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Differential Receptors for Advanced Glycation End-Products (RAGE) Expression in Preeclampsia, Intrauterine Growth Restriction and Gestational Diabetes

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ABSTRACT

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Preeclampsia (PE), intrauterine growth restriction (IUGR) and gestational diabetes (GDM) increase the risk of maternal and fetal morbidity and mortality. The roles of Advanced Glycation End-products (AGEs) are already well documented concerning inflammation, hypoxia and oxidative stress. AGEs bind to its receptor, Receptor for Advanced Glycation End-products (RAGE), and activate an inflammatory pathway. This pathway alters the efficacy of invasive trophoblast cells and in the placenta and can result in placental dysfunction. We hypothesized that the placental dysfunction found in PE, IUGR, and GDM resulted from an over activation of the RAGE-mediated inflammatory pathway. Using human placental samples, we found that RAGE protein expression via western blotting was increased in PE and decreased in IUGR while GDM remained similar to that of control placentas. We then wanted to determine the efficacy of RAGE activation to alter the invasive nature of invasive cytotrophoblasts cells. We found that the addition of AGEs to SW71 cells decreases invasion through the activation of JNK and ERK cellular signaling pathways. Altogether these findings suggest that RAGE activation in trophoblast cells seems result in insufficient placental pathogenesis causing PE, however the IUGR and GDM samples we obtained did not seem to have resulted from RAGE activation. We also found that RAGE activation can alter the ability of invasive trophoblasts to invade, thus limiting the ability of the placental cells to remodel the maternal spiral arteries. We believe that further research into specific triggers of IUGR (smoking-induced) and un-treated diabetes could result in RAGE stimulated placental insufficiency.

Keywords: RAGE, preeclampsia, intrauterine growth restriction, gestational diabetes, AGEs, pregnancy

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CHAPTER 1: Introduction

Failure to deliver oxygen and nutrients through the placenta to a developing fetus can result in life threatening conditions for both the fetus and mother. There are a variety of pregnancy pathologies that can result in placental dysfunction including: preeclampsia (PE), intrauterine growth restriction (IUGR), and gestational diabetes (GDM). These pathologies affect the ability of the placenta to transport vital nutrients and oxygen important for fetal development. In ischemic conditions the ability of maternal spiral arteries to mature allowing greater blood flow is significantly reduced (Roberts, 2015; Pardi, Marconi, & Cetin, 2002). These pregnancy pathologies involve a developed hypoxic condition in the uterus/placenta reducing the ability of the fetus to obtain necessary components for proper growth. Although each pathology is rooted in ischemia, there are many compounding variables making each disease unique and complicated. There is not one single pathophysiological disturbance that causes these pathologies, but a combination of factors that compound to each disease. Because pregnancy complications are often life threatening to the mother and/or fetus, pathologies such as PE, IUGR and GDM have been studied profusely. Despite the plethora of knowledge obtained in recent years, much is left to be understood especially the role of inflammation in ischemic conditions.

Preeclampsia

Preeclampsia is a pregnancy complication that arises in 6-8% of all pregnancies with a higher occurrence in women with diabetes mellitus, hypertension or a history of preeclampsia (Sibai, Caritis, & Hauth, 2003). If left untreated, preeclampsia can cause severe morbidity to the mother and fetus by causing preeclampsia, HELLP syndrome, intrauterine growth restriction, brain hemorrhage or cardiovascular complications (Naruse, *et. al.,* 2012). Severe preeclampsia and eclampsia account for 15% of maternal deaths in the United States and significantly higher

rates in third world countries (Sibai, Caritis, & Hauth, 2003). The phenotype of preeclampsia is well classified as hypertension combined with proteinuria after 20 weeks gestational age, however the cause of preeclampsia has not been elucidated. There have been a variety of pathophysiological processes that have been linked to preeclampsia such as endothelial dysfunction, oxidative stress, angiogenic and inflammatory factor imbalance and activation of the receptor for advanced glycation end products (RAGE) (Oliver, *et al.*, 2010). Despite the plethora of physiological explanations for the development of preeclampsia, there are many holes of understanding that need to be filled.

Intrauterine Growth Restriction

Intrauterine growth restriction/retardation (IUGR) occurs in 12% of all pregnancies. The key physiological effect of IUGR is a small-for-gestational age infant accompanied by a small placenta and a link of the fetus developing coronary heart diseases (Heinonen, Taipale, & Saarikoski, 2001). A little over three decades ago, it was hypothesized that the small placental size resulted from decreased migration of trophoblasts when they invade to restructure the maternal spiral arteries in order to allow greater nutrient and oxygen accessibility to the placenta and fetus (Pijnenborg, Bland, Robertson, & Brosens 1983). If the maternal spiral arteries are insufficiently remodeled there is a reduction of blood flow to the placenta compared to controls (Pijnenborg, Bland, Robertson, & Brosens 1983). The inability for the placenta to transport necessary nutrients to the fetus results in an undernourished fetus and can lead to fetal death. Despite the understanding that insufficient trophoblast invasion is utilized in intrauterine growth restriction, there are no therapeutic targets or methods available to prevent it (Friedman, & Cleary, 2014). Although the role of trophoblast invasion has been well defined in IUGR, the cause of trophoblast dysfunction is still being elucidated.

Gestational Diabetes

In 2014 it was estimated that diabetes affected 387 million people worldwide—which is 8.3% of the global population—with the occurrence of type 2 diabetes mellitus increasing globally (International Diabetes Federation, 2014). Occurring in 3%-30% of pregnancies, gestational diabetes is the principal metabolic disorder during gestation (Aires, & de Santos, 2015). Gestational diabetes is glucose intolerance of any degree, which arises (or is diagnosed) during pregnancy. The maternal glucose intolerance results in an increase in placental weight and a decrease in fetal/placental weight ratio at birth (Taricco, Radaelli, de Santis, & Cetin, 2003). In gestational diabetes pathologies, it has been shown that there is an increase in cell proliferation rate and decrease in apoptosis which results in a larger placenta (Zorn, *et al.*, 2011; Benirschke, Kaufmann, Baergen, 2006; Belkacemi, Kjos, Nelson, Desai, & Ross, 2013). The immature villi are unable to successfully transport oxygen and nutrients to the fetus resulting in a hypoxic state (Benirschke, Kaufmann, Baergen, 2006). Not only is the function of trophoblast cells altered, but also the trophoblast layers are thicker than normal placentas and have significantly higher lipid droplets and glycogen (Gewolb, Merdian, Warshaw, & Enders, 1986). Gestational diabetes has an adverse effect on trophoblast function, but recent studies have demonstrated detrimental epigenetic changes in the fetus associated with maternal hyperglycemia (Ronald, Tutino, Lillycrop, Hansen, & Tam, 2015). The underlying mechanism for these pathophysiological effects are unknown and research is needed to elucidate the causes for these adverse effects.

Receptor for Advanced Glycation End Products (RAGE)

The receptor for advanced glycation end products (RAGE) is a 35kd transmembrane protein receptor expressed on multiple subtypes of tissues and is known to be part of the immune-inflammation pathway (Ramasamy, Yan, Herold, Clynes, & Schmidt, 2008). Ligand

binding of RAGE stimulates multiple cellular signaling cascades: the MAPK activation has been demonstrated in smooth muscle, myoblasts, osteoblasts, monocytes, and tubular epithelial myofibroblasts (Lander, Taurus, Ogiste, Hori, Moss, & Schmidt, 1997; Shanmugam, Kim, Lanting, & Natarajan, 2003; Li, Wang, Huang, Oldfield, Schmidt, Cooper, Lan, 2004; Cortizo, Lettieri, Barrio, Mercer, Etcheverry, & McCarthy, 2003; Sorci, Ruizzi, Agneletti, Marchetti, & Donato, 2004). RAGE has also been implicated in a variety of physiological pathologies such as aging, diabetes, cardiomyopathy, neurodegeneration, nephropathy, retinopathy, rheumatoid arthritis, chronic kidney disease, inflammation, polycystic ovarian syndrome, preeclampsia, and obesity (Ramasamy, Vannucci, Yan, Herold, Yan, & Schmidt, 2005; Rojas, Pérez-Castro, González, Delgado, Romero, & Rojas 2014; Alghasham, & Rasheed, 2014; Singh, Bali, Singh, & Jaggi, 2014; Merhi, 2013). Not only are there many diseases associated with RAGE, but there are multiple ligands that can bind and activate RAGE thus perpetrating the disease state such as: amyloid beta peptide and the family of beta sheet fibrils, amphoterin, S100/calgranulins and advance glycation end-products (Bucciarelli, *et al.*, 2002, Gugliucci, & Menini, 2014, Bierhaus, *et al.*, 2005). Advanced glycation end-products (AGEs) are a common ligand for RAGE and have been studied extensively in pathological conditions. AGEs are produced via the Maillard reaction when the carbonyl group of a carbohydrate reacts with a primary amino group of a protein in a non-enzymatic reaction (Brownlee, Cerami, & Vlassara 1988). AGEs are produced endogenously by our body and can be formed as a byproduct of foods (Poulsen, *et al.,* 2013). In situations of chronic inflammation, proteins and lipids are modified which also produce AGEs (Sado, *et al.,* 2011). The regulation of RAGE is dependent on the concentration of receptors in the membrane of the cell, as well as the concentration of soluble RAGE (sRAGE). The molecular structure of sRAGE does not contain the transmembrane region; therefore it can bind

the ligands of RAGE, such as AGEs, but does not activate cellular signaling inflammatory pathways. Many studies have examined the role of RAGE in pregnancy, specifically with the concentrations of sRAGE in circumstances of infection, preeclampsia, and IUGR (Oliver, *et al.,* 2010; Naruse, *et. al.,* 2012). It has been found that AGEs change the formation of the vascular beds in the placenta which reduces the ability of the placenta to deliver sufficient nutrients and gas to the fetus (Guedes-Martins, Matos, Soares, Silva, & Almeida, 2013). Despite the research previously done, there are significantly more questions than answers concerning the effect of AGEs and RAGE on pregnancy pathologies as well as activation of RAGE in other pregnancy complications.

Summary of Research

The study outlined in the following chapter is the first to observe RAGE expression and MAPK activation in preeclampsia (PE), intrauterine growth restriction (IUGR) and gestational diabetes (GDM). It is also the first to look the effect of AGEs on altering the phenotype of invasive first trimester trophoblast cells. Because PE, IUGR and GDM can stem from chronic placental inflammation, we wanted to test if placental insufficiency resulted from a RAGE-induced inflammatory response. If RAGE activation was the culprit behind placental dysfunction in these pregnancy pathologies, then we could ameliorate the effects of RAGE by the addition of a small molecule that down-regulates or has competitive binding, thus limiting RAGE activation. We also wanted to test if RAGE activation in invasive cytotrophoblasts cells could alter the efficacy of these cells to invade which could possibly result in an inhibition of trophoblast cells to remodel the maternal spiral arteries. The inability of the placenta to remodel the spiral arteries results in decreased blood flow to the placenta which can result in decreased nutrients and gas to the fetus, resulting in a small for gestational age fetus, which can be the case in PE and IUGR.

However, the activation of the MAPK pathway in trophoblast cells can also result in a significantly larger placenta which then delivers too much glucose to the fetus, resulting in a large for gestational age fetus, which occurs often in GDM. Determining the role of RAGE in altering the invasive properties of trophoblast cells will enable us to seek alternative therapies to restore vital properties to placental cells.

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CHAPTER 2: Differential Receptor for Advanced Glycation End-Products (RAGE) Expression in Preeclamptic, Intrauterine Growth Restricted, and Gestational Diabetic Placentas

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Abstract

The Receptor for Advanced Glycation End-Products (RAGE) is a cell surface receptor implicated in the modulation of diverse inflammatory pathways. Inflammation has been associated with the development of pregnancy pathologies including preeclampsia (PE), intrauterine growth restriction (IUGR), and gestational diabetes mellitus (GDM) that can centrally impact diverse maternal and fetal complications. Our objective was to examine placental RAGE expression in PE, IUGR, and GDM complications. We hypothesized that RAGE would be activated in PE, IGUR and GDM. We also hypothesized that RAGE activation by stimulation of AGEs would result in a decrease of invasion, increase in JNK, ERK and p38 activation as well as an increase in cytokine release. Compared to control placental samples we observed: 1) a 3.4-fold increase in placental RAGE gene expression during GDM-D, 2) a 2.2-fold increase in placental RAGE gene expression during GDM-I, 3) a 2-fold increase of placental RAGE protein expression in the PE placenta, and 4) a 3-fold decreased of placental RAGE protein expression in the IUGR placenta. In trophoblast cells exposed to 25 ug of AGEs, a common RAGE ligand, we observed: 1) decreased trophoblast invasion, 2) a 10-fold increase in JNK activation, 3) a 8-fold increase in ERK activation, and 4) significantly increased TNF-α and IL-1β secretion. We conclude that placental RAGE is activated in PE situations and that RAGE-mediated inflammation in the trophoblast involves increased TNF-α and IL-1 β secretion through the activation of ERK and JNK signaling.

Abbreviations:

PE, Preeclampsia
IUGR, Intrauterine Growth Restriction
GDM-I, Gestational Diabetes Mellitus treated with Insulin

GDM-D, Gestational Diabetes Mellitus treated with diet (GDM-D)

ERK, Extracellular signal-regulated kinases

JNK, c-Jun N-terminal kinases

p38, p38 mitogen-activated protein kinase

TNF-α, Tumor Necrosis Factor Alpha

IL-1β, Interleukin-1 Beta

Introduction

Inflammation plays a key role in maintaining homeostasis; however exaggerated inflammation can cause tissue damage and lead to disease (Challis, Lockwood, Myatt, Norman, & Strauss, 2009). Abnormal inflammation has been associated with various pregnancy pathologies such as preeclampsia (PE), pre-term birth (PTB), spontaneous abortion and intrauterine growth restriction (Challis, Lockwood, Myatt, Norman, & Strauss, 2009; Challis, Lye, Gibb, Whittle, Patel, & Alfaidy, 2001).

Toll-like receptors (TLR) are expressed on the plasma membrane of macrophages and are associated with inflammatory responses (Challis, Lockwood, Myatt, Norman, & Strauss, 2009). Previous research has suggested that TLRs may be responsible for enhancing the risk of preterm birth (Challis, Lockwood, Myatt, Norman, & Strauss, 2009; Challis, Lye, Gibb, Whittle, Patel, & Alfaidy, 2001). Like TLRs, Receptors for Advanced Glycation End-products (RAGE) are cell surface pattern recognition receptors detected in many cell types including endothelium, smooth muscle, macrophages and several specific epithelial cell types (Buckley, & Ehrhardt, 2010; Warburton, *et al.*, 2005). Of interest, RAGE is also expressed in the human placenta (Holmlund, *et al.*, 2007). RAGE was initially characterized for its ability to bind to non-enzymatically glycated macromolecules, advanced glycation end-products (AGEs); however, RAGE ligation also occurs following the binding of a diversity of ligands of various types (Morbini, Villa, Campo, Zorzetto, Inghilleri, & Luisetti, 2006). When AGEs bind to receptors, MAP kinases (MAPK, JNK, p44/42 and p38) and intermediates of the Jak/STAT pathway are activated (Morbini, Villa, Campo, Zorzetto, Inghilleri, & Luisetti, 2006). While RAGE activation is implicated in inflammation and cell migration, the expression of RAGE is relatively low in most physiological settings. Interestingly, inflammatory responses up regulate RAGE and enhance its

activation culminating in increased activity of several MAPKs and NF-κB mobilization to the nucleus (Xie, Mendez, Mendez-Valenzuela, & Aguilar-Hernandez, 2013). NF-κB is then capable of coordinating augmented expression and secretion of a variety of inflammatory response-related molecules such as tumor necrosis factor α (TNFα), interferon γ (INF-γ) and interleukin 6 (IL-6) (Xie, Mendez, Mendez-Valenzuela, & Aguilar-Hernandez, 2013).

RAGE is up regulated in disease states such as diabetes, lung fibrosis, acute respiratory distress syndrome (ARDS), polycystic kidney disease, chronic obstructive pulmonary disease (COPD), and in the myometrium of women affected with preeclampsia (Bohlender, Franke, Sommer, & Stein, 2005; Cooke, Brockelsby, Baker, & Davidge, 2003; Ferhani, *et al.*, 2010; He, *et al.,* 2007; Nakamura, *et al.,* 2011; Oliver, *et al.,* 2011; Park, Seo, & Park, 2010; Robinson, Johnson, Bennion, & Reynolds, 2012; Wu, Merrilees, Young, & Black, 2011). In conditions associated with abundant RAGE ligands, positive feedback loops may also exacerbate persistent inflammation observed in chronic disease conditions (Schmidt, Yan, Yan, & Stern, 2001). While RAGE has been implicated in a few studies involving PE, little research has been conducted that involves RAGE and other pregnancy pathologies.

The placenta often functions insufficiently in terms of fulfilling the needs of the developing fetus during pregnancy complications. Accordingly, a properly functioning placenta is critical for pregnancy success. Specifically, the placenta is vital for fetal development as it mediates gas and nutrient exchange between mother and fetus. During implantation, the placental cytotrophoblasts differentiate and invade into the uterine endometrium and myometrium and remodel the uterine spiral arteries to become low resistant, high capacity vessels (Arroyo, & Winn, 2008; Pijnenborg, *et al.*, 1991; Reuvekamp, Velsing-Aarts, Pouline, Capello, & Duits, 1999; Zhou, Damsky, & Fisher, 1997). The remodeling of these arteries allows increased oxygen

and nutrient exchange (Arroyo, & Winn, 2008; Pijnenborg, *et al.*, 1991; Reuvekamp, Velsing-Aarts, Pouline, Capello, & Duits, 1999; Zhou, Damsky, & Fisher, 1997). In many pregnancy pathologies, the trophoblast remodeling is insufficient and the placenta is unable to sufficiently nourish the maturing fetus (Saito, & Nakashima, 2014). The processes that lead to decreased placental function are multifactorial and may vary between different pathologies as well as among patients. Given the role of RAGE in diseased inflammatory tissues, our initial goal was to identify the expression profile of RAGE in several of the most common placental pathologies. Through the identification of RAGE expression, impaired invasion, and inflammatory signaling, our research suggests a role for RAGE and its activation in the biology of placental trophoblast cells.

Material and Methods

Human Placental Tissues

Placental biopsies and paraffin embedded placental tissues slides for PE, IUGR, GDM-I (gestational diabetes mellitus treated with insulin), GDM-D (gestational diabetes mellitus treated with diet) and control placenta were purchased from the Research Center for Women's and Infant's Health BioBank, Ontario, Canada. Placentas were collected shortly after delivered either vaginally or by C-section. Ten samples were analyzed for each group. PE was confirmed by increased maternal blood, pressure, and increased proteinuria. IUGR placentas were confirmed by ultrasound showing placental insufficiency with uterine Doppler and absent end diastolic flow (AEDV). IUGR samples also came from patients with an estimated fetal weight below the 10^{th} percentile. Sample demographics are shown in Table 1.

Real Time PCR

Real time PCR was performed to determine gene activation of the RAGE receptor in the placenta of normal and complicated pregnancies. cDNA was synthesized using Oligo (dT) and SuperScript II Reverse Transcriptase (both from Invitrogen by Life Technologies, Carlsbad, CA) by following the protocol suggested by the manufacturer. RT-PCR was performed using SsoFast EvaGreen Supermix (Bio-rad Laboratories, Hercules, CA), which contains a cocktail of all necessary components excluding primers and templates. Sso7d-fusion polymerase was used as the enzyme. Primers for RAGE (Fwd- ACTACCGAGTCCGAGTCTACC, Rev-GTAGCTTCCCTCAGACACACA), were utilized with 18S primers (Fwd.-GGGAGGTAGTGACGAAAAATAACAAT, Rev.-CCCTCCAATGGATCCTCGTT) as a baseline control for the various experiments. Results were tested for significance against control placentas. Cycling conditions were as follows: 95°C for 30 seconds; 95°C for 5 seconds; 60°C for 30 seconds; melt curve, 65°C for 2 seconds and 95°C for 5 seconds.

Immunofluorescence

IF was performed on paraffin embedded placental samples. In summary, slides were de-waxed, washed in a 1x Tris buffer solution (TBS), and blocked with Background Sniper (Biocare Medical, Concord, CA) for one hour. This was followed by incubation overnight with a primary RAGE antibody (Cell Signaling Technology, Danvers, MA), or Cytokeratin 7 (Dako, Carpinteria, CA,). Slides were then incubated for 1 hr with donkey anti rabbit TR or donkey ant-mouse TR (Biocare Medical, Concord, CA). 4', 6-diamidino-2-phenylindole, dihydrochloride (DAPI) was used for counterstaining prior to mounting with glass coverslips. Slides were viewed using a Texas Red excitation and emission filter.

Trophoblast Cell Culture

The first-trimester cytotrophoblast cell line Swan71 (SW71) was used for cytotrophoblast experiments. SW71 cells were cultured in RPMI medium (Mediatech, Manassas, VA) supplemented with 10% fetal bovine serum (FBS) and 1%penicillin and streptomycin.

Cell Treatments

SW71 cytotrophoblast cells were incubated in normal culture medium in a 6 well plate. At 50% confluency cells were cultured overnight at 2% serum to create quiescence. Cell cultures were exposed to media supplemented with 25 μg/ml of advanced glycation end products ((Carboxymethyl)-lysine-BSA, Circulex) or untreated media for 24 hours. After treatment, cell medium was collected and stored at -20°C for ELISA, and cells were lysed and stored at -80°C for molecular studies.

Real-Time Cell Invasion Determination

Real-time invasion of SW71 cells was performed using an xCELLigence RTCA DP (Real-Time Cell Analysis Dual Plate) instrument as previously described.[23] Briefly, cells were plated in the top chamber at a concentration of 20,000 cells/well in 2% FBS RPMI in a total volume of 100 μL in the presence or absence of 25 μg/ml of AGEs. The bottom chamber wells were filled with 160 μL of 10% FBS RPMI. The cells were then place in the RTCA DP instrument, and invasion readings were taken every 15 min for the next 24hr.

Western Blot Analysis

Western blot analysis was used to assess the expression of RAGE and a collection of cell signaling proteins in control and diseased placenta. Cell lysates (50 μg) were separated on 4–12% Bis-Tris gel SDS-PAGE and transferred to nitrocellulose membranes. Membranes were incubated with antibodies against RAGE, and phospho- and total ERK, p38 and JNK proteins (all from Cell Signaling Technology). Membranes were then incubated with a secondary horseradish peroxidase (HRP)-conjugated antibody for one hour at room temperature. The membranes were incubated with ECL substrate, and the emission of light was detected using x-ray film. To determine loading consistencies, each membrane was stripped and re-probed with an antibody against mouse β-actin (Sigma Aldrich, St. Louis, MO). Expression levels of the proteins were quantified by densitometry normalized to β-actin expression and changes in the expression of the various targets were compared to the untreated controls.

Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA was used to assess the concentration of tumor necrosis factor alpha (TNF-α) and interleukin 1 beta (IL-1β) cytokines secreted by trophoblast following treatment with AGEs. Briefly, media was removed before cell lysis, and equal volumes of cell culture media (50 ul) were assessed in each experimental group for concentrations of cytokines in triplicate using a TNF-α ELISA kit (Ray Biotech, Norcross, GA) or an IL-1β ELISA kit (R&D Systems) as directed by the manufacturer.

Statistical Analysis

Comparisons of the following endpoints were made between control and treated samples: ELISA, mRNA, and protein levels of RAGE, p-JNK, p-ERK. All data were assessed for normality and treatment effects were determined by using Mann-Whitney test, with $p<0.05$ considered significant.

Results

Placental (RAGE) Expression Profile

While RAGE is notably expressed in lung and immune tissues, basal expression of the receptor is also detected in several systemic tissues as well (Schmidt, Yan, Yan, Stern, 2001). We initially evaluated the transcriptional activation of the RAGE gene in control placentas and those affected by PE, IUGR and GDM. When compared to the control placenta, we discovered no significant differences in the levels of RAGE mRNA in the PE and IUGR placenta (Figure 2.1A). In contrast, RAGE mRNA expression was significantly decreased in the GDM-D (3.4-fold; $p<0.0007$) and GDM-I (2.2-fold; $p<0.004$) placenta (Figure 2.1A). We next investigated protein levels of placental RAGE in the control and pathological placentas. Interestingly we observed that placental RAGE protein expression was significantly increased (2-fold; $p<0.03$) in the PE placenta when compared to controls (Figure 2.1B). In contrast there was a significant decrease in RAGE expression (3-fold; $p<0.02$) in IUGR samples when compared to controls (Figure 2.1B). No differences in RAGE expression were observed when comparisons were made between the control and GDM placentas. We next performed qualitative immunofluorescence for RAGE in order to determine whether altered RAGE expression in affected placentas localized to trophoblast cells. RAGE was notably present in the villi trophoblast of control placenta (Figure

2.2). When compared to controls, RAGE was increased in the villi trophoblast of the PE placenta while decreased in placentas identified as IUGR, GDM-D, and GDM-I (Figure 2.2).

RAGE in Invasive Trophoblast Cells

A hallmark of PE placenta is decreased invasion of trophoblast cells. We next investigated if, like the villi trophoblast, invasive trophoblast cells expressed RAGE. Western blot experiments showed expression of RAGE in invasive trophoblast cells (Figure 2.3A). To determine the mechanistic effects of R activation in the invasion of trophoblast cells, we treated cells with (carboxymethyl)-lysine-BSA (25 ug/ml), a type of AGEs which are a common RAGE ligand, for 24 hours in culture. Trophoblast cell invasion was significantly decreased (1.5-fold; $p<0.05$) after 24 hour exposure (Figure 2.3B). In terms of RAGE signaling, our *in vitro* experiments resulted in a significant increase in the activation of JNK (10-fold; $p<0.006$) and ERK (8.0-fold; $p<0.002$) following the addition of AGEs (Figure 2.4A-B). In contrast, the addition of AGEs was not sufficient to induce an appreciable expression of active p38.

AGEs-RAGE Ligation and Cytokine Release

The activation of RAGE following AGEs incubation has been shown to induce the release of cytokines such as TNF-α and IL1B; however, no such studies have been performed to date in placental cells (Schmidt, Yan, Yan, & Stern, 2001). RAGE activation by AGEs induced increased synthesis and secretion of TNF-α (1.8-fold; $p<0.05$) and IL-1β (1.3-fold; $p<0.05$) by invasive trophoblast cells in culture (Figure 2.5A-B).

Discussion

In the present study, we assessed RAGE expression in the placenta of patients affected by PE, IUGR, and GDM and evaluated signaling molecules in a cytotrophoblast cell line exposed to RAGE ligands. The biology of RAGE has been a centerpiece of inflammation research for several years and despite clear functions in the pulmonary apparatus, focus has also turned to several non-pulmonary tissues. Notwithstanding a more inclusive research approach, clarifying potential functions in placentas from normal and complicated pregnancies has been limited. A role for RAGE in normal placentation is supported by research that identified RAGE localization to the developing human trophoblast (Konishi, *et al.*, 2004). Specific placental roles for RAGE have been enigmatic, but a plausible function relates to RAGE activation during the coordination of apoptosis (Konishi, *et al.*, 2004).

In relation to the diseased placenta, our research confirms previous studies that identified increased RAGE levels in the serum of patients with severe preeclampsia, and in patients with gestational diabetes but studies centered on its expression during IUGR are still inadequate (Oliver, *et al.*, 2011; Tang, Qin, Xie, & He, 2015). Interestingly, RAGE mRNA transcription was not different in placentas affected with PE or IUGR; however, RAGE protein was disparately regulated leading to elevated RAGE protein in PE placentas and significantly decreased protein availability in the IUGR placenta. These findings suggested differential posttranscriptional regulation of RAGE in cases of these diseases. Such posttranscriptional and posttranslational regulation of RAGE has already been presented, particularly in cases of defective immune responses and apoptosis (Brune, *et al.*, 2013; Grimm, Ott, Horlacher, Weber, Hohn, & Grune, 2012). While no differences in RAGE expression were observed in the GDM placenta, our

results are potentially impacted by specific treatment regimen involving insulin or diet, two modalities known to diminish diabetic symptoms (Magon, & Seshiah, 2011; Poomalar, 2015).

Elevated RAGE detection in the PE placenta also corroborates a recent discovery that soluble RAGE is increased in the serum of PE patients (Oliver, *et al.*, 2011; Germanova, *et al.,* 2010). As observed in other disease states, circulating soluble RAGE may provide evidence of RAGE-mediated coping mechanisms in the inflammatory profile of PE (Basta, Del Rurco, Navarra, & Lee, 2015; Benjyo, Smarason, Redman, Sims, & Conrad, 2001; Cai, *et al.,* 2015; Ciccocioppo, *et al.*, 2015; Lee, *et al.,* 2015; Redman, & Sargent, 2003). Studies have previously demonstrated RAGE immunoreactivity in both the syncytiotrophoblast and cytotrophoblast of normal pregnancies. Our research goes further by localizing RAGE to villi and robust expression of RAGE in the villi of PE placentas provides important insight into probable RAGE-mediated effects that warrant further investigation.

The *in vitro* assessment of invasive trophoblasts exposed to AGEs resulted in the implication of RAGE signaling as a modulator of placental apoptosis, hindered invasion, and inflammatory cytokine elaboration observed in complicated pregnancies (Lunghi, Ferretti, Medici, Bionidi, & Vesce, 2007). In fact, our findings confirm elevated inflammatory responses and compromising apoptosis are both mediated, at least in part, by RAGE signaling (Konishi, *et al.,* 2004). Apoptosis and hindered invasion are characteristics that distinguish placentas of complicated pregnancies. Accordingly, a higher apoptotic index in cells required in the establishment of villi, coupled with decreased invasion, suggested AGEs-RAGE interactions may assist in the derivation of compromised placentas. Much like the administration of lipopolysaccharide (LPS) and the activation of TLRs in a rat model of PE, the availability of RAGE ligands at critical periods of placentation may deregulate immune responses at the

maternal-fetal interface contributing to poor early placentation (Xue, *et al.,* 2015). It is also likely that placental abnormalities stem from the elaboration of cytokines such as TNF-α and IL-1β following the activation of RAGE signaling. TNF-α secretion by trophoblasts has previously been documented to threaten pregnancy outcome, and it likely cooperates with an array of local cytokines including IFN-γ, IL-6 and IL-1β that exacerbate PE-related inflammatory responses (Diaz, *et al.,* 2009; Noyola-Martinez, *et al.,* 2013). Taken together, the current research opens the door to an important pro-inflammatory signaling program that when targeted, may be effective in ameliorating obstetrics complications.

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Table 2.1: Demographical Data for Human Placenta Samples. Groups were analyzed for statistical significance ($p < 0.05$) using the Kruskal-Wallis test. There was no difference in maternal age. PE and IUGR groups were statistically lower from control in gestational and fetal weight.

	Control	PE	IUGR	GDM-D	GDM-I	P value
Maternal Age	34±1.6	36±2.2	30±1.6	33±1.5	35±1.4	0.326
Gestational Age (wks)	39±0.07	32±1.3	32±0.7	38±0.3	39±0.8	0.007
Fetal Weight (g)	3498±59	1893±344	1005±47.3	3245±151	3453±217	0.004
% C-section /Vaginal	90%/10%					

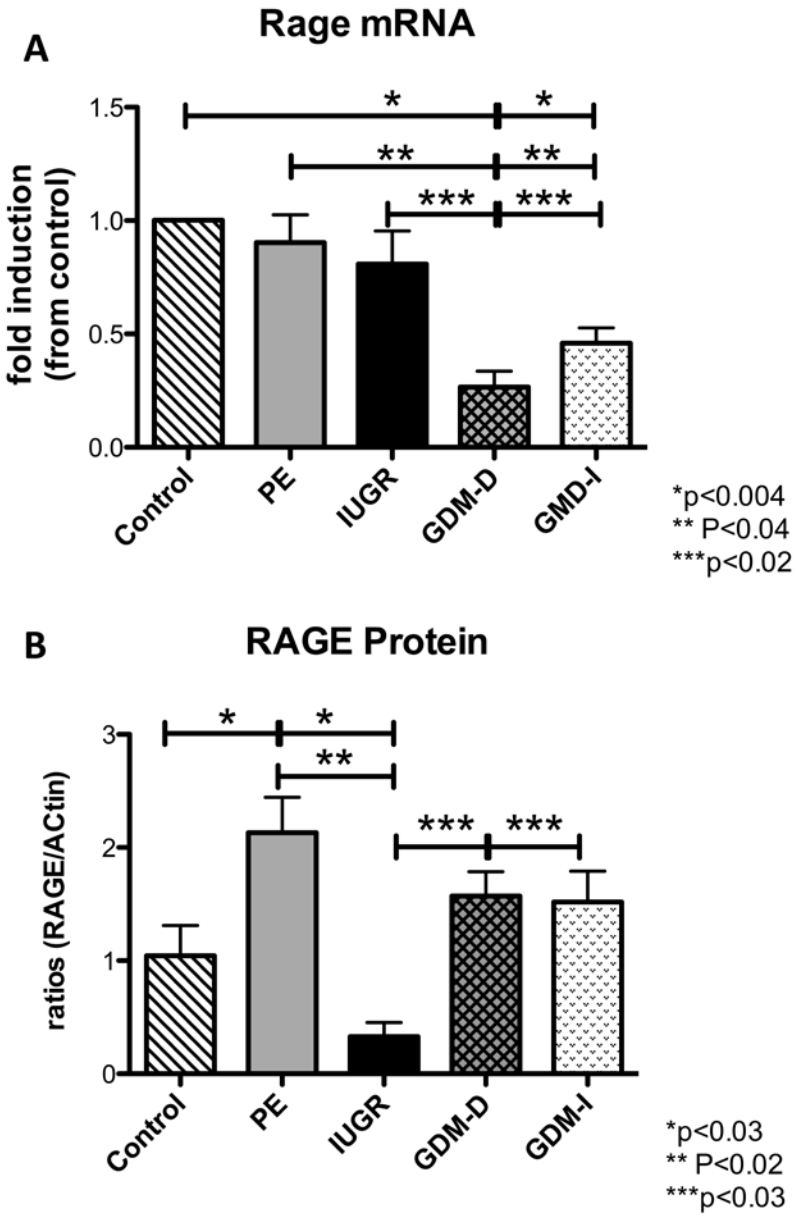

Figure 2.1: RAGE Profile in Pregnancy Pathologies. Real time PCR showed deceased RAGE gene transcription ($p<0.004$, $n=6$) in GDM-D and GDM-I placentas when compared to controls (A). Western blot analysis showed increased placental RAGE protein expression during PE ($p<0.03$, $n=6$) while there was a significant decreased in RAGE expression in the IUGR placenta when compared to controls.

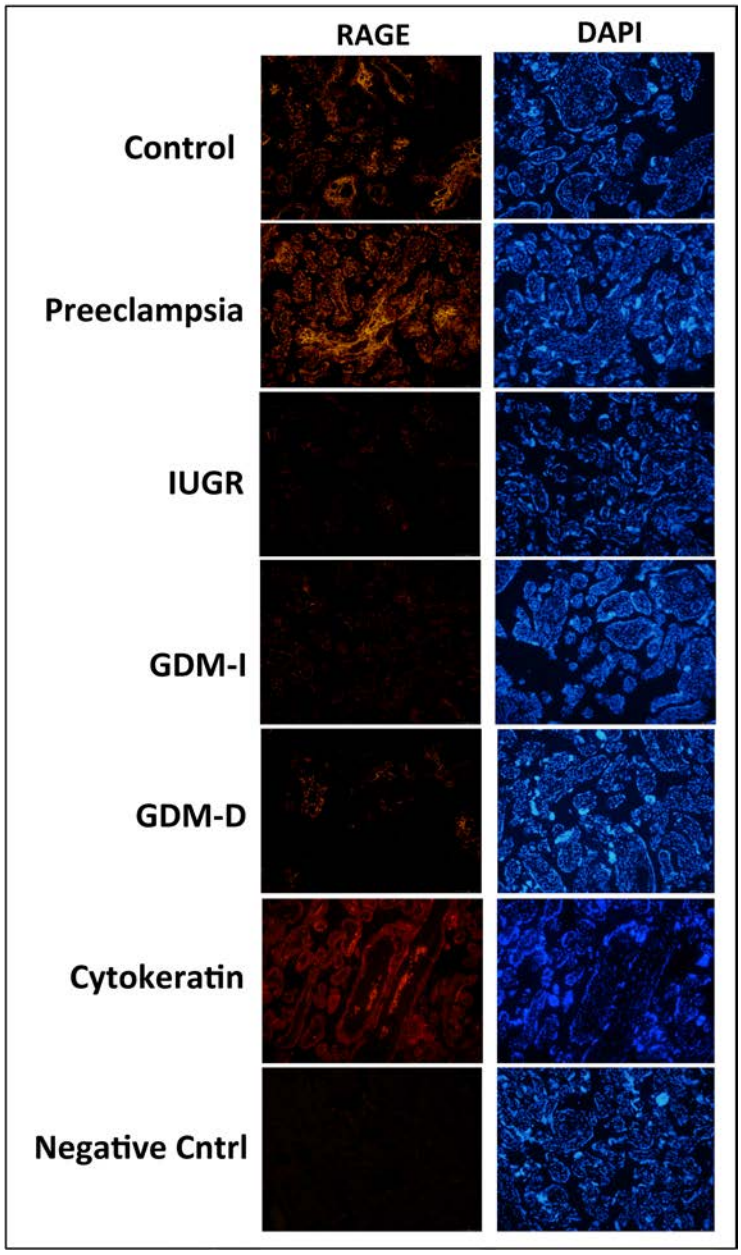

Figure 2.2: RAGE Immunofluorescence in Pregnancy Pathologies. RAGE levels were elevated in preeclampsia affected placenta samples. Immunofluorescence (IF) staining for RAGE in placental tissue samples; Control, Preeclampsia (PE), Intrauterine Growth Restriction (IUGR), Gestational Diabetes treated with diet (GDM-D), Gestational Diabetes treated with Insulin, CK7 (for trophoblasts localization) and negative control. Immunofluorescence confirmed increased RAGE in the villi trophoblast in PE placentas and decreased expression in the villi trophoblast of IUGR placenta. 20x scale.

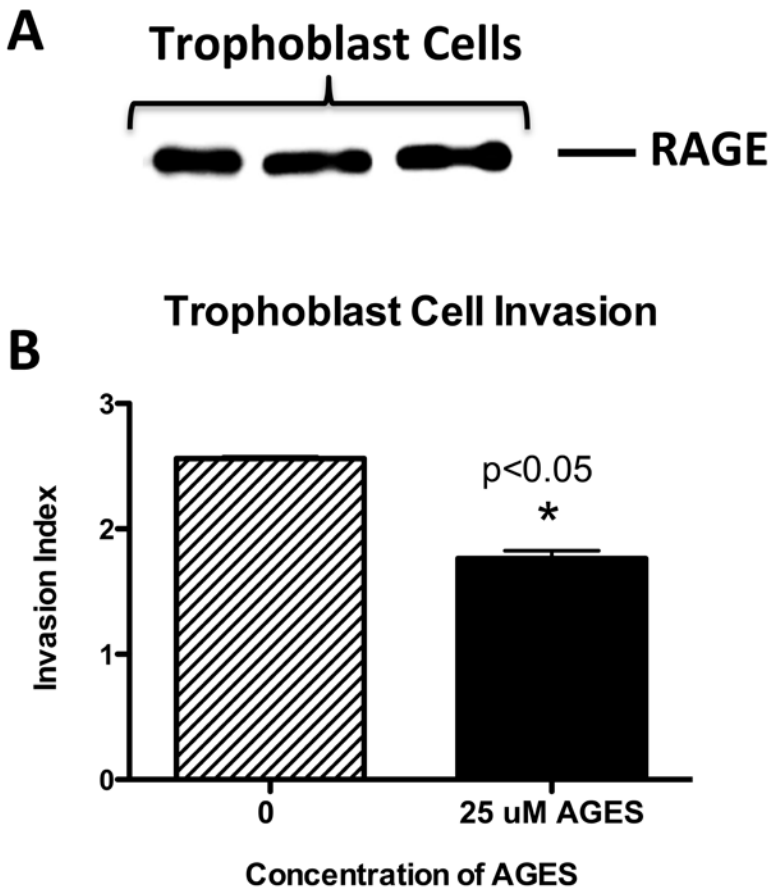

Figure 2.3: AGEs-induced Alteration of Trophoblast Invasion. Immunoblotting showed expression of RAGE in invasive trophoblast cells (A). Invasive properties of these cells were reduced ($p<0.05$, $n=6$) when RAGE was activated by treatment with 25 μg/ml of AGEs.

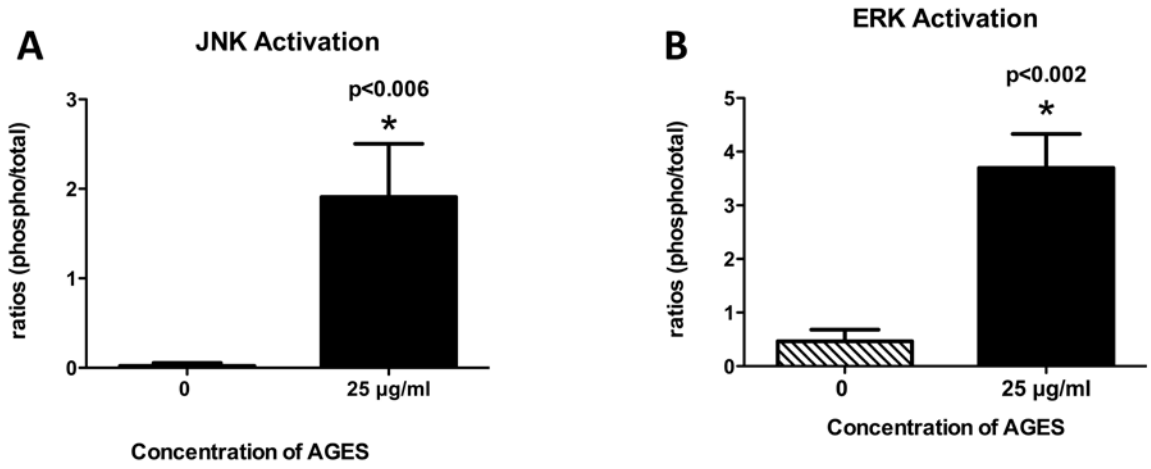

Figure 2.4: AGEs-induced MAPK Regulation. JNK was activated ($p<0.006$, $n=6$) in invasive trophoblast cells when treated with 25 µg/ml AGEs for 24 hours (A). Similarly, ERK was significantly activated ($p<0.002$; $n=6$) in treated trophoblast cells (B).

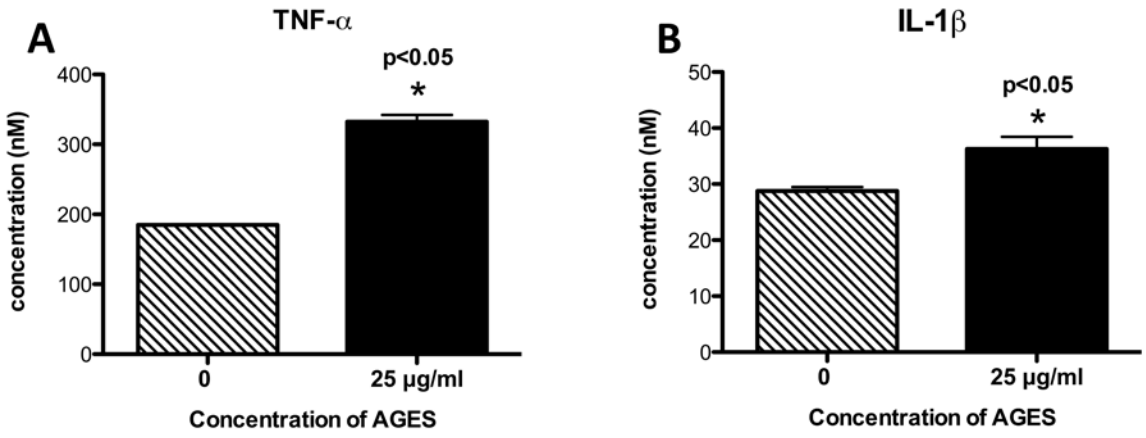

Figure 2.5: AGEs-induced Cytokine Release. TNF-α and IL-1β release by trophoblast cells treated with AGEs (n = 6). There was an increase ($p<0.05$) in the release of the inflammatory cytokines TNF-α and IL-1β when RAGE was activated by AGEs in trophoblast cells (A-B).

CHAPTER 3: General Discussion and Future Directions

Our data from the previous chapter reinforce other studies implicating RAGE as a mediator to the pathogenesis of preeclampsia. It has been previously shown that the AGEs-RAGE system is up regulated in preeclampsia and can contribute to vascular dysfunction due to oxidative stress (Chekir, *et al.,* 2006). It had been hypothesized that RAGE is increased in preeclampsia due to a slurry of hypoxia, oxidative stress, and chronic inflammation (Sado, *et al.,* 2011). What has not been demonstrated before our study is an in-depth examination of RAGE expression and the MAPK downstream signaling cascade. We studied this pathway and were able to demonstrate that it is through activation of the MAPK pathway that we see decreased invasion leading to placentation failure.

The first point presented in our research analyzes the RAGE expression levels in a variety of pregnancy pathologies: preeclampsia (PE), intrauterine growth restriction (IUGR) and gestational diabetes treated with insulin or diet (GDM-I or GDM-D respectively). Previous research has demonstrated that inflammation during pregnancy is key to pregnancy pathologies (Girard, Heazell, Derricott, Allan, Sibley, Abrahams, & Jones, 2014). They found that serum levels of inflammatory markers in the mother represented biomarkers of placental inflammation. Because RAGE is associated with the inflammatory pathway, we investigated RAGE expression in PE, IGUR, GDM-I and GDM-D. We found that RAGE protein levels are increased in conditions of PE and decreased in conditions of IUGR although the mRNA levels remain unchanged. These conclusions enhance the research previously published that sRAGE levels are decreased in pregnant women but the levels are significantly higher in women with severe preeclampsia suggesting that the RAGE system is activated in severe preeclampsia (Oliver, *et al.*, 2010). Because no previous research has been done with regards to IUGR, this is novel information that the mRNA levels are similar to PE, but protein expression is decreased even though there is similar trophoblast dysfunction in IUGR. We have shown through protein and mRNA analysis

that RAGE expression is higher in women with PE. We have also demonstrated that the increase of RAGE is centralized to the trophoblasts in the placenta. This information supports our hypothesis that women with PE have higher RAGE expression levels. However, we found that in GDM-I and GDM-D the mRNA levels are significantly lower, but the protein levels are unchanged compared to the control placentas. Because the gestational diabetes patients were undergoing treatment of either insulin or diet, we believe that the treatment of the diabetic symptoms could alleviate the inflammatory pathway and could potentially be the reason why RAGE mRNA levels were decreased (Magon, & Seshiah, 2011; Poomalar, 2015). To the best of our knowledge, this is the first study examining the expression of RAGE in IUGR and GDM. We have found that although RAGE is increased in PE, it is decreased in IUGR and not significantly changed in GDM-I or GDM-D. More studies need to be done to identify the RAGE expression in GDM patients not undergoing treatment as well as more experiments to understand what is causing the trophoblast dysfunction in IUGR if RAGE is not up regulated.

The second conclusion reached in the previous chapter is that the addition of Advanced Glycation End-Products (AGEs) to stimulate RAGE expression is sufficient to decrease cell invasion. AGEs are a product of the Maillard reaction and results in a carbonyl reacting with an amino group of a protein (Brownlee, Cerami, & Vlassara, 1988). This reaction occurs endogenously, in oxidative stress, and is even produced when some foods are heated (Poulsen, Hedegaard, Andersen, Courten, Bügel, Nielsen, Skibsted, & Dragsted, 2013). AGEs are abundant in our lifestyle and are known to be a ligand to RAGE (Bierhaus, Humpert, Morcos, Wendt, Chavakis, Arnold, Stern, & Nawroth, 2005). When we stimulated RAGE by adding its ligand, we saw a markedly decreased rate of invasion in first trimester trophoblast cells. Because PE and IUGR develop when proper placentatation fails (usually due to insufficient invasion of the trophoblasts), this further substantiates the theory that RAGE activation is a component in these pregnancy pathologies (Pijnenborg, Bland, Robertson, & Brosens, 1983). Not only

did we find that AGEs stimulation decreases invasion, but we found that it occurs through the MAPK pathway.

In order to further understand the role of RAGE in PE, IUGR and GDM we investigated the protein signaling cascade activated by RAGE. When ligands bind to R, multiple cellular signaling cascades are stimulated; we focused specifically on MAPK pathway activation. After AGEs stimulation, we saw that the trophoblast cells had a remarkable increase in JNK and ERK activation while p38 was undetectable. The production of AGEs resulted in oxidative stress, when you mimic oxidative stress in trophoblast cells by adding hydrogen peroxide, there is an increase in JNK, p-38 and ERK expression (Tang, Liang, Oian, Lin, Du, Li, & Li, 2014). This is similar to our findings that JNK and ERK are activated when AGEs stimulates the expression of RAGE. Increased regulation of JNK results in dimerization and the ability of JNK to regulate gene transcription in the nucleus (Kyriakis, 1999). Through gene regulation, JNK activates physiological changes such as apoptosis, inflammation, tumorigenesis and cell migration. ERK is a similar kinase as JNK; once activated, ERK can translocate into the nucleus and regulate transcription, or it can target proteins in the cytosol. ERK activation generally results in a variety of effects such as adhesion, proliferation, differentiation, and survival (Huttenlocher, *et. al,* 1998). Because we notice that AGEs stimulated RAGE activation triggers the JNK and ERK cellular signaling pathways to be up regulated, we can hypothesize that it is through RAGE activation that the trophoblast cells lose their innate ability to migrate and invade thus resulting in placental dysfunction.

After having confirmed that RAGE expression is increased after AGEs stimulation in trophoblast cells and having found that it activates the JNK and ERK signaling pathways, we looked at the release of cytokines from the trophoblasts indicating the cells are sending inflammatory signals. We found that both TNF-α and IL-1β levels are increased after RAGE activation. This demonstrates that the cells

recognize the inflammatory state they are in and actively communicate with surrounding cells to stimulate an inflammatory response tissue wide.

This research has broad implications on the prevention of pregnancy pathologies. Because RAGE activation initiates an inflammatory pathway that can result in trophoblast dysfunction, if we can prevent the activation and stimulation of RAGE then we could alleviate the root cause of ineffective placentation and restore function of trophoblast cells to modify maternal spiral arteries. By understanding the AGEs-activated RAGE signaling cascade, we will be able to produce novel small molecules to decrease RAGE activation.

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- Design and instigate lessons based on individual students needs
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Emergency Room Volunteer (January 2011 – January 2012)
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- Assist nurses and doctors in helping patients back to exam rooms and take vital measurements
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Primary School Teacher Volunteer (June 2009- August 2009)
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Submitted manuscripts

Alexander, K., Nelson, M., Howell, B., Mejia, C., Jones, C., Reynolds, P., and Arroyo, J. Differential receptor for advanced glycation end-products (RAGE) expression in preeclampsia, intrauterine growth restriction and gestational diabetes.

ABSTRACTS:

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