Phosphatidylglycerol Suppresses Influenza A Virus Infection

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Phosphatidylglycerol antagonism of Influenza A infection

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Abstract

Influenza A virus (IAV) is a worldwide public health problem causing 500,000 deaths each year. Palmitoyl-oleoyl-phophatidylglycerol (POPG) is a minor component of pulmonary surfactant, which has recently been reported to exert potent regulatory functions upon the innate immune system. In this report we demonstrate that POPG acts as a strong anti-viral agent against IAV. POPG markedly attenuated IL-8 production and cell death induced by IAV in cultured human bronchial epithelial cells. The lipid also suppressed viral attachment to the plasma membrane and subsequent replication in MDCK cells. Two virus strains, H1N1-PR8-IAV and H3N2-IAV bind to POPG with high affinity but exhibit only low affinity interactions with the structurally related lipid palmitoyl-oleoyl-phosphatidylcholine. Intranasal inoculation of H1N1-PR8-IAV in mice, in the presence of POPG, markedly suppressed the development of inflammatory cell infiltrates and the induction of IFN-γ recovered in bronchoalveolar lavage, and viral titers recovered from the lungs after 5 days of infection. These findings identify supplementary POPG as a potentially important new approach for treatment of IAV infections.

Key words: Antiviral, innate immunity, pulmonary surfactant
INTRODUCTION

IAV is one of the most common viruses causing global health problems and life-threatening infections, resulting in an estimated 500,000 deaths each year (1). In the US, 5-20% of the population is infected annually, producing 200,000 hospitalizations and 36,000 deaths (1-3). Patients with chronic pulmonary disease (e.g. chronic obstructive pulmonary disease, asthma) are more susceptible to IAV infection and typically develop more severe symptoms requiring hospitalization, or ICU admissions (4-6). In 2009-2010, the pandemic influenza A outbreak was caused by a novel IAV of swine origin and the H1N1 subtype. This pandemic spread rapidly and is illustrative of the problems of emergence of new strains (2, 7). Vaccination is the standard strategy for prevention of influenza, but this effect varies and depends upon successful matching of the vaccine antigen with the epidemic, or pandemic virus, and population compliance with vaccination programs (1). Vaccine shortages for rapidly spreading pandemic viruses can also limit population coverage and further intensify disease outbreaks and persistence.

Two classes of drugs currently available for treatments of influenza in non-immune individuals are the ion channel inhibitors (e.g. amantadine, remantadine), and the neuraminidase inhibitors (NAI) (e.g. Oseltamivir, Zanamivir, Peramivir) (1). The near complete loss of efficacy of the ion channel inhibitors has led to heavy reliance upon NAIs (8), which are currently standard drugs of choice for both prophylaxis and the early treatment of IAV infection. The frequency of NAI resistant IAV is approximately 1% in adults, and 4-8 % in children (9). This resistance usually develops from prior application, or prophylaxis treatment with NAIs. However, the CDC recently reported that seasonal oseltamivir-resistant IAV has appeared independently of oseltamivir use. The oseltamivir-resistant IAV is a more frequent and serious problem in children.
The growing frequency of NAI-resistant IAV strains (9-11) highlights the importance of developing new agents for the treatment of influenza infection with novel mechanisms of action.

Pulmonary surfactant is a lipid and protein complex that regulates biophysical properties of the alveoli, and innate immune responses in the lung (12). It is well recognized that the hydrophilic surfactant proteins (SP-A and SP-D) play multiple roles in regulating host defense. SP-A and SP-D bind to a variety of bacteria, fungi, and viruses with high affinity, and regulate the innate immune responses to these pathogens in the lung (12, 13). Surfactant proteins are minor components of the surfactant complex accounting for approximately 10% of the material. The major constituents of pulmonary surfactant are phospholipids, with phosphatidylcholines (PC) as the dominant molecular class. Dipalmitoyl-PC is the most abundant lipid molecular species in surfactant and is the lipid most responsible for the reduction of surface tension at the air-tissue interface, within the alveolar compartment (13). Phosphatidylglycerol (PG) is also present in surfactant and comprises approximately 10-mole % of the lipids. In humans, palmitoyl-oleoyl PG (POPG) is the most abundant molecular species present within the PG class (14). The concentration of phospholipids in the extracellular pulmonary surfactant present in the alveolar hypophase, is estimated to be approximately 35 mg/ml (15). These extraordinarily high extracellular phospholipid levels are not found in any other organ system. In addition, no other organ has such high levels of PG, although trace levels of PG are found in numerous organs where this lipid primarily functions at the subcellular level as a precursor to mitochondrial cardiolipin. The functions of such high levels of extracellular PG within the lung have been unclear, but recent studies now provide evidence that this lipid plays an important role in regulating innate immunity and viral infection (16-21). We recently reported that POPG,
suppresses LPS induced-inflammatory responses in vivo and in vitro through direct interactions with CD14 and MD2 (16). Previous studies have also reported that PG antagonizes ligand recognition by LPS binding protein (LBP), and CD14; and reduces LPS-induced inflammatory responses (17-19). In addition to regulating cellular responses to LPS, CD14 has been implicated in the innate immune response to respiratory syncytial virus (RSV) (22). This latter connection prompted recent examination of the effects of POPG upon RSV induced inflammation and infection (20). These studies produced the unanticipated finding that POPG blocks RSV infection in vitro and in vivo by disrupting viral attachment to epithelial cell surfaces. An additional unanticipated finding was that supplemental POPG administered intranasally, markedly attenuated RSV infection in vivo, in mice (20). This unexpected anti-viral activity of surfactant lipid led us to examine the effect of POPG as an IAV antagonist. The goals of this study were to determine if POPG could, 1) suppress the inflammatory response and cell death induced by IAV infection in epithelial cells in vitro, 2) inhibit viral attachment and subsequent replication in epithelial cells, 3) directly interact with IAV, and 4) attenuate IAV infection in vivo. Our findings demonstrate that supplemental POPG is a potent anti-viral agent against IAV. These findings strongly suggest that POPG and related compounds play an important role in pulmonary innate immunity and could be developed and used as a novel therapy against IAV infection.

**MATERIAL AND METHODS**

**Viruses, tissue culture, infection and surfactant lipid treatments.**

Influenza A viruses, Philippines 82/H3N2 and H1N1/PR8, were prepared as previously described (23-25). Madin-Darby Canine Kidney (MDCK) cells and Beas2B cells were obtained
from ATCC (Manassas, Virginia, USA). Phospholipids were obtained from Avanti (Alabaster, AL, USA), and uni-lamellar liposomes were prepared as previously reported (16, 20). To examine the effects of phospholipids on H3N2-IAV infection, cells were pretreated for 1h with POPG or POPC liposomes (20).

**Viral protein expression.**

MDCK cells were grown in 24-wells plates, and pretreated for 1h with phospholipids prior to virus addition. Viruses were added to cells in the presence or absence of phospholipids, and the total well lysates were subjected to immunoblotting after 36hrs using Goat polyclonal anti IAV antibody (Millipore, Billerica, MA, USA) and β-actin (Cell Signaling Technology, USA). Quantification of MP and NA protein expression was performed using NIH Image J1.34 software.

**HA mRNA analysis by qRT-PCR.**

MDCK cells were grown in 24-well plates, and H3N2-IAV adsorption was performed using a multiplicity of infection (MOI) of 0.5-1.0 for 2 hr at 37°C. Immediately following the adsorption, and at 24h, total well contents were processed for RNA extraction using a Qiagen RN-easy kit (Quagenm, Maryland, USA). HA mRNA expression was quantified using a qRT-PCR kit (Invitrogen, Camarillo, CA, USA).

**Binding of Influenza A viruses to phospholipids and MDCK cells.**

To examine the direct interactions between IAV and phospholipids, solid phase binding assays were performed (20). Phospholipid coated wells were incubated for 2 hr at 37°C with varying
concentrations of viruses. Viral attachment was detected with goat anti IAV antibody added with 3% BSA at 37°C. The bound viruses were quantified by absorbance at 450nm, or 490nm.

For cellular binding studies, MDCK cells were grown in 24-well plates and IAV was adsorbed to the monolayers, for 2 hr at 19°C, either in the absence, or presence of phospholipids. At 19°C endocytosis by MDCK cells is minimal, and this temperature allows viral binding to reach equilibrium within 2 h. The cell monolayers were processed at 0 °C for subsequent analysis by quantitative immunoblotting.

**In vivo suppression of Influenza A infection.**

Female BALB/c mice at 6 weeks old, were obtained from Jackson Laboratory (Bar Harbor, Maine, USA). Mice were anesthetized with 0.25g/kg avertin introduced intraperitoneally (20). Anesthetized mice were inoculated intranasally with a total volume of 50 µl of PBS in groups consisting of sham infection, IAV infection (80pfu/mouse), IAV infection plus POPG, and POPG alone. POPG liposomes were prepared in PBS (16, 20), and mice were inoculated with 3 mg of the lipid premixed with the virus. On specific days, mice were euthanized by intraperitoneal injection of 0.25 ml of Nembutal (10 mg/ml). Bronchoalveolar lavage fluid (BALF) was used for differential cell quantification and IFN-γ analysis (20). Homogenates of the left lungs were used for IAV plaque assays (26). The right lungs were processed for lung histopathology score (20, 27). Animal studies followed all prescribed guidelines and were approved by the Institutional Animal Care and Use Committee.

**RESULTS**

**POPG attenuates H3N2-IAV induced cytokine production in human bronchial epithelial**
cells.

We first examined the effects of POPG upon IL-8 production induced by H3N2-IAV in a human bronchial epithelial cell line (BEAS2B). IL-8 is a typical early alarm cytokine released by tissues to recruit neutrophils to sites of injury and infection. Cells were pretreated with POPG, in the form of small unilamellar vesicles, for 1 hr, and challenged with H3N2-IAV at an MOI of 2/cell. As shown in Figure 1, H3N2-IAV induced a 6000-fold increase of IL-8 compared with uninfected cells. POPG (200 µg/ml) treatment inhibited H3N2-IAV induced IL-8 production by 91%. A control lipid, palmitoyl-oleoyl-phosphatidylcholine (POPC), did not alter the virally induced IL-8 production. POPG and POPC contain identical hydrophobic domains, but differ in their hydrophilic domains, which contain phosphoglycerol and phosphocholine, respectively. Treatment of BEAS 2B cells with either POPG or POPC, in the absence of virus had no effect upon basal IL-8 production. From these experiments we conclude that POPG acts as a potent inhibitor of the inflammatory response elicited by H3N2-IAV in cultured human epithelial cells. These results also indicate that the polar portion of POPG plays a major role in dictating the specificity of the lipid as an antagonist of H3N2-IAV induction of IL-8 production.

The concentrations of POPG used in these experiments are less than 10% of the PG levels found in pulmonary surfactant, suggesting that in vivo resident PG pools may provide significant protection from the virus. Previous studies have shown that the actions of POPG are not broadly pleiotropic for inhibition of IL-8 production, since the lipid does not suppress the expression of the cytokines induced by the TLR5 agonist, flagellin (20). Additional control experiments demonstrate that POPG does not alter cellular protein synthesis, or growth. These findings clearly demonstrate that POPG can significantly suppress H3N2-IAV induced inflammatory cytokine production.
**POPG prevents cell death and suppresses IAV protein expression.**

We next investigated the action of POPG upon the cytopathic effects of the virus against MDCK cells, which are routinely used for plaque assays and in vitro propagation of many strains of IAV. As shown in Figure 2, untreated MDCK cells form monolayers with a typical cobblestone appearance. Infection of the cells with H3N2-IAV, at an MOI of 1, destroys the cell monolayer after 36 hr. In contrast, treatment of the cells with virus in the presence of 1 mg/ml POPG completely protects the cells from the cytopathic effects of IAV. At 200 µg/ml POPG also provides significant protection of the monolayer from the lytic effects of the virus, although a few cytopathic foci are evident. Treatment of the cultures with virus in the presence of 200 µg/ml POPC fails to prevent cell death by IAV infection. From these data we conclude that POPG acts early in the infectious cycle to protect cells from IAV.

To estimate phospholipid antagonism of virus propagation in MDCK cells, we examined the effects of POPG upon the expression of M1 Protein (MP) and neuraminidase (NA), which were measured following infection of the cells with H3N2-IAV for 36 hr. As shown in Figure 3A, at 2 hr after infection, using viral MOIs of either 0.5 or 1.0, neither MP, nor NA protein were detectable; but after 36hr infection, MP and NA protein expression clearly increased and was readily measurable. POPG treatment attenuated both MP and NA protein expression in a dose dependent manner with 1 mg/ml being significantly more effective than 200 µg/ml; (Figure 3A, 3B). At 1mg/ml, POPG inhibited NA expression by 80% and MP expression by 75%. In contrast to POPG, POPC was completely ineffective. In addition to the H3N2 strain, we also performed experiments with the mouse adapted H1N1-PR8-IAV strain, because it is routinely used to
perform in vivo studies with mice. As shown in Figure S1, infection of MDCK cells with H1N1-PR8-IAV at an MOI of 0.5, for 36hr, in the presence of 1 mg/ml POPG or POPC; produced results similar to that found for H3N2-IAV. The treatment with POPG at 1 mg/ml attenuated MP expression by 70% whereas POPC at 1 mg/ml did not alter MP expression. From these results, we conclude that POPG can suppress IAV protein expression from both H3N2-IAV and H1N1-PR8 strains, and the effect is dependent upon phospholipid structure.

We also examined the mRNA expression for the hemagglutinin (HA) gene using quantitative RT-PCR. The results from these experiments are shown in Figure 4. After 2 hr of viral adsorption, RNA for HA was not detectable at MOIs of either 0.5, or 1.0. After 24 hr, a robust RT-PCR signal was obtained from cells infected at an MOI of 0.5 (Figure 4A). POPG treatment significantly attenuated the HA-mRNA signal, but POPC did not alter the HA-mRNA signal. Quantitative analysis of the RT-PCR data in Figure 4B, showed that the inclusion of 200 µg/ml POPG during infection suppressed HA gene expression by 75%, and 1mg/ml POPG suppressed the expression by 88%. In contrast to the findings with POPG, the treatment with POPC was ineffective. These data demonstrate that POPG suppresses IAV HA mRNA expression in MDCK cells. Collectively, the experiments examining cytopathology, protein expression and mRNA expression demonstrate that POPG disrupts the IAV infection process at an early stage, and consequently prevents viral replication and cell death. These findings suggested that POPG might directly interact with the virus and interfere with cell binding, and additional experiments were conducted to test this idea.

**POPG binds to influenza A virus with high affinity.**
To investigate the mechanism of the anti-viral effect of POPG, we examined the binding interaction between IAV and the lipid. Figure 5A shows the direct binding of H3N2-IAV to a POPG solid phase adsorbed to an ELISA plate. This binding interaction is virus concentration dependent, high affinity and saturable. In contrast to the virus binding to POPG, the binding to POPC has the characteristic of a low affinity, non-specific interaction. Figure 5B shows the results of similar experiments using H1N1-PR8 IAV with POPG, and also demonstrates high affinity, concentration dependent, and saturable binding. Compared with POPG, the lipid POPC is a weak-binding ligand for H1N1-PR8 IAV and the interaction is non-saturable and non-specific.

Additional experiments examined whether POPG could interrupt binding of H3N2-IAV to cell surfaces. In these studies H3N2-IAV was adsorbed to MDCK cells at varying multiplicities of infection for 2 hr at 19°C (to block endocytosis), in either the absence, or presence of POPG. Following viral adsorption, cell monolayers were washed with PBS to remove unbound viruses, and processed to detect attached viruses by immunoblotting for MP. The results presented in Figure 5C demonstrate that MP detection increased with increasing H3N2-IAV titer. The attachment of the virus to the cell surface was high affinity and saturable. The recovery of MP was inhibited 75% by 200 µg/ml POPG, and inhibited 93% by 1mg/ml POPG, at viral MOIs as high as 10/cell (Figure 5D). POPC failed to block the binding between MDCK cells and IAV. These data provide clear evidence that POPG binds directly to IAV and disrupts viral adsorption to epithelial cell surfaces, thereby suppressing infection (Figures 2, 3 and 4) and the inflammatory response (Figure 1).

**POPG antagonism of H3N2-IAV infection is reversible and dependent upon the timing of**
lipid addition.

The data presented in Figure 5 provide compelling evidence that POPG directly binds to IAV and this interaction inhibits cell surface attachment of the virus. However, these findings do not provide any information about the consequences of POPG binding to IAV upon the integrity of the virus. In order to examine this issue in more detail and assess whether the lipid has direct virucidal activity, we conducted two types of experiments. In the first line of experimentation, H3N2-IAV (MOI of 1) was preadsorbed to MDCK cells in culture for 4hrs at 8C° (a temperature that inhibits all viral endocytosis), and then the adsorbed virus was exposed to POPG at either 200 µg/ml, or 1000 µg/ml, for an additional 4hr at 8C°. As a control phospholipid, POPC was added to separate MDCK monolayers also harboring preadsorbed virus. Following the incubation with lipid, the cultures were washed and warmed to 37C° to allow the viral infection to proceed. We reasoned that if the POPG acted as a virucidal agent to compromise the integrity of the virus, the lipid treatment should reduce the subsequent progress of the infection. As shown in Figure 6A, B and C, treatment of cell surface associated virus with POPG failed to disrupt cell infection, as monitored by the production of the viral proteins NA and MP, at 36hrs following the infection.

A second approach to examining whether POPG was directly acting as a virucidal agent, tested the reversibility of the interaction of POPG with IAV. In these experiments H3N2-IAV (10^8 pfu/ml) was incubated with 1mg/ml POPG at 37C° for 1hr. Following the incubation, the virus and lipid were diluted 10^3-fold in either the absence, or presence of 1 mg/ml POPG, and then used to infect monolayers of MDCK cells. As a control for these manipulations, identical aliquots of H3N2-IAV were incubated at 37C for 1hr in the complete absence of phospholipid.
The infectivity of the IAV in these experiments was examined by quantifying fluorescent foci of viruses formed on the MDCK monolayers 6 hrs after infection, detected by antibody. The results presented in Figure 6D show that H3N2-IAV alone produced $1.01 \pm 0.25 \times 10^4$ fluorescent foci per well, and virus transiently exposed to 1mg/ml lipid and then diluted $10^3$-fold produced $0.87 \pm 0.23 \times 10^4$ foci per well, whereas virus exposed to a constant level of POPG produced $0.23 \pm 0.04 \times 10^4$ foci per well. These results demonstrate that the effects of POPG upon H3N2-IAV are reversible. Together, the data in panels 6A-D provide strong evidence that POPG is not directly virucidal.

An additional conclusion from the data in panels 6A-C is that POPG must act prior to viral attachment to the cell surface, because virus already bound to cells is resistant to the antagonistic effects of the lipid. To further examine this latter conclusion, we applied a viral challenge to MDCK monolayers that were exposed to POPG for various periods before and after the addition of virus. To quantify the effects of viral infections in these experiments we measured cell viability at 36 hrs after adding viruses to the cultures. The results of these experiments are presented in Figure 6E. The viability of uninfected cells, $98.7 \pm 0.24\%$, and cells treated with POPG alone, $98.6 \pm 0.3\%$, was equivalent. The addition of H3N2-IAV at an MOI of 0.5 reduced cell viability to $45.2 \pm 6.8\%$. Pretreatment of MDCK cells with POPG followed by washout of the lipid prior to addition of virus, resulted in $44.7 \pm 3.9\%$ viability; whereas omission of the lipid washout resulted in $79.3 \pm 1.7\%$ viability. Simultaneous addition of lipid and virus provided significant protection to the monolayer and produced $70.8 \pm 3.0\%$ viability. The addition of lipid 1hr after infection showed marginal protection and yielded $57.1 \pm 2.5\%$ viability. When the lipid was added at 2hrs after infection, there was no significant protection of the
cultures from the extent of cell death produced by virus alone. Collectively, these data show that: 1) preincubation of cells with POPG followed by washout, prior to viral challenge, does not block infection, 2) the anti-viral effects of POPG are nearly the same whether cells are pre-incubated with lipid or simultaneously incubated with lipid at the time of viral challenge; and 3) the protective effects of the lipid diminish rapidly with time following viral challenge. All of these conclusions are consistent with the lipid acting prior to viral binding to the cell surface. These in vitro properties of POPG suggested that the lipid might function as an effective anti-viral agent in vivo, and further experiments were conducted to examine in vivo efficacy.

**Intranasal administration of POPG suppresses IAV infection in mice.**

We examined the potency of POPG as an anti IAV-agent using a mouse model of viral infection. 6 week old female BALB/c mice were inoculated intranasally, with the mouse adapted influenza strain, H1N1-PR8-IAV (80 pfu/mouse) either in the absence, or presence of 3 mg of POPG. Five days after infection, the animals were euthanized and the lungs were lavaged and harvested, and analyzed for the effects of viral infection (26). The results presented in Figure 7A demonstrate the POPG treatment clearly suppressed viral propagation in the lung by a factor 10 (IAV infection = 6.21 ± 0.6 x 10^5 pfu, IAV + POPG = 0.6 ± 0.2 x 10^5 pfu). No plaques were obtained from uninfected animals, or animals challenged with UV inactivated IAV, or animals treated with POPG alone. Lavage from control mice produced a total cell number of 5.3 ± 1.6 x 10^4 cells/ml, and H1N1-IAV increased the total cells recovered in lavage to 15.3 ± 1.5 x 10^4 cells/ml (Figure 7B). The POPG treatment reduced the total cell number in lavage, induced by virus by 50%. The data presented in Figure 7C demonstrate that POPG significantly suppressed the proportional
increase in lymphocytic and neutrophilic cellular infiltrates in lavage by 60%. IFN-γ production was undetectable in lavage from control animals, and was 111.5 ± 24.7 pg/ml following virus infection. The POPG treatment suppressed the virally induced IFN-γ response by 81% (Figure 7D).

Lung tissue from experimental animals was examined and assigned a histopathology score (20), and the data are shown in Figure 7E. H1N1-IAV infected animals had a 3-fold higher histopathology score than sham infected controls; and animals receiving virus plus POPG were not significantly different from sham-infected animals. Representative micrographs are shown in Figure 7E and reveal H1N1-IAV infection elicited a significant influx of inflammatory cells in alveolar and peribronchial areas, and pneumonia. POPG treatment markedly attenuated these virus-induced inflammatory changes, and the lipid treatment alone did not cause significant histological changes. From the data shown in Figures 6 and 7, we conclude that POPG suppresses H1N1-PR8 IAV infection and viral replication in vivo, and markedly reduces the inflammatory responses to the virus. These findings strongly suggest that supplementary POPG could be an important and novel approach for prevention and treatment of IAV infections.

DISCUSSION

In this report we provide strong evidence that supplementary POPG, the major molecular species of PG present in pulmonary surfactant, potently suppresses the infection of epithelial cells by IAV in vitro and in vivo. By interfering with the initial infective process, the lipid also disrupts the release of inflammatory cytokines, such as IL-8, by the epithelium. The in vitro doses of POPG capable of disrupting IAV infection are similar to in vitro doses of the lipid
effective against RSV (20). In contrast, the in vivo doses of POPG required to attenuate IAV infection (3 mg/mouse) are much higher than those effective against RSV (150 µg/mouse). The reasons for these differences are not yet completely understood. One important difference between RSV and mouse adapted IAV, is the efficiency of the infection. Based on our experimental data, only 80 pfu of IAV are required to produce a robust in vivo infection yielding, 2 x 10^3 pfu/ left lung on day 1, 3 x 10^5 pfu/ left lung on day 3, and 6 x 10^5 pfu/ left lung on day 5 after infection, whereas 10^7 RSV are required to produce an in vivo yield of 5 x 10^3 pfu/ left lung, after 5 days (20). The infectivity, thermal stability and replication kinetics of IAV make this virus less susceptible to POPG than RSV. However, the 3 mg dose of POPG used in mice in these experiments did not produce any deleterious effects in either animal behavior or tissue histopathology.

We are currently examining the delivery and turnover of POPG administered to mice. The turnover rate of POPG will be an important determinant of the window of efficacy of the exogenously applied lipid. The turnover of POPG is expected to be very high in mice because of their respiratory rate (250-300/min). By comparison, the respiratory rate in human newborns is 25-30 and the estimated t_{1/2} of PG is 30h (28). Thus the anticipated anti-viral effects of exogenous POPG in humans may be achievable with reasonable dosing and can reasonably be expected to be relatively long lived.

The role of PG in pulmonary surfactant has long been enigmatic. Our recent work and earlier studies (16, 18-21) provide strong evidence that PG plays an important role in suppressing TLR-mediated inflammatory processes. We propose that the inhibitory actions of PG against TLRs, function to set a high threshold for the engagement of inflammatory cascades in the lung.
Fundamentally, this threshold prevents inflammation by casual environmental stimuli such as ambient levels of microparticulate LPS, but enables engagement of inflammatory processes once the threshold is exceeded by sufficient quantities of TLR agonists. During established infections by bacteria and viruses the quantity of TLR agonists produced is expected to exceed the inhibitory threshold that results from POPG, thereby allowing a robust inflammatory response to proceed.

In addition to inhibiting TLR activation, emerging research, including this report, now demonstrates that PG can interfere with viral infections involving the respiratory tract. Our work demonstrates the action of POPG against RSV and IAV. Studies by Perino et al (29), demonstrate that dipalmitoyl-PG also disrupts vaccinia virus infection in vitro and in vivo. Interestingly, the major route of infection for vaccinia virus is respiratory. The principal mechanism of anti-viral action of POPG against RSV and IAV is by inhibition of viral attachment to epithelial cell surfaces, and dipalmitoyl-PG antagonizes vaccinia virus in the same manner. In this report we provide direct evidence that POPG binds to IAV with high affinity, (Figs 5A, B) and that this binding reaction disrupts the adsorption of viral particles to cell surfaces (Figs 5C, D). In mice the net effects of POPG treatment are to reduce the viral burden by a factor of 10, suppress the influx of inflammatory cells by a factor of 2, reduce the neutrophilic and lymphocytic populations by a factor of 4, and limit IFN-γ production by 80%. We interpret the reduction of IFN-γ levels as a measure of success in reducing the viral burden in vivo. The POPG treatment also reduced the lung histopathology to the level of uninfected animals. Collectively, these findings demonstrate POPG is an important lead compound for developing new classes of anti-viral agents.
The anti-viral actions of POPG are likely to be complementary to those of the surfactant proteins, SP-A and SP-D, which also bind IAV and markedly attenuate the host inflammatory response. In mouse models of IAV infection, the absence of SP-A is associated with reduced viral clearance and elevated inflammatory responses (relative to wild type strains); both of which are rectified by providing supplementary SP-A at the time of infection (30). Likewise, genetic ablation of SP-D results in increased viral loads and higher inflammatory cytokine responses, compared to wild type strains, that are alleviated by adding SP-D at the time of infection (31). In general, the action of SP-D against IAV appears more robust than that of SP-A. The binding of SP-D to IAV is critically dependent upon N-linked glycosylation of asparagine 165 of the viral HA1 protein, whereas the binding of SP-A to IAV is dependent upon the presence of sialic acid in the N-linked oligosaccharide in the C-terminal domain of the SP-A protein. The potential therapeutic application of POPG, or related lipids has some advantages over the use of surfactant proteins, insofar as POPG: 1) is of low molecular weight, 2) likely to be non-immunogenic, 3) can be chemically synthesized in large amounts and 4) is chemically stable. The recently discovered activity of different molecular species of PG against multiple viruses (RSV, IAV and vaccinia) with structurally different surface proteins, suggests that single point mutations to viral surface proteins (e.g asparagine 165 of HA1) may be insufficient to enable viral evasion of the inhibitory effects of the phospholipid.

Although the anti-viral properties of PG were not anticipated, it is clear that in vitro administration of this lipid, which is part of the intrinsic pulmonary surfactant system, can disrupt the infective processes of RSV and IAV, which are serious, problematic pathogens,
worldwide. Most importantly, our data demonstrate that supplemental POPG introduced via the airways can significantly attenuate IAV infection in mice. This property of POPG suggests that the lipid has significant potential for preventing viral infections in at-risk human populations, and perhaps treating viral infections after they have become established.

**STATISTICAL ANALYSIS**

All results are shown as mean ± SE. ANOVA was used to determine the level of significant difference among all groups. Differences among groups were considered significant at $P < 0.05$. 
REFERENCE


FIGURE LEGENDS

**Fig 1.** POPG attenuates H3N2-IAV induced IL-8 production by bronchial epithelial cells.

IL-8 production by Beas2B cells was determined by ELISA after a 48 h H3N2-IAV challenge, in either the absence, or presence of 200 µg/ml of POPG, or POPC. The cells were either sham treated (UN) or challenged with virus at an MOI of 2. Values shown are means ± SE for 3 independent experiments and the asterisks indicate p < 0.05.

**Fig. 2.** POPG prevents the cytopathic effects of H3N2 upon cultured MDCK cells.

Monolayers of MDCK cells were either uninfected (CONL), or treated with H3N2-IAV at an MOI of 1 (IAV), in either the absence, or presence of POPG (200 µg /ml, or 1000 µg /ml), or POPC (200 µg /ml), as indicated. After 36 hr, the cultures were examined by light microscopy.

**Fig 3.** POPG suppresses NA and M1 protein expression elicited by H3N2-IAV infection of MDCK cells.

(A) Monolayers of MDCK cells were either uninfected (UN), or infected with H3N2-IAV at MOIs of 0.5, or 1, for 2 h, in either the absence, or presence of POPG (200 µg/ml, or 1000 µg/ml), or POPC (200 µg/ml). After 36 h the wells were harvested and analyzed for the expression of NA and M1 (M-P) by SDS-PAGE and immunoblotting. Control experiments also included incubation with POPG (1000 µg/ml) and POPC (200 µg/ml) in the absence of viral infection. (B) Quantification of NA and M1 expression from 3 independent experiments. Values shown are means ± SE. Asterisks indicate p < 0.05, upon comparison of virally infected cells
without PG addition to those with PG addition.

**Fig 4.** POPG inhibits HA mRNA expression in H3N2-IAV infected MDCK cells.

(A) Monolayers of MDCK cells were either uninfected (UN), or infected at MOIs of 0.5, or 1, for 2 h, in either the absence, or presence of POPG (200 µg/ml, 1000 µg/ml), or POPC (200 µg/ml). After 24h the wells were harvested and processed for RNA extraction and subjected to quantitative RT-PCR (qRT-PCR) and gel electrophoresis. (B) The results from three independent experiments are shown. Values are means ± SE and single and double asterisks indicate, p < 0.02., or p < 0.001, respectively.

**Fig 5.** POPG binds IAV with high affinity and inhibits cell surface binding of H3N2-IAV.

(A) Aliquots of 1.25 nmole phospholipid (POPG, or POPC) were adsorbed onto microtiter wells, and the indicated concentrations of H3N2-IAV were added and incubated at 37°C for 2 h. The viral binding was detected by ELISA and quantified by A490. (B) Solid phase lipids were prepared as in panel A and the binding of H1N1-PR8-IAV was performed at 37°C for 2 h. The viral binding was detected by ELISA and quantified by A450. (C) Monolayers of MDCK cells, at 19°C, were challenged with H3N2-IAV at MOIs of 0-10, as indicated; in either the absence, or presence of POPG (200 µg/ml, or 1000 µg /ml), or POPC (200 µg /ml). The cultures were harvested and processed for SDS-PAGE and immunoblotting. (D) Quantification of three immunoblotting experiments performed as described for panel C. Values shown for panels A, B and D, are means ± SE for 3 independent experiments.
**Fig 6.** The POPG effect upon IAV infection is reversible and dependent upon the timing of lipid and virus addition.

A) Monolayers of MDCK cells were infected with IAV at an MOI of 1, at 8°C for 4hr; and then treated with POPG (200ug/ml, 1000 ug/ml), or POPC (1000ug/ml), at 8°C for 4hr. The cultures were shifted to 37°C and the infections were allowed to proceed for 36hr. The contents of each tissue culture well were recovered and processed for immunoblotting with polyclonal goat anti-IAV, and rabbit anti-β-actin antibodies. Immunoblots from a representative experiment are shown. B), C) Quantification of NA and MP expression, normalized to β-actin expression from three experiments are summarized. D) Aliquots of H3N2-IAV (10⁸ pfu/ml) were incubated for 1hr at 37C in either the absence, or presence of 1000 ug/ml POPG. Subsequently, the viral aliquots were diluted 10³-fold in either the absence (Reversal), or presence of POPG (POPG) and used to infect monolayers of MDCK cells at an MOI = 0.05. At 7 hrs after the initiation of infection, the cultures were washed with cold PBS and the monolayers were fixed with paraformaldehyde, permeablized and stained for the presence of viral antigens with polyclonal goat anti-IAV antibody and rabbit anti-goat Alexa 548 antibody. Fluorescent foci were scored using a Zeiss 200-M microscope and Slidebook software, at 10 x magnification. E) Cells were either uninfected (UN), or treated with 1000 ug/ml POPG alone (POPG), or challenged with virus (+ H3N2) in either the absence, or presence of POPG, as indicated. POPG was added either 0.5 hr, or 0 hr before (designated by -), or 1 hr, or 2 hr following (designated by +) viral addition. In one group of 0.5 hr lipid treatment prior to viral addition, the POPG was washed out (WO) of the culture well, before viral infection. In all other groups the POPG level was maintained until cell harvest. In each case viral adsorption was conducted for 1 hr at 37°C, followed by removal of unbound virus. Culture wells were harvested and the cells present in both the supernatant and
the adherent monolayer were counted and stained for viability using 0.02% trypan blue. Viral infection at an MOI = 0.05, occurred at 0 hr.

Values shown in bar graphs are means ± SE for 3 independent experiments; * indicates p < 0.05 and § indicates p<0.001.

**Fig 7.** POPG inhibits H1N1-PR8 IAV infection and inflammation in vivo and suppresses the histopathology elicited by the virus. BALB/c mice were infected with 80 pfu H1N1-PR8 (H1N1) by intranasal inoculation, in either the absence, or presence of 3 mg of POPG, as indicated. Sham (CONL) and lipid only treatments (POPG) were also performed. After 5 days of infection, the animals were euthanized. (A) Amount of virus present in the left lung was quantified using plaque assays. (B) Lavage fluid collected from animals was used to quantify total cells recovered from the bronchoalveolar compartment. (C) Cytospin preparations were used to quantify the percentage of lymphocytes (Lymph.) and neutrophils (Neut.) present in the lavage fluid. (D) The production of IFN-γ was measured in cell free lavage fluid by ELISA. Values shown are means ± SE for 3 independent experiments; * indicates p < 0.01 and §§ indicates p < 0.001. Each group contains 4-6 animals per individual experiment. (E) Paraffin sections (4 µm) were stained with hematoxylin-eosin, analyzed by light microscopy and assigned a histopathology score. The groups consisted of sham treated (CONL), virus infected (H1N1) and lipid treated (POPG) mice. (F) Representative micrographs from the experiment. Scale bar Values shown in A are means ± SE for 3 independent experiments; * indicates p < 0.05. Bar inset in CONL panels indicates 200µm.
Fig.2

237x145mm (300 x 300 DPI)
Fig. 3

A.

<table>
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<tr>
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<th>2Hr</th>
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</tr>
<tr>
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<td>+ POPC 200</td>
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224x176mm (300 x 300 DPI)
Fig. 3

B.

Relative fold expression / β-Actin

UN IAV 0.5 IAV 1 IAV 0.5 IAV 0.5 IAV 0.5 PG200 PG1000 PC200

* *
Fig. 4

A.

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<tr>
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<tr>
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<td>MOI 1</td>
<td>Un</td>
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</table>

- HA
- β-Actin

202x93mm (300 x 300 DPI)
Fig. 4

B.

HA mRNA expression / β-actin (% of Stimulated)

H3N2
H3N2
H3N2
H3N2
POPG200
POPG1000
POPC200

*  
**
Fig. 5

A.

![Graph showing the relationship between H3N2-IAV Titer (X10^7 pfu/ml) and A490 for POPG and POPC.](image)

241x188mm (300 x 300 DPI)
Fig. 5

B.

![Graph showing the relationship between A450 and H1N1-IAV Titer (X10^8 pfu/ml) for POPG and POPC.](image)

241x190mm (300 x 300 DPI)
Fig. 5

C. 

<table>
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<tbody>
<tr>
<td>0</td>
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<tr>
<td>MP</td>
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<tr>
<td>β-Actin</td>
</tr>
</tbody>
</table>

+ POPG 200
+ POPG 1000
+ POPC 200

D. 

% of maximally adsorbed/β-actin

IAV
IAV+POPG200
IAV+POPG1000
IAV+POPC200

IAV titer (X10^5 p.f.u/well)

250x186mm (300 x 300 DPI)
Fig. 6

A. 36Hr, MOI 1

UN

POPG POPG POPC POPG POPC
200 1000 1000 1000

NA

MP

β-Actin

B. MP expression/β-actin (% of Stimulated)

UN IAV IAV IAV IAV IAV POPG POPC
200 1000 1000

C. NA expression/β-actin (% of Stimulated)

UN IAV IAV IAV IAV IAV POPG POPC
200 1000 1000

253x188mm (300 x 300 DPI)
Fig. 7

E. Lung pathology score (Day5)

F. Histopathological images

CONL  H1N1  H1N1+POPG  POPG
Phosphatidylglycerol Suppresses Influenza A Virus Infection

Mari Numata, Pitchaimani Kandasamy, Yoji Nagashima, Janelle Posey, Kevan Hartshorn, David Woodland and Dennis R. Voelker

ONLINE DATA SUPPLEMENT
Supplemental Materials and Methods

Sources of reagents:

MDCK cells and BEAS2B cells: ATCC, Manassas, Virginia, USA
Phospholipids: Avanti Polar Lipids, Alabaster, AL, USA
Goat polyclonal anti-IAV: Millipore, Billerica, MA, USA
Qiagen RN-easy kit: Qiagen, Germantown, MD, USA
qRT-PCR kit: Invitrogen, Camarillo, CA, USA
BALB/C mice: Jackson Laboratory, Bar Harbor, ME, USA
Dulbecco’s Modified Eagle Medium (DMEM) and Bronchial Epithelial Cell Growth Medium (BEGM): Lonza, Walkersville, MD
AlexaFluor 568-rabbit-anti goat antibody: In Vitrogen, Carlsbad, CA, USA
Bovine Growth Serum (BGS): Hyclone, South Logan, UT, USA
Human IL-8 ELISA kit: In Vitrogen, Camarillo, CA, USA
Mouse interferon γ (IFNγ) ELISA kit: BD Biosciences, San Diego, CA, USA
Polyclonal rabbit Anti β-actin: Cell Signaling Technology, Danvers, MA, USA
Paraformaldehyde: Electron Microscopy Science, Hatfield, PA, USA

Tissue Culture Methods:

MDCK cells were cultured in DMEM plus 10% bovine growth serum, penicillin (50U/ml) and Steptomycin (50µg/ml). Beas2B cells were cultured in Bronchial Epithelial Cell Growth Medium (BEBM) supplemented with antibiotics.
Phospholipid Preparation

Organic solvents were removed from stock phospholipids by evaporation under a stream of N\textsubscript{2}. The dried film was suspended in methanol and dried again under N\textsubscript{2} to remove traces of chloroform. The lipid films were hydrated in phosphate buffered saline (PBS) at 37°C for 1hr at phospholipid concentrations of 10-40 mg/ml. After hydration the lipid preparations were bath sonicated until the solutions clarified. The stock liposome suspensions were diluted to final concentrations in media, or PBS and filter sterilized prior to use.

Lipid antagonism of viral infection and cell binding

In tissue culture experiments phospholipid liposomes at concentrations of 0.2-1.0 mg/ml were either pre-incubated with cells, or pre-incubated with viral preparations as specified in Figures. For in vivo experiments phospholipids were preincubated with IAV for 15 min at 22°C prior to intranasal inoculation.

Two types of experiments were devised to test for a direct virucidal effect of phospholipids upon IAV. In one series of experiments IAV at an moi = 1.0 was incubated with a monolayer of MDCK in DMEM at 8°C for 4hr, to allow cell surface binding without endocytosis. Following this incubation the monolayers were washed three times at 0C with cold PBS to remove unbound virus. Next the cells with adsorbed virus were incubated with 0.2-1.0 mg/ml phospholipid (POPC, or POPG) and incubated for another 4 hours at 8°C in DMEM. Subsequently the phospholipid was removed by washing three times with cold PBS. The cultures were shifted to 37°C and the viral infection was allowed to proceed for 36 hr. Progress of the viral infection was monitored by
immunoblotting of cell extracts separated by gel electrophoresis using β-actin for standardization. Quantification of NA, MP and β-actin was performed using NIH Image J1-34 software.

In a second type of experiment we examined the reversal of POG antagonism of viral infection. Aliquots of IAV (1x10^8 pfu/ml) were incubated in either the absence or presence of 1mg/ml POPG for 1h at 37°C. Subsequently, the viral preparations were diluted 10^3 fold in either the absence or presence of POPG, and used to infect monolayers of MDCK cells either without or with POPG in the culture medium. After 1h of viral adsorption the unbound virus was removed by washing three times with PBS and then culturing in DMEM, either without, or with POPG. The cells were further incubated for 6h and then washed with PBS and fixed in 3.2% paraformaldehyde. The cells were washed five times with PBS and permeabilized with PBS/0.2% triton X-100 for 15 min. The cells were next washed with PBS five times and blocked overnight at 4°C with 10% BGS. After blocking, the cells were washed five times with PBS and then incubated with goat anti-IAV antibody (1:200) for 2 hrs at 37°C. The unbound primary antibody was removed by PBS washing and Alexa Fluor 568 conjugated rabbit anti goat antibody (1:200) was incubated with the samples for 1 hr at room temperature. Finally the cells were washed with PBS and analyzed by quantitative immunofluorescence using a Mariana’s microscope and Slide book software. Single cell fluorescent foci were scored for each sample at 10x magnification. The time window for the anti-viral action of POPG was examined by quantifying cell viability 36 hrs after IAV infection (moi=0.5) conducted in the absences or presence of 1 mg/ml POPG. The POPG was added to
MDCK monolayers either 0.5 hr prior to virus or simultaneously with the virus, or 1 hr following virus addition, 2 hr following virus addition. Both adherent and floating cells were harvested from the wells and 0.02% trypan blue dye exclusion was used to score cell viability.

**Histopathology Scoring**

The histopathology scoring used the methods described by Cimolai et al (current ms reference 27) that apply weighted evaluation of: A, the percentage of perbronchiolar and bronchial infiltrates (scored 0-3); B, the quality of peribronchiolar/bronchial infiltrates (scored 0-3); C, bronchiolar/bronchial luminal exudate (scored 0-2); D, perivascular infiltrate (scored 0-3) and E, parenchymal pneumonia (scored 0-5). The weighting is described by the formula: $A + 3(B + C) + D + E$. The final numeric score by this method ranges from 0-26. The scoring was performed for each group of animals (saline control, + RSV, + RSV + POPG, and + POPG alone) in 3 independent experiments.
Fig E1. POPG suppresses M protein expression by H1N1-PR8 in MDCK cells.

(A) Monolayers of MDCK cells were either uninfected (UN), or infected with H1N1-IAV at an MOI = 0.5, in either the absence, or presence of POPG (1000 µg/ml), or POPC (1000 µg/ml). After 36 h the wells were harvested and analyzed for the expression of M1 (MP), or β-actin by SDS-PAGE and immunoblotting. (B) Quantification of MP expression normalized to β-actin levels from 3 independent experiments. Values shown are means ± SE. Asterisk (*) indicates p < 0.05.
Figure E1

A.

H1N1 5X10^4 pfu
UN
MOI 0.5
POPG 1000
POPC 1000

MP
β-Actin

B.

Relative fold Expression /β-actin

<table>
<thead>
<tr>
<th></th>
<th>UN</th>
<th>H1N1</th>
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<tbody>
<tr>
<td>MP</td>
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