8-14-2015 12:00 AM

Characterization of the nicotine-induced endoplasmic reticulum stress response in the rat placenta in vivo and in vitro

Michael Ka Chun Wong
The University of Western Ontario

Supervisor
Dr. Daniel B. Hardy
The University of Western Ontario

Graduate Program in Physiology and Pharmacology
A thesis submitted in partial fulfillment of the requirements for the degree in Master of Science

CHARACTERIZATION OF THE NICOTINE-INDUCED ENDOPLASMIC RETICULUM STRESS RESPONSE IN THE RAT PLACENTA *IN VIVO* AND *IN VITRO*

(Thesis format: Integrated Article)

by

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The School of Graduate and Postdoctoral Studies
The University of Western Ontario
London, Ontario, Canada

Abstract

Nicotine exposure during pregnancy leads to adverse health outcomes, including compromised placental development. Although the molecular mechanisms remain elusive, recent studies identified that endoplasmic reticulum (ER) stress may underlie poor placentation. Therefore, we were interested in investigating the effects of nicotine exposure on the ER stress response in the placenta. A well-established maternal nicotine exposure rat model and Rcho-1 trophoblast giant cell model were utilized to address the research questions. Maternal nicotine exposure *in vivo* led to elevated ER stress in association with impaired disulfide bond formation and hypoxia. Nicotine exposure *in vitro* further differentiated that ER stress may be augmented directly through nicotinic acetylcholine receptor activation and indirectly through nicotine-induced hypoxia. Moreover, we relieved nicotine-induced ER stress in Rcho-1 cells *in vitro* using Tauroursodeoxycholic acid. In conclusion, this thesis provides novel mechanistic insight and contributes to the development of innovative therapeutic approaches to ameliorate nicotine-induced injury in pregnancy.

Keywords

Nicotine, NRT, cigarette, smoking, e-cigarette, pregnancy, maternal, placenta, endoplasmic reticulum stress, ER stress, unfolded protein response, UPR, PERK, disulfide bond formation, Tauroursodeoxycholic acid, TUDCA, hypoxia, rat, Rcho-1, trophoblast giant cell

Co-Authorship Statement

Michael K. Wong primarily conducted all the experimental work, study designs, data analyses, and interpretations of results found in the following thesis.

Catherine J. Nicholson and Dr. Alison C. Holloway contributed to animal care and drug administration at McMaster University, Hamilton, Ontario. Dr. Alison C. Holloway further contributed to editing the written content of this thesis.

Dr. Daniel B. Hardy contributed to study design, interpretations of results, supplying of necessary materials, and editing the written content of this thesis.

Nicole Edwards, Dr. Patrick Swan, Dr. Nicole Barra, Dr. John Ciriello, and Dr. Andy Babwah contributed to editing the written content of this thesis.

This thesis is dedicated to my loving parents, **Donald and Violet Wong**.

Thank you for your willingness to give me everything,
So that I could grow up with the freedom to pursue anything.

Acknowledgments

First and foremost, I must offer my sincerest gratitude for my supervisor, Dr. Daniel B. Hardy. Your patience throughout the *many* expensive mistakes I made truly helped build my confidence in my own work. Your sympathy and understanding during my more pre-occupied times (a.k.a. wedding planning) will always be remembered. Ultimately, what I remain most appreciative of is the trust that you placed in me when first accepting me first as a volunteer, then as a 4th year thesis student, and finally as a Masters student – had you not welcomed me into your lab, I may never have developed my passion for research. I hope to make you proud with the scientific questions I continue ask in the next stages of my career. Your additional loves for wrestling, Machete, and your family of Lisa and Mark revealed to me that scientists can, and must, enjoy life outside the laboratory. Your regular meets with your own father remind me that we cannot forget those who were most important to us despite becoming busier. I hope that age will not restrict your growth as you continue to advance in this life.

A huge thanks goes out to the rest of the DDT(AB) lab: Peter Vo – Thanks for teaching and befriending me when I first joined the lab. Witnessing your tenacious pursuit for medical school was inspiring and I know you will make a great doctor someday. Andrew Revesz – Everyone hates training undergrads, but you were more than patient and willing to help me out. Though life has not been easy on you, I hope that you may continue to find the rare joys that it offers. Gurjeev Sohi –In hindsight, I have really come to admire your hard work and the tough decisions you had to make. Best of luck with your future plans. Noelle Ma – Thanks for taking the initial pilot project to publication. Elaine Chan – Thank you for putting in the good word for me at the beginning and getting me into the lab, and also for our many years of friendship! To the newest Hardy lab member, Nicole Barra – you are one of the kindest individuals I have ever had the pleasure to meet. I hope your early research career (and marriage!) flourishes richly and takes you where you wish to go. Dr. Lin Zhao – an enormous thanks to the most talented, knowledgeable, and conversationally-controversial lab technician I will probably ever meet. Best wishes for you and your family, and may you find the answers that you seek in life. Jacky Chiu, Ian Tobias, Nicole Edwards, Andrew Ghaly, Master Elias, Joohwan Kim, Kristyn Dunlop, and all others – Thank you for the many good years of friendship, conversation, and long hours spent together at the lab. I wish the best for each and every one of you in whatever path you choose to take.

To my advisory committee, Drs. John Ciriello, Rommel Tirona, and Andy Babwah – Thank you for your guidance and support during the many hours we have spent together discussing my project. Dr. Patrick Swan – Thank you so much for sharing all your knowledge (and antibodies) with me. Congratulations on your recent accomplishment and I wish the best for you in the next step of your career.

To my mother and father, Violet and Donald Wong – Thank you for your many years of unconditional love. You have always supported me wherever I wandered to, and continue to do so. More than anyone else, you have taught me the value of working hard for the sake of your family. To my only sister, Nicole – I wish nothing but the best for you in the next big steps of your life. I know I have been tough on you at times, but know that I love you very much and that it brings me great joy to see you begin to take your own steps in this life. To my in-laws, James, Wendy, Valerie, and Nolan – Thank you for always supporting me in whatever wild endeavor I shared with you over the dinner table. You taught me that tactful encouragement will always contribute more to growth than careless criticism.

And finally, to my beautiful wife, Tiffany – After over 10 years of friendship and more, you still chose to stand next to me in marriage. And now, you share with me in the completion of this milestone as my better half. I know that I have not always been the most caring or patient husband, especially in times of frustration during graduate school. Yet, you still joyfully supported me the best that you knew how, and more than that, you encouraged me to pursue what I love. May whatever next steps we take, in research and in life, we take together.

"For he endured as seeing him who is invisible."

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List of Abbreviations

ATF4	Activating Transcription Factor 4
ATF6	Activating Transcription Factor 6
ATP	Adenosine Triphosphate
BAK	BCL2-Antagonist/Killer
BAX	BCL2-Associated X protein
BCL2	B-cell CLL/lymphoma 2
BH3	BCL2-homology 3
BIM	BCL2-Interacting Mediator of cell death
BiP/GRP78	Glucose-Regulated Protein 78
ChIP	Chromatic Immunoprecipitation
CHOP	C/EBP-homologous protein
CYP	Cytochrome P450
eIF2	Eukaryotic Initiation Factor 2
ER	Endoplasmic Reticulum
ERAD	Endoplasmic Reticulum-Associated Degradation
ERO	ER oxidoreductase
DAI	Double-stranded RNA-Activated Inhibitor
FRAP	Fluorescence Recovery after Photobleaching
GCN2	General Control Nonderepressible 2
GFP	Green Fluorescence Protein
GLUT1	Glucose Transporter 1
GTP	Guanosine Triphosphate
HAND1	Heart and Neural Crest Derivatives Expressed 1
HSP	Heat Shock Protein
HRI	Heme-Regulated Inhibitor
IRE1	Inositol-Requiring Enzyme 1
IUGR	Intrauterine Growth Restriction
nAChR	Nicotinic Acetylcholine Receptor
NOXA	Phorbol-12-myristate-13-acetate-induced protein 1
NRT	Nicotine Replacement Therapy
PBA	4-Phenyl Butyric Acid

PDI	Protein Disulfide Isomerase
PERK	Protein kinase-like Endoplasmic Reticulum Kinase
PGF	Placental Growth Factor
Pl-1	Placental Lactogen 1
PKR	Protein Kinase RNA
PUMA	p53-Upregulated Modulator of Apoptosis
RIDD	Regulated IRE1-Dependent Decay
ROS	Reactive Oxygen Species
SERCA	Sarco/Endoplasmic Reticulum Calcium-ATPase
TG	Trophoblast Giant
TUDCA	Tauroursodeoxycholic Acid
UPR	Unfolded Protein Response
VEGF	Vascular Endothelial Growth Factor
XBP1	X-box Binding Protein 1

1 LITERATURE REVIEW

Excerpts of this chapter have been submitted for publication and are currently in revision: **Wong MK, Barra NG, Alfaidy NN, Hardy DB, and Holloway AC.** Adverse effects of perinatal nicotine exposure on reproductive outcomes. *Reproduction*, in revision.

1.1 Nicotine

1.1.1 Prevalence and concerns regarding nicotine exposure during pregnancy

It is well established that cigarette smoke contains many teratogens and exposure during pregnancy increases the risk of adverse fetal developmental outcomes, such as placental abruption [1], placenta previa [2], sudden infant death syndrome [3], spontaneous abortion [4], stillbirth [5], low birth weight [6], and fetal growth restriction [7]. Despite increased awareness of these detrimental effects, approximately 10-28% of women continue to smoke during pregnancy worldwide [8-11]. Canada is no exception, reporting an average prevalence of smoking during pregnancy between 10.5-23%, with rates as high as 59.3% in some communities in the Northern Territories [8, 11]. Amongst those who attempt to quit smoking, it is suggested that only half will successfully abstain; the steep rates of relapse attributable in part to the highly addictive nature of nicotine [9, 12, 13]. However, maternal exposure to nicotine during pregnancy is not only restricted to cigarette smoking. Nicotine-based pharmacotherapies, such as nicotine replacement therapy (NRT), have been developed for smoking cessation and its usage has been considered beneficial for those struggling with heavy dependence [14]. NRTs are most commonly found in the form of chewing gums, transdermal patches, inhalers, and nasal sprays. The US FDA has currently assigned NRT gums under pregnancy category C (adverse effects seen in fetus in animal studies, but inconclusive evidence in humans; potential benefits still outweigh risks) and NRT transdermal patches, inhalers, and sprays are under pregnancy category D (adverse effects seen in human studies, but potential benefits still outweigh risks) [15]. Non-combustible, nicotine-containing smoking alternatives, such as e-cigarettes, have also garnered increased popularity within recent years especially among adults of reproductive age, regardless of history of cigarette use [16, 17]. E-cigarettes are perceived to be safer than cigarettes in that they utilize flavoured vapour and nicotine [18], but depending on the user's experience and puff intervals, nicotine absorption can be similar to that of conventional cigarettes [19-21]. It has generally been assumed that the benefits of NRT/e-cigarette use during pregnancy outweigh the risks, as users are able to avoid the thousands of other chemicals found in cigarette smoke [22, 23]; however, there is currently

insufficient data to verify the safety and efficacy claims of these nicotine-based products during pregnancy [24-26].

Nonetheless, the usage of these nicotine-based products are still considered to be most strongly influenced by clinical "approval from their healthcare professional" [27]. Alarmingly, 47% of obstetricians/gynecologists stated in a recent study to inconsistently screen their pregnant patients for exposure to these non-combustible tobacco products, and only 5% felt fully informed of the potential side-effects [28]. Furthermore, 43% of pregnant women reported to not even know that e-cigarettes may contain nicotine [29]. Considering that research is increasingly suggesting that even maternal exposure to nicotine alone may lead to deleterious consequences in the fetus, a more comprehensive evaluation of the potential effects of maternal NRT and e-cigarette use is required [30].

1.1.2 Nicotinic acetylcholine receptors (nAChRs)

Nicotine exerts its effects, as first characterized by John Langley in 1905, through binding to nAChRs [31-33]. nAChRs are ligand-gated, transmembrane, ionotropic cation channels consisting of five distinct subunits (two α-, one δ-, and one β-, γ-, or ε-subunit) that interact in unique combinations to form different subtypes [34-36]. nAChRs have been demonstrated to be expressed in both neuronal and non-neuronal cell types throughout the body, including the placenta, and may regulate a large range of physiological functions (*e.g.*, cell differentiation, migration, viability, and transmitter release) depending on receptor subtype and location [37-39]. nAChR binding stimulates the opening of nonselective cation channels, which subsequently instigates sodium influx, membrane depolarization, and activation of voltage-gated calcium channels [35, 40]. The resulting calcium influx then activates calcium-dependent kinases (*e.g.*, protein kinase C, calmodulin-dependent protein kinase II, extracellular signal-regulated kinases, etc.) and may trigger various transcription factors (*e.g.*, cAMP response element binding protein, etc.) responsible for governing the aforementioned physiological functions [41-43]. Endogenous agonists, such as acetylcholine, normally bind nAChRs to regulate these cellular pathways [35]. However, exogenous agents, such as nicotine or α-bungarotoxin, may also competitively or noncompetitively bind to nAChRs and exert alternative, and potentially pathological, effects [35].

1.1.3 Pharmacology of Nicotine

1.1.3.1 Absorption and distribution

To better appreciate the physiological impact of nAChR activation via nicotine, we must also review the pharmacological absorption, distribution, and metabolism of nicotine within the body. Nicotine ($C_{10}H_{14}N_2$) can easily traverse membrane barriers due to its lipophilic nature and activate nAChRs within various organ systems [32]. Whole body nicotine distribution is rapid, occurring within seconds to minutes from exposure, with highest affinities in the brain, lung, liver, kidney, spleen, and skeletal muscle [44, 45]. As a weak base, nicotine is also susceptible to ion-trapping and accumulation in acidic bodily fluids/tissues – up to 2.9-fold in breast milk, placental tissue, amniotic fluid, and fetal blood compared to maternal serum levels, increasing risk of fetal or neonatal exposure to maternal nicotine [31, 46-49].

Consistent reports of the typical steady-state plasma nicotine concentrations in human smoking populations are difficult to find due to vast genetic diversity in the metabolism of individuals (to be discussed in greater detail later in this thesis), and between-study variations in mode of nicotine administration and measuring techniques used [50]. Several pharmacokinetic studies targeting non-pregnant smokers and/or NRT users reported ranges of serum nicotine concentrations between 25nM-25μM (for consistency in our comparisons, doses were converted from original units to molar concentration, based on 162.23 $g \cdot mol^{-1}$ molecular weight of nicotine) [51-55]. Fewer studies were conducted in pregnant human smokers/NRT users, though one report identified average serum nicotine concentrations of ~500nM in pregnant women who smoked approximately 20 cigarettes/day or wore 21-mg transdermal nicotine patches [56]. Difficulty in accurately assessing serum nicotine levels may be due to its relatively shorter half-life (~2 hours), thus its metabolite, cotinine, may be measured to quantify nicotine exposure (~17 hours) [57].

1.1.3.2 Metabolism

Nicotine is primarily metabolized by the liver into six metabolites, of which cotinine constitutes 70-80% of the product [44, 58]. The conversion of nicotine to cotinine occurs through the two-step C-oxidation pathway, where nicotine is first converted to nicotine-

$\Delta^{1'(5')}$-iminium ion by CYP2A6, then converted to cotinine by aldehyde oxidase [58-62]. Cotinine may be further metabolized into six other primary metabolites (*e.g.,* 3'-hydroxycotinine, etc.) [63]. Nicotine and its metabolites are eventually cleared from the body chiefly through renal excretion, but traces have been detected in sweat and breast milk [64].

During pregnancy, the clearance of nicotine and cotinine was reported to be increased by 60 and 140%, respectively, compared to non-pregnant women [65, 66]. Increased drug clearance during pregnancy is not unique to nicotine and cotinine, as similar effects were also seen in the metabolism of penicillin, phenytoin, and other drugs [67]. This was suggested to occur due to accelerated oxidation via CYP2A6 alongside faster glucuronide formation, though the exact mechanisms are not well understood and very unpredictable between individuals [66]. Interestingly, this may help explain why pregnant women were reported to experience decreased NRT efficacy for smoking cessation [65]. However, due to nicotine's potentially harmful effects on pregnancy, caution would be warranted in increasing its dose and/or duration to compensate for decreased cessation efficacy [25].

1.1.4 Long-term health outcomes of prenatal nicotine exposure

Since pharmacological studies have verified that nicotine directly affect various systems within the mother and fetus, we must next address the health consequences of exposure during pregnancy. There are currently no human studies available reporting the long-term health outcomes of adults exposed to nicotine *in utero* through maternal NRT or e-cigarette usage. However, emerging animal studies clearly demonstrate that maternal nicotine exposure alone may lead to deleterious long-term health outcomes in the offspring. These included compromised neural development [68-70], increased risk of metabolic disorders (*e.g.,* increased hepatic and circulating triglycerides, impaired β-cell function and insulin resistance, obesity [71-80]), impaired fertility (*e.g.,* transient testicular and epididymal changes, impaired ovarian function [76, 81, 82]), and poor cardiovascular and respiratory health (*e.g.,* increased blood pressure, risk of hypertension, impaired alveolarization [74, 83, 84]) (Figure 1.1).

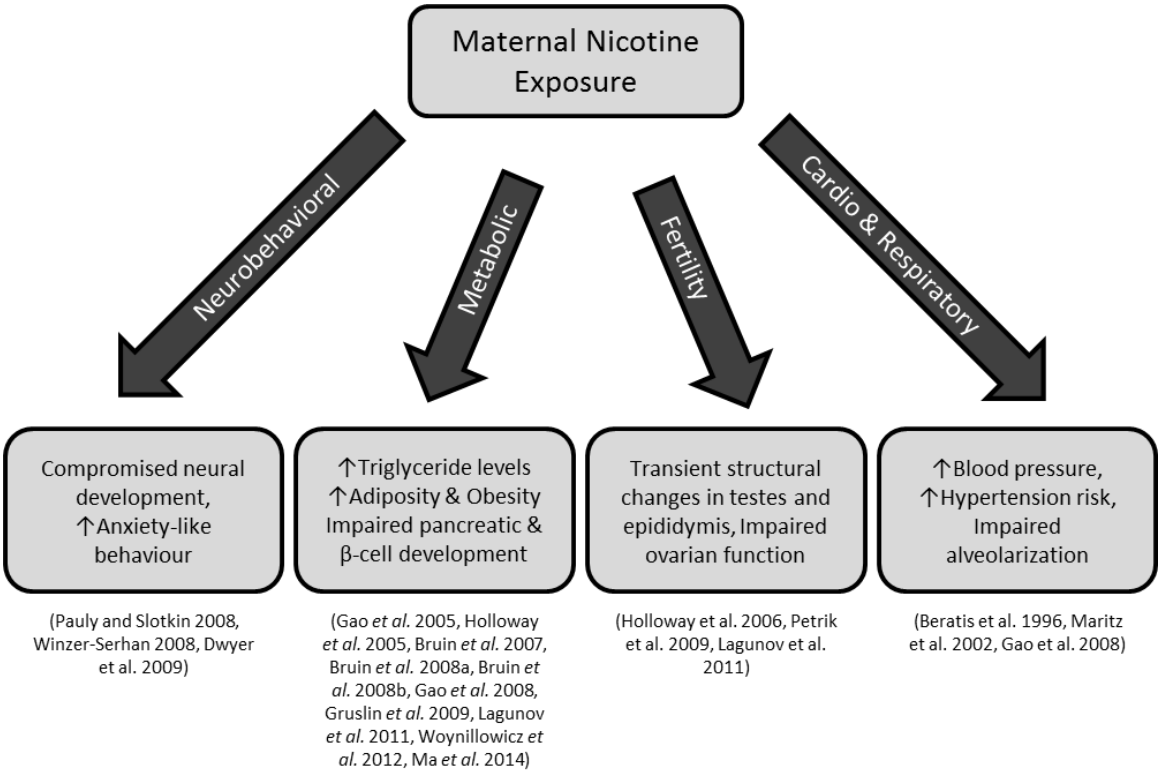

Figure 1.1. Maternal exposure to nicotine alone may lead to many deleterious long-term health outcomes in the offspring.

1.1.5 Short-term health and pregnancy outcomes of prenatal nicotine exposure

In addition to its impact on long-term health in the offspring, maternal nicotine exposure may also impact short-term health and pregnancy outcomes. Findings from human studies, however, have generally reported minimal health effects on the offspring exposed to NRTs during pregnancy. A series of studies from the Smoking, Nicotine, and Pregnancy (SNAP) Trial Team reported no short-term postnatal health impairments from maternal NRT use compared to placebo after delivery and a two-year follow-up [24, 85]. Dhalwani *et al.* (2015) also did not report any change in risk of major congenital abnormalities in offspring, with the exception of increased risk of respiratory system anomalies [86]. Similarly, a 2012 Cochrane review analyzing six randomized controlled trials of NRT use during pregnancy did not report any significant differences in rates of stillbirth, preterm labour, or low birthweight [24]. Yet, there are several major limitations to note across these studies: (1) Compliance and adherence rates were generally quite low, limiting researchers from consistently assessing the full effects. (2) Intervention studies relied mainly on self-report measures, increasing risk of bias or inaccuracy in data. (3) NRT use did not improve prolonged abstinence from smoking in mothers compared to placebo groups, suggesting that the doses provided were perhaps not strong enough to induce any effects at all, including effects in the offspring. Interestingly, another study found that the simultaneous use of more than one NRT product during pregnancy, which resulted in a higher dose of nicotine, was associated with mild decreases in birth weight in human offspring, suggesting that higher nicotine doses may indeed carry some risks for the offspring [87]. Further research addressing these limitations are required before conclusions may be made regarding the safety and efficacy of NRT and/or e-cigarette use in human pregnancy.

The short-term and pregnancy effects of nicotine exposure in animal studies have been more robustly demonstrated. Nicotine exposure during pregnancy led to significant reductions in birth weight in rodent offspring [72, 77, 88]. This is concerning, as low birthweight has been associated with long-term chronic disease development (to be discussed in greater detail later in this chapter.) Furthermore, low birthweight in the offspring may implicate some degree of placental impairment, as the placenta plays a

critical role in governing the growth of the fetus [89-91]. Indeed, pregnant rats exposed to chronic nicotine injections demonstrated compromised placental development and function at embryonic day 15 [92], to be followed by low fetal birthweight at term [77].

1.2 The Placenta and its Role in Fetal Development

The placenta is anatomically defined as the discoid-shaped, fetal-derived organ comprised of a fusion of fetal and maternal tissues, essential for the establishment and maintenance of pregnancy [93, 94]. It is important to note that the human fetus is transiently, but entirely, dependent on the placenta for survival through nutrient exchange, waste removal, immune and barrier protection, and endocrine regulation [93, 94].

1.2.1 Human placental development

Placental and fetal development begins with the blastocyst, an early developmental structure comprising the inner cell mass, blastocoel cavity, and outer trophectoderm layer of trophoblast cells. The blastocyst implants into the epithelial layer of the maternal uterine endothelium at approximately 6-7 days post-fertilization [95]. The placenta originates from the polar trophectoderm layer, and placentation is initiated when this endometrial-invading layer differentiates into the mononucleated cytotrophoblast and multinucleated syncytiotrophoblast layers. The syncytiotrophoblasts are responsible for initial enzyme-mediated digestion and invasion into the maternal uterine endometrium, whereas the cytotrophoblasts rapidly divide and fuse to expand the syncytiotrophoblast layer [96, 97].

Fluid-filled spaces known as syncytial lacunae develop within the syncytiotrophoblast layer and eventually house maternal blood as the invading syncytiotrophoblasts penetrate and rupture maternal capillaries. At approximately two weeks of pregnancy, finger-like projections known as primary chorionic villi are rapidly established, comprised of a solid core of cytotrophoblast cells surrounded by the outer syncytiotrophoblast layer. At day 16, mesenchymal cells from the underlying extraembryonic mesoderm penetrate the cytotrophoblast core transforming the primary chorionic villi into secondary chorionic villi. At day 21, these mesenchymal cells rapidly differentiate into embryonic blood vessels at the center of the villi, transforming the secondary chorionic villi into tertiary chorionic villi; this allows for greater exchange between embryonic and maternal blood. At

approximately five weeks of pregnancy, the cytotrophoblasts expand and breach the syncytiotrophoblast layer to reach the decidua basalis of the uterus. As this occurs, syncytial lacunae become more pronounced giving rise to the intervillous space between the chorionic and basal plates of the placenta, which borders the fetus and maternal endometrium, respectively [94]. Thirty to forty chorionic villous "trees" project from the chorionic plate through this intervillous space; each globular-shaped projection existing as an individual maternal-fetal exchange unit between the mother and fetus. Anchoring villi from the villous tree attach to the decidua basalis of the uterus to further secure the placenta and embryo, while terminal villi, richly vascularized with embryonic vessels, branch from the villous trees to serve as main sites of exchange [98]. Due to successful invasion and remodeling of spiral arteries in the maternal uterus by extravillous trophoblasts (EVTs; discussed in more detail below), maternal blood pools into the intervillous space from maternal spiral arteries, immersing the villous trees to allow for exchange to occur. Maternal blood then exits the intervillous space of the placenta through uterine veins to return to maternal circulation. Newly enriched blood within the embryonic capillaries of the villous trees merge at the umbilical vein to travel to the fetus; two umbilical arteries carry deoxygenated blood from the fetus back to the placenta to be exchanged [94].

Perfusion of maternal blood into the intervillous space is made possible by EVTs, which derive from the tips of the anchoring villi and invade into the maternal myometrium to access and remodel the maternal uterine spiral arteries [97]. Two subpopulations of EVTs are responsible for invading the maternal myometrium, interstitial and endovascular trophoblasts. Interstitial trophoblasts invade the maternal decidual stroma and remodel the spiral arteries from the outside-in; whereas the endovascular trophoblasts infiltrate and colonize the actual vascular walls of spiral arteries, regulating blood flow to the placenta initially through plug formation within the lumen, and later through replacement of the vascular walls with trophoblast cells [99]. The EVT invasion and migration process is complexly regulated by a variety of factors, including growth factors [100], immune cells (*e.g.*, uterine natural killer cells) [101], and many others (extensively reviewed in [102-104]). Poor invasion and migration may contribute to the development of pregnancy disorders such as preeclampsia and intrauterine growth restriction (IUGR) [102].

Considering the indispensable role of the placenta in maintaining a healthy pregnancy, there remains a need to further understand the intricate physiologies and pathologies associated with this organ. Due to obvious ethical concerns, human studies on pregnancy and placentation have been limited, necessitating the development of suitable experimental animal and *in vitro* models to further the study of this multifaceted organ.

1.2.2 The rat as a model for human placentation

The rat serves as an appropriate model for studying many aspects of human placentation due to similarities in hemochorial placental structure, deep intrauterine invasion through both endovascular and interstitial pathways, and trophoblast-directed remodeling of uterine spiral arteries [105, 106]. Advantageous as well is the abundance of existing molecular tools to analyze rat physiology, whereas antibodies and primer sequence databases are not as readily available or reliable for other animal models, such as sheep, guinea pigs and hamsters. The mouse has also been extensively used in placental research; however, mouse trophoblast invasion remains far more superficial than humans, making the rat a better selection for *in vivo* studies [107, 108]. Despite the similarities, some of the major differences in rats compared to humans include lower number of spiral arteries, relatively shallow implantation, shorter gestation (21 days), and polytocous pregnancies [105]. These differences may limit the study of certain specific placental processes, such as decidualization during implantation, and prevent accurate modeling of certain pregnancy disorders. However, the similarities in trophoblast-directed roles [105], conserved cellular pathways between humans and rats [109], and ability of nicotine to cross into both species' placentas [92, 110], permit usage of the rat as a model for our study.

During rat placentation, trophoblast stem cells are established from the trophectoderm of the blastocyst and may derive at least five cell types (trophoblast giant (TG) cells, invasive trophoblasts, spongiotrophoblast cells, syncytial trophoblast cells, and glycogen cells). Each cell type adopts an important role in pregnancy, but the one of interest to our study will be the TG cell due to their large population in the rat placenta and cardinal involvements throughout placentation. TG cells are the first trophoblast cell lineage to differentiate in the early "choriovitelline" placenta, enabling uterine invasion and anastomosis with maternal vasculature. The mature "chorioallantoic" placenta consists of

the decidua, junctional, and labyrinth zones, throughout which TG cells continue to provide chief invasion and endocrine functions [107, 111, 112]. Similar to human extravillous cytotrophoblasts, rat TG cells facilitate both interstitial and endovascular invasion pathways in remodeling the myometrium and spiral arteries of the maternal decidua to perfuse the placenta [106, 113, 114]. Uterine natural killer cells and other immune cells are similarly suggested to direct TG cell migration and invasion [107]. The junctional zone is located between the decidua and labyrinth zone and consists of TG cells, spongiotrophoblast cells, and glycogen cells; it is mainly involved in endocrine functions and facilitating maternal blood to and from the placenta. The labyrinth zone is located at the fetal interface and consists of TG cells, syncytiotrophoblast cells, and fetal mesenchymal cells; it is mainly involved in maternal-fetal nutrient exchange [105, 107].

1.2.3 *In vitro* models to investigate mechanisms

In addition to *in vivo* animal models, numerous useful *in vitro* models have been developed to study placentation. Though *in vitro* systems are sometimes criticized for lack of translatability due to current sub-physiologically-relevant culture conditions, a major advantage is that the simplified setting allows for more direct investigation of underlying cellular mechanisms. A common *in vitro* approach involves the use of isolated primary cell cultures. Primary placental cultures may be derived from either human [115, 116] or rat [117] placentas, and are advantageous as they are directly sourced from the organ. However, procedures involved with isolation are often very tedious and stressful for the cells, and cultures respond poorly to repeated passaging. An alternative option is the use of placental cell lines derived from choriocarcinoma cells (*e.g.,* JEG-3 [118], Rcho-1 [119]) or immortalized extravillous trophoblast cells (*e.g.,* HTR-8/SVneo [120]). Placental cell lines are relatively easy to handle and endure longer-term culturing and repeated passaging [121]; however, they may exhibit phenotypic differences due to the malignancy or immortalization. It is essential to conduct comparative experiments in order to verify the appropriateness of the choriocarcinoma cell line as a model. The Rcho-1 cell line has been extensively studied and determined to share many similarities with true placental TG cells in terms of cell cycle regulation, differentiation, gene transcription profile, transport processes, hormone production, and others (please refer to [122] for full reference list on

studies). All in all, the establishment of suitable *in vivo* and *in vitro* models alongside clinical observations have enabled deeper study into compromised placental development and the consequences that may follow.

1.2.4 The role of compromised placental development in the "Developmental Origins of Health and Disease"

To better comprehend the unparalleled role of the placenta in regulating a suitable prenatal environment for the fetus, we must also address the importance of the prenatal environment in determining postnatal and long-term health. Pioneering studies by David J. Barker and colleagues proposed that the detrimental health outcomes seen in later life may be rooted in programming that occurred during an individual's fetal life. This theory is better known as the "Developmental Origins of Health and Disease" [123]. One of the earliest studies revealed that geographical regions across England and Wales with high rates of infant mortality in 1921-25 strongly correlated with high rates of ischemic heart disease mortality later on in 1968-78 [124]. The high rates of infant mortality were attributed to nutritional deprivation in the area in 1921-25, and surviving individuals from these same areas who later developed ischemic heart disease in 1968-78 were suggested to have been particularly susceptible to the rich diet in later life because of their poor diet in early life. Later studies confirmed the association between low birth weight and the development of cardiovascular and metabolic disorders in adulthood [125-129]. However, it was by comparison of the Leningrad and Dutch famines during World War II that would serve to further advance our understanding of the Developmental Origins theory [130, 131]. There were no differences found in glucose tolerance in adults born during the 28-month Leningrad famine compared to those born outside the famine, whereas glucose intolerance was found in adults born during the 6-month Dutch famine. Correspondingly, these infants born during the long Leningrad famine would likely have experienced continued famine into their postnatal lives, whereas infants born during the short Dutch famine would have received an immediate reintroduction of nutrition as the famine ended, experiencing a stark "mismatch" between prenatal and postnatal environments [130, 131]. Therefore, it appears that an affluent postnatal environment is necessary to amplify the effects of a poor prenatal environment in later-life health outcomes. Moreover, animal models demonstrated that

rapid “catch-up” growth, which was induced by the return to a normal postnatal diet after prenatal protein restriction, led to later life development of diabetes [132]. This collectively revealed the crucial relationships between a poor prenatal environment, a mismatched affluent postnatal environment, and the adult onset of chronic diseases.

However, many factors along with maternal diet/nutrition may also influence the quality of the prenatal environment and intrauterine growth of the fetus, including maternal hypoxia [133, 134], glucocorticoid stress levels [135], and drug exposure [26, 30]. In addition to maternal factors, “placental insufficiency” was found to precede many instances of abnormal fetal development and IUGR in humans [89-91]. Placental insufficiency will be defined in the context of this thesis as compromised growth, development, or function of the placenta, leading to an inability to meet the nutritional and other growth-related needs of the fetus. As demonstrated by Holloway *et al.* (2014), nicotine-induced placental insufficiency may be specifically caused by reduced growth in the decidua and junctional zones, impaired markers of cell proliferation and vascularization in the labyrinth zone, and inhibition of trophoblast cell differentiation, invasion, and migration [92]; however, the underlying cellular mechanisms have yet to be fully elucidated. Several mechanisms have been proposed to underlie placental insufficiency at the cellular level, including increased oxidative stress [136, 137] and inflammation [138, 139]. Another mechanism that was more recently proposed to underlie placental insufficiency is an intracellular protein folding disturbance known as endoplasmic reticulum (ER) stress [140-143].

1.3 ER Stress and the Unfolded Protein Response (UPR)

The ER is the organelle primarily responsible for protein synthesis, folding and maturation, and secretion within the cell [144]. Protein folding capacity may be affected by many factors, such as calcium levels in the ER, disulfide bond formation, asparagine (N)-glycosylation, and ATP/glucose availability [109]. Calcium plays an important role in protein folding by both directly interacting with nascent proteins and enabling the activity of ER-resident calcium-binding chaperones, such as calreticulin [145]. However, ER calcium stores fluctuate rapidly through the inositol 1,4,5-triphosphate/ryanodine receptors and sarco/endoplasmic reticulum calcium-ATPases (SERCAs) during intracellular signaling, which may influence protein folding efficiency [146]. Successful co- and post-

translational protein modifications also depends on disulfide bond formation [147]. Occurring through a series of redox reactions, disulfide bonds are initially introduced by protein disulfide isomerase (PDI) through thiol oxidation. ER oxidoreductase (Ero1-Lα/β) subsequently reoxidizes PDI to continue the redox relay [148]. Various alternative ER oxidoreductases, VKORC1 [149], GPx-7/8 [150], QSOX1 [151], have also been discovered to potentially assist with PDI reoxidation, or may directly oxidize thiols of nascent proteins. ER oxidases are then reoxidized by FAD, and finally by a terminal electron acceptor such as oxygen [147]. Therefore, adequate oxygen levels and a proper redox environment are essential in the formation of disulfide bonds and protein maturation [109, 147]. Alongside disulfide bond formation, N-glycosylation is another key modification required in most proteins. This process involves covalent oligosaccharide attachment to the asparagine residue of nascent proteins, upon which they may then be associated with lectin chaperones, calnexin and/or calreticulin, to undergo further folding [152, 153]. Moreover, sufficient ATP and glucose availability are required at various steps during many of these processes (*i.e.,* ATP required for SERCA-directed calcium influx to ER, and glucose required for ATP generation and synthesis of oligosaccharide for N-glycosylation [154, 155]). Properly matured proteins may then be exported to the Golgi complex for additional alterations, or to the cytosol to fulfill their functions within the cell.

ER stress is classically defined as the perturbation of any of these functions leading to an accumulation of misfolded or unfolded proteins within the ER lumen [109]. Human studies revealed a strong association between ER stress and placental insufficiency [141, 142]. Yung *et al.* (2008) found significantly increased ER stress in growth-restricted placentas from IUGR pregnancies compared to control placentas from normal pregnancies [141]. Women who newly migrated to high altitude regions (3100m), which is known to induce placental insufficiency and fetal IUGR due to decreased oxygen availability, also exhibited placental growth restriction and ER stress compared to control sea-level placentas [142]. Experimental induction of ER stress using the drug tunicamycin, which inhibits N-linked glycosylation to cause protein misfolding, decreased proliferation in JEG-3 and JAR trophoblast cells *in vitro* [141]. Kawakami *et al.* (2014) further explicated these findings by demonstrating that chronic tunicamycin-induced ER stress in pregnant mice *in vivo* leads to compromised placentation and low birth weight offspring [156]. Mice that were

especially vulnerable to ER stress, due to genetically-modified elevations in basal protein translation, also demonstrated placental insufficiency and low birth weight offspring [143]. Altogether, these studies strongly suggest that augmented placental ER stress may underlie placental insufficiency, leading to IUGR in the offspring.

The UPR aims to alleviate ER stress and restore homeostasis by activating a series of transcriptional and translational quality-control events that increase protein folding capacity and decrease incoming protein traffic [109]. Additional quality-control mechanisms also exist to further relieve ER stress in more dire situations, such as ER-associated degradation (ERAD) and autophagy. The ERAD pathway retrotranslocates terminally misfolded proteins from the ER lumen into the cytosol where they are ubiquitinated for proteasomal degradation [157, 158]. Severe accumulation of misfolded protein aggregates may further initiate autophagy of the ER, which involves sequestering of the targeted organelle and associated cytoplasm into membrane-bound compartments for lysosomal degradation [159, 160]. However, for the purposes of this thesis, we will focus our review on the three canonical pathways of the UPR.

Each UPR pathway is governed by an ER-resident transmembrane protein transducer, including activating transcription factor 6 (ATF6), inositol-requiring enzyme 1 (IRE1), and protein kinase-like ER kinase (PERK). Though the sensory mechanisms underlying the activation of the UPR in response to ER stress remain somewhat unclear, Ron and Walter (2007) have compiled the current evidence into three possible theoretical models [160]. The first model, termed the direct recognition model, proposes that each ER stress transducer (ATF6, IRE1, and PERK) directly interacts and senses misfolded/unfolded proteins via their luminal domains, though the evidence is mainly drawn from structural studies on IRE1 [161, 162]. The second model, termed the indirect recognition model, proposes that glucose-regulated protein 78 (BiP/GRP78), an ER chaperone protein, normally remains bound to the three transducers to maintain inactivity. In the presence of ER stress, BiP disassociates from the transducers to chaperone the misfolded/unfolded proteins, and thus initiates activation of the UPR pathways [163-165]. The third model, termed the hybrid recognition model, acknowledges the valid culmination of data from both theories and proposes that luminal binding and BiP disassociation are mutually

involved in activating the UPR [160]. Regardless of the mode of sensing stress, each UPR transducer is known to differentially respond to distinct forms and causes of ER stress (*e.g.*, inhibition of N-linked glycosylation, dysregulation of calcium homeostasis, etc.) [166], thus there is insult-dependent specificity involved in the activation of each UPR pathway.

1.3.1 ATF6 pathway of the UPR

In the first pathway of the UPR, ATF6 translocates to the Golgi bodies to become cleaved from 90 to 50 kDa by site-1 and site-2 proteases. Cleaved ATF6 may then enter the nucleus to facilitate transcription of genes involved in protein folding/chaperoning (*e.g.*, Grp78), ERAD, and lipid biogenesis [109, 167, 168]. Two ubiquitously-expressed isoforms of ATF6 exist in eukaryotes, ATF6α/β, and both are involved in the same processes [169]. However, ATF6α is a rapidly-degraded, potent transcriptional activator of ER stress response genes [170], whereas ATF6β is a stably-expressed, weak activator [171]. Due to these properties, ATF6β is suggested to hinder ATF6α-mediated gene transcription, and balanced expression of the two isoforms are important for fine-tuning the ATF6-dependent ER stress response [171, 172]. In experimental models, Atf6α-knockout mice were more susceptible to hyperinsulinemia after high-fat diet feeding [173], and increased ATF6 pathway activation was seen in β cells of diabetes-modeling Akita mice [174], revealing a potential role in diabetes. ATF6 was also shown to synergistically upregulate transcription of X-box binding protein 1 (Xbp1) and C/EBP-homologous protein (Chop) alongside the other two UPR transducers [175, 176], and may share various downstream gene targets with XBP1 [177], demonstrating cross-talk between the pathways.

1.3.2 IRE1 pathway of the UPR

In the second pathway of the UPR, IRE1 homodimerizes and undergoes autophosphorylation at its cytoplasmic domain [178, 179]. Phosphorylation of IRE1 activates an endoribonuclease domain which unconventionally splices Xbp1 mRNA transcript. Translation of spliced Xbp1 transcript yields active XBP1, a transcription factor that enters the nucleus to up-regulate the transcription of genes involved in protein folding/chaperoning, ERAD, lipid biogenesis, and autophagy [180-182]. The IRE1 pathway was previously reported to play essential roles in pancreatic development and

function [183-185], neurological disorders [186], and angiogenesis in tumour formation [187]. Furthermore, two isoforms of IRE1 exist in higher eukaryotes, where IRE1α is ubiquitously expressed [188], and IRE1β is uniquely expressed in the gut [189]. Ire1α knockouts in mice led to embryonic lethality due to dysfunctional placental labyrinth organization, which is crucial for maternal-fetal exchange. Interestingly, embryonic viability was maintained when Ire1α knockout was conditionally restricted only to the embryo without affecting the placenta, implicating an integral role for IRE1α in placenta-dependent survival [180]. Ire1β knockout did not affect viability in mice, though it increased susceptibility to dextran sodium sulfate-induced inflammatory bowel disease compared to controls [190].

In an alternate pathway known as the regulated IRE1-dependent decay (RIDD) pathway, IRE1 also functions to rapidly degrade a select subset of mRNAs under conditions of ER stress in order to decrease the availability of transcripts for translation [191, 192]. The RIDD pathway has been shown to carry important roles in inflammation/immunity [193] and lipid metabolism [194].

1.3.3 PERK pathway of the UPR

In the third pathway of the UPR, PERK homodimerizes and autophosphorylates its cytoplasmic domain. Phosphorylated PERK further phosphorylates eukaryotic initiation factor (eIF) 2 at Ser51 of the α-subunit (eIF2α) [195]. Normally, eIF2, GTP and methionyl-initiator tRNA comprise the ternary complex involved in protein translation. The ternary complex mediates the binding of methionyl-initiator tRNA with the 40S ribosomal subunit to initiate translation, alongside various other eIFs and ribosomal complexes [196]. When the initiation codon AUG of the targeted mRNA is detected by methionyl-initiator tRNA, the GTP coupled to eIF2 is hydrolyzed to GDP, as catalyzed by eIF5, to assist with binding of 60S ribosomal subunit to the initiation complex. eIF2-GDP is then released, allowing for the remainder of translation to occur. eIF2B catalyzes the exchange of GDP for GTP on eIF2 to permit the formation of new ternary complexes for successive rounds of translation [197]. However, phosphorylation of eIF2α by PERK inhibits disassociation from eIF2B, thus preventing subsequent eIF2B-catalyzed GDP-GTP exchange from occurring. Therefore, initiation of translation is attenuated for most mRNA transcripts

during ER stress, reducing incoming protein traffic into the ER lumen [196, 198]. Phosphorylated eIF2α, however, paradoxically elevates the translation of select transcripts involved in mediating ER stress, such as Atf4, Chop, and Grp78 [199-204]. Acute activation of the PERK pathway was previously reported to play a protective role in organs involved with high protein secretory activity, such as the pancreas or placenta [143, 205]. Furthermore, PERK may be involved in hypoxic adaptation of tumour cells via translational regulation of proangiogenic genes, implicating a potential role in cancer [206].

In addition to PERK, there are three other known kinases that may phosphorylate eIF2α depending on the insult. Double-stranded RNA-activated inhibitor (DAI), also known as Protein Kinase RNA (PKR), is activated in the presence of viral infection [207]. Heme-regulated inhibitor (HRI) is activated during iron or heme deficiency, arsenite, or osmotic/heat shock [208]. General control nonderepressible 2 (GCN2) is activated during amino acid starvation or UV irradiation [209, 210]. These pathways are collectively known as the Integrated Stress Response, commonly converging at eIF2α phosphorylation to regulate translation and related downstream responses under diverse stress conditions.

1.3.4 The consequences of chronic ER stress and UPR activation

If ER stress persists and the UPR remains chronically activated, several downstream apoptotic pathways may become amplified to initiate cell death. One of the major pathways of ER stress-induced cell death involves mitochondrial apoptosis through the pro-apoptotic BCL2 family members (*i.e.*, BCL2-Associated X protein (BAX), BCL2-Antagonist/Killer (BAK), BCL2-Interacting Mediator of cell death (BIM), p53-Upregulated Modulator of Apoptosis (PUMA), phorbol-12-myristate-13-acetate-induced protein 1 (NOXA)). Though the apoptotic involvement of BCL2 family member proteins in the placenta remains unclear, they have been well-studied in various other cell types. Firstly, ER stress may lead to down-regulation of anti-apoptotic B-cell CLL/lymphoma 2 (BCL2) expression, allowing BAX/BAK to translocate and perforate the mitochondrial membrane for cytochrome c release and apoptosis [211]. Indeed, tunicamycin and thapsigargin, a selective SERCA inhibitor that causes ER stress, down-regulated Bcl2 expression and led to increased cell death in several fibroblast cell lines [212, 213]. Also, overexpression of Bcl2 [212, 213] and/or double knock-out of Bax/Bak [211] protected against ER stress-

induced cell death. Secondly, ER stress may lead to increased activation of BCL2-homology 3 (BH3)-only proteins (*e.g.*, BIM, PUMA, NOXA, etc. [214]). BIM was shown to be a critical initiator of apoptosis after tunicamycin and thapsigargin-induced ER stress in various cell types (thymocytes, macrophages, renal tubular epithelial cells, breast cancer derived cells, and kidney epithelial cells), and Bim knockout increased survival during ER stress [214]. ER stress may also lead to increased expression of PUMA and/or NOXA, which were demonstrated to play important roles in ER stress-induced apoptosis in mouse embryonic fibroblasts [215] and primary cortical neurons [216]. This may occur through either p53-dependent or -independent mechanisms during ER stress [215, 216]. Some other mediators of ER stress-induced cell death include the caspase pathway (caspase 12, 9, and 3), tribbles homolog 3, and death receptor 5, amongst others [217].

Importantly, most ER stress-related apoptotic pathways are understood to be triggered by CHOP, a transcription factor regulated by ATF4 and phosphorylated eIF2α of the PERK pathway [204, 218]. Chop deletions/knockouts attenuated BIM and PUMA induction [214, 216], restored Bcl2 expression [213], increased cell survival during ER stress in various cell types/organs [219-222], improved glucose regulation and beta cell mass in diabetic mouse models [223], and reduced neuronal cell loss in ischemic brain injury [224]. In summary, ER stress and UPR-mediated apoptosis and cell death, as predominantly mediated by CHOP, may lead to placental insufficiency, and ultimately compromise fetal development (Figure 1.2).

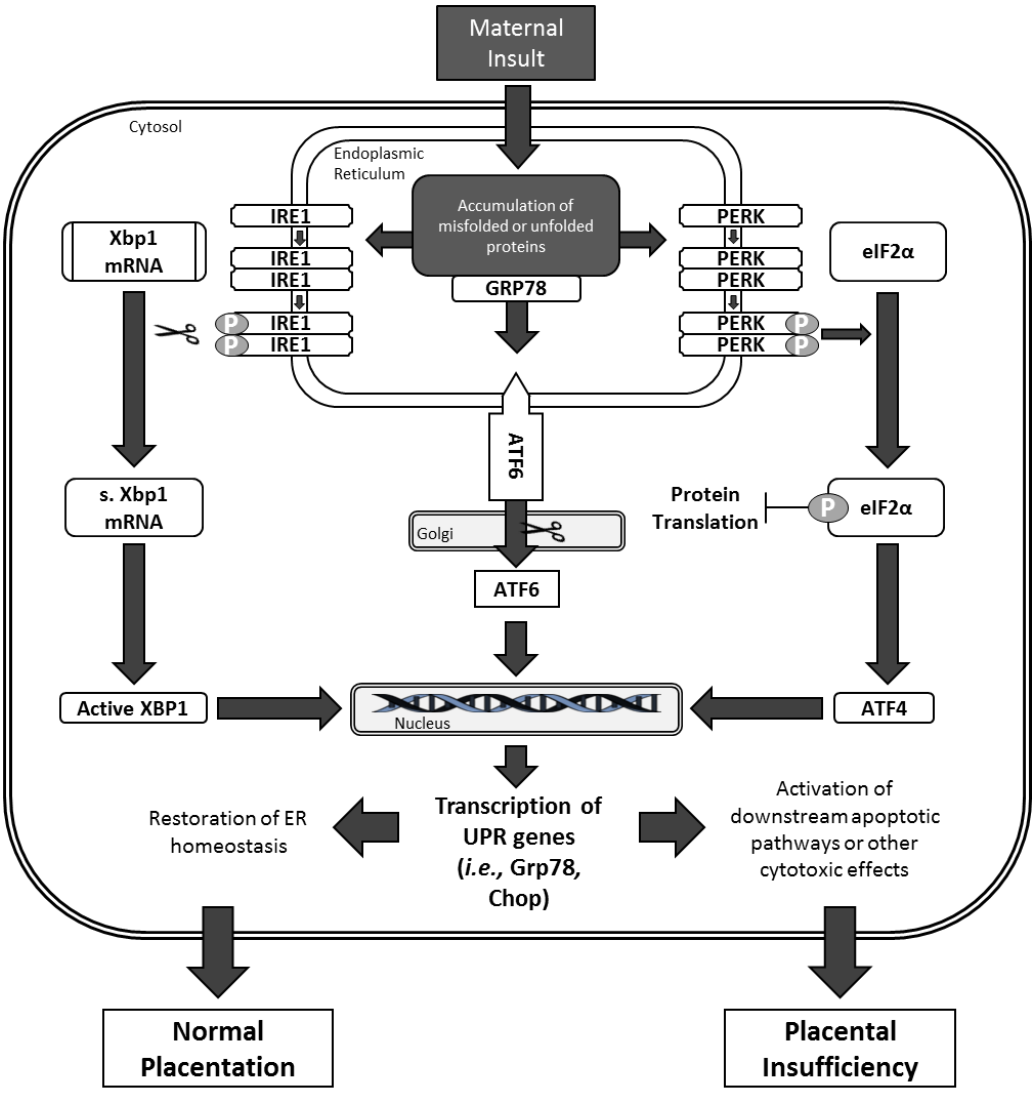

Figure 1.2. Maternal insults may augment ER stress and induce activation of the UPR. Chronic activation of the UPR may lead to downstream apoptosis or other cytotoxic effects, potentially underlying placental insufficiency.

1.4 Rationale, Hypothesis, and Objectives

1.4.1 Rationale and Hypothesis

Therefore, the rationale for this Master's thesis was founded upon two major premises (Figure 1.3):

(1) Maternal nicotine exposure in pregnancy leads to increased hypoxia [92, 110, 225] and placental insufficiency [92].

(2) Both hypoxia and placental insufficiency have been demonstrated to be strongly associated with ER stress [141, 143, 147, 156, 226].

Therefore, we hypothesize that nicotine exposure leads to augmented ER stress in the rat placenta *in vivo* and *in vitro*.

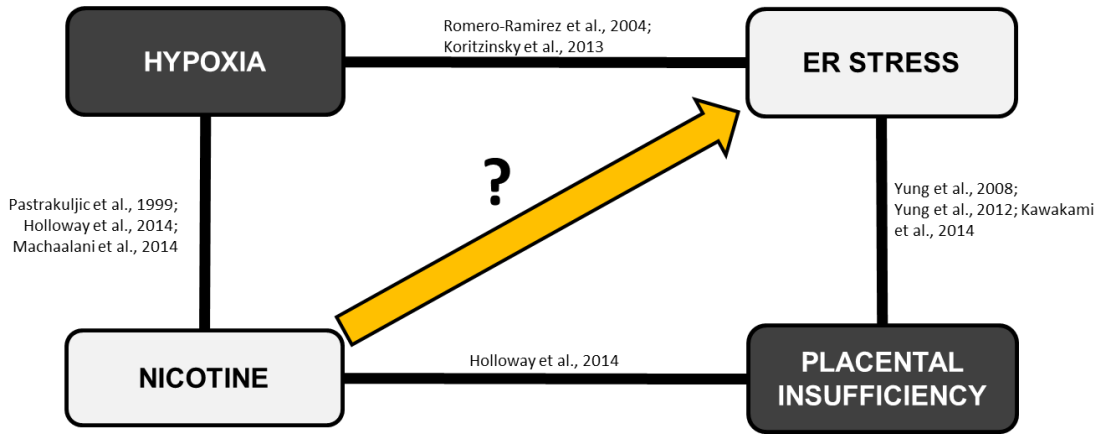

Figure 1.3. Schematic diagram illustrating the rationale of our study, summarizing what associations are currently known (connecting lines) and what we are interested in investigating (arrow).

1.4.2 Objectives

The *long-term objective* of this study is to characterize the nicotine-induced ER stress response in the rat placenta. In other words, this study aims to elucidate the impact of maternal nicotine exposure on placental development and function through the augmentation of ER stress and the UPR. By identifying the cellular mechanisms underlying the adverse outcomes of nicotine exposure, we may contribute to the development of innovative therapeutic interventions to alleviate the deleterious outcomes of nicotine exposure on placental development – ultimately benefiting both maternal and fetal health during pregnancy.

The *short-term objectives* are:

(i) To investigate the effects of maternal nicotine exposure on ER stress in the rat placenta *in vivo*.

(ii) To investigate the direct and indirect effects of nicotine exposure on ER stress in rat placental trophoblast giant cells *in vitro*.

(iii) To ameliorate nicotine-induced ER stress and UPR activation in rat placental trophoblast giant cells *in vitro* using Tauroursodeoxycholic acid (TUDCA).

Objective (i) will be addressed in Chapter 2 of this thesis. Objectives (ii) and (iii) will be addressed in Chapter 3 of this thesis.

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2 MATERNAL NICOTINE EXPOSURE LEADS TO IMPAIRED DISULFIDE BOND FORMATION AND AUGMENTED ENDOPLASMIC RETICULUM STRESS IN THE RAT PLACENTA

A version of this chapter has been previously published: **Wong, M.K., Nicholson, C.J., Holloway, A.C., and Hardy, D.B.** (2015) Maternal nicotine exposure leads to impaired disulfide bond formation and augmented endoplasmic reticulum stress in the rat placenta. *PLoS One* 10(3): p. e0122295.

2.1 Introduction

Despite increased awareness and education, approximately 10-28% of women reported to continue smoking during pregnancy [1-4]. Cigarette smoke contains many teratogens, and exposure during pregnancy increases the risk of adverse pregnancy and neonatal outcomes, including placental abruption, placenta previa, sudden infant death syndrome, spontaneous abortion, stillbirth, low birth weight, and fetal growth restriction [5-11]. Although many pregnant women want to quit smoking, recent studies suggest that only 50% successfully abstain from smoking during pregnancy, due in part to the highly addictive nature of nicotine [12, 13].

Nicotine replacement therapies (*e.g.*, nicotine patches and gums) were developed to assist with smoking cessation while concurrently allowing the smoker to avoid the thousands of chemicals in cigarette smoke [14]. Although nicotine replacement therapies are often considered to be safer than smoking, there is still great concern regarding the effects of nicotine on fetal and postpartum health (please refer to [15] for a review).

Due to the lipid-soluble nature of nicotine, it easily traverses membrane barriers to enter the placenta, where it can bind to many subtypes of nicotinic acetylcholine receptors (nAChR) previously reported to be expressed in human and rat placental syncytiotrophoblasts, cytotrophoblasts, Hofbauer cells, visceral yolk sac epithelium, and amniotic epithelium [16, 17]. Emerging animal studies demonstrate that nicotine *alone* during pregnancy can lead to compromised placental and fetal development, with many detrimental health outcomes in the offspring [18-24]. Furthermore, nicotine exposure *in utero* results in low birth weight pups, implicating placental insufficiency [25-27]. Specifically, nicotine *in utero* led to compromised placental development in pregnant rat dams (*e.g.*, compacted decidual and junctional zones, decreased labyrinth vascularization and cell proliferation, increased placental hypoxia, and impaired trophoblast differentiation) at embryonic day 15, prior to any observable fetal growth deficiencies [17]. Given that compromised placental development is a strong predictor of fetal growth restriction in humans, elucidation of the underlying mechanisms would be therapeutically beneficial for offspring exposed to nicotine *in utero* [28]. However, to date, mechanisms linking maternal nicotine exposure to compromised placental function remain elusive.

Recent studies have suggested that endoplasmic reticulum (ER) stress, the perturbation of ER homeostasis due to the accumulation of misfolded or unfolded proteins, plays a critical role underlying compromised placentation [29-33]. The unfolded protein response (UPR) activates to alleviate the stress and restore ER homeostasis through three major signaling pathways governed by activating transcription factor 6 (ATF6), inositol-requiring enzyme 1α endoribonuclease (IRE1α), and protein kinase-like endoplasmic reticulum kinase (PERK) [34-36]. However, if the ER remains severely debilitated, C/EBP-homologous protein/Gadd153 (CHOP) activates downstream apoptosis [37, 38], which has been associated with compromised placental growth both *in vivo* and *in vitro* [31, 39]. Since the ER is the primary site of protein synthesis and maturation within the cell, prolonged disturbance of its function through ER stress could negatively impact essential signaling and transport function in the placenta (*e.g.*, Vegf and Glut-1 expression) [29, 40-43]. Moreover, one of the key processes underlying protein maturation and folding within the ER lumen is the disulfide bond formation of nascent proteins through protein thiol oxidation, and deterrence of its function has been demonstrated to augment ER stress [40, 44-46]. It is important to note that due to high protein secretory activity in the placenta, low-moderate basal levels of UPR activation may occur even under normal physiological conditions; however, chronic pathological augmentation may rear consequences in placental development [29, 33, 47].

Nicotine is known to induce vasoconstriction in placental and umbilical vasculature, thus restricting oxygen and nutrient (*e.g.*, amino acids) supply [48, 49]. Interestingly, hypoxia and low amino acid supply have been demonstrated to both hinder disulfide bond formation and induce ER stress *in vitro* [50-54]. Exposure to cigarette smoke or nicotine has also been shown to cause ER stress in several tissue/cell types, but little is known about the mechanisms underlying maternal nicotine exposure *in vivo* on ER stress and disulfide bond formation in the developing placenta [55-61]. Therefore, the aim of this study was to determine whether maternal nicotine exposure *in vivo* leads to augmented placental ER stress and impaired disulfide bond formation in the rat placenta.

2.2 Materials and Methods

2.2.1 Experimental model

All animal experiments were approved by the Animal Research Ethics Board at McMaster University in accordance with the guidelines of the Canadian Council for Animal Care. Nulliparous female Wistar rats (200–250 g, Harlan, Indianapolis, IN, USA) were randomly assigned to receive daily subcutaneous injections of either saline (vehicle) (n=6) or nicotine bitartrate (1 mg/kg, Sigma-Aldrich) (n=5) for 14 days prior to mating and during pregnancy. This dose has previously resulted in maternal and neonatal serum cotinine concentrations similar to either moderate female smokers and/or low-dose nicotine replacement therapy users [24, 62-64]. Mating (embryonic day (e) 0) was confirmed by the presence of sperm in a vaginal flush. At necropsy (e15) whole placentas were harvested, immediately flash-frozen in liquid nitrogen, and stored at -80 °C until molecular analyses were performed. Each placenta used in molecular analyses was taken from a different dam.

2.2.2 RNA extraction and Real Time-Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from homogenized whole placentas using TRIzol reagent (Invitrogen). Chloroform (Sigma-Aldrich) was added to the solution, and then centrifuged at 12,500rpm. Supernatant was transferred to a fresh tube with an equal volume of isopropanol (Sigma-Aldrich) and centrifuged again at 12,500rpm. Total RNA was then collected from the pellet and dissolved in DEPC-treated water. Deoxyribonuclease I, Amplification Grade (Invitrogen) was added to the RNA to digest contaminating single- and double-stranded DNA. Four μg of RNA were reverse-transcribed to cDNA using random hexamers and Superscript II Reverse Transcriptase (Invitrogen). Primer sets directed against gene targets of interest were designed through National Center for Biotechnology Information's primer designing tool and generated via Invitrogen Custom DNA Oligos (Table 1). Quantitative analysis of mRNA expression was performed via RT-PCR using fluorescent nucleic acid dye SsoFast EvaGreen supermix (BioRad) and BioRad CFX384 Real Time System. The cycling conditions were 95 °C for 10 min, followed by 43 cycles of 95 °C for 15 sec and 60 °C for 30 sec and 72 °C for 30 sec. The cycle threshold was set so that exponential increases in amplification were approximately level between

all samples. Relative fold changes were calculated using the comparative cycle times (Ct) method, normalizing all values to the geometric means of three housekeeping genes (β-Actin, 18S, and Gapdh). Suitable housekeeping genes were determined using algorithms from GeNorm [65], Normfinder [66], BestKeeper [67], and the comparative ΔCt method [68] to provide an overall ranking of the most stable housekeeping genes (available online at http://www.leonxie.com/referencegene.php). (Please refer to Appendix I, Supplemental Figure 1 to see all mRNA targets normalized to individual housekeeping genes). Given all primer sets had equal priming efficiency, the ΔCt values for each primer set were calibrated to the experimental samples with the lowest transcript abundance (highest Ct value), and the relative abundance of each primer set compared with calibrator was determined by the formula $2^{\Delta\Delta Ct}$, in which ΔΔCt was the normalized value.

2.2.3 Xbp1 splicing assay

Quantitative analysis of Xbp1 mRNA splicing was performed as previously described [69]. Briefly, primers were designed to span the unique exon-exon border formed by unconventional IRE1 splicing to target spliced Xbp1 mRNA. Primers were also designed to target total Xbp1 mRNA (Table 1). Results were normalized to the geometric means of three housekeeping genes (β-Actin, 18S, and Gapdh), and then a ratio of spliced to total Xbp1 was taken to quantify the splicing of Xbp1.

Table 2.1. Forward and reverse sequences for the primers used for quantitative Real-Time PCR.

Gene	*Forward*	*Reverse*	*GenBank/ Reference*
Atf6	GGATTTGATGCCTTGGGAGTCAGAC	ATTTTTTTCTTTGGAGTCAGTCCAT	NM_001107196.1
Xbp1	GAGCAGCAAGTGGTGGAT	TCTCAATCACAAGCCCATG	NM_001004210.2
Spliced Xbp1	GAGTCCGCAGCAGGTG	GCGTCAGAATCCATGGGA	[69]
Grp78	AACCCAGATGAGGCTGTAGCA	ACATCAAGCAGAACCAGGTCAC	NM_013083.2
Atf4	CCTGACTCTGCTGCTTATATTACTCTAAC	ACTCCAGGTGGGTCATAAGGTTTG	NM_024403.2
Chop	CCAGCAGAGGTCACAAGCAC	CGCACTGACCACTCTGTTTC	NM_001109986.1
Prdx4	TCCTGTTACAGACTGAAGCTTTGC	GTGATCTGCGACCGAAACCC	NM_053512.2
Gpx-7	CCTGCCTTCAAATACCTAACCC	TGTAATACGGGGCTTGATCTCC	NM_001106673.1
Vkorc1	GCTGGTGGAGCATGTGTTAGG	CAACGTCCCCTCAAGCAACC	NM_203335.2
Qsox1	AGCCACTGCCCTAGATGTACC	TGAGGCCTGCGTTTAGTTCC	NM_001109898.1
Bax	AGGATCGAGCAGAGAGGATGG	GACACTCGCTCAGCTTCTTGG	NM_017059.2
Bcl-2	TGTGGATGACTGAGTACCTGAACC	CAGCCAGGAGAAATCAAACAGAGG	NM_016993.1
β-Actin	CACAGCTGAGAGGGAAAT	TCAGCAATGCCTGGGTAC	NM_031144
18S	TTGCTGATCCACATCTGCTGG	ATTGCCGACAGGATGCAGAA	M11188.1
Gapdh	GGATACTGAGAGCAAGAGAGAGG	TCCTGTTGTTATGGGGTCTGG	NM_017008.4

2.2.4 Protein extraction and Western Blot

Whole placentas were homogenized in RIPA buffer (50 mM Tris-HCL, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P40, 0.25% $C_{24}H_{39}NaO_4$, supplemented with phosphatase inhibitors (20 mM NaF, 40mM Na-pyrophosphate, 40mM Na_3VO_4, 200mM β-glycerophosphate disodium salt hydrate), and a protease inhibitor cocktail (Roche)). The solution was sonicated at 30% amplitude for 5 sec total, 1 sec per pulse. It was then mixed in a rotator for 10 min at 4 °C and centrifuged at 300g for 15 min at 4 °C. The supernatant was collected and centrifuged at 16000g for 20 min at 4 °C. The resulting supernatant was collected as the total cellular protein extract and quantified by colorimetric DC protein assay (BioRad). Loading samples were prepared with fresh total cellular protein extract (avoiding repeated freeze-thaw cycles), NuPAGE LDS Sample Buffer (4X) (Invitrogen), NuPAGE Reducing Agent (10X) (Invitrogen), and deionized water, and heated at 70 °C for 10 min to denature the proteins. Proteins (20μg/well) were separated by size via gel electrophoresis in gradient polyacrylamide gels (Novex), and transferred onto polyvinylidene difluoride membrane (Millipore). Membranes were blocked in 1x Tris-buffered saline-Tween 20 buffer with 5% non-fat milk (blocking solution), and then probed using primary antibodies of the protein targets of interest, all diluted in the blocking solution (Table 2). Secondary antibodies were used to detect the species-specific portion of the primary antibody, all diluted in the blocking solution (Table 3). Immuno-reactive bands were visualized using SuperSignal West Dura Chemiluminescent Substrate (Thermo Scientific). Relative band intensity was calculated using BioRad ImageLab software and normalized to the quantified protein levels of β-Actin for each respective membrane, as β-Actin served as an appropriate housekeeping gene for this study.

2.2.5 Statistical analysis

All statistical analyses were performed using GraphPad Prism 5 software. All results were expressed as means of normalized values ± SEM. The significance of the differences ($p<0.05$) between normalized mean values was then evaluated using the two-tailed, nonparametric Mann-Whitney test.

Table 2.2. Western Blot primary antibodies, dilutions used in experiments, and company and catalogue information.

Antibody name	*Source*	*Dilution*	*Company (#Catalogue)*
KDEL (GRP78) (10C3)	Mouse monoclonal	1:300	Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA (#sc-58774)
ATF6	Mouse monoclonal	1:600	Novus Biologicals, Oakville, ON, Canada (NBP1-40256)
Phospho-PERK (Thr980) (16F8)	Rabbit monoclonal	1:500	Cell Signaling Technology Inc., Danvers, MA, USA (#3179)
PERK (D11A8)	Rabbit monoclonal	1:500	Cell Signaling Technology Inc., Danvers, MA, USA (#5683)
Phospho-eIF2α (Ser51) (119A11)	Rabbit monoclonal	1:1000	Cell Signaling Technology Inc., Danvers, MA, USA (#3597)
eIF2α	Rabbit monoclonal	1:1000	Cell Signaling Technology Inc., Danvers, MA, USA (#9722)
CREB-2 (ATF4) (C-20)	Rabbit polyclonal	1:5000	Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA (#sc-200)
CHOP (D46F1)	Rabbit monoclonal	1:500	Cell Signaling Technology Inc., Danvers, MA, USA (#5554)
ERO1-Lα	Rabbit polyclonal	1:1000	Cell Signaling Technology Inc., Danvers, MA, USA (#3264)
PDI (C81H6)	Rabbit monoclonal	1:1000	Cell Signaling Technology Inc., Danvers, MA, USA (#3501)
VKORC1 (D-17)	Rabbit polyclonal	1:500	Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA (#sc-54456-R)
Quiescin Q6 (QSOX1) (G-12)	Goat polyclonal	1:500	Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA (#sc-160084)
GPx-7 (S-12)	Goat polyclonal	1:500	Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA (#sc-160062)
Caspase-3 (8G10)	Rabbit monoclonal	1:1000	Cell Signaling Technology Inc., Danvers, MA, USA (#9665)
Caspase-6	Rabbit polyclonal	1:1000	Cell Signaling Technology Inc., Danvers, MA, USA (#9762)
Caspase-7 (D2Q3L)	Rabbit monoclonal	1:1000	Cell Signaling Technology Inc., Danvers, MA, USA (#12827)
Lamin A/C (4C11)	Mouse monoclonal	1:1000	Cell Signaling Technology Inc., Danvers, MA, USA (#4777)
BAX	Rabbit polyclonal	1:500	Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA (#sc-493)
BCL-2	Rabbit polyclonal	1:100	Abcam Inc., Toronto, ON, Canada (#ab7973)
HIF1α	Rabbit polyclonal	1:2000	Novus Biologicals, Oakville, ON, Canada (NB100-479)

GCN2	Rabbit polyclonal	1:1000	Cell Signaling Technology Inc., Danvers, MA, USA (#3302)
β-Actin	Mouse monoclonal	1:50000	Sigma-Aldrich Co., St. Louis, MO, USA Canada (#A3854)

Table 2.3. Western Blot secondary antibodies, dilutions used in experiments, and company and catalogue information.

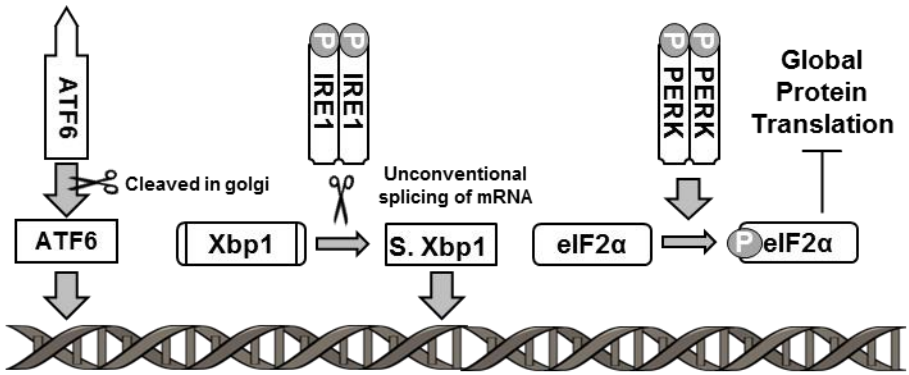
Antibody name	*Dilution*	*Company (#Catalogue)*
Donkey Anti-Rabbit IgG (H+L)	1:10000	Jackson ImmunoResearch Laboratories, West Grove, PA, USA (#711-001-003)
Donkey Anti-Mouse IgG (H+L)	1:5000	Jackson ImmunoResearch Laboratories, West Grove, PA, USA (#715-001-003)
Donkey Anti-Goat IgG (H+L)	1:5000	Jackson ImmunoResearch Laboratories, West Grove, PA, USA (#705-001-003)

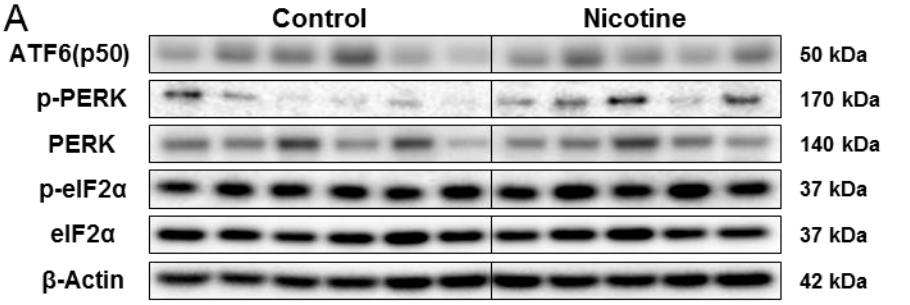
2.3 Results

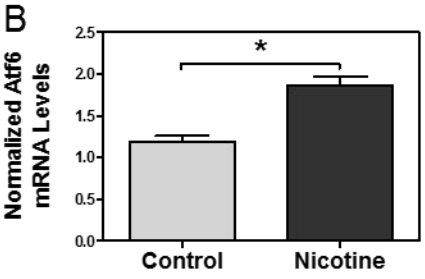
2.3.1 Maternal nicotine exposure leads to augmented ER stress and unfolded protein response activation in embryonic day 15 placenta

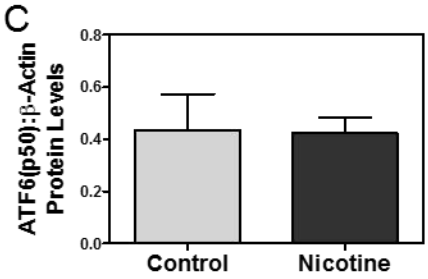
To determine the presence of ER stress in nicotine-exposed placenta, we assessed mRNA and protein levels of the main players involved in the three branches of the UPR (ATF6, IRE1α, and PERK) via Real-Time PCR and Western blot, respectively. Activation of the UPR indicates the presence of ER stress [35]. With respect to the ATF6 branch of the UPR, the steady-state mRNA levels of Atf6 were found to be significantly elevated in e15 nicotine-treated placentas compared to controls ($p<0.05$), however, the protein levels of active ATF6(p50) remained unaltered (Figure 2.1A-C**)**. To determine the activation of the IRE1α branch, splicing of its downstream target, Xbp1 mRNA, was measured and found to be unaltered (Figure 2.1D). However, nicotine exposure led to activation of the PERK branch of the UPR as demonstrated through significantly increased ratios of phosphorylated PERK [Thr980]: total PERK protein levels in nicotine-exposed placentas compared to controls at e15 ($p<0.05$, Figure 2.1A, E). Nicotine exposure also led to significantly increased ratios of phosphorylated eukaryotic initiation factor (eIF) 2α [Ser51]: total eIF2α protein levels in the placenta ($p<0.05$), suggesting global protein translation attenuation (Figure 2.1A, F).

Since activation of the PERK pathway of the unfolded protein response was demonstrated, we decided to next investigate the expression of its potential downstream targets, ATF4, GRP78, and CHOP. ATF4 protein levels were significantly elevated in e15 nicotine-exposed placentas compared to controls ($p<0.01$) with unchanged steady-state mRNA levels (Figure 2.2A-C). GRP78 protein levels were also significantly elevated in nicotine-exposed placentas ($p<0.05$) with unchanged mRNA levels (Figure 2.2A, D-E), revealing post-transcriptional ER-stress-related increases in protein expression. However, nicotine exposure led to increased expression of CHOP ($p<0.05$), indicating prolonged ER stress and potential activation of ER-stress-related apoptotic pathways in e15 placentas (Figure 2.2A, F-G). Collectively, these results confirm the presence of augmented ER stress and unfolded protein response activation of the PERK pathway in nicotine-treated placentas.


ATF6
Cleaved in golgi
ATF6
IRE1
IRE1
Unconventional
splicing of mRNA
Xbp1
S. Xbp1
PERK
PERK
eIF2α
eIF2α
Global
Protein
Translation


A
Control
Nicotine
ATF6(p50)
p-PERK
PERK
p-eIF2α
eIF2α
β-Actin
50 kDa
170 kDa
140 kDa
37 kDa
37 kDa
42 kDa


B
Normalized Atf6
mRNA Levels
2.5
2.0
1.5
1.0
0.5
0.0
*
Control
Nicotine


C
ATF6(p50):β-Actin
Protein Levels
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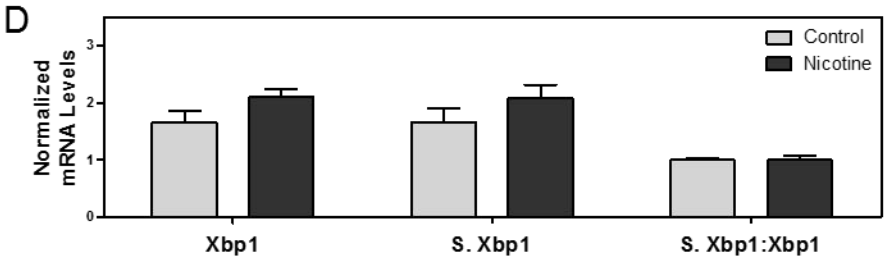
0.6
0.4
0.2
0.0
Control
Nicotine


D
Normalized
mRNA Levels
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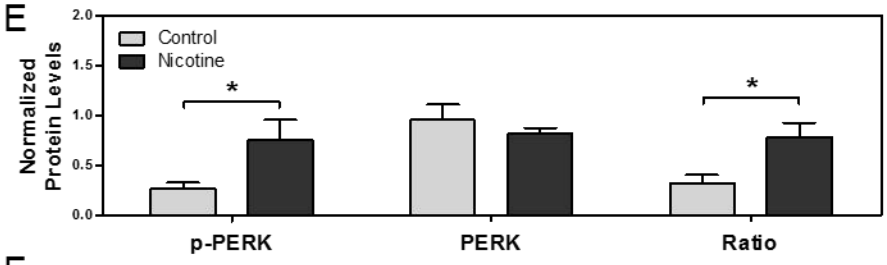
2
1
0
Control
Nicotine
Xbp1
S. Xbp1
S. Xbp1:Xbp1


E
Normalized
Protein Levels
2.0
1.5
1.0
0.5
0.0
Control
Nicotine
*
*
p-PERK
PERK
Ratio

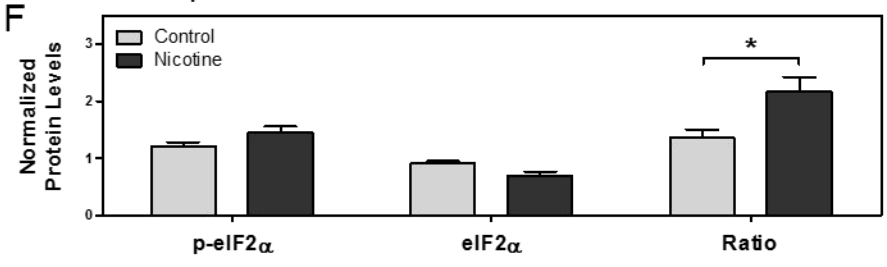

F
Normalized
Protein Levels
3
2
1
0
Control
Nicotine
*
p-eIF2α
eIF2α
Ratio

Figure 2.1. The effect of maternal nicotine exposure on the three branches of the unfolded protein response (ATF6, IRE1α, PERK) in e15 rat placentas. Protein and mRNA levels of targets of interest were determined via Western blot and RT-PCR, respectively. (A) Specific targeted protein bands as detected by respective antibodies via Western blot. (B) Atf6 mRNA levels. (C) ATF6 protein levels. (D) mRNA levels of spliced Xbp1, unspliced Xbp1, and ratio of spliced: unspliced Xbp1. (E) Protein levels of p-PERK, PERK, and ratio of p-PERK:PERK. (F) Protein levels of p-eIF2α, eIF2α, and ratio of p-eIF2α:eIF2α. All protein levels were expressed as means normalized to β-Actin ± SEM (n=5-6/group). All mRNA levels were expressed as means normalized to the geometric mean of three stable housekeeping genes (β-Actin, 18S, and Gapdh) ± SEM (n=5-6/group). *, Significant difference ($p<0.05$). **, Significant difference ($p<0.01$).

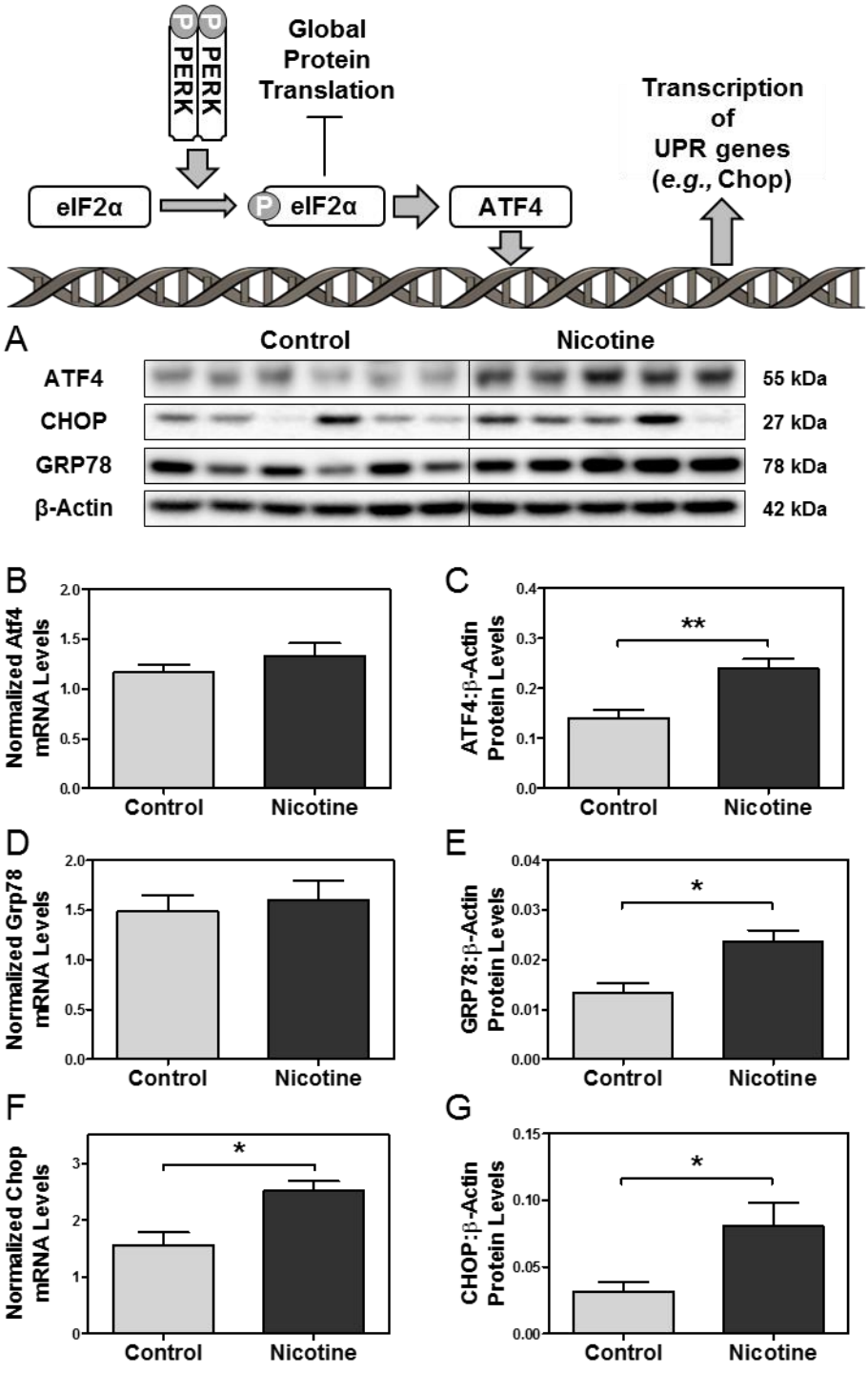

PERK
PERK
Global
Protein
Translation
Transcription
of
UPR genes
(e.g., Chop)
eIF2α
eIF2α
ATF4
A
Control
Nicotine
ATF4
CHOP
GRP78
β-Actin
55 kDa
27 kDa
78 kDa
42 kDa
B
Normalized Atf4 mRNA Levels
Control
Nicotine
C
ATF4:β-Actin Protein Levels
**
D
Normalized Grp78 mRNA Levels
E
GRP78:β-Actin Protein Levels
*
F
Normalized Chop mRNA Levels
G
CHOP:β-Actin Protein Levels

Figure 2.2. Nicotine exposure leads to activation of downstream targets in the PERK branch of the unfolded protein response in e15 rat placentas. Protein and mRNA levels of targets of interest were determined via Western blot and RT-PCR, respectively. (A) Specific targeted protein bands as detected by respective antibodies via Western blot. (B) Atf4 mRNA levels. (C) ATF4 protein levels. (D) Grp78 mRNA levels. (E) GRP78 protein levels. (F) Chop mRNA levels. (G) CHOP protein levels. All protein levels were expressed as means normalized to β-Actin ± SEM (n=5-6/group). All mRNA levels were expressed as means normalized to the geometric mean of three stable housekeeping genes (β-Actin, 18S, and Gapdh) ± SEM (n=5-6/group).*, Significant difference ($p<0.05$). **, Significant difference ($p<0.01$).

2.3.2 The effects of nicotine-induced activation of CHOP on downstream apoptotic pathways

Due to elevated expression of CHOP in nicotine-exposed placentas, we next wanted to determine the expression of downstream apoptotic targets, pro-apoptotic Bax and anti-apoptotic Bcl-2, which are known to synergistically orchestrate apoptosis [37, 71]. To assess the level of apoptotic activation, the ratio of Bax:Bcl-2 mRNA levels were quantified; however, we found no significant difference between groups (Figure 2.3A, B). There was a slight increase in BAX protein and decrease in BCL-2 protein in nicotine-exposed placentas compared to controls, however, neither the markers nor their ratio reached statistically significant differences (Figure 2.3A, C). Maternal nicotine exposure also did not alter the mRNA levels of Bim or Puma (Appendix I, Supplemental Figure 2).

2.3.3 Maternal nicotine exposure does not induce caspase-mediated apoptosis

To further investigate the severity of the ER stress, we measured another major ER stress-related apoptotic pathway, the caspase-mediated apoptosis pathway. We found no significant differences in the protein levels of cleaved caspase-3, 6, 7, nor their substrate Lamin A, between control and nicotine-exposed placentas at e15 (Figure 2.4).

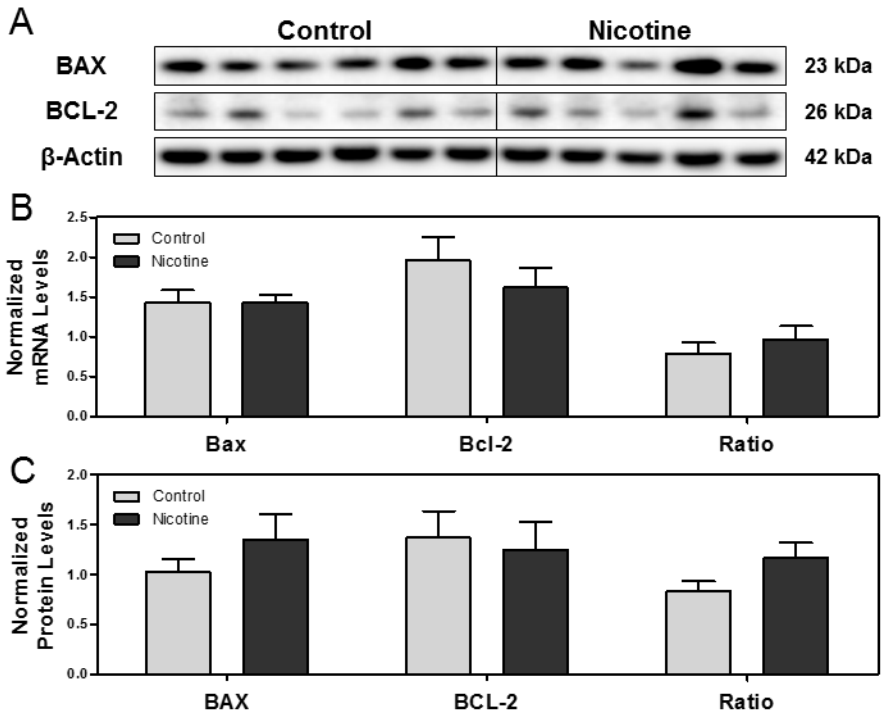


Figure 2.3. The effect of maternal nicotine exposure on downstream CHOP-mediated apoptotic pathways. Protein and mRNA levels of targets of interest were determined via Western blot and RT-PCR, respectively. (A) Specific targeted protein bands as detected by respective antibodies via Western blot. (B) mRNA levels of Bax, Bcl-2, and ratio of Bax: Bcl-2. (C) Protein levels of BAX, BCL-2, and ratio of BAX: BCL-2. All protein levels were expressed as means normalized to β-Actin ± SEM (n=5-6/group). All mRNA levels were expressed as means normalized to the geometric mean of three stable housekeeping genes (β-Actin, 18S, and Gapdh) ± SEM (n=5-6/group).

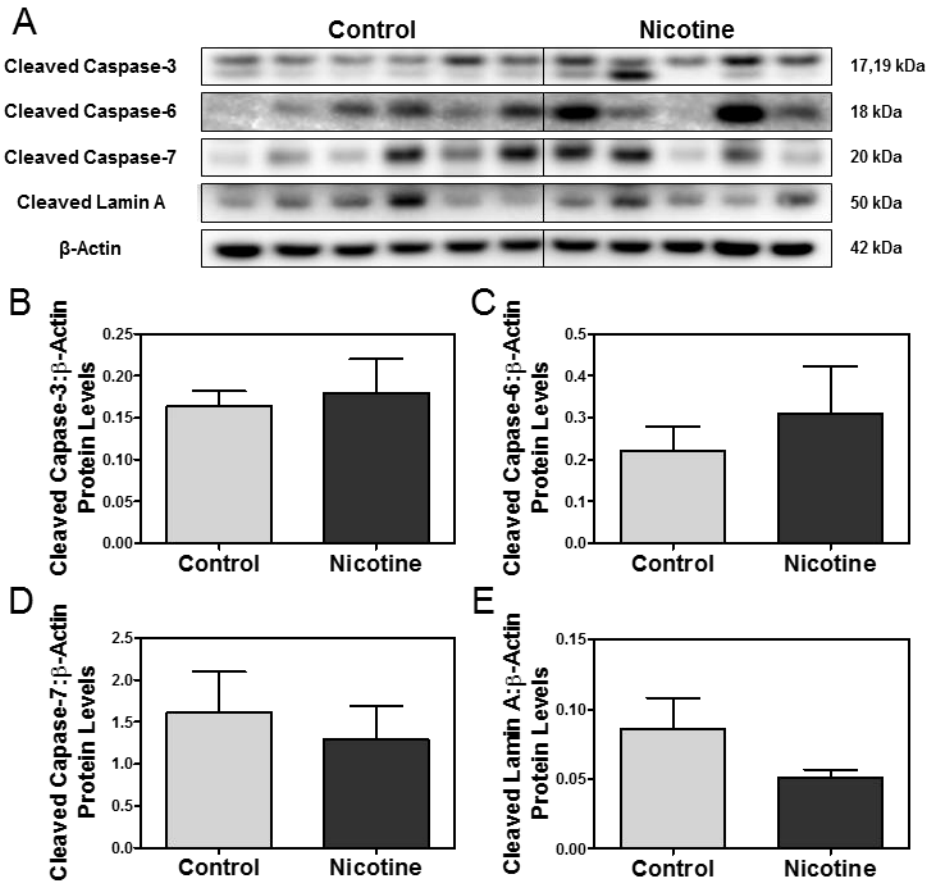


Figure 2.4. The effect of maternal nicotine exposure on downstream caspase-mediated apoptotic pathways. Protein levels of targets of interest were determined via Western blot. (A) Specific targeted protein bands as detected by respective antibodies via Western blot. (B) Cleaved caspase-3 protein levels. (C) Cleaved caspase-6 protein levels. (D) Cleaved caspase-7 protein levels. (E) Cleaved Lamin A protein levels. All protein levels were expressed as means normalized to β-Actin ± SEM (n=5-6/group).

2.3.4 Maternal nicotine exposure down-regulates expression of protein disulfide isomerase and ER oxidoreductases

In order to elucidate the underlying mechanisms of nicotine-induced ER stress in the placenta, we further examined disulfide bond formation, a process intimately connected with ER homeostasis and known to cause ER stress when compromised [52]. Specifically, we were interested in looking at the effects of nicotine on expression of the key isomerase and oxidoreductases that carry out disulfide bond formation. Protein disulfide isomerase (PDI) mediates protein folding by introducing disulfide bonds to nascent proteins through thiol oxidation. ER oxidoreductase, ERO1-Lα/β, will then subsequently reoxidize PDI to continue the redox relay [40, 44, 45]. Interestingly, PDI protein levels were found to be significantly decreased in nicotine-exposed placentas at e15 ($p<0.05$, Figure 2.5A, B**)**. In contrast, the main ER oxidoreductase, ERO1-Lα, demonstrated no significant change between treatment groups (Figure 2.5A, C).

These results provoked further exploration of the expression of alternative ER oxidoreductases (*e.g.*, PRDX4, GPx-7, VKORC1, and QSOX1) recently found to be involved in PDI reoxidation and/or direct thiol oxidation of nascent proteins [72-77]. Real-time PCR revealed decreases in the steady-state mRNA levels of Gpx-7, Vkorc1 ($p<0.05$) and Qsox1 ($p<0.05$, Figure 2.6A, B, D, F). Additionally, QSOX1 was significantly decreased at the protein level in nicotine-treated placentas compared to the controls ($p<0.05$, Figure 2.6A, G).

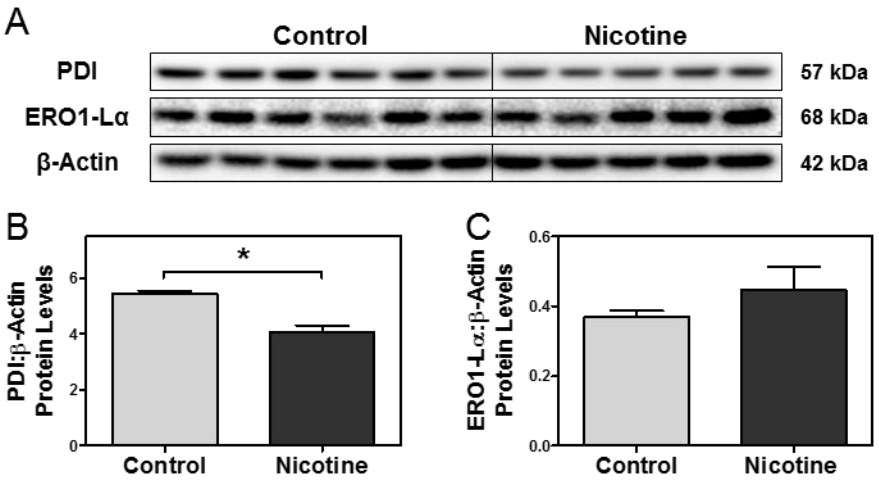


Figure 2.5. Nicotine decreases PDI expression in e15 rat placentas. Protein levels of targets of interest were determined via Western blot. (A) Specific targeted protein bands as detected by respective antibodies via Western blot. (B) PDI protein levels. (C) ERO1-Lα protein levels. All protein levels were expressed as means normalized to β-Actin ± SEM (n=5-6/group). *, Significant difference ($p<0.05$).

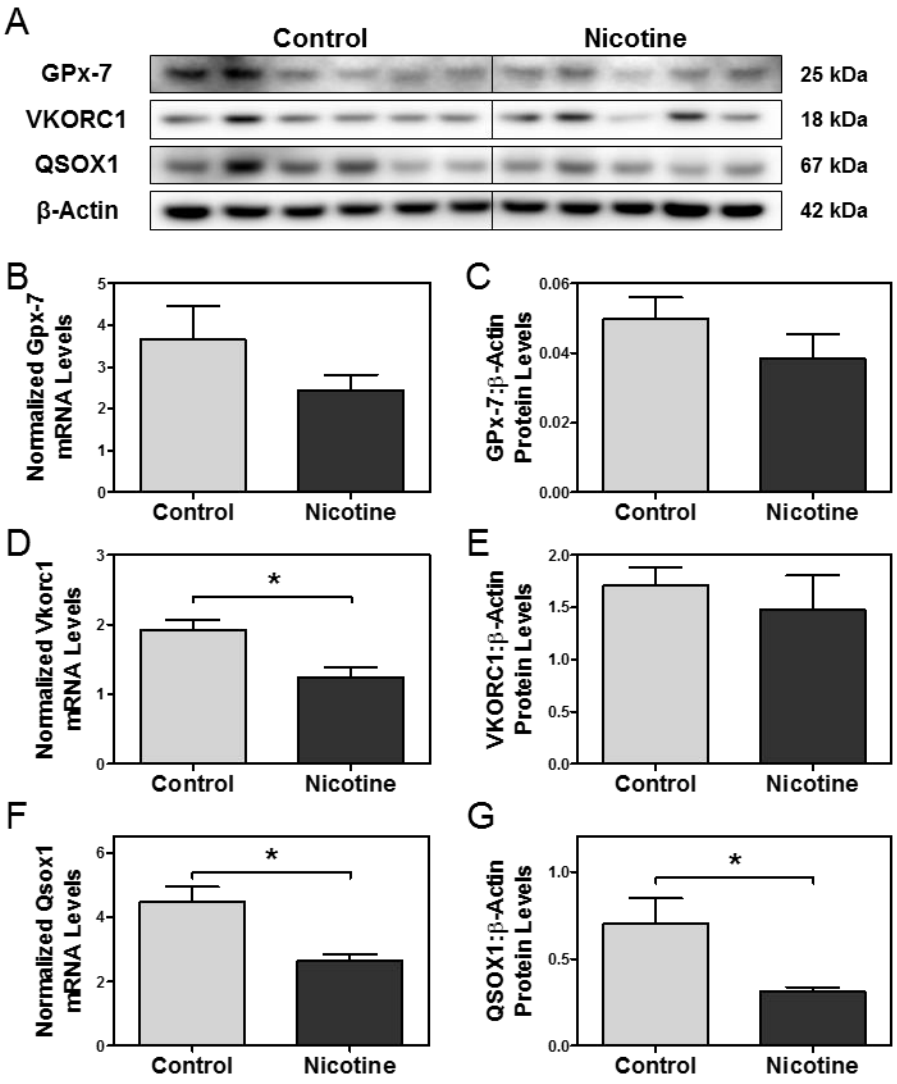


Figure 2.6. The effect of maternal nicotine exposure on various ER oxidoreductases in e15 rat placentas. Protein and mRNA levels of targets of interest were determined via Western blot and RT-PCR, respectively. (A) Specific targeted protein bands as detected by respective antibodies via Western blot. (B) Gpx-7 mRNA levels. (C) GPx-7 protein levels. (D) Vkorc1 mRNA levels. (E) VKORC1 protein levels. (F) Qsox1 mRNA levels. (G) QSOX1 protein levels. All protein levels were expressed as means normalized to β-Actin ± SEM (n=5-6/group). All mRNA levels were expressed as means normalized to the geometric mean of three stable housekeeping genes (β-Actin, 18S, and Gapdh) ± SEM (n=5-6/group). *, Significant difference ($p<0.05$).

2.3.5 Maternal nicotine exposure increases markers of hypoxia and amino acid deprivation

Given that oxygen is the final electron acceptor in post-translational disulfide bond formation [52], we next investigated whether hypoxia was induced by nicotine exposure by measuring placental protein levels of hypoxia-inducible factor (HIF) 1α [78, 79]. Western blot revealed that HIF1α protein levels were significantly elevated in e15 nicotine-treated placentas compared to the controls ($p<0.05$, Figure 2.7A, B). We were also interested in exploring whether additional insults induced by the vasoconstrictive effects of nicotine were present (*e.g.*, low amino acid supply) [48]. General control non-depressible 2 (GCN2) is a protein kinase that responds to amino acid starvation by up-regulating transcription factors (*e.g.*, GCN4) to mediate nutrient deprivation. GCN2 also acts as an alternative kinase to phosphorylate eIF2α alongside PERK to attenuate protein translation [80, 81]. Western blot revealed that GCN2 protein levels were strongly elevated in e15 nicotine-treated placentas compared to the controls ($p<0.01$, Figure 2.7A, C).

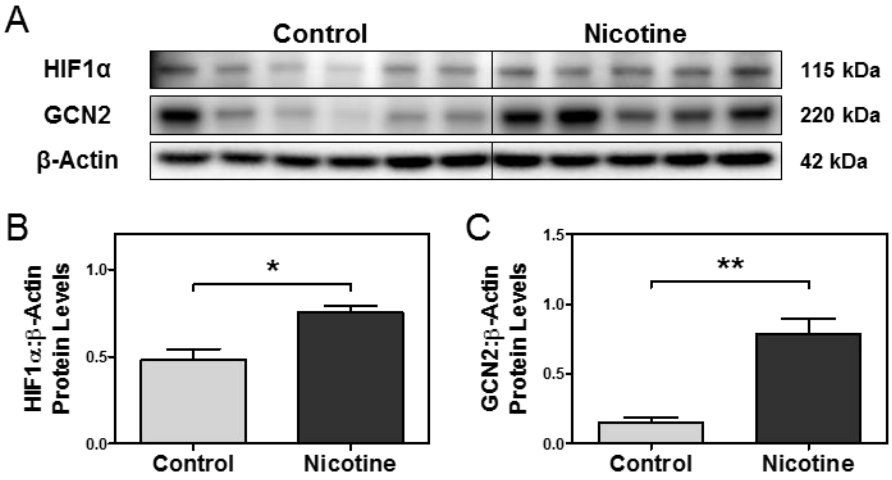


Figure 2.7. Nicotine-induced vasoconstriction leads to both hypoxia and reduced amino acid supply in e15 placenta. Protein levels of targets of interest were determined via Western blot. (A) Specific targeted protein bands as detected by respective antibodies via Western blot. (B) HIF1α protein levels. (C) GCN2 protein levels. All protein levels were expressed as means normalized to β-Actin ± SEM (n=5-6/group). *, Significant difference ($p<0.05$). **, Significant difference ($p<0.01$).

2.4 Discussion

In the current study, we have demonstrated that nicotine exposure in pregnant rats leads to augmented ER stress in the e15 placenta. We were interested in selecting a time-point during pregnancy when nicotine exposure was previously shown to cause structural and morphological aberrations in the rat placenta, prior to exhibiting any observable fetal growth deficit [17, 25]. Given that ER stress and placental insufficiency were observed to precede human intrauterine growth restriction [26, 27, 29, 31, 33], the presence of augmented ER stress exhibited in e15 nicotine-exposed rat placentas reveal a potential mechanism through which nicotine may cause adverse placental and fetal outcomes in pregnant mothers who are smoking or undergoing nicotine replacement therapy.

An elegantly conducted study by DuRose et al. (2006) revealed intrinsic differences between the three UPR pathways (ATF6, IRE1α, and PERK) in their abilities to sense and recognize distinct types of ER perturbations [82]. We demonstrated that maternal nicotine exposure selectively activates the PERK unfolded protein response pathway in e15 rat placentas. Increases in the steady-state levels of Atf6 mRNA proposes possible involvement of the ATF6 branch, however, the lack of change in the protein levels and transcript levels of downstream target (*e.g.,* Grp78), do not strongly support this at this particular time-point. Activation of PERK induces phosphorylation of eIF2α, which attenuates global protein translation to reduce the incoming protein load [83-85]. Phosphorylated eIF2α also paradoxically elevates translation of mRNA transcripts with conserved upstream open reading frames, such as Atf4, which was demonstrated through unchanged Atf4 mRNA levels, but significantly increased protein levels in our nicotine-treated placentas compared to controls [86-88]. Interestingly, GRP78 protein levels were also found to be significantly up-regulated in nicotine-treated placentas compared to controls, amidst unchanged mRNA levels. Grp78 mRNA transcription is more commonly regulated by ATF6 and IRE1α branches of the UPR; however, various post-transcriptional mechanisms (*e.g.,* alternative translation initiation due to eIF2α phosphorylation) have been demonstrated to independently regulate protein levels of GRP78 in the presence of ER stress regardless of transcript levels [89, 90].

CHOP, which may be up-regulated by ATF4 and/or phosphorylated eIF2α, is known to amplify various downstream apoptosis pathways (*e.g.*, down-regulation of anti-apoptotic BCL-2 expression, translocation of BCL-2-associated X protein (BAX) to mitochondria to amplify death pathway, etc.) [38, 71, 91-95]. However, the minimal changes seen in the ratio of BAX: BCL-2, Bim, and Puma expression despite significantly increased CHOP expression perhaps suggests an early stage of CHOP activation in e15 nicotine-exposed placentas, when downstream apoptosis have not yet been fully elicited. The other possibility is that although CHOP is involved in the translocation of BAX to the mitochondria, it may not be involved in up-regulating the transcription of Bax [94]. The lack of change seen in other caspase markers further indicates the absence of nicotine effects on these specific apoptosis pathways at this particular time point. However, the expression of placental genes previously found to be influenced by nicotine (*e.g.*, up-regulation of Vegf and down-regulation of Glut-1) are also altered in the same manner by tunicamycin (a known inducer of ER stress), without any reported activation of apoptosis [17, 29, 96, 97]. This collectively suggests that the structural and morphological aberrations in nicotine-exposed placentas may be due to the ER stress-induced alterations in gene expression caused by nicotine, instead of pathological apoptosis [17]. Therefore, the nicotine-induced unfolded protein response at e15 may possibly be attempting to avoid apoptosis by re-establishing some manner of sub-optimal placental homeostasis to adapt to the ER stress experienced.

Activation of the unfolded protein response also reveals possible dysfunction of protein maturation. Disulfide bond formation is critical for successful co- and post-translational modifications during protein maturation, and impairment is known to lead to ER stress [35, 98]. Traditionally, PDI and/or QSOX1 expression increases during hypoxia, tunicamycin or thapsigargin-induced ER stress to further assist with protein folding and disulfide bond formation [99-102]. PDI has also been found to be up-regulated in the lungs of chronic smokers, perhaps as a protective response against the oxidative damage of chronic cigarette smoke exposure [103-105]. However, down-regulation of mRNA transcripts of essential isomerases and oxidoreductases in disulfide bond formation (*e.g.*, Vkorc1, Qsox1) was seen in the nicotine-exposed rat placentas at e15. Protein levels were also seen to be significantly down-regulated in a few markers (*e.g.*, PDI, QSOX1), though to a lesser

degree compared to the change in mRNA levels. It is possible that these transcripts initially down-regulated by nicotine are being subsequently stabilized at the protein level by the unfolded protein response, which seeks to post-transcriptionally up-regulate their protein levels in the presence of ER stress, as seen in previous studies [99-105]. This may explain the milder changes seen in protein levels of VKORC1 and QSOX1, despite strong decreases in their transcript levels. Interestingly, cigarette smoke has recently been demonstrated to also lead to excessive posttranslational oxidation of PDI, abating its functionality in the formation of disulfide bonds [60]. Given that inhibition of PDI is also known to disrupt protein folding and augment ER stress, it may be stipulated that the nicotine-induced down-regulation of PDI and other oxidoreductases at e15 may still be contributing in part to the augmentation of ER stress, despite the adaptive efforts of the unfolded protein response to stabilize their protein levels [46]. In addition to the impairment on disulfide bond formation, the influence that nicotine may have on nicotinic acetylcholine receptor-directed calcium signaling may also influence ER homeostasis and stress activation (please refer to section 1.1.2 for background on nicotinic acetylcholine receptor activation.) Regardless, more studies must be conducted on the effects of nicotine on calcium signaling in UPR activation and PDI and oxidoreductase regulation to further address these speculations.

It is noteworthy that increased expression of HIF1α, alongside previously reported increases in CA-IX expression, jointly reveals hypoxia in nicotine-exposed placentas [17, 78, 79]. The increase in hypoxia may be due to nicotinic antagonism of nAChR α9, which induces vasoconstriction of placental vasculature to reduce oxygen supply [48, 49]. Koritzinsky *et al.* (2013) recently identified oxygen as the terminal electron acceptor in post-translational disulfide bond formation, further implicating the impairment of protein maturation in hypoxia-induced ER stress [52]. Additionally, vasoconstriction is known to reduce nutrient and amino acid supply to the placenta [48]. Nicotine has also been documented to depress amino acid transport system A and block acetylcholine-mediated nutrient delivery in trophoblasts, collectively hindering the maternal transport of many essential amino acids to the feto-placental unit [106-108]. Significantly increased expression of GCN2 indeed reveals amino acid starvation in nicotine-exposed placentas [80, 81]. Furthermore, GCN2 is an alternative kinase of eIF2α and may be partially

responsible for its phosphorylation alongside PERK to cooperatively initiate an integrated stress response to hypoxia/ER stress and amino acid starvation [109]. However, future research is directed to further investigate the relationships between low amino acid supply and impaired disulfide bond formation.

In summary, this study has demonstrated that nicotine alone can induce ER stress and evoke an integrated stress response in the rat placenta, as revealed through PERK- and GCN2-activation of the p-eIF2α-ATF4-CHOP axis (summarized in Figure 2.8). Recent studies have demonstrated the induction of ER stress via super-physiological nicotine dosages or cigarette smoke [55-61]; however, our study was the first to induce ER stress in the rat placenta through an *in vivo* model of maternal nicotine exposure using physiological nicotine dosages. Furthermore, we provide novel insight by demonstrating this in association with impairment of the disulfide bond formation pathway, as shown through nicotine-induced down-regulation of PDI and QSOX1 expression and increased hypoxia. By elucidating that maternal nicotine exposure is linked to placental ER stress and impaired disulfide bond formation, this may contribute to the development of more efficacious interventions (*e.g.*, Tauroursodeoxycholic acid to relieve ER stress [110]). More importantly, given that nicotine alone exerts severe effects on placental function, and consequently, on fetal and postnatal health, this study further implicates that greater caution is required for women considering nicotine replacement therapy for smoking cessation in pregnancy.

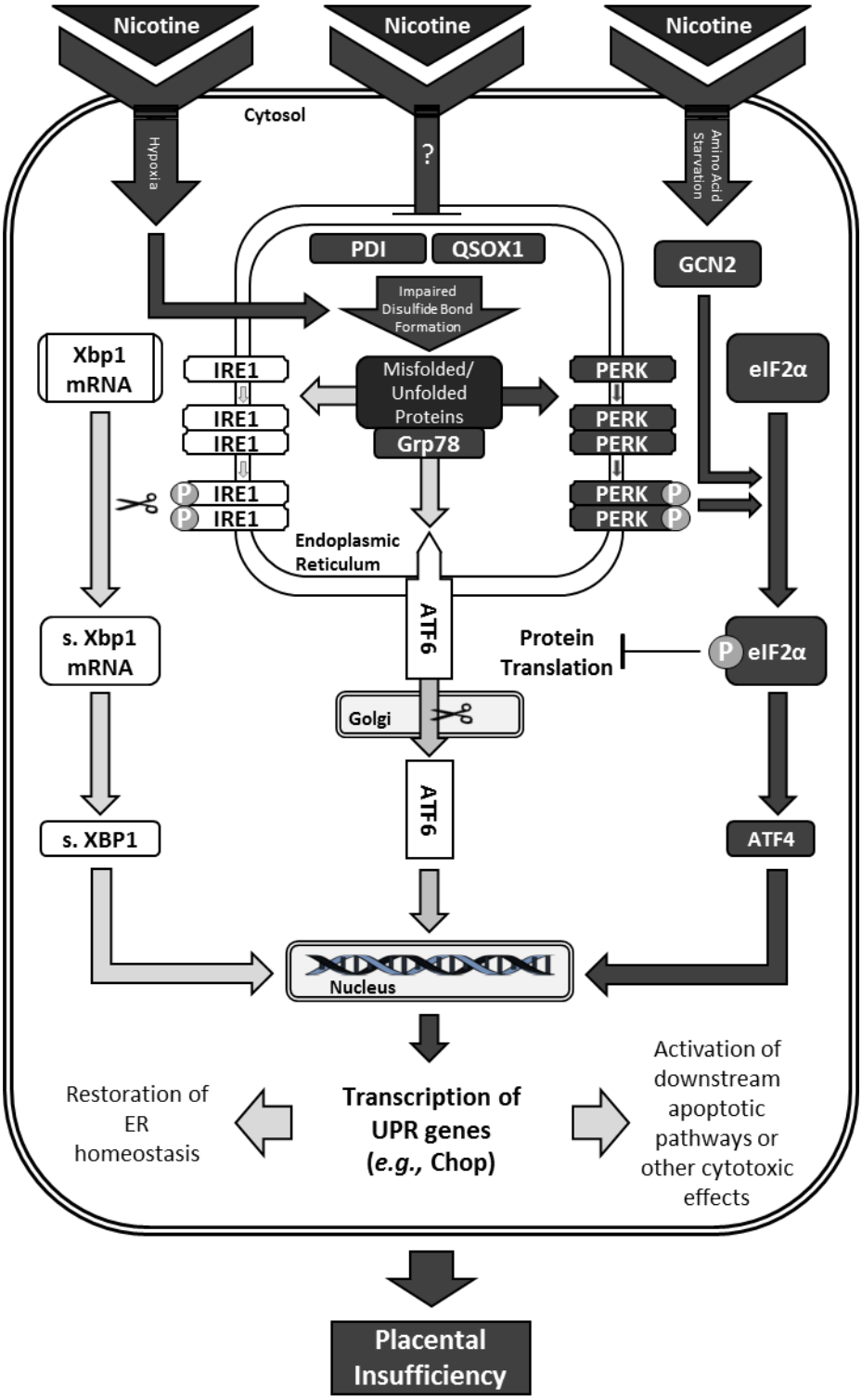

Nicotine
Nicotine
Nicotine
Cytosol
Hypoxia
?
Amino Acid Starvation
PDI
QSOX1
GCN2
Impaired Disulfide Bond Formation
Xbp1 mRNA
IRE1
IRE1
IRE1
P IRE1
P IRE1
Misfolded/ Unfolded Proteins
Grp78
PERK
PERK
PERK
PERK P
PERK P
eIF2α
Endoplasmic Reticulum
ATF6
Protein Translation
P eIF2α
s. Xbp1 mRNA
Golgi
ATF6
s. XBP1
ATF4
Nucleus
Restoration of ER homeostasis
Transcription of UPR genes (e.g., Chop)
Activation of downstream apoptotic pathways or other cytotoxic effects
Placental Insufficiency

Figure 2.8. Proposed schematic of the effect of nicotine on ER stress and the unfolded protein response in the e15 placenta. Pathways affected by nicotine are indicated by the darkened arrows and boxes. In summary, nicotine exposure was shown to augment ER stress and activate the unfolded protein response in the e15 placenta. Activation was most prominent in the PERK branch and was demonstrated in association with impaired disulfide bond formation. Nicotine is proposed to impair disulfide bond formation through direct or indirect down-regulation of PDI and other oxidoreductases. Disulfide bond formation is further impaired through increased hypoxia as caused by nicotine-induced vasoconstriction. Additionally, up-regulation of GCN2 suggests amino acid starvation and activation of the integrated stress response to further phosphorylate eIF2α. However, the lack of BAX and caspase activation seen at e15 suggests that the nicotine-induced ER stress response may possibly be attempting to avoid apoptosis by re-establishing some manner of sub-optimal placental homeostasis to adapt to the ER stress experienced.

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3 EXPOSURE TO NICOTINE AND HYPOXIA *IN* VITRO INDUCES THE ENDOPLASMIC RETICULUM STRESS RESPONSE IN RCHO-1 PLACENTAL TROPHOBLAST GIANT CELLS

A version of this chapter is currently being prepared for submission: **Wong MK and Hardy DB**. Nicotine exposure directly induces the endoplasmic reticulum stress response in Rcho-1 placental trophoblast giant cells.

3.1 Introduction

Nicotine exposure during pregnancy remains a rampant issue worldwide. Recent studies reported that approximately 10-28% of pregnant women across various countries continued to engage in cigarette smoking [1-3], but exposure to nicotine can also occur through nicotine replacement therapies (NRTs), e-cigarettes, and other forms of non-combustible tobacco products [4-6]. The risk of nicotine exposure through NRTs or e-cigarettes during pregnancy had been long overlooked in comparison to the known hazards of traditional cigarette smoking. However, findings from animal studies revealed many deleterious long-term health consequences in the offspring exposed to nicotine alone *in utero*, demanding a careful reevaluation of the impact of maternal nicotine exposure [6, 7].

Maternal nicotine exposure leads to structural, morphological, and functional defects in rat placentation *in vivo* [8]. The lipid-soluble nature of nicotine allows it to rapidly traverse past membrane barriers into the placenta where it competes with endogenous acetylcholine for binding to nAChRs. Many nAChR subtypes are expressed in both human and rat placentas, and nAChRs in rat placental trophoblast cells were previously demonstrated to be responsive to a large range of nicotine concentrations (10^{-3} to 10^{-9} M) [8-10]. Nicotine can impair trophoblast cell differentiation, migration, and invasion *in vitro* in a dose-dependent manner, revealing a potential *direct* effect of nicotine exposure that may be responsible for compromised placental development [11]. Alternatively, nicotine can also lead to vasoconstriction in placental vasculature *in vivo*, which may induce hypoxia in placental cells, revealing a potential *indirect* effect of nicotine exposure on poor placental development [10, 12]. Nonetheless, the mechanisms by which these different nicotine-induced effects compromise placentation have yet to be elucidated.

Augmented endoplasmic reticulum (ER) stress was recently suggested to underlie the detrimental phenotype seen in nicotine-exposed placentas [13]. ER stress is generally defined as the cellular perturbation of ER homeostasis leading to the accumulation of misfolded or unfolded proteins [14]. The unfolded protein response (UPR) is activated to alleviate the stress through various signaling pathways that either decreases global protein translation or increases protein folding capacity [15]. However, under prolonged ER stress and UPR activation, the cell will activate downstream apoptosis, which has been well-

associated with compromised placental growth both *in vivo* and *in vitro* [16-20]. Due to the broad scope of effects that nicotine can elicit in the placenta that may potentially contribute to ER stress *in vivo*, we were interested in further investigating the potential underlying mechanisms at the cellular level.

The rat serves as a suitable model for studying placentation due to its similarities to humans in hemochorial placental structure, deep intrauterine invasion through both endovascular and interstitial pathways, close analogy of cell types, and trophoblast-directed remodeling of uterine spiral arteries [21, 22]. During rat placentation, trophoblast stem cells are established from the blastocyst and may give rise to at least five cell types [21]. The one of interest to our study will be the trophoblast giant (TG) cell due to their large population in the rat placenta, cardinal involvements in early establishment through uterine invasion and anastomosis of maternal blood supply, and later maintenance of a healthy pregnancy through key endocrine roles in local and systemic physiological adaptations [23-25]. Given that nicotine negatively impacts TG cell function and differentiation [8], and ER stress underlies poor placentation [16, 19, 20], we were interested in determining the direct and indirect effects of nicotine exposure on ER stress in placental TG cells.

Consequently, we were also interested in investigating whether we could relieve nicotine-induced ER stress via treatment with Tauroursodeoxycholic acid (TUDCA), an endogenous taurine-conjugated ursodeoxycholic bile acid produced by intestinal bacteria [26]. Exogenous application of TUDCA has previously relieved ER stress and stabilized UPR activation in several cell and tissue types [27-29], but its efficacy as a therapeutic agent in the placenta and on nicotine-induced ER stress have yet to be examined.

Therefore, this study aims to investigate: (1) the *direct* effect of nicotine exposure through nAChR activation on ER stress, (2) the *indirect* effect of nicotine exposure through hypoxia on ER stress, and (3) the capacity of TUDCA to prevent nicotine-induced ER stress. We hypothesize that nicotine exposure will directly induce ER stress in Rcho-1 TG cells through nAChR activation, the indirect effects of nicotine exposure through placental hypoxia will also induce ER stress, and there will be an interaction effect between nicotine

and hypoxia to jointly augment placental ER stress. We further hypothesize that TUDCA will prevent nicotine-induced ER stress and UPR activation.

3.2 Materials and Methods

3.2.1 Experimental model

Rcho-1, a cell line derived from rat choriocarcinoma, was used to study the effects of nicotine exposure on ER stress in placental TG cells. Rcho-1 cells may be maintained in either proliferative stem cell-like or differentiated TG cell state based on the culture conditions [30]. Rcho-1 trophoblast stem (TS) cells were plated at 1.5 x 10^6 cells/mL and cultured at 37ºC in 95% room air/5% CO_2 [30]. Proliferation was maintained by growing Rcho-1 TS cells in RPMI-1640 media supplemented with 20% fetal bovine serum. At 80-90% confluency (after 3 days of proliferation), TG cell differentiation was induced by switching to NCTC-135 media supplemented with 1% horse serum. Removal of essential nutrients in the fetal bovine serum halted proliferation and encouraged differentiation into TG cells. NCTC-135 media was used as it was experimentally determined to provide better pH regulation and decreased toxicity during differentiation [30].

3.2.2 RNA extraction and Real Time-Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from cells using TRIzol reagent (Invitrogen). Chloroform (Sigma-Aldrich) was added to the solution, and then centrifuged at 12,500rpm. Supernatant was transferred to a fresh tube with an equal volume of isopropanol (Sigma-Aldrich) and centrifuged again at 12,500rpm. Total RNA was then collected from the pellet and dissolved in DEPC-treated water. Deoxyribonuclease I, Amplification Grade (Invitrogen) was added to the RNA to digest contaminating single- and double-stranded DNA. Four µg of RNA were reverse-transcribed to cDNA using random hexamers and Superscript II Reverse Transcriptase (Invitrogen). Primer sets directed against gene targets of interest were designed through National Center for Biotechnology Information's primer designing tool and generated via Invitrogen Custom DNA Oligos (Table 3.1). Quantitative analysis of mRNA expression was performed via RT-PCR using fluorescent nucleic acid dye SsoFast EvaGreen supermix (BioRad) and BioRad CFX384 Real Time System. The

cycling conditions were 95 °C for 10 min, followed by 43 cycles of 95 °C for 15 sec and 60 °C for 30 sec and 72 °C for 30 sec. The cycle threshold was set so that exponential increases in amplification were approximately level between all samples. Relative fold changes were calculated using the comparative cycle times (Ct) method, normalizing all values to the geometric mean of two housekeeping genes (β-Actin and Gapdh). Suitable housekeeping genes were determined using algorithms from GeNorm [31], Normfinder [32], BestKeeper [33], and the comparative ΔCt method [34] to provide an overall ranking of the most stable housekeeping genes (available online at http://www.leonxie.com/referencegene.php). Given all primer sets had equal priming efficiency, the ΔCt values for each primer set were calibrated to the average of all control Ct values, and the relative abundance of each primer set compared with calibrator was determined by the formula $2^{\Delta\Delta Ct}$, in which ΔΔCt was the normalized value.

Table 3.1. Forward and reverse sequences for the primers used for RT-PCR.

Gene	*Forward*	*Reverse*	*GenBank/ Reference*
Pl-1	TGACTTTGACTCTTTCGGGCT	GCTCTGAATACACCGAGAGCG	[35]
Pgf	GTGAGTATGCTGAGCCTAAGGG	AGACCTTACAAGACATGGATTCCC	NM_053595.2
Hand1	GCGAAAGCAAGCGGAAAAGG	CAGCCAGTGCGTCCTTTAATCC	NM_021592.2
β-Actin	CACAGCTGAGAGGGAAAT	TCAGCAATGCCTGGGTAC	NM_031144
Gapdh	GGATACTGAGAGCAAGAGAGAGG	TCCTGTTGTTATGGGGTCTGG	NM_017008.4

3.2.3 Protein extraction and Western Blot

Cells were homogenized in RIPA buffer (50 mM Tris-HCL, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P40, 0.25% $C_{24}H_{39}NaO_4$, supplemented with phosphatase inhibitors (20 mM NaF, 40mM Na-pyrophosphate, 40mM Na_3VO_4, 200mM β-glycerophosphate disodium salt hydrate), and a protease inhibitor cocktail (Roche)). The solution was sonicated at 30% amplitude for 5 sec total, 1 sec per pulse. It was then mixed in a rotator for 10 min at 4 °C and centrifuged at 16000g for 20 min at 4 °C. The resulting supernatant was collected as the total cellular protein extract and quantified by colorimetric DC protein assay (BioRad). Loading samples were prepared with fresh total protein extract (avoiding repeated freeze-thaw cycles), NuPAGE LDS Sample Buffer (4X) (Invitrogen), NuPAGE Reducing Agent (10X) (Invitrogen), and deionized water, and heated at 70 °C for 10 min to denature the proteins. Proteins (20μg/well) were separated by size via gel electrophoresis in gradient polyacrylamide gels (Novex), and transferred onto polyvinylidene difluoride membrane (Millipore). Membranes were blocked in 1x Tris-buffered saline-Tween 20 buffer with 5% non-fat milk (blocking solution), and then probed using primary antibodies of the protein targets of interest, diluted in the blocking solution (Table 3.2). Secondary antibodies were used to detect the species-specific portion of the primary antibody, diluted in the blocking solution (Table 3.3). Immuno-reactive bands were visualized using SuperSignal West Dura Chemiluminescent Substrate (Thermo Scientific). Relative band intensity was calculated using BioRad ImageLab software and normalized to the quantified total protein on each respective membrane, as determined through Amido black staining.

3.2.4 Statistical analysis

All statistical analyses were performed using GraphPad Prism 5 software. All results were expressed as means of normalized values ± SEM. All experiments were replicated four times (n=4). The significance of the differences ($p<0.05$) between normalized mean values were then evaluated using either one-way ANOVA followed by Tukey's post-test, or two-way ANOVA followed by Bonferroni's post-test to compare individual and interactive effects of treatments where appropriate.

Table 3.2. Western Blot primary antibodies, dilutions used in experiments, and company and catalogue information.

Antibody name	*Source*	*Dilution*	*Company (#Catalogue)*
BiP (GRP78)	Rabbit polyclonal	1:1000	Cell Signaling Technology Inc., Danvers, MA, USA (#3183)
P-PERK (Thr981)	Rabbit polyclonal	1:800	Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA (#sc-32577)
PERK (D11A8)	Rabbit monoclonal	1:500	Cell Signaling Technology Inc., Danvers, MA, USA (#5683)
Phospho-eIF2α (Ser51) (119A11)	Rabbit monoclonal	1:1000	Cell Signaling Technology Inc., Danvers, MA, USA (#3597)
eIF2α	Rabbit monoclonal	1:1000	Cell Signaling Technology Inc., Danvers, MA, USA (#9722)
CHOP (D46F1)	Rabbit monoclonal	1:500	Cell Signaling Technology Inc., Danvers, MA, USA (#5554)
Caspase-3 (8G10)	Rabbit monoclonal	1:1000	Cell Signaling Technology Inc., Danvers, MA, USA (#9665)
HIF1α	Rabbit polyclonal	1:2000	Novus Biologicals, Oakville, ON, Canada (NB100-479)
Phospho-mTOR (Ser2448)	Rabbit polyclonal	1:1000	Cell Signaling Technology Inc., Danvers, MA, USA (#2971)
mTOR	Rabbit polyclonal	1:1000	Cell Signaling Technology Inc., Danvers, MA, USA (#2972)
Akt1	Rabbit monoclonal	1:1000	Cell Signaling Technology Inc., Danvers, MA, USA (#2938)
β-Actin	Mouse monoclonal	1:50000	Sigma-Aldrich Co., St. Louis, MO, USA Canada (#A3854)

Table 3.3. Western Blot secondary antibodies, dilutions used in experiments, and company and catalogue information.

Antibody name	*Dilution*	*Company (#Catalogue)*
Donkey Anti-Rabbit IgG (H+L)	1:10000	Jackson ImmunoResearch Laboratories, West Grove, PA, USA (#711-001-003)

3.3 Results

3.3.1 Verification of Rcho-1 TG cell differentiation

TG cells differentiation was confirmed either through phase contrast microscopy of distinct morphological traits (*i.e.*, multiple nuclei, large cell body) (Figure 3.1A) and/or through quantitative measurement of prolactin-I (Pl-1) mRNA levels via RT-PCR, which are uniquely expressed by TG cells (Figure 3.1B**)**.

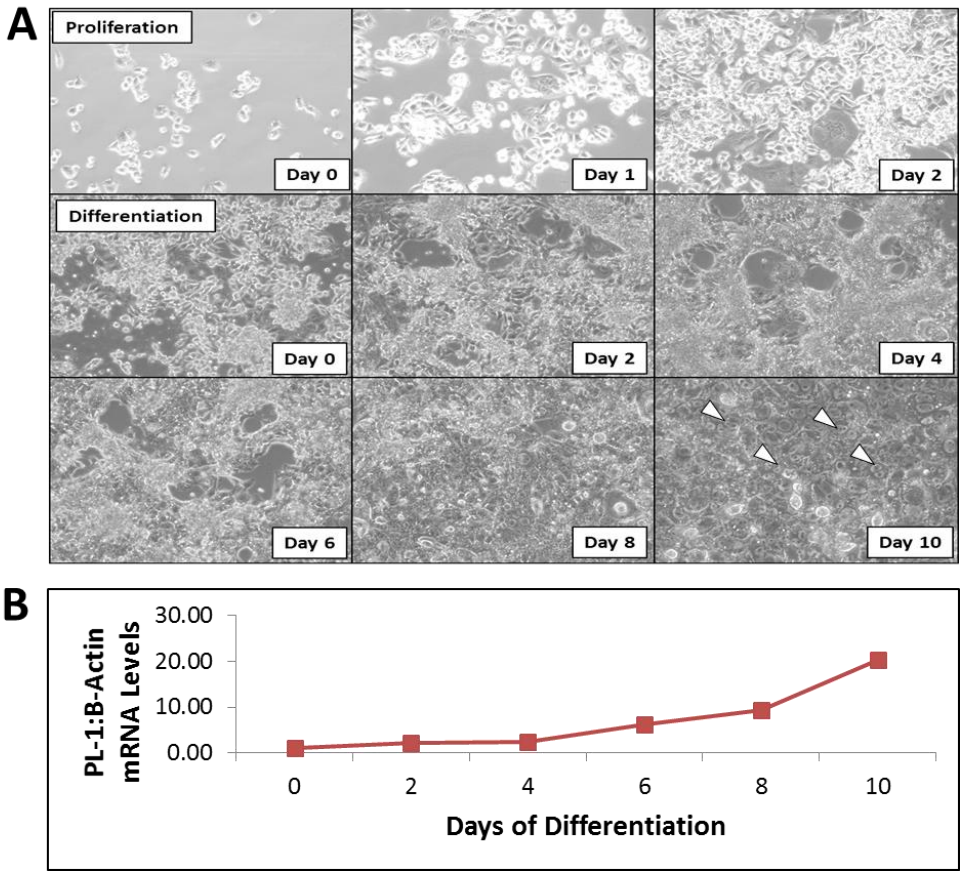


Figure 3.1. Different methods used to detect the presence of differentiated Rcho-1 TG cells. (A) Phase contrast microscopic images (10X) throughout 10 days of differentiation. (B) mRNA levels of Pl-1 as measured through RT-PCR. White triangles identify several representative TG cells.

3.3.2 Nicotine exposure dose-dependently increased PERK activation in Rcho-1 placental TG cells

After 10 days of differentiation, Rcho-1 TG cells were treated with various doses of nicotine (0.1-100μM) for 6 or 24 hours. The nicotine doses encompassed (and surpassed, at 100μM) the average serum concentrations of nicotine (25nM-25μM) previously reported in pharmacokinetic studies of cigarette smoking and/or NRTs [36-41]. The 6 hour time-point ensured detection of rapid protein phosphorylation events (*e.g.,* phosphorylation of PERK, eIF2α) and the 24 hour time-point allowed detection of later changes in protein and mRNA expression. Activation of the UPR indicated the presence of ER stress [14].

Increasing nicotine concentration (0.1-100μM) led to a dose-dependent activation of the PERK branch of the unfolded protein response in Rcho-1 TG cells. This was demonstrated through the increasing ratio of phosphorylated (P)-PERK:PERK protein levels with increasing nicotine dose at 6 hours, reaching significantly elevated levels at 10μM and 100μM nicotine exposure compared to control ($p<0.05$, $p<0.001$, respectively; Figure 3.2A, B). At 24 hours, the ratio of P-PERK:PERK protein levels were still significantly changed with different nicotine doses, but increases were no longer dose-dependent ($p<0.01$; Figure 3.2A, C).

Downstream of PERK, a similar response to nicotine was seen in the phosphorylation of eIF2α. At 6 hours, the ratio of P-eIF2α:eIF2α dose-dependently increased with nicotine concentration, reaching significantly increased levels at 100μM nicotine compared to control ($p<0.01$; Figure 3.3A, B). However, protein levels were no longer significantly different from one another at 24 hours (Figure 3.3A, C). Protein levels of CHOP, a transcription factor downstream of P-eIF2α involved in activating ER stress-related apoptotic pathways during chronic ER stress [42-48], remained unaltered at 6 hours. Levels appeared to be trending towards an increase at 24 hours, but remained non-significantly changed (Figure 3.3A, D-E).

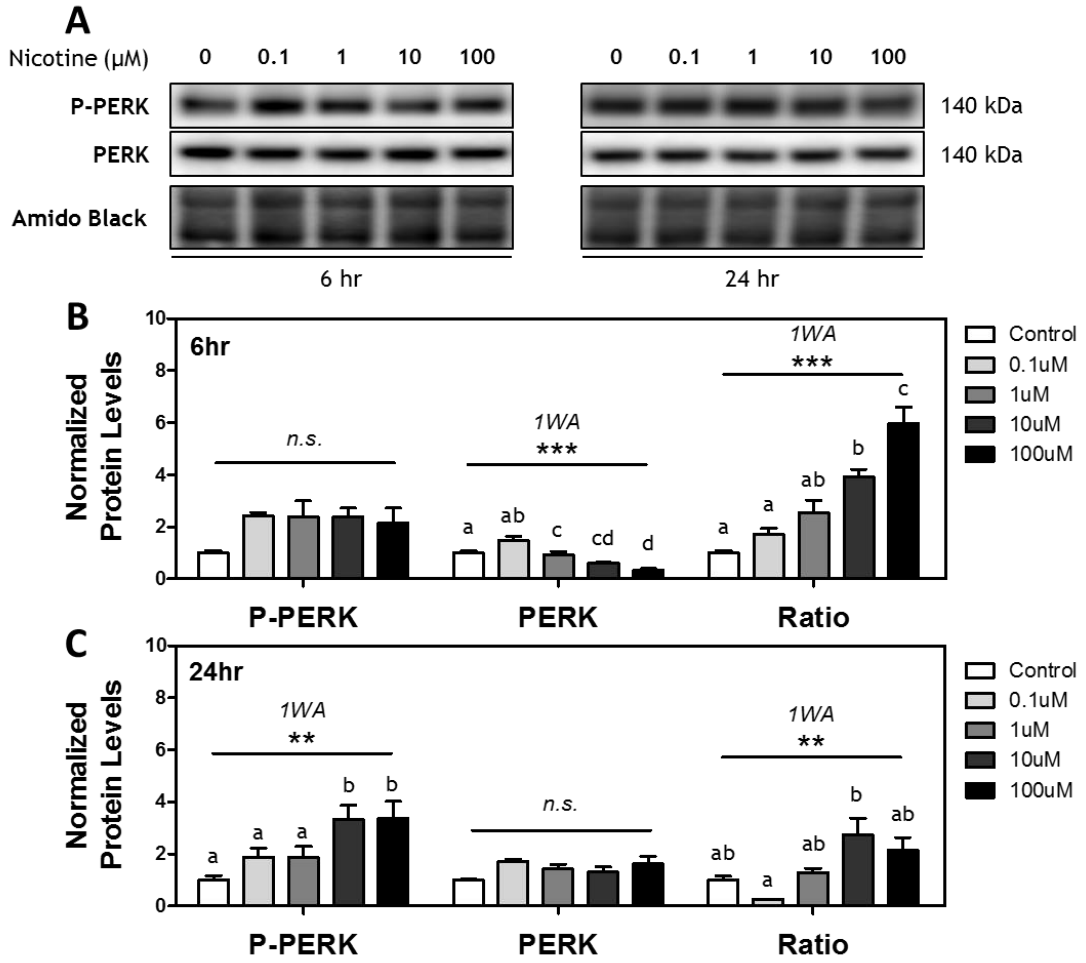


Figure 3.2. The effect of various doses of nicotine (0.1-100μM) on phosphorylation of PERK after 6 and 24 hours in Rcho-1 TG cells. (A) Specific targeted protein bands as detected by respective antibodies via Western blot. (B) Protein levels of P-PERK, PERK, and ratio of P-PERK:PERK at 6 hours and (C) 24 hours of nicotine exposure. All arbitrary values were expressed as means normalized to Amido Black ± SEM. All experiments were performed in quadruplicates (n=4). Significant differences between treatment groups as determined by one-way ANOVA (1WA) indicated by ** ($p<0.01$) or *** ($p<0.001$). Different letters represent means that are significantly different from one another according to Tukey's post-test ($p<0.05$). Non-significant differences ($p>0.05$) indicated by n.s.

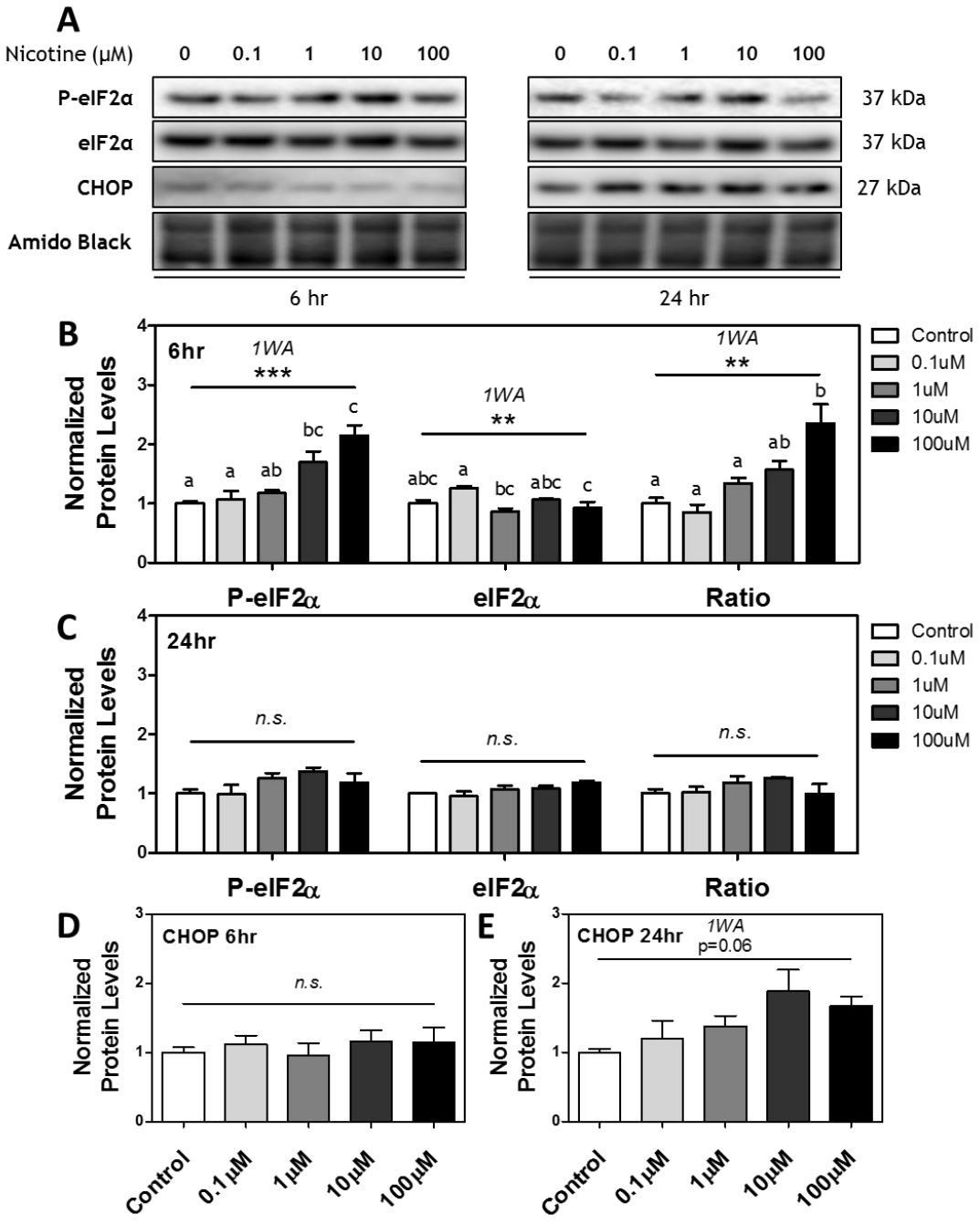

A
Nicotine (μM)
0
0.1
1
10
100
P-eIF2α
eIF2α
CHOP
Amido Black
37 kDa
37 kDa
27 kDa
6 hr
24 hr
B
6hr
1WA
***
1WA
**
1WA
**
Normalized Protein Levels
P-eIF2α
eIF2α
Ratio
Control
0.1uM
1uM
10uM
100uM
C
24hr
n.s.
n.s.
n.s.
D
CHOP 6hr
n.s.
E
CHOP 24hr
1WA
p=0.06
Control
0.1μM
1μM
10μM
100μM

Figure 3.3. The effect of various doses of nicotine exposure (0.1-100μM) on UPR targets downstream of PERK after 6 and 24 hours in Rcho-1 TG cells. (A) Specific targeted protein bands as detected by respective antibodies via Western blot. (B) Protein levels of P-eIF2α, eIF2α, and ratio of P-eIF2α:eIF2α at 6 hours and (C) 24 hours of nicotine exposure. (D) Protein levels of CHOP at 6 hours and (E) 24 hours of nicotine exposure. All arbitrary values were expressed as means normalized to Amido Black ± SEM. All experiments were performed in quadruplicates (n=4). Significant differences between treatment groups as determined by one-way ANOVA (1WA) indicated by ** ($p<0.01$) or *** ($p<0.001$). Different letters represent means that are significantly different from one another according to Tukey's post-test ($p<0.05$). Non-significant differences ($p>0.05$) indicated by n.s.

To investigate several other markers of the UPR, we measured protein levels of two chaperone proteins: BiP/GRP78, a chaperone protein up-regulated during ER stress to assist with protein re-folding [49], and PDI, a key enzyme and chaperone protein involved in disulfide bond formation in protein folding [50-53]. Both markers were found to be unaltered by nicotine after 6 and 24 hours (Figure 3.4A-E).

To assess general placental TG cell function and differentiation in response to varying doses of nicotine (0, 1, 10μM), we measured the steady-state mRNA levels of Pl-1, placental growth factor (Pgf), and heart-and-neural-crest-derivatives-expressed 1 (Hand1) via RT-PCR. Pl-1 is a member of the placental lactogen/pro-lactin family of hormones, uniquely expressed by TG cells, and carries many biological roles in pregnancy (*i.e.*, mammary gland development, immune regulation, stimulation of progesterone biosynthesis in corpus luteum) [30, 54]. Pgf is a member of the vascular endothelial growth factor family and carries important roles in placental vasculogenesis and angiogenesis [55]. Hand1 is a transcription factor essential for TG cell differentiation and placental development [24]. Pl-1, Pgf, and Hand1 mRNA levels did not demonstrate any significant changes in response to nicotine compared to the control after 24 hours (Figure 3.5A-C).

In addition, we also measured the effect of varying doses of nicotine (1-100μM) on protein levels of ATF4, GCN2, and VKORC1. There were no significant differences across the nicotine doses in these targets (Appendix I, Supplemental Figure 3).

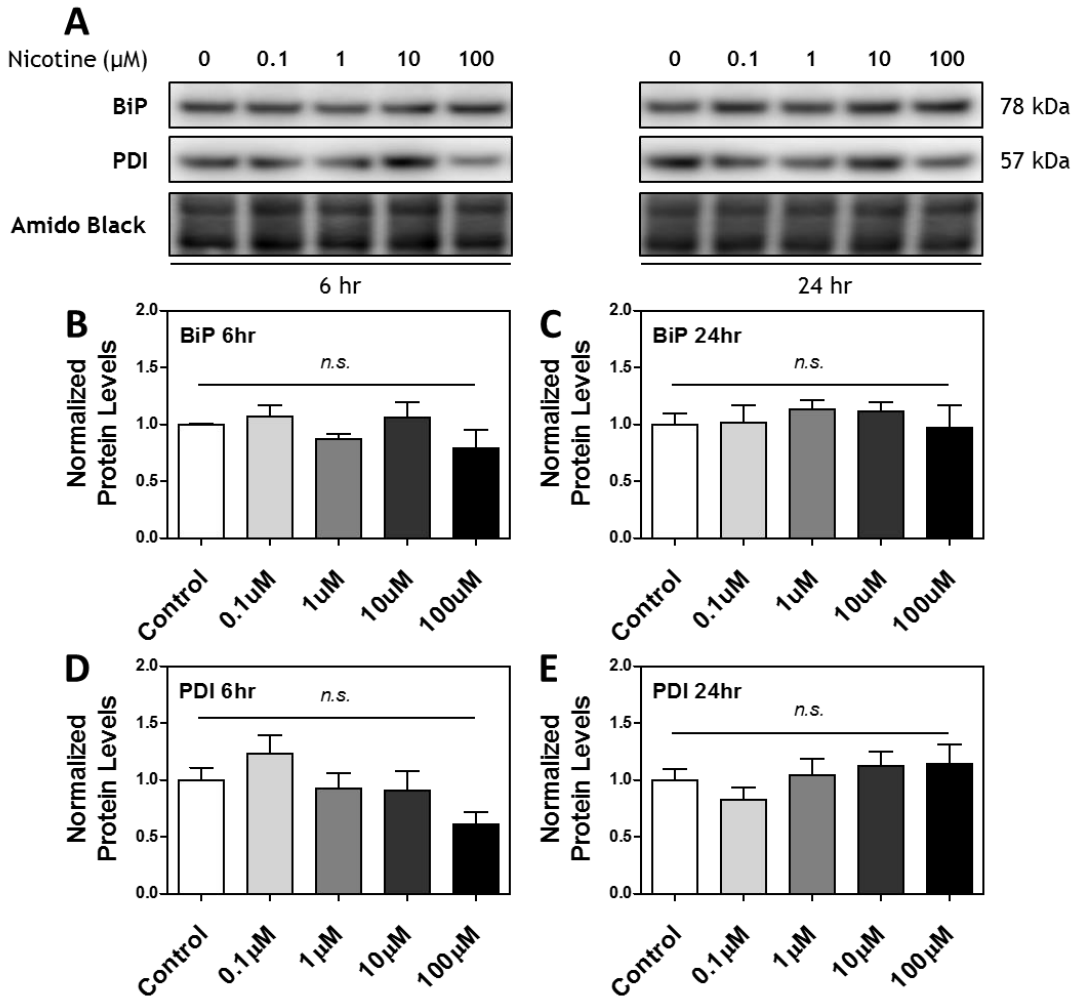


Figure 3.4. The effect of various doses of nicotine exposure (0.1-100μM) on markers of the unfolded protein response after 6 and 24 hours in Rcho-1 TG cells. (A) Specific targeted protein bands as detected by respective antibodies via Western blot. (B) Protein levels of BiP at 6 hours and (C) 24 hours of nicotine exposure. (D) Protein levels of PDI at 6 hours and (E) 24 hours of nicotine exposure. All arbitrary values were expressed as means normalized to Amido Black ± SEM. All experiments were performed in quadruplicates (n=4). Non-significant differences ($p>0.05$) indicated by n.s.

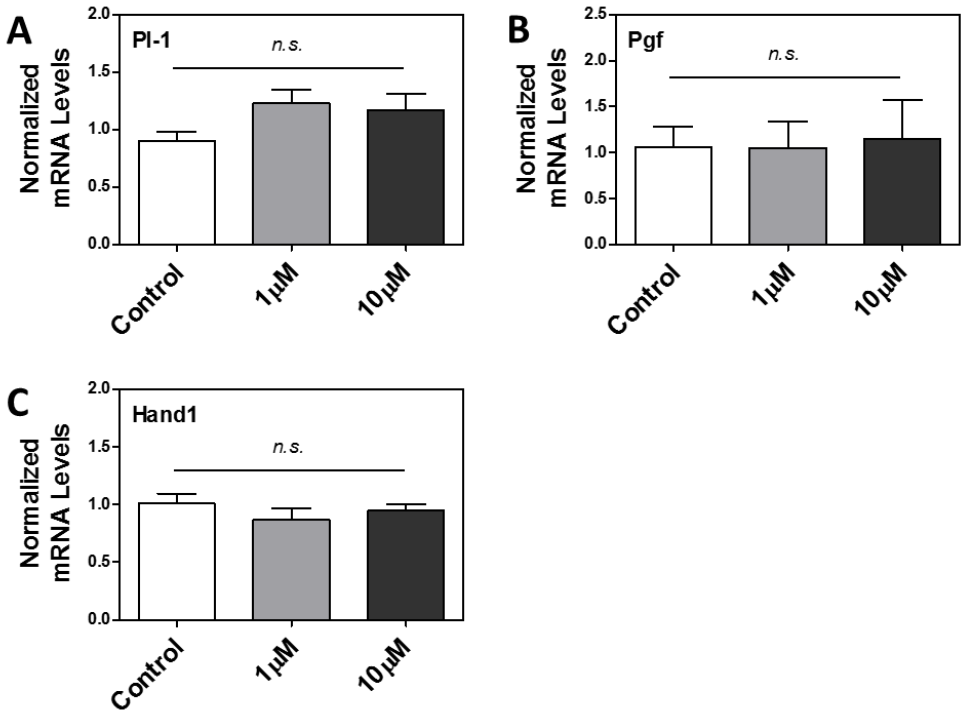


Figure 3.5. The effect of various doses of nicotine (0, 1, 10μM) on markers of placental TG cell function and differentiation after 24 hours in Rcho-1 TG cells. mRNA levels of (A) Pl-1, (B) Pgf, and (C) Hand1. All arbitrary values were expressed as means normalized to the geometric mean of β-Actin and Gapdh ± SEM. All experiments were performed in quadruplicates (n=4). Non-significant differences ($p>0.05$) indicated by n.s.

3.3.3 Nicotine-induced PERK phosphorylation can be blocked by nAChR antagonist

To further verify the direct binding of nicotine to nAChRs, Rcho-1 TG cells were pre-treated for 1 hour with 10μM mecamylamine hydrochloride (MH; a non-competitive, total nAChR inhibitor), and then treated with 10μM nicotine for 6 or 24 hours. MH dose was chosen based on previously published findings demonstrating effective nAChR blocking against nicotine [56-58]. We selected PERK phosphorylation as the protein target to quantify the effect due to the previously observed robust changes to nicotine. Treatment with 10μM nicotine led to significantly increased PERK phosphorylation ($P<0.05$), and pre-treatment for 1 hour with 10μM MH completely blocked nicotine-induced increases in PERK phosphorylation ($p<0.05$; Figure 3.6A, B), verifying involvement of direct binding of nicotine to nAChRs in the induction of ER stress. MH pre-treatment alone did not induce any effects on PERK phosphorylation.

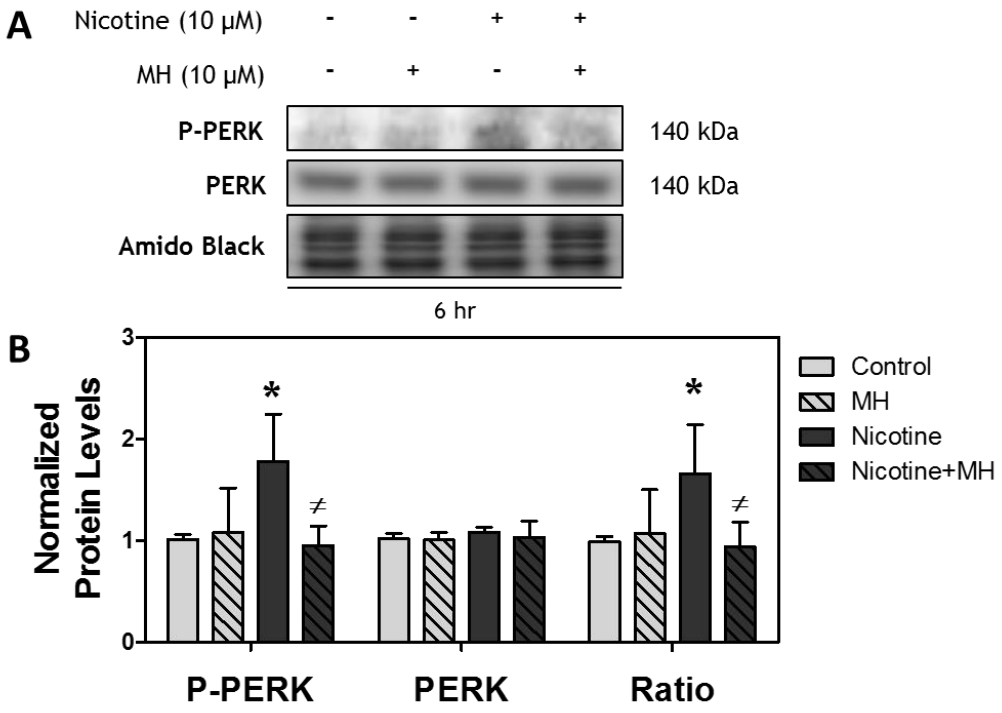


Figure 3.6. Pre-treatment with 10μM MH blocked nicotine-induced PERK phosphorylation after 6 hours in Rcho-1 TG cells. (A) Specific targeted protein bands as detected by respective antibodies via Western blot. (B) Protein levels of P-PERK, PERK, and ratio of P-PERK: PERK at 6 hours. All arbitrary values were expressed as means normalized to Amido Black ± SEM. All experiments were performed in quadruplicates (n=4).

3.3.4 Hypoxia dose-dependently increased the phosphorylation of PERK in Rcho-1 rat placental TG cells *in vitro*

We previously demonstrated that maternal nicotine exposure can induce hypoxia (*e.g.*, increased HIF1α, CA-IX) in the rat placenta *in vivo* [8, 13]. Given that hypoxia is also capable of augmenting ER stress [59, 60], we sought to next investigate the possible effects of hypoxia on placental ER stress in Rcho-1 TG cells. After 10 days of differentiation in NCTC-135 + 1% horse serum at 37ºC in 95% room air/5% CO_2 [30], Rcho-1 TG cells were exposed to either 20%, 8%, or 1% oxygen for 6 or 24 hours. The range of oxygen concentrations included: the recommended *in vitro* oxygen tension for growing Rcho-1 cells (20%), the representative physiological oxygen tension for second or third trimester placentas (8%; considered mild hypoxia), and the oxygen tension previously shown to effectively induce hypoxia in trophoblast cells (1%; considered moderate hypoxia) [61]. Oxygen levels were regulated through the use of individual hypoxic culturing chambers. To prevent the effects of hypoxic re-oxygenation, cells were sealed inside appropriately acclimated Ziploc bags during transfer from hypoxic chamber and instantly lysed in RIPA or TRIzol for protein or RNA isolation, respectively.

To verify the effective induction of hypoxia through decreased oxygen levels in Rcho-1 TG cells, we measured HIF1α protein levels at 20%, 8%, and 1% oxygen. Rcho-1 TG cells exposed to 8% and 1% oxygen demonstrated significantly increased HIF1α protein levels after 6 hours ($p<0.001$) and 24 hours ($p<0.05$) compared to 20% oxygen (Figure 3.7A-C), verifying increasing hypoxia with decreasing oxygen.

Decreasing oxygen levels also led to significant dose-dependent increases in the ratio of P-PERK:PERK at both 6 hours ($p<0.05$) and 24 hours ($p<0.01$; Figure 3.8A-C), suggesting increasing PERK activation and ER stress induction with increasing hypoxia.

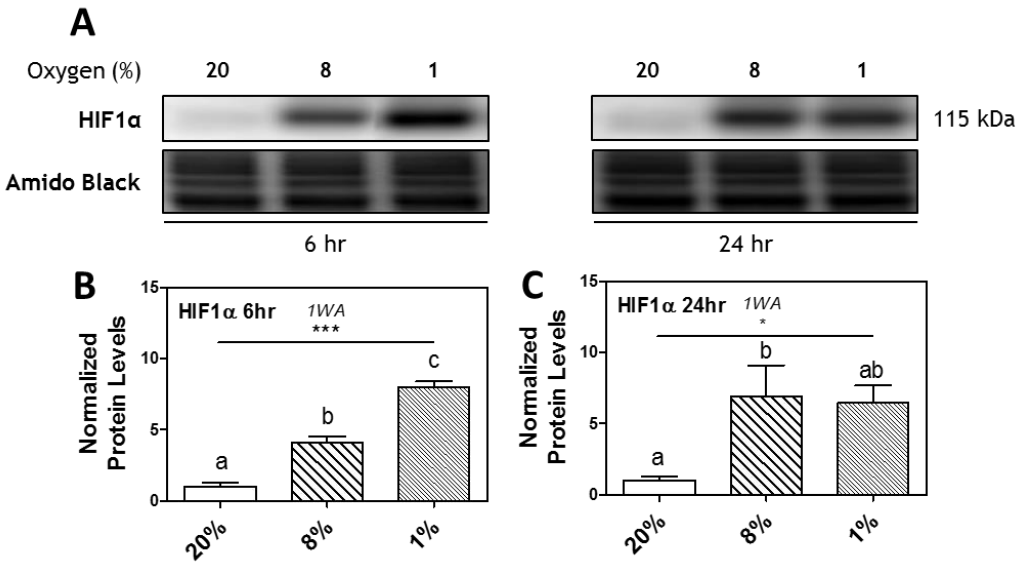


Figure 3.7. Decreasing oxygen levels induced HIF1α protein levels after 6 and 24 hours in Rcho-1 TG cells. (A) Specific targeted protein bands as detected by respective antibodies via Western blot. (B) Protein levels of HIF1α after 6 hours, and (C) 24 hours. All arbitrary values were expressed as means normalized to Amido Black ± SEM. All experiments were performed in quadruplicates (n=4). Significant differences between treatment groups as determined by one-way ANOVA (1WA) indicated by * ($p<0.05$) or *** ($p<0.001$). Different letters represent means that are significantly different from one another according to Tukey's post-test ($p<0.05$).

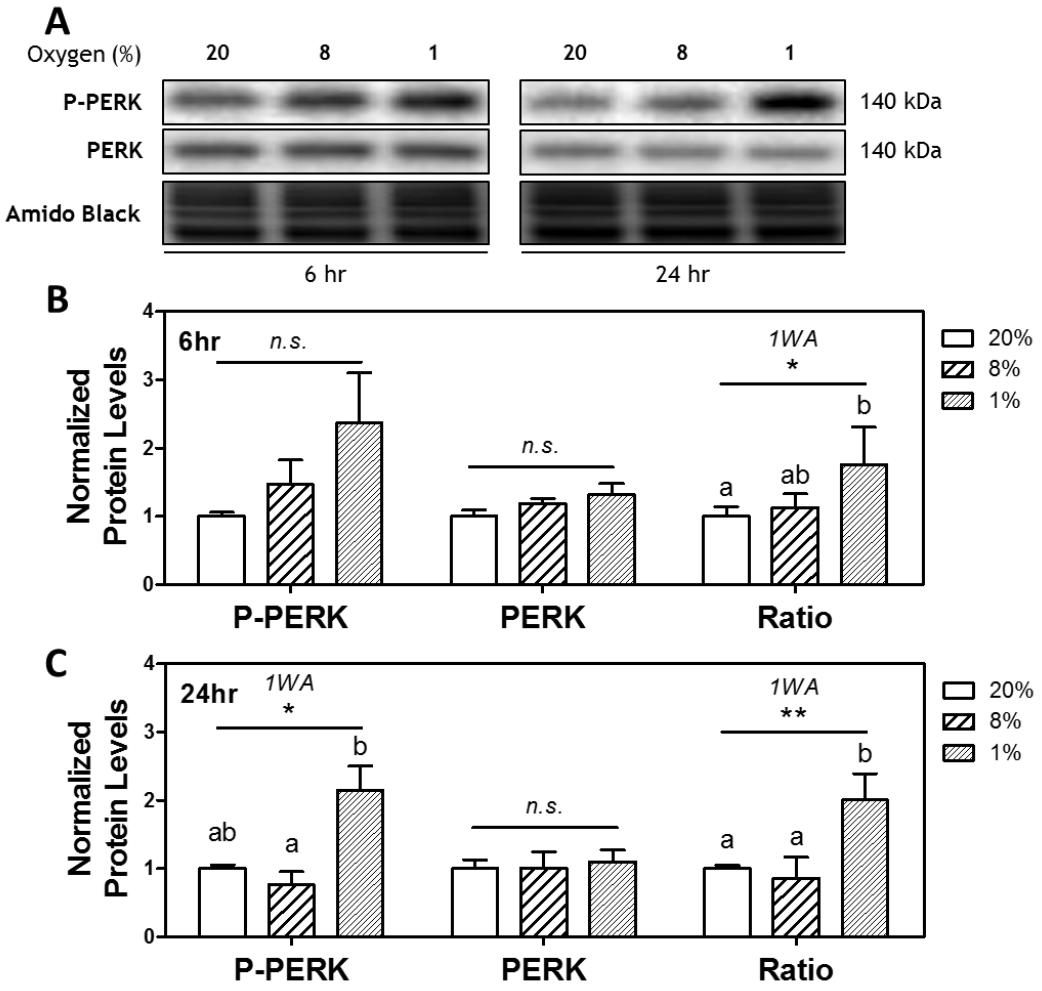
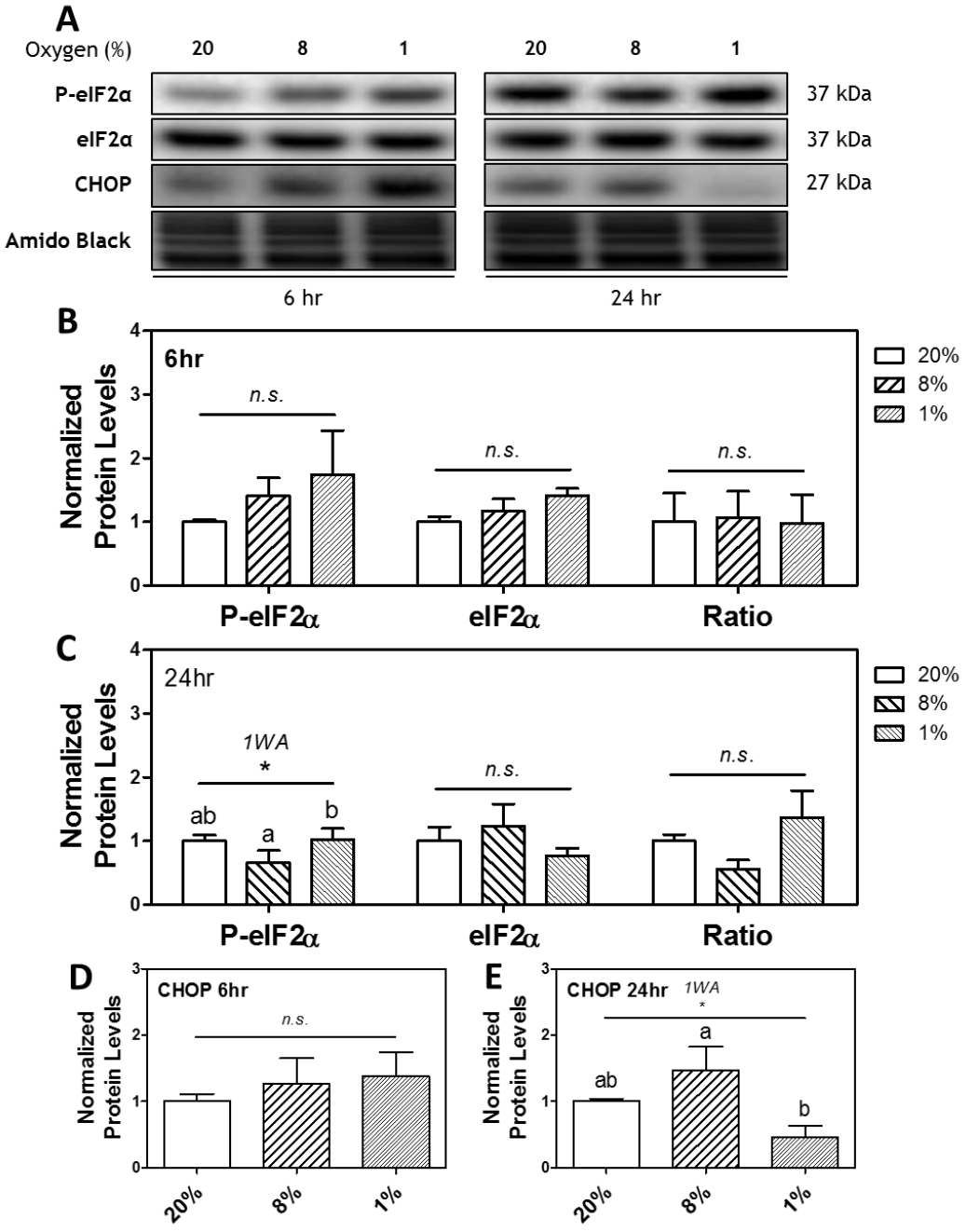


Figure 3.8. Decreasing oxygen levels induced dose-dependent increases in phosphorylation of PERK after 6 and 24 hours in Rcho-1 TG cells. (A) Specific targeted protein bands as detected by respective antibodies via Western blot. (B) Protein levels of P-PERK, PERK, and ratio of P-PERK:PERK after 6 hours, and (C) 24 hours. All arbitrary values were expressed as means normalized to Amido Black ± SEM. All experiments were performed in quadruplicates (n=4). Significant differences between treatment groups as determined by one-way ANOVA (1WA) indicated by * ($p<0.05$) or ** ($p<0.01$). Different letters represent means that are significantly different from one another according to Tukey's post-test ($p<0.05$). Non-significant differences ($p>0.05$) indicated by n.s.

Downstream of PERK, 1% oxygen led to a significant increase in protein levels of P-eIF2α compared to 8% oxygen, though neither were significantly different from 20% oxygen (Figure 3.9A, C). There were no significant changes in the ratio of P-eIF2α:eIF2α with changing oxygen levels at 6 or 24 hours (Figure 3.9A-C). Protein levels of CHOP appeared to be trending towards a dose-dependent increase with decreasing oxygen levels at 6 hours, though it did not reach statistical significance. Varying oxygen levels also led to significant changes in CHOP protein levels at 24 hours ($p<0.05$) (Figure 3.9A, E). Interestingly, CHOP protein levels were not significantly different at 8% nor 1% O_2 compared to 20% oxygen. However, CHOP protein levels were significantly decreased at 1% oxygen compared to 8% oxygen. This was surprising, as CHOP expression was previously demonstrated to be selectively up-regulated in the presence of ER stress and UPR activation in rat placentas *in vivo* and mouse embryonic fibroblasts *in vitro* [13, 62]. Therefore, we wondered if this decrease in CHOP was perhaps attributable to some non-UPR-directed regulation of protein expression (*i.e.,* Akt1-mTOR pathway) in response to moderate hypoxia [63].

To inquire into the possible involvement of non-UPR-directed translational regulation of CHOP at 24 hours, we measured the protein levels of Akt1 and the phosphorylation of mTOR due to their canonical role in protein translation [63]. We found significant changes in protein levels of Akt1 ($p<0.05$) and the phosphorylation of mTOR ($p<0.05$) with varying oxygen levels (Figure 3.10A-C). Trends mirrored the changes on CHOP, where neither 8% nor 1% oxygen caused any changes in protein levels compared to 20% oxygen, but 1% oxygen led to significantly decreased protein levels compared to 8% oxygen.

To investigate several other markers of the UPR, we measured protein levels of BiP and PDI. BiP protein levels appeared to have a trending increase with decreasing oxygen levels, however, levels did not reach statistical significance (Figure 3.11A-C). PDI protein levels demonstrate significant dose-dependent increases with decreasing oxygen levels at 6 hours (Figure 3.11D-F), collectively suggesting mild acute increases in chaperone proteins in response to hypoxia.


A
Oxygen (%)
20
8
1
20
8
1
P-eIF2α
eIF2α
CHOP
Amido Black
37 kDa
37 kDa
27 kDa
6 hr
24 hr
B
6hr
Normalized Protein Levels
n.s.
n.s.
n.s.
20%
8%
1%
P-eIF2α
eIF2α
Ratio
C
24hr
Normalized Protein Levels
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ab
a
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n.s.
20%
8%
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D
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n.s.
20%
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1%
E
CHOP 24hr
1WA
*
Normalized Protein Levels
ab
a
b
20%
8%
1%

Figure 3.9. The effects of decreasing oxygen levels on UPR targets downstream of PERK after 6 and 24 hours in Rcho-1 TG cells. (A) Specific targeted protein bands as detected by respective antibodies via Western blot. (B) Protein levels of P-eIF2α, eIF2α, and ratio of P-eIF2α:eIF2α after 6 hours, and (C) 24 hours. (D) Protein levels of CHOP after 6 hours and (E) 24 hours. All arbitrary values were expressed as means normalized to Amido Black ± SEM. All experiments were performed in quadruplicates (n=4). Significant differences between treatment groups as determined by one-way ANOVA (1WA) indicated by * ($p<0.05$). Different letters represent means that are significantly different from one another according to Tukey's post-test ($p<0.05$). Non-significant differences ($p>0.05$) indicated by n.s.

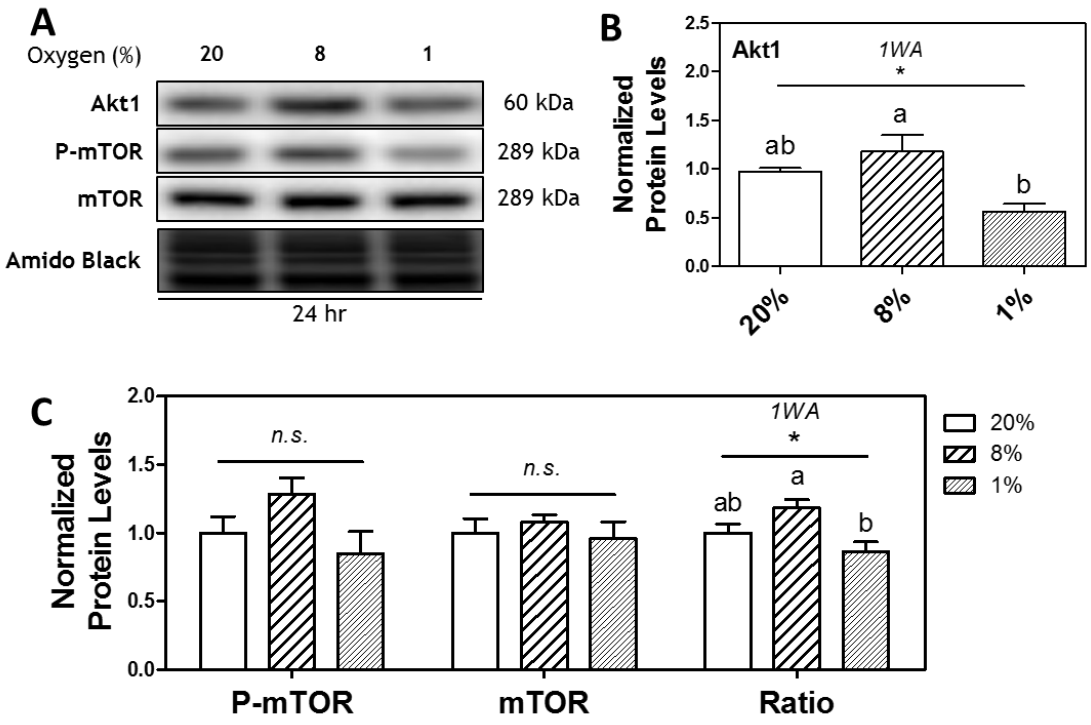

Figure 3.10. The effects of decreasing oxygen levels on the Akt-mTOR pathway after 6 and 24 hours in Rcho-1 TG cells. (A) Specific targeted protein bands as detected by respective antibodies via Western blot. Protein levels of (B) Akt1 and (C) P-mTOR, mTOR and ratio of P-mTOR:mTOR after 24 hours. All arbitrary values were expressed as means normalized to Amido Black ± SEM. All experiments were performed in quadruplicates (n=4). Significant differences between treatment groups as determined by one-way ANOVA (1WA) indicated by * ($p<0.05$). Different letters represent means that are significantly different from one another according to Tukey's post-test ($p<0.05$). Non-significant differences ($p>0.05$) indicated by n.s.

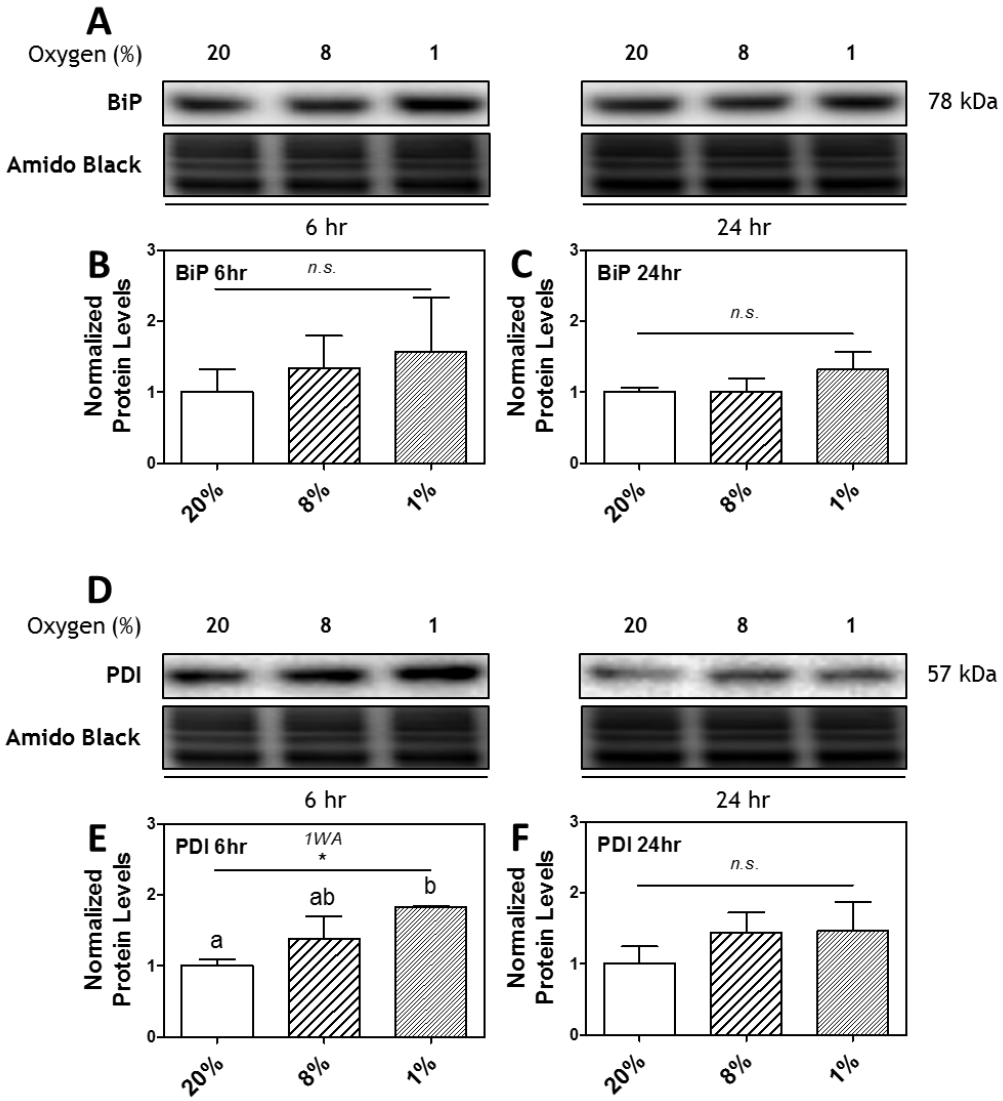

Figure 3.11. The effects of decreasing oxygen levels on BiP and PDI protein levels after 6 and 24 hours in Rcho-1 TG cells. (A) Specific targeted protein bands as detected by respective antibodies via Western blot. (B) Protein levels of BiP after 6 hours, and (C) 24 hours. (D) Specific targeted protein bands as detected by respective antibodies via Western blot. (E) Protein levels of PDI after 6 hours, and (C) 24 hours. All arbitrary values were expressed as means normalized to Amido Black ± SEM. All experiments were performed in quadruplicates (n=4). Significant differences between treatment groups as determined by one-way ANOVA (1WA) indicated by * ($p<0.05$). Different letters represent means that are significantly different from one another according to Tukey's post-test ($p<0.05$). Non-significant differences ($p>0.05$) indicated by n.s.

To assess general placental TG cell function and differentiation in response to hypoxia, we measured the steady-state mRNA levels of Pl-1, Pgf, and Hand1 via RT-PCR. Pl-1 mRNA levels did not demonstrate any significant changes in response to decreasing oxygen (Figure 3.12A). However, Pgf and Hand1 mRNA levels decreased significantly at 8% and 1% oxygen compared to 20% oxygen ($p<0.001$; Figure 3.12B, C).

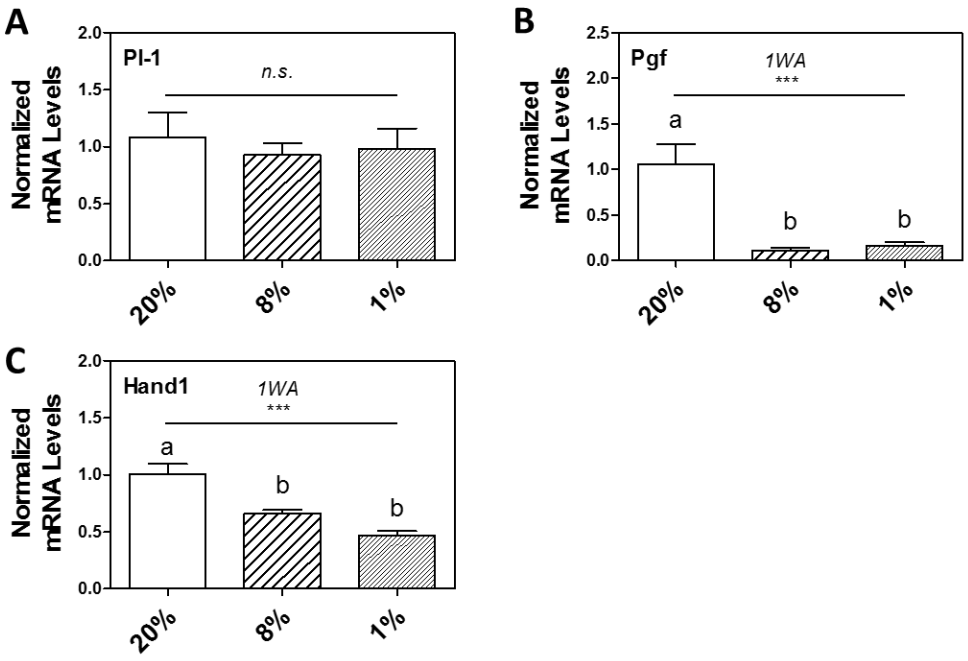

Figure 3.12. The effects of decreasing oxygen levels on markers of placental TG cell function and differentiation after 24 hours in Rcho-1 TG cells. mRNA levels of (A) Pl-1 after 24 hours, (B) Pgf after 24 hours, and (C) Hand1 after 24 hours. All arbitrary values were expressed as means normalized to the geometric mean of β-Actin and Gapdh ± SEM. All experiments were performed in quadruplicates (n=4). Significant differences between treatment groups as determined by one-way ANOVA (1WA) indicated by *** ($p<0.001$). Different letters represent means that are significantly different from one another according to Tukey's post-test ($p<0.001$). Non-significant differences ($p>0.05$) indicated by n.s.

3.3.5 The combinative effects of nicotine and hypoxia on ER stress and PERK phosphorylation

Since we observed that nicotine and hypoxia both independently have the ability to induce ER stress and UPR activation in Rcho-1 TG cells, we were next interested in investigating whether nicotine and hypoxia might have an interaction effect. We selected PERK and eIF2α phosphorylation as protein targets to quantify the effect, as they previously responded robustly to either nicotine or hypoxia. Rcho-1 TG cells were then treated with nicotine (0, 1, or 10μM) at either 20%, 8%, or 1% oxygen for 6 or 24 hours.

To first verify the effectiveness of our hypoxic conditions and to ensure that nicotine exposure has no effect on the induction of hypoxia *in vitro*, we measured protein levels of HIF1α. Rcho-1 TG cells exposed to decreased oxygen levels demonstrated significantly increased HIF1α protein levels after both 6 hours ($p<0.001$) and 24 hours ($p<0.05$) compared to 20% oxygen, verifying the induction of hypoxia. Nicotine caused no significant treatment effect on the protein levels of HIF1α (Figure 3.13A-C).

Decreasing oxygen levels also led to a significant treatment effect on the phosphorylation of PERK at both 6 hours ($p<0.05$) and 24 hours ($p<0.001$), with 1% oxygen causing the greatest increase in PERK phosphorylation (Figure 3.14A-C). Trends within the 20% O_2 group paralleled previous findings (Figure 3.2), with nicotine dose-dependently increasing PERK phosphorylation at 6 hours, though the two-way ANOVA analysis did not identify any statistically significant changes. However, the trending nicotine effect no longer held at 8% or 1% oxygen. There were no significant interaction effects between nicotine and hypoxia on the phosphorylation of PERK according to two-way ANOVA analysis.

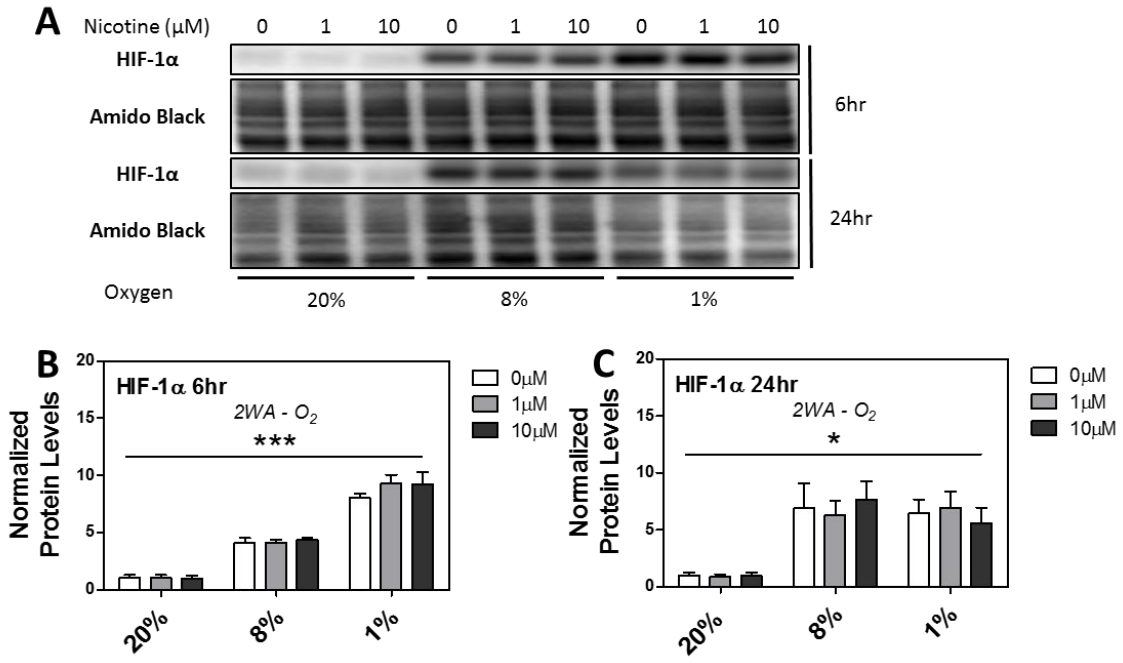

Figure 3.13. The combinative effects of nicotine and hypoxia on HIF1α protein levels after 6 and 24 hours in Rcho-1 TG cells. (A) Specific targeted protein bands as detected by respective antibodies via Western blot. (B) Protein levels of P-PERK, PERK, and ratio of P-PERK:PERK after 6 hours, and (C) 24 hours. All arbitrary values were expressed as means normalized to Amido Black ± SEM. All experiments were performed in quadruplicates (n=4). Significant differences between treatment effect as determined by two-way ANOVA (2WA) indicated by * ($p<0.05$) or *** ($p<0.001$). Treatment effect due to oxygen on protein levels indicated by *2WA - O_2*.

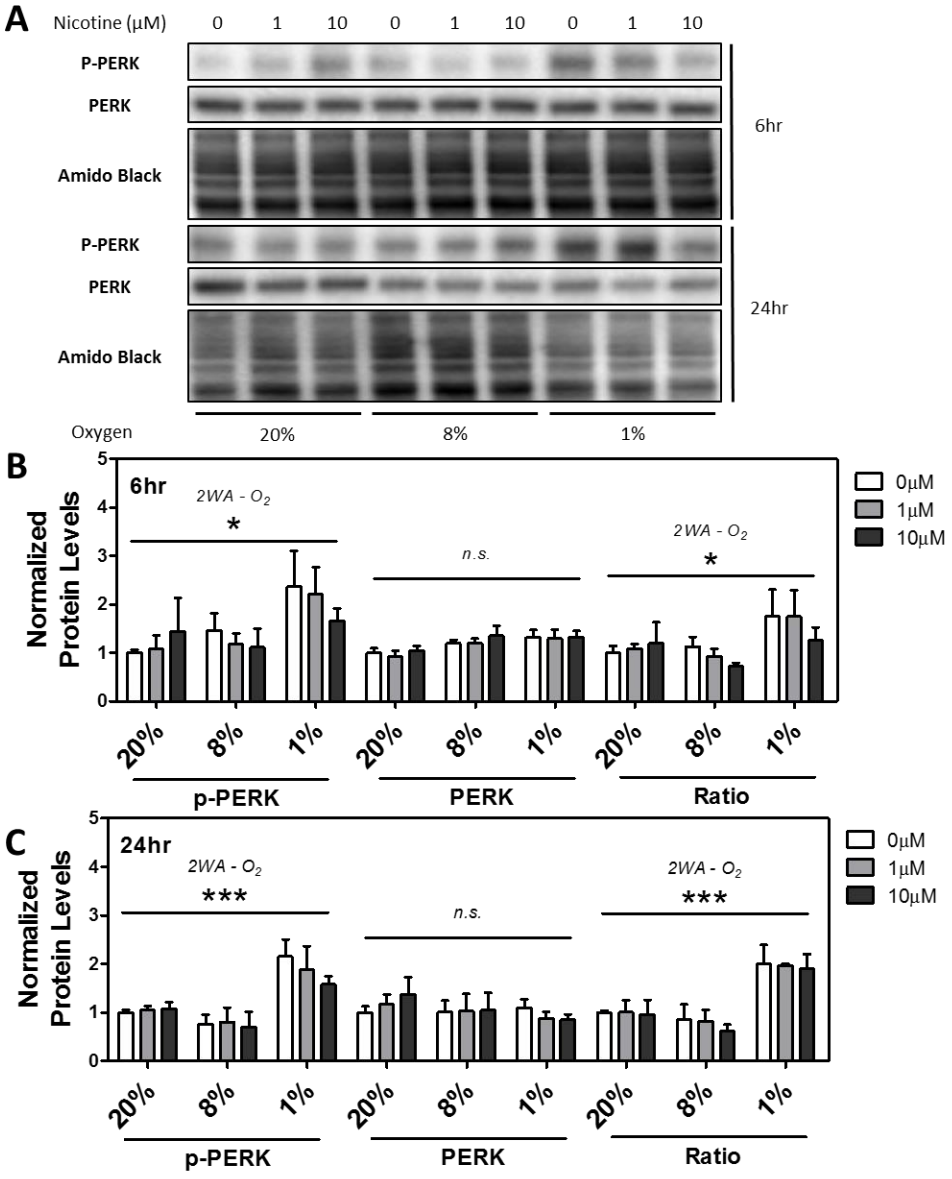

Figure 3.14. The combinative effect of nicotine and hypoxia on phosphorylation of PERK after 6 and 24 hours in Rcho-1 TG cells. (A) Specific targeted protein bands as detected by respective antibodies via Western blot. (B) Protein levels of P-PERK, PERK, and ratio of P-PERK:PERK after 6 hours, and (C) 24 hours. All arbitrary values were expressed as means normalized to Amido Black ± SEM. All experiments were performed in quadruplicates (n=4). Significant differences between treatment effect as determined by two-way ANOVA (2WA) indicated by * ($p<0.05$) or *** ($p<0.001$). Treatment effect due to oxygen on protein levels indicated by *2WA - O_2*. Non-significant differences ($p>0.05$) indicated by n.s.

Downstream of PERK, varying oxygen levels significantly affected the phosphorylation of eIF2α at 24 hours ($p<0.05$; Figure 3.15A-C). Nicotine did not induce any significant treatment effect according to two-way ANOVA analysis. Similar to PERK, trends within the 20% oxygen group paralleled previous findings (Figure 3.3) with nicotine dose-dependently increasing the phosphorylation of eIF2α at 6 hours, though the two-way ANOVA analysis did not identify significant changes. The nicotine effects also no longer held at 8% or 1% oxygen. There were no significant interaction effects between nicotine and hypoxia on the phosphorylation of eIF2α according to two-way ANOVA analysis.

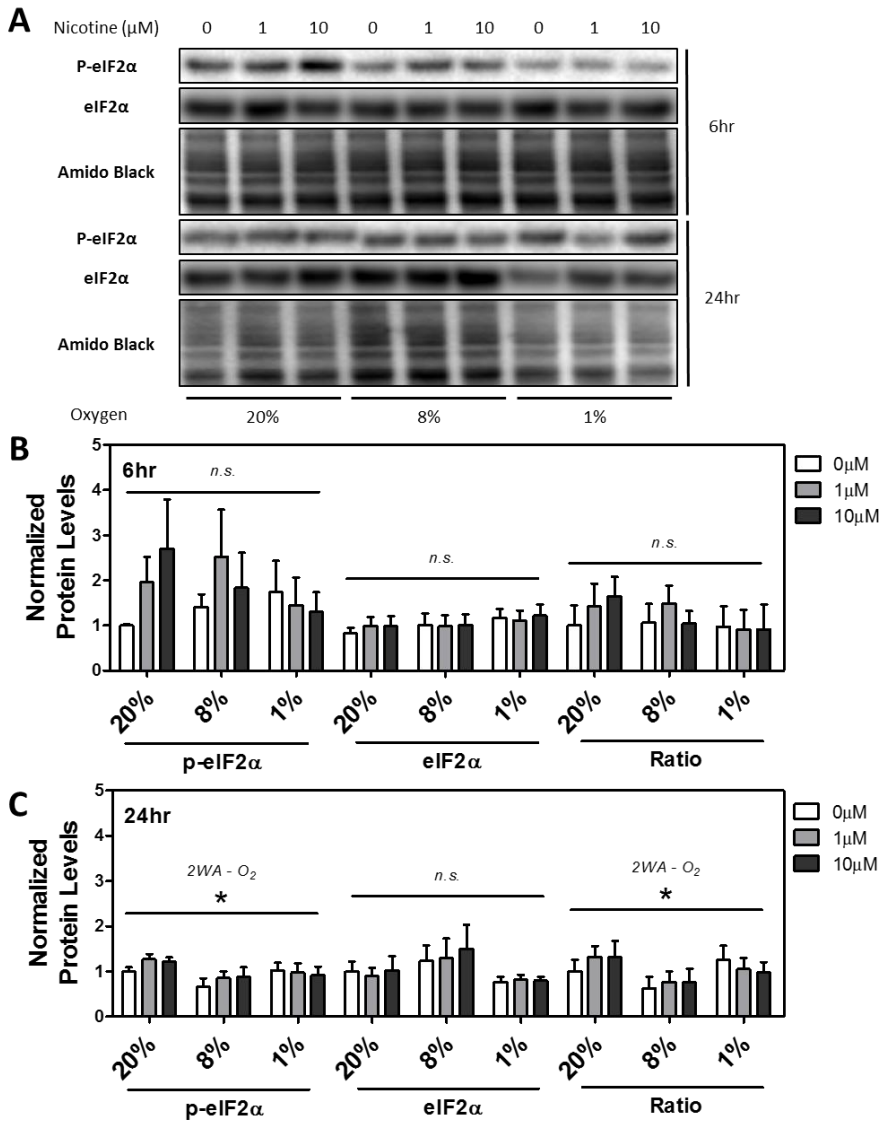

Figure 3.15. The combinative effects of nicotine and hypoxia on phosphorylation of eIF2α after 6 and 24 hours in Rcho-1 TG cells. (A) Specific targeted protein bands as detected by respective antibodies via Western blot. (B) Protein levels of P-eIF2α, eIF2α, and ratio of P-eIF2α:eIF2α after 6 hours, and (C) 24 hours. All arbitrary values were expressed as means normalized to Amido Black ± SEM. All experiments were performed in quadruplicates (n=4). Significant differences between treatment effect as determined by two-way ANOVA (2WA) indicated by * ($p<0.05$). Treatment effect due to oxygen on protein levels indicated by *2WA - O_2*. Non-significant differences ($p>0.05$) indicated by n.s.

3.3.6 The effects of Tauroursodeoxycholic acid (TUDCA) on nicotine-induced ER stress, PERK activation, and downstream placental TG function

Lastly, we were interested in assessing TUDCA as a potential novel therapeutic approach to protect against nicotine-induced ER stress in the placenta. After 10 days of differentiation in NCTC-135 + 1% horse serum in 95% room air/5% CO_2 [30], Rcho-1 TG cells were pre-treated for 1 hour with 100μM TUDCA, and then treated with 10μM nicotine for 6 or 24 hours. TUDCA dose was selected based on previously published findings that effectively reduced ER stress *in vitro* [64, 65]. We selected PERK phosphorylation as the protein target to quantify the effect due to the previously observed robust changes to nicotine. Treatment with 10μM nicotine led to significantly increased PERK phosphorylation ($P<0.05$) in Rcho-1 TG cells, and pre-treatment with 100μM TUDCA completely inhibited nicotine-induced increases in PERK phosphorylation ($P<0.05$; Figure 3.16A, B). TUDCA pre-treatment alone did not induce any effects on PERK phosphorylation.

To assess general placental TG cell function and differentiation in response to TUDCA, we measured the steady-state mRNA levels of Pl-1, Pgf, and Hand1 via RT-PCR. Pl-1 and Pgf mRNA levels did not demonstrate any changes in response to TUDCA pre-treatment nor nicotine treatment (Figure 3.17A, B). However, pre-treatment with TUDCA led to significantly decreased Hand1 mRNA levels at 24 hours compared to both control and nicotine treatment groups ($p<0.01$ and $p<0.05$, respectively; Figure 3.17C).

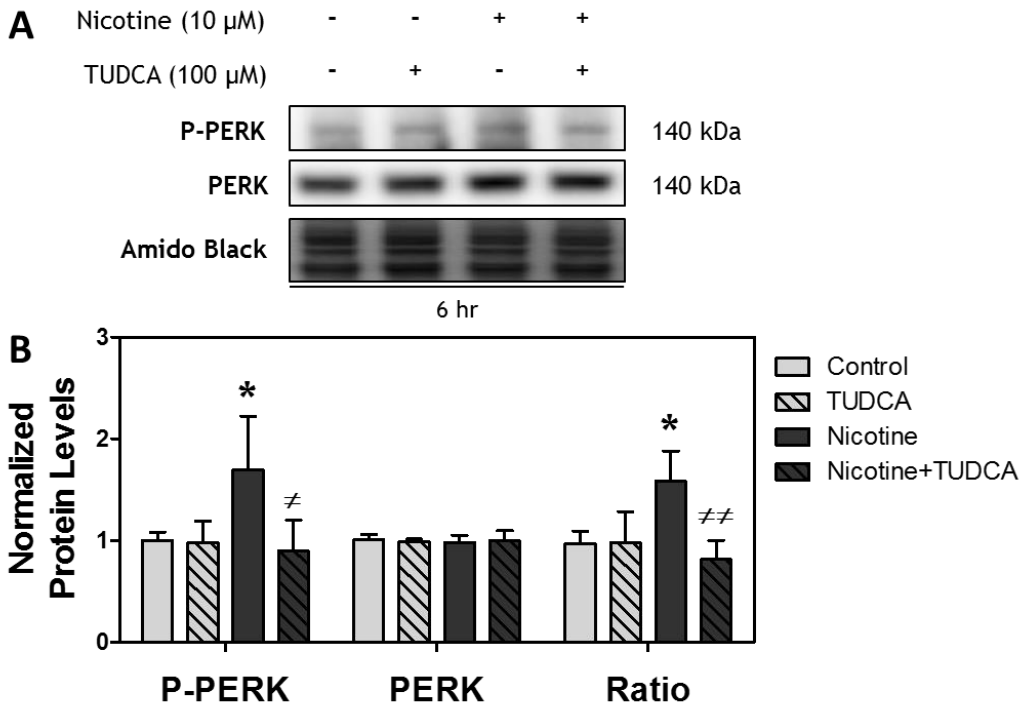

Figure 3.16. Pre-treatment with 100μM TUDCA prevents nicotine-induced PERK phosphorylation after 6 hours in Rcho-1 TG cells. (A) Specific targeted protein bands as detected by respective antibodies via Western blot. (B) Protein levels of P-PERK, PERK, and ratio of P-PERK: PERK at 6 hours. All arbitrary values were expressed as means normalized to Amido Black ± SEM. All experiments were performed in quadruplicates (n=4). Significant differences between treatment groups determined by one-way ANOVA (1WA) followed by Tukey's post-test were indicated by * ($p<0.05$; significant difference from control), ≠ ($p<0.05$; significant difference from nicotine), or ≠≠ ($p<0.01$; significant difference from nicotine).

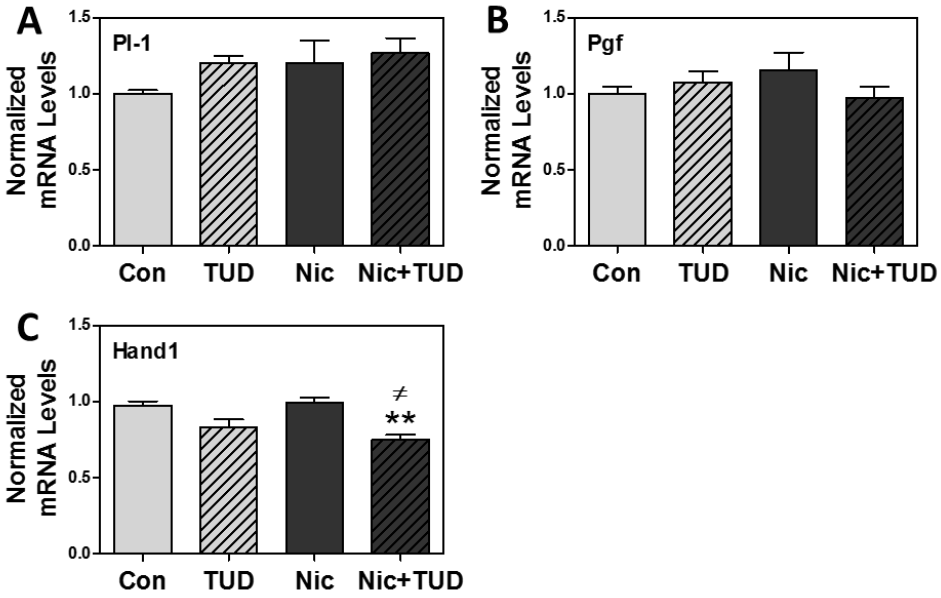

Figure 3.17. The effects of TUDCA (100µM) and/or nicotine (10µM) on various markers of placental TG cell function and differentiation after 24 hours in Rcho-1 TG cells. mRNA levels of (A) Pl-1, (B) Pgf, and (C) Hand1 after 24 hours. All arbitrary values were expressed as means normalized to the geometric mean of β-Actin and Gapdh ± SEM. All experiments were performed in quadruplicates (n=4). Significant differences between treatment groups determined by one-way ANOVA (1WA) followed by Tukey's post-test indicated by ** ($p<0.01$; significant difference from control) and ≠ ($p<0.05$; significant difference from nicotine).

3.4 Discussion

Maternal nicotine exposure was recently demonstrated to augment ER stress in the rat placenta *in vivo*, however, it had yet to be discriminated the degree to which the direct and/or indirect cellular effects of nicotine were responsible. In this study, we firstly demonstrated that nicotine can directly augment ER stress and UPR activation in Rcho-1 placental TG cells, as seen through dose-dependent increases in PERK and eIF2α phosphorylation. This was further verified by successful blocking of nicotine-induced PERK phosphorylation using MH, a nAChR antagonist. Secondly, we revealed that hypoxia, which is a potential indirect effect of nicotine exposure on the placenta *in vivo* [8, 13], can also dose-dependently induce ER stress in Rcho-1 TG cells *in vitro*. However, no statistically significant interaction effect was seen in the combined experiments using both nicotine and hypoxia. Lastly, we utilized TUDCA in a novel manner to prevent nicotine-induced PERK phosphorylation in placental TG cells. Though choriocarcinoma cell lines are admittedly limited in some ways due to their transformed phenotype, they may still serve as good models to dissect the cellular mechanisms and relationships underlying complex *in vivo* situations. Rcho-1 cells are very well characterized and determined to exhibit many similarities to true placental TG cells in cell cycle regulation, differentiation, gene transcription profile, transport processes, hormone production, and others (please refer to [66] for full reference list on conducted studies). Furthermore, Rcho-1 cells are remarkable in their versatile ability to fully differentiate into TG cells, allowing our study of the nicotine-induced ER stress response in that particular trophoblast cell type.

Placental TG cells exposed to nicotine *in vivo* likely experienced the effects of both nAChR activation and hypoxia [13], though the degree to which they individually and synergistically induced ER stress remained largely enigmatic. Repo *et al.* (2014) had preliminarily assessed the direct effects of nicotine on ER stress in BeWo cells; however, the single super-physiological dose of nicotine used (15μM) and minimal markers of ER stress measured (only BiP and IRE1α) urged for a more comprehensive investigation [67]. Our findings in Rcho-1 TG cells now elucidate two possible mechanisms by which nicotine may induce ER stress in placental TG cells: (1) directly through nAChR activation and (2) indirectly through hypoxia. These *in vitro* results also identify the underlying mechanisms

of nicotine-induced ER stress augmentation previously seen in the rat placenta *in vivo* [13]. However, unlike previous *in vivo* results [13], downstream UPR activation of targets such as ATF4, CHOP, BiP, PDI, VKORC1, and GCN2 were far less prominent in response to nicotine and/or hypoxia, despite the strength of PERK and/or eIF2α activation. Longer and more chronic exposures to nicotine and/or hypoxia may be required to alter these downstream targets (*e.g.*, previous studies performed month-long daily nicotine injections *in vivo* [13], or 3 days of chronic hypoxia *in vitro* [18] to demonstrate effects). This also provides a possible explanation for the lack of change seen in markers of TG cell function and differentiation (*e.g.*, Pl-1, Pgf, and Hand1), despite previously reported changes upon exposure to nicotine or ER stress in rodent placentas *in vivo* [8, 20]. However, our study was mainly purposed to investigate the activation the UPR via acute nicotine exposure, which we were indeed able to examine through PERK phosphorylation.

Furthermore, our findings indicating that hypoxia increased ER stress and PERK phosphorylation were consistent with Yung *et al.* (2012), who demonstrated increased UPR activation in JEG-3 and BeWo cells after 3 days of culture at 1% oxygen [18]. In our experiments, however, CHOP protein levels unexpectedly decreased from 8% to 1% oxygen after 24 hours despite dose-dependent increases in PERK activation. Conversely, other studies have shown that protein levels of CHOP increased in response to 1% oxygen after 24 hours in human melanoma cells *in vitro* [68]; though, changes were not seen rat myocardial tissue exposed to hypobaric hypoxia *in vivo* [69]. Also, Chop mRNA levels strongly increased in response to severe hypoxia (0% oxygen) in HeLa cells *in vitro*; yet, specific translation of Chop transcripts demonstrated little to no change as determined through ribosomal-transcript interaction [59]. This inconsistency seen across various studies suggests that CHOP expression may be differentially regulated dependent on cell/tissue type, severity of hypoxia, and experimental model. We further investigated the Akt-mTOR protein translation pathway and saw parallel changes across CHOP, Akt1, and P-mTOR:mTOR protein levels, suggesting that the changes seen in CHOP protein levels may be due to altered protein translation regulation via the Akt-mTOR pathway in response to hypoxia. Hypoxia is known to inhibit mTOR complex 1-regulated protein translation initiation [63], which may specifically suppress the translation of various transcripts [59], perhaps including Chop mRNA. Nonetheless, these findings are only correlative and no

studies have been conducted to date investigating the direct effect of Akt-mTOR-regulated protein translation on CHOP protein levels, thus more experiments are needed to verify a causal relationship.

Strikingly, hypoxia strongly suppressed the mRNA levels of Hand1 and Pgf. Murine hearts exposed to hypoxia *in vivo* led to increased Hand1 mRNA levels, which contrasted with our findings [70]; however, there are no published studies to date reporting the effects of hypoxia or ER stress on Hand1 expression in the placenta. More consistently, primary human syncytiotrophoblasts exposed to 1% oxygen demonstrated strongly suppressed Pgf mRNA levels, though the underlying regulatory mechanisms remained unclear [71]. Maternal exposure to tunicamycin, a drug that induces ER stress through inhibition of protein N-glycosidic linkage formation, also impaired placental Pgf expression alongside compromised placental development [20]. Considering that ER stress and UPR activation were seen in association with impaired Pgf expression in Rcho-1 TG cells exposed to acute hypoxia in our study, it is possible that hypoxia-induced ER stress may underlie the impairment of Pgf expression. This is an important preliminary finding warranting further investigation *in vivo*, as the reestablishment of ER function may be linked to the desirable restoration of Pgf expression under hypoxia.

Despite the individual effects of nicotine and hypoxia on ER stress, we did not detect any statistically significant interaction effects between the two insults. However, we no longer saw dose-dependent effects of nicotine on PERK and eIF2α phosphorylation at decreased oxygen levels of 8% or 1%. One possible explanation is that the severity of hypoxia in comparison to the milder nAChR activation masked any noticeable nicotine-induced effects on ER stress, though this is merely speculative. A more likely explanation is that moderate hypoxia down-regulated nAChR expression, which has been seen in other tissue types [72, 73], thus leading to a decreased effect of nicotine on ER stress under hypoxia. This also suggests that the nicotine-induced ER stress augmentation previously seen in the whole placenta *in vivo* may be predominantly caused by nicotine-induced hypoxia and less so by nAChR activation, since placental tissues exposed to nicotine were found to be hypoxic [13]. Future studies are directed to investigate the effects of hypoxia on the expression of various nAChR subtypes in the placenta to clarify these results.

Findings from our study are the first to reveal that TUDCA can prevent nicotine-induced PERK activation in placental TG cells. TUDCA is suggested to ameliorate ER stress either through direct assistance with protein folding or increasing the expression of molecular chaperones [28]. Exogenous supplementation has provided beneficial effects in treating neurodegenerative and metabolic diseases, and other protein folding disorders (*e.g.*, obesity, type 2 diabetes, Alzheimer's disease) [29, 74]. Both TUDCA and UDCA, the non-taurine-conjugated counterpart of TUDCA, are approved by the FDA for treating primary biliary cirrhosis; however, TUDCA has been demonstrated to possess more favourable metabolic properties for longer-term use [75, 76]. Recent developmental studies further demonstrated that postnatal TUDCA injections can reverse prenatal ethanol-induced ER stress damage in liver and skeletal muscle of rat offspring [77, 78]. Alongside the favourable effects against ER stress, TUDCA may also inhibit ROS production and protect against mitochondrial-mediated and caspase-mediated apoptosis [79-81], perhaps collectively enriching global cellular health. These properties of TUDCA may be especially desirable during pregnancy, as the placenta is particularly susceptible to oxidative and ER stress augmentation due to high protein folding and secretory activity [19]. However, mild decreases seen in Hand1 mRNA levels following TUDCA treatment *in* vitro suggest that TUDCA may potentially influence placental trophoblast cell differentiation; though more extensive experimentation would be required before conclusions may be made. Overall, given the effectiveness of TUDCA in relieving nicotine-induced ER stress in the current study, it will be of great interest to examine the potential benefits of TUDCA on hypoxia-induced ER stress in Rcho-1 TG cells *in vitro,* nicotine-induced ER stress in the placenta *in vivo*, and ultimately, as a novel therapeutic agent to remedy the consequences of maternal nicotine exposure in the clinical setting.

In conclusion, our current *in vitro* study provides strong mechanistic insight on the direct and indirect effects of nicotine exposure on placental ER stress and trophoblast cell function and differentiation (summarized in Figure 3.18). Our findings further demonstrate that TUDCA supplementation may indeed be a promising therapeutic option to consider for treating the negative outcomes of maternal nicotine exposure, though more studies are needed in order to assess its safety in pregnancy. With the high rates of maternal nicotine exposure that continue to occur worldwide, research in intervention development is

urgently required for those afflicted. Nonetheless, the compliant usage or abstinence from these nicotine-containing products remain most heavily influenced by "approval from healthcare professional[s]" [82], thus we must continue to raise the clinical voice in this transgenerational battle against nicotine use during pregnancy.

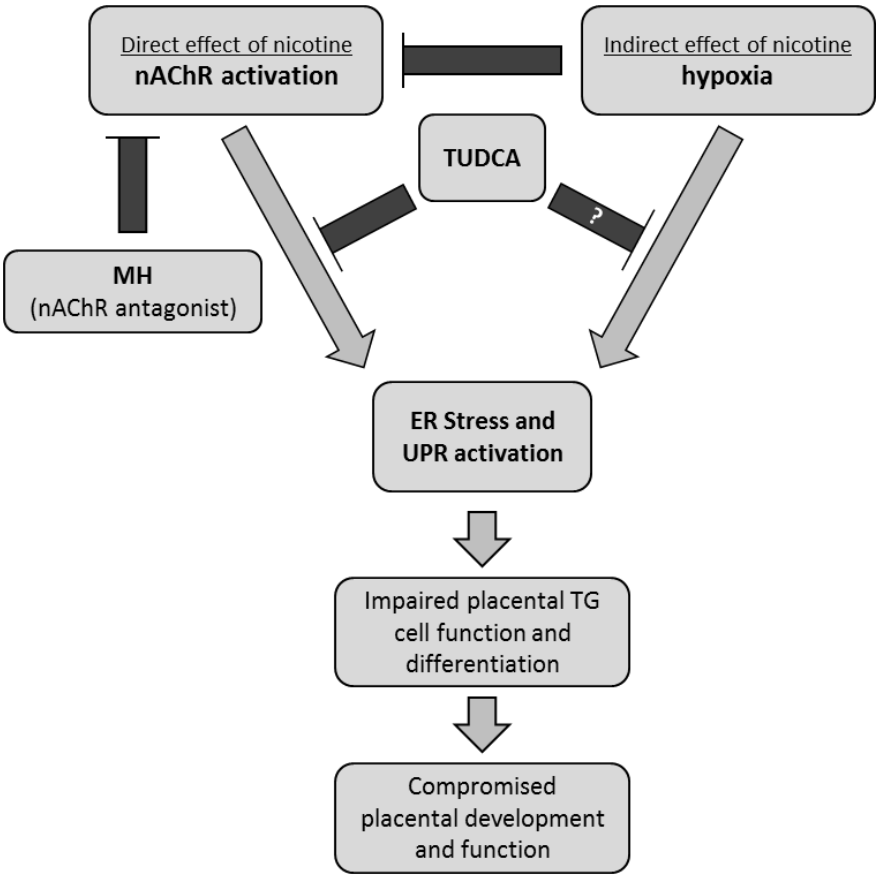

Figure 3.18. Direct nAChR activation and indirect hypoxia both induce ER stress and UPR activation in placental TG cells. The effects of nAChR activation were less prominent at increased hypoxia possibly due to either a masking effect or nAChR down-regulation. TUDCA also inhibited nicotine-induced PERK activation, but its potential effects on hypoxia-induced ER stress have yet to be determined. Chronic ER stress and UPR activation may lead to impaired placental TG cell function and differentiation, which may in turn compromise development and function in the whole placenta.

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4 GENERAL DISCUSSION

Excerpts of this chapter have been submitted for publication and currently in revision: **Wong MK, Barra NG, Alfaidy NN, Hardy DB, and Holloway AC.** Adverse effects of perinatal nicotine exposure on reproductive outcomes. *Reproduction*, in revision.

4.1 Summary

It is now becoming better understood that even maternal nicotine exposure alone can lead to deleterious outcomes in pregnancy and long-term health. Nicotine is known to directly pass through the placental barrier, accumulate in fetal serum, and exert its effects on developing tissues [1-3]. Alternatively, we are now aware that nicotine may also impair development of the placenta [4], which *indirectly* compromises the well-being of the growing fetus. However, many of the cellular mechanisms underlying the effects of nicotine on the placenta had yet to be discovered. To reiterate the rationale for this thesis, recent findings demonstrated that nicotine exposure leads to increased hypoxia in the placenta [4-6] and placental insufficiency [4]. Furthermore, hypoxia and placental insufficiency were both strongly associated with ER stress [7-11]. Based on this, we were interested in determining whether nicotine exposure leads to ER stress in the placenta.

In the present studies, nicotine exposure led to augmented ER stress and UPR activation in the rat placenta both *in vivo* and *in vitro*, supporting the hypothesis. This was associated with impaired disulfide bond formation, hypoxia, and amino acid starvation at e15 *in vivo*, though it remained unclear how nicotine exposure induced ER stress at the cellular level [12]. Upon further investigation using the well-established Rcho-1 TG cells, we concluded that ER stress may be augmented in the placenta both directly through nAChR activation and indirectly through hypoxia *in vitro*. Additionally, since hypoxia masked the effects of nAChR activation on ER stress in the combined hypoxia and nicotine experiments *in vitro*, we deduced that the placental ER stress occurring *in vivo* may be predominantly attributed to nicotine-induced hypoxia. After identifying the involvement of ER stress in nicotine-induced placental injury, we were interested in next investigating potential therapeutic interventions. By pre-treating Rcho-1 TG cells with TUDCA, we were the first to successfully prevent nicotine-induced ER stress and UPR activation in the placenta *in vitro*, encouraging future studies to assess its full potential for alleviating the detrimental outcomes of maternal nicotine exposure in pregnancy. In this final chapter, we will identify some limitations of our work and conclude by discussing the implications from our studies for future research directions in this field.

4.2 Limitations and Improvements

In order to improve the quality of future studies, we will address some of the major limitations of our current work. Firstly, the molecular techniques used to quantify ER stress and UPR activation in both studies were limited to quantification of protein and mRNA levels via Western blot and Real-time PCR. Though these methods are theoretically sound, since quantifying expression changes in the UPR pathways indicate the presence of ER stress [13, 14], it is unable to directly measure the accumulation of misfolded proteins which truly defines ER stress, nor does it allow for real-time, live cell monitoring [15, 16]. One recently discovered technique that may overcome both these limitations involves the use of a fluorescently-tagged chaperone protein, such as green fluorescence protein-tagged BiP (BiP-GFP), paired with Fluorescence Recovery After Photobleaching (FRAP) [17]. The molecular chaperone BiP inherently detects and binds misfolded proteins within the ER lumen [18], which slows down its diffusional mobility. FRAP then allows for the quantification of BiP-GFP diffusional mobility, which provides real-time information on changes in misfolded protein burden and ER stress in live cells [17].

Secondly, our analysis of ER stress and UPR activation *in vivo* was limited to whole rat placentas. Although it is undoubtedly important to determine the effects on the organ as a whole, the study could be improved in two ways. Given the heterogeneous anatomy of the placenta, we should further analyze the ER stress response within specific regions [19]. Nicotine was previously demonstrated to induce region-specific effects within the rat placenta (*e.g.*, nicotine exposure only impaired growth in decidua and junctional zone, but not in labyrinth zone [4]). Experimental augmentation of ER stress in murine placenta also led to region-specific inductions of the UPR (*e.g.*, ER stress mouse model led to PERK activation in junctional zone, but not labyrinth zone [9]), thus it is possible that nicotine-induced ER stress may be differentially augmented in specific placental regions. Furthermore, to extend the relevance of our findings back to the clinical setting, it would be very informative to pair our examination of the ER stress response in the rat placenta with human placentas exposed to NRTs/e-cigarettes during pregnancy.

Thirdly, our *in vivo* study was limited to only one time-point (e15) and nicotine dose (1mg/kg). This time-point was specifically chosen as nicotine exposure previously led to structural and morphological aberrations in the rat placenta at e15, prior to any observable fetal growth deficits [12]. However, it was known that rat offspring exposed to nicotine exposure *in utero* would be eventually have low birth weights at term compared to controls [20]. Given that ER stress and placental insufficiency were observed to precede IUGR in humans [10, 11, 21, 22], determining the presence of ER stress at the e15 time-point would reveal a potential mechanism through which nicotine may cause adverse placental and fetal outcomes in pregnant mothers who are smoking or using NRTs. However, the study could be improved by analyzing several other time-points to observe the progression of ER stress in response to chronic nicotine. Furthermore, we found significantly increased CHOP expression at e15 with no changes in markers of apoptosis (*e.g.*, ratio of BAX to BCL2, Bim, Puma, caspase pathway) [12]. Though we have speculations that CHOP activation may have been at an early stage and apoptosis might be amplified later on in pregnancy, measurements at a later time-point would have provided concrete evidence to confirm this. Furthermore, using more than one nicotine dose would have allowed us to determine the dose-dependent relationship between nicotine and ER stress in the placenta *in vivo*. We attempted to overcome this by treating Rcho-1 TG cells with a large range of nicotine doses (0.1-100μM) in our *in vitro* experiments that encompass the average serum levels found in humans (25nM-25μM) [23-28]; of which we were indeed able to observe dose-dependent increases in PERK and eIF2α phosphorylation with increasing nicotine dose. Although these findings do not fully extrapolate what might occur in the whole placenta *in vivo*, these *in vitro* experiments still provide strong support [19].

4.3 Implications and Future Directions

Regardless of our findings, there are still many unanswered questions surrounding the complex issue of maternal nicotine exposure. In the following section, we will discuss our results in light of other recent breakthroughs to highlight future directions for the field.

4.3.1 From adaptation to apoptosis: the delicate balance of PERK pathway activation

It was apparent from both our *in vivo* and *in vitro* results that the PERK pathway of the UPR played a crucial role in responding to nicotine exposure in the placenta. Acute PERK activation is a necessary and adaptive response to relieve ER stress [13, 15], however, chronic activation is known to conversely amplify apoptosis [4, 12, 29]. As mentioned, this was believed to be regulated downstream of PERK by CHOP, which triggers many ER stress-related apoptotic pathways [15, 30]. Indeed, acute nicotine exposure *in vitro* did not affect CHOP expression in Rcho-1 TG cells, but chronic nicotine exposure *in vivo* elevated CHOP expression in rat placentas. Though we did not yet see activation of apoptosis at e15 in association with increased CHOP, we speculated that continued nicotine exposure may perhaps lead to apoptosis at a later time-point [12]. The precise timing and regulation of this molecular switch from adaptation to apoptosis still remains unclear, however, recent publications reveal a growing interest in ER stress research towards the development of small molecule PERK pathway modulators. As future studies are directed to further examine the influence of the PERK pathway in nicotine-induced ER stress, we will highlight two recently developed compounds, GSK2656157 and ISRIB.

GSK2656157 is a potent, highly-selective, ATP-competitive inhibitor of PERK enzyme activity [31, 32]. Treatments with GSK2656157 (or GSK2606414, a slightly older and less specific PERK inhibitor) prevented PERK phosphorylation both *in vitro* and *in vivo,* and inhibited subsequent downstream up-regulation of p-eIF2α, ATF4, and CHOP [31]. We conducted several preliminary *in vitro* experiments using GSK2656157 and indeed found effective blocking of nicotine-induced PERK activation in Rcho-1 TG cells (Appendix I, Supplemental Figure 4A, B). However, GSK2656157 was also demonstrated to possess anti-tumour properties by reducing the vascular density, perfusion, and growth of

xenografted tumours in mice [31-33]. PERK is involved in up-regulating pro-angiogenic transcripts [31]; microarray analyses revealed several pro-angiogenic transcripts that were preferentially translated in a PERK-dependent manner, such as VEGF and type I collagen inducible protein and matrix metallopeptidase 13 [34]. Although the alleviation of tumour growth is beneficial for combating cancer, PERK-mediated angiogenesis and perfusion is essential to placental development [35]. Dysregulation may lead to detrimental conditions such as pre-eclampsia or IUGR [36]. Though there were no adverse effects from our initial GSK2656157 experiments on Pl-1, Pgf, or Hand1 expression in Rcho-1 TG cells (Appendix I, Supplemental Figure 4C), the anti-angiogenic features of PERK-specific inhibition may render GSK2656157 inappropriate to treat nicotine-induced ER stress in the placenta during pregnancy. However, *in vivo* studies would be required to verify this.

Recently, GSK2656157 was also criticized for not consistently blocking the activation of the associated downstream pathways [37]. In fact, PERK inhibition via GSK2656157 treatment in human tumour cells *in vitro* paradoxically increased eIF2α phosphorylation, perhaps as a compensatory mechanism for losing the adaptive features of acute PERK activation [37]. Thus, though PERK activation was blocked, its downstream targets may still be activating. This may be due to involvement of the integrated stress response (as reviewed in Chapter 1). Several other kinases (PKR, HRI, and GCN2), in addition to PERK, converge to phosphorylate eIF2α under varying stress stimuli, hence PERK-specific inhibition alone may not be sufficient to prevent prolonged activation of the downstream targets (*i.e.,* eIF2α, ATF4, CHOP). A novel compound known as ISRIB was recently discovered to render cells resistant to the effects of eIF2α phosphorylation both *in vivo* and *in vitro,* leading to the antagonism of the integrated stress response altogether [38]. This restored global protein translation, inhibited ATF4/CHOP induction, and facilitated stress granule dissolution to free mRNA transcripts for translation [38, 39]. ISRIB was demonstrated to act as a potent enzyme activator of eIF2B by promoting eIF2B dimerization and GEF activity [40], reinstating ternary complex formation for protein translation even under states of stress-induced eIF2α phosphorylation. However, whether ISRIB releases the non-productively sequestered eIF2B from phosphorylated eIF2α or simply enhances the activity of residual unbound eIF2B has yet to be distinguished. However, its use may not be appropriate if the ER stress is still being actively augmented,

as co-treatment of ISRIB with tunicamycin synergistically decreased cell viability [38]. All in all, ISRIB may serve as an attractive therapeutic option to alleviate the negative outcomes of chronic PERK activation following nicotine exposure. Future studies determining the timing and frequency of use will be crucial to advancement of its applications in pregnancy.

4.3.2 Alteration of gene expression by ER stress and the UPR

In addition to ER stress-induced apoptosis, there are also target genes indirectly related to the UPR pathways that are undesirably altered by ER stress and UPR activation. One example mentioned in the third chapter was Pgf, a growth factor essential for placental angiogenesis [41]. Maternal tunicamycin treatments led to severe ER stress induction in association with decreased placental Pgf mRNA levels, and compromised placental development [11]. Our *in vitro* experiments exposing Rcho-1 TG cells to hypoxia also led to ER stress in association with impaired Pgf mRNA levels (see Figure 3.12). Though the exact regulatory factors responsible for these changes in expression have not yet been identified, augmented ER stress and UPR activation appears to be a common cellular state across both these models. Other examples of target genes altered in parallel manners by both tunicamycin and nicotine include Vegf (increased expression) and Glut1 (decreased expression), without any reported activation of apoptosis [4, 11, 42, 43]. Using chromatin immunoprecipitation (ChIP), future studies may determine the binding of specific UPR transcription factor proteins (*i.e.*, ATF6, XBP1, ATF4, CHOP) to promoter/DNA binding sites of these target genes of interest (*i.e.*, Pgf, Vegf, Glut1) to elucidate the transcriptional mechanisms that may be involved during ER stress. Though this was suggested to occur for stress-adaptive purposes in order to escape apoptosis [12], the changes in expression are often unideal long-term due to their functional importance. Moreover, altered transcriptional outcomes were suggested to potentially underlie the structural and morphological aberrations seen in nicotine-exposed placentas [12].

4.3.3 Therapeutic agents to remedy ER stress-induced injury

Therefore, there is incredible value in discovering therapeutic agents that may improve protein folding to safely protect against ER stress-related apoptosis and other impairments.

Normally in the body, a family of "molecular chaperones" known as heat shock proteins (HSPs) promote proper protein folding under situations of cellular stress, such as elevated temperatures, inflammation, and hypoxia. In the ER, several groups of calcium-dependent molecular chaperones reside to assist with protein folding, including HSP70 (also known as GRP78/BiP), HSP90 (also known as GRP94), calreticulin, and others [44]. Resembling molecular chaperones in their analogous ability to assist with protein folding comprises a class of non-selective small molecules known as "chemical chaperones". While their exact mechanism of action remains elusive and varies across compounds, they are generally understood to either directly interact with misfolded proteins to facilitate their refolding, or indirectly increase the expression or activity of existing molecular chaperones [44].

The endogenous taurine-conjugated bile acid, TUDCA, has received much attention due to its safety *in vivo*, and ability to relieve ER stress and associated downstream consequences in various metabolic and neurodegenerative experimental models [45-47]. More interestingly, postnatal TUDCA injections had recently been demonstrated to reverse the effects of prenatal ethanol-induced ER stress damage in rat liver and skeletal muscle [48, 49]. However, its efficacy against nicotine-induced ER stress had yet to be assessed. Our study provided novel findings revealing the capacity of TUDCA to prevent nicotine-induced ER stress and PERK activation in placental TG cells *in vitro*, directing future studies to verify its promising effectiveness in treating nicotine-induced ER stress in the placenta *in vivo* (please refer to Chapter 3 for a more comprehensive discussion regarding TUDCA). However, this ability to reduce ER stress through improving protein folding is not unique to TUDCA. Another popular chemical chaperone is 4-phenyl butyric acid (PBA), a short-chain fatty acid effective in preventing protein aggregation and promoting refolding through binding to hydrophobic surfaces of misfolded proteins [44]. PBA possesses similar effectiveness as TUDCA in the reduction of ER stress and apoptosis both *in vitro* and *in vivo*, and has also provided beneficial effects against various ER stress-related metabolic and neurodegenerative disorders [45, 50, 51]. In addition, PBA is currently FDA-approved for treatment of urea-cycle disorders, and has demonstrated promise to treat thalassemia [52], beta-globin disorders [53], and potentially serve as a chemotherapy agent [54]. As previously mentioned, the placenta should theoretically benefit from the effects of these chemical chaperones due to its high susceptibility to ER

stress [9]. The current caveat inhibiting more widespread usage of these chemical chaperones is that very high doses are often required for sufficient efficacy against ER stress *in vivo*, carrying risk of side-effects [44]. Though TUDCA and PBA are safe and approved in humans for their current applications, future clinical studies must carefully determine a safe and effective dose for use in pregnancy to treat placental insults.

4.3.4 Integrating ER stress, oxidative stress and inflammation

Beyond ER stress and the UPR, nicotine also invokes other cellular disturbances in the placenta, such as oxidative stress and inflammation. Maternal smoking increased markers of oxidative stress in the human placenta [55]; however, nicotine exposure alone has yet to demonstrate evidence of increased placental ROS production or oxidative damage [4, 56, 57]. Interestingly, placentas from nicotine-treated animals exhibited evidence of hypoxia, which is known to increase ROS production [58]. Moreover, nicotine-induced deficits in placental function may be partly ameliorated by co-administration with Vitamin C, an antioxidant, suggesting that oxidative stress underlies aspects of nicotine-induced placental deficiency [56]. The relationship between nicotine and inflammation during pregnancy remains controversial, where some studies proposed pro-inflammatory (PI) roles for nicotine [59, 60], and others proposed anti-inflammatory (AI) [61]. The discrepancies between studies were possibly due to variations in nicotine dose (*e.g.*, PI: 2 mg/kg/day; AI: 1 mg/kg/day) and duration of exposure (*e.g.*, PI: Gestational day (GD) 1-21; AI: GD 14-15 only) [59, 61]. However, these multidimensional effects of nicotine provoke inquiry into whether these three cellular mechanisms may be interconnected.

Indeed, current research suggests that ER stress and the UPR may be further linked with oxidative stress and inflammation due to organelle proximity and pathway cross-talk [62]. The mitochondria and ER are tightly physically interconnected through mitochondrial-associated membranes, allowing for close signalling interactions [63]. This likewise allows for perturbations in one organelle to affect the other (*i.e.*, ER stress and calcium imbalance in the ER may also induce ROS production and oxidative stress in the mitochondria.) Extensive cross-talk also occurs between the pathways of these three mechanisms, such that their activation rarely occurs in isolation (*e.g.*, ER stress-related PERK branch activation can potentially trigger inflammatory mediator NF-κB, and UPR-induced

oxidative protein folding can also increase downstream ROS production to augment oxidative stress [62]). Recent work has identified that nicotine exposure may also affect the epigenetic profile by altering microRNA expression [64]. microRNAs are small, non-coding RNA molecules that fine-tune gene expression, usually through mRNA silencing. They have been demonstrated to be involved in the regulation of oxidative stress, inflammation, and ER stress, adding further intricacy to the relationship between these three mechanisms (*e.g.*, PERK-NF-κB pathway up-regulates miR-30c-2*, which is known to degrade Xbp1 transcripts [65, 66]; ATF4 may upregulate miR-211 which inhibits CHOP expression [67]). Indeed, we have preliminary data suggesting that maternal nicotine exposure significantly upregulates miR-211 levels in the rat placenta *in vivo*, however, the downstream effects have yet to be studied ($p<0.05$; Appendix I, Supplemental Figure 5). Considering all these findings, future research investigating the mechanistic underpinnings of maternal nicotine exposure must take an integrative approach to understand the synergistic involvements of oxidative stress, inflammation, ER stress, and the underlying epigenetic factors involved (Figure 4.1).

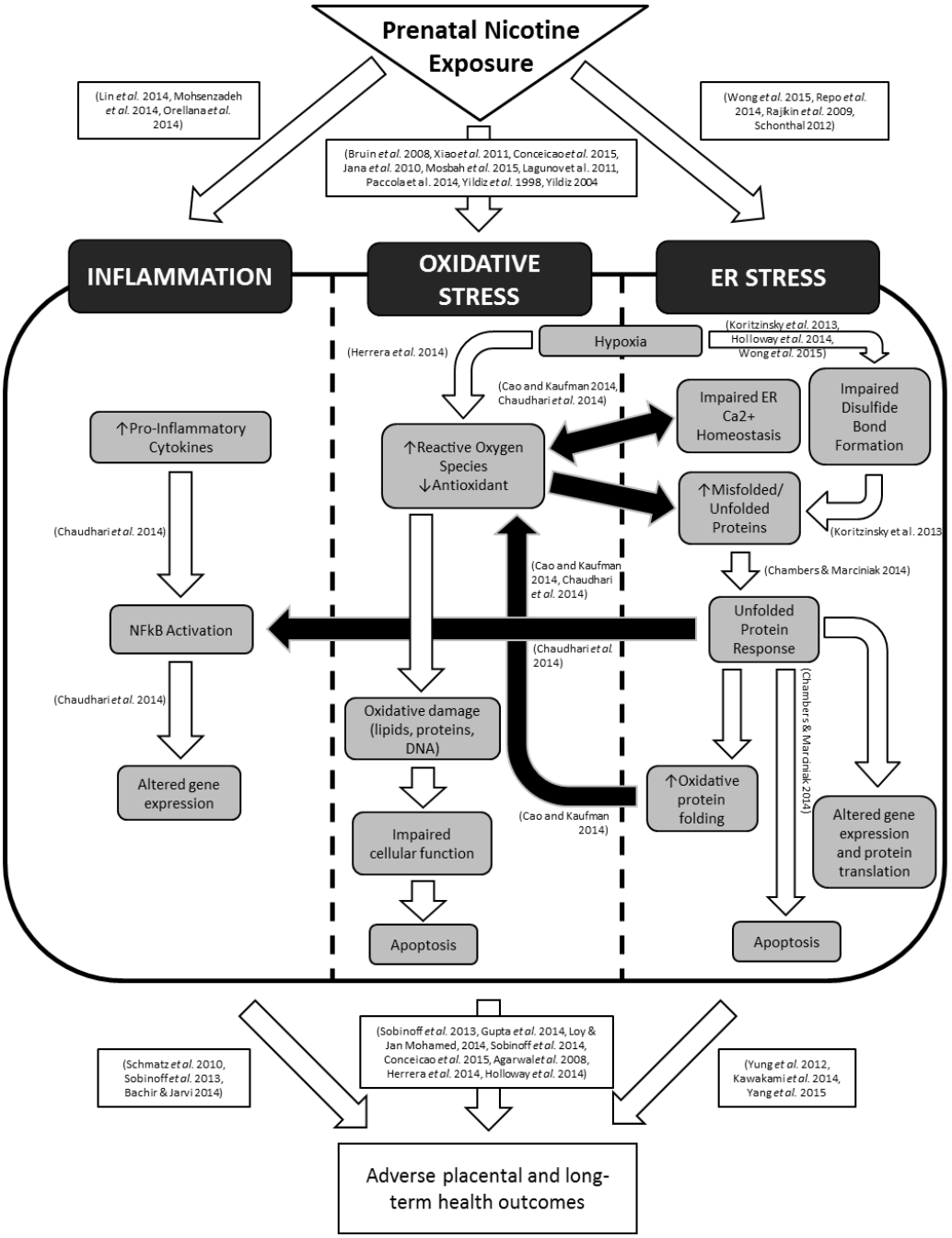

Figure 4.1. A proposed schematic illustrating the adverse effects of prenatal nicotine exposure on placental and long-term health outcomes, as mediated through the integrated induction of inflammation, oxidative stress, and ER stress. White arrows indicate the normal pathway within a specific mechanism, whereas black arrows indicate interconnected pathways between the mechanisms. miRNAs also regulate many aspects of these pathways (not indicated in figure).

4.3.5 Nicotine-induced sensitization to postnatal insults: Revisiting the developmental origins of health and disease theory

In addition to the adverse effects on pregnancy and health outcomes in the offspring, maternal nicotine exposure may also sensitize the offspring to greater injury from other insults in later life. Postnatal high fat/high cholesterol diet at eleven weeks of age led to a greater elevation in serum IL-1β inflammatory response when mice were prenatally treated with nicotine, compared to mice not prenatally treated [60]. In other studies, rats postnatally exposed to chlorpyrifos, an organophosphate pesticide, demonstrated heightened and altered neurotoxic effects in their serotonergic and cholinergic systems when prenatally treated with nicotine, compared to rats not prenatally treated [68, 69].

Therefore, prenatal nicotine exposure appears to create a subpopulation that is especially vulnerable to particular postnatal insults. By applying our understanding of the Developmental Origins of Health and Disease theory (as extensively reviewed in the first chapter), we may hypothesize that this sensitization occurs due to altered fetal programming and a mismatched postnatal environment. Expanding on this idea, since nicotine exposure is known to affect cellular nAChR expression and responsiveness, chronic prenatal exposure may alternatively "program" the nAChR profile of the offspring [70]. Hence, postnatal insults that involve nAChR signaling may exhibit an altered response; this likely underlies the unique postnatal chlorpyrifos-induced cholinergic system response seen in prenatal nicotine-exposed rats [69]. To further apply the "mismatched postnatal environment" element of the theory, we must also recall how nicotine-exposed rat offspring experience decreased birthweight [20], perhaps due to placental insufficiency [4], thus providing opportunity for subsequent rapid catch-up growth to occur. Beyond the increased risk of cardiovascular and metabolic disease that Barker discovered to follow combined IUGR and rapid postnatal catchup growth [71], an additional consequence that nicotine-exposed offspring may face is an augmented vulnerability to postnatal insults that might otherwise have been adaptable. Relating this back to the molecular focus of this thesis, would altered cellular pathways be responsible for the nicotine-induced sensitizations to postnatal injury? More specifically, is the greater vulnerability to postnatal insult after prenatal nicotine exposure attributed to increased

susceptibility to ER stress augmentation? Ongoing research from our lab is attempting to answer this question by investigating the ER stress response in livers and other organs of offspring that were prenatally treated with nicotine and later fed a postnatal high fat diet.

4.3.6 The influence of maternal genetics in determining the degree of nicotine exposure

To this point, our discussion has mostly focused on the effects of nicotine on the placenta or the fetus. However, the extent to which nicotine may induce *any* effect at all in these systems is largely determined by the initial maternal metabolism of nicotine [72]. As previously alluded to, there is substantial variability between individuals' metabolomic capacity for nicotine due to genetic polymorphisms of essential metabolizing enzymes, such as CYP2A6 [73, 74]. These genetic polymorphisms have profound effects on individual variations in smoking behaviour and therapeutic responses to pharmacotherapies, since the degree of metabolism strongly influences the amount of nicotine available [75, 76]. Based on this, we must also consider the role of maternal genetics in affecting the placental and fetal exposures to nicotine *in utero*. More specific to our research, to what degree might maternal genetic polymorphisms in CYP2A6 influence placental exposure to nicotine, and successively affect ER stress induction in placental trophoblast cells? Studies are currently suggesting that maternal polymorphisms in CYP2A6 and other nicotine metabolizing enzymes may differentially impact the well-being of offspring exposed to nicotine *in utero* [77-79], yet, the effects on the placenta have not been addressed. This further suggests that distinct maternal polymorphisms may place certain individuals more at risk to nicotine exposure during pregnancy, conveying an urgent need to identify, study, and treat these unique populations accordingly.

4.4 Conclusion

Though there are still many complex facets left to be investigated, our findings have provided novel contributions to characterizing the nicotine-induced ER stress response in the placenta (summarized in Figure 4.2). Importantly, by elucidating these underlying molecular mechanisms, we may better understand the clinical pathologies associated with maternal nicotine exposure. Our *in vitro* experiments using TUDCA have also revealed some promising results, promoting exciting future avenues of research in the development of therapeutic interventions. Bearing in mind the considerable risks that still follow the maternal use of conventional NRTs, e-cigarettes, and other emerging forms of nicotine delivery [80], there remains an unprecedented need for continued research on the consequences of nicotine exposure during pregnancy.

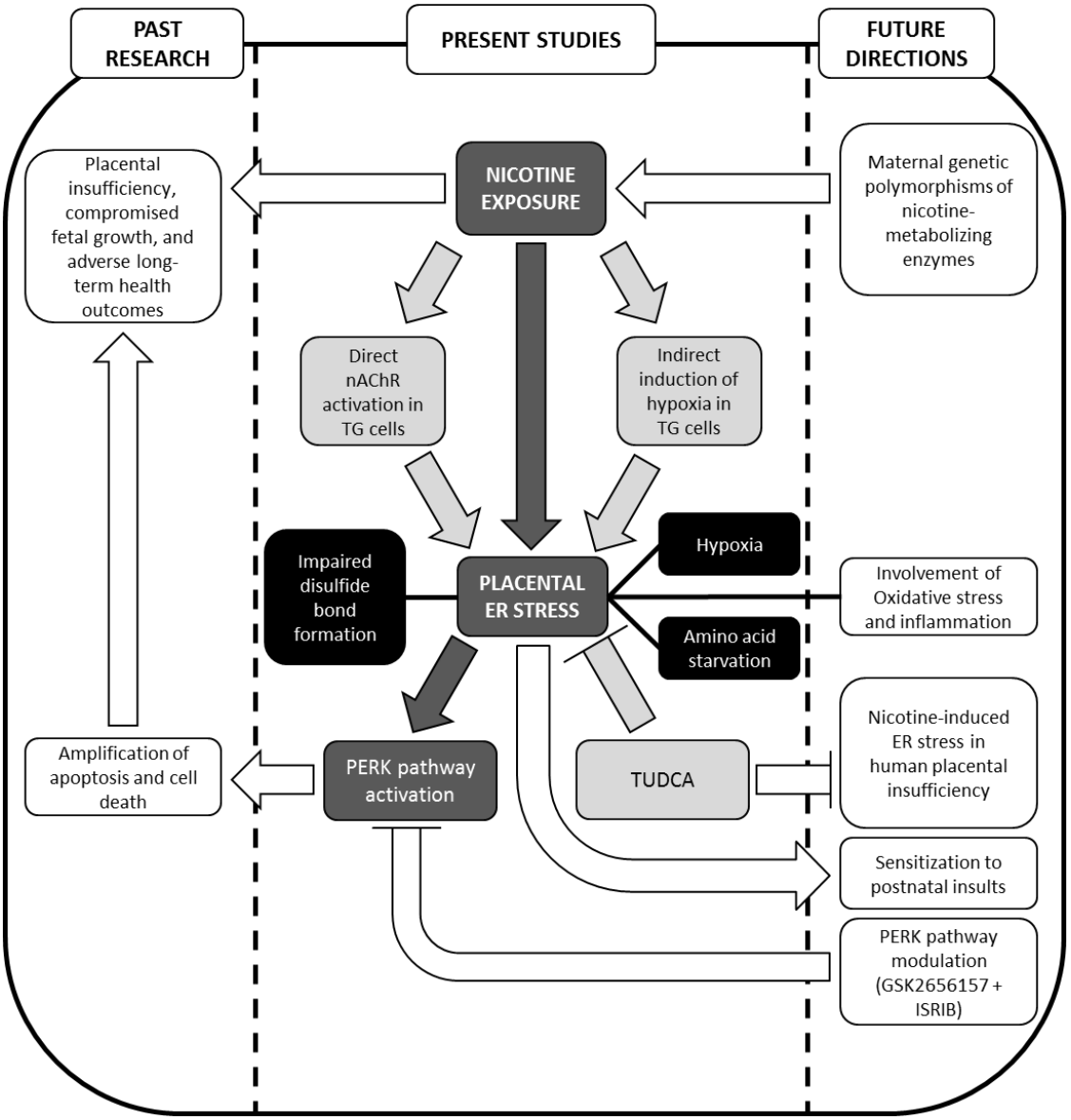

Figure 4.2. Characterization of the nicotine-induced ER stress response in the rat placenta. Light grey boxes indicate findings from our *in vitro* experiments. Black indicates findings from our *in vivo* experiments. Medium grey indicates findings from both *in vivo* and *in vitro* experiments. White indicates findings from either past research or proposed future areas of study. Pointed arrows indicate a potentially causal relationship. Flat arrows indicate an inhibitory relationship. Connecting lines indicate associations between findings.

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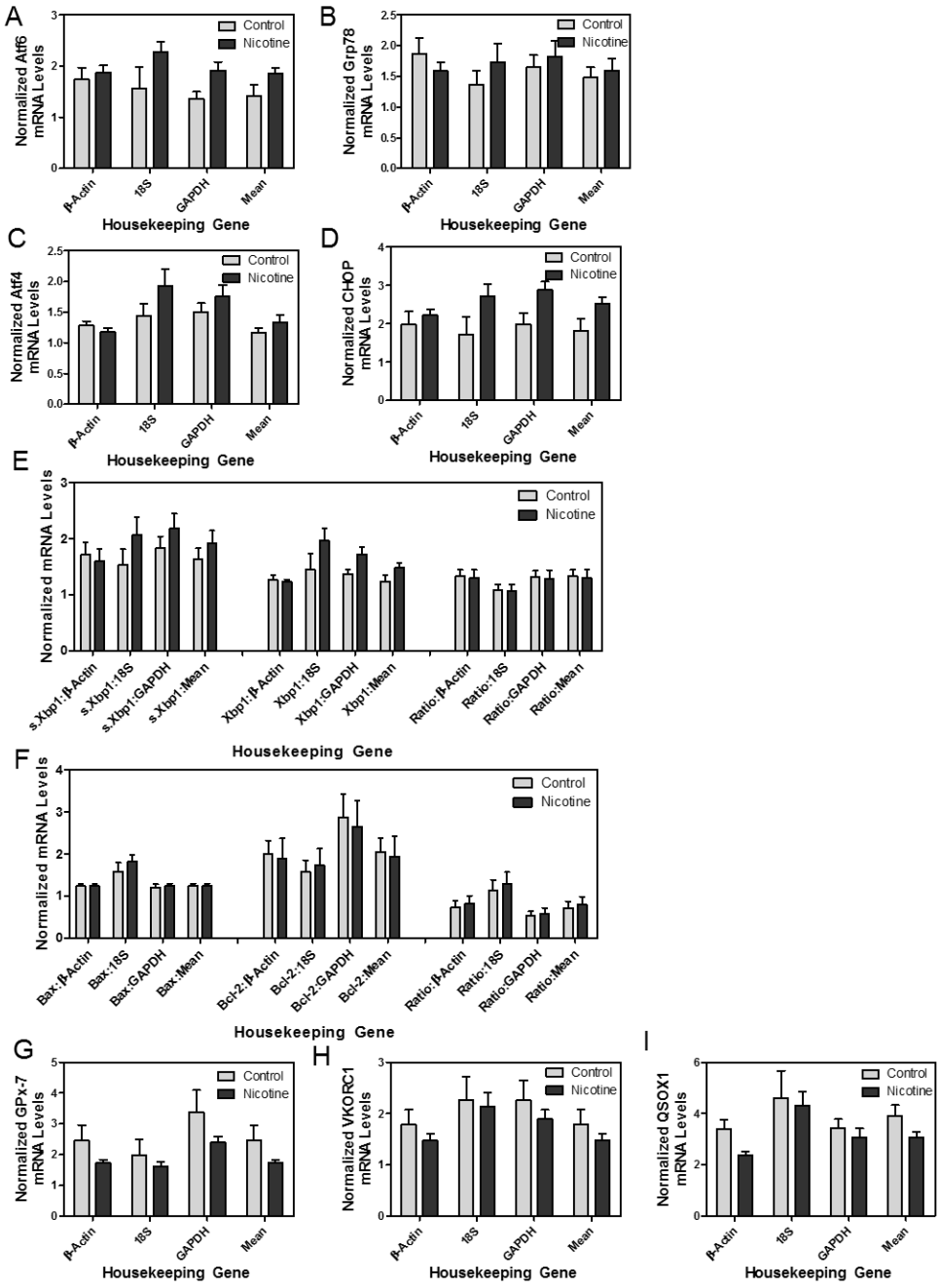
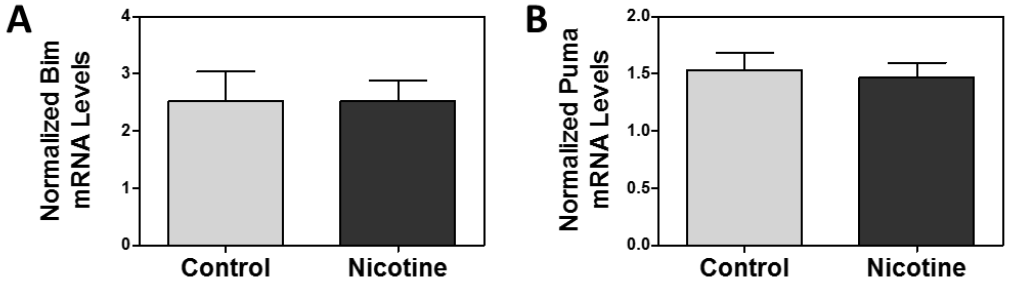
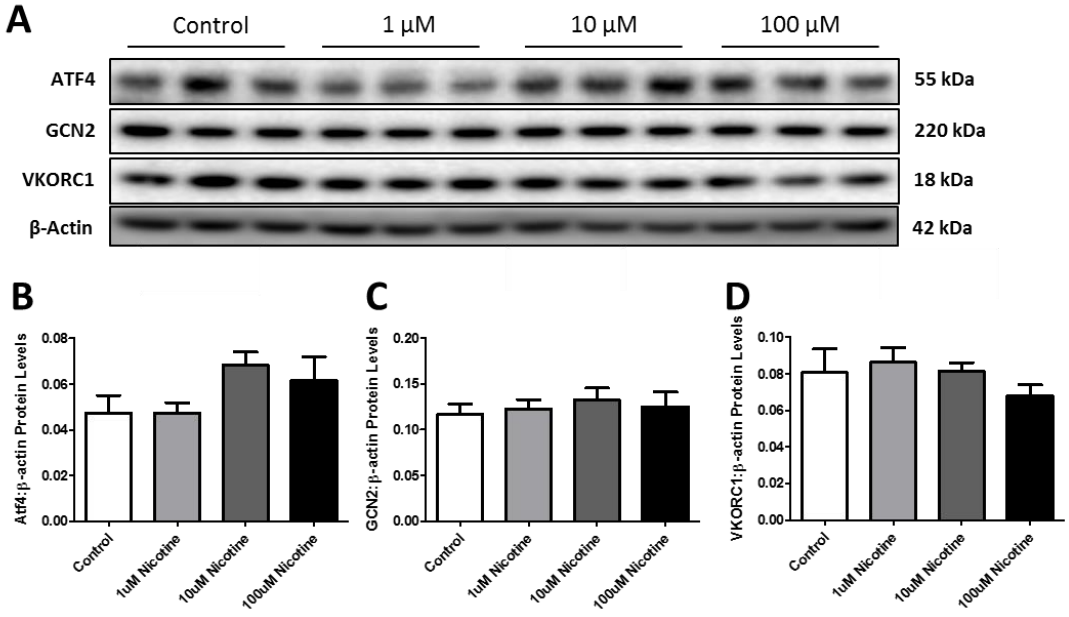
Appendix I: Supplemental Figures



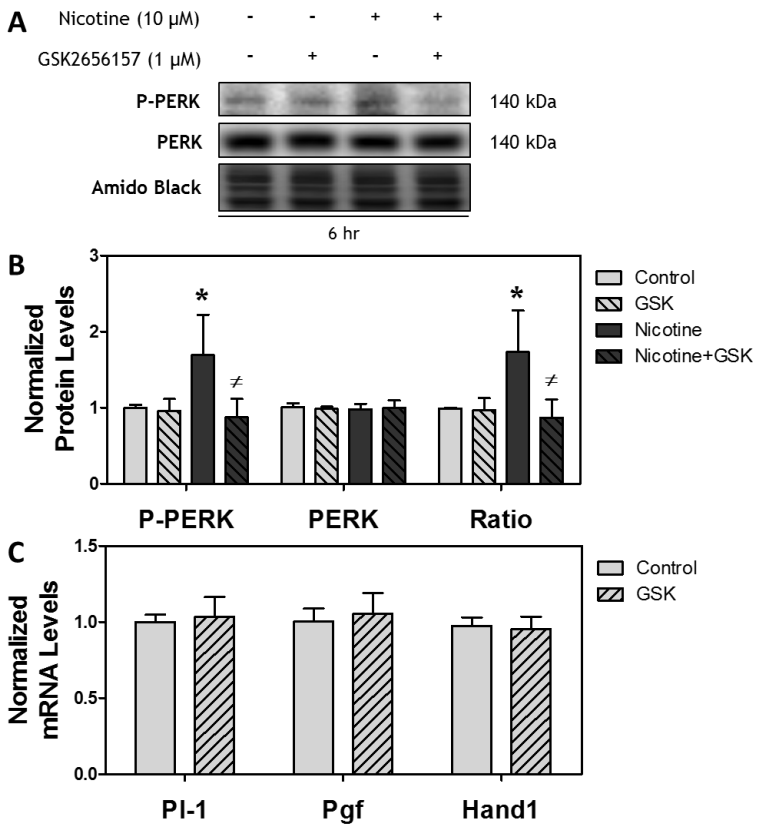
Supplemental Figure 1. mRNA targets all normalized to individual housekeeping genes selected for geometric mean. Trends remain across all normalizations to individual housekeeping genes. All mRNA levels were expressed as means normalized to either β-Actin, 18S, Gapdh, or the geometric mean ± SEM (n=5-6/group). Statistical analyses were not performed in these graphs.



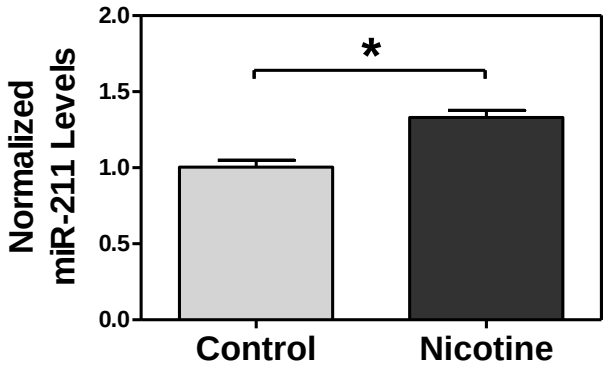
Supplemental Figure 2. The effect of maternal nicotine exposure on Bim and Puma mRNA levels. mRNA levels of targets of interest were determined via RT-PCR. mRNA levels of (A) Bim and (B) Puma. All mRNA levels were expressed as means normalized to the geometric mean of three stable housekeeping genes (β-Actin, 18S, and Gapdh) ± SEM (n=5-6/group).



Supplemental Figure 3. The effect of various doses of nicotine (1-100µM) on ATF4, GCN2, and VKORC1 after 24 hours in Rcho-1 TG cells. (A) Specific targeted protein bands as detected by respective antibodies via Western blot. Protein levels of (B) ATF4, (C) GCN2, and (C) VKORC1. All arbitrary values were expressed as means normalized to β-Actin ± SEM. All experiments were performed in triplicates (n=3). There were no significant differences across treatment groups.



Supplemental Figure 4. Preliminary experiments assessing the effect of GSK2656157 on nicotine-induced PERK activation and downstream TG cell function. (A) Specific targeted protein bands as detected by respective antibodies via Western blot. (B) Protein levels of P-PERK, PERK, and ratio of P-PERK: PERK at 6 hours. All arbitrary values were expressed as means normalized to Amido Black ± SEM. (C) mRNA levels of Pl-1, Pgf, and Hand1 after 24 hours. All arbitrary values were expressed as means normalized to either Amido Black ± SEM, or the geometric mean of β-Actin and Gapdh ± SEM. All experiments were performed in quadruplicates (n=4). Significant differences between treatment groups determined by one-way ANOVA (1WA) followed by Tukey's post-test were indicated by * ($p<0.05$; significant difference from control) or ≠ ($p<0.05$; significant difference from nicotine).



Supplemental Figure 5. The effect of maternal nicotine exposure on miR-211 levels. Levels of miR-211 were determined via RT-PCR. miRNA levels were expressed as means normalized to RNU6B ± SEM (n=5-6/group). *, Significant difference ($p<0.05$).

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McMaster University

APPROVED

ANIMAL Utilization Protocol

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Approval Date: 11 (Day) - June (Month) - 2014 (Year)

Expiry Date: 11 (Day) - June (Month) - 2018 (Year)

AUP #: 14 - 02 - 09

Replaces AUP #: 10 - 06 - 48

University Veterinarian's Signature — Date: June 11/14

AREB Chair's Signature — Date: June 11/14

Principal Investigator's Signature — Date: 05 06 14

Prepared By:
Name: Dr Alison Holloway
Email Address:

Refer to the Guide for Preparation of AUPs for assistance with completing this form
(available at http://www.fhs.mcmaster.ca/healthresearch/areb_forms.html)

Section 1 — Project Title

Title: Effect of fetal and neonatal chemical insults on postnatal health

Type of Project (Check all applicable):
- ☒ Research
- ☐ Teaching*
- ☐ New Project
- ☒ Ongoing Project
- ☐ Research Pilot Study
- ☐ Other

*Attach Teaching Addendum available at http://www.fhs.mcmaster.ca/healthresearch/areb_forms.html.

Section 2 — Principal Investigator

Last Name	Holloway	First Name	Alison
Title	Associate Professor	Department	Obstetrics and Gynecology
Business Phone		Home Phone	
Emergency Phone		Pager Number	N/A
Laboratory Room #		Laboratory Phone	
Institutional Email			
Mailing Address			

Section 3 — Associate Scientists, Research Staff & Training/Experience

Provide at least one after hours emergency contact number.

Name	Department	Title (Co-PI, Tech, Undergrad Student, Grad)	Extension	After Hours Emergency Contact Number
Dr Sandeep Raha	Pediatrics	Co-PI		
Dr Colin Nurse	Biology	Co-PI		

Identify AF courses *completed including Principal Investigator*
CCAC Guidelines require that all individuals listed on the AUP take Orientation.
A print-out verifying the training listed below must be attached to this document (contact AF, Ext. 22365).

Name	OR	AH	Inj An	Gas An	SS	IM	TN	EP	BL	Brd	Bio	Fish	Experience and/or other training
Dr Alison Holloway	☒	☒	☒	☒	☒	☐	☐	☒	☒	☒	☐	☐	18 years experience with animal models (fish, sheep and rodents)
Dr Sandeep Raha	☒	☒	☐	☒	☐	☐	☐	☒	☒	☐	☐	☐	
Nicole De Long	☒	☒	☐	☒	☐	☐	☐	☒	☒	☐	☐	☐	
Harriet Law	☒	☒	☐	☒	☐	☐	☐	☒	☒	☐	☐	☐	
Taylor Van Duzer	☒	☒	☐	☒	☐	☐	☐	☒	☐	☐	☐	☐	

Curriculum Vitae

MICHAEL WONG

Department of Physiology and Pharmacology
The University of Western Ontario
London, Ontario, Canada
N6A 5C1

EDUCATION

MSc in Physiology and Pharmacology '15
Western University, London ON Canada
BMSc in Physiology '13
Honors Specialization, Western Scholars, Dean's Honour List
Western University, London ON Canada

RESEARCH EXPERIENCE

Master's Research Thesis *2013 – Present*
Western University, Department of Physiology & Pharmacology, Obstetrics & Gynaecology
Supervisor: Dr. Daniel B. Hardy

- Project title: Characterization of the nicotine-induced endoplasmic reticulum stress response in the rat placenta *in vivo* and *in vitro.*
    - Objective 1: To investigate the effects of maternal nicotine exposure on ER stress in the rat placenta *in vivo*, in collaboration with Dr. AC Holloway (McMaster University).
    - Objective 2: To investigate the direct and indirect effects of nicotine exposure on ER stress in rat placental trophoblast giant cells *in vitro*.
    - Objective 3: To investigate the effects of ameliorating nicotine-induced ER stress and UPR activation in rat placental trophoblast giant cells *in vitro.*

Undergraduate Research Assistant *2011 – 2013*
Western University, Department of Physiology & Pharmacology, Obstetrics & Gynaecology
Supervisor: Dr. Daniel B. Hardy

- Investigated the effects of nicotine exposure on the serotonergic pathway in the rat placenta and HTR8/SVneo human trophoblast cells.
- Investigated the effects of *in utero* nicotine exposure on rat pancreatic and liver function, in collaboration with Dr. AC Holloway (McMaster University).

LABORATORY AND PROFESSIONAL SKILLS

- *Molecular techniques*: Extraction and quantification skills (Protein, RNA, miRNA, histones), Real-Time PCR, Western blot, microscopy.
- *Physiology techniques*: Cell culturing, animal handling/injections, motor point stimulation i*n vivo*, frog sciatic nerve extraction and ex vivo stimulation, compound action potential nerve recording, subject recruitment, human static and dynamic exercise experiments, and auditory localization experiments.
- *Computer skills:* Statistical analysis (GraphPad Prism), Microsoft Office, online search tools (PubMed, etc.), Adobe Photoshop, InDesign, Quantity One, Image Lab.
- *Additional professional skills:* Public speaking, scientific writing and knowledge translation, project design/management.

PUBLICATIONS

- M. K. Wong, N. G. Barra, N. Alfaidy, D. B. Hardy, A. C. Holloway. Prenatal nicotine exposure and postnatal health outcomes. Review. *Reproduction* (Submitted).
- M. K. Wong, C. J. Nicholson, A. C. Holloway, D. B. Hardy. Maternal nicotine exposure leads to impaired disulfide bond formation and augmented endoplasmic reticulum stress in the rat placenta. *PloS one* **10**, e0122295 (2015).
- N. Ma, C. J. Nicholson, M. Wong, A. C. Holloway, D. B. Hardy, Fetal and neonatal exposure to nicotine leads to augmented hepatic and circulating triglycerides in adult male offspring due to increased expression of fatty acid synthase. *Toxicology and applied pharmacology* **275**, 1-11 (2014).

AWARDS/SCHOLARSHIPS/GRANTS

- Public Service Alliance of Canada Academic Achievement Scholarship $500 *(2015)*
- Paul Harding Research Day Oral presentation 1st place *(2015)*
- Southern Ontario Reproductive Biology Oral presentation award *(2015)*
- Best New Investigator Award Society of Reproductive Investigation *(2015)*
- Western Libraries Open Access Fund Publication Grant $1689 *(2015)*
- Ontario Graduate Scholarship $15,000/year *(2014-2015)*
- Graduate Teaching Assistantship $4,550/year *(2013-2015)*
- Graduate Research Scholarship $4,500/year *(2013-2015)*
- Physiology and Pharmacology Research Day Poster Presentation 1st place *(2014)*
- Paul Harding Research Day Oral presentation 2nd place *(2014)*
- Public Service Alliance of Canada Academic Achievement Scholarship $500 *(2014)*
- Obstetrics and Gynaecology Graduate Scholarship $15,500/year *(2013-14)*
- Dean's Honour List 5 years *(2009-13)*
- Western's Excellence in Leadership Award *(2011)*
- Western Scholarship of Distinction $1,000 *(2008-2009)*
- Harold Botsford Memorial Award $300 *(2008)*
- Writer's Craft Highest Graduating Average *(2008)*

PRESENTATIONS

1. Wong M, Nicholson CJ, Holloway AC, and Hardy DB. Characterization of the endoplasmic reticulum stress response in nicotine-induced placental insufficiency. *Oral presentation* at Paul Harding Research Day, London ON, May 2015.
2. Wong M, Nicholson CJ, Holloway AC, and Hardy DB. Characterization of the endoplasmic reticulum stress response in nicotine-induced placental insufficiency. *Oral presentation* at Southern Ontario Reproductive Biology Meeting, Hamilton ON, May 2015.
3. Wong M, Nicholson CJ, Holloway AC, and Hardy DB. Characterization of the endoplasmic reticulum stress response in nicotine-induced placental insufficiency. *Oral presentation* at London Health Research Day, London ON, April 2015.
4. Wong M, Nicholson CJ, Holloway AC, and Hardy DB. Maternal nicotine exposure leads to impaired disulfide bond formation and endoplasmic reticulum stress in the rat placenta. *Poster presentation* at Society of Reproductive Investigation, San Francisco CA, March 2015.
5. Wong M, Nicholson CJ, Holloway AC, and Hardy DB. Characterization of the endoplasmic reticulum stress response in nicotine-induced placental insufficiency. *Oral presentation* at Lawson Health Research Institute Talks on Fridays, London ON, February 2015.
6. Wong M, Nicholson CJ, Holloway AC, and Hardy DB. Maternal nicotine exposure leads to impaired disulfide bond formation and endoplasmic reticulum stress in the rat placenta. *Poster presentation* at Canadian National Perinatal Research Meeting, Montebello QC, February 2015.
7. Wong M, Nicholson CJ, Holloway AC, and Hardy DB. Maternal nicotine exposure leads to impaired disulfide bond formation and endoplasmic reticulum stress in the rat placenta. *Poster presentation* at Physiology and Pharmacology Research Day, London ON, November 2014.
8. Wong M, Nicholson CJ, Holloway AC, and Hardy DB. Maternal nicotine exposure leads to augmented endoplasmic reticulum stress in the rat placenta. *Oral presentation* at Paul Harding Research Day, London ON, May 2014.
9. Wong M, Nicholson CJ, Holloway AC, and Hardy DB. Maternal nicotine exposure leads to augmented endoplasmic reticulum stress in the rat placenta. *Oral presentation* at Southern Ontario Reproductive Biology Meeting, Toronto ON, April 2014.
10. Wong M, Nicholson CJ, Holloway AC, and Hardy DB. Maternal nicotine exposure leads to augmented endoplasmic reticulum stress in the rat placenta. *Poster presentation* at London Health Research Day, London ON, March 2014.
11. Wong M, Nicholson CJ, Holloway AC, and Hardy DB. Exposure to nicotine or low-protein diet in pregnancy leads to ER stress in the rat placenta. *Poster presentation* at Physiology and Pharmacology Research Day, London ON, November 2013.
12. Wong M, Nicholson CJ, Holloway AC, and Hardy DB. Nicotine exposure in vitro leads to decreased expression of Tryptophan Hydroxylase 1 (TPH1) in pancreatic and placental cell lines. *Poster presentation* at Physiology Thesis Poster Day, London ON, March 2013.