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Anionic pulmonary surfactant lipid regulation of innate immunity

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“...palmitoyl-oleoyl-phosphatidylglycerol ... may have significant therapeutic potential for treating both acute and chronic lung diseases, especially in the setting of bacterial and viral infections.”

Pulmonary surfactant is a protein- and lipid-rich complex synthesized by alveolar type 2 cells and secreted onto the air/tissue interface of the alveoli [1]. Lipids constitute approximately 90% of pulmonary surfactant and proteins are approximately 10% by weight. The phospholipid content of surfactant is remarkably high, with concentrations estimated to be 35–50 mg/ml [2]. Phosphatidylcholine is the most abundant lipid class present in surfactant; and an unusual lipid molecular species, dipalmitoyl-phosphatidylcholine, is essential for reducing the alveolar surface tension and preventing collapse during the respiratory cycle [3, 4]. Approximately 10% of the phospholipid pool of surfactant consists of the anionic, phosphatidylglycerol (PG) class, with the major molecular species being palmitoyl-oleoyl-PG (POPG) [5, 6]. The PG content of surfactant (~3–5 mg/ml) is the highest concentration of this lipid found for any mammalian organ or tissue system [7, 8].

“...palmitoyl-oleoylphosphatidylglycerol provides broad-spectrum suppression of proinflammatory stimuli within the lung elicited by bacterial infection.”

Historically, the presence of PG in pulmonary surfactant has been a curiosity of the lung that seemed to lack tangible function. A growing body of data now implicates PG as a major regulator of innate immunity within the lung. Both *in vitro* and *in vivo* studies reveal that

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POPG can suppress the activation of the proinflammatory Toll-like receptors (TLRs), TLR2 and TLR4 [9–12]. In addition, POPG appears to have novel antiviral properties against respiratory syncytial virus (RSV) and influenza A virus (IAV) [13, 14]. These properties of POPG suggest that it may have significant therapeutic potential for treating both acute and chronic lung diseases, especially in the setting of bacterial and viral infections.

POPG attenuates bacterial lipopolysaccharide-induced inflammation from the TLR4 pathway

Lipopolysaccharide (LPS), derived from Gram-negative bacteria, is a potent stimulator of inflammatory processes. Although inflammation is essential for combating pathogen invasion, dysregulation of the process can produce excessive tissue damage that is especially pernicious within the gas exchange regions of the lung [15, 16]. Excessive LPS is a major contributor to acute lung injury and acute respiratory distress syndrome resulting from both pulmonary and nonpulmonary infections [17, 18]. LPS interacts with a quartet of molecules to elicit proinflammatory responses from leukocytic cells within the lung [19, 20]. Temporally, macrophages and dendritic cells are first stimulated by LPS, followed by recruitment of neutrophils and lymphocytes [21]. LPS binds to plasma or interstitial LPS-binding protein and is transferred to a cell-associated protein, cluster of differentiation 14 (CD14). CD14 subsequently transfers LPS to a TLR4/lymphocyte antigen 96 protein (MD2) complex at the plasma membrane of responding cells. The activated TLR4/MD2 engages intracellular signaling cascades that elicit transcription of genes encoding the proinflammatory cytokines, IL-8 (or KC and MIP2 in mice), TNF- α , IL-1 and IL-6 [22].

In vitro experiments demonstrate that POPG liposomes effectively antagonize LPS activation of the inflammatory process [9, 10]. For both macrophage cell lines and freshly prepared primary human alveolar macrophages, treatment with POPG prevents the LPS-dependent production of TNF- α and inflammatory eicosanoids such as PGD2 and thromboxane A2. POPG acts at multiple extracellular sites to disrupt the LPS activation of the TLR4 pathway. Binding studies reveal that POPG interacts with the LPS binding site of CD14 and prevents the protein from recognizing LPS [9, 12]. In addition, POPG binds MD2 and inhibits the ability of this protein to recognize both LPS and TLR4 [9]. *In vivo* studies with mice that are challenged with LPS, delivered either intratracheally or intravenously, produce results consistent with the *in vitro* studies. Intratracheal POPG, administered along with LPS, suppresses the appearance of neutrophils and the production and secretion of the inflammatory cytokines TNF- α , KC and MIP2 [9]. Thus, POPG acts to inhibit the earliest steps in the inflammatory cascade elicited by LPS within the intact lung.

POPG inhibits TLR2-dependent inflammatory processes

Activation of TLR2 engages the same intracellular signaling processes as TLR4 and produces the same downstream inflammatory mediators [23]. The microbial ligands that activate TLR2 are principally lipopeptides such as the *Mycoplasma fermentans* derived MALP-2, and the synthetic agonist, Pam3CysK4 [24, 25]. TLR2 oligomerizes with either TLR6 or TLR1; and the TLR2/6 complex recognizes MALP-2, whereas the TLR2/1 complex recognizes Pam3CysK4. *In vitro* studies demonstrate that POPG inhibits the

production of TNF- α , PGD₂, thromboxane A₂ and thromboxane B₂, elicited by the activation of TLR2 complexes with MALP2 and Pam3CysK4. In addition, the activation of TLR2 by *Mycoplasma pneumoniae* and membranes derived from this bacterium is also inhibited by POPG [10]. *M. pneumoniae* attaches to cell surface sialic acid residues via the bacterial P1 adhesin protein; and binding studies reveal that POPG does not disrupt this binding reaction, but prevents the surface determinants on the bacteria from engaging the TLR2 receptor after the bacteria attaches to the cell surface [10]. TLR2 ligands are found on Gram-negative and Gram-positive bacteria in addition to mycoplasmas [26]. Thus, POPG provides broad-spectrum suppression of proinflammatory stimuli within the lung elicited by bacterial infection.

POPG blocks infection by RSV

As described earlier, POPG binds to the CD14 protein that participates in the TLR4 signaling pathway. Experiments with genetically modified mice provided evidence that the innate immune response to RSV was significantly diminished in animals lacking CD14 and TLR4 [27]. These findings suggested that POPG might also have some influence on the innate immune response to RSV. *In vitro* studies have demonstrated that primary human airway epithelial cells and an airway epithelial cell line (BEAS2B) produced a robust proinflammatory IL-8 response to RSV, which was completely suppressed by the addition of POPG liposomes concomitant with the virus [13]. In addition, RSV-binding studies with epithelial cells revealed that the high-affinity association of the virus with the cell surface was almost completely blocked by inclusion of POPG in the reaction. The action of POPG on RSV attachment to epithelial cells is sufficient to prevent productive infections and also protects uninfected cells from the spread of virus from preinfected neighboring cells. Extension of these studies to a mouse model of infection provided additional important findings [13]. When mice were challenged by intranasal inoculation with RSV, significant histopathology occurred with infiltration of neutrophils and lymphocytes around the small airways and within the alveolar compartment. The inclusion of POPG, along with the virus at the time of infection, almost completely attenuated the inflammatory histopathology and reduced the amount of virus recovered from the lung tissue by a factor of 1500. The reduction in viral infection resulted in the elimination of IFN- γ response, and suppressed the increase in surfactant protein (SP)-D expression, normally produced in response to infection.

“...palmitoyl-oleoyl-phosphatidylglycerol may be effective for short-term application against rapidly emerging viral strains for which vaccines have not been developed, or that lag behind viral outbreaks.”

These findings suggest that POPG, or related compounds could provide an important new approach for preventing and treating RSV infection. Currently, there is no approved vaccine for RSV, and immunity to the virus elicited by natural infections is incomplete, resulting in reinfection throughout life [28]. Although almost all individuals are infected with RSV within the first 2 years of life, the high frequency of reinfection presents significant medical difficulties to adults with chronic lung diseases such as asthma and COPD [29, 30].

POPG suppresses IAV infection

The discovery that POPG inhibited RSV infection was unanticipated and raised the issue of whether this pulmonary surfactant lipid might also be effective against other respiratory viruses. *In vitro* studies with IAV-H3N2 demonstrated that POPG liposomes effectively inhibited the IL-8 response of BEAS2B cells elicited by viral challenge [14]. Microscopy revealed that the cytopathic effect of IAV-H3N2 on cultured cells was almost completely suppressed by the addition of POPG. Binding studies demonstrated that POPG effectively inhibited the attachment of IAV-H3N2 to epithelial cells, thereby preventing viral invasion and replication. *In vivo* studies with mice, using a mouse-adapted version of influenza, IAV-H1N1-PR8, demonstrated that POPG provided significant protection from infection [14]. The simultaneous addition of POPG liposomes, along with IAV-H1N1-PR8, suppressed inflammatory cell infiltration into the lungs and reduced the histopathology score from high levels to values indistinguishable from those of uninfected animals. The quantity of infectious viral particles recovered from lung tissue was reduced by a factor of 10, and the IFN- γ production was reduced by 80%. These findings provide evidence that POPG is also effective against IAV, although the antagonism of this virus is not quite as robust as that found for RSV.

The above studies suggest that POPG or compounds with a similar structure and function may provide important approaches to complement large-scale annual vaccination programs for IAV. POPG treatment could potentially be applied to nonvaccinated individuals or those who failed to produce effective antibody titers despite vaccination [31]. In addition, POPG may be effective for short-term application against rapidly emerging viral strains for which vaccines have not been developed, or that lag behind viral outbreaks.

Therapeutic applications of POPG & related lipids

The recognition of POPG as a natural constituent of pulmonary surfactant with significant innate immune function is a very recent development. The broad-spectrum activity of this lipid as an antagonist of inflammatory processes and respiratory viral infection provides a rational explanation for its presence and relative abundance in bronchoalveolar secretions of the lung. The breadth of antimicrobial action of POPG is not dissimilar to that found for the pulmonary collectins SP-A and SP-D [1], and is consistent with a homeostatic molecular infrastructure that sets a relatively high threshold for engaging inflammatory processes within the alveolar compartment. Such an infrastructure should ensure that casual exposure of the bronchoalveolar compartment to ambient pyrogens, such as airborne LPS, does not continually elicit a robust inflammatory response. Thus, the pulmonary innate immune system appears to be actively suppressed until a critical quantitative threshold of proinflammatory mediators is reached. If this general hypothesis is correct, it seems probable that the intrinsic threshold for engaging inflammatory cascades could be artificially increased by simply providing supplemental amounts of POPG. This approach may provide an important new direction for both the prevention and treatment of uncontrolled inflammatory processes that occur in acute lung injury/acute respiratory distress syndrome, or that accompany bacterial and viral infections in chronic lung diseases such as asthma and COPD.

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