-leucyl-phenylalanine (fMLP), a synthetic bacterial surrogate peptide that binds to its receptor on PMN with subsequent generation of intracellular second messages (e.g. intracellular calcium influx) that stimulate as well as inhibit (e.g. formation of cyclic AMP) PMN [7]. Although several classes of intracellular lipids are known to activate leukocytes (e.g. diacylglycerols, phosphatidic acid and arachidonic acid), few counterregulatory lipid-derived mediators are known [2]. To this end, we screened for the presence of novel mediators in neutrophil lipid extracts that regulate superoxide anion generation, a critical phagocyte response important during microbial killing, inflammation and tissue injury [8]. Neutrophil-derived lipid extracts initiated the generation of substantial amounts of superoxide anions (2.71 nmol O_2^-/mg protein/min) by cell sonicates (fig. 1 inset). To identify novel regulatory signals, we eliminated intact phospholipids with ester and/or amide bonds by saponification hydrolysis before extraction and CC4 silica chromatography as in [9, 10]. The initial chloroform:methanol (2:1, v/v) elutions (denoted fraction A) contained a mixture of fatty acids and neutral lipids that gave similar rates of superoxide anion generation (2.41 nmol/mg protein/min). O_2^- production, in these experiments, was comparable to that observed with known lipid activators in this system, namely arachidonic acid [75 µM (C20:4), 1.38 nmol/mg protein/min] and phosphati-dic acid [100 µM, 1,2-dicapryl-*sn*-glycero-3-phosphate (C10:0), 1.34 nmol/mg protein/min] [8]. In contrast, subsequent elutions with chloroform:methanol:water (10:10:3, v/v) (fraction B), containing phosphorylated lipids that resist saponification, stimulated significantly less superoxide anion generation (0.62 nmol/mg protein/min, $P < 0.05$) than fraction A, suggesting that fraction B carried an inhibitor (fig. 1 inset). Furthermore, the lipids present in fraction B potently inhibited O_2^- production stimulated by either phosphatidic acid (PA) (52%, fig. 1) or arachidonic acid (56%) [8]. These findings indicated that material present within fraction B counteracts the known intracellular lipid signals (i.e. PA and arachidonic acid) that are held to be involved in the initial activation and assembly of the neutrophil oxidase.

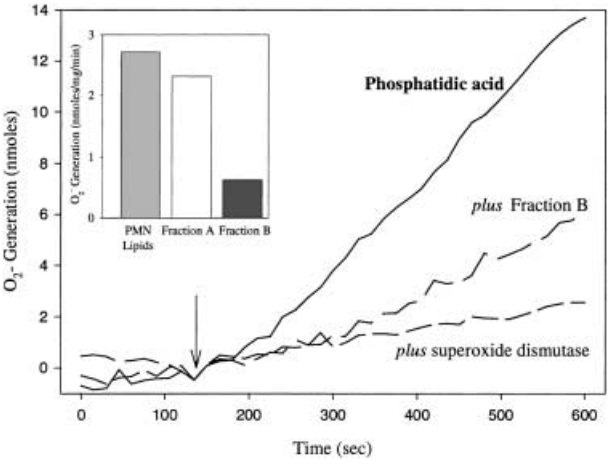


Figure 1. PMN-derived lipids that resist saponification regulate superoxide anion generated by PMN sonicates. O_2^- generation by PMN sonicates (0.1–0.5 mg protein/ml) in the presence of PMN lipid extracts (inset) and a representative time course for PMN sonicate O_2^- generation in the presence of fraction B or superoxide dismutase [phosphatidic acid (C10:0); 100 µM; $n = 5$]. The arrow indicates the time of β-NADPH (200 µM) addition to initiate assembly of the oxidase. Superoxide-dismutase inhibitable cytochrome c reduction was monitored by absorbance at 550 nm (15-s intervals or 10 min, 37 °C).

Structural elucidation of the putative 'stop signal' present in PMN lipid fraction B

To identify this inhibitor, materials in fraction B were isolated from stimulated neutrophils and initially examined using thin layer chromatography (TLC) ($n = 3$). Lipids from sequential 5 mm fractions (from origin to solvent front) were eluted and assessed for phosphorus content and activity. Regions 10–15 mm from the origin carried phosphorus (11.3 nmol phosphorus/10^8 cells) and gave ~52% inhibition of PA-stimulated superoxide anion generation (fig. 2). To elucidate structure, this material was initially taken directly to gas chromatography/mass spectrometry (GC/MS) and gave base peaks at *m/z* 69 with a fragmentation pattern of repeating C_5H_8 (*m/z* 68) units, ions that are characteristic of isoprenoids [11]. Fraction B's lipids were chromatographed by TLC in parallel and stained with iodine to identify unsaturated lipids and molybdenum blue to visualize phosphorus. Four major compounds were observed [8] with compound II in the region 10–15 mm from the TLC origin. Rf values in this TLC system were determined for isoprenoids, including isopentenyl diphosphate, geranyl diphosphate, geranylgeranyl diphosphate (GGDP), dolichyl monophosphate (DOL-MP), isoprenyl alcohols, squalene or cholesterol [8]. Compounds I and III (Rf values of 0.05 ± 0.01 and 0.17 ± 0.01) matched farnesyl diphosphate (FDP) and farnesyl monophosphate (FMP), respectively. Compounds II and IV carried the most phosphorus/cell and gave Rf values (0.10 ± 0.01 and 0.25 ± 0.02) that differed

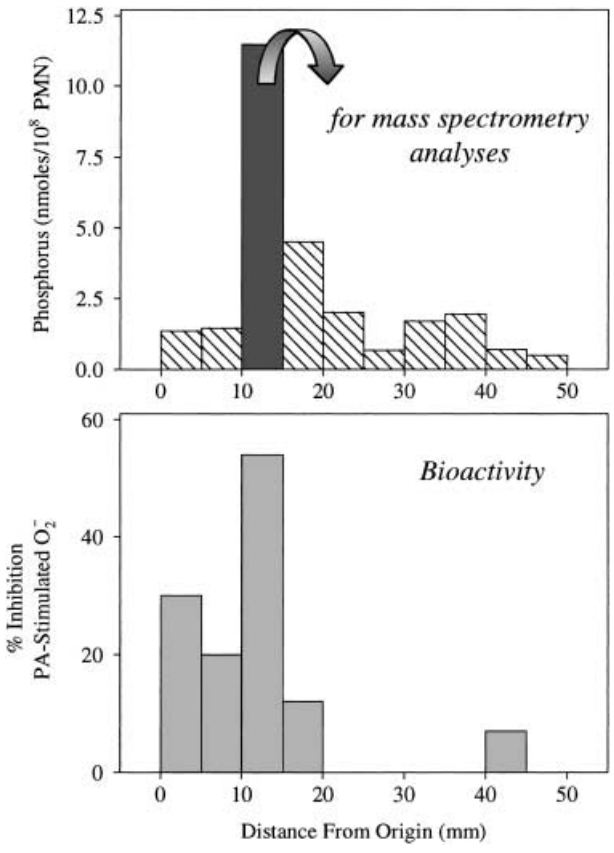


Figure 2. Identification of an inhibitor of superoxide anion generation. Phosphorus content (upper panel) and biological activity (lower panel) were determined for sequential 5 mm TLC segments after chromatography ($CHCl_3$:MeOH:dH_2O 65:25:4, v/v/v, 50 min, RT) of fraction B. Each segment was analyzed (as in [8]) for inorganic phosphorus content and the capacity to inhibit PA-initiated O_2^- generation in PMN sonicates.

from these isoprenoids [8]. Based on Rf values and the relationship between phosphorus content and mass (measured by scanning densitometry of charred TLC plates), compound II was found to contain two phosphates, and compounds III and IV one phosphate each. Together, these findings established the presence of four major phosphorylated lipids in neutrophils that resist saponification with physical properties consistent with polyisoprenyl phosphates.

To address the identification of these four lipids before assessment of their bioactivity, each was isolated and taken for analyses using GC/MS using either direct injection or analyses after conversion to Me_3Si derivatives [8]. Each of the four lipids possessed the characteristic fragmentation pattern of isoprenoids [11]. Compounds I and III gave retention times and prominent ions consistent with FDP and FMP that were confirmed by direct comparison to authentic material. Compound II gave a fragmentation pattern resembling that of squalene [molecular ion (M^+) = 410 amu]. It is important to note that it displayed anions $m/z > 410$ (fig. 3) and had a retention different than squalene [8]. This observation combined with compound II's properties in TLC analyses indicated a phosphorus containing isoprenoid with ≥ 6 C_5H_8 units.

To distinguish di- from monophosphorylated isoprenoids, compound II was treated with acid, which liberates 80% of the phosphate from polyisoprenyl diphosphates without substantial destruction of polyisoprenyl monophosphates [10], and converted compound II into squalene [8]. Compound IV's retention and fragmentation pattern in GC/MS were similar to that seen with compound II; however, compound IV was not susceptible to

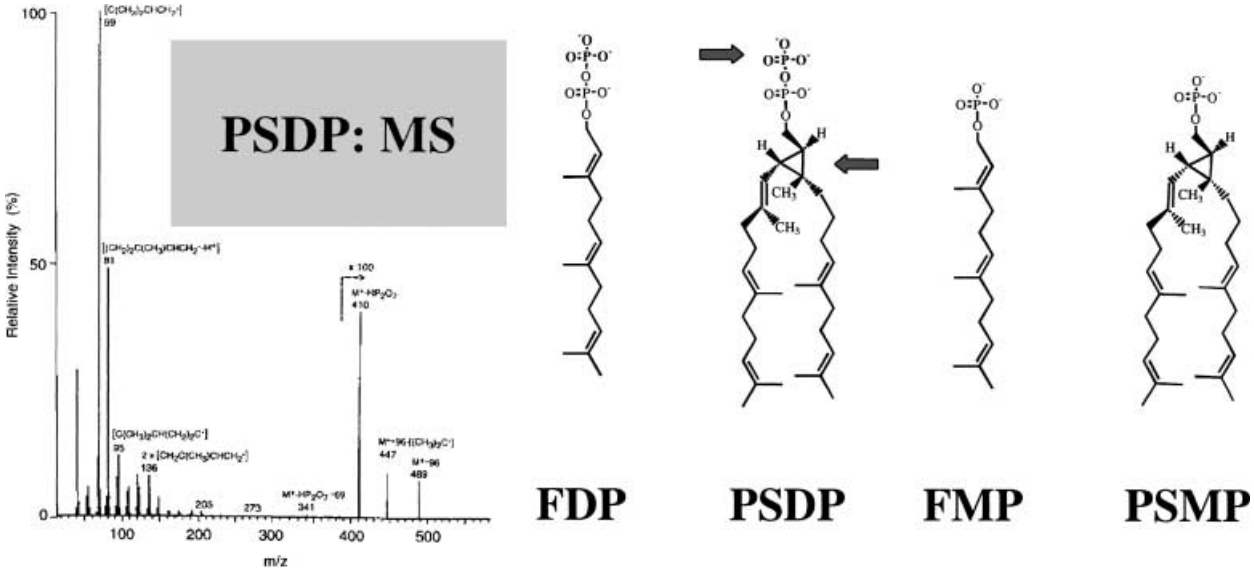


Figure 3. Mass spectrum of PSDP – PMN polyisoprenyl phosphates: structure activity relationship. Mass spectrum (MS) of PSDP (compound II) (left). Prominent PMN polyisoprenyl phosphate structures are depicted (right). Arrows denote the presence of both the diphosphate and cyclopropylcarbinyl ring that make PSDP unique amongst these closely related compounds.

acid hydrolysis. Molecular ions were not readily apparent for either compound II or IV, a trait consistent with GC/MS analysis of polyisoprenyl phosphates [12, 13]. Based on these physical properties as well as evidence obtained from material prepared by total organic synthesis (vide infra), the assigned structure of compound II was presqualene diphosphate (PSDP) (fig. 3) and of compound IV, presqualene monophosphate (PSMP). In yeast, plants and mammalian liver, squalene synthase condenses two molecules of farnesyl diphosphate to form PSDP, a cyclopropylcarbinyl intermediate that in the presence of NADPH is further converted to squalene [14–16]. Human PMN generate squalene from endogenous sources but lack the enzymes required for further conversion to cholesterol [17]. FDP, PSDP, FMP and PSMP represented 0.1, 1.8, 0.4 and 0.5%, respectively, of total phosphorylated lipids, which was 92.4 nmol phosphorus/10^7 unstimulated cells, $n = 3$ [8]. These results indicated that among the human neutrophil lipids that resist saponification, a series of biosynthetically related polyisoprenyl phosphates were present (fig. 3) that carried a novel inhibitory activity.

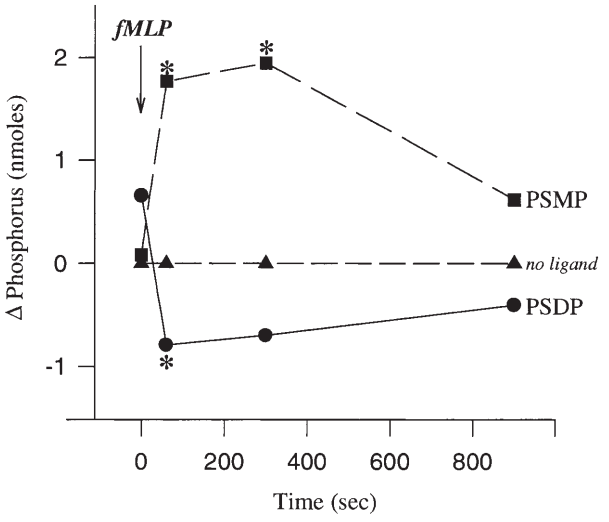


Figure 4. Receptor activation by a pro-inflammatory agonist triggers remodeling of PSDP and PSMP. Time course of fMLP (100 nM) stimulated (20×10^6 PMN/ml) changes in inorganic phosphorus content of PSDP (●) and PSMP (■) (without agonist ▲). * $P < 0.03$ by Student's paired t test.

Do compounds I–IV rapidly remodel upon receptor-ligand interaction in a fashion consistent with signaling events?

Resting human PMN, exposed to γ-$^{32}PO_4$-ATP to enable mass quantitation and identify phosphate turnover, selectively incorporated radiolabel into compounds II (PSDP) and IV (PSMP) [8]. The bacterial peptide analog fMLP stimulates neutrophil superoxide anion generation via interaction with a G-protein-coupled, seven-transmembrane-spanning fMLP receptor [18]. Exposure of neutrophils to fMLP resulted in changes in compound PSDP and PSMP's phosphorus content which were rapid (within 60 s) and reciprocal (decrements in PSDP paralleled increments in PSMP) (fig. 4). Cytokines such as granulocyte/monocyte-colony stimulating factor (GM-CSF) prime neutrophils for inflammatory responses to fMLP by interacting with receptors whose signaling mechanisms remain to be fully elucidated [19]. Of interest, GM-CSF enhanced neutrophil $^{32}PO_4$ incorporation into both PSDP (45%) and PSMP (84%) [8]. Thus, remodeling of PSDP and PSMP temporally overlaps the initial rate of O_2^- production triggered by receptor activation, and physiologically relevant stimuli increase PSDP and PSMP phosphate turnover in neutrophils.

Bioactivity and role of PSDP and PSMP

To test the impact of PMN-derived compounds PSDP and PSMP on superoxide anion generation, these lipids isolated from PMN were introduced into cells by electroporation prior to determining agonist-induced O_2^- production. PSDP gave ~60% inhibition of fMLP-stimulated superoxide anion generation, while O_2^- production in the presence of PSMP appeared unaltered. Importantly, neither of these compounds alone stimulate O_2^- production in the nanomolar range [8]. Prenyl cysteine analogs can disrupt G protein interaction with activated FMLP receptors [20]; therefore, to determine PSDP's level of action, neutrophil superoxide anion generation was next examined in the presence of phorbol 12-myristate 13-acetate (PMA), a potent agonist for O_2^- production that bypasses cell surface receptors to stimulate protein kinase C and subsequent oxidase assembly [18]. PMA-activated superoxide anion generation was inhibited by ~45% with PSDP (1 µM) (fig. 5) in a concentration-dependent fashion, with significant inhibition present at 10 nM (fig. 6). PMA-stimulated O_2^- production was not significantly blocked by PSMP, FDP or other closely related compounds that possess similar hydrophobicity (fig. 5). These results clearly establish the critical structural basis for inhibition by PSDP since its monophosphate containing form (PSMP) was devoid of activity at equimolar concentrations. In addition, these findings indicate that PSDP selectively inhibits O_2^- production at site(s) post G-protein-receptor interactions. Inhibition of the oxidase activity by PSDP, not observed with PSMP or other closely related isoprenoids (fig. 3), suggested a unique role for PSDP as a lipid carrying signaling information that is in sharp contrast to the simple structural roles assumed by other isoprenoids, such as cholesterol, in the relay of signals as part of lipid rafts within cell membranes [21].

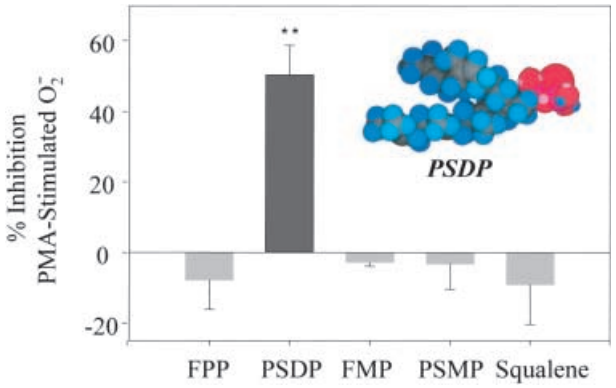


Figure 5. PSDP selectively inhibits PMN superoxide anion generation. The impact of PSDP and closely related polyisoprenoids (1 μM) on O_2^- stimulated PMA (100 nM) with electroporated PMN. CS Chem3D Pro software (CambridgeSoft Corp., Cambridge, MA) was used to calculate an energy-minimized model of PSDP (inset). ** $P < 0.01$.

Matching the physical and biological properties of PSDP and PSMP isolated from PMN to materials derived from total organic synthesis

To confirm that PSDP indeed carries inhibitory actions observed with biologically derived material, both PSDP and PSMP were prepared via total organic synthesis [8]. Each synthetic compound matched the physical and biological properties of neutrophil-derived PSDP and PSMP. Again, synthetic PSDP, but not synthetic PSMP, inhibited superoxide anion generation in a concentration-dependent fashion in the presence of PA, a physiologically relevant endogenous stimulus (fig. 6). Maximal inhibition (~60%) was observed at 10^{-7} M PSDP. Submicromolar concentrations of PSDP also resulted in significant inhibition of both fMLP (10^{-7} M) and PMA (10^{-8} M) stimulated oxidase activity in electroporated PMN (fig. 6). While the concentration response for inhibition of O_2^- generation by PSDP was overlapping for PA and fMLP as agonists, PSDP was approximately one log order less potent in blocking PMA, suggesting that PMA initiates this response, in part, via a mechanism independent of the other agonists in PMN. Of interest, concentrations of PSDP > 10^{-6} M did not further inhibit O_2^- production. At 10^{-5} M, PSDP gave a bell-shaped dose response curve, similar in pattern to responses observed with some other bioactive lipids [2]. In this case, PSDP aggregates in buffer alone at a concentration of 10^{-5} M, evidenced by a dramatic increase in absorbance. Thus, at supraphysiologic concentrations, physical interactions precluded PSDP from inhibiting O_2^- production. These findings indicate that PMN-derived PSDP and PSMP matched the physical and biological properties of synthetic PSDP and PSMP, and that PSDP, in nanomolar to micromolar amounts, regulates proinflammatory agonist-induced PMN responses.

Biological impact of lipids carrying information

Isoprenoids are pivotal intermediates in the generation of diverse classes of compounds, including sterols, retinoids, dolichols, ubiquinone and prenylated proteins [14]. In PMN, we identified the major polyisoprenyl phosphates, and in response to receptor activation, two of these compounds, PSDP and PSMP, rapidly remodel. Physiologic levels of PSDP regulated cellular events relevant during inflammation and tissue injury. The finding

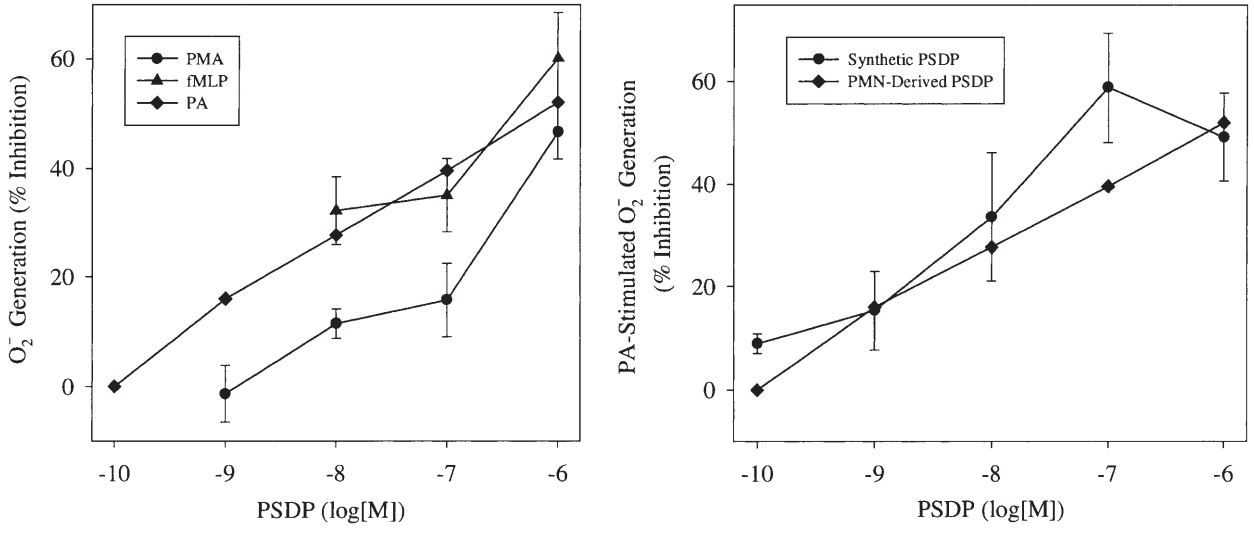


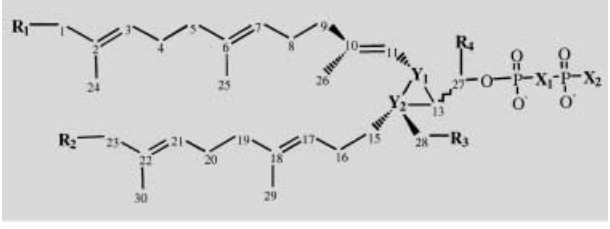
Figure 6. Concentration response for inhibition of superoxide anion generation by PSDP: synthetic materials match PMN-derived PSDP. Concentration-dependent inhibition by PSDP of agonist-induced O_2^- generation in PMN sonicates (phosphatidic acid ◆) or electroporated PMN (PMA ● and fMLP ▲) (left panel). Direct comparison of synthetic (●) and biologically derived (◆) PSDP in the concentration-dependent inhibition of phosphatidic acid-stimulated O_2^- generation (right panel).

that PSDP serves as a negative regulator in PMN does not preclude PSDP from exerting stimulatory actions with other immune effector cells, as exemplified by the lipoxins which inhibit PMN yet stimulate monocyte motility [22]. Together, these results indicate that PSDP represents a novel, potent lipid-derived signal and provide evidence for isoprenoid remodeling and a link between cholesterol metabolism and immune function.

Polyisoprenyl phosphates as templates for novel anti-inflammatory drugs by design

Based upon these findings, a series of polyisoprenyl phosphate analogs that resist rapid inactivation were designed and proposed as pharmacophores that may be superior in controlling the aberrant release of injurious agents from activated neutrophils (fig. 7). Although nonsteroidal antiinflammatory drugs help to alleviate some of the symptoms of chronic inflammatory illness, current disease-remitting agents are most often ineffective, and no currently available medication can prevent ischemia-reperfusion injury. Also, reactive oxygen species generated by neutrophil NADPH oxidase are associated with the pathogenesis of these inflammatory conditions.

Structural analogs of PSDP may offer several advantages [23]. The analogs would be more resistant to degradation or alternatively may inhibit degradation of natural PSDP. Since these are small molecules, they may find utility as pharmaceuticals for treating or preventing conditions associated with inappropriate inflammatory mediated cellular responses because they are likely to be bioavailable.



To block hydrolysis, add substituents:
X_1, X_2 = O, N, CH_2 or CH_3, azide, and S in combination grid series

To resist P450 oxidation and slow first pass metabolism:
R_{1-4} = halogenation and methylation

To stabilize the conformation to determine the site of action:
Y_1, Y_2 = R,S; S,R; R,R; S,S respectively

Figure 7. Novel analogs of PSDP: delay metabolic inactivation. A series of PSDP analogs was designed with substituents added to block hydrolysis (X_1 and X_2), to resist P450 oxidation and slow first-pass metabolism (R_{1-4}) and to stabilize the structure's conformation (Y_1 and Y_2).

In addition, the capacity of PSDP to regulate NADPH oxidase assembly is a convenient means to screen structural analogs for bioactivity.

Polyisoprenyl phosphates (PIPP) as antiinflammatory lipid mediators

Regulation of PMN responses by autacoid natural products

In response to inflammatory stimuli, PMN phospholipases are activated to remodel cell membranes and generate bioactive lipids that serve as intra- or extracellular mediators in the transduction of functional responses [2]. Important components of microbicidal and acute inflammatory responses include reactive oxygen species and granule enzymes that are targeted to phagocytic vacuoles, but aberrant release of these potentially toxic agents can lead to amplification of inflammation as well as tissue injury and are implicated in a wide range of diseases [24]. To prevent an overexuberant inflammatory response and limit damage to the host, these PMN programs are tightly regulated. Bioactive lipids are rapidly generated by activation of cell surface receptors that carry either specific positive or negative signals to modulate cellular responses. This is exemplified by the related eicosanoids, LTB_4, a potent chemoattractant [25], and LXA_4, an endogenous 'stop signal' for PMN recruitment [4]. LTB_4 and LXA_4 interact with highly specific and distinct G-protein-coupled membrane receptors [5, 6] to evoke opposing PMN responses, including LXA_4 inhibition of LTB_4-initiated chemotaxis, adhesion and transmigration [4].

Widely used clinically for its antiinflammatory properties, aspirin is known to affect biosynthesis of lipid mediators. Mechanisms responsible for aspirin's antiinflammatory actions continue to be of great interest, as new 'super-aspirins' are sought that do not possess its deleterious side effects [26]. In addition to inhibiting prostanoid formation, aspirin triggers the endogenous generation of novel carbon 15 epimers of LX by transcellular routes (see fig. 8) during inflammation in vivo (e. g. between tissue-resident cells and infiltrating leukocytes) [27]. These aspirin-triggered lipoxins (15-epi-LX) are even more potent than the native LX as inhibitors of PMN responses, in part because they are active longer [28]. PMN inhibition by LX and 15-epi-LX is evoked by specific receptor-activation of 'inhibitory' signals and not via direct receptor level antagonism at LTB_4 receptors [29]. The recent finding that LTB_4 receptors also serve as novel human immunodeficiency virus-type 1 (HIV-1) coreceptors has heightened interest in the regulation of the LTB_4 receptor activation [30]. Despite its common use over more than a century, complete knowledge of aspirinís therapeutic impact is still evolving, with many newly discovered clini-

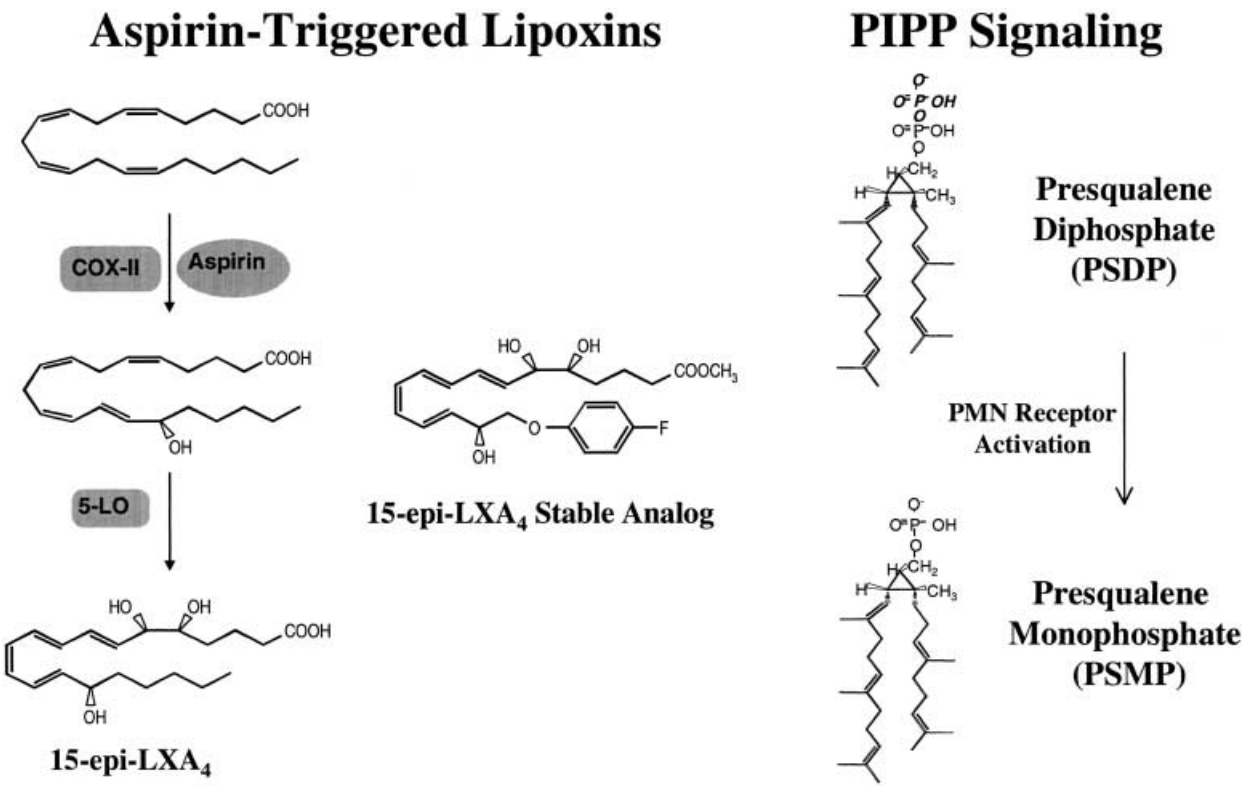


Figure 8. Formation of antiinflammatory lipid signals. Scheme for aspirin-triggered 15-epi-LXA_4 biosynthesis and structure of the stable analog, 15-epi-16-para-fluoro-phenoxy-LXA_4-methyl ester (left) and hypothetical scheme for PIPP signaling (right).

cal utilities [31]. Regular ingestion of aspirin decreases the incidence of myocardial infarction, colorectal carcinoma and Alzheimerís disease (reviewed in [32]), but side effects from aspirin, such as gastrointestinal ulceration, can limit its use. The recent discovery of a second isoform of cyclooxygenase (COX) that is induced during inflammation has led to a search for super-aspirins that can selectively inhibit COX-2 without disrupting the protective constitutive functions of COX-1 [26, 33]. Of particular interest in this regard, 15-epi-LX, which inhibit PMN migration, are endogenous products of aspirinís acetylating ability that may underly some of the salutary benefits of aspirin. These findings suggest several novel strategies for using 15-epi-LX mimetics as new antiinflammatory agents designed after endogenous mediators. In this context, LX and 15-epi-LX stable analogs were designed, like 15-epi-16-para-fluoro-phenoxy-lipoxin A_4-methyl ester, a synthetic analog of 15-epi-LXA_4 (fig. 8) that resists rapid inactivation, acts via the LXA_4 receptor [28, 29] and when applied topically, inhibits PMN infiltration and vascular permeability during mouse ear skin inflammation [34].

Phospholipase D is a candidate signaling pathway target for receptor-operated blockade in PMN responses

Phospholipase D (PLD) signaling plays a pivotal role in mounting cellular responses. PLD hydrolyzes membrane phosphatidylcholine (PC) within seconds of exposure to ligands to generate PA [35]. Formation of PA temporally antecedes functional responses, including vesicle secretion and assembly of the NADPH oxidase [36, 37]. Several isozymes of PLD1 and PLD2 were cloned and characterized [38], with PLD1b identified as a prominent isoform in human granulocytes (reviewed in [39]) that localizes to granule membranes [40]. With the identification of a new polyisoprenyl phosphate (PIPP) signaling pathway [8] (fig. 8) that regulates PMN superoxide anion generation and because PLD activation is linked to the assembly and activation of the PMN NADPH oxidase [41], we reasoned that PIPP signaling might also modulate phospholipase activity critical to global cellular activation [42].

LTB_4 stimulates rapid remodeling of PIPP and degradation of PSDP

LTB_4 interacts with its surface receptor to rapidly activate phospholipases, such as PLD, and signal cellular re-

sponses [6]. To determine whether LTB_4 receptor activation leads to remodeling of PIPP and specifically PSDP, cellular phosphate pools were steady-state-labeled with [γ-^{32}P]-ATP and exposed to either LTB_4 (100 nM) or vehicle (0.1% ethanol) alone. Aliquots were removed at timed intervals from 0 to 300 s (37 °C), and nonsaponifiable phosphorylated lipids were isolated and quantitated by phosphoimager for [^{32}P] incorporation. PSDP levels in unstimulated PMN are ~1.7 nmol/10^7 PMN (~50 nM) [8]. PSDP and PSMP, but not FDP, incorporated [^{32}P] from ATP (vide supra). LTB_4 initiated a rapid (evident within 30 s) and statistically significant decrease in [^{32}P]-PSDP (28%) within 60 s (fig. 9). [^{32}P]-PSDP levels returned to baseline amounts within 270 s. Changes in [^{32}P]-PSDP after LTB_4 receptor activation reflected changes in PSDP mass. These findings demonstrated a marked decrement in PSDP (fig. 9) concurrent with LTB_4's kinetics of PMN activation [8, 25]. In PMN, PSDP is enriched in subcellular fractions containing nuclei (39% of total PSDP) and granules (36%) with lesser amounts in plasma membrane (16%), and its topography changes when cells are activated by LTB_4 [42a].

Aspirin-triggered 15-epimer LX analog switches the LTB_4 program accumulating PSDP

Interacting with the ALXR on PMN, both LXA_4 and 15-epi-LXA_4 stable analogs can inhibit organ and tissue infiltration in vivo [29]. To determine whether ALXR activation mediates inhibitory actions via PIPP signaling, the impact of a 15-epi-LXA_4 analog (fig. 8) (100 nM, 5 min, 37 °C) on LTB_4 (100 nM)-stimulated changes in PSDP was examined using [^{32}P] labeling of PMN lipids (vide supra, in parallel incubations). Alone, the 15-epi-LXA_4 analog did not affect the rate of PIPP remodeling (fig. 9). Of interest, exposure to LTB_4 in the presence of equimolar 15-epi-LXA_4 analog not only prevented the LTB_4-initiated decrease in PSDP but additionally stimulated a significant increase (~72%) in [^{32}P]-PSDP at 60 s (fig. 9). PSDP levels continued to rise for at least 300 s after exposure to LTB_4 (fig. 9). Native LXA_4 and its related LXA_4 receptor agonist, 16-phenoxy-LXA_4-methyl ester, gave qualitatively similar responses as the 15-epi-LXA_4 analog with a rank order of potency of 15-epi-LXA_4 analog > 16-phenoxy-LXA_4 > LXA_4, with 15-epi-LXA_4 analog 1–2 orders of magnitude more potent [42]. These results indicate that 15-epi-LXA_4, which inhibits LTB_4 responses in vivo [29], dramatically switches LTB_4-initiated PIPP signaling. Moreover, increases in PSDP levels evoked by coactivation of the LXA_4 and LTB_4 receptors correlates with the time course for regulation of LTB_4's biological actions by LXA_4 and 15-epi-LXA_4 (*vide infra*).

15-epi-LXA_4 inhibits PLD activity and O_2^- generation in PMN

LTB_4-stimulated PLD activation temporally antecedes and accompanies morphologic changes, degranulation and O_2^- production in PMN [37, 43]. To determine whether LT and LX-mediated remodeling of PIPP correlated with specific cell-signaling events, we monitored PLD activity in cell lysates. LTB_4 increases PLD activity that is maximal by 60 s (fig. 10). These values for LTB_4 and PLD activation are consistent with those reported earlier [43, 44]. In the presence of 15-epi-LXA_4 analog, LTB_4-stimulated PLD activity and O_2^- generation were potently inhibited (>80%) (fig. 10). The inverse relationship between [^{32}P]-PSDP levels and PLD activity ob-

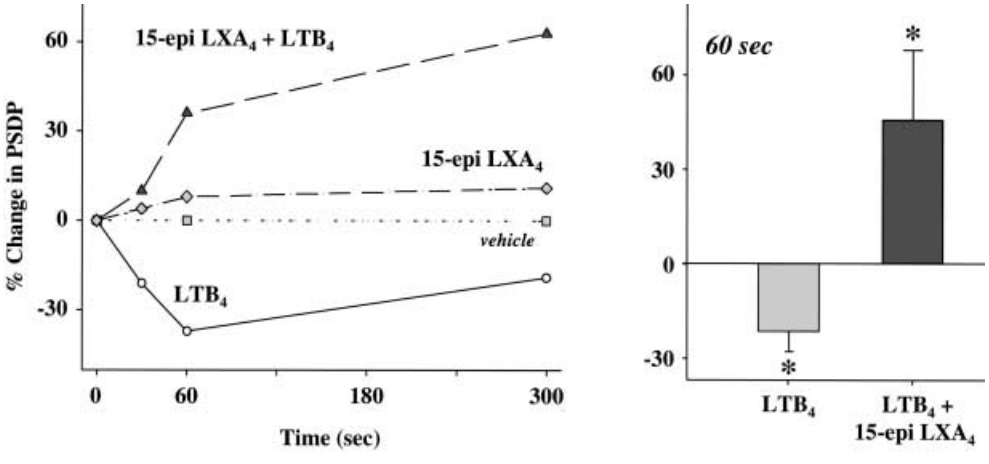


Figure 9. LTB_4 rapidly remodels PSDP in human PMN: impact of a 15-epi LXA_4 analog. PMN were labeled with [γ-^{32}P]-ATP and incubated (12.5×10^6/ml, 37 °C) with LTB_4 (○, 100 nM), 15-epi-LXA_4 analog (◆, 100 nM), vehicle (■, 0.1% ethanol) or 15-epi-LXA_4 analog (100 nM, 5 min) followed by LTB_4 (▲, 100 nM). Nonsaponifiable lipids were extracted and separated by TLC, and [^{32}P]-incorporation was quantitated by phosphorimaging [42]. Values are densitometric measurements. A 30% change in PSDP represents ~0.5 nmol/10^7 PMN. A representative time course ($n = 5$) (left) and the mean ± SE for the change in PSDP at 60 s (right) are shown. * $P < 0.05$ by Student's t test.

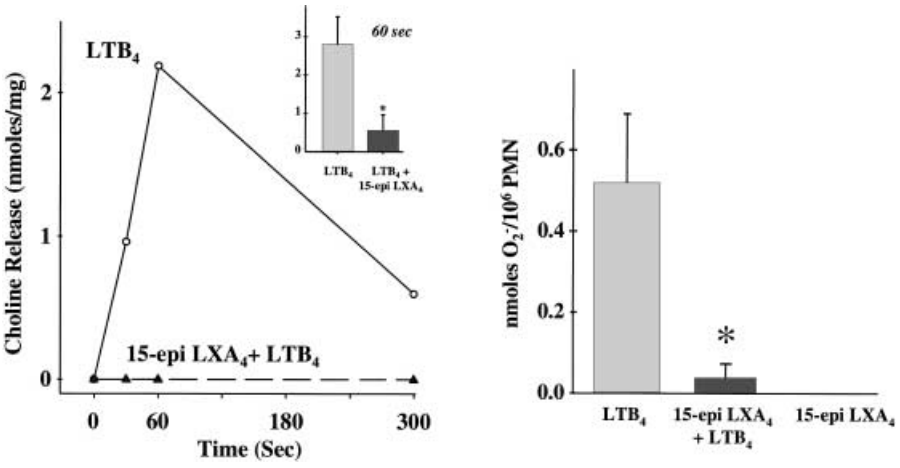


Figure 10. 15-epi LXA_4 analog inhibits human PMN PLD activity and O_2^- generation. PLD activity was determined using cell lysates ($2-5 \times 10^6$ cells, 90–130 µg protein) as in [42]. Representative time course for the impact of the 15-epi LXA_4 analog on choline release ($n = 5$, $d = 4$) (left), and mean ± SE for the change in PLD activity at 60 s (inset) are shown. Superoxide anion generation by freshly isolated human PMN (right) was determined (10 min, 37 °C) for LTB_4 (100 nM), 15-epi-LXA_4 analog (100 nM) or 15-epi-LXA_4 analog (100 nM, 5 min, 37 °C) followed by LTB_4 (100 nM). Mean ± SE for $n \geq 3$. * $P < 0.05$ by Student's t test.

served with ligand-receptor interactions that signal opposing cellular responses suggested that PSDP might regulate PLD.

PSDP: a direct inhibitor of both plant and mammalian PLD

To analyze polyisoprenyl phosphates, direct impact on PLD, PSDP and closely related lipids were incubated with purified plant enzyme (EC 3.1.4.4; $V_m = 0.29$ nmol/s, $K_m = 1.4$ mM). PSDP inhibited cabbage PLD (cPLD) in a concentration-dependent fashion (10–1000 nM) with a K_i of 20 nM ([PSDP] = 10 nM) [42]. Lineweaver-Burk analyses were consistent with a competitive inhibition model. Closely related lipids, such as PSMP (minus only one phosphate), showed a greater than 100-fold loss in inhibitory potency compared with PSDP (table 1). Comparable inhibition was not evident with other polyisoprenoids (i.e. FDP and squalene) or PA. We questioned whether PSDP could also inhibit mammalian PLD, and determined recombinant human PLD1b kinetics in vitro with PSDP. The recombinant enzyme ($V_m = 0.36$ nmol/s, $K_m = 13.8$ mM) was also dramatically inhibited by PSDP, with a K_i of 6 nM (table 1). Because PLD activation occurs in vivo in the presence of many cofactors which modulate its activity, we also determined the impact of PSDP on PLD activity in PMN lysates. Sixty seconds following LTB_4, PSDP levels decreased (28%, fig. 9), and PLD activity was maximal (fig. 10). Addition of PSDP (100 nM) to PMN lysates at this time (60 s, LTB_4 100 nM) gave 89.5 +/– 9.7% inhibition of PLD activity [42]. Collectively, these results indicate PSDP's potent inhibition of both plant and mammalian PLDs and establish a critical role for both the terminal phosphate and isoprenoid chain length (fig. 3) in PSDPs regulation of PLD.

A wide range of receptor classes initiate PMN responses via PLD-mediated hydrolysis of PC to PA [37]. Both G-protein-linked receptors and receptor tyrosine kinases can activate PLD. In leukocytes, several factors, including PKCα (in a kinase-independent manner) and increased intracellular calcium, can activate PLD1 [45]. FMLP-stimulated PLD activity in PMN is increased by membrane association of the ADP-ribosylation factor (ARF) and small GTPase RhoA [46]. Of considerable interest here, PSDP directly inhibited recombinant hPLD1b in the absence of regulatory proteins (table 1). These results suggest that PSDP may inhibit PLD at its catalytic center and is likely to act at other PLD isoforms, such as PLD1a and PLD2 isoforms where the catalytic centers

Table 1. PSDP inhibits PLD: structure-activity relationship*.

Lipid	PLD	$K_{m\ app}$ (mM)	$V_{m\ app}$ (nmol/s)	K_i (nM)
PSDP	cabbage	2.1	0.25	20
	rhPLD1b	3.1	0.03	6
PSMP	cabbage	3.1	0.36	3210
Squalene	cabbage	4.0	0.46	0
FDP	cabbage	0.9	0.25	0
PA	cabbage	3.6	0.43	0

* Enzyme kinetics for PLD were determined in the presence of PSDP or related lipids. Purified or isolated PLD (3u cPLD or 0.3 u rhPLD1b/reaction) activity in the presence of the test compounds was determined as in [53] ($n \geq 3$). For c PLD, $K_m = 1.4$ mM and $V_m = 0.29$ nmol/s, and for rhPLD1b, $K_m = 13.8$ mM and $V_m = 0.36$ nmol/s. K_i was calculated using the formula slope = $K_m/V_m\ (1 + [I]/K_i)$. Absence of inhibition is reported as $K_i = 0$.

are conserved. PSDP's ability to serve as an endogenous inhibitor of PLD likely results from PSDP's unique three-dimensional and physical chemical properties, which can serve as a template for the preparation of more potent PLD inhibitors by design to fulfill the structure activity relationship reviewed here.

Additional intracellular targets: PSDP interactions with SH2 domains

Phosphoproteins interacting with Src homology domains are critical to regulated tyrosine kinase activity and related PMN responses, such as NADPH oxidase assembly [47]. We determined the capacity of PSDP to interact with these potential regulatory sites by using an in vitro equilibrium binding method that employs the adaptor protein Grb2 which contains one SH2 and two SH3 domains [48]. [^{32}P]-PSDP, isolated from labeled human PMN, associated with Grb2 (fig. 11). Displacement of [^{32}P]-PSDP occurred in the presence of excess unlabeled PSDP and delipidated albumin, while the SH3 bearing hSOS inhibitory peptide showed no demonstrable impact (fig. 11). These results provide evidence for an interaction between PSDP and the SH2 domain of Grb2. To confirm interaction between PSDP and SH2 domains, we next determined whether PSDP would interact with PLCγ1 (amino acids 530–850), which bears an SH2 and SH3 domain. Binding of [^{32}P]-PSDP was subject to competition by pp60c-src C-terminal phosphoregulatory peptide (amino acids 521–533), which binds to SH2 domains, but not the unphosphorylated (i.e. control) peptide (data not shown). Together these findings indicate that PSDP interacts with SH2 domains in a model system. Interaction between PIPP and SH2 domains represents additional potential targets for these compounds to regulate cell function.

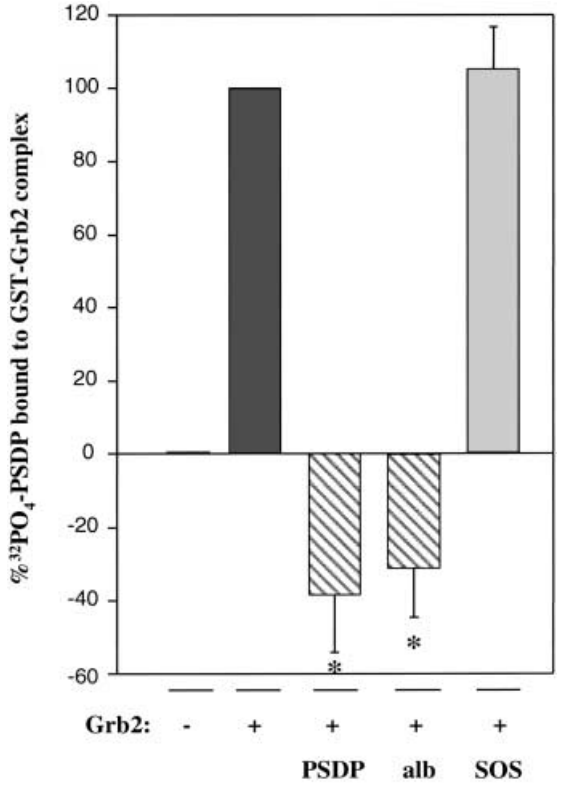


Figure 11. Additional intracellular targets: PSDP interacts with SH2 domains. Radiolabeled PSDP was exposed (20 min, 37 °C) in saline (0.9%) to GST-Grb2 complexed to agarose beads (2 µg/reaction). Bound material was separated from unbound material by spin filtration (0.2 µ nylon filter, 4000 g, 5 min). Competitive binding was determined in the presence of excess unlabeled PSDP, delipidated albumin (alb) of hSOS (amino acids 1143–1162) peptide (50 µg/reaction).

PIPP remodeling: a rapid switch for 'stop' signaling used by aspirin-triggered 15-epi-LXA$_4$

LTB$_4$ receptor activation initiated a rapid and transient ~30% decrement in PSDP (fig. 9) that was concurrent with increased PLD activity (fig. 10). As PSDP levels returned toward baseline values, PLD activity decreased, revealing an inverse relationship and suggested a role for PSDP in the regulation of this pivotal lipid-modifying enzyme. Cells exposed to an LXA$_4$ receptor agonist prior to LTB$_4$ showed a dramatic switch in PSDP remodeling to now give increased [^{32}P]-PSDP and marked inhibition of both PLD activity and superoxide anion generation (figs 9, 10). In addition, synthetic PSDP was a selective and potent inhibitor of isolated PLD (table 1). Collectively, the reciprocal relationship between PSDP levels and PLD activity as well as direct inhibition of recombinant human PLD1b, purified cPLD and PLD activity in PMN lysates support a role for PSDP as an endogenous lipid regulator of PMN PLD activity. The different temporal profiles of PIPP remodeling initiated upon receptor activation by PMN ligands with opposing actions (i.e. stimulation and inhibition) suggest that PIPP remodeling and PSDP itself may serve as important signaling components, in particular as intracellular stop signals.

Is receptor-activated PIPP remodeling unique to human PMN?

Unlike most cells, including monocytes and lymphocytes, PMN lack a mixed function oxidase and cyclase necessary for endogenous formation of cholesterol from acetate [17]. The resultant biosynthetic termination at squalene in PMN suggests that products such as squalene's direct precursor, PSDP, carries functions distinct from cholesterol biosynthesis. Hence it is likely that the PIPP signaling pathway uncovered here in human PMN may extend to other cell types. The presence of PSDP in peripheral blood PMN despite their inability to generate

cholesterol from endogenous sources, its rapid remodeling in response to receptor-mediated inflammatory stimuli of diverse classes of receptor agonist, and its ability to inhibit PLD activity and NADPH oxidase at nanomolar levels are supportive evidence for a role for PSDP as a novel negative intracellular signal. Thus, this newly uncovered PIPP signaling might function to decrease negative signal levels, which contrasts with the well-appreciated phosphotidylinositol signaling pathways (reviewed in [49]) that, when activated, rapidly generate positive intracellular signals (e.g. inositol trisphosphate, diacylglycerol and Ca^{2+}) to initiate cellular responses.

Accumulation of PSDP by a 15-epimer LX analog implicates PIPP remodeling as a component for aspirin's therapeutic impact

In addition to its actions as the lead nonsteroidal antiinflammatory drug, aspirin also affects cholesterol biosynthesis by mechanisms that remain to be completely elucidated [50]. Beyond its well-appreciated inhibition of COX, aspirin can pirate this system to set in place an antiinflammatory circuit generating 15-epi-LX, carbon 15-*R*-epimers of the natural 15-*S*-containing-LX, during cell-cell interactions by aspirin-acetylated COX-2 and 5-lipoxygenase (fig. 8). These aspirin-triggered LX carry antiinflammatory and antiproliferative properties [51, 52] and may mediate a component of aspirin's beneficial therapeutic actions. Since the 15-epi-LXA_4 analog reversed the LTB_4 receptor-initiated PSDP turnover and inhibited both PLD activity and superoxide anion generation (figs 9, 10), these results implicate PIPP remodeling as a component of the cellular basis for aspirinís inhibition of excessive inflammatory responses. This novel mechanism of inhibition of LTB_4 receptor signaling may have wider implications in host defense because of this receptor's recent identification as a coreceptor for HIV-1 [30].

Summary

Regulation of PMN activation in complex host responses is controlled in part by soluble mediators and, in particular, by autacoids with opposing actions [2], such as LT and LX. These eicosanoids give markedly different profiles for PIPP remodeling. As an agonist, LTB_4 initiates a marked decrement in PSDP. When ALXR is activated in conjunction with the LTB_4 receptor, there is a dramatic switch in PIPP remodeling that leads to an accumulation of PSDP (fig. 9). In most human cell types, PSDP is appreciated as a biosynthetic intermediate in cholesterol production by microsomal squalene synthase, which catalyzes head-to-head condensation of two FDP [53]. Ligand-operated rapid remodeling of PSDP in PMN is likely to occur in membranes in proximity to LTB_4 and LXA_4 receptors, suggesting a nonmicrosomal pool of PSDP that may result from (i) novel biosynthetic and/or metabolic pathways or (ii) intracellular trafficking of PIPP with proteins from endoplasmic reticulum to membrane domains. Incorporation of [^{32}P] from ATP into PSDP but not FDP [8, 42] is further evidence in support of a novel route for PSDP formation in PMN. Our results suggest that PIPP remodeling is linked to cell surface receptor activation and is involved in the intracellular transmission of extracellular signals with opposing biological actions. In our working model (fig. 12), a 'negative lipid signal' (i.e. PSDP) is held at a set point, like a ratchet, in 'resting' cells. Incoming positive signals (LTB_4, fMLP, and so on) initiate the degradation and inactivation of this inhibitory lipid (e.g. remodeling PSDP to the inactive monophos-

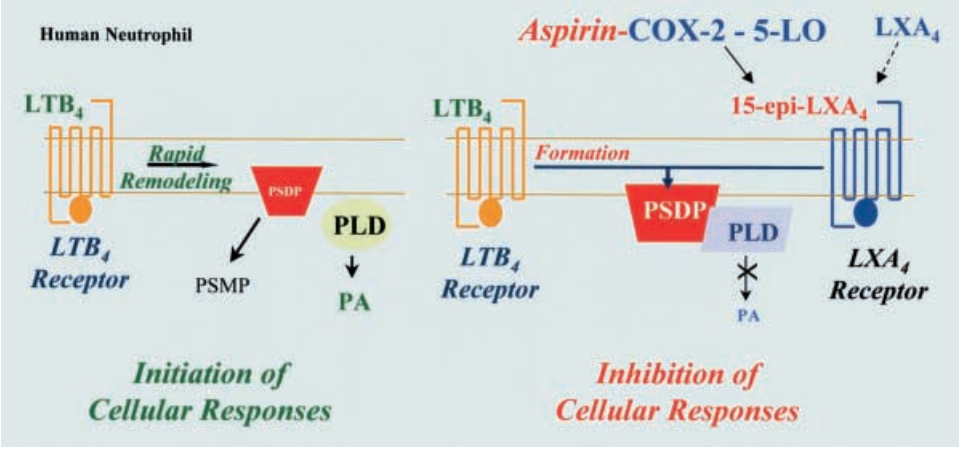


Figure 12. Ligand-operated PIPP remodeling: 'stop' signaling. PSDP undergoes rapid remodeling upon receptor activation by LTB_4, a proinflammatory agonist for PMN (left). During inflammation, cell-cell interactions lead to the generation of LX and 15-epi LX (in the presence of aspirin) as 'stop' signals for PMN responses that reverse-stimulated PMN remodeling of PSDP to give an accumulation of PSDP that in turn inhibits PLD and O_2^- generation (right). (See text for details.)

phate species, PSMP) (figs 4, 8). Thus, PIPP remodeling enables mounting of intracellular positive signals that threshold for activation of select cellular processes. This type of signaling may explain the selectivity and tight coupling required by agonists such as LTB_4 that stimulate highly specialized functional responses of PMN such as chemotaxis, granule mobilization and superoxide anion generation. Generalization of this model of cell signaling, namely receptor-initiated degradation of negative lipid signals, to other receptors and cell types remains for future studies.

Conclusion

Ligand-operated rapid remodeling of PIPPs in human PMN and direct inhibition of PLD activity at nanomolar levels support a new role for PSDP as an intracellular signal [8] and also provide novel intracellular targets by which PSDP and related compounds can regulate cellular responses. Given the wide occurrence of PIPP and critical role of PLD in the plant and animal kingdoms [39, 54], ligand-operated PIPP remodeling and direct inhibition of PLD first established in the experiments reviewed here may have wider implications in cell signaling in cell types other than human PMN. Our results show direct inhibition of a phospholipase involved in signal transduction by an endogenous intracellular lipid and set forth a new paradigm for lipid-protein interactions in the control of cellular responses, namely receptor-initiated degradation of a repressor lipid that is also subject to regulation by aspirin ingestion via the actions of aspirin-triggered 15-epimer LX. The PIPP signaling cascade reviewed here appears to serve as an intracellular counterbalance to the well-known phosphoinositide-phospholipase C pathway for cell activation [55]. Collectively, these findings suggest that PIPP signaling pathways are of interest in designing new therapeutic interventions and, specifically, that the conformation of PSDP serves as a template for developing novel inhibitors.

Acknowledgments. We thank Mary Halm Small for skillful assistance in manuscript preparation. These studies were supported in part by National Institutes of Health grants GM-38765 and DK-50305 (to C.N.S.) and NHLBI-HL-56383. B.D.L. is a recipient of a mentored clinical scientist development award from the National Institutes of Health (NHLBI-K08-HL03788).

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