ABSTRACT

BIOLOGICAL SCIENCES

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IDENTIFICATION OF MECHANISM(S) BY WHICH THE COMPLEX
SPHINGOLIPID, C2-CERAMIDE, INFLUENCES CYP1A1 INDUCTION BY 3-
METHYLCHOLANTHRENE

Advisor: Dr. Juarine Stewart

Thesis dated July 2005

Both sphingolipids and Cytochrome P450s play vital roles in cellular survival. Sphingolipids facilitate cell growth, differentiation and signaling. P450s, in general, aid in xenobiotic transformation, vascular autoregulation in the brain and the formation of sterols like cholesterol and steroids. CYP1A1 acts on polycyclic aromatic hydrocarbons making them more soluble and easier for cell secretion. Earlier studies in this laboratory had found that the complex sphingolipid, C2-ceramide, modulates CYP1A1 induction by 3-Methylcholanthrene. Using Western Blot analysis, confocal microscopy, and Electrophoretic Mobility Shift Assays, we have determined the mechanism C2-ceramide uses for this modulation. Electrophoretic Mobility Shift Assays and Western Blots
Analysis revealed no significant change in 3MC-AhR-ARNT triplex binding to cyp1a1 XRE1 or AhR and ARNT protein concentrations in the presence of C2-ceramide, respectively. It is the ability of C2-ceramide to form large stable pores in the plasma membrane, allowing more 3MC to enter, that modulates CYP1A1 induction by 3MC.
IDENTIFICATION OF MECHANISM(S) BY WHICH THE COMPLEX SPHINGOLIPID, C2-CERAMIDE, INFLUENCES CYP1A1 INDUCTION BY 3-METHYLCHOLANTHRENE

A THESIS SUBMITTED TO THE FACULTY OF CLARK ATLANTA UNIVERSITY IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

BY

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DEPARTMENT OF BIOLOGICAL SCIENCES

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<tr>
<td>3MC</td>
<td>3-Methylcholanthrene</td>
</tr>
<tr>
<td>AhR</td>
<td>Aryl hydrocarbon receptor</td>
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<tr>
<td>ARNT</td>
<td>Aryl receptor nuclear translocator</td>
</tr>
<tr>
<td>CYP1A1</td>
<td>Cytochrome P450 1A1</td>
</tr>
<tr>
<td>CYP1B1</td>
<td>Cytochrome P450 1B1</td>
</tr>
<tr>
<td>DIG</td>
<td>Detergent insoluble glycolipid-enriched</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle medium</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediamine-tetraacetic acid</td>
</tr>
<tr>
<td>FB1</td>
<td>Fumonisin B1</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>GPI</td>
<td>Glycosylphosphatidylinositol</td>
</tr>
<tr>
<td>GST</td>
<td>Glutathione s-transferase</td>
</tr>
<tr>
<td>HRP</td>
<td>Horse radish peroxidase</td>
</tr>
<tr>
<td>ISP1</td>
<td>Intracellular serine proteinase</td>
</tr>
<tr>
<td>NADP(H)</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>PAH</td>
<td>Polycyclic aromatic hydrocarbon</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PDVF</td>
<td>Polyvinylidene fluoride</td>
</tr>
<tr>
<td>TEMED</td>
<td>N, N, N=, N-Tetramethylethylenediamine</td>
</tr>
<tr>
<td>UDP</td>
<td>Uridine dinucleotide phosphate</td>
</tr>
<tr>
<td>XRE</td>
<td>Xenobiotic responsive element</td>
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CHAPTER 1

INTRODUCTION

Biotransformation

Biotransformation is defined as the process by which a foreign compound, xenobiotic, is subjected to chemical change(s) by living organisms (1-3). The purpose of this process is to reduce the amount of compounds that are potentially toxic in the body. A final product of biotransformation is a compound that is chemically distinct from and usually more hydrophilic than the parent compound. The major organs that are involved in biotransformation reactions are the liver (most important), kidneys, lungs, intestine, skin, and testes. Biotransformation reactions occur in two main phases: I and II.

Phase I enzymes are usually located in the endoplasmic reticulum and are membrane bound. Phase II enzymes typically have a cytosolic localization (1-3). Phase I reactions typically combine nonpolar compounds, such as polycyclic aromatic hydrocarbons, with a functional group like carboxyl (-COOH), hydroxyl (-OH), amino (-NH₂) or thiol (-SH). The addition of one of these groups occurs through either oxidation, reduction or hydrolysis reactions (1-2). These reactions prime the xenobiotic for Phase II reactions. Phase II consists of conjugation reactions that link the product from Phase I with water soluble moieties and make the foreign compound more easily excretable. The types of Phase II reactions include: acetylation, methylation, and sulfate, amino acid, and glucuronic acid conjugation (1-2). There are several factors that affect the efficiency of biotransformation reactions. These factors are: nutrition, age, sex,
species, genetics, dose, presence of disease and enzyme induction or inhibition (1-4).

Biotransformation may cause the bioactivation of xenobiotics. This occurs when the resulting compound is more reactive/toxic than the starting compound.

**Cytochrome P450s**

Cytochrome P450s are a superfamily of proteins containing heme groups that catalyze the metabolism of drugs, pesticides, environmental pollutants and some endogenous compounds (5-8). P450s are involved in the beginning stage of biotransformation. They add a hydroxyl or oxygen group to hydrophobic substrates, such as polycyclic aromatic hydrocarbons (PAH), to form a compound that can be made more soluble in later stages of biotransformation. These biotransformation metabolites are usually then excreted from cells. A disadvantage of biotransformation is that some PAHs are made into more reactive compounds that become mutagenic/carcinogenic. Many of these mutagenic compounds are made in the first stage of biotransformation by P450 enzymes (8-10).

There are a myriad of isoforms of cytochrome P450s known (11-12). CYP1A1 is a 58 kDa isoform of P450 that is induced by aromatic hydrocarbons. The cyp1a1 gene is located on chromosome 15 at 15q22q24. It is comprised of 7 exons 2601 base pairs long that translate into 512 amino acids. CYP1A1 has a region of 40-500 amino acids that is homologous to other P450 isoforms that confers function and gives the protein an overall alpha helical structure. CYP1A1 is bound to the membrane of the endoplasmic reticulum (13). Although the endogenous substrate of CYP1A1 is unknown, it is known to metabolize many dioxins and PAHs, such as the carcinogen and environmental pollutant
methylcholanthrene (3MC) (Figure 1)(5,14,15). Thus, this enzyme system is critical in environmental carcinogenesis.

![Structure of 3-methylcholanthrene](image)

Figure 1. Structure of 3-methylcholanthrene

An induction pathway of CYP1A1 by polycyclic aromatic hydrocarbons has been elucidated. A ligand, e.g. PAH, enters a cell and binds to an aryl hydrocarbon receptor (AhR). Binding of a PAH to AhR releases hsp90, which stabilizes AhR in the cytoplasm. The ligand-AhR duplex moves to and translocates across the nuclear membrane and binds aryl-hydrocarbon receptor nuclear translocator, ARNT. This triplex then becomes a transcription factor which binds to one of many xenobiotic responsive
elements (XREs) on the cyp1a1 gene. Binding to the XRE activates transcription (16-18) (Figure 2).

**Sphingolipids**

Sphingolipids are a large class of lipids that are essential to structure, function and overall cellular integrity (19). There are approximately 300 types of sphingolipids present in various cell types. These lipids are found in membranes, with the plasma membrane having the highest concentration and the nuclear membrane containing the lowest (19-20). Sphingolipids facilitate numerous cellular functions including signaling, apoptosis, and growth. Other cellular functions of sphingolipids are immune recognition, modulation of protein phosphorylation and membrane fluidity. Many important second messengers in signal transduction, such as inositol phosphates, diacylglycerol and leukotrienes, are sphingolipid breakdown products and metabolites (19).

Sphingolipid biosynthesis begins with the synthesis of 3-ketosphinganine from palmitoyl CoA and serine via serine palmitoyltransferase. 3-Ketosphinganine reductase then acts on 3-ketosphinganine to form sphinganine. Ceramide synthase utilizes a proton from NADPH to create dihydroceramide from sphinganine. A desaturase then acts on the dihydroceramide to make ceramide which is modified to form the various types of sphingolipids (Figure 3).
Figure 2. Schematic representation of the signal transduction pathway for CYP1A1 induction by a PAH
Figure 3. Sphingolipid synthesis pathway
Sphingolipids differ from most biological phospholipids in containing long, largely saturated acyl chains, allowing them to readily pack tightly together. This property gives sphingolipids much higher melting temperatures ($T_m$) than membrane glycerophospholipids, which are rich in kinked unsaturated acyl chains (21). With this property in mind, Dr. Kai Simons and colleagues developed the “raft hypothesis” (22). It postulates the existence of lateral assemblies (microdomains), termed rafts, of sphingolipids and cholesterol. It also predicts that these rafts float in glycerophospholipid-rich environments, associating with specific proteins, such as glycosylphosphatidylinositol (GPI)-anchored proteins (22-24).

Rafts are thought to form by self-association of sphingolipids (lipid-lipid interactions) because of their long, mostly saturated hydrocarbon chains. The interaction between glycosphingolipids can be enhanced by hydrogen bonds between their head groups. Voids between the hydrocarbon chains caused by the rather bulky head groups are filled by cholesterol, which might also participate in the hydrogen bonding to the sphingolipids. Sphingolipids, specifically glycosphingolipids and sphingomyelin, have a higher gel to liquid transition temperature than do glycerophospholipids. Sphingolipids thus exhibit stronger lateral cohesion that is thought to be a consequence of van der Waals interactions (22).

Biochemically, the components of lipid rafts are characterized by their insolubility in the detergent Triton X-100 at 4°C, forming detergent insoluble glycolipid-enriched complexes (DIGs) that are enriched in cholesterol, glycosphingolipids,
sphingomyelin, and saturated glycerophospholipids (23). Glycosphingolipids are insoluble by themselves, and sphingomyelin is resistant to detergent extraction in the presence of cholesterol. In contrast, the majority of the cell's glycerophospholipids are soluble in Triton X-100 (25).

The integrity of rafts depends on the presence of both cholesterol and sphingomyelin. Lipid rafts bind proteins, such as GPI-anchored and transmembrane proteins, as well as doubly acylated tyrosine kinases of the Src family, which have been found to associate with rafts and are incorporated into DIGs. The membrane protein caveolin, also found in DIGs, localizes to caveolae (plasma membrane structures enriched in cholesterol and sphingomyelin containing a variety of distinct proteins) and to post-Golgi transport vesicles binding cholesterol, which alludes to its association with the DIGs (22, 26).

**HepG2**

HepG2 cells are cancerous human hepatocytes that were obtained from a Caucasian male 15 years of age (27). HepG2 are epithelial cells that express insulin and insulin-like growth factor II (IGF II) receptors. They have a karyotype range from 50-60 chromosomes and a rearranged chromosome 1 (27). These cells have been well characterized and are used predominantly in drug metabolism studies. HepG2 cells are useful in our studies because they have been shown in various studies to express the induction of CYP1A1 by aromatic hydrocarbons.
Rationale

Preliminary studies in our laboratory have shown that in HepG2 cells, 3MC increased CYP1A1 activity 20-30 fold. However, this induction was significantly suppressed by the inhibitors of sphingolipid synthesis, Fumonisin B1 (FB1) and intracellular serine proteinase (ISP1). Neither of these compounds affected basal CYP1A1 activity (28). Subsequent studies have shown that induction of CYP1A1 activity by 3MC that was inhibited by ISP1 was restored by the addition of C2-ceramide to cells (Figure 4). Therefore, these studies uncovered a requirement for complex sphingolipids during the induction of CYP1A1 by polycyclic aromatic hydrocarbons. We have also shown that C2-ceramide causes a synergistic induction of CYP1A1 when used in conjunction with 3MC in either the presence or absence of a sphingolipid synthesis inhibitor (Figure 4 & 5). This study examined whether complex sphingolipids modulate CYP1A1 induction by 3MC via a specific mechanism. The specific aims of this study were to:

1: To determine whether C2-ceramide modulates ARNT and/or AhR concentrations in the PAH induction pathway of CYP1A1.

2: To determine whether C2-ceramide affects 3MC-AhR-ARNT binding to XREs on the CYP1A1 gene.

3: To determine whether C2-ceramide enhances cellular uptake of 3MC.
Figure 4. Modulation of CYP1A1 activity.
CYP1A1 activity in HepG2 cells treated with 3-Methylcholanthrene (M), M + Isp1 (I), or M+I+C2-ceramide (C2). Untreated (U) cells were used as the control.
Figure 5. Synergistic induction of CYP1A1 activity by 3MC+C2-ceramide. CYP1A1 activity was assessed after HepG2 cells were either untreated (U) or treated with Methylcholanthrene (M), Methylcholanthrene and C2-ceramide (M+C2) or C2-ceramide alone (C2).
CHAPTER 2
EXPERIMENTAL PROTOCOLS

Specific Aim 1: To determine whether C2-ceramide modulates ARNT and/or AhR concentrations in the PAH induction pathway of CYP1A1.

To investigate this specific aim, HepG2 cells were grown and treated with 3MC, 3MC+C2-ceramide or C2-ceramide alone. The amount of ARNT and AhR present in the cell after each treatment was assessed via Western Blot analysis. The methods used are described below.

Western Blot Analysis. Cell lysate preparation for AhR and ARNT western blots were prepared according to Pollenz (29). Cells were rinsed twice with PBS and 500 μl lysis buffer was added directly to the confluent plates. The resulting lysate was transferred to a 1.5 ml microcentrifuge tube (29). The lysate was then boiled for 10 min Protein concentration was determined using the Bradford method (30). Loading samples were prepared containing 10 mg protein and 1x loading buffer [60 mM Tris-HCl, pH 6.8, 25% glycerol, 2% SDS, 14.4 mM 2b-mercaptoethanol, and 0.1% bromophenol blue].

Protein samples were subjected to electrophoresis in a denaturing polyacrylamide gel. The resolving gel was 10% acrylamide:bisacrylamide (29:1) and the stacking gel was 5% acrylamide:bisacrylamide (29:1). The gel was run with a constant current of 120 volts for 2 h. The gel was transferred to a PVDF membrane (Millipore Bedford, MA) for 1 h at 350 mA with the cold pack and prechilled transfer buffer [0.025 M tris base, 0.192M glycine, 20% methanol, and 0.1% SDS] using the “sandwich” method. After this process, the membrane was immersed in blocking buffer [PBS, 0.1% Tween-20, and 5% bovine
serum albumin, (BSA)] and blocked for 1 h at room temperature.

Primary antibodies for AhR and ARNT (Abcam, Great Britain) at 1:5000 and 1: 236 dilutions, respectively, were used. The membrane was then washed three times for 5 min with PBS-T [PBS with 1% tween-20] and blocked for 5 min. The membrane was then incubated with the secondary antibody, horse radish peroxidase (HRP)-conjugated anti-goat IgG (Sigma, St. Louis, MO) at 1:320,000 dilution in 5% fatty acid-free BSA for 1 h at room temperature on an orbital shaker. The membrane was then washed with PBS-T three times for 10 min each. The membrane was developed by film exposure to detect the chemiluminescent substrate, Amersham ECL plus kit (Piscataway, NJ). The membrane was exposed to the chemiluminescent substrate for 5 min and then to the film for 1 min. The film was developed to display bands formed. Densitometric analysis was used to determine the relative amount of protein present in each lane.

Specific Aim 2: To determine whether C2-ceramide affects 3MC-AhR-ARNT binding to XREs on the CYP1A1 gene.

To investigate this aim, the electromobility mobility shift assay (EMSA) was used to determine whether C2-ceramide enhances 3MC-AhR-ARNT triplex binding to XRE 1. Nuclear Extraction. After treatment with 3MC, 3MC+C2-ceramide or C2-ceramide for 18 h, cells were rinsed twice and harvested with PBS. The samples were pelleted at 12000 rpm for 3 min and the supernatant was removed. Nuclear extraction was done according to the instructions in the Pierce NE-PER Nuclear and Cytoplasmic Extraction Reagent kit (31). Briefly, a hypotonic solution was added to the pelleted samples and the
samples were incubated for 10 min on ice. Next, lysis buffer was added and the samples were vortexed and incubated again on ice for 1 min. Samples were then centrifuged for 5 min at 13000 rpm. The supernatant was removed and nuclear extraction buffer was added. Samples were incubated on ice for a total of 40 min with vortexing at 10 min intervals. After incubation, samples were centrifuged for 10 min at 13000 rpm. The supernatant was collected and stored at -80°C until needed. Protein concentration was determined by the Bradford method (30).

DNA Labeling. Oligonucleotide primers, forward: 5'-TCGAGCTGGGGGACGTTGACATTAC-3' and reverse: 3'-TCGAGGTATGTCACGCAATGCCCCAGC-5' were purchased from Operon (Valencia, CA). The biotin labeling reaction consisted of 25 ul of ddH2O, 10 ul of 5X Terminal Deoxynucleotidyl Transferase (TdT), 5 ul of unlabeled oligonucleotide (1 uM), 5 ul of Biotin-N4-CTP (5uM) and 5 ul of diluted TdT (2U/ul). The final reaction volume was 50 ul. The reaction was incubated at 37°C for 30 min then stopped with the addition of 2.5 ul of 0.2M EDTA. The labeled DNA was extracted using a 24:1 chloroform:isoamyl alcohol solution. After centrifuging the samples for 2 min at 13000 rpm, the top aqueous phase containing the labeled oligonucleotides was removed. The labeled forward and reverse oligonucleotides were combined and allowed to anneal for 2 h at room temperature (32).

Binding Reactions. The binding reactions contained 3 ul of 10X binding buffer, ddH2O, 10 ug of HepG2 nuclear extract, 1.5 ul of poly (dI-dC) and 2 ul of labeled oligonucleotide. Reactions were incubated for 20 min at room temperature. Gel loading buffer (5 ul) was added and the total reaction volume was applied to the gel (33).
The gel was a 6% nondenaturing gel of 40:1 acrylamide:bisacrylamide. It consisted of 2 ml of 10X TBE [890mM Tris base, 890mM boric acid, 20mM EDTA], 2.5 ml of 29:1acrylamide:bisacrylamide, 2 ml of acrylamide, 1.25ml 80% glycerol, 300 ul of 10%APS, 20ul of TEMED and 32 ml of ddH$_2$O. After polymerizing for 2 h, the gel was pre-run in 0.5X TBE for 1 h at 200V. Samples were loaded and the gel ran at 200V at 4°C until the dye was three-fourths down the gel. The samples were then transferred to a positively charged nylon membrane for 1 h at 360mA at 4°C. After the transfer, the membrane was placed under an ultraviolet light to hybridize the transferred DNA to the membrane. The membrane was then washed three times with 1X wash buffer, equilibrated with substrate-equilibration buffer and band shifts were detected via chemiluminescence (33).

**Specific Aim 3: To determine whether C2-ceramide enhances cellular uptake of 3MC.**

3MC uptake by HepG2 cells was determined by use of both a radiolabled assay and confocal microscopy. The goal of these experiments was to determine whether C2-ceramide enhances 3MC uptake by HepG2 cells.

**Radiolabled Assay.** $[^3]$H$^3$MC (+/- C2-ceramide) was added to cell culture media once the cells had reached confluency. The amount of 3MC that traversed the plasma membrane was determined by measuring the amount detected in harvested cells. HepG2 cells were grown in labeled 3MC for 0.25, 0.5, 1, 4 and 18 h, after which the cells were detached from the plate by trypsinization (0.025% for approximately 5 min). The loosed cells
were then pipetted into centrifuge tubes and centrifuged at 2500 rpm for 5 min to pellet the cells. The resultant supernatant was removed. The cells were washed again by resuspension in 1 ml of 0.1 M sodium phosphate(pH 7.6) and pelleting. The pelleted cells were homogenized in 0.1 M sodium phosphate(pH 7.6) to release cell contents. An aliquot of this homogenate was assayed by liquid scintillation counting to determine the amount of the labeled 3MC that went into the cell or was attached to or trapped in the cell membrane.

Confocal Microscopy. HepG2 cells were plated on 4 well glass slides (Nunc Lab Tek II CC2 treated) at a density of 2.0x10^3 cells/ml. One hour prior to treatment, cells were fed DMEM without fetal bovine serum. Treatment times were 1 min, 5 min, 30 min, 1h and 2h. After treatment, cells were rinsed twice with PBS and fixed using a 3% formaldehyde solution for 10 min. Fixed cells were kept in PBS+10% FBS until staining. The cells were stained with 50ul of Sytox Green (1:10,000) and 300ul of Alexa Fluor 568 phalloidin (Molecular Probes, Eugene, OR) for 10 min and 30 min, respectively. Once the cells were stained, the slides were mounted using Flouromount-G (Southern Biotechnology Associates, Birmingham, AL) and observed using a Zeiss LSM 510 Confocal Microscope.
CHAPTER 3

RESULTS

Western blot analysis for AhR [Figure 6] and ARNT [Figure 7] showed no significant change in protein concentration either between treatment groups or over a 4 h time period. However, there were fluctuations within untreated and treated groups over the 4 h time period.

Electromobility mobility shift assay [Figure 8] results showed a shift in untreated, 3MC, 3MC +C2 ceramide and C2 ceramide treatment groups. However, the shift was more intense in 3MC and 3MC +C2 ceramide than in untreated. Between 3MC and 3MC +C2 ceramide treatments, there was no significant difference in intensity of the shift. C2 ceramide treatment only resulted in a slight shift.

Permeability studies using [$^3$H] 3MC showed that between [$^3$H] 3MC treated and [$^3$H] 3MC+ C2 ceramide treated cells, there was more [$^3$H] 3MC accumulation in the cells in the presence of C2-ceramide [Figure 10]. A time course permeability study was done using [$^3$H] 3MC and revealed consistent [$^3$H] 3MC accumulation with C2-ceramide treatment over [$^3$H] 3MC treatment alone over a time period of 4 h [Figure 11].

Confocal microscopy was used to confirm the movement of 3MC into HepG2 cells. These studies yielded similar results to the permeability studies [Figure 12]. 3MC treatment alone showed 3MC entrance into cells. However, in the presence of C2 ceramide, significantly more 3MC was present in the cells. 3MC uptake within HepG2 cells occurred within 1 min of treatment. Another noticeable feature was that 3MC
accumulation was not dispersed throughout the cells. Small "pockets" or possibly vesicles containing 3MC were seen aggregating around the nucleus.

Figure 6. Electrophoretic mobility shift assay results. Actual gel and graphical depiction of shifted band intensity.
Figure 7. AhR western blot
Figure 8. ARNT western blot
Figure 9. C2-ceramide enhances cell membrane permeability to 3-Methylcholanthrene. HepG2 cells were treated with either [\(^3\)H] Methylcholanthrene or with [\(^3\)H] Methylcholanthrene and C2-ceramide. The amount of [\(^3\)H] Methylcholanthrene incorporated into cells was assessed at the end of 18 h.
Figure 10. Timed uptake of $[^3H]$ 3MC by cells treated with 3MC alone or with 3MC+C2-ceramide.
Figure 11. Confocal micrographs of HepG2 cells after 5 minute treatments. Legend: Red - Cytoskeleton; Blue 3-Methylcholanthrene; Green - Nucleus
CHAPTER 4

DISCUSSION

The goal of this study was to determine how the complex sphingolipid, C2-ceramide, caused the synergistic induction of CYP1A1 by 3-Methylcholanthrene. The specific aims of the study were to examine three steps in the known signal transduction pathway to determine whether C2-ceramide caused changes there that could account for its overall affect on CYP1A1 induction. Those steps are: 1) changes in the amounts of AhR and ARNT, two critical components of the signal transduction pathway; 2) changes in the binding of the transcription factor to the XRE1 of the cyp1a1 gene; and 3) changes in the permeability of the plasma membrane to 3MC.

The results of the experiments performed to determine possible modulations in the AhR and ARNT amounts showed no significant change in AhR and ARNT concentrations between untreated, 3MC, 3MC + C2 ceramide and C2-ceramide treatment groups or over a 4 h treatment time (Figures 7 & 8). AhR is an indiscriminate receptor. It is a part of other enzyme transcription pathways. Examples of such enzymes are CYP1A2, CYP1B1, NADP(H)-oxidoreductase, GST-Ya and UDP-glucuronosyltransferase (34). Other studies have shown that AhR is involved in cell cycle regulations and cell proliferation (35). Because AhR and ARNT are involved in several different signaling pathways, they are probably constitutively expressed.

Studies on transcription factor binding to the cyp1a1 gene (Figure 6) showed that there was no significant difference in binding between 3MC and 3MC + C2-ceramide
treated cells. Therefore, C2-ceramide does not seem to directly influence the 3MC-AhR-ARNT triplex binding to XRE1 of the cyp1a1 gene to modulate CYP1A1 transcription. Though C2-ceramide has been shown to cross the plasma membrane and incorporate into other organelle membranes (36), the nuclear envelope is a highly specialized membrane that contains the smallest portion of sphingolipids of any other cellular membrane. Because the nuclear envelope is so selective in what it is permeable to, C2-ceramide may be unable to cross it. Another possibility is that C2-ceramide is incorporated into other organelles or vesicles before it is able to reach the nuclear envelope.

The final step examined was the possible enhanced permeability of the plasma membrane to 3MC in the presence of C2-ceramide. Such an enhancement would lead to a higher intracellular concentration of 3MC which could increase the rate of the signal transduction, resulting in the observed increased production of CYP1A1 protein by 3MC in the presence of C2-ceramide. The results showed that C2-ceramide increases the permeability of the plasma membrane and allows more 3MC to enter the cell (Figure 12). Thus, it is at the beginning of the CYP1A1 induction pathway that C2-ceramide has its effect. Short chain ceramides, specifically C2-ceramide, have been shown to form a hexagonal, non-bilayer, inverted micellar structure that disrupts phospholipid membranes (36). Other studies have shown that C2-ceramide has the ability to form stable channels in membranes (37).
SUMMARY

In conclusion, it appears that the mechanism used by C2-ceramide to cause the synergistic induction of CYP1A1 by 3MC is its membrane pore-forming ability. This biophysical property of ceramide increases membrane permeability to 3MC, allowing more to enter cells. Though the elements that comprise the CYP1A1 induction pathway did not significantly change with C2-ceramide treatment, the increased amount of 3MC entering cells through C2-ceramide permeabilized membranes maximizes the usage of the CYP1A1 induction pathway.
Figure 12. CYP1A1 induction pathway with C2-ceramide pore
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