Antiviral Activity of Bioactive Sphingolipids

A Senior Honor Thesis

Presented in Partial Fulfillment of the Requirements for graduation
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by

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Abstract

Human cytomegalovirus (HCMV) causes severe diseases in those with immature or compromised immune systems, including transplant patients and congenitally infected infants. Even in normal healthy adults, this virus has been linked to chronic diseases such as atherosclerosis and cancer. Research has shown that HCMV is able to utilize a range of cellular signaling pathways to promote its replication. Sphingolipid signaling impacts several important physiological processes, including cell growth, migration, and survival (1). Based on this, we hypothesized that sphingolipids are regulated by virus infection. When we examined the accumulation of sphingolipids in cells infected with HCMV, we observed that sphingolipid metabolism is dynamically regulated according to the temporal phase of infection. In this thesis, we set out to study the consequences of this regulation. In these studies, we found that cells exposed to bioactive sphingolipids before infection show reduced synthesis of viral proteins. These findings provide evidence that activation of sphingolipid signaling pathways may play a role in cellular antiviral defense.

Introduction.

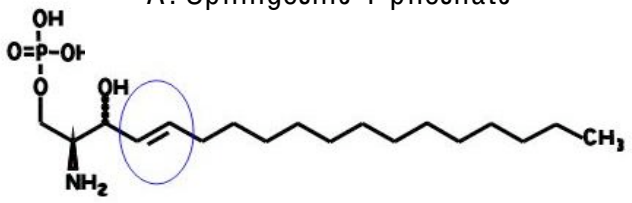
Human cytomegalovirus: HCMV is classified as herpesvirus 5, which is the prototype of the beta herpesvirus subfamily. The herpesvirus family also includes viruses that cause oral and genital lesions (herpes simplex virus), chicken pox (varicella-zoster virus) and infectious mononucleosis (Epstein-Barr virus). HCMV infections are widespread within the human population with estimates ranging from 50-80% of the population as carrying this virus (5). Cytomegaloviruses share many characteristics with

other herspesviruses, including the structure of the virus. The HCMV genome consists of a double-stranded linear DNA, which is enclosed in a capsid. The 230 kb genome includes both protein-coding and noncoding genes (3). Viral gene expression is organized in temporal phases. The immediate early genes are required for both early and late gene expression. The early genes encode for the factors that the virus requires for replication and the late gene expression occurs once the virus has begun replicating its genome and these genes encode for structural components of the virion. The capsid is surrounded by a tegument, and these components are then enclosed in envelope in which is inserted numerous viral glycoproteins (4). Another characteristic of HCMV and all human herpesviruses is that they establish a life-long infection. Initial or primary HCMV infections can be "silent," meaning there are no signs or symptoms if infected or may manifest as symptoms that include fever, sore throat, fatigue, and swollen glands (4, 5). During primary infection, infectious virus can be isolated from saliva, urine, blood, semen, vaginal secretions, milk, and stool (4, 5). After primary infection, however, the virus establishes a state of clinical latency, in which the genome remains present in tissues, but infectious virus is no longer detectable. During latency, the virus remains persistent at low-levels or in a quiescent latent state. In healthy adults, the virus is usually benign, but severe complications of HCMV infections are seen in immunosuppressed individuals, congenitally infected infants, and transplant recipients. These individuals can suffer from graft loss, vascular disease, hepatitis, pneumonitis, and growth retardation (4). Recent studies also provide evidence that HCMV infections are linked to chronic diseases such as atherosclerosis, coronary restenosis, and cancer (4). Currently, no effective vaccine is available to prevent HCMV infections. There are five licensed

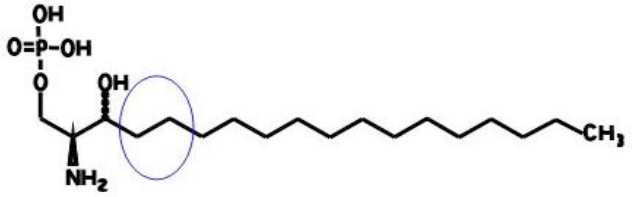
antiviral drugs known to treat HCMV, including ganciclovir, its prodrug valganciclovir, foscarnet, cidofovir, and formivirsen. All except formivirsen, which is only licensed to treat infections of the eye, are known to cause toxicity to humans, and are not always effective due to emergence of drug-resistant strains. Thus our ultimate goal is to find an effective treatment for this virus.

Fig. 1.

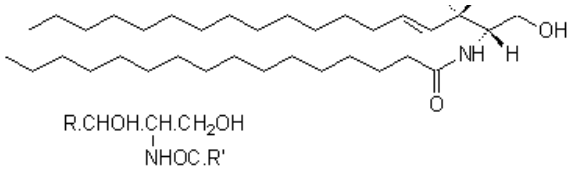
A: Sphingosine-1-phoshate



B: Dihydrosphingosine-1-phosphate



C: Ceramide



Sphingolipid metabolism and signaling. The biology of sphingolipids has advanced significantly in the past few years. Sphingolipids are present as constituents in membranes of all eukaryotic cells, and contribute to the protection of the cell against harmful environmental factors by the plasma membrane lipid bilayer (1, 6). More recently, sphingolipids have been shown to function as signaling molecules. Most work has focused on the bioactive sphingolipids, sphingosine-1-phosphate (S1P) and ceramide. S1P, Figure 1A, is a polar sphingolipid metabolite which has been discovered to act both as an extracellular mediator via a family of G-protein-coupled receptors (GPCRs), $S1P_{1-5}$, and intracellularly as second messenger important for regulation of calcium homeostasis (6). S1P is synthesized in a variety of cells in response to a range of stimuli including growth

factors and cytokines. S1P tends to promote cell survival, proliferation, differentiation, and migration, suppresses apoptosis, and may also have important pathophysiological roles in diseased states such as, atherosclerosis, cancer, and inflammation (6). In addition, S1P is known to influence immune cell function and lymphocyte trafficking (2). S1P is produced from the phosphorylation of sphingosine, which is synthesized by the deacylation of ceramide in the degradative pathway of sphingolipid metabolism (6).

Ceramide, Figure 1C (8), which serves as precursor for the synthesis of S1P is known to have opposing effects, which often lead to cessation of cell proliferation and apoptosis. This suggests that inhibiting the conversion of ceramide into S1P may induce cell death in diseases, such as cancer (6). Ceramide is synthesized in the de novo pathway.

Dihydrosphingosine-1-phosphate (dhS1P), Figure 1B, is similar to S1P and differs only in that it lacks the 4, 5-*trans* double bond, as shown in Figure 1A and B. Dihydrosphingosine-1-phosphate is produced from the phosphorylation of dihydrosphingosine, which is an intermediate of the de novo pathway (1). Altogether, much less is known about the biological activity of dhS1P, though it is presumed to use the same receptors as S1P.

In previous studies, it was established that HCMV modulates lipid-signaling pathways (2). In this study, we set out to explore the consequences of this modulation. This area of research brings us great fascination considering how little is known regarding the role of lipids in virus infection. Ultimately, further research in this area may suggest novel treatments for viral diseases. A recent publication by the laboratories of Dr. Joanne Trgovcich and Dr. James Van Brocklyn demonstrated that sphingolipid

metabolism is dynamically regulated by HCMV infection (2). Specifically, it was observed that levels of the bioactive lipid dhS1P were elevated at early times of infection, suggesting activation of the de novo pathway of sphingolipid metabolism. Activation of the de novo pathway appears to promote viral gene expression during the immediate early temporal phase of infection. At later times of infection, the levels of both S1P and dhS1P were decreased. In this study, we wanted to explore more fully the consequence of decreased bioactive lipids observed at later times of infection. This decrease in S1P and dhS1P abundance may represent a cellular response to infection. Alternatively, it may represent a viral strategy to optimize growth or spread. To begin to determine the biological significance of these observations, we performed studies to activate sphingolipid-signaling pathways prior to infection.

We observed that viral protein accumulation was reduced when cells pre-treated with S1P and dhS1P were infected with HCMV. This led us to hypothesize that activation of sphingolipid receptors may contribute to cellular antiviral responses. Next, we set out to test if this involved activation of genes associated with antiviral defense, namely, interferon-stimulated genes (ISGs). We provide very preliminary data suggesting that antiviral genes can be up-regulated by activation of lipid signaling pathways. However, much more work needs to be done to establish the mechanism that leads to the reduction in viral protein accumulation.

Experimental Procedures

Cell Culture and infection. MRC-5 human primary fibroblasts were obtained

from ATCC. MRC-5 cells were preserved in modified Eagle's medium supplemented with 10% fetal calf serum, 1.7mM sodium bicarbonate, 1.4mM sodium chloride, essential and non-essential amino acids, vitamins, and sodium pyruvate at manufacturers' recommended concentrations (Sigma). Cells were passed weekly by trypsin digestion at a ratio of 1:3 and used in experiments at passages 5-7. Cells were maintained at 37 ºC in 95% air, 5% CO_2 (2, 12). MRC-5 cells were serum-starved for 48 hours prior to infection. Prior to infection, 24 hours, 9 six-well plates containing MRC-5 cells were pretreated with 1000nM of dhS1P, 1000nM of S1P, and vehicle alone (DMEM containing 10% fatty acid-free bovine serum albumen). Cells were then exposed to1.0 or 0.5 plaque-forming units per cell (PFU/cell) of HCMV strain AD169, which was purchased from ATCC.

Plaque Assay: MRC-5 cells were serum-starved for 48 hours prior to infection. Prior to infection, 24 hours, 9 six-well plates containing MRC-5 cells were pretreated with 1000nM of dhS1P, 1000nM of S1P, and vehicle alone (DMEM containing 10% fatty acid-free bovine serum albumen). Cells were then exposed to1.0 or 0.5 plaque-forming units per cell (PFU/cell) of HCMV, which is diluted in medium containing 1% serum of 199V. At 0 and 96 hrs after infection, cells were then scraped, sonicated, and subjected to freeze and thaw. Virus titers were determined in MRC-5 cells by standard plaque assay (2). Briefly, lysates were used as inoculums in fresh MRC-5 cells. After the infection period, cells were overlaid with 1% solution of agarose (SeaKem LE, FMC Corp.) in dH_20 and 1X B medium. Plates were fixed by adding 3 mL of 10% neutral buffered formalin per well and the agar was gently pulled away from the plastic. Plates were then air dried and stained with 0.1% crystal violet prepared in 25% methanol. The

stain was then removed and washed gently with water.

Lipid and Drug Treatment: Prior to infection, 24 hours, six-well plates containing MRC-5 cells were pretreated with 1000nM of dhS1P, 1000nM of S1P, 100 units (U) of interferon beta as a positive control, vehicle alone (10% fatty acid-free bovine serum albumen) as a negative control, or 20ug/mL of FTY720, or the vehicle for FTY720, dimethylsulfoxide (DMSO). FTY720 is both an agonist and antagonist of the S1P receptor that binds to the receptor and leads to its down regulation from the cell surface.

Immunoblotting: At 24, 48, and 72 hours (hrs) after infection, cells were disrupted in lysis buffer containing Triton X-100, 50 mM Tris, 150 mM NaCl and 1% (v/v) protease inhibitor mixture (Sigma). Proteins in cell lysates were separated by size using denaturing polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred to a nitrocellulose membrane. The abundance of viral proteins, immediate early protein 1 (IE1), UL44, pp150, and the cellular protein glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (used as a loading control), was then analyzed by immunoblot analysis by using specific antibodies and visualized by chemiluminescence and exposure to film. The viral proteins were chosen based on the fact that they are representative proteins of different temporal phases of infection including immediate early (IE1), early (UL44), and late (pp150).

Reverse-Transcriptase Polymerase Chain Reaction (RT-PCR): Reverse transcription PCR was performed as described by ThermoScript RT-PCR System according to manufactures instructions (Invitrogen). MRC-5 cells were pretreated with 1000nM of S1P and dhS1P and 100 units (U) of interferon. RNA was then harvested at 6 and 24hrs after treatment. RNA was extracted from cultured cells using Trizol Reagent

(Invitrogen) according to manufacturer's instructions, followed by treatment of DNase 1 for 15 min at 37°C. cDNA was created using ThermoScript RT-PCR System (Invitrogen) according to manufacture's instructions. PCR reactions were setup using ThermoScript System (Invitrogen). PCR analysis was performed for 30 cycles using 60 °C for annealing and 72°C for extension. PCR products were observed by agarose gel electrophoresis. Primer sequences purchased from Integrated DNA Technologies (IDT) are provided as follows: annealing temperature (60°C): interferon-stimulated gene 56 (ISG56) forward 5'-ACGGCTGCCTAATTTACAGC-3' and reverse 5'AGTGGCTGATATCTGGGTGC-3', glyceraldehyde-3-phosphate dehydrogenase (GAPDH) forward 5'CGGAGTCAACGGATTTGGTCGTA-3' and reverse 5'AGCCTTCTCCATGGTGGTGAAGAC-3'.

Real Time Quantitative PCR: Real time PCR analysis was performed by total RNA extraction using Trizol (Invitrogen, Carlsbad, CA), followed by DNase treatment according to manufactures instructions (Ambion, Austin, TX), using 100ng of RNA per reaction. cDNA was created using the 5x cDNA synthesis buffer (Invitrogen) according to manufacturers instructions. PCR reactions were set up using Taqman® Universal PCR Master Mix (Applied Biosystems, Foster City, CA). Real time PCR analysis was performed using BioRad iCycler Sequence Detection System Instrument for 40 cycles. (7).

Primer sequences of Applied Biosystems Assays-on-Demand™ gene expression assays are proprietary, though RefSeq accession numbers for genes, location of binding sites for fluorescent probes, and amplicons are as follows: ISG56 (catalog # HS01675197_m1) based on RegSeq NM001548.3. Probe was located at base 76, exon1/

exon2 boundary. Oligoadenylate synthetase-like (OASL) (catalog # HS00984390_m1) based on RefSeq NM198213.1. Probe was located at base 811, exon4/exon5 boundary. GAPDH (catalog # HS99999905_m1) based on RefSeq NM.002046.3 Probe was located at base 157, exon3/exon3 (7).

Results

Activation of sphingolipid signaling pathways reduces viral protein accumulation. Previously, it was shown that at early times after infection, dhS1P levels are elevated in infected cells but, at later times, both S1P and dhS1P levels were decreased (data not shown). To begin to investigate this decrease in bioactive sphingolipids at late times, we activated sphingolipid signaling prior to infection. Our initial step was to measure viral protein accumulation in cells previously exposed to S1P and dhS1P. MRC-5 cells were first treated with 1000nM of each lipid then exposed to 0.5 PFU per cell of AD169 strain of HCMV. Cells were harvested on a time course of 24, 48, and 72hrs. The cells were then solubilized and assayed by immunoblotting. As shown in figure 2, we observed a decreased accumulation of viral proteins IE1, UL44, and pp150 when pretreated with S1P and dhS1P. In total, we performed four experiments showing consistent results for UL44 and pp150. IE1 levels were reduced in 2 out of 4 experiments. Further studies are needed to determine if the effect of these bioactive lipids on IE1 is related to confluency of cells or the multiplicity of infection. The observation that IE1 levels recover at late times may be indicative that the lipids have the greatest effect at early times when viral replication occurs. Based on these observations, we suggested that S1P and dhS1P

signaling activates antiviral responses in cells.

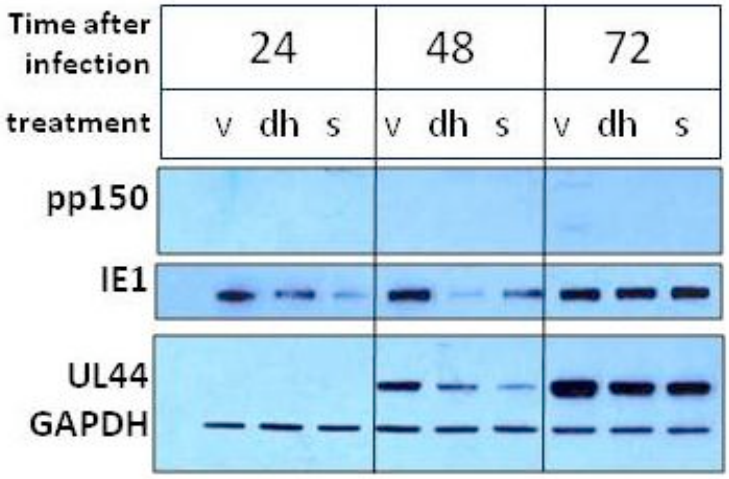


Fig. 2. Pre-treatment of MRC-5 cells with S1P and dhS1P showed a decreased accumulation of viral proteins. Film image of immunoblot of viral proteins immediate early (IE1), early (UL44), late (PP150) and the cellular protein (GAPDH) from lysates of cells treated with vehicle (v), dhS1P (dh), or S1P (s) at various times of infection, 24, 48 and 72hrs.The viral proteins chosen are based on the fact that they representative of different temporal phases of infection.

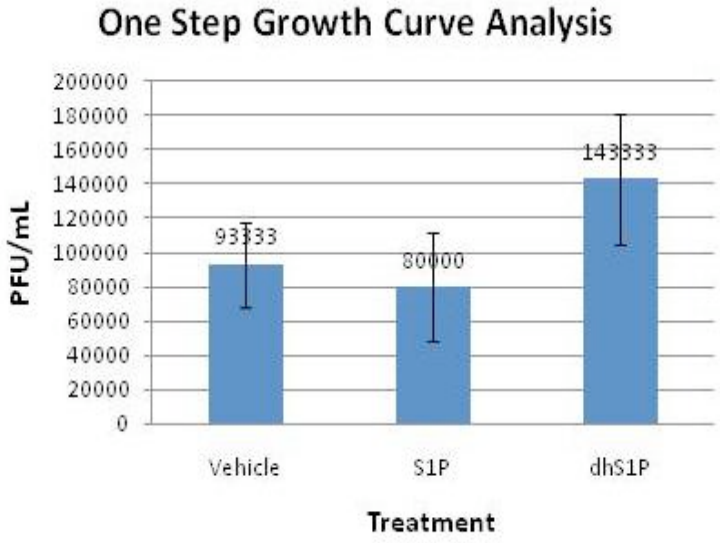


Fig. 3. Growth curve analysis. The graph depicts PFU/mL between vehicle, S1P and dhS1P for 0 and 96 hours after infection.

One Step Growth Curve Analysis in Cells Pre-Treated with Bioactive Sphingolipids. In order to determine if the decrease in viral protein accumulation will translate into a decrease in virus growth, we performed a one-step growth-curve study in MRC-5 cells pre-treated with S1P and dhS1P. MRC-5 cells were first treated with 1000 nM of each lipid then exposed to 1.0 PFU per cell of AD169 strain of HCMV. Cells were then scraped, sonicated, and subjected to freeze and thaw at 0 and 96 hours after

infection. These lysates were used as inoculums in a plaque assay. As shown in figure 3, we did not observe a significant change between virus growth in cells exposed to vehicle, S1P, and dhS1P, although we do see a slight decrease for S1P. We conclude that under these conditions, pretreatment of cells with S1P and dhS1P does not reduce viral growth in vitro. It may be that the virus is able to overcome the effects of these bioactive lipids at later times of infection. However, it is also important to note that many viral mutants that show no growth defects in vitro exhibit severe defects in vivo. Therefore, it may still be useful to perform in vivo studies using the mouse model of CMV infection.

Bioactive sphingolipid signaling may lead to induction of interferon-stimulated genes known to possess antiviral activity. After examining a decrease in viral protein accumulation, our next step was to investigate the mechanism by which sphingolipid treatment affected viral protein production. One possible explanation for the decrease in accumulation of viral proteins is that activation of signaling pathways mediated by bioactive sphingolipids leads to the direct induction of antiviral gene expression or the induction of interferons, which would indirectly activate antiviral gene expression. To test this, cells were pretreated with S1P, dhS1P, and interferon beta as previously stated. RNA was then harvested for 6 and 24hrs and RT-PCR was carried out as described. Figure 4 demonstrates that, upon treatment of S1P and dhS1P, there is expression of interferon-stimulated gene 56 (ISG56) in cells treated with dhS1P, but not S1P. Also, these results were not completely conclusive due to a low level of expression in our negative control, vehicle-treated MRC-5 cells. Given these results, we question if there is a low level of expression of ISG56 normally observed in MRC-5 fibroblasts. Thus, in order to confirm our results, our next step was to carry out Real Time Quantitative PCR

to more precisely quantitate levels of ISGs 6 hrs after exposure to S1P and dhS1P. A preliminary experiment is shown in Figure 5 and Table 1. In this experiment, we amplified GAPDH in samples in which reverse transcriptase (RT+) was included but not those in which RT was omitted indicating we did not have DNA contamination. We could only amplify ISG56 in cells treated with INF and even here only at a very high Ct cycle. This indicates this probe is not sensitive enough at the template levels used in these reactions. We could amplify OAS in INF-treated cells as well as cells treated with S1P and dhS1P, although again the Ct values were very high. We conclude we need to repeat these studies with better probes or with more templates. However, these preliminary findings generally support the notion that ISG induction may be a consequence of activated lipid-signaling pathways.

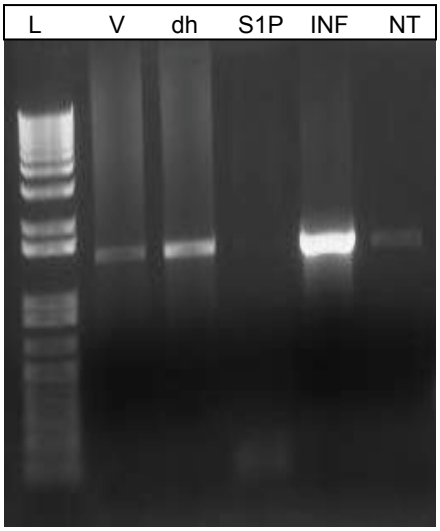
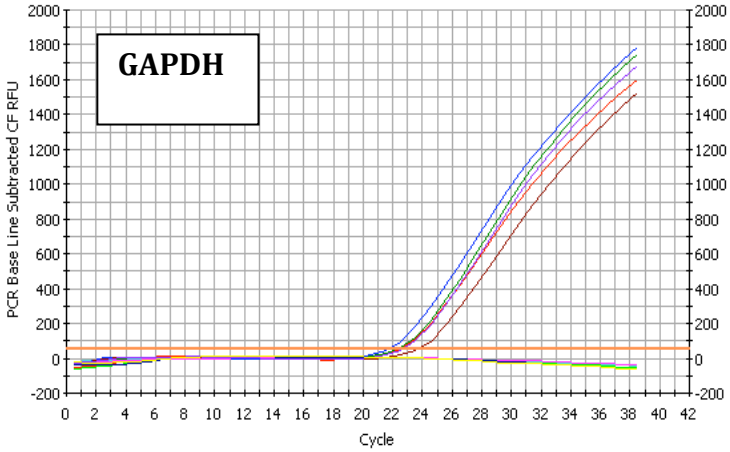
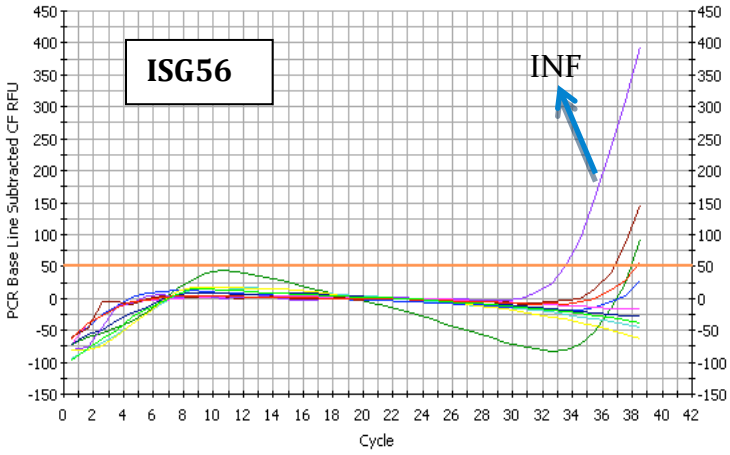


Fig. 4. Possible ISG induction at lipid treatment: Digital Image of agarose gel used to separate PCR products specifically for ISG56 transcripts. MRC-5 cells in six-well tissue culture plates after serum starved were pre-treated with 1000nm of dhS1P, S1P and 100 units interferon. After treatment cells were harvested at 6 h. Total RNA was isolated. RT-PCR of ISG56 transcripts identified. Labels are as followed: L-ladder, dh-dhS1P, S1P-S1P, INF-interferon, NT-no test (cell only)

Table 1. Ct Values of OAS and ISG 56 in Cells Treated with S1P and dhS1P

Well	Identifier (+/- RT)	Ct
A01	GAPDH_S1P+	22.5
A02	GAPDH_S1P-	N/A
B01	GAPDH_dhS1P+	22.2
B02	GAPDH_dhS1P-	N/A
C01	GAPDH_v-bsa+	21.7
C02	GAPDH_v-bsa-	N/A
D01	GAPDH_IFN+	23.6
D02	GAPDH_IFN-	N/A
E01	GAPDH_NT+	22.6
E02	GAPDH_NT-	N/A
A03	ISG56_SIP+	N/A
A04	ISG56_SIP-	N/A
B03	ISG56_dhS1P+	N/A
B04	ISG56_dhS1P-	N/A
C03	ISG56_v-bsa+	36.8
C04	ISG56_v-bsa-	N/A
D03	ISG56_IFN+	33.5
D04	ISG56_IFN-	N/A
E03	ISG56_NT+	N/A
E04	ISG56_NT-	N/A
A05	OAS_S1P+	36
A06	OAS_S1P-	N/A
B05	OAS_dhS1P+	37.4
B06	OAS_dhS1P-	N/A
C05	OAS_v-BSA+	N/A
C06	OAS_v-BSA-	N/A
D05	OAS_IFN+	31.5
D06	OAS_IFN-	N/A
E05	OAS_NT+	N/A
E06	OAS_NT-	N/A





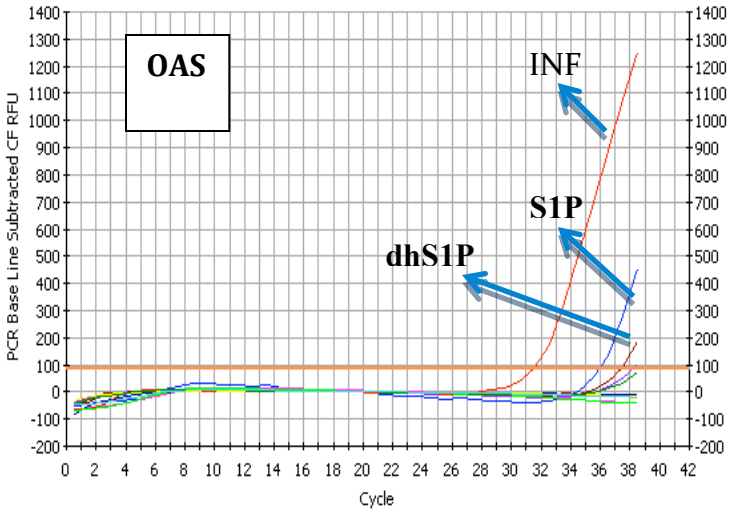


Figure 5. PCR Amplification/Cycle Graphs. Cells were exposed to the indicated lipids and RNA was isolated 6 hrs after treatment and subjected to RT Real-Time PCR.

Discussion

Over the past few years, it has become clear that sphingolipids are key signaling molecules. From our previous studies, we show evidence that sphingolipids are modulated during infection of HCMV. In this study, we show the following results: (1) There is a decreased accumulation of viral proteins IE1, UL44 and pp150 in cells exposed to S1P and dhS1P prior to infection; (2) This decrease does not cause a substantial decrease in viral growth in vitro, though there is a very slight decrease in viral growth in cells pre-treated with S1P; (3) there is a possible induction of the interferon-induced genes, ISG56 and OAS, in cells pre-treated with S1P and dhS1P.

Earlier studies performed by our laboratory demonstrated that sphingolipid metabolism is regulated dynamically by HCMV infection. At early times of infection, 24 hrs, we did not observe a significant change in S1P between mock-infected cells and HCMV-infected cells, although we did see a drastic increase in dhS1P, which was indicative of a viral strategy to optimize growth and also suggests that *de novo* sphingolipid synthesis is stimulated by HCMV during 24 hrs of infection and is later decreased (2). The data specifically illustrated that levels of the bioactive lipids S1P and dhS1P were diminished at later times of infection. This may represent a cellular response to infection. Alternatively, it may represent a viral strategy to optimize growth or spread.

In our current study, we aimed to determine why we saw a decrease at late times of infection. In order to test this phenomenon, we looked at the accumulation of viral proteins in cells exposed to S1P and dhS1P before infection. Interestingly, we saw that there was a decreased accumulation of IEI, UL44, and pp150 during the three phases of infection. These results alluded to an antiviral activity of bioactive sphingolipids. This

guided us to our next step, which was to learn the mechanism by which bioactive lipids may exert antiviral activity.

A common cellular response to challenges against viral infection, parasites, or foreign invaders is the production of interferon's (IFNs), which belong to a class of glycoproteins known as cytokines. These natural cell-signaling proteins, aide in inhibiting viral replication in cells, inducing resistance of cells to viral infection and ultimately cellular defense mechanisms against viruses. Thus, our initial strategy to learn the mechanism used by the lipids was to look at induction of interferon-stimulated genes (ISGs). We specifically looked at ISG56, a known antiviral gene. From our data, we demonstrated that dhS1P, but not S1P, induced ISG56, although we can not conclude if this is a direct approach of the lipids acting on GPCRs to stimulate ISGs, or an indirect effect by means of the interferon pathway. In order to answer these questions, we began to look with quantitative PCR. Although not conclusive, the quantitative PCR suggests that OAS is induced by S1P and dhS1P. From preliminary results, we conclude the necessity to improve the assay conditions and for a more detailed time-course experiment to establish whether activation of S1P and dhS1P signaling directly or indirectly (through interferons) stimulates antiviral gene expression.

Overall we conclude that sphingolipid metabolism is dynamically regulated upon HCMV infection. We have shown a novel idea that sphingolipids are known to possess antiviral activity, yet further research is needed in order to understand the differences seen at early and late time points, as well as the mechanistic approach used by these bioactive sphingolipids. We have only begun to understand the significance of sphingolipids, though future studies will help us answer if sphingolipid synthesis is a

method used by the virus to regulate replication, or a cellular mechanism to antagonize virus growth. *Our future aims for this study include the following:*

1. To test the hypothesis that bioactive sphingolipids activate expression of interferon-induced genes by specifically analyzing other interferon-stimulating genes including ISG54 and oligo 2A synthetase. We also need to improve the RT-PCR method and analyze later time points. We will also repeat this experiment in the presence of neutralizing antibodies to interferons.
2. Preliminary results do not show a decrease in virus growth, although early time points need to be analyzed. We would also like to test the hypothesis that activation of sphingolipid signaling pathways will impair not only HCMV protein production, but also virus replication using a plaque reduction assay.
3. To test the hypothesis that exposing cells to these bioactive sphingolipids elicits a general antiviral virus replication. This is an RNA virus with very different properties from the DNA response by measuring Sindbis virus protein accumulation and herpes viruses.

Together these studies will help us learn more about the biology of sphingolipids and lay the groundwork for understanding the potential antiviral activity of bioactive sphingolipids.

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