The Dynamic Role of Sphingolipids and their Regulatory Enzymes in Preeclampsia

by

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Abstract

Sphingolipids act as regulators of cell fate decisions. Ceramides (CERs) are key effector molecules regulating cell death. CERs metabolism is controlled by a balance between its breakdown and synthesis by the enzymes acid ceramidase (AC) and acid sphingomyelinase (ASM), respectively. We hypothesized that sphingolipid metabolism plays a role in proper placental development by establishing trophoblast cell fate and alterations in the pathways contribute to preeclampsia. CERs levels were increased in preeclamptic relative to normotensive preterm control placentae and this associated with decreased AC and ASM expression levels and altered glycosylation and activity of these enzymes, an effect caused by oxidative stress. Furthermore, C-16 ceramide or inhibiting AC activity induced autophagy in placental cells. In conclusion, altered expression of AC and ASM in preeclamptic placentae, induced by oxidative stress, are responsible for changes in the sphingolipid rheostat which may contribute to increased autophagy and trophoblast cell turnover characteristic of this disorder.
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Abbreviations

AC acid ceramidase
ACOG American College of Obstetrics and Gynaecology
ASM acid sphingomyelinase
Bcl-2 B-cell lymphoma-2
Bok Bcl-2-related ovarian killer
CERs ceramides
CT cytortrophoblast
C1P ceramide 1-phosphate
DHCers dihydro-ceramides
DMEM Dulbecco’s modified Essential Medium
EVT extravillous trophoblast
ECL enhanced chemiluminescence
Fas-L Fas ligand
FBS fetal bovine serum
FFA free fatty acid
H&E hematoxylin and eosin
HIF-1 Hypoxia inducible factor 1
ICM inner cell mass
IP immunoprecipitation
LC3B-11 mitochondrial-associated protein-1 light chain 3
Mcl-1 myeloid cell leukemia factor 1
Mtd matador
M6P mannose 6-phosphate
M6PR mannose 6-phosphate receptor
NO nitric oxide
PBS phosphate buffered saline
PE preeclampsia
PKA protein kinase A
PKC protein kinase C
PP2A phosphoprotein phosphatase 2A
PTC pre-term control
PVDF polyvinylidene fluoride
RIPA radioimmunoprecipitation assay buffer
ROS reactive oxygen species
Sa sphinganine
SAP sphingolipid activator protein
SDK1 sphingosine dependent kinase 1
SEM standard error of the mean
sFLT-1 fms-like tyrosine kinase
siRNA small-interfering ribonucleic acid
SPH sphingosine
SM sphingomyelin
SMS sphingomyelin synthase
SNP sodium nitroprusside
SphK sphingosine kinase
SPP sphingosine 1-phosphate phosphatases
ST syncytiotrophoblast
S1P  sphingosine 1-phosphate
TBST  tris-buffered saline with Tween-20
term control
TGFβ  transforming growth factor β
tumour necrosis factor α
VEGF  vascular endothelial growth factor
WB  western blot
3-KSR  3-ketosphinganine reductase
Chapter 1

1 Introduction

Sphingolipids comprise a complex group of lipids with an extreme range of functional versatility. The root term “sphingo” was coined from the Egyptian sphinx, which was typically designed with the fierce body of a lion and a head representing one of the many faces of Egyptian royalty. Similar to the sphinx, sphingolipids are built with common molecular core of ceramide, a “fierce” renowned messenger of death upon which are different head groups that distinguish each sphingolipid species. Though classically known as important structural elements of the cell membrane, sphingolipids have recently been characterized as signaling molecules involved in executing a variety of cell fate events.

Human placental development requires a tight regulation of trophoblast cell differentiation events including death and proliferation that are largely dependent upon a dynamic intertwining of pro-apoptotic and pro-survival factors. The goal of my thesis is to characterize sphingolipid metabolism and assess the role of sphingolipids in regulating trophoblast cell fate in normal placental development and in preeclampsia (PE).

1.1 Sphingolipids

Sphingolipids, classically thought to be purely inert structural elements of the cell membrane, have been recognized as bioactive signalling mediators in modulating a variety of fundamental cellular processes (1). This group of lipids is built on a common sphingoid base backbone. In humans this backbone is most commonly but not exclusively sphingosine (SPH), which is an 18 carbon amino-alcohol with an
unsaturated hydrocarbon chain. Sphingoid bases can fuse with various fatty acids generating ceramides (CERs), the central branch point for biosynthesis of hundreds of sphingolipids (Figure 1.1). CERs can be modified by phosphorylation or glycosylation to ceramide-1-phosphate (C1P) or glucosylceramide respectively. Alternatively CERs also serve as metabolic and structural precursor for complex sphingolipids such as sphingomyelin (SM), the most abundant sphingolipid in mammals (2). The role of each individual sphingolipid is multidimensional in terms of its subcellular localization, and mechanism of action.

1.1.1 Sphingolipid Metabolism

Central to the sphingolipid metabolism pathway are CERs, a class of sphingolipids that have been a subject of intense investigation because of their ability to respond to diverse stress stimuli and mediate pro-apoptotic responses such as cell cycle arrest, apoptosis, and autophagy (3,4). CERs are the only sphingolipids that can be synthesized de novo while all other sphingolipids originate from the breakdown or modification of CERs. The de novo pathway consists of a series of 4 reactions beginning with the condensation of serine and palmitoyl CoA by the enzyme serine-palmitoyl CoA transferase (SPT), leading to the synthesis of 3-ketosphinganine, which is then converted to sphinganine (Sa) by 3-ketosphinganine reductase (3KSR). Ceramide synthase (CerS) then couples fatty acyl-CoAs of various lengths to Sa forming dihydroceramides (DHCers), which are then converted to CERs by the enzyme desaturase (DES) (Figure 1.2). Sa can fuse with various fatty acyl-CoAs generating a huge variety of CERs with anywhere between 2 and 28 carbon atoms; however the most common have between 16-24 carbons (C16-C24) (2). All steps leading to CERs synthesis are localized to the cytosolic side of the endoplasmic reticulum (ER). However, it is noteworthy that CERs can also be produced from the catabolism of complex sphingolipids in a variety of other
Ceramides consist of a sphingosine (sphingoid) base (black box) linked to fatty acyl-CoA via an amide bond (red arrow). The fatty acyl-CoA is either saturated or unsaturated and can vary in length from 2 to 28 carbon atoms. Ceramides are formed as key intermediates for the biosynthesis of all complex sphingolipids, in which the terminal primary hydroxyl group (purple box) is linked to a variety of different head groups. Shorthand nomenclature uses the length of the fatty acids to denote the molecular species of ceramide.
cellular compartments (Figure 1.3) (5). Because CERs are extremely hydrophobic and cannot exist in the cytosol, they exert their main actions at the membrane level. The impact of CERs on cell function depends on the length of the fatty acyl-CoA that is attached to the sphingoid base (Figure 1.1). CERs with specific chain lengths have differing effects on physiological and pathophysiological processes (6). Of particular interest, long chain CERs are important for apoptosis induction. Studies have shown that C16 and C24 CERs originating from de novo synthesis are important mediators of apoptosis in response to cellular stress such as ionizing radiation (7). Furthermore, the induction of apoptosis by sodium nitroprusside involves the accumulation of C22 and C24 CERs (8). However, short chain CERs may also influence apoptosis by increasing the permeability of the mitochondrial membrane (9). Hence, the pathway to cell death is dependent on specific species of CERs.

In the de novo pathway, the enzyme CerS catalyzes the formation of DHCer from sphinganine (Sa), however this enzyme is also responsible for the reacylation of SPH into CERs. Inhibition of CerS will therefore cause accumulation of Sa and SPH as well as depletion of CERs and further complex sphingolipids. (10). CerS activity is inhibited by the fumonisins, a family of mycotoxins that cause a wide range of diseases including cancer and birth defects (11). For instance, Fumonisin B1 competes with binding of the fatty acyl-CoA to Sa leading to excess Sa and blockage of the de novo synthesis of CERs. Previous studies have reported that inhibition of CerS and subsequent accumulation of both Sa and SPH are toxic to cells and have similar effects as CERs on cell growth and apoptosis (12).

CERs are a substrate for sphingomyelin synthase (SMS), an enzyme that adds a phosphorylcholine moiety to the terminal primary hydroxyl group of CERs forming SM (Figure 1.2) (13). The SMS enzyme plays a crucial role in controlling CERs levels
Central to the sphingolipid metabolism pathway is ceramide. Ceramide can be generated \textit{de novo}, in a series of 4 reactions beginning with serine and palmitoyl CoA. Ceramide levels are regulated by a balance between the rates of its synthesis from SM, catalyzed by acid sphingomyelinase (ASM) and its breakdown by acid ceramidases (AC). AC catalyzes the hydrolysis of ceramide into sphingosine and free fatty acids (FFAs). Sphingosine in turn can be phosphorylated to form sphingosine-1-phosphate (S1P).
and can therefore influence functions mediated by this bioactive lipid. Because SM is synthesized from a unit of CER and CERs can be composed of various lengths of fatty acyl-CoAs, this chain length carries over leading to a variety of SMs. Like CER, SM can be synthesized in different cellular sites including the plasma membrane and cis- or medial-golgi and this localization is accompanied by SMS enzyme expression at these sites (Figure 1.3). SM is the most abundant sphingolipid in mammals and plays a very important role in cell membrane formation (14). Mounting evidence suggests that inhibition of SMS activity alters membrane composition through SM depletion, perturbing intracellular signaling pathways. Also, the interaction of SM with cholesterol drives the formation of plasma membrane rafts that play important roles in protein sorting and signaling microdomains as they lead to the clustering of receptors and signaling molecules (3). Additionally, SM has recently been implicated as an adaptor molecule in the functioning and processing of membrane proteins. Specifically, SM18 interacts directly and specifically with the transmembrane domain of p24, a coat protein (COPI) implicated in bidirectional transport processes at the ER-golgi interface. Here, SM aids in regulating this proteins rheostat between an inactive monomeric and active oligomeric state (15). Thus, the complexity of membrane SM composition may have significant functional interactions and influences with membrane proteins.

On the other side of the sphingolipid metabolism pathway, CERs can alternatively be broken down or de-acylated into SPH and free fatty acids (Figure 1.2). SPH is composed of a hydrophobic sphingoid long chain base and, like CERs, has also shown to be a negative regulator of cell proliferation and a promoter of apoptosis (16). SPH has also been demonstrated to be a potent inhibitor of protein kinase C (PKC), a group of isoenzymes involved in cell growth and survival (17). Furthermore, SPH recently has also been shown to inhibit or activate a variety of other kinase activities
Figure 1. 3 Pools of ceramide involved in cellular response to stress

Metabolic pathways responsible for ceramide synthesis and degradation in response to cellular stressors such as hypoxia or oxidative stress. The two main pathways for generation of excess CER during stress response are 1) de novo synthesis restricted to the ER and 2) turnover of SM in the plasma membrane and endo/lyso compartment. Various stress agonists activate these pathways leading to elevation of CER. The magnitude of CER accumulation also depends on the action of the ceramidases and sphingomyelinases, also known to be activated by stress agonists. Stimulation of the de novo pathway during stress leads to newly generated CER, which is then transported to the golgi, where it serves as the rate-limiting substrate in the synthesis of other complex sphingolipids including SM.
such as sphingosine dependent kinase 1 (SDK1) and protein kinase A (PKA), kinases involved in cell death (16).

SPH in turn can be phosphorylated to form sphingosine-1-phosphate (S1P) a functionally counteractive metabolite of CER and SPH known to promote cell proliferation, differentiation and survival (18). Following its synthesis, S1P can be exported out of cells into the circulation; however due to its hydrophobic structure, it is highly bound to lipoprotein carriers, such as high-density lipoproteins, which greatly decreases its active concentration (19). S1P regulates signals through 5 different G-protein coupled receptors, S1P1-S1P5 and has potent and diverse biological effects on the vascular system (20,21). Notably cellular levels of CER are significantly higher than those of SPH, which in turn are greater than those of S1P (22). S1P is reversibly degraded through the action of specific intracellular S1P phosphatases (1 and 2) back into SPH. Alternatively, S1P can be cleaved in an irreversible manner by S1P lyase to ethanolamine phosphate and hexadecenal. Hence, S1P lyase provides an exit point from the dynamic interconversion of sphingolipids by catalyzing sphingolipid degradation (23).

A more complex mechanism regulating levels of CERs involve the recycling or salvage pathway (Figure 1.2). The salvage pathway involves the recycling of SPH whereby complex sphingolipids, mainly SM, are degraded in the lysosome to form SPH and subsequently CERs (24). SPH can then be used as a substrate for CerS leading to the re-synthesis of CERs without the DHDer intermediates. This salvage pathway generally leads to the accumulation of C14-C26 CERs (6). Studies in vitro have shown that applying exogenous short chain CERs (C6) to cells can activate this salvage pathway, leading to biochemical recycling of the SPH backbone and generation of endogenous long-chain CERs. Interestingly, this pathway is upregulated by oxidative stress and has
been shown to generate specifically C16 and C24 CERs in response to this insult (25).

Taken together, bioactive sphingolipids form a network of metabolically interrelated lipid mediators. A clear dichotomy exists between sphingolipids with opposing roles in cell fate events, particularly CERs and S1P. Their ability to be interconverted suggests that balance between them is related to cell death and survival (Figure 1.4).

1.1.2 AC and ASM: Sphingolipid Regulatory Enzymes

CERs levels are regulated by a balance between the rates of its hydrolysis from SM, catalyzed by the enzyme acid sphingomyelinase (ASM) and its breakdown by the acid ceramidase (AC) enzyme. Various physiological and stress stimuli such as cytokines and oxidative stress result in CERs accumulation, which is often the result of ASM activation or may also be due to inhibition of CERs clearance by the ceramidases (4) (Figure 1.3).

The enzyme AC, a key mediator of CER metabolism, is a heterodimeric glycoprotein that catalyzes the hydrolysis of CER into SPH and free fatty acyl-CoA. Lysosomal cleavage of CER in vivo requires the combined efforts of AC and SAP-C or SAP-D, a sphingolipid activator protein that allows the amide bond of CERs to become more accessible to the AC enzyme active site (26). Biosynthesis of AC begins in the ER with a precursor polypeptide of 55kDa, which is then processed and cleaved to the 53kDa mature heterodimeric enzyme in the lysosomes. Proper shuttling of AC to the lysosomes, along with enzyme activation are heavily influenced by its N-linked oligosaccharide chains. AC is composed of an alpha and beta subunit that has 6 potential N-glycosylation sites. Complete deglycosylation of AC using N-glycanases reduces the molecular weight of the β-subunit to 28kDa, whereas the α-subunit is not glycosylated (27). Previous studies using site directed mutagenesis leading to impairment of
Ceramides are involved in mediating pro-apoptotic responses whereas its counteractive metabolite, S1P plays pivotal roles in cell proliferation and survival. These sphingolipids can be interconverted within cells suggesting that a balance between them plays a role in cell fate. Importantly, because CERs are the only sphingolipid that can be synthesized de novo, S1P levels are highly dependent on the breakdown of CERs by AC, making this enzyme a key regulator of cell fate decisions.

**Figure 1. 4 Sphingolipid Rheostat**

Ceramides are involved in mediating pro-apoptotic responses whereas its counteractive metabolite, S1P plays pivotal roles in cell proliferation and survival. These sphingolipids can be interconverted within cells suggesting that a balance between them plays a role in cell fate. Importantly, because CERs are the only sphingolipid that can be synthesized de novo, S1P levels are highly dependent on the breakdown of CERs by AC, making this enzyme a key regulator of cell fate decisions.
glycosylation prevented proteolytic maturation of AC. Consistently, removal of specific glycosylation sites resulted in diminished enzymatic activity due to protein misfolding and retention within the ER (26). Intracellular targeting of AC to the lysosomes is dependent on mannose 6-phosphate (M6P) receptor-mediated sorting (26). The construction of the M6P recognition signal requires specific mannose residues present on N-linked oligosaccharide chains. Newly synthesized lysosomal glycoproteins are carried from the ER to the golgi where a phosphate residue is added to mannose forming M6P. Once phosphorylated, these phosphomannosyl residues are bound by M6P receptors and the enzymes are shuttled in vesicles to the lysosomes where the low pH mediates dissociation of the complex (Figure 1.5). Improper glycosylation of the AC enzyme would also lead to failed formation of M6P residues preventing correct targeting to the lysosome, and instead retention of this enzyme in the ER (28).

An alternate pathway leading to CERs accumulation is by the enzyme acid sphingomyelinase. The ubiquitous, lysosomal ASM is a soluble hydrolase that in response to a variety of apoptotic stimuli causes SM hydrolysis and subsequent CERs generation. ASM is synthesized as a 75kDa propolypeptide, which undergoes differential trafficking to produce two unique enzymes. This single protein precursor is cleaved into a 72kDa precursor, which is transported to the lysosome where it further undergoes cleavage into a 70kDa active enzyme. Alternatively, the 75kDa precursor can be cleaved into a 57kDa form that is secreted extracellularly (29). Extracellular ASM may have a role in non-lysosomal hydrolysis of SM in the outer plasma membrane and plasma lipoproteins (30). Similar to AC, ASM possesses 6 potential N-linked oligosaccharide chains that are important for enzyme trafficking and activation. ASM also undergoes mannose-6-phosphate (M6P) dependent targeting to the lysosomes (Figure 1.5). Previous studies have shown that eliminating these glycosylation sites
Figure 1. 5 AC and ASM have six N-linked oligosaccharide chains

A hypothetical model of AC and ASM protein trafficking. Both AC and ASM are synthesized in the ER, post-translationally modified in the golgi, then shuttled to the lysosome where they are cleaved into active enzymes. Both enzymes have 6 N-linked oligosaccharide chains. Within an N-linked oligosaccharide chain are mannose residues, which upon phosphorylation form Mannose-6-phosphate (M6P). Binding of M6P receptor then targets these enzymes to the lysosomes. Modifications to the glycosylation would therefore prevent the M6P receptor from binding and prevent proper targeting to the lysosome.
significantly diminishes ASM activity, which may be due to disruption of proper ASM targeting to the lysosome (31).

Hence, both AC and ASM are glycoproteins synthesized in the endoplasmic reticulum (ER) that undergo extensive post-translational modification during their transport to the lysosomes, where they are then cleaved into active enzymes in the acidic environment (31). Studies on other glycoproteins conducted in vitro have examined glycosylation using an antibiotic known as tunicamycin. Exposing cells to tunicamycin disrupts glycosylation of newly synthesized proteins due to blockage of the transfer of oligosaccharide from a donor molecule to certain asparagine (Asn) residues present on the protein. Tunicamycin can be thus be used to investigate both the glycosylation status of newly synthesized proteins as well as their trafficking through the cytoplasmic organelles (32).

1.1.3 Mechanisms Regulating Sphingolipid Metabolism

Upstream of CERs, apoptosis inducing molecules, such as TNF-α, chemotherapeutic agents and ischemia/reperfusion, modulate one or more enzymatic activities involved in sphingolipid metabolism, leading to an accumulation of CERs (33).

1.1.3.1 Reactive Oxygen Species and cytokines

Recently, reports have established a link between the production of reactive oxygen species (ROS) and sphingolipid generation (34). Aerobic metabolism is linked to the generation of toxic byproducts called ROS whose rate of synthesis is positively correlated to the prevailing oxygen concentration (35). ROS include superoxide anion, hydrogen peroxide, nitric oxide and peroxynitrate and can act as intracellular signaling molecules regulating cell death and autophagy (36). Evidence indicates that ROS can...
alter protein structure and function by oxidative modifications by superoxide anion interaction with cysteine residues on proteins. Additionally, alterations to the redox state within the intercellular environment can cause intra-molecular disulfide bridges altering protein conformation (35). Notably, a variety of conditions leading to increased cellular levels of ROS are also known to increase CERs levels and cell death (37).

One example of the interaction between ROS and sphingolipids is hydrogen peroxide, which causes increases CERs levels and that this is due to activation of the de novo synthetic pathway. Furthermore, ASM activity remained unchanged in response to hydrogen peroxide, suggesting CERs accumulation was not from SM breakdown via ASM (34). Interestingly, through Gi protein involvement, S1P activates NADPH oxidase (nicotinamide adenine dinucleotide phosphate-oxidase), an enzyme involved in the generation of superoxide, leading to deregulation of oxidative status within cells (38). Studies have indicated that the NADPH oxidase enzyme is increased in disorders complicated by oxidative and nitrative stress including preeclampsia (39). Thus, alterations in S1P can also contribute to a status of oxidative stress.

Nitrative stress induced by the release of reactive nitrogen species, can also impinge on protein structure and function leading to cell death. Nitric oxide (NO\textsuperscript{-}) can react together with superoxide can to generate the pro-oxidant peroxynitrite (ONOO\textsuperscript{-}), which in turn is capable of inducing protein oxidation (40). An agent such as sodium nitroprusside (SNP), which is a NO\textsuperscript{-} donor, is capable of inducing nitrile stress while also catalyzing the Fenton reaction resulting in hydroxyl radical (OH\textsuperscript{-}) generation leading to oxidative stress. In osteoblasts, it has been reported that, SNP induces apoptosis by increasing levels of C22 and C24 CERs (8). Hence, SNP-mediated osteoblast cell death is mediated through elevation of these long chain CERs.

Aside from ROS, a variety of other agents’ known to induce cell death and
environmental stressors also alter the sphingolipid rheostat. Transforming growth factor-β (TGFβ), a member of a superfamily of growth factors, is implicated in a variety of cellular processes including cell proliferation, differentiation and apoptosis. TGFβ has previously been established to be an oxygen-regulated gene and has been shown to stimulate ROS production in a variety of cell types. Recent evidence suggests an interaction between TGFβ and the sphingolipid signaling pathways leading to the induction of endogenous CER levels (41). Additionally, studies have shown cooperation between TGFβ and S1P signaling. TGFβ has also been demonstrated to induce SphK synthesis thus leading to increased S1P production. Hence, SphK1/S1P may contribute to the mitogenic effects of TGFβ (42).

Apoptotic inducers such as tumor necrosis factor (TNFα) and Fas ligand (Fas-L) cause a rapid accumulation of CERs and SPH in a variety of cell lines. Binding of TNFα and Fas-L to their receptors leads to stimulation of the sphingomyelinases, thus catalyzing the degradation of SM to CERs (43). Interestingly, SMS, the enzyme responsible for converting CER into SM, is also inhibited by TNFα and this inhibition is caspase dependent (44). Both mechanisms of CER regulation were initiated prior to the onset of TNFα-induced apoptosis. TNFα is thus a powerful inducer of apoptosis as it has the ability to inhibit degradation and promote formation of CERs.

Besides cytokines and oxidative stress, many reports have shown that ultraviolet radiation (UV) plays a critical role as an upstream mediator of sphingolipid metabolism. Studies using breast cancer cells have shown that UV radiation leads to activation of ASM through its phosphorylation by PKCδ at ser508. This in turn is responsible for increased CERs levels, a mechanism by which UV-damaged cells are eliminated by apoptosis (45). Conclusively, a variety of stress inducing agents are able to trigger a shift in sphingolipid metabolism favouring an accumulation of CERs.
1.1.4 Sphingolipids in Development and Disease

The ceramide signaling pathway has previously been shown to have a critical role in early vertebrate development (46). Recently, ceramide species have been shown to be vital for the formation and differentiation of embryonic epithelia (47). Particularly, depleting embryoid bodies of CERs was shown to prevent formation of the primitive ectoderm layer due to failed organization of cell polarity. CERs interact with polarity proteins such as Par6 to regulate cell polarity, a crucial process involved in the organization of the three germ layers (48). Moreover, complete knockout of the AC gene (Asah1) in mice leads to an early embryonic lethal phenotype due to elevated CERs levels leading to enhanced apoptotic cell death. Conversely, heterozygous AC knockouts display progressive lipid storage disease causing drastic lipid-filled inclusions in most organs that prevent normal mouse development (49). Interestingly, homozygous ASM knockout mice can survive up to 8 months of age, suggesting that this enzyme is only transiently required for development (50). Furthermore, mutations in the S1P receptor gene prevent primordial heart tissue from migrating properly leading to cardia bifida, a lethal disease of defective heart development (46). Thus, sphingolipid metabolism, particularly AC, is essential for mammalian development.

Sphingolipid accumulation in a variety of cells and tissues characterizes several human disorders. Additionally, the subcellular compartment in which sphingolipids are generated, the pathway involved in their synthesis generation, and the cell type, further influences the role of sphingolipids in disease (5).

While complete knockouts are lethal, inherited deficiencies caused by mutations in Asah1, the gene encoding AC results in Farber disease. In this disorder, a profound reduction in AC enzymatic activity leads to the accumulation of CER in the lysosomes causing abnormalities including swollen and deformed joints, subcutaneous nodules, and
progressive hoarseness (51).

Niemann-Pick disease is a lysosomal storage disorder caused by mutations in the *SMPD1* gene encoding for ASM (52). This leads to disruption of membranes and causes SM to accumulate in the lysosomes of parenchymal cells of a variety of organs causing progressive abnormalities in the liver, spleen, brain and bone marrow.

While ASM absence leads to an established pathological state, activation of ASM has been suggested to play an important role in the development of several conditions including cystic fibrosis, a genetic disorder associated with chronic bacterial airway infection (53). Models of cystic fibrosis present with increased long-chain CER (C16-C20) levels in bronchial epithelial cells that exhibit increased susceptibility to apoptotic cell death (54). Studies have shown that CERs increase most likely originates from increased ASM activity since treatment with amitriptyline, an ASM inhibitor, normalized pulmonary CER levels thereby preventing development of cystic fibrosis symptoms (6). Hence interfering with CERs metabolism by targeting its regulatory enzymes may be a useful target for both cystic fibrosis and several lung diseases.

An increasing body of evidence implicates altered mechanisms of CERs regulation in cancer pathogenesis and prognosis (55). The regulation of CERs generation and expression of the regulatory enzymes involved in sphingolipid metabolism are altered in several types of tumor tissues and can be correlated with invasion and metastasis (55). As such, sphingolipids and their regulatory enzymes provide novel therapeutic targets with implications for a variety of cancer therapies (22). Particularly, several anti-cancer drugs have been found to function by elevating cellular CER and triggering apoptosis by activating the ASM/CER pathway (56). Previous studies have also shown that altered sphingolipid metabolism is responsible for chemotherapeutic drug resistance. Specifically, S1P receptors are highly expresses in chemotherapy-
resistant prostate cancer leading to an increased proliferation rate (57). Accordingly, deficiency in CERs production in prostate cancer cells conferred resistance to radiation-induced apoptosis (58). Thus evading the apoptotic effects of endogenous sphingolipids such as CER and SPH has evolved as a mechanism of cancer progression.

Studies have demonstrated that sphingolipids are also involved in the regulation of vascular tone via cross-talk with intrinsic vasoactive factors released by the endothelium. A specific role has been defined for sphingolipids in regulating both vasoconstriction and vasodilation responses in different types of blood vessels (59). More recently, elevation of vascular CERs in spontaneously hypertensive rats have been shown to lead to vasoconstriction of vessels due to increased thromboxane A2 (TXA2) release, a molecule known to constrict vascular smooth muscle (60). Interestingly, increased plasma CER levels have also been found in humans affected by essential hypertension (61). Hence, upregulation of CERs may contribute to increased vascular tone and endothelial dysfunction, a known contributor to hypertension observed in preeclampsia (PE). S1P, the sphingolipid opposing CERs function has also been reported to be involved in the regulation of vascular tone by activating vasorelaxation or vasoconstriction depending on the expression of difference receptor subtypes (20). Interestingly, in various microcirculatory beds, S1P binds to its receptors on vascular endothelial cells and smooth muscle cells of blood vessels and induces vasoconstriction (62). Both CERs and S1P are potent vasoactive factors and genome wide associations have established the involvement of the CER/S1P rheostat in blood pressure regulation in hypertension (63). Thus, alterations in these sphingolipid levels are likely another causative factor of hypertension.

1.1.5 Sphingolipids Dynamic Role in Cell Fate

Accumulation of CERs, produced in response to various stress stimuli, leads to
its processing into SPH and subsequently to the pro-survival S1P. The ability of these bioactive sphingolipids to interconvert creates a rheostat upon which a variety of both intrinsic and extrinsic stimuli can act. As such, the balance between CERs and its metabolites is an important determinant of cell fate (64). Importantly, both SPH and S1P cannot be synthesized de novo and S1P is highly dependent on the availability of SPH generated by the AC enzyme. Thus AC is critical in regulating not only the hydrolysis of CERs but also the generation of both SPH and consequently S1P in cells, making this enzyme a key regulator of cell fate (Figure 1.4) (43).

Additionally, ASM is a key enzyme involved in the “sphingomyelin cycle” which involves the hydrolysis of SM yielding CERs (65). Therefore, one can see that accumulation of CER while reducing S1P would impinge on cell fate by promoting a state of growth arrest and cell death. Sphingolipid metabolism is highly dependent upon AC and ASM function, thereby highlighting their pivotal role as regulators of cell fate. Regulation of cell fate is critical in the development of a variety of organs, including the human placenta.

1.1.5.1 Sphingolipids in cell death and autophagy

The accumulation of CERs during the progression of cell death involves several sphingolipid enzymes and metabolic pathways including de novo synthesis of CERs, SM hydrolysis, loss of SphK and generation of CERs through the SPH salvage pathway (66). CERs have long been known as key players in the induction of apoptosis in response to a variety of stimuli such as oxidative stress, cytokines and radiation (6) (22). In particular, under pro-apoptotic conditions, endogenous levels of C16 CER increase thereby inducing cell death. Intrinsic and extrinsic pathways can trigger apoptosis; in particular, mitochondria though essential for cellular life, are considered deadly organelles involved in the intrinsic cell death pathway induced in response to a variety
of stimuli. Central to this process are the pro-apoptotic B-cell lymphoma-2 (Bcl-2) family members including, Bax and Bak, which are able to pierce the mitochondrial outer membrane thereby inducing the release of pro-apoptotic proteins such as cytochrome c, which once in the cytosol initiates caspase activation, a hallmark of apoptotic cell death (67). A model of apoptosis has been proposed whereby following cytotoxic stimuli, Bak (located in the mitochondria) binds to CerS, thereby activating de novo synthesis of CERs, which leads to formation of ceramide-rich domains in the mitochondria and subsequent Bax activation, facilitating mitochondrial outer membrane permeabilization. Studies from others point towards activation of the de novo sphingolipid synthesis as the primary source of C16 CER (3).

Endogenous long-chain ceramides can also be generated in the acidic organelles from hydrolysis of complex sphingolipids through the action of ASM. Accumulation of CERs in the endolysosomal compartment has been identified to interact with the lysosomal aspartic protease, cathepsin D (Figure 1.6) (68). Once activated cathepsin D is known to mediate apoptosis through cleavage and activation of Bid, a pro-apoptotic protein that has a role in inducing mitochondrial membrane permeabilization leading to activation of caspase 3 and 9 (55,69).

A well-established target of CERs include phosphoprotein phosphatase 2A (PP2A), a multimeric protein that performs critical functions in apoptosis (70). CERs induced activation of PP2A triggers apoptosis by inactivating pro-survival targets Akt (also known as protein kinase B) and Bcl-2 while activating pro-apoptotic proteins, Bad and Bax. Most interestingly, CERs precursors DHCers do not activate PP2A, indicating that PP2A is a direct and specific target of CERs (3).

The hydrophobic properties of CER allow it to cluster in membranes, which can lead to structural changes and the formation of lipid microdomains (rafts) (71). These
Figure 1. 6 Ceramide induces both cell death and autophagy

A hypothetical model that integrates CERs modulation of autophagy and autophagic cell death. Elevation of CERs may promote formation of autophagosomes that will be converted into autophagolysosomes. Autophagy may proceed by breaking down cytoplasmic constituents, providing nutrients to the cell and favouring cell survival. Alternatively Accumulation of CER may destabilize the membrane resulting in release of cathepsins (including cathepsin D, which is also directly activated by CERs) to ultimately activate caspases and cell death.
rafts are enriched in sphingolipids and promote assembly of receptors and proteins that favour signaling activation of FasL and caspase activation, both inducers of apoptosis (72). CER can alternatively induce membrane leakiness to aqueous solutes, which may play a role in release of pro-apoptotic mediators from mitochondria (3).

More recently, the complexity of SPHs effects on signaling has become apparent. SPH specifically activates a kinase, known as sphingosine dependent kinase 1 (SDK1) that phosphorylates 14-3-3 proteins, which are inhibitors of apoptosis, which function by controlling pro-apoptotic mediators (73). SDK1 phosphorylation disrupts the dimeric structure of this protein, preventing it from carrying out its anti-apoptotic functions and ultimately leading to sphingosine-induced cell death (16).

A growing body of evidence has established the involvement of CERs in autophagy (6). Autophagy is a process by which cells recycle intracellular material under different contexts to maintain a state of homeostasis (74). Though often considered a cellular response to stress, autophagy is also an alternative cell death pathway upon which sphingolipids can exert their influence. Macropathology (hereafter referred to as autophagy) is the process for degradation of cytoplasmic constituents via a lysosomal pathway through engulfment into double-membraned vacuoles termed autophagosomes (4). Autophagosomes mature and fuse with lysosomes where acidic hydrolases mediate degradation of their cargo in the acidic environment. Under starvation and stress conditions, autophagy has been recognized both as an adaptive mechanism for cell survival, however, excessive autophagy can lead to an alternative cell death pathway, termed type 2 cell death (75). Only a fine line exists between the conversion of safe to lethal autophagy and conversion from one to the other may be mediated by CER (3).

Interestingly, Spassieva et al. demonstrated that down regulation of CerS, the
enzyme involved in CERs synthesis, by siRNA induces autophagy concomitant with a significant increase in C14 and C16 ceramide levels (76). One of the various molecular targets of CER is the B-cell lymphoma-2 (Bcl-2) family of proteins and previous studies have linked Bcl-2 expression to increased CER levels (77). It has been shown that elevation of CERs lead to dissociation of a complex formed by the pro-death inducer beclin1 and pro-survival Bcl-2. Dissociation of the beclin:Bcl-2 complex and subsequent accumulation of beclin-1 initiates formation of autophagosomes. Elongation of the autophagosomal membrane requires the incorporation of the lipid conjugated membrane-bound form of microtubule-associated protein-1 light chain 3 (LC3B-II) among many others. LC3B transcription and recruitment to autophagosomes are both mechanisms shown to be upregulated by CERs. Hence, CERs induce autophagy by all the accepted criteria (3).

Once initiated, the autophagic process acts as a stress adaptation to avoid cell death, however, excessive autophagy can lead to cellular demise or autophagic cell death (Figure 1.6). The “double-edged sword” is therefore an appropriate term to describe autophagy, as autophagy ties together cellular metabolism and death and is therefore recognized as an important mediator of cell fate decisions. After autophagosomes fuse with lysosomes, they acquire lysosomal enzymes such as AC that will hydrolyze CERs to SPH; which in turn may be effluxed from the autophagolysosome to the cytosol where it may be phosphorylated to form S1P by the enzyme SphK. Interestingly, it has been shown that increased content of CERs can lead to an autophagic response associated with cell death, whereas S1P has been shown to stimulate autophagy in an attempt to save the cell (77). Consistent with this, induction of autophagy in DU145 cells is also thought to involve \textit{de novo} sphingolipid synthesis, with a particular role CERs intermediate, DHCers in the autophagic process (78). Accumulation of CER, DHCer and
SPH in the autophagolysosome could cause membrane destabilization resulting in the release of cathepsins and cytotoxic factors. Furthermore, CER is a potent activator of cathepsins, which are able to activate caspases and induce apoptosis (75) (Figure 1.6).

Because sphingolipids play dynamic roles in both membrane organization and cellular signaling, they are involved in a variety of biological functions responsible for cell fate decisions. Hence, assessing their role in placental development and related disorders is valuable since the balance between cell proliferation and death must be tightly regulated in order to guide proper organogenesis.

1.2 Human Placental Development

The human placenta is a transient organ supporting the developing fetus by creating a fetal-maternal interface required to ensure the exchange of gas and nutrients. Following fertilization the blastocyst, made up of an inner cell mass (ICM), also known as the embryoblast, gives rise to the embryo, umbilical cord and placental mesenchyme. Overlying the ICM is the trophectoderm, which is directly in contact with the uterine epithelium. The trophectoderm gives rise to the trophoblast cells during pregnancy, which as gestation advances, will form the placenta and fetal membranes. Trophoblast cells of the primitive placenta undergo fusion, generating a population of highly invasive layer termed the syncytium, which penetrate the maternal decidua thereby ensuring the connection between the mother and developing embryo. Mononucleated trophoblast cells, termed cytotrophoblasts (CTs), considered the stem cells of the placenta, continue to proliferate throughout gestation and comprise a subset of cells that will differentiate to generate both the syncytiotrophoblast (ST) layer and extravillous cytotrophoblast cells (EVTs). Rapid cell divisions of these CTs partake in syncytial fusion to form the overlying syncytiotrophoblast layer. The continuous expansion of the
syncytiotrophoblast layer allows it to increase in size, promoting further invasion into
the wall of the uterus. Fluid-filled spaces known as lacunae appear in the
syncytiotrophoblast layer, which fuse and anastomose with maternal capillaries giving
rise to the intervillous space. Subsequent rupture of the maternal endometrial capillaries
by the invading STs allows maternal blood to fill the intervillous space giving rise to
primitive uteroplacental circulation, which allows the embryo to receive nutrition in the
first few weeks of gestation (79-81).

As gestation progresses, the chorionic villi denote the functional units of the
placenta providing the site of exchange between fetal and maternal circulation. Initially,
CT cells proliferate and bulge into the syncytiotrophoblast layer creating sprouts called
primary trophoblast villi. Extra-embryonic mesodermal cells grow into the primary villi
transforming these into secondary villi. The formation of blood vessels is key for
differentiating secondary villi into tertiary villi, the site where the majority of gas and
nutrient exchange between fetal and maternal circulations take place (81).

The chorionic villi can be further differentiated into floating and anchoring villi.
The anchoring villi are responsible for maintaining the physical connection of the
placenta to the uterus wall (82). The cytotrophoblast cells of the anchoring villi in
contact with the uterine wall proliferate and aggregate into multilayered columns of cells
that comprise the extravillous trophoblast (EVT) cells. EVT cells at the proximal end of
the column continue to proliferate, whereas at the distal end of the columns, EVT cells
lose their proliferative capability, acquire an invasive and migratory phenotype, and
invade the uterine endometrium and associated endometrial spiral arteries. As EVTs
invade the decidua, they can be further classified into interstitial EVTs (iEVTs), which
accumulate around maternal spiral arteries and replace smooth muscle cells with a
fibroid material. A subset of these cells termed, endovascular EVTs (eEVTs) target and
enter maternal spiral arteries, invade the muscular arteriolar wall thereby replacing the existing endothelium with trophoblast cells (83). Importantly, during early gestation as the endovascular EVT's migrate along the lumen of the arteries, they form endovascular plugs of EVT, thereby restricting flow of maternal blood into the intervillous space. Subsequent invasion of the maternal vessels leads to the destruction of the smooth muscle layer and to a partial replacement of the endothelial cells leading to a change in elasticity of the spiral arteries (Figure 1.7). Remodeling of the spiral arteries leads to compliant, dilated arterioles and blood circulation, which is no longer under the vasomotor control of the maternal endothelium and thus a larger blood flow is allowed in the placenta. This accompanied by the destruction of the endovascular plugs at 10-12 weeks of gestation, leads to a dramatic rise in intervillous perfusion (84,85).

Floating villi consist of two epithelial layers an inner cytotrophoblast cell layer and outer syncytiotrophoblast cell layer and enclose fetal capillaries that sit in the mesenchymal core. The inner cytotrophoblast cells have stem-like properties as they undergo rapid division and following detachment from the basement membrane, fuse to form the overlying syncytiotrophoblast layer. This multinucleated syncytiotrophoblast layer is in direct contact with the maternal blood and provide the primary site for gas and nutrient exchange (86,87) (Figure 1.7).

Throughout pregnancy, aging content within the cytoplasm of the syncytiotrophoblast layer and accumulation of condensed nuclei is shed as membrane enclosed vesicles called syncytial knots. The proliferation of cytotrophoblasts, fusion into the syncytiotrophoblast layer and extrusion of syncytial knots is in part dependent on trophoblast cell death. Increased syncytial knots are often associated with conditions of disrupted utero-placental perfusion (88).
Figure 1. 7 Illustration of human chorionic villi during human placental development

The floating villi are in contact with the maternal blood in the intervillous space (IVS). The floating villi, underlying cytotrophoblastic cells (CT) (yellow) differentiate and fuse to become the overlying syncytiotrophoblast layer (ST) (red). In the anchoring villi, proliferating CT cells break through the overlying syncytiotrophoblast layer forming columns of extravillous trophoblast cells. These cells begin to differentiate and acquire a migratory and invasive phenotype and invade the decidua and first third of the myometrium. Here, they remodel the maternal uterine arteries from outside and inside the arterial lumen. (Figure modified from (86))
1.2.1 Placentation and Oxygen

Early placentation occurs in a relatively hypoxic environment that has proven to be both crucial for proper embryonic and placental development and protective from the harmful effects of oxygen (89). Trophoblast proliferation, differentiation and invasion are all temporally and spatially regulated by a variety of factors including cytokines, signaling cascades, growth factors and oxygen tension. During early placental development (<8-10 weeks), plugs of endovascular cytotrophoblasts obstruct uteroplacental arteries, causing limited uterine blood flow to the placenta (90). Studies have shown that low pO$_2$ maintains trophoblast cells in a non-invasive, undifferentiated, proliferative phenotype. Dislocation of the endovascular plugs allows maternal blood to flow into the intervillous space, causing a dramatic increase in oxygen tension from <20mmHg or 2-3% O$_2$ to approximately >50mmHg equivalent to 8-10% O$_2$, an event which occurs at 10-12 weeks (91). Oxygen has been shown to be a key regulator of proper trophoblast differentiation and in particular controlling the switch from a proliferative to an invasive phenotype, an essential aspect of early placental development that involves controlled cell death (87).

Oxygen has been shown to regulate trophoblast function by a well-known transcription factor named, hypoxia inducible factor 1 alpha (HIF-1α) (92). Under normoxia, very little HIF-1α is present in cells; however, in hypoxic conditions HIF-1α protein is stabilized and free to become transcriptionally active (93). HIF-1α modulates the expression of genes by binding to hypoxia responsive elements in promoter or enhancer regions of the gene (94). In the placenta, a variety of genes are under the regulation of HIF-1α including vascular endothelial growth factor (VEGF), transforming growth factor beta-3 (TGFβ3) and integrin-α6, all essential to maintain the immature phenotype of cells present in low oxygen conditions of early placental development. HIF-1α is highly expressed in the placenta before 10 weeks, when oxygen tension is
low, however its levels decrease at 10-12 weeks when the trophoblastic plugs are displaced and blood flow begins. The result is a switch in expression in a variety of HIF-1 regulated genes. Following the change in oxygen levels, inhibitors of invasion decline while expression of genes required for migration increase (95). This allows extravillous trophoblasts to acquire a migratory and invasive phenotype along the anchoring column and enables the transformation of the maternal spiral arteries. Also noteworthy is the increase in trophoblast cell production of antioxidant enzymes to counteract the increase in pO2 and accompanying oxidative stress and ROS faced at this time (96).

Altered oxygenation causes impairment of these events leading to improper placentation and trophoblast turnover and, consequently, leading to the development of pregnancy-related disorders. Accordingly, alterations in trophoblast cell death and survival are a contributing cause to a variety of placental pathologies. The mechanisms underlying these cellular processes is essential for understanding these disorders and will aid in prevention, diagnosis and treatment of such conditions.

1.2.2 Preeclampsia

Preeclampsia (PE) is devastating placenta-related disorder that affects 5-8% of all pregnancies and is the most common cause of fetal and maternal mortality and morbidity (97). The cause of PE remains a mystery, however, it is established that the placenta is responsible for the development of the disease. In PE, lack of remodeling of the maternal spiral arteries is believed to be the cause for insufficient utero-placental circulation leading to placental hypoxia and trophoblast cell death (98). Excessive trophoblast cell death leads to altered trophoblast turnover and shedding of syncitial fragments into the maternal circulation which then cause systemic vascular endothelial injury leading to the clinical symptoms of PE. Patients present themselves with sudden maternal hypertension (systolic: \( \geq 140 \text{mmHg} \) diastolic: \( \geq 90 \text{mmHg} \)) and proteinuria
(≥30mg/day) (97). In addition, edema in the extremities and brain is another common symptom. PE often requires pre-mature delivery of the baby because symptoms only remit after the placenta has been removed. In addition, reduced perfusion caused by abnormal placentation also presents a major impairment on fetal development (97,99).

As stated above, a key histopathological characteristic is shallow invasion and failed transformation of maternal spiral arteries by EVTs predisposing the placenta to a chronic state of hypoxia and oxidative stress (96). Gene array studies have shown by comparing human villous explants cultured in 3% oxygen and placentation in high-altitude pregnancies to PE, that reduced oxygenation leads to aberrant changes in global placental gene expression. Increased expression of HIF-1α regulated genes including integrin-α6 and TGFβ-3 consequently inhibits trophoblast cell differentiation (100). Through the activation and repression of these and many other genes, trophoblasts are arrested in an immature phenotype characterized by hyperproliferation (101,102). Additionally, ischemia-reperfusion injury to the placenta creates a powerful stimulus for the generation of reactive oxide species (ROS) (84).

Placentally derived circulating anti-angiogenic factors are thought to be the culprit for maternal endothelial dysfunction and hypertension observed in PE. Soluble fms-like tyrosine kinase (sFLT-1) is a potent inhibitor of pro-angiogenic molecules such as VEGF and placental growth factor and has a causal role in the maternal manifestations of PE. This molecule is elevated in response to placental hypoxia and is produced by the placenta and released into the maternal circulation (103). Also, overexpression of TGF-β3 in women with early-onset PE has been shown to cause a dramatic rise in soluble endoglin (SEng), another anti-angiogenic protein. Studies have shown that SEng blocks TGF-β1-induced vasodilation. Interestingly, administering sFLT-1 and sEng to an animal model induces the clinical manifestation of PE (104). Measuring serum levels of
the above markers from mothers may allow the prediction of PE before the onset of clinical symptoms.

Placental trophoblast changes, including increased apoptosis and trophoblast cell turnover seen in PE are in part due to the oxidative stress status characteristic of this disorder (105). Oxygen plays a key role in the transcriptional regulation of a variety of the Bcl-2 family members. Particularly, Mcl-1 (myeloid cell leukemia factor 1) is an important anti-apoptotic factor, while its family member, matador/Bcl-2-related ovarian killer (Mtd/Bok), induces mitochondrial apoptotic events (106). Reduced oxygenation has been shown to alter the apoptotic rheostat between Mtd/Bok and Mcl-1 and is detrimental to cell fate. Increased expression of Mtd/Bok and caspase mediated cleavage of prosurvival Mcl-1 into death inducing isoforms are both oxygen dependent events and result in elevated trophoblast cell death. Similarly, alterations of the Mtd/Mcl-1 rheostat induced by oxidative stress have been shown to lead to aberrant levels of autophagy in PE pregnancies (Kalkat et al. 2012 Submitted to CDD).

1.2.3 Sphingolipids and Placentation

Little is known about sphingolipid metabolism in human placental development and disease status. Recent evidence suggests that in mice, the sphingolipid metabolism pathway is highly active in the deciduum during pregnancy, particularly sphingosine kinase (SphK), the enzyme that catalyzes the phosphorylation of SPH into S1P. Mizugishi et al. have shown that SpkK1 knockouts are infertile. These mice exhibit reduction in S1P and accumulation of SPH, which contributes to pregnancy loss due to defective decidualization and impaired uterine blood vessels (107). S1P has also been identified as an inhibitor of cytotrophoblast differentiation. Through binding with G(i)-coupled S1P receptors, S1P inhibits adenylate cyclase lowering cAMP levels within cytotrophoblast cells causing in inhibition of functional markers of differentiation (108).
Previous studies have also identified differential distribution of sphingolipids in stem and terminal villi of the human placenta at term. Specifically, SM was found to be distributed in stem villi but not in terminal villi (109).

Interestingly, CERs levels increase when autophagy is induced by oxidative stress. Also, a variety of external stimuli (e.g. pro-inflammatory cytokines, oxidative stress) representative of PE milieu, are closely related to CERs signaling and sphingolipid metabolism. Previous studies have shown that ASM and AC enzymes both have key roles in the regulation of CERs levels and thus cell fate, however, the impact of these regulatory enzymes in regulating autophagic responses and trophoblast cell death remains to be established.
1.3 Rationale, Hypothesis and objectives

Since CER and its metabolites have opposing functions in regulating cellular events such as death and proliferation in a variety of biological systems, we hypothesized that sphingolipid metabolism plays a pivotal role in proper placental development by regulating trophoblast cell fate (autophagy and apoptosis) and its alterations contribute to the pathogenesis of preeclampsia. Growing evidence suggests that ceramide is virtually a universal feature of cell death (110), thus examination of the enzymes that regulate the balance between ceramide and its synthesis and breakdown to other metabolites may shed light into the mechanism responsible for CERs accumulation and its impact on cell fate. A variety of stimuli, including hypoxia and oxidative stress, both hallmarks of PE, can offset the balance between CER and S1P, promoting a state of growth arrest and cell death (111). However, the exact mechanisms leading to aberrant levels of trophoblast cell death in preeclampsia remain elusive. Despite impressive advances in our understanding of sphingolipid function, little is known about their involvement in controlling the transition between mitotic potential and cell death or autophagy. Hence, the objective of my thesis was to investigate the contribution of sphingolipid metabolism focusing on the relationship between expression, function and processing of key enzymes that regulate sphingolipid metabolism in the human placenta in physiological conditions and in disease status. Altered expression of sphingolipids and their rate limiting enzymes in preeclampsia (PE) may suggest differential regulatory mechanisms and associated downstream cellular events and contribute to altered trophoblast cell death and autophagy typical of this pathology.

Overall, in this study, we report that the oxidative stress status characteristic of PE causes a decrease in AC protein levels and alters ASM levels favouring an increase in CER levels which may contribute to increased cell death typical of this
disorder.
Chapter 2

2 Materials and Methods

2.1 Placental tissue collection.

Informed consent was obtained from each individual and tissue collection was carried out in accordance with participating institutions ethics’ guidelines (Ethics guidelines of the University of Toronto’s Faculty of Medicine and Mount Sinai Hospital) by Biobank of Mount Sinai Hospital and by the O.I.R.M- Sant’ Anna Hospital, University of Turin, Italy. First trimester human placental tissues (5-8 weeks of gestation, n=24) were obtained immediately following the elective termination of pregnancies by dilation and curettage, or suction evacuation. Gestational age was determined by the date of the last menstrual period and first trimester ultrasound measurement of crown-rump length. Preeclamptic group (PE, n= 68) was selected on the basis of ACOG clinical and pathological criteria. Calcified, necrotic and visually ischemic areas of the placental tissue were omitted from sampling. Placental tissues obtained from age-matched preterm deliveries included as controls (n= 56) from healthy pregnancies with normally grown fetuses that did not have signs of preeclampsia or placental dysfunction. Birth weight, gestational age, laboratory values and clinical observations relevant to the health of the mother were taken from the clinical records. The PE patients’ group was characterized by an average gestational age of 32 weeks: average blood pressure 168/105 (systolic/diastolic), and the presence of proteinuria (average 3g/day). One out of eight PE pregnancies was associated with intrauterine growth restriction. The AMC and TC patients were delivered at an average of 31.5 and 38 weeks respectively and exhibited normal blood pressure (116/73) with no sign of proteinuria. Immediately following delivery, tissues were either snap-frozen in liquid
nitrogen for protein and mRNA analysis or fixed in 4% paraformaldehyde and processed for histochemical analysis.

2.2 First trimester villous explant culture.

Following elective termination of pregnancies, first trimester human placentas (5-8 weeks gestation) were obtained by dilation and curettage. Placental tissue was placed in ice-cold PBS and processed within 2 hours of collection. The tissue was washed in sterile PBS and aseptically dissected using a microscope to remove decidual tissue and fetal membranes. Small fragments of placental villi (15–25mg wet weight) were teased apart and cultured in serum-free DMEM/F12 (GIBCO-BRL, Grand Island, New York, USA), supplemented with 10,000 units/ml of penicillin /streptomycin. Villous explants were placed at 37°C in either standard tissue culture condition (5% CO₂ in 95% air) or maintained in an atmosphere of 3% O₂ /92% N₂ /5% CO₂ for 24 and 48 hours to establish whether different oxygen tensions affected the sphingolipid and enzyme profile. Explants were also treated with 2.5, 5.0 and 10mM of SNP (Sigma, St. Louis, MO, USA) as an inducer of oxidative stress. In addition, to examine the effect of oxidative stress on AC/ASM enzyme glycosylation, explants were treated with SNP with and without glycosylation inhibitor, tunicamycin (Invitrogen, Carlsbad, CA, USA) while maintained in standard tissue culture conditions. Following treatments, explants were washed in cold PBS, collected into eppendorf tubes and snap frozen on dry ice. Frozen tissue was sent directly for sphingolipidomic analysis. For protein extractions, 50-100µL of RIPA buffer with protease inhibitor was added to the eppendorf tube, followed by homogenization on ice with a glass pestle (Wilmad, Vineland, NJ) until all tissue was dissolved. Tissue was vortexed and allowed to sit in RIPA buffer for 1 hour before centrifugation and protein concentration analysis using Bradford protein assay (Bio-Rad, Hercules, CA).
2.3 Cell line culture and analysis

Human choriocarcinoma JEG-3 cells (ATCC, Manassas, VA, USA) were cultured in EMEM media (ATCC, Manassas, VA, USA) supplemented with 10% heat-inactivated fetal bovine serum and 10,000 units/ml of penicillin/streptomycin in 75cm²-flasks and kept in humidified incubators at 37°C in standard conditions (20% O2, 5% CO2 in 95% air). When 100% confluent, cells were washed with PBS, trypsonized with 0.25% Trypsin-EDTA (Invitrogen, Carlsbad, CA, USA) and passaged to a dilution of 1:4 into a new flask. Before plating cells for in vitro experiments, cells were counted with a hematocytometer in trypan blue dye (Invitrogen, Carlsbad, CA, USA) and then seeded into 35mm x 6-well plates.

2.4 In Vitro Experiments

2.4.1 SNP treatments

JEG3 cells were seeded at a density of 1.5 x 10⁵ cells/well into a six-well plate. Human villous explants were cut and set to float in 24 well plates with 500µL of DMEM/F12 media. After 24 hours, cells and explants were treated with SNP (2.5, 5.0 and 10mM) for 6 and 24 hours. A control well was treated with only EMEM media, the SNP vehicle. Cells were then either collected at each time-point on ice in RIPA buffer solution with protease inhibitor cocktail or fixed in 3.7% formaldehyde for 15 minutes at room temperature to perform staining. Explants were washed in cold PBS, placed into eppendorf tubes and snap frozen on dry ice for protein extraction.

2.4.2 2-Oleylethanolamine treatments

JEG3 cells were seeded at a density of 1.5 x 10⁵ cells/well into a six-well plate and treated the following day with 2-OE (Invitrogen, Carlsbad, CA, USA) in doses of 25, 50 and 100µM for 6 and 24 hours. Cells were either collected at each time-point on ice in RIPA buffer solution with protease inhibitor cocktail or fixed in 3.7% formaldehyde for
15 minutes at room temperature to perform staining.

2.4.3 Oxygen Studies

JEG3 cells were seeded at a density of $1.5 \times 10^5$ cells/well into a six-well plate. Human villous explants were cut and set to float in 24 well plates with 500µL of DMEM/F12 media. The following day, cells and explants were maintained in either standard conditions or in an atmosphere of $3\% O_2/92\% N_2/5\% CO_2$ for 6 hours and 24 hours at $37^\circ C$. Cells were either collected at each time-point in RIPA buffer solution with protease inhibitors or fixed in 3.7% formaldehyde for 15 minutes at room temperature to perform staining. Explants were washed in cold PBS, placed into eppendorf tubes and snap frozen on dry ice for protein extraction.

2.4.4 Localization of AC/ASM to endoplasmic reticulum and lysosome.

JEG3 cells ($1.5 \times 10^5$ cells/well) were seeded on sterile glass coverslips and allowed to adhere overnight. Cells were then incubated with 2.5 and 5.0mM SNP for 6 and 25 hours. For the final hour of treatment, 50nM Lysotracker® Red DND-99 (Invitrogen, Carlsbad, CA, USA), a dye taken up by active lysosomes was added to the media. Cells were fixed for staining in 3.7% formaldehyde in EMEM media for 15 min at room temperature. Incubation with primary antibody against AC or ASM was carried out overnight and secondary detection was performed as described above.

2.5 Lipid profiles.

Preeclamptic placental tissue and normotensive age-matched controls were collected as described above. Placental tissue and sera from pre-term normotensive women were collected for controls as described above. All placental tissue was processed for lipid extraction. JEG-3 cells and human villous explants were cultured with sodium nitroprusside (SNP, 5.0mM) and at 3% oxygen and collected as described above and processed for lipid extraction. Lipid extraction was performed according to
the Bligh and Dyer method (112). Following extraction, sphingolipids were separated using high performance liquid chromatography and then analyzed by tandem mass spectrometry at a Mass Spectrometry facility in the Hospital for Sick Children, Toronto, Ontario. This analysis permits the detection of ceramides (CERs) with different fatty acid chain lengths, sphingomyelin (SM) containing CER cores with various fatty acyl-CoA chain lengths, sphingosine (SPH), sphinganine (Sa) and dihydroceramides (DHCers) were reported.

2.6 RNA Analysis

RNA was extracted from frozen placental tissue using an RNeasy Mini Kit (Qiagen, Valencia, CA, USA). All samples were treated with DNase I to remove genomic DNA contamination; reverse transcribed using random hexamers (Applied Biosystems, Foster City, CA, USA). The resulting templates (2µL cDNA for AC and ASM) were amplified by 20 cycles of PCR (5 min at 95°C, cycle: 30s at 95°C, 30s at 55°C and 1.5 min at 72°C). Analysis was carried out using the DNA Engine Opticon 2 System (MJ Research, Waltham, MA, USA) as previously described. Taqman Universal MasterMix and specific Taqman primers and probe for ASM (encoded by SMPD1), AC (encoded by ASAH1) and 18S were purchased from ABI as Assays-on-Demand form human genes. Data were normalized against expression of 18S ribosomal RNA using the 2ΔΔCt formula as previously described (113).

2.7 Antibodies and HRP Substrates

Antibodies were purchased from Santa Cruz Biotechnology, Santa Cruz, CA: AC (T-20) goat polyclonal [IF 1:200, WB 1:350], ASM (H-181) rabbit polyclonal [IF 1:200, WB 1:350], β-actin (I-19) goat polyclonal [WB 1:1000]; and Cell Signaling Technology, Inc. Beverly, MA: LC3B-II rabbit polyclonal [WB 1:1000], Cleaved Caspase-3 (D175) rabbit polyclonal [WB 1:500] and Beclin1 (D40C5) rabbit polyclonal [WB 1:1000] and
Abcam (Cambridge, MA, USA): Calreticulin (FMC 75) mouse monoclonal [IF 1:2000]. Secondary antibodies: horseradish peroxidase-conjugated donkey anti-goat, goat anti-rabbit, and goat anti-mouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA); Alexa Fluor® 594: donkey anti-goat/-rabbit/-mouse and Alexa Fluor® 488: donkey anti-goat/-rabbit [IF 1:200, Invitrogen, Carlsbad, CA]. Concanavalin A was purchased from Sigma Aldrich [WB: 3µg/mL].

2.8 Western blot analysis

Frozen placental tissue chunks were crushed using a mortar and pestle in liquid nitrogen followed by homogenization with a homogenizer (Ultra-Turrax T25 basic, IKA, Wilmington, NC, USA) in 1ml of RIPA buffer (150mM NaCl, 50mM Tris at pH 7.5, 1% NP-40) with a protease inhibitor cocktail (Roche, Indianapolis, IN, USA). Alternatively, JEG3 cells were collected on ice in RIPA buffer with protease inhibitor cocktail and protein was extracted.

Following quantification of protein concentration by Bradford assay, 50µg of total protein from placental tissue lysates and 25µg of protein from cell lysates was added to sample buffer and loaded into 15% SDS-PAGE acrylamide gel in a solution of running buffer (25mM Tris base, 192mM glycine and 0.1% sodium dodecylsulfate (SDS)). After electrophoresis at 180 volts until sufficient separation was noted, proteins were transferred to a methanol-hydrated PVDF (polyvinylidene fluoride) membrane for 1 hour and 30 minutes in transfer buffer (25mM Tris base, 192mM glycine and 20% methanol). For blocking, the PVDF membrane was then incubated in 5% nonfat dry milk in tris-buffered saline solution containing 0.1% Tween-20 (TBST) for 1 hour to prevent non-specific binding. The tris buffered saline solution contained 20mM Tris-Cl, 136mM NaCl and 0.1% Tween 20. Membranes were incubated overnight at 4°C with a primary antibody against the protein of interest in the dilutions stated above. After
overnight incubation, membranes were washed three times for 10 minutes in TBST and incubated with a secondary horseradish peroxidase (HRP)-conjugated polyclonal antibody against the primary antibody species diluted at room temperature in 5% non-fat dry milk in TBST. Following incubation with HRP-conjugated secondaries, membrane blots were washed three times in TBST for 10 minutes and visualized using enhanced chemiluminescence (PerkinElmer Inc., Waltham, MA, USA) and imaged on x-ray film (GE Healthcare). All western blots were confirmed for equal protein loading by probing for β-actin after developing the blots for the initial protein of interest. Membranes were stripped twice for 30 minutes each in stripping buffer (0.2 M glycine, ddH2O, pH=2.2) followed by several washes with ddH2O. Membrane was incubated in 5% non-fat dry milk for 1 hour then probed with a 1:1000 dilution of primary goat antibody against β-actin at 4°C overnight and was similarly processed for developing. For quantification purposes, bands of interest were analyzed using CanoScanLiDE20 image scanner (Canon Canada Inc. Mississauga, ON).

2.9 Immunoprecipitation of AC/ASM

Following SNP treatment, JEG3 cells were washed with PBS and collected with RIPA buffer with protease inhibitor. Following exposure to SNP or tunicamycin, explants were washed in PBS, collected and snap frozen on dry ice. RIPA buffer was added and the tissue was homogenized with a Wheaton glass tissue grinder. Cell and tissue lysates were pre-cleared using a combination of Protein A agarose (10µL) and Protein G agarose (10µL) for 1 hour on an end-over-end rotator at 4°C. The lysate was then incubated overnight with antibodies against AC or ASM with anti-goat normal IgG or anti-rabbit normal IgG used as a negative respectively was added at a concentration of 1 µg per 100-500ug of protein and incubated over night on a rotator at 4°C. A combination of Protein A agarose and Protein G agarose was used to precipitate the
immune complexes. The immune complexes were washed once in ice-cold RIPA buffer and twice in ice cold PBS. The immune complexes were dissociated by heating for 5 min in sample buffer (10% [vol/vol] glycerol, 2% [vol/vol] SDS, 5% [vol/vol] ,6-mercaptopethanol, 0.0025% [wt/vol] bromophenol blue, 0.06M Tris, pH 8.0). The immunoprecipitated lysates were subsequently immunoblotted with 30% loaded to immunoblot for the input and 70% loaded for the interaction of interest.

2.10 ASM Enzyme Activity Analysis

ASM enzyme activity was assessed using an Echelon Acid Sphingomyelinase Assay Kit (K-3200) (Echelon Bioscience, Salt Lake City, UT) as described in the manufacturer's protocol. Briefly, cells were harvested, washed with PBS and collected in RIPA buffer with protease inhibitor cocktail. Fifty µg of protein from placenta tissue and 30 µg of protein form JEG3 cells and human villous explants were used to determine ASM activity. Reaction was stopped after incubation with the ASM substrate at 37°C for 3 hours and analyzed using a 96-well fluorescence microtiter plate reader (Tecan Infinite M200) at 360 nm excitation and 460 nm emission. The enzyme activity was calculated from the slope of the graph of intrinsic fluorescence plotted against time.

2.11 Immunofluorescence staining

Placenta tissues were fixed in 4% formaldehyde and then embedded in paraffin wax. Following, tissues were sectioned with a thickness of 5µM and mounted on glass microscope slides. Every 20th section was stained with hematoxylin and eosin (H&E) to allow evaluation of the quality of the placenta and selection of the most representative sections. Sections of placental tissue were deparaffinized by xylene in three 5-minute washes, followed by hydration through a decreasing graded concentration of ethanol/water. Antigen retrieval technique was performed using 10mM sodium citrate, pH 6.0. The slides were placed in the microwave for 5 minutes, removed and kept at 95-
99°C for 15 minutes. They were then placed in the microwave again for an additional 3 minutes followed by a 30 minute cool down. Because red blood cells are a common source of autofluorescence, slides were treated with Sudan Black (0.3% sudan black in 70% ethanol) for 30 minutes, which quenches endogenous fluorescence. Sudan black was rinsed off by three 5-minute washes in PBS.

Alternatively, JEG3 choriocarcinoma cells, plated on glass cover slips, were fixed in 3.7% formaldehyde mixed in media for 15 minutes at room temperature followed by 3 5-minute washes in PBS. Cells were then permeabilized with 0.2% Triton X-100 (Bishop Canada Inc., Burlington, ON) in PBS on a gentle rotator and washed 3 times again in PBS.

Slides/cells were then pre-incubated for 1 hour at room temperature with 5% normal horse serum diluted in PBS to block non-specific binding sites. Following blocking, slides/cells were incubated with primary antibodies diluted in 5% blocking serum and antibody dilutant (0.4% sodium azide, 0.625% gelatin in PBS filtered with 0.22mm syringe-driven filter) in a 1:1 ratio, overnight at 4°C. The following primary antibody dilutions were used AC (goat polyclonal anti-human AC; Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:200 (for cells) dilution), ASM (rabbit polyclonal anti-human ASM; Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:100 (for sections) 1:200 (for cells) dilution), Cytokeratin (rabbit anti-human cytokeratin, Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:250 dilution), Calreticulin (rabbit polyclonal anti-human calreticulin; Cell Signaling Technology, Inc. Beverly, MA, 1:1000 (for sections and cells) dilution). The following day, sections/cells were washed 3 times for 8 minutes in PBS then incubated with an Alexa fluorochrome-conjugated secondary antibody against the IgG of the primary antibody (Alexa Fluor® 594: donkey anti- goat/-rabbit/-mouse and Alexa Fluor® 488: donkey anti- goat/-rabbit, Invitrogen,
Carlsbad, CA, USA) diluted 200-fold in antibody dilutant for 1 hour at room temperature. Sections and cells were covered in tin foil from this point onwards to prevent light from diminishing the intensity of the fluorophore. For co-staining experiments, an alternate colour of Alexa fluorochrome-conjugated secondary antibody was used. Following secondaries, sections/cells were washed 3 times for 8 minutes in PBS. For negative controls, the primary antibody was replaced by a corresponding concentration of mouse, goat or rabbit IgG. Sections/cells were treated with DAPI (4’,6-diamino-2-phenylindole) for 12 minutes at room temperature for nuclear detection. Finally, a 20µL drop of mounting solution (50% glycerol diluted in PBS) was dropped onto sections followed by a cover slip, which was then sealed with nail polish. For cells, fine tweezers were used to lift the cover slips and place them onto glass microscope slides with a 20µL drop of mounting solution followed by sealing with nail polish. Fluorescence images were viewed and captured using the DeltaVision Deconvolution microscopy with z-stacking (Applied Precision, LLC, Issaquah, WA, USA).

2.12 Lysotracker® Red Staining

Lysotracker® Red (Invitrogen, Carlsbad, CA, USA) was used to monitor lysosomal activity as a marker for autophagy. Lysotracker® Red dye at a concentration of 1mM in DMSO was mixed with cell media to create a mixture at a final concentration of 50nM. Cells were incubated in this mixture for 1 hour at 37°C. Following formaldehyde fixation, cells were washed 3x5 minutes in PBS with gentle rotation and nuclei were subsequently counterstained with 4’,6-diamidino-2-phenylindole (DAPI, Invitrogen, Carlsbad, CA, USA) for 12 minutes. Coverslips were mounted onto microscope slides using a drop of 50% glycerol in PBS as a mounting medium, and adhered to the coverslip with nail polish.
2.13 Transmission Electron Microscopy

JEG-3 cells were seeded at a density of 1.5x10^5 cells/well into six-well plates and allowed to attach by overnight incubation. The following day, cell were treated with either 100µM 2-OE, DMSO (control), 50µM C-16 CER or 100% ethanol (control) for 6 hours at 37°C. Cells were washed in ice-cold PBS and scrapped using 0.25% trypsin and underwent centrifugation. Samples were processed for TEM by fixing in 2% glutaraldehyde in 0.1M sodium cacodylate buffer, rinsed in buffer, post-fixed in 1% osmium tetroxide in buffer, dehydrated in a graded ethanol series followed by propylene oxide, and embedded in Quetol-Spur resin. Sections 100nm thick were cut on an RMC MT6000 ultramicrotome, stained with uranyl acetate and lead citrate and viewed in an FEI Tecnai 20 TEM.

2.14 Statistical analysis

Quantification of western blot was accomplished by densitometry using Image Quant 5.0 software (Molecular Dynamics, Piscataway, NJ, USA). Expressions of all proteins of interest were normalized to the house-keeping gene, β-actin. Statistical analysis was performed using GraphPad Prism 4 software (San Diego, CA, USA). For comparison of data between 2 groups we used the Mann-Whitney, Kruskal-Wallis and paired or unpaired t-test, where applicable. For comparison between multiple groups, One-way analysis of variance (ANOVA) with post-hoc Dunnett’s or Newman-Keuls test was performed, where applicable. Statistical significance was defined as p<0.05. Results are expressed as the mean±S.E.M.
Table 2.1. 1 Clinical parameters of control and preeclamptic patients

<table>
<thead>
<tr>
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<th>Preterm (n=56)</th>
<th>Preeclampsia (n=68)</th>
<th>Term (n=28)</th>
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</thead>
<tbody>
<tr>
<td>Mean gestational age at delivery (weeks)</td>
<td>31±3</td>
<td>30±2</td>
<td>38.4±0.6</td>
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<tr>
<td>Blood Pressure (mmHg)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Systolic</td>
<td>118±4</td>
<td>175±13</td>
<td>122±5</td>
</tr>
<tr>
<td>Diastolic</td>
<td>69±5</td>
<td>109±10</td>
<td>74±5</td>
</tr>
<tr>
<td>Proteinurea (g/24hr)</td>
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<td>3.1±1.5</td>
<td>N/A</td>
</tr>
<tr>
<td>Fetal sex</td>
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<td>M: 52%</td>
<td>M: 43%</td>
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<tr>
<td></td>
<td>F: 67%</td>
<td>F: 48%</td>
<td>F: 57%</td>
</tr>
<tr>
<td>Fetal Weight</td>
<td>1672±388</td>
<td>1462±423</td>
<td>3423±232</td>
</tr>
<tr>
<td>Mode of delivery</td>
<td>CS: 44%</td>
<td>CS: 89%</td>
<td>CS: 100%</td>
</tr>
<tr>
<td></td>
<td>VD: 56%</td>
<td>VD: 11%</td>
<td>VD: 0%</td>
</tr>
</tbody>
</table>

Data represented are mean ± SEM

CS: Caesarean section delivery

VD: Vaginal delivery
Chapter 3

3 Results

Advancements in the field of lipidomics using high performance liquid chromatography linked to tandem mass spectrometry (MS/MS) now permits the simultaneous analysis of the complete sphingolipid profile within a cell, tissue or organ. Using this sophisticated technology, we sought to examine the sphingolipid profile in the human placenta, in normal conditions and in preeclampsia (PE). Moreover, investigating the contribution of the sphingolipid regulatory enzymes will provide insight into the mechanisms leading to altered sphingolipid dynamics.

3.1 Sphingolipid levels are altered in PE.

Numerous reports have shown that trophoblast cell death is increased in PE (114). Because the sphingolipid rheostat plays a well defined role in cell death and survival (115), herein we sought to investigate sphingolipid levels in PE and assess whether their alterations would contribute to increased cell death characteristic of this disorder. In this study we used MS/MS to evaluate the sphingolipid profiles in preeclamptic and age-matched preterm control (PTC) placentae. MS/MS analysis revealed significant increases in CERs with various fatty acyl-CoA lengths (C-16, 18, 20 and 24) in PE placentae compared to PTC. Levels of C-22 CER did not exhibit changes. Notably, the relative abundance of different CERs was similar both in PE and PTC placentae with regards to the individual amounts of each CER (Figure 3.1). Intermediates of the de novo synthesis pathway, sphinganine (Sa) and dihydroceramide (DHCer) were also significantly increased in PE compared to PTC placental tissue indicating activation of that excess CERs may be originating from the de novo synthesis pathway (Figure 3.2a). Interestingly, CERs metabolites, sphingosine (SPH) and sphingomyelin 18
(SM18) levels were also significantly increased in PE placentae (Figure 3.2b). Because S1P is expressed at such minute levels within cells, MS/MS analysis could not detect levels of S1P in both PE and PTC placentae.

3.2 AC expression is decreased in PE.

Metabolism of CERs is tightly regulated by their rate of synthesis and breakdown and is exquisitely dependent upon the action of specific enzymes including AC and ASM. AC is the enzyme that hydrolyzes CERs into SPH and free fatty acyl-CoA. Accumulation of CERs in PE suggests that the machinery involved in CERs breakdown may potentially be altered, hence we first mRNA and protein examined expression of AC. Quantitative PCR analysis revealed that AC mRNA levels were significantly decreased in PE compared to PTC (PE: 0.613 ± 0.037 fold decrease vs PTC: 1 ± 0.189, p=0.0014) (Figure 3.3a). Western blot analysis identified the AC enzyme as a double band, which represents a 55kDa precursor protein synthesized in the ER that is then proteolytically processed into heterodimeric 53kDa active enzyme within lysosomes (26). In line with these mRNA data, protein expression of AC was also markedly decreased in PE compared to PTC and TC (Figure 3.3b). (PE: 0.434 ± 0.047 fold decrease vs PTC: 1 ± 0.088, p=0.0001; PE: 0.533 ± 0.083 fold decrease vs TC: 1 ± 0.131, p=0.0088). No changes in AC expression between PTC and TC were found (PTC vs TC: 1.12 fold increase, p=ns).

3.3 Hypoxia/oxidative stress decreases AC enzyme expression.

PE is a pathology associated with placental hypoxia and oxidative stress (101) (116). Likewise during early gestation, the placenta develops in a relatively hypoxic environment, which is followed by a dramatic rise in oxygen tension at 8-10 weeks when the intervillous space opens up to the maternal circulation. Though essential for embryonic development, changes in oxygen tension also generates oxidative stress, with
which the placenta has been shown to cope by establishing an effective antioxidant enzyme profile. Hence, in order to understand AC regulation throughout normal placental development and in response to hypoxia and oxidative stress we performed western blot analysis on placental tissues across gestation. Notably, hypoxia and oxidative stress are not mutually exclusive as intermittent or continuous hypoxia can lead to ROS production and oxidative stress. Western blot analysis showed a unique pattern of AC protein expression whereby AC levels were low during early gestation (5-8wks) followed by an increase at 10-16 weeks after which levels remain high to term (40 weeks) (Figure 3.4a). AC mRNA levels, measured by qPCR analysis follow a similar trend throughout placental development (Figure 3.4b). Interestingly, decreased AC expression levels found in PE correlate to low expression observed during early first trimester.

We next assessed the spatial localization of AC in first trimester human placental tissue by immunofluorescence staining. At 5 weeks of gestation, a positive cytoplasmic signal for AC was detected in the syncytiotrophoblast layer with very little expression in the cytotrophoblast layer and mesenchyme of the chorionic villi. Expression is also seen in the extravillous trophoblast cells in the proximal part of the anchoring column (Figure 3.5a).

To establish the mechanisms leading to the observed changes in the sphingolipid regulatory enzymes, we tested the effects of oxidative stress and hypoxia on AC expression to mimic a preeclamptic phenotype. We treated human villous explants from 6-8 week human placentas with SNP, a nitric oxide donor that also activates the Fenton reaction inducing a state of oxidative stress. Twenty-four hour exposure of villous explants with 2.5 and 5.0mM SNP resulted in a significant decrease in AC active enzyme expression when compared to untreated controls (SNP 2.5mM: 0.274 ± 0.065
fold decrease vs Control (veh): 1.19 ± 0.39, P=0.0369, SNP 5.0mM: 0.416 ± 0.105 vs. Control (veh): 1.19 ± 0.39, P=0.0369). As well exposure of explants to SNP resulted in accumulation of the inactive 55kDa form at the expense of the active enzyme (53kDa) (Figure 3.6a left).

Similar to SNP, AC expression was markedly decreased in villous explants cultured at 3% O₂ relative to 20% O₂ standard conditions, with a complete loss of the active enzyme form (53kDa) and to a lesser extent the precursor form (Figure 3.6a right). Our in vitro observations of reduced AC levels following exposure to 3% O₂ are in line with observation with the expression profile of AC in placental development when oxygen conditions are low.

Because hypoxia/oxidative stress caused similar changes in the regulatory enzymes to those observed in PE, we next assessed the sphingolipid profile in explants treated with SNP and/or maintained at 3% O₂. Sphingolipidomic analysis showed that SNP (5.0mM) lead to significant increases in C-16 and C-18 CERs compared to untreated control explants maintained at standard conditions. Furthermore, C-20, 22 and 24 CERs showed a trend towards an increase in response to SNP compared to untreated controls. Similarly, culturing explants at 3% O₂ also resulted in a trend towards an increase in CERs levels (C-16, 18, 22 and 24) (Figure 3.6b).

3.4 Hypoxia/oxidative stress increases CERs in JEG-3 cells.

We also examined AC expression and spatial localization in JEG3 choriocarcinoma cells. These human placental cells are easily manipulated and provide a suitable model for studying intracellular enzyme trafficking. Similar to explants, exposure of JEG3 cells to 5.0mM of SNP lead to significant increases in the various CERs (C14, 16, 18, 20 and 24) (Figure 3.7a). Interestingly, increased CERs levels were accompanied by a decrease in AC protein expression and SNP treatment induced
accumulation of the inactive 55kDa form to the account of the active enzyme (53kDa) (**Figure 3.7b**). Hence, decreased AC expression levels seen in our *in vitro* studies using hypoxia and oxidative stress follow a similar pattern of AC expression in PE.

Because AC is synthesized in the ER as a 55kDa precursor protein, which is then shuttled to the lysosomes where it is cleaved to become active, we were inclined to investigate whether the 55kDa band observed in response to oxidative stress was the result of an accumulation of the precursor in the ER. To assess the spatial localization of AC in response to oxidative stress, immunofluorescence analysis was carried out in JEG3 cells following treatment with 5.0mM SNP and immunostained for AC and calreticulin, an ER resident protein. In untreated control cells, positive AC immunoreactivity was restricted to the cytoplasm and the cell boundaries. Following treatment with SNP, AC localized with calreticulin, indicated by arrows. Thus, this data indicates that oxidative stress induced by SNP treatment causes an accumulation of this enzyme in the ER (**Figure 3.7c**).

Similar to SNP, hypoxia also exhibited detrimental effects on AC protein expression and subcellular localization. Culturing JEG3 cells in 3% O$_2$ caused a decrease in AC active enzyme levels (53kDa) and this was accompanied by an increase in the precursor form (55kDa) (**Figure 3.8a**). Immunofluorescence staining was used to establish the spatial distribution and thus the shuttling of AC between the ER and lysosomes in response to hypoxia. In control cells cultured in standard conditions, AC resides in the cytoplasm and cell boundaries. Additionally, AC positive immunoreactivity is also seen in the lysosomes as identified by lysotracker staining. However, following 24-hour incubation at 3% O$_2$, AC accumulates in the ER shown by increased colocalization of AC and calreticulin (yellow) and decreased expression is seen in the lysosomes (**Figure 3.8b**). Therefore, these data demonstrate that hypoxia
affects the shuttling of the AC enzyme from the ER leading to decreased active AC levels.

3.5 Glycosylation of AC is impaired in PE.

The six N-linked oligosaccharide chains on the AC enzyme have a profound influence on lysosomal trafficking and enzyme activation (26). Hence, we sought to examine the glycosylation status of this enzyme and if oxidative stress had an effect on ACs N-linked oligosaccharide chains. As shown above, explants treated with SNP exhibited a decrease in active AC expression. With the addition of a glycosylation inhibitor, tunicamycin, there was a build up of the 55kDa precursor form of AC, similar to that found with SNP, suggesting that deglycosylation prevents trafficking and subsequent cleavage of the AC enzyme (Figure 3.9a). Immunoprecipitation of AC from JEG3 cells treated with increasing concentrations of SNP followed by immunoblotting for Concanavalin A, a mannose binding lectin that binds specifically to N-linked oligosaccharide chains, displayed increases in concanavalin A, which is indicative of an increase in AC glycosylation (Figure 3.9b). Thus, oxidative stress may be leading to abnormal glycosylation of AC that, in turn, may affect the shuttling and bioactivity of this enzyme.

Glycosylation status of AC was further examined by immunoprecipitation from PE and PTC placenta tissue lysates followed by immunoblotting with concanavalin A. In contrast to what we found in JEG3 cells, Concanavalin A showed a reduction of glycosylation in PE compared to PTC tissue (Figure 3.9c). Tunicamycin, which inhibits N-linked glycoprotein synthesis, was used as a positive control and clearly shows a decrease in concanavalin A expression (Figure 3.9c). Using PNGase F, an amidase that cleaves oligosaccharide from N-linked glycoproteins, we found that AC is glycosylated in tissue from both PE and PTC placentae. Following deglycosylation, the
weight of AC is reduced to 28kDa as detected following PNGaseF treatment (Figure 3.9d). Hence, glycosylation of AC is decreased in PE.

3.6 AC contributes to autophagy in a model of PE.

Currently, there are no assays available to examine AC enzyme activity; hence we used an experimental approach where we employed a pharmacological inhibitor of AC enzyme activity, 2-oleoyl ethanolamine, to JEG3 cells. Decreased active AC levels would prevent the breakdown of CERs therefore mimicking a preeclamptic scenario. CERs act on several cellular processes involving cell death regulation such as apoptosis and autophagy (3). Autophagy is a mechanism to supply essential nutrients to a cell during starvation, however autophagy can also be utilized as a pathway to cell death (75). Following 2-OE treatment in JEG3 cells, we examined autophagy and apoptosis, both perturbed events typical of this pathology (106). Western blot analysis showed that treatment with 2-OE in JEG-3 cells for 6 and 24 hours revealed significant increases in LC3B-II following 6 hours of treatment with 2-OE (2-OE 100µM: 1.66 ± 0.17 fold increase vs Control (veh): 1 ± 0.18, P=0.042). Beclin1 is a Bcl-2-homology (BH)-3 domain only pro-autophagic protein, which is increased during periods of cell stress (117). Beclin1 protein expression was also significantly increased with 2-OE treatment (2-OE 100µM: 1.33 ± 0.16 fold increase vs Control (veh): 1 ± 0.077, P=0.042) (Figure 3.10a,b). Here, we also confirmed the role of CER in apoptosis by seeing an increase in cleaved caspase 3 (CCasp3) protein expression following 2-OE treatment for 6 hours. (Figure3.10a).

Since autophagy proceeds through fusion of autophagosomes with lysosomes where degradation of cytoplasmic constituents is completed, we analyzed lysosomal activity using lysotracker red staining, a dye taken up by lysosomes. Lysotracker staining increased in a concentration dependent manner with 25 and 100µM of 2-OE
(Figure 3.11a). Finally, using transmission electron microscopy (TEM) we examined the formation of autophagosomes, the gold standard for detecting autophagy. Figure 12 depicts transmission electron microscopic images of JEG-3 cells following 6 hour treatment with DMSO (control) or 2-OE (100µM). DMSO-treated control JEG-3 cells exhibited healthy looking cellular structures with normal appearing subcellular structures and few vesicles. Conversely, exposure to 2-OE for 6 hours resulted in appearance of double-membrane vacuoles resembling autophagosomes compared to little cytoplasmic vacuolization observed in vehicle treated controls (Identified by black arrows in Fig. 3.12). In addition, these autophagosome structures appear to be fusing with lysosomes, which then may form autolysosomes where cargo is recycled. Dense material was seen within cytoplasm of the autophagolysosomes (Figure 3.12a). Thus, inhibition of AC promotes an autophagic response associated with cell death.

3.7 Ceramide induces autophagy directly.

To determine the impact of CER itself on trophoblast cell fate, we treated JEG-3 cells and human villous explants with a synthetic C-16 CER, a specific CER known to induce cell death (72). C-16 CER caused a significant increase in LC3B-11 expression after 6 hours of treatment (C-16 50µM: 1.54 ± 0.22 fold increase vs Control (veh): 1 ± 0.028, p=0.039); however no response was seen at 24 hours (Figure 3.13a top). Interestingly, after 24 hours, we found an increase in CCasp3 expression suggesting an early induction of autophagy followed by a later occurrence of apoptosis at 24 hours (Figure 3.13a bottom). Increased lysosomal activity using lysotracker red staining was detected after 6 hours of C-16 CER treatment (Figure 3.13b). TEM imaging of JEG-3 cells treated with C-16 CER for 6 hours showed an astounding number vacuoles and the presence of autophagosomes. We also noted an increase in the number of lysosomes consistent with increased lysotracker staining (Figure 3.14a).
3.8 ASM expression is altered in PE.

SM is hydrolyzed into CERs and phosphocholin via the enzyme acid sphingomyelinase (ASM). Because sphingolipidomic analysis revealed increased SM levels in PE placentae compared to normotensive controls, we chose to assess ASM in PE. Furthermore, increased CER levels also leads to elevated SM through conversion via the enzyme sphingomyelin synthase (118). ASM mRNA levels did not change in PE relative to PTC (Figure 3.15a). Studies have reported that ASM is synthesized as a 75kDa propolypeptide in the ER. Processing by cleavage in the acidic compartments of the cell finally yields the prevalent, mature ASM 70kDa form (30). In PE, ASM showed an altered pattern of protein expression exhibiting the appearance of an additional, slightly heavier band that was much more predominant than in PTC (Figure 3.15b). Based on previous evidence, we speculated that this band could represent the ASM precursor protein of 75kDa.

ASM enzyme activity in normal and preeclamptic tissue using an enzyme coupled assay that measures ASM activity in biological samples through the direct hydrolysis of a fluorogenic substrate. Notably, we observed a significant decrease in ASM enzyme activity in PE as compared to PTC (PE: 0.133 ± 0.041 fold decrease vs PTC: 1 ± 0.413, P=0.041) (Figure 3.15c). Hence ASM protein processing and its activity are altered in placentae from PE pregnancies.

3.9 Glycosylation of ASM is affected in PE.

Like acid ceramidase, the enzyme ASM also contains 6 N-linked oligosaccharide chains, which have previously been determined to affect the activity, targeting and processing of this enzyme (31). To analyze the glycosylation status of ASM in the human placenta in physiological conditions and in PE, we immunoprecipitated ASM from PE and PTC placental lysates and by using a sugar-specific binding lectin, we
found that ASM in PTC placentae contained a higher amount of N-linked oligosaccharide chains detected by concanavalin A than in PE (Figure 3.16a). Furthermore, deglycosylation of PE placental lysates using PNGase F, an amidase that cleaves oligosaccharides from N-linked glycoproteins at the very base of the sugar, resulted in a greater decrease of ASM in PTC than in PE, suggesting that the glycosylation status is impaired in placentae from preeclamptic pregnancies (Figure 3.16b). Overall our data show that ASM N-glycosylation, which is essential for regulating it’s targeting to the lysosome and subsequent enzyme activation, is altered in PE. To determine whether the appearance of this heavier band was an accumulation of the precursor in the ER, we examined by immunofluorescence staining if ASM was associated with ER resident protein, calreticulin in placental tissue sections from PE and PTC. Strong positive immunoreactivity of ASM and dual labeling of ASM with calreticulin was also more evident in PE compared to PTC sections (Figure 3.18a).

3.10 Hypoxia/oxidative stress alters ASM in JEG-3 cells.

As the human placenta experiences physiological changes in oxygenation across the first trimester of gestation, to establish the role of oxygen on ASM expression and activity we first examined its expression throughout placental development. To determine the pattern of ASM expression throughout placental development, we performed western blot analysis on placental lysates across gestation. Similar to the AC enzyme, ASM expression is low in early first trimester and it increases with advancing gestation, (Figure 3.18a). Immunofluorescence staining revealed that ASM is more prominently expressed in the syncytiotrophoblast layer in early gestation (5-7wks) where it exhibited a punctate cytoplasmatic expression. Strong ASM positive immunoreactivity is also seen in the EVTs of the anchoring column. Moving distally through the anchoring column, expression decreases (Figure 3.18b).
As ASM expression is low during early gestation, a time of physiological low oxygen, we examined its regulation in first trimester placental explants cultured from 6-8 week human placentas exposed to 3% O$_2$, standard 20% O$_2$. ASM precursor protein (75kDa) levels were increased after 24 hour culture at 3% O$_2$, compared to 20% O$_2$, while the active form (72kDa) of the enzyme is decreased (Figure 3.19a). Next, we used SNP treatment to induce oxidative stress in villous explants. SNP treatment caused an overall increase in ASM protein expression and induced the expression of the slightly heavier 75kDa precursor band (Figure 3.19b). We also observed a significant decrease in ASM enzyme activity in explants treated with SNP 5.0mM (SNP 5.0mM: 0.634 ± 0.056 fold decrease vs control (veh): 1 ± 0.099, p=0.016) (Figure 3.19c).

In line with our in vitro observations using explants, we found that exposure of JEG-3 cells to SNP for 24 hours also resulted in the appearance of an additional band around 75kDa, much like what is observed in PE placentae (Figure 3.20a). Similarly, with 2.5mM SNP treatment, ASM enzyme activity in JEG3 cells showed a trend towards a decrease and at 5mM of SNP treatment, activity was significantly decreased (SNP 5.0mM: 0.202 ± 0.096 fold decrease vs Control (veh): 1 ± 0.257, P=0.011) (Figure 3.20b). Hence, both hypoxia and oxidative stress cause accumulation of ASM precursor protein and decrease ASM enzyme activity in villous explants and JEG3 cells.

Glycosylation of ASM in vitro was assessed in human villous explants. Explants treated with 5.0mM SNP showed an increase in ASM expression, however following addition of tunicamycin, ASM expression decreased (Figure 3.21a). Additionally immunoprecipitation of ASM from explants, followed by western blotting with ConA showed a decrease in ConA expression indicating SNP was reducing glycosylation of ASM (Figure 3.21b).

Next, it was important to determine the subcellular localization of CERS regulatory
enzyme, ASM following oxidative stress and low oxygen tension, two hallmarks of preeclampsia. ASM enzyme is synthesized as a precursor in the ER, which is then shuttled to the lysosome where it is processed into active enzymes (31). We assessed ASM localization in JEG-3 cells following treatment with SNP for 6 and 24 hours. To determine whether ASM localized to the lysosome, we subjected JEG-3 cells to Lysotracker staining, a specific lysosomal tracer dye, and assessed for ASM colocalization by IF. ASM is an acidic enzyme that resides in the lysosomes, and thus we observed colocalization of ASM and lysostracker in control untreated cells. Following SNP treatment, decreased colocalization of ASM with lysosomes was observed (Figure 3.22). ER resident protein, calreticulin was used to depict localization to the ER. Twenty-four hour exposure to SNP lead to increased colocalization of ASM with calreticulin compared to control untreated cells. Thus, oxidative stress, induced by SNP is halting this shuttling process of the ASM enzyme.
Figure 3. CERs levels in placental tissue from preeclamptic and normotensive control pregnancies

(a) Levels of CERs measured by MS/MS showed a significant increase in C16, C18, C20 and C24 CER in placenta and from preeclamptic women compared to age-matched controls from normotensive pregnancies. No differences were seen in C22 CER. Numbers indicated fatty acyl-CoA chain length. Statistical significance was determined as *p-value<0.01 using one-way ANOVA with post-hoc Newman-Keuls test (n=21 PE, n=18 PTC).
Levels of sphingolipids measured by MS/MS from PE and PTC placentae. (a) Cer DiHy24 and Sa levels, both intermediates in the *de novo* synthesis pathway of CERs, are significantly increased in placentae from preeclamptic women compared to normotensive controls. (b) PE placentae also demonstrated elevation of SM18 levels as well as SPH levels. Statistical significance was determined as *p*-value<0.01 using one-way ANOVA with post-hoc Newman-Keuls test (n=21 PE, n=18 PTC).
Figure 3. 3 AC in preeclampsia

(a) In PE placentae, AC mRNA are significantly decreased compared to PTC placentae as detected by qPCR and normalized to 18S (n=9 PE, n=10 PTC). (b) Tissue lysates analyzed by western blot analysis showed significant decreases in AC protein expression in PE compared to PTC placenta. No significant difference was seen between PTC and TC placentae. Densitometric analysis of AC expression normalized to actin and expressed as a fold change compared to PTC. Statistical significance was determined as *p-value<0.01 and **p-value<0.001 using one-way ANOVA with post-hoc Newman-Keuls test. (n=23 PE, n=15 PTC, n=12 TC).
Figure 3. 4 AC expression throughout gestation

(a) Immunoblot of AC protein expression throughout placental development. AC expression is low from 5-9 weeks gestation followed by an increase at 10-15 weeks and remained elevated through to term. Actin demonstrates equal protein loading. (b) Relative transcript levels of AC in placental development as detected by qPCR. AC mRNA levels also increased significantly at 10-15 weeks of gestation relative to 5-9 weeks followed by a further increase at term. Statistical significance was determined as *p-value<0.01 using one-way ANOVA with post-hoc Newman-Keuls test. (n=18)
Figure 3. Spatial localization of AC in 5 week placental sections

(a) Early on in gestation, AC localized to the cytoplasm of the ST layer and in the EVT cells within the anchoring column. AC (green), E-cadherin (red) and the nuclei counterstained with DAPI (blue). ST=syncytiotrophoblast layer; CT= cytotrophoblast cells; EVTs= extravillous trophoblast cells. (Magnification: 20X and 100X)
Figure 3. 6 Expression of AC in PE is reduced by hypoxia and oxidative stress

(a) Exposure of human villous explants to SNP (SNP: 2.5, 5.0mM) caused a significant decrease in active AC (53 kDa) protein levels while it increases the AC precursor protein (55 kDa) relative to control vehicle treatment. Exposure of human villous explants to 3% O₂ decreases AC compared to explants maintained in standard conditions (20% O₂). (n=3 experiments run in triplicates) (b) CERs levels in human villous explants measured by MS/MS were increased in response to SNP treatment and 3% oxygen conditions. Statistical significance was determined as *p-value<0.05 using one-way ANOVA with post-hoc Newman-Keuls test. (n=3 experiments run in triplicates)
Figure 3. 7 Effect of SNP on CERs levels and AC protein expression and subcellular localization in JEG3 cells

(a) Exposure of JEG3 cells to SNP significantly increased C14, 16, 18, 20, 22 and 24 CERs expression levels measured by MS/MS compared to control vehicle treated cells. (n=3 experiments run in triplicates) (b) Exposure of JEG3 cells to SNP caused a concentration dependent decrease in AC active protein levels relative to control vehicle-treated cells. (n=4 experiments run in duplicates) (c) Immunofluorescence staining of AC in JEG3 cells treated with or without SNP for 24h stained for AC (green), Calreticulin (red) and the nuclei counterstained with DAPI (blue). Cells exhibit signs of nuclear condensation. AC accumulates in the ER following SNP-induced oxidative stress. Magnification: 100X (n=3 experiments run in duplicates)
Figure 3. 8 Hypoxia leads to altered AC expression and subcellular localization

(a) Representative immunoblot of AC in cells maintained at 3% O₂ and 20% O₂ for 24hrs. Culturing JEG3 cells in 3% O₂ decreases active AC protein expression (53kDa), while increasing the precursor form (55kDa), compared to cells cultured in 20% O₂. (n=4) (b) Co-localization of AC and calreticulin/lysotracker in JEG-3 cells cultured at 3% and 20% O₂. At 20% O₂, AC expression is seen at the cell boundaries and in the lysosomes. Following hypoxia, AC localizes to the ER and is seen less in the lysosomes. AC (green), Calreticulin/lysotracker (red) and nuclei counterstained with DAPI (blue). Magnification: 100X
Figure 3. Glycosylation status of AC in placental explants and in PE
(a) Human villous explants treated with increasing concentrations of SNP followed by immunoprecipitation of AC. Western blotting with ConA shows increased glycosylation with SNP treatment. (b) Human villous explants treated with 5.0mM of SNP with the addition of tunicamycin. (c) Immunoprecipitation of AC from PE and PTC placenta followed by western blotting with sugar-specific binding lectin, ConA. AC glycosylation is reduced in PE as compared to PTC placenta. (d) PE and PTC placental lysates treated with PNGaseF. The deglycosylated protein (28kDa) of AC is indicated.
Figure 3. 10 Inhibition of AC with 2-OE treatment activates autophagy in JEG3 cells

(a) JEG-3 cells were cultured for 6 hours in the presence or absence of 2-OE (25µM or 100µM) or vehicle (DMSO) as indicated. Markers of autophagy, LC3B-II and Beclin-1 increased in JEG-3 cells treated with 2-OE. CCasp3 is also increased indicating apoptotic cell death. (b) Densitometric analysis of LC3B-II and Beclin-1 expression normalized to actin and expressed as a fold change compared to control. Statistical significance was determined as *p-value<0.05 using Kruskal-Wallis test with post-hoc Dunn’s multiple comparison test. (n=3 experiments run in duplicate)
Figure 3. 11 Inhibition of AC activity with 2-OE treatment activates autophagy in JEG3 cells

(a) JEG-3 cells were cultured for 6 hours in the absence or presence of an inhibitor of AC enzyme activity, 2-oleoylethanolamine (2-OE, 25µM or 100µM) or vehicle (DMSO). Lysosomal activity, indicated by lysotracker staining (red) is increased in 2-OE treated JEG-3 cells. Nuclei counterstained with DAPI (blue). Magnification: 20X and 100X. (n= 3 experiments run in duplicates)
Figure 3. 12 Inhibition of AC with 2-OE treatment activates autophagy in JEG3 cells

(a) JEG-3 cells treated with 100uM 2-OE were examined by transmission electron microscopy. Autophagy was indicated by increased number of lysosomes and formation of vacuoles depicting autophagosomes. Representative micrographs are shown and arrows indicate autophagosomes. N = nucleus. Scale bars, 2µM.
Figure 3. 13 C-16 CER directly induces autophagy in JEG3 cells

(a) JEG-3 cells were treated with C-16 CER (20µM or 50µM) or vehicle (100% ethanol diluted in EMEM media to a concentration of 50µM) as indicated. C-16 increased LC3B-II expression after 6 hours of treatment; no response was seen at 24 hours. After 24 hours, CCasp3 expression increased suggesting an early induction of autophagy followed by a later occurrence of apoptosis induced by C-16. (n=3) (b) Lysosomal activity, indicated by lysotracker staining (red) is increased in C-16 treated JEG-3 cells. Nuclei counterstained with DAPI (blue). Magnification: 20X and 100X.
Figure 3. 14 C-16 CER directly induces autophagy in JEG3 cells

(a) JEG-3 cells treated with C-16 or vehicle (100% ethanol diluted in EMEM media to a concentration of 50µM) and were examined by transmission electron microscopy. Representative micrographs are shown and arrows indicate autophagosomes. Lysosome accumulation was also seen in C-16 treated cells. N = nucleus. Scale bars, 2µM.
Figure 3. 15 Expression and activity of ASM is altered in PE placentae

(a) Relative mRNA levels of ASM in PE and PTC as detected by qPCR. Values are normalized to and do not change in PE compared to PTC placentae. (n=18 PE, n=18 PTC)

(b) Protein expression of ASM in PE is detected as a double band, which shows an ASM 70kDa active band and the precursor protein (75kDa) that is significantly increased in PE compared to PTC and TC placentae. Densitometric analysis of ASM precursor expression normalized to actin and expressed as a fold change compared to PTC (right panel).

(c) ASM enzyme activity is significantly decreased in PE as compared to PTC. Statistical significance was determined as *p-value<0.05 using one-way ANOVA with post-hoc Dunn’s multiple comparison test, n=18 PE, n=11 PTC.
Figure 3. 16 Glycosylation of ASM is deficient in PE

(a) Immunoprecipitation of ASM from PE placental lysates followed by western blotting using Concanavalin A showed a reduction of N-linked oligosaccharide chains in PE than in PTC. (N = negative IgG) (n=15 PE, n=12 PTC) (b) Deglycosylation of PE placental lysates using PNGase F resulted in a greater decrease of ASM in PTC than in PE, suggesting that ASM glycosylation in PE is impaired. (n=6)
Figure 3. 17 Spatial localization of ASM in PE

(a) Spatial localization of ASM and calreticulin in placental tissues from PE and PTC placentae. ASM positive immunoreactivity is increased in PE compared to PTC placentae. ASM also colocalizes with calreticulin to a greater extent in PE. ASM (green), calreticulin (red) and nuclei counterstained with DAPI (blue). Magnification: 20X and 100X (n=3)
Figure 3. 18 Expression and spatial localization of ASM in placental development

(a) Representative western blot analysis of ASM protein expression throughout pregnancy. ASM protein expression increased with advancing gestation. Actin demonstrates equal protein loading. (b) ASM is prominently expressed in ST in early gestation and in EVT cells within the proximal part of the anchoring column. E-cadherin staining identifies cytotrophoblast cells. ASM (green), E-cadherin (red) and the nuclei counterstained with DAPI (blue). ST=syncytiotrophoblast layer; CT= cytotrophoblast cells; EVTs= extravillous trophoblast cells. Magnification: 20X and 100X
Figure 3. 19 Hypoxia and oxidative stress affect ASM protein expression and activity in human villous explants

(a) Culturing human villous explants at 3% oxygen tension resulted in an increase in ASM precursor (75kDa) expression and a decrease in ASM active enzyme (70kDa) expression. (n=3 experiments run in duplicates) (b) Exposure of explants to SNP led to an accumulation of ASM 75kDa precursor and active 70kDa form compared to explants treated with vehicle. (n=3 experiments run in triplicates) (c) A significant reduction in ASM enzyme activity was also observed in explants treated with 5.0mM compared to control untreated explants. Expressed in Relative Fluorescence Units (RFU) (n=5). Statistical significance was determined as *p-value<0.02 using Mann-Whitney test or One-way ANOVA with post-hoc Dunnett’s multiple comparison test.
Figure 3. 20 Hypoxia and oxidative stress affect ASM protein expression and activity in JEG3 cells

(a) Exposure of JEG-3 cells to SNP caused an increase in ASM precursor protein levels relative to control, vehicle treated cells (n=3 experiments run in duplicates). (b) A significant reduction in ASM enzyme activity was also observed in cells treated with 5.0mM of SNP (n=3 experiments run in duplicates). Statistical significance was determined as *p-value<0.02 using One-way ANOVA with post-hoc Dunnett’s multiple comparison test.
Figure 3. 21 Glycosylation status of ASM is altered in response to oxidative stress

(a) Human villous explants cells treated with or without SNP for 24h. Addition of tunicamycin leads to a reduction of ASM expression. (b) Immunoprecipitation of ASM followed by western blotting for concanavalin A shows decreased glycosylation with increasing SNP treatment. Inhibitor of glycosylation, tunicamycin was used as a positive control. (C= untreated control, T= tunicamycin, N= negative IgG)
Figure 3. 22 Subcellular localization of ASM in JEG3 cells in response to SNP treatment

JEG3 cells treated with or without SNP for 6 and 24h. Cells were labeled with lysotracker, a dye taken up by lysosomes, or immunostained for calreticulin, an ER resident protein and both immunostained for ASM. (a) ASM accumulates in the ER following SNP-induced oxidative stress indicated by the co-localization of ASM and calreticulin (yellow). (b) ASM colocalizes with calreticulin following SNP treatment with little expression seen in the lysosomes. ASM (green), Calreticulin/lysotracker (red) and nuclei counterstained with DAPI (blue). Magnification: 100X (n=3 experiments run in duplicates)
Chapter 4

4 Discussion

Sphingolipids, classically considered strictly structural components of the cell membrane, have recently emerged as bioactive signaling molecules capable of activating or inhibiting downstream targets that mediate the fate of a cell in response to cellular stress. As CERs have evolved as key mediators of cell death, their generation or hydrolysis by the enzymes ASM and AC, are also of great importance in regulating cell fate. Herein, we examined sphingolipid metabolism in PE, a disorder of pregnancy where trophoblast differentiation and invasion are compromised by increased cell death. We found that disruption in AC and ASM in PE contribute to offsetting of the intricate balance between CER and its metabolites. As the contribution of excess CERs to increased trophoblast cell turnover has never been examined, we demonstrate for the first time alterations in the sphingolipid rheostat between placental development and PE.

Our data demonstrate that AC and ASM expression levels follow a unique pattern throughout placental development whereby expressions are lowest at <8-10 weeks when the placenta is developing in a relatively hypoxic environment. Another major finding of the present work was that we have shown a robust increase in the various CERs in PE placental tissue and sera. Our in vitro studies using human JEG3 choriocarcinoma cells and villous explants demonstrated that oxidative stress conditions lead to a sphingolipid profile exemplifying that of PE. Likewise, our in vitro data also indicated that hypoxia and SNP hindrance are a contributing cause of decreased AC levels and impairment of ASM glycosylation and activation leading to alterations of the rheostat between CERs and their metabolites. Additionally, we have shown that accumulation of CERs in PE upregulate autophagy, which may contribute to increased trophoblast cell turnover.
characteristic of this disorder (Figure 4.1b).

During placental development, syncytial membrane fragments are physiologically released from the apical syncytiotrophoblast layer into the maternal circulation (88). This turnover aids in continuously replenishing the syncytiotrophoblast layer to ensure its functionality. Interestingly, our data demonstrated that both AC and ASM localize predominantly to the ST layer, and may therefore be involved in the placental disposal system to remove aged apoptotic syncytial fragments. In line with this, AC and ASM are crucial enzymes regulating levels of death inducing CERs. Previous evidence has established a role for CERs in mediating apoptosis in trophoblast cells (119). Hence, it is tempting to speculate that dysregulation of the sphingolipid metabolic pathway leading to increased CERs levels, as observed in PE, may be a contributing factor increased apoptotic shedding of syncytial fragments.

Sphingolipid accumulation in a variety of cells and tissues forms the basis of many human diseases (3,22). Patients with cystic fibrosis have increased long-chain CERs (C16-C20) in lower airway epithelium and this was shown to increase susceptibility of bronchial epithelial cells to apoptosis (53). Additionally, intracellular generation and accumulation of CERs have been shown to decrease proliferation of prostate cancer cells whereas S1P upregulation plays a role in resistance of prostate cancer cells to chemotherapy (120). CERs are also important regulators of tumor cell death following exposure to stress stimuli such as hypoxia and nutrient deprivation (121). In line with this, increased levels of CERs are also known to be an established step in reactive oxygen species (ROS)-induced apoptosis (8). Previous studies using human blood neutrophils that accumulation of C-16 and C-24 CERs increases cell sensitivity to apoptosis. In agreement with these observations, C16 and C24 CERs were the most abundant in our PE placentae, which may contribute to increased levels of trophoblast
cell death seen in this disorder. Several lines of evidence support a role for placentally derived circulating factors in maternal endothelial dysfunction. Preliminary data have demonstrated that the profile of CERs in the placenta is similar to that of the sera of preeclamptic women. Both placenta and sera had increased levels of CERs; hence it is plausible that these CERs may be being shed from the placenta into the maternal circulation. Recently, elevation of vascular CER in spontaneously hypertensive rats have been shown to lead to vasoconstriction of vessels (60). Furthermore, humans with essential hypertension have increased plasma CER levels compared to normotensives (61). Interestingly, a striking difference we noticed in the sera was reduced S1P levels in preeclamptic women compared to normotensive controls. However, because S1P was immeasurable in the placenta, the origin of S1P is currently under investigation. Reduced S1P levels may also contribute to altered endothelial function seen in these women. In the endothelium, S1P is known to stimulate vasodilation through activation of endothelial nitric oxide synthase (eNOS) and release of the potent vasodilator NO (122). PE is associated with reduced circulating NO, which contributes to the increase in peripheral resistance seen in these women (124). Thus, increased CER and decreased S1P may contribute to reduced NO, increased vascular tone and endothelial dysfunction, a known contributor to hypertension observed in PE.

AC is a lipid hydrolase that directly regulates CERs metabolism by catalyzing its degradation into SPH and fatty acyl-CoA (26). Herein, we demonstrated that AC mRNA and protein expression is decreased in PE placentae. Previous evidence has demonstrated that AC is essential for mammalian development, as the generation of AC knockout mice resulted in embryonic lethality in homozygotes. Knockouts experience increased pools of CERs leading to enhanced cell death (49). At the transcriptional level, AC is regulated by a variety of transcription factors including cAMP-responsive
element-binding protein (CREB), which is involved in regulating a variety of trophoblast genes, however little is known about the effect of hypoxia or oxidative stress on AC gene expression (127). Imposing an environment of hypoxia/oxidative stress using SNP in JEG3 cells and human villous explants caused similar effects on CERs and its regulatory enzyme, AC, to those observed in PE. Intriguingly, AC expression levels were also decreased in early gestation (5-8wks) when oxygen tension is physiologically low. This was followed by an increase in expression at 10-16 weeks, the time when oxygen tension rises in placental development. Thus, we provide evidence for the first time that hypoxia/oxidative stress may have a detrimental effect on AC, leading to the decreased expression contributing to increased levels of CERs.

Another important facet of this work is that we established a critical role for CERs in autophagy, an alternative cell death pathway previously shown by our laboratory to be upregulated in PE (Kalkat et al. 2012, Submitted to CDD). CERs have been shown to cause activation of the c-Jun N-terminal kinase (JNK1) pathway, thereby controlling dissociation of the Beclin 1:Bcl-2 complex, allowing Beclin1 to perform its role in the initiation of the autophagic response (128). This is consistent with our finding showing that exposure of JEG3 cells to C-16 CER increases autophagy. Interestingly, inhibition of the AC enzyme, using 2-OE, also lead to activation of autophagy suggesting that the CERs responsible for induction of autophagy come from lack of degradation by AC. Hence, CERs have both a indirect and direct effect on autophagy.

Autophagy can be a stress adaptation to avoid cell death, however, excessive autophagy can bring to cellular demise (75). We saw an increase in autophagic markers at 6 hours of C-16 treatment, hence we speculate that C-16-induced autophagy in cells may increase in an attempt to rapidly metabolize CERs accumulation. Previous studies have shown that accumulation of CERs, DHCers and SPH in the autophagolysosome
can cause membrane destabilization resulting in the release of cathepsins and cytotoxic factors leading to cleavage and activation of caspase 3 and subsequent apoptosis (3). In line with this, following 24-hour treatment with C-16, we saw activation of caspase 3, which may be indicative of the progression of autophagy into apoptotic cell death. Thus, CER may play the role of a gauge that senses the level of cell injury and depending on downstream factors, determines specific biological outcome. Autophagy observed in PE placentae may be a defense mechanism to recycle damaged organelles and aid the cells in the harsh oxidative stress environment, however, there is a fine line between protection and death.

Our present observation of elevated CERs levels was accompanied by a significant increase in SM18 levels, a species of sphingolipids from which CERs are derived through the action of ASM (118). Previous evidence has shown parallel accumulation of CER and SM as a result of various stressors (LPS, heat shock) (3). Likewise, Wharton’s jelly, the gelatinous connective tissue that surrounds the umbilical cord, from preeclamptic mothers, shows increases in SM, CERs, SPH and Sa. This altered sphingolipid content has been linked to remodeling of the jelly composition contributing to altered umbilical cord compression and twisting (129). Also, studies report that SM is a key component in the membranes of the cells composing the stem villi of the placenta (109). SM drives the formation of raft domains, which play important roles in protein sorting and signaling. Therefore, disrupting SM levels may compromise placental functions of villous membranes, including gas exchange (3).

Following synthesis, AC and ASM undergo extensive post-translational modification during their transport to the lysosomes. The enzymatically inactive precursor (75kDa) of ASM contains six N-linked oligosaccharide chains and is rapidly converted to a predominant 70kDa glycoenzyme active form after transport to acidic
organelles (31). Removal of an N-terminal segment consisting of 36 amino acids is thought to be the proteolytic processing event (130). Likewise, AC is first synthesized as a 55kDa precursor protein that is then proteolytically processed into heterodimeric 53kDa active enzyme within lysosomes. For both enzymes, the 6 N-glycosylation sites are essential for the processing, trafficking and activity of these enzymes. Previous studies using site directed mutagenesis have shown that removal of N-glycosylation leads to impairment of lysosomal targeting and maturation of these enzymes and almost completely abolishes AC and ASM activity (26,31). Herein we demonstrated that glycosylation of both AC and ASM is decreased in PE as compared to PTC placentae. Additionally, in PE, the precursor form of ASM increases, suggesting failure of this enzyme to undergo maturation and shuttling to the lysosome where it is activated and required for break down of SM into CER, and thus we see an accumulation of SM. Hence, reduced glycosylation of the ASM precursor may lead to accumulation in the ER while preventing shuttling to the lysosome (Figure 4.1a). Hypoxia has been previously shown to affect the enzymes involved in N-linked oligosaccharide synthesis. These proteins may therefore be mis-folded and retained in the ER preventing post-translational modifications required for enzyme activity. Our data suggests that SNP-induced oxidative stress may be contributing to the build up of the precursor form by disrupting glycosylation. Likewise, in cells treated with increasing SNP concentrations, we see the formation of an ASM double band pattern and colocalization with the ER, much like that seen in PE. Furthermore, specific mannose residues present on N-linked oligosaccharide chains of ASM is required to allow binding of mannose-6-phosphate receptors and subsequent targeting to the lysosome (31). Improper N-glicosylation leading to decreased M6P residues prevents correct targeting of lysosomal proteins, and instead these proteins are retained in the ER. In line with this, Guibourdenche et al. have
previously shown that oxidative stress seen in trophoblasts of trisomy 21 pregnancies may be associated with modification of glycosylation of proteins, leading to a lower bioactivity (131). Additionally, the compound SNP while inducing oxidative stress is degraded into cyanide, ferrous ion and NO. Nitric oxide is a potent vasodilator with dual actions on venous and arterial smooth muscle causing decreased systemic vascular resistance and increased cardiac output (123). By directly targeting the contractile machinery in cells, NO reduces calcium release leading to inactivation of myosin and reduced myosin-based motility (125). The ability of NO to halt myosin motility may help to explain the SNP-induced inhibition of AC/ASM transport between cellular organelles we observed. Also, cyanide toxicity can induce deleterious effects on cells and may also contribute to altered sphingolipid regulation in response to SNP (126).

Abundant evidence indicates that ER-stressors and the unfolded protein response (UPR) are potent triggers of autophagy (132). When mis-folded proteins accumulate in the ER a variety of stress signaling cascades are initiated that can contribute to the formation of autophagosomes (133). Overall, there may be a link between sphingolipid homeostasis, ER homeostasis and the induction of autophagy (Figure).

ASM is a key enzyme in generating CER in response to cellular stress (111). Active ASM protein and mRNA levels do not change in PE, however, it has been previously reported in cells that ASM enzyme activity is triggered by hypoxic conditions and thus also contributing to CER generation and the apoptotic response (24). In contrast, we see accumulation of SM in PE and a down regulation of ASM enzyme activity. One possible explanation may be that the harsh environment of PE and chronic oxidative stress insult forces the system to advance to an adaptive state and decrease ASM enzyme activity in attempt to decrease the excess CER levels.

Two pathways of CERs formation include the de novo synthesis pathway and SM
hydrolysis both of which are important in mediating apoptotic effects (6). Our data infer that the excess CER in PE was not coming from SM and instead implicate a participation of de novo generated CERs. We found that DHCers and Sa, both intermediates in the de novo pathway were upregulated in PE (Figure 4.1b). In line with this, the various enzymes involved in the de novo synthesis of CERs are in the ER and known to play a prominent role in cellular response to stress and induction of apoptosis (66). In accordance, the salvage pathway involves the recycling of SPH whereby complex sphingolipids, mainly SM, are degraded in the lysosome to form CERs and subsequently SPH. This pathway is regulated by oxidative stress and generates specifically long-chain CERs, C16 and C24 (25). This supports our findings of increased SPH levels in association with CERs and SM.

To conclude, our data suggests that in preeclamptic placentae altered AC and ASM expression and activity is likely induced by the oxidative stress status, favours an increase in CERs, which may contribute to increased cell death typical of this disorder (Figure 4.1b). Our results support the idea that the rheostat between CER and its metabolites governs cell fate and both hypoxia and oxidative stress impinge on sphingolipid homeostasis. Sphingolipid metabolism extends its relevance to many complex pathophysiological processes, thus further analysis will elucidate how processes as relevant as apoptosis and autophagy are controlled. These enzymes also provide novel approaches to therapeutic strategies that aim to influence sphingolipid-mediated physiologic responses, such as cell death, that are disrupted in PE.
Figure 4.1

Oxidative Stress / ↓ pO2

ER

↓ Activation

↓ Shutting

Lysosomes

ASM AC Precursor

ER STRESS ?

↑ Trophoblast Cell death/Autophagy

De novo synthesis

↑ Sa

↑ DHCer

↑ ASM Activity

↑ SM

↑ ? CerS

↑ AC

? CerS

S1P

SPH

CERs

Salvage

↑ Trophoblast Cell death/Autophagy

Figure 4.1
Figure 4.1  Putative model of AC and ASM processing and sphingolipid profile in preeclampsia

(a) In PE, the hypoxic/oxidative stress status impairs AC and ASM enzyme glycosylation and this may contribute to failure of enzyme shuttling, accumulation in the ER and lack of activation. Accumulation of improperly glycosylated enzyme precursors in the ER may lead to ER stress. (b) CERs levels are significantly increased in placentae from preeclamptic pregnancies and this is accompanied by a significant decrease in AC enzyme expression. ASM enzyme activity was decreased which may account for increased SM levels. Additionally, intermediates of the de novo synthesis pathway, Sa and DHCer were also increased suggesting that excess CERs are being generated by this pathway and not coming from conversion of SM. Excess SM may, in turn be recycled through the salvage pathway generating excess SPH, also observed in PE. Sphingosine can then be re-converted into CERs by the enzyme CerS contributing to increased CERs levels in PE.
Chapter 5

5 Future Directions

5.1 Does the sphingolipid metabolism profile change throughout placental development?

Our data on CERs regulatory enzymes during pregnancy indicate that both AC and ASM have unique patterns of expression throughout placental development. During early gestation (5-8wks), low expression levels of AC and ASM were followed by an increase at 10-14 weeks after which remained high through gestation. Using sphingolipidomic analysis (MS/MS), it would be interesting to evaluate levels of CERs and its metabolites throughout placental development to see if the sphingolipid profile follows the expression of lipid regulatory enzymes. The placenta experiences a rapid increase in oxygenation at 8-10 weeks which is accompanied by a burst of cytokines and ROS (105), both of which are known to affect levels of CERs. Development of the human placenta requires strict regulation of cell fate to control structural and functional changes. Because sphingolipids are key mediators of cell fate decisions, they may play an important role in directing trophoblast cell death and proliferation. When AC increases during this change in oxygen tension, CERs levels should decrease perhaps reflecting maturation of the various cell layers within the placenta. In addition, we would also like to evaluate the spatial localization of CER and its regulatory enzymes. S1P has previously been shown to be involved in the inhibition of differentiation of CTs into STs (108). Additionally, epithelial growth factor (EGF), which plays an important role in trophoblast cell growth regulation and differentiation, has been found to lower CERs levels in trophoblasts (119). However, the complete sphingolipid profile and its
physiological relevance with respect of trophoblast differentiation events remain to be established.

5.2 From which sphingolipid metabolic pathway do CERs originate in PE?

The most profound observation reported in this thesis is the robust increase in CERs in PE placentae. Interestingly, levels of SM18 were also increased and ASM activity decreased implying that CERs are not coming from SM breakdown. We also observed increased Sa and DHCers, both intermediates involved in the de novo synthesis of CERs, which suggests that excess CERs in PE are coming from the de novo synthesis pathway. We have recently begun analysis on other sphingolipid metabolites in JEG3 cells and human villous explants. Examination of the enzymes involved in the de novo synthesis of CERs would help establish if this pathway is upregulated in PE. It would also be of interest to establish if the oxidative stress status of PE plays a role in CERs synthesis. In line with our data, previous evidence has indicated that oxidative stress is capable of increasing cellular CERs levels via stimulation of the de novo synthetic pathway. CERs generated via this pathway play a role in ROS-induced apoptosis (34). Selective inhibitors of the de novo synthesis pathway, such as Fumonisin B1, a known inhibitor of the CerS enzyme, would aid in establishing how this pathway is upregulated. If oxidative stress is having an effect on de novo synthesis of CERs, by culturing cells or explants in the presence of SNP and Fumonisin B1 should result in decreased CERs levels. Furthermore, it would be of great value to investigate if inhibiting de novo synthesis prevented CERs downstream effects including cell death and autophagy.

5.3 Where are CERs spatially localized in PE placentae?

Preliminary data showed increases of CERs in the sera from preeclamptic women. It would therefore be of interest to define the spatial localization of CERs in placentae from PE women relative to normotensive controls. Analysis of the spatial
distribution of sphingolipids in organs is difficult due to the lack of anti-sphingolipid antibodies. Recently, an anti-ceramide antibody has been made commercially available which could be used in immunofluorescent staining to identify where CERs are expressed in preeclamptic placenta sections relative to normotensive controls. Fusion of CTs into STs is followed by turnover and accumulation of condensed nuclei into syncytial knots, which are extruded into the maternal circulation (86). It is tempting to speculate that CERs are involved in accelerating trophoblast turnover and involved in the progression of syncytial knots since CERs are prominent bioactive mediators of cell death (4). Furthermore, PE is characterized by increased release of syncytial knots, a phenomenon that supports the idea that placental debris is contributing to increased CERs levels in the sera of preeclamptic women (134). However, it is also plausible that activated maternal leukocytes may also contribute to increase CERs in the sera.

5.4 Is AC gene regulation altered in PE?

Although the work presented in this thesis demonstrates reduced AC mRNA and protein levels in PE placentae, the precise mechanism underlying AC reduction requires further investigation. Gene regulation can be achieved at a genetic or epigenetic level. Previous studies have shown that the AC promoter-enhancer region has a Kruppel-like Factor 6 (KLF6) binding motif (135). KLF6 is a transcription factor that functions as a tumor suppressor gene and is mutated in prostate cancer, which leads to an up-regulation of AC. Similarly, our laboratory is interested in performing further in silico analysis on the AC promoter to establish if there are regulatory sites such as a hypoxia response element (HRE) or putative SMAD-binding elements, which is a binding site for TGF-β. It would be interesting to follow up this analysis with promoter bashing, whereby we would mutate the regulatory elements followed by measurement of transcription. Additionally, we are also inclined to examine epigenetics, which are modifications to
chromatin structure or DNA induced mostly by the environment. Previous studies have described epigenetic rearrangements across gestation as a critical factor for placental development and function (136). Epigenetic marks including DNA methylation and acetylation result in gene alterations that play an active role placental physiology. Moreover, disturbances in placental epigenetics leading to aberrant gene expression have been reported to be involved in the pathogenesis of PE (137). PE is characterized by hypoxia and oxidative stress, both states known to lead to epigenetic alterations including hypermethylation (138). Additionally, reduced oxygenation has been shown to alter placental gene expression in PE (101). Thus, it would be of interest to evaluate whether the AC gene is epigenetically altered in PE and if hypoxia/oxidative stress has an effect on this regulation.

5.5 Are there differences in sphingolipid metabolism of IUGR compared to PE placentae?

IUGR is defined as fetal birth weight below the tenth percentile for gestational age (139). PE is often complicated by IUGR, however IUGR can manifest in the absence of preeclamptic symptoms. These two pathologies are similar with regards to placental insufficiency due to defects in trophoblast differentiation along with excessive trophoblast proliferation and death (140). However, it’s important to note that although characteristically similar in structural and cellular abnormalities, each has a unique molecular signature (141). In IUGR, there is a decrease in exchange surface area that prevents the placenta from providing sufficient nutrients, thus the fetus fails to achieve its growth potential (98). Impaired fetal growth has long-term clinical consequences and emerging evidence suggests that low birth weight is linked to increased risk of developing adult diseases such as type 2 diabetes, cardiovascular disease and hypertension (142).
This prompts investigation of the expression of CERs, its metabolites, and regulatory enzymes in IUGR as compared sphingolipid profiles observed in PE. Preliminary data from our lab using MS/MS, has demonstrated in contrast to preeclampsia, levels of CERs were decreased in IUGR placentae further underscoring the differences in perturbed sphingolipid metabolism in placental pathologies (Figure 5.1a). To corroborate this finding we have also shown by western blot analysis, a trend towards an increase in AC enzyme levels (Figure 5.1b). This data opens many avenues for investigation into the roles of sphingolipid metabolism in regulating trophoblast cell fate in the pathogenesis of IUGR. Opposing sphingolipid profiles seen in IUGR versus PE may suggest differential regulatory mechanisms and associated downstream cellular events by which death and proliferation changes in these pathologies.

5.6 Do CERs play a role in trophoblast cellular senescence in IUGR or PE?

Studies have previously shown that sphingolipids, specifically CERs, have been implicated in senescence (143). Senescence is functionally defined as irreversible growth arrest with increased levels of cell cycle inhibitory proteins, such as p21. It is also characterized by shortened telomeres, which are tandem DNA repeats that cap the ends of chromosomes (144). Telomerase directs telomere length by adding on telomeric repeats to the ends of chromosomes. Decreased telomerase prevents maintenance of telomeres causing progressive shortening resulting in growth arrest and senescence. Telomerase is activated in 80-85% of cancers and highly correlated with proliferation(145). It has been shown that in senescent fibroblasts, conversion of CERs to sphingomyelin is reduced, while the reverse pathway is increased (143). Exogenous CERs result in the inhibition of telomerase activity, thus inducing senescence in cancer fibroblasts. Recent studies suggest that stress induced senescence may lead to impaired
tissue function (146). Hence, it is of interest to establish the impact of CERs on senescence in preeclamptic and IUGR placentae. Preliminary data demonstrated an increase in telomerase expression in IUGR placentae suggesting an anti-senescence state (Figure 5.2a). This data nicely corroborates the decreased levels of CERs observed in IUGR. Because hypoxia is a central feature of IUGR (147), we also tested the effect of hypoxia on telomerase expression in JEG3 choriocarcinoma cells. Preliminary data shows increases in telomerase after being cultured in hypoxic conditions suggesting that hypoxia may play a role in the regulation of this enzyme (Figure 5.2b). This data warrants further investigation behind the contribution of increased telomerase and its contribution to aberrant levels of proliferation in IUGR.
Figure 5.1 CERs levels and expression of AC in IUGR

(a) CER levels measured by MS/MS showed a significant decrease in C18, C20 and C24 CERs in IUGR placentae compared to PTC. (b) Placental tissue lysates analyzed for protein expression of AC showed significant decreases in PE compared to PTC.
Figure 5. 2 Telomerase expression in IUGR and hypoxic conditions

(a) Placental tissue lysates analyzed for protein expression of telomerase showed significant increases in IUGR compared to PTC. (b) Telomerase expression increased in JEG3 cells cultured at 3% O₂ over 48 hours compared to cells cultured in 20% O₂.
References


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