DEVELOPMENT OF A GESTATIONAL MEMBRANE TRANSWELL SYSTEM
FOR MEASUREMENT OF PROSTAGLANDIN AND CYTOKINE RESPONSES

by

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to my loving husband, Adam

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INTRODUCTION

Despite its medical importance, our understanding of the mechanisms and regulation of human parturition remains limited. Births occurring before 37 weeks gestation are considered preterm and account for significant neonatal morbidity and mortality in this country (Saigal and Doyle 2008). Gestational membranes, which provide a barrier between the fetus and mother, may be an important source of prostaglandins and inflammatory cytokines for signaling the onset of parturition in humans (Keelan, Blumenstein et al. 2003). Bacterial infection is a leading cause of preterm birth, and along with cases of unknown etiology, is often associated with a generalized inflammatory response (Romero, Espinoza et al. 2007). Because human gestational membranes are normally discarded following birth, they provide a unique opportunity to probe mechanisms of environmental modulation of term and preterm parturition in humans.

Public Health Significance of Preterm Birth

Preterm birth is a significant public health concern in the USA and worldwide. The incidence of preterm births, those occurring before 37 weeks gestation, has risen in the USA despite advances in prenatal care. In the US in 2005, 12.79% of infants were born prematurely, which is an increase of more than 20% from 10.6% of all births in 1990 (Martin, Hamilton et al. 2007). In 2004, prematurity-related outcomes were responsible for 36.5% of all neonatal deaths (Mathews and MacDorman 2007). In

addition, preterm delivery results in complications such as respiratory distress syndrome, apnea, intraventricular hemorrhage, patent ductus arteriosis, necrotizing enterocolitis, retinopathy, jaundice, anemia, bronchopulmonary dysplasia, and infections (Saigal and Doyle 2008). Many of these disorders develop into chronic health problems such as blindness, deafness, cerebral palsy, lower IQ, and subnormal height.

The majority of preterm deliveries are caused by spontaneous preterm labor (45%) or preterm premature rupture of the membranes (25%); together they are referred to as spontaneous preterm birth (Goldenberg, Culhane et al. 2008). Maternal risk factors for spontaneous preterm birth include previous preterm delivery, African-American or Afro-Caribbean heritage, low socioeconomic and educational status, low and high maternal ages, stressful work conditions (long hours or hard physical labor), short interpregnancy interval, low body-mass index, undernutrition, multi-fetal gestation, periodontal disease, uterine infection, infection at remote sites from the uterus, and cigarette smoking (Goldenberg, Culhane et al. 2008). For the most part, these risk factors do not suggest a mechanism. However, inflammation may be an underlying factor in many of these circumstances (Goldenberg, Culhane et al. 2008).

Gestational Membranes

The gestational membranes extend from the placenta proper and surround the fetus during pregnancy, making a water tight barrier to enclose the fetus and amniotic fluid. These membranes are comprised of three fused tissue layers that are of maternal and fetal origins (Fig.1.1). The amnion is an epithelial barrier of fetal origin that faces the fetal compartment. The subepithelial mesenchymal layer is comprised of a compact collagen layer that lends strength to the membranes. The collagen matrix is interspersed

with fibroblast cells and has both amnion and chorion components. The middle layer, called the chorion, is comprised primarily of trophoblast cells of fetal origin. The decidua is maternal in origin and is adjacent to the uterine myometrium. The amnion can separate naturally or be blunt dissected from the choriodecidua. Hereafter, "intact" gestational membranes refers to membranes in which the three layers remain fused to one another. The close proximity of the gestational membranes to the uterine myometrium may allow for passage of biochemical signals between these tissues.

Role of Prostaglandins in Parturition

Parturition is highly regulated by a variety of interdependent mechanistic checks and balances. Myometrial contractions arise from a combination of the release from inhibitory controls and acquisition of the ability to respond to contraction promoting stimuli (Gibb and Challis 2002). Phase one of parturition involves the activation of the myometrium, which is driven by maturation of the fetal hypothalamic-pituitary-adrenal axis and mechanical stretch of the uterus (Gibb and Challis 2002). Upon activation of the uterus, multiple stimulatory pathways result in myometrial contractility. Interdependent effectors of myometrial contractility include prostaglandins, estrogens, oxytocin, and corticotrophin releasing hormone (Weiss 2000).

The prostaglandin pathway is an important component of parturition that is susceptible to perturbation by the environment. Prostaglandins are made in human placenta, gestational membranes and myometrium and cause uterine contraction and cervical ripening. Prostaglandin E_2 and $PGF_{2\alpha}$ are potent stimulators of myometrial contractility and are elevated in women experiencing pre-term and term labor (Kinoshita, Satoh et al. 1984; McCoshen, Hoffman et al. 1990);(Kredentser, Embree et al. 1995).

The central role of prostaglandins in regulating myometrial contractility is evidenced by their common usage to induce labor clinically. Although prostaglandin inhibitors could theoretically be utilized as tocolytics to treat preterm labor, clinical use is limited because of unwanted fetal side effects (Loudon, Groom et al. 2003).

The prostaglandin synthesis and metabolic pathway is depicted in Figure 1.2. Prostaglandins are derived from arachidonic acid (AA), which is released from phospholipids in the cell membrane by phospholipases. Arachidonic acid is converted to prostaglandin H (PGH) by prostaglandin endoperoxide H synthase (PGHS; also commonly known as cyclooxygenase or COX). Although both PGHS-1 and PGHS-2 contribute to PGH synthesis, PGHS-2 is the principle enzyme induced in human gestational tissues at the time of parturition and is responsible for the increased PGH formation seen then (Gibb and Challis 2002). PGH is converted to PGE_2 or $PGF_{2\alpha}$ by PGE synthase or PGF synthase, respectively (Sales and Jabbour 2003). PGHS is located throughout the membranes, but predominance of PGE synthase in the amnion and both PGE synthase and PGF synthase in the choriodecidua determines the isoforms released from either side of the membranes (Sawdy, Dennes et al. 1999). Prostaglandin isomers are inactivated by 15-hydroxy-prostaglandin dehydrogenase (PGDH), which is present in the chorion. It has been proposed that localization of PGDH in the chorion may prevent passage of prostaglandins from the gestational membranes to the underlying myometrium (Challis, Patel et al. 1999).

The enzymes in the prostaglandin pathway are subject to a variety of regulatory mechanisms. As depicted in Figure 1.2, hormones, inflammatory cytokines, and growth factors influence the activity of the phospholipases PGHS-2 and PGDH (Sales and

Jabbour 2003). For instance, the inflammatory cytokine interleukin 1β (IL-1β) enhances PGE_2 production in human amnion cells. Cells treated with IL-1β showed an increase in both PLA_2 and PGHS-2 mRNA and a decrease in PGDH mRNA (Sales and Jabbour 2003). It is thought that nuclear factor-κB (NF-κB) mediates the effects of inflammatory cytokines such as IL-1β on enzyme mRNA expression (Patel, Funder et al. 2003). Glucocorticoids increase PGE_2 production in the chorion and amnion (Whittle, Patel et al. 2001). Biologically relevant concentrations of estradiol inhibit PGE_2 production from perfused human term placentas in a concentration-dependent manner. The inhibitory effect of estradiol is reversed with the concurrent administration of progesterone (Siler-Khodr, Kang et al. 1996).

Infection, Inflammation and Lipopolysaccharide

Gestational tissues collected from preterm births show overt signs of microbial infection in 25-40% of cases (Goldenberg, Culhane et al. 2008). Microbial infection causes an immune response with release of inflammatory cytokines, and activation and mobilization of immune cells. Most often, intrauterine microorganisms result from ascending infections where bacteria or yeast present in the vagina traverse the cervix and colonize the decidua (Pararas, Skevaki et al. 2006). Microorganisms can cross the gestational membranes, in some cases resulting in intraamniotic and/or fetal infection (Romero, Espinoza et al. 2007). Bacterial DNA is detected in as many as 70% of gestational membranes from elective term cesarean deliveries indicating that presence of bacteria alone is not enough to cause preterm delivery (Steel, Malatos et al. 2005). Other factors that promote inflammation or inhibit the ability of the immune system to fight off infection must contribute to preterm births.

Lipopolysaccharide (LPS) is a cell membrane component from gram negative bacteria that elicits a strong immune response and may be an important signaling molecule in cases of intrauterine infection and preterm birth (Romero, Espinoza et al. 2007). It is commonly used as a model inflammatory agent. In mouse models of preterm labor and birth, LPS injected into the cervix causes preterm expulsion of the fetuses (Reznikov, Fantuzzi et al. 1999).

Lipopolysaccharide stimulates prostaglandin synthesis by inducing enzymes of the prostaglandin synthetic pathway, particularly PGHS-2 (Brown, Alvi et al. 1998). LPS may also affect prostaglandin production indirectly by inducing production of inflammatory cytokines such as tumor necrosis factor-α. This cytokine induced PGHS-2 and decreases PGDH resulting in increased prostaglandin levels (Sato, Keelan et al. 2003). Experimental usage of LPS presents an opportunity to manipulate gestational membranes ex vivo in order to understand signals generated by the gestational membranes under inflammatory conditions.

Polychlorinated Biphenyls

The persistent organic toxicants, polychlorinated biphenyls (PCBs), are ubiquitously present in the soil, groundwater and sediment, and consequently bioaccumulate in the food chain (Patandin, Koopman-Esseboom et al. 1998). Body burdens above typical background levels are due to occupational exposures or consumption of specific food products such as Great Lakes sport fish (Stewart, Darvill et al. 1999). In humans these toxicants have long half-lives (~10 years) because they are lipophilic and poorly metabolized (Jacobson, Fein et al. 1984).

Previous research has established that PCBs are present in the placenta and uterus during pregnancy. In one study, the extracted lipids of the uterine muscle, placenta and amniotic fluid had relatively high levels of PCBs compared to maternal serum, with the highest levels being in the lipids of the amniotic fluid (Polishuk, Wassermann et al. 1977). A more contemporary study of women exposed to background levels of environmental pollutants found one in three amniotic fluid samples contained detectable levels PCBs (Foster, Chan et al. 2000). The immediacy of these toxicants to sites of uteroplacental prostaglandin synthesis, metabolism and action creates the potential for toxicant disruption of the concentration, distribution and activity of prostaglandins.

Known actions of PCBs establish plausible mechanisms for disruption of prostaglandins during pregnancy. 2,4,5,2',4',5-hexachlorinated biphenyl (PCB 153) is a predominate coplanar congener found in the environment (Dabrowska, Fisher et al. 2006). A coplanar PCB mixture including PCB 153 induced expression of prostaglandin endoperoxide H synthase-2 (PGHS-2) mRNA and reduced expression of interleukin-1β (IL-1β) mRNA in rabbit pre-implantation embryos (Kietz and Fischer 2003). In human mast cells, PCB 153 caused induction of PGHS-2 and intrleukin-6 (IL-6) mRNA by a nuclear factor-κB (NF-κB) dependent mechanism (Kwon, Lee et al. 2002). Because PGHS-2, IL-1β, IL-6 and NF-κB are important mediators of prostaglandin production in human pregnancy, their modulation by PCBs suggests a mechanism for toxicant disruption of parturition.

The high incidence of parturition complications, combined with the negative health impacts associated with these events, point to the need to investigate the underlying causes. Gestational membranes may be a key regulator of the inflammatory

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status with in the uterus. Understanding the underlying causes of preterm birth will help

researchers design more effective intervention and prevention strategies.
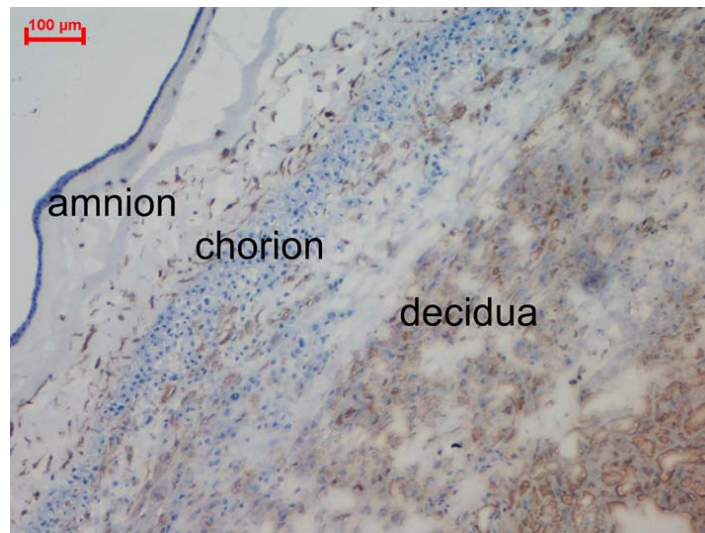
Figure 1.1 Hematoxylin and eosin stained section of human gestational membranes from Cesarean section delivery. The human gestational membranes are compromised of three main tissue layers. The amnion, adjacent to the fetal compartment, has a single layer of epithelial cells. The mesenchymal layer underlying the epithelium is comprised of a collagen matrix interspersed with fibroblast cells. Chorion trophoblast cells stain blue, and decidua is adjacent to the uterine myometrium. Amnion and chorion are of fetal origin and decidua is of maternal origin.
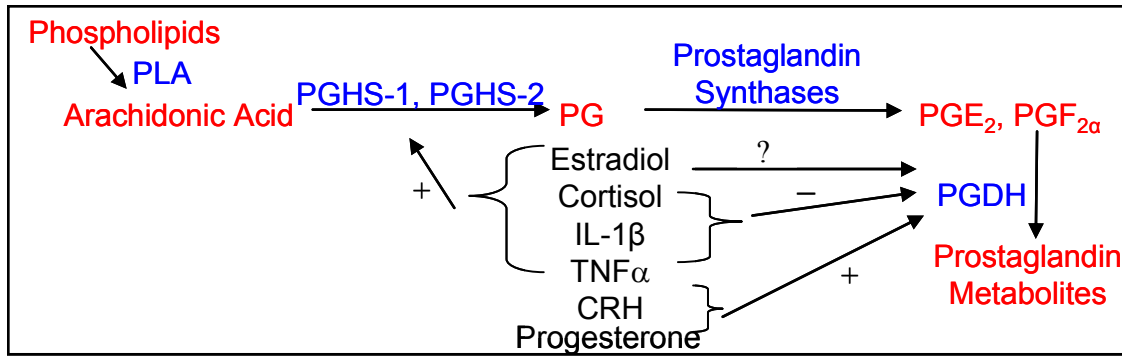
Figure 1.2 Prostaglandin synthesis and regulation. Membrane phospholipids are cleaved by phospholipases (PLA) to release arachidonic acid. Arachidonic acid is further metabolized by prostaglandin endoperoxide H synthase (PGHS) to prostaglandin H (PGH). Species-specific prostaglandin synthases convert the PGH to prostaglandin E_2 (PGE$_2$) or prostaglandin $F_{2\alpha}$ (PGF$_{2\alpha}$). 15-Hydroxy prostaglandin dehydrogenase (PGDH) catatabolizes terminal prostaglandins into inactive metabolites. Prostaglandins and pathway intermediates are shown in red, enzymes are in blue and potential modulators of the pathway are shown in black.

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CHAPTER 2

HUMAN GESTATIONAL MEMBRANE TRANSWELL CULTURE METHOD

ABSTRACT

A Transwell system was designed to study the dynamics of uterotonic prostaglandin synthesis from the fetal and maternal sides of the gestational membrane after in vitro exposure to lipopolysaccharide or environmental toxicants. In this system, intact gestational membranes comprised of amnion, chorion and decidua were mounted on a Transwell frame to create distinct chambers on either side of the membranes. This culture system allowed for selective exogenous stimulation and sampling of either side of the membrane for studying stimulated release of prostaglandins from the amnion or choriodecidua. Establishment of the in vitro Transwell culture of human gestational membranes required attainment of three goals: 1) optimization of specimen collection and transport to the laboratory, 2) optimization of culture and treatment conditions allowing discrete measurements of secreted prostaglandins and cytokines from the amnion and choriodecidua, and 3) determination of appropriate methods of data analysis. During initial work, high levels of between-subject and within-subject variability in response to treatment were seen. To combat these problems, procedures for prompt acquisition of the tissue specimens after delivery were established. Furthermore, optimization of the culture technique included culture with 1% fetal bovine serum, frequent media changes, and an 18-24 h equilibration period before experimental

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conditions were started. These approaches resulted in improved health of the tissue and reduced prostaglandin variability of response to treatments. Three samples from each subject were exposed to each treatment in separate culture wells and prostaglandin concentrations from the culture medium were normalized to the weight of the sample. Other methods of normalization did not improve the variability or statistical power. This optimized gestational membrane Transwell culture system and data analysis allowed ex vivo stimulation of human gestational membranes while maintaining cell-to-cell and tissue-to-tissue interactions important for processing of inflammatory signals and enabled side-specific measurement of prostaglandins.

INTRODUCTION

A Transwell system was designed to study the dynamics of uterotonic prostaglandin synthesis from the fetal and maternal sides of the gestational membranes after in vitro exposure to lipopolysaccharide or environmental toxicants. Distinguishing which layer(s) of the gestational membranes produce prostaglandins is of fundamental importance for understanding the role of gestational membranes in mediating uterine contractions and parturition. Furthermore, this system enables the study of signal transduction across the membranes when only the fetal or maternal side of the membranes is stimulated.

Gestational membranes extend from the placenta proper and surround the fetus during pregnancy, making a water tight barrier to enclose the fetus and amniotic fluid. These membranes are comprised of three fused tissue layers which are of maternal and fetal origins. The amnion is an epithelial barrier of fetal origin that faces the fetal compartment. The middle layer, called the chorion, is comprised primarily of trophoblast

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cells of fetal origin. The decidua is of maternal origin and is adjacent to the uterine myometrium. The amnion can separate naturally or be blunt dissected from the choriodecidua. Hereafter, "intact" gestational membranes refers to membranes in which the three layers remained fused to one another.

The close proximity of the gestational membranes to the uterine myometrium may allow for passage of biochemical signals between these tissues. In particular, gestational membranes make and secrete uterotonic prostaglandins such as prostaglandin E_2 (PGE_2) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$). Experiments comparing prostaglandin secretion from term gestational membranes collected from non-laboring Cesarean section deliveries and laboring vaginal deliveries show that membranes from laboring situations have increased prostaglandin production (Kinoshita, Satoh et al. 1984; McCoshen, Hoffman et al. 1990; Kredentser, Embree et al. 1995). This may indicate that gestational membranes are a source of prostaglandins during labor.

Current knowledge regarding the importance of gestational membrane prostaglandin signaling in myometrial contractility is limited. A predominating theory to explain preterm labor is that PGE_2 from the amnion prematurely crosses the chorion and decidua to cause uterine contractions (Challis, Patel et al. 1999). However, this widely published theory does not explain how prostaglandins cross the gestational membranes given that they are anionic at physiological pH, which hinders passive diffusion across cellular membranes. Determining the source of prostaglandins from within gestational membranes may shed light on this conundrum.

The strategy I adopted made use of a Transwell device to create distinct chambers on the amnion or choriodecidual side of the membranes (Fig. 2.1). In this system, intact

gestational membranes comprised of amnion, chorion and decidua were mounted on a Transwell frame to create a water-tight barrier. The Transwell device with the gestational membranes attached was placed in a culture dish with medium on both sides of the membrane. This culture system allowed for selective exogenous stimulation of the membranes and independent sampling of prostaglandins in the culture medium on the amnion or choriodecidual sides of the membrane.

Previous development of gestational membrane dual chamber-type systems was limited. Nakla, et al. (1986) mounted gestational membranes on a 4 cm perspex cylinder with a Teflon band and cultured them in a beaker. Though their system was limited by the crude culture device, they were able to measure passage of radiolabeled prostaglandins and arachidonic acid from one side of the laboring or non-laboring gestational membranes to the other.   McCoshen et al. (1987; 1990) mounted 8 cm^2 discs of separated or intact gestational membranes in a dual-chamber perfusion device. Synthetic amniotic fluid was continuously circulated around the membranes. Experimental conditions were started after a 2 h pre-incubation period, and prostaglandin production and transport across labored or non-labored membranes was measured.

The Vadillo-Ortega group from Mexico City was the first to use Transwell devices for gestational membrane culture (Zaga, Estrada-Gutierrez et al. 2004). The main objective of their research was to study mechanisms responsible for preterm premature rupture of membranes. They mounted membranes on the Transwell frame and secured them with silicone bands. Culture medium included 10% fetal calf serum and membranes were incubated under standard tissue culture conditions for an equilibration period of 3 days before proceeding with the experiment. This group measured cytokines

from the gestational membranes such as interleukin-1β and tumor necrosis factor-α after stimulation with bacteria or bacterial products.

No research groups have optimized a system for studying independent side-specific prostaglandin synthesis due to exogenous stimulus. Furthermore, there has been no discussion of the inherent variability within the system. The method described here addresses these limitations to allow for measurement of lipopolysaccharide (LPS) induced side-specific secretion of PGE_2 and $PGF_{2\alpha}$ (Chapter 3), LPS-induced side-specific secretion of cytokines and cheomokines (Chapter 4) and 2,2',4,4',5,5'-hexachlorobiphenyl induced side-specific secretion of PGE_2 and $PGF_{2\alpha}$ (Chapter 5).

Because of the limitations of the previous methods for gestational membrane Transwell culture, further characterization and optimization of the method was needed before prostaglandin synthesis could be studied. In particular, establishment of the in vitro Transwell culture of human gestational membranes required attainment of three goals: 1) optimization of specimen collection and transport to the laboratory, 2) optimization of culture and treatment conditions allowing discrete measurements of secreted prostaglandins and cytokines from the amnion and choriodecidua, and 3) determination of appropriate methods of analysis for data obtained.

In addition to developing the gestational membrane Transwell culture, I explored several alternative techniques. The Transwell culture method developed here has distinct advantages over monocellular isolation and culture, separated amnion and choriodecidual floating tissue cultures, and intact gestational membrane floating tissue cultures. A brief review of the advantages and disadvantages of each technique are presented in the Results section of the Chapter.

MATERIALS AND METHODS

Chemicals, Reagents and Antibodies

Tissue culture reagents including high glucose Dulbecco's Modified Eagle's Medium (DMEM) without phenol red, penicillin/streptomycin antibiotic, and heat-inactivated fetal bovine serum were purchased from Invitrogen (Carlsbad, CA). Lipopolysaccharide (LPS) from Salmonella Typhinurium (Lot #225) was purchased from List Biological Laboratory (Campbell, CA) and 2,2',4,4',5,5'-hexachlorobiphenyl (PCB 153) was purchased from AccuStandard, Inc. (New Haven, CT). The Vectastain Elite ABC immunohistochemical staining kit, DAB Substrate Kit (3,3'-diaminobenzidine) and hematoxylin counterstain were purchased from Vector Laboratories (Burlingame, CA). Prostaglandin E_2 enzyme immunoassay kits were purchased from Cayman Chemical Co. (Ann Arbor, MI). Antibodies (anti-cyotokeratin 7 and anti-vimentin) were purchased from Dako North America, Inc. (Carpinteria, CA). Transwell frames with no micropore membrane insert were a gift from Corning Corporation (Corning, NY).

Tissue Acquisition

Methods pertaining to human tissue were reviewed and approved by the local institutional review board prior to initiation of experiments; review and approval were updated yearly. Full-thickness gestational membranes comprised of amnion, chorion and decidua were collected at 37-39 weeks from non-laboring elective Cesarean sections following healthy pregnancies. Exclusion criteria included smoking, multi-fetal gestation, complications of pregnancy such as gestational diabetes or hypertension, use anti-inflammatory drugs (e.g., aspirin, Singulair®), or any other condition which would require the tissue to be sent to pathology. Within 1-60 min after delivery, full-thickness

extraplacental membranes were sharp dissected from the placenta, placed in phosphate buffered saline solution and transported to the laboratory. Alternatively, the entire placenta and attached membranes were transported to the laboratory.

Tissue Culture

In the laboratory, under sterile conditions, membranes were checked for integrity to ensure that amnion and choriodecidua remained attached to one another and that the collagen compact layer between the amnion and the choriodecidua was not degraded. The membranes were rinsed with culture medium (DMEM supplemented with 1% heat inactivated-fetal bovine serum and 100 units penicillan/100 μg streptomycin per milliliter) to remove excess blood clots. The membranes were cut into approximately 2 x 2 cm^2 pieces (Fig. 2.1B) and affixed by latex rubber bands onto ethylene oxide sterilized Transwell frames (without a micropore membrane) to create two distinct chambers on either side of the mounted membranes (Fig. 2.1C-G) (Zaga, Estrada-Gutierrez et al. 2004). The choriodecidual side of the tissue faced the inner chamber of the device. Extra tissue was removed with a scalpel. Transwell frames with attached tissue were placed in 12-well plates with culture medium on both sides of the membranes (Fig. 2.1H). Mounted membranes were cultured in a 5% CO_2 tissue culture incubator for 2-4 h. To maintain optimal culture conditions and health of the tissue, the medium was changed after the first equilibration period and then tissues were left overnight. Prior to beginning the treatment protocol, the overnight culture medium was removed and the membranes were pretreated with fresh culture medium for 1-2 h. Various culture conditions were tested to maintain optimal health of the tissue (see Results).

Treatment

LPS was diluted in sterile deionized water to make a primary stock of 100 μg/ml. Just prior to the experiment, the LPS primary stock was further diluted with culture medium to achieve the experimental concentration (100 ng/ml). To initiate exposure, medium was removed and fresh medium with or without LPS was added to the chambers of the culture dish, depending on the exposure conditions of the experiment. Mounted intact gestational membrane pieces were assigned randomly to treatment groups with three pieces of tissue included in each treatment. Membranes were incubated with the treatments for 4 or 8 h. After the specified treatment time, treatment medium was recovered from each side of the membrane and stored at -20°C or -80°C for subsequent enzyme immunoassay analysis of secreted PGE_2. The tissue was removed from the frame, weighed, embedded in freezing medium and frozen in tissue molds for subsequent immunohistochemistry analysis.

Enzyme Immunoassay

Prostaglandin E_2 in the culture medium was assayed by enzyme immunoassay according to the manufacturer's protocol (Cayman Chemical Co., Ann Arbor, MI). Prior to the assay, samples were thawed and diluted to concentrations that fell within the range of the standard curve. Each sample was assayed in duplicate. PGE_2 concentrations were calculated relative to the values from the standard curve. Mean PGE_2 concentrations were divided by the wet weight of gestational membrane tissue pieces and the mean of the treatment replicates was calculated. Mean results and standard error of the mean were calculated from multiple subjects and graphed. Various methods for normalizing the data, decreasing variance and improving statistical power were tested (see Results).

Immunohistochemistry

After tissue was removed from the Transwell frames, it was rolled, embedded in freeze medium, frozen in liquid nitrogen-cooled isopentane and stored at -80° C. The tissue was sliced with a cryotome into 8 μm sections, and the cryosections were mounted on slides and stored at -80° C. On the day of staining, slides were warmed to room temperature, fixed with 4% paraformaldehyde and stained using the Vectastain Elite ABC Kit according to the manufacturer's protocol (Vector Labs). Sections were incubated with primary antibodies (anti-cytokeratin 7 or anti-vimentin), labeled with a biotinylated secondary antibody and an avidin:biotinylated enzyme complex, visualized with 3,3-diaminobenzidine and counterstained with hematoxylin. Stained sections were visualized with an upright light microscope and recorded digitally.

Statistical Analyses

Intraclass correlation coefficients (ICC) were calculated to evaluate between-subject variability compared to within-subject variability for tissues (amnion and choriodecidua) and treatment (non-treated control or lipopolysaccharide.) Between-subject and within-subject variance components were obtain using the PROC MIXED procedure (with 'covtest' option) in SAS 9.1 for Windows.

RESULTS

Tissue Acquisition

Gestational membranes were collected from non-laboring healthy cesarean section deliveries at the University of Michigan Birth Center, with approval from the University of Michigan Institutional Review Board (IRB). These cesarean deliveries were scheduled because of a previous cesarean or breech presentation of the baby, and

met the established exclusion criteria for our studies. Because the tissue would normally have been discarded and no identifying patient information was linked with the collected specimens, requirement of informed consent was waived by the IRB.

At the beginning of this project, we found that delays in specimen acquisition from the operating room resulted in degraded tissue and irregular in vitro tissue responses. Initially, operating room staff transported the specimen out of the operating room and we received the entire placenta with attached membranes after surgery was completed. Because of variations in surgery time, we received the placenta and attached membranes 30 min to over 1 h after it was delivered. During the intervening time between delivery and laboratory staff acquisition, blood associated with the placenta proper clotted on the membranes. The blood clots along with the lack of moisture and oxygenation decreased the quality of the tissue for culture.

To minimize these unwanted effects on the membranes, we worked with hospital staff to establish a more optimal procedure for obtaining the tissue. Laboratory staff donned protective clothing and appropriate gear, and then waited within the surgical suite for the placenta with attached membranes to be delivered and placed in a sterile container. Once the attending physician declared that the tissue did not need to be sent to pathology, the container was moved out of the surgical field and laboratory staff sharp dissected the extraplacental gestational membranes. In most cases, the tissue was obtained almost immediately after delivery. The gestational membranes were quickly placed in sterile, room temperature phosphate buffered saline solution for transport to the laboratory. Tissue obtained in this manner had fewer blood clots and less overt histopathology, and yielded cultures with improved response to stimuli.

Establishment of Cultures

In the laboratory, the health and integrity of the tissue was assessed by gross examination before samples were taken for tissue culture. Criteria were established for tissue sampling from within a single specimen to ensure consistency across subjects and to minimize variability. In particular, tissue samples were not taken from within 2 cm of the placenta or from any tissue showing signs of amnion and choriodecidual separation or degradation of collagen in the mesenchymal layer. Additionally, tissue regions that were much thicker or thinner than the typical membranes were not used.

Selected gestational membrane samples were mounted on Transwell frames. Initially, pre-sterilized Transwell devices were purchased from Corning. These devices were designed by the manufacturer to be used for cellular co-cultures, and are commercially available with a micropore membrane insert attached to the frame. Using the Transwell frames for the two-compartment gestational membrane tissue culture system required removal of the micropore membrane insert. Later, we received Transwell frames that had no micropore membrane insert as a gift from Corning. The Transwell frames were lightly sanded to enhance tissue attachment to the device and subsequently sterilized by ethylene oxide. Membranes were attached to Transwell frames with sterile latex bands.

Efforts were undertaken to ensure that the tissue remained healthy during culture for consistent prostaglandin measurements. In particular, frequent media changes during the 18-24 h equilibration period improved tissue health. In as little as 24 h in culture, the pH of the choriodecidual culture medium was 6.5, indicating that the tissue metabolism was exhausting the buffering capacity of the medium. Time course experiments

indicated consistent prostaglandin secretion into culture medium under non-treated and LPS-stimulated conditions for up to 8 h of treatment (see chapter 3 for LPS time-course data). However, little to no prostaglandins could be measured in culture medium after 24 h. Upon replacement of culture medium with fresh medium, these membranes resumed prostaglandin secretion. The pH and prostaglandin time-course results suggested that accumulation of metabolites or depletion of nutrients in the medium was detrimental to the tissue and prostaglandin synthesis. For these reasons, equilibration and treatment times were limited to 24 h or less.

Immunohistochemistry

Immunohistochemistry with anti-cytokeratin 7 or anti-vimentin antibodies identified specific cell types within fresh, intact gestational membrane tissue (Fig. 2.3). Amnion epithelial cells, mesenchymal fibroblast cells, and chorion trophoblast cells were identified by this method. After equilibration and treatment periods, the health of the tissue was evaluated by immunohistochemistry (data not shown). Tissue remained intact and morphologically healthy (similar to fresh, intact tissue in Fig. 2.3) after 18-24 h equilibration time and 1-24 h treatment time under control or stimulated treatment conditions.

Variation of Prostaglandin Release

Data from five subjects were used to assess variability in the model system. Prostaglandin E_2 data from tissue exposed for 4 h to non-treated control conditions (NT) or 100 ng/ml LPS were analyzed. In these experiments, secreted PGE_2 response was measured in medium from the amnion or choriodecidual side of the Transwell by enzyme immunoassay. The data were graphed and analyzed to assess the nature of variability

Plotting raw values for PGE_2 release into amnion or choriodecidua media for each subject (duplicate or triplicate) revealed tissue-specific patterns of within-subject and between-subject variability (Fig. 2.4). In the amnion (Fig. 2.4A), the within-subject variability was minimal, but the between-subject variability in response to LPS stimulus was large. For example, subjects 2 and 4 were high responders to LPS, subjects 1 and 3 were medium responders to the stimulus, and subject 5 did not respond at all. In comparison to amnion, the choriodecidua showed increased within-subject variability (Fig. 2.4B). Furthermore, the choriodecidua response was not correlated with the amnion response. For example, subject 5 produced no excess PGE_2 in response to LPS treatment on the amnion side compared to the non-treated control samples, although this subject had a large PGE_2 response on the choriodecidual side.

To quantify within-subject and between-subject variability, intraclass correlation coefficients (ICC) were calculated. The ICC can be estimated from the between-subject variance ($\sigma^2(b)$) and the within-subject variance ($\sigma^2(w)$):

$$ICC = \frac{\sigma^2(b)}{\sigma^2(b) + \sigma^2(w)}$$

Thus, the ICC is the ratio of the between-subject variance to the total variance. As the ratio approaches 1, the variance is increasingly attributed to between-subject variance. A lower ratio corresponds to higher within-subject variance relative to between-subject variance. Table 2.1 shows the ICC calculated for the data from the five gestational membranes shown in Fig. 2.4. The amnion NT value of 0.62 and the amnion LPS value of 0.97 indicated high between-subject variability compared to within-subject variability. In contrast, the choriodecidua NT value of 0.38 and the choriodecidua LPS value of 0.26 indicated high within-subject variability for the tissue and treatment group. This finding

correlates with the visual interpretation of Figure 2.4 and indicates distinct pathways within each tissue that determine response to LPS and distinct causes of variability.

The same data plotted in Figure 2.4 were re-plotted as control or treatment means with the unit of analysis as the specimen (subject). The overall scheme for this analysis is presented in Figure 2.5. Three pieces of tissue from a single subject were exposed to the same treatment or control conditions. PGE_2 concentrations in media from each replicate were analyzed by enzyme immunoassay in duplicate. Enzyme immunoassay means were calculated and further combined to calculate replicate means. This overall mean was representative of the response from the subject and combined with results from other subjects with n referring to total number of subjects.

Various methods of data adjustment were investigated to reduce within-subject and between-subject variability that may have arisen due to inconsistencies in sampling or culture methods. Mean PGE_2 concentrations for non-treated control (NT) and stimulated (LPS) conditions were graphed in Fig. 2.6A without adjustment. An alternative approach was to graph the mean PGE_2 concentrations from the EIA for each sample divided by the sample wet weight. These values were then combined for all subjects and graphed to give the PGE_2 produced per gram of tissue (Fig 2.6B). This manipulation had little effect on the interpretation of the data or the between-subject variability. In a second approach, the PGE_2 per gram tissue values were normalized to non-treated control levels. Within data from a single subject, mean PGE_2 concentrations were divided by the mean PGE_2 concentrations from the amnion non-treated control and multiplied by 100 to give percent non-treated control. As can be seen in Fig. 2.6C the variability and statistical power were not improved by this manipulation.

An alternative to the methods above was tested on samples from a single subject. Basal PGE_2 secretion was measured on each of 4 control or treated (PCB 153) samples prior to the 4 and 24 h measurements. These data were then normalized to pretreatment concentrations (Fig. 2.7). To do this, basal PGE_2 concentrations were measured from the tissue during a pretreatment incubation (2 h). Fresh control or treatment medium was applied to the tissue and collected after 4 h. The treatment medium was then replenished and samples were incubated for an additional 20 h before the final medium collection. Raw PGE_2 concentrations for basal, 4 and 24 hour time points are shown in Fig. 2.7A. To normalize to basal levels, PGE_2 concentrations measured at 4 and 24 h from individual pieces of gestational membrane were divided by basal PGE_2 concentrations from the same piece of membrane. Means and standard error of the means were calculated for treated and non-treated control samples for each time point and graphed (Fig. 2.7B). Dividing by basal PGE_2 levels changed the relative values of some of the means. However, no improvement was seen in the within-subject variability (Fig. 2.7).

Alternative Culture Systems

Alternative experimental designs and model systems to gestational membrane Transwell culture were considered to study prostaglandin response to stimulus in gestational membranes. Primary cell monocultures of gestational membrane cell types including the amnion epithelium, mesenchymal fibroblast and chorion trophoblast cells were evaluated for studying the dynamics of prostaglandin synthesis and metabolism. Primary cell cultures have a number of drawbacks including being labor intensive due to the long isolation and purification procedures, cell yields that are insufficient for many of the experimental designs and poor culture purity and variability among subjects. In

addition, the cell-to-cell or tissue-to-tissue interactions that occur in vivo are disrupted in cell cultures, yet may be important for shaping the prostaglandin response. The alternative approach of using full-thickness gestational membranes in floating cultures was considered unsatisfactory because the amnion and choriodecidual contribution to the response cannot be differentiated.

Another alternative is to separate the amnion from the choriodecidua by blunt dissection. By analyzing the responses of separated membranes in floating tissue culture one may be able to discern tissue-specific sensitivity and response to a stimulus. However, I found that the amnion folded up on itself in culture, leading to variable and suboptimal culture conditions. In addition, immunohistochemical analysis of the post-culture tissue indicated that the amnion epithelium of separated tissue degraded in culture. Furthermore, the blunt dissection of amnion and choriodecidua may produce erroneous results if the chorion mesenchyme partitions with the amnion, and if the intermediate collagen layer is distributed unevenly to either the amnion or chorion side (Fig 2.8). Furthermore, this experimental model does not allow exploration of amnion and choriodecidual interactions or the ability of amniotic prostaglandins to pass or signal through the choriodecidua.

DISCUSSION

Because of significant differences between species in pregnancy implantation, placentation and parturition, there are no satisfactory animal models for research on mechanisms of human parturition. Fortunately, human placentas and gestational membranes are normally discarded and thus, can be relatively easily obtained for research purposes. In this work, I have optimized methods to make use of this tissue to

develop the gestational membrane Transwell system for studying gestational membrane responses to exogenous stimuli (Fig 2.9). This system was designed to address unanswered questions about prostaglandin production in the gestational membranes.

Previously, two laboratories published research using a Transwell gestational membrane model or a similarly principled model. Early attempts were undertaken to culture gestational membranes in a dual chamber system and study differences in prostaglandin release under laboring or non-laboring conditions (Nakla, Skinner et al. 1986; McCoshen, Johnson et al. 1987). More recently, a group in Mexico City headed by Vadillo-Ortega has expanded and improved upon the model. The culture system described here is based on the model of Vadillo-Ortega (Zaga, Estrada-Gutierrez et al. 2004). Previous research in this field did not take into account differences in response to stimulation of the amnion and choriodecidua sides of the gestational membranes. The system I describe in this work addresses this important gap.

I have refined the technique established by the Vadillo-Ortega group to optimize for prostaglandin measurements. In contrast to their practice of equilibrating gestational membrane Transwell cultures for 3 days prior to exposing to experimental conditions, I equilibrated the explant cultures for 18-24 h. Their report that it took 3 days for the tissue to regain health and integrity after commencing the culture was not supported by my data.   Instead, I found that the health of the tissue decreased over time in culture. The decrease in pH over 24 h combined with gross examination of the tissue and IHC results led to our practice of conducting the experiment as quickly as possible after a reasonable recovery period. Our goal was to keep the tissue as healthy as possible while reducing the number of changes and adaptations the tissue makes in culture. During the 18-24 h

30

equilibration period, the medium was changed twice to maintain correct pH of the medium. Experimental treatments of 1-24 h were conducted following the equilibration period. Although we cannot mimic a pregnant uterus in culture, this system maintains the tissue in a physiologic state that more closely resembles that of pregnancy.

Another difference in culture practices from the Vadillo-Ortego group is our use of 1% heat inactivated fetal bovine serum (FBS) for the duration of the experiment compared to their use of 10% FBS during the equilibration period and no FBS during the experimental period (Zaga, Estrada-Gutierrez et al. 2004). Using 1% FBS allowed for continuity of culture medium between equilibration and treatment periods.

At the onset of this project, it became clear that though there were many advantages to working with this tissue in lieu of animal models, there were also some disadvantages. The foremost concern was the high variability seen in the prostaglandin response to stimuli (Fig. 2.4) that made it difficult to draw meaningful conclusions about the data. We needed to determine whether variability was due to sampling and culture methodology or due to biological differences in samples from different subjects or from different regions of the membranes from a single subject.

There are meaningful explanations for a portion of the variability. High between-subject variability may be related to the genetics of the mother or the baby as well as the environment to which they are exposed. Between-subject variability may be due to proximity to the onset of labor. There is certain to be biochemical differences in tissues taken from subjects that may have been within hours of the onset of natural labor compared to others may not have delivered for weeks. This notion is corroborated by

research reporting increased cytokine response to LPS stimulus from early gestational age samples compared to term gestational age samples (Jung, Yoon et al. 2005).

It is interesting to note that the within-subject variability differs from samples taken from the amnion side compared to the choriodecidua side of the same piece of tissue. This could be due to culture conditions or to inherent differences in the tissue. Overall, the amnion had a more uniform response to stimulus. High variability in pieces of choriodecidua from one subject may be due to placement within the uterus. For example, choriodecidua that is near a cervix undergoing dilation and effacement may respond differently to LPS stimulation than choriodecidua adjacent to the upper fundus which undergoes tonic uterine contractions to expulse the fetus. Though variability in the tissue is frustrating for certain experimental designs, differences in choriodecidual responses from various places within the uterus may be physiologically important for understanding of the interactions between decidua and myometrium during labor.

There has been no discussion in the scientific literature of methods to deal with inherent variability of prostaglandin production in gestational membrane tissues. This work is the first to examine the variability of human gestational membranes, investigating how different data manipulations affect this variability. Neither normalizing the stimulated values to non-treated control values (Fig. 2.6C) nor normalizing to pretreatment prostaglandin values (Fig. 2.7) improved the variability. Given the inadequacy of these approaches, we decided that the simple normalization to tissue wet weight was the best approach. This approach was likely more precise than normalizing to surface area as previously used (Zaga, Estrada-Gutierrez et al. 2004) since mass measurements on an analytical balance are more precise than surface area measurements.

Compared to cellular isolations or other forms of tissue culture, the gestational membrane Transwell system is most adept at understanding dynamics of side-specific prostaglandin secretion in response to stimulus. Alternative in vitro systems, such as intact and separated floating gestational membrane cultures, have been used to study stimulated responses of human gestational membranes. However, each of these approaches is limited in the ability to differentiate the side of the tissue that secretes prostaglandins. An immeasurable benefit of the Transwell system is the ability to maintain cell-to-cell and tissue-to-tissue interactions that may be important for processing inflammatory signals while detecting side-specific production of prostaglandin species. Gestational membrane Transwell experiments will be useful for determining under what conditions prostaglandins are released from choriodecidua side of the membranes with potential to modulate uterine contractions and parturition.

Research on the role of gestational membranes in parturition is at an early stage. Most previous research has utilized isolated cell cultures or floating gestational membrane explant cultures to focus on differences in cells and tissue before and after parturition. Additionally, cell and tissue prostaglandin responses to LPS or bacterial stimulation have been demonstrated but without the context of the intact gestational membrane side-specific prostaglandin output could not be determined. The development of the gestational membrane Transwell system allows new research to explore the mechanisms of prostaglandin dynamics in the gestational membranes through measurement of side-specific tissue responses.

This system can be used to study release of various factors, such as cytokines, and is amenable to a wide variety of stimulants. The present study characterized the system

using the model inflammatory stimulant LPS, but experiments have also been conducted

in our laboratory with environmental toxicants such as polychlorinated biphenyls,

polybrominated diphenyl ethers, and phthalate metabolites. Other possibilities for study

in this system include signal transduction, nutrient transport or the mechanisms by which

the membrane functions as a barrier to infectious disease.

Transswell frame

choriodecidual
chamber

gestational
membrane
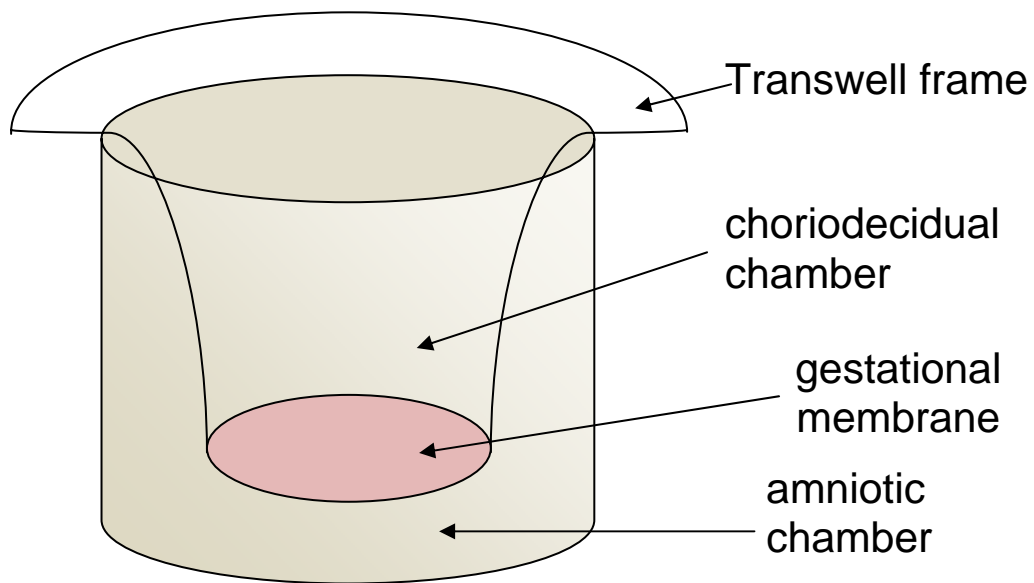
amniotic
chamber

Figure 2.1. Diagram of gestational membrane mounted on Transwell frame. The choriodecidual side of the membrane faces the inner chamber and the amniotic side of the membrane faces the outer chamber. This design allows for discrete stimulation and measurement of secreted products from the two sides of the membrane. *Illustration by Mark Miller.*
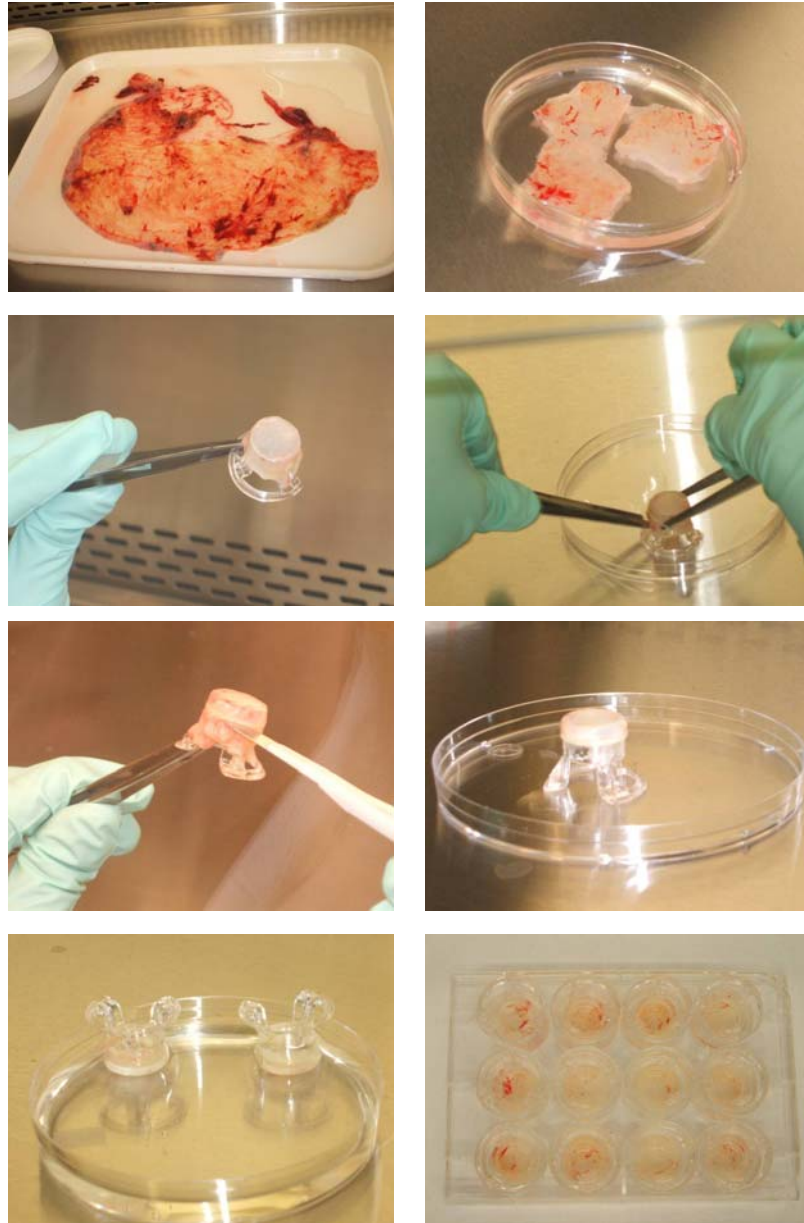
Figure 2.2. Step-by-step process of mounting full-thickness gestational membranes on Transwell frames. A) Gestational membrane tissue after sharp dissection from the placenta proper. Excessive blood clots or regions where membranes are coming apart or disintegrating are removed before selecting samples for culture. B) Gestational membrane samples of 2 cm x 2 cm were cut off of the main specimen and floated in culture medium. C) One sample was placed on top of a sanded, sterilized Transwell device. D) Gestational membranes were affixed to the Transwell device with sterilized latex bands. E) Excess membranes were removed with a scalpel leaving no membranes free beneath the latex band. F) One gestational membrane affixed to a Transwell device. G) Transwell devices with affixed gestational membranes in culture medium. H) Gestational membranes on Transwell devices are cultured in 24-well plates with medium in separate chambers on both the amnion and choriodecidual surfaces of the membrane.
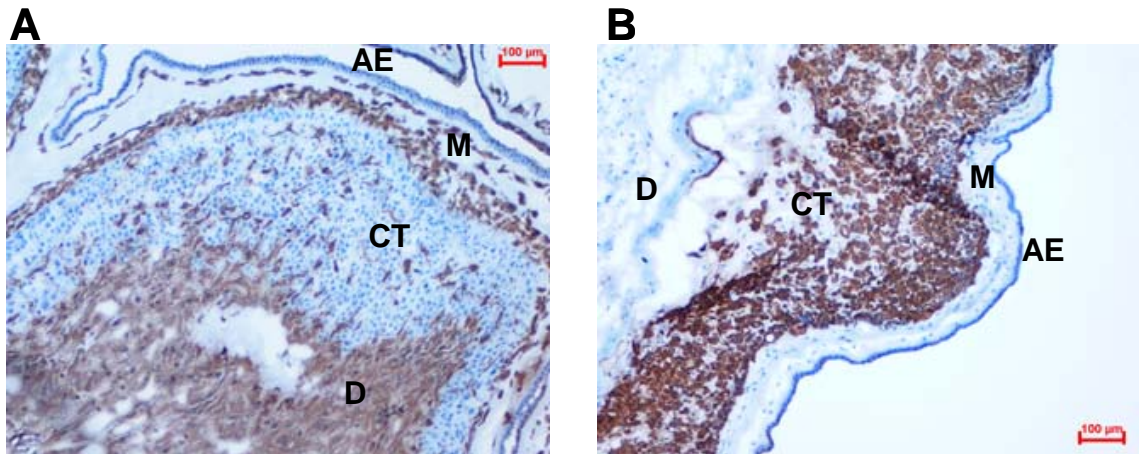
Figure 2.3. Immunohistochemical staining of intact gestational membranes.
A.) Cryosections of intact, fresh gestational membranes were stained with anti-
vimentin antibodies. B.) Cryosection of intact, fresh gestational membranes were
stained with anti-cytokeratin 7 antibodies. In both panels sections were
counterstained with hematoxylin (blue) to identify specific cell types and study
health and integrity of the tissue. A single layer of amnion epithelial cells (AE)
stain blue (hematoxylin) in both panels. Decidual cells (D) and fibroblast cells of
the mesenchymal layer (M) stain positively (brown) for vimentin but not
cytokeratin 7. Chorion trophoblast cells (CT) stain positively for cytokeratin 7
(brown), but not vimentin.
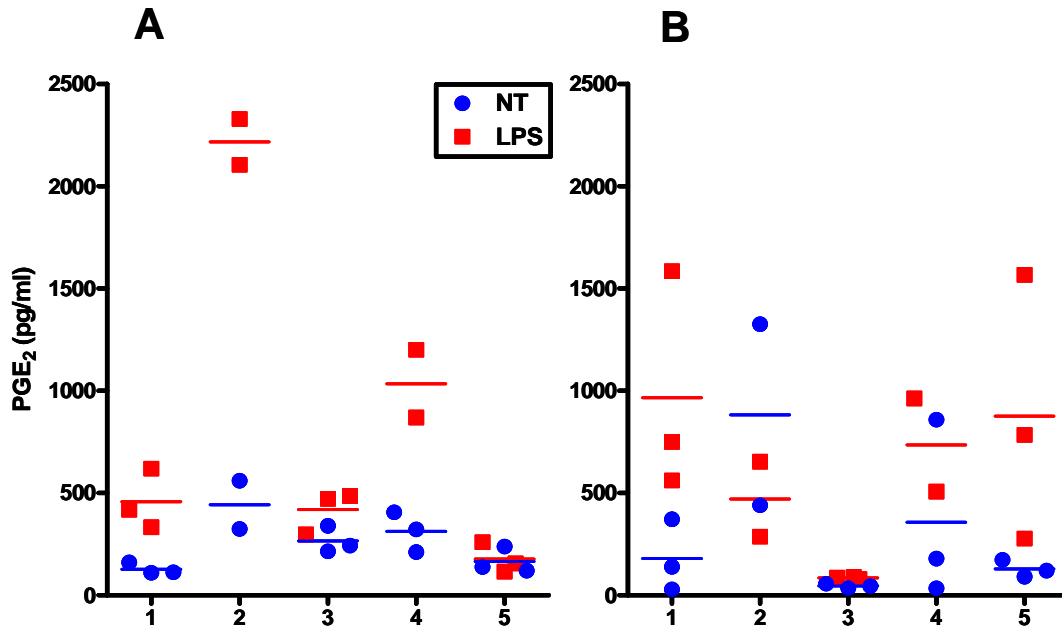
Figure 2.4. Graphs depicting within-subject and between-subject variability.
Gestational membranes mounted Transwell frames were exposed for 4 h to 100
ng/ml lipopolysaccharide (LPS) on both sides of the membrane to assess
variability in prostaglandin response to treatment. PGE_2 concentrations from (A)
amnion culture medium or (B) choriodecidual culture medium were analyzed.
For each subject (designated by a number on the x-axis), LPS-treated (red square)
or non-treated control (NT, blue circle) replicates were graphed. Means for NT
and LPS-treated PGE_2 values are indicated by a bar of the corresponding color.
Each subject had two or three replicates per treatment; subject numbers in (A)
correspond to those in (B).

Table 2.1. Interclass correlation coefficient

	non-treated control	LPS
amnion	0.62	0.97
choriodecidua	0.38	0.26
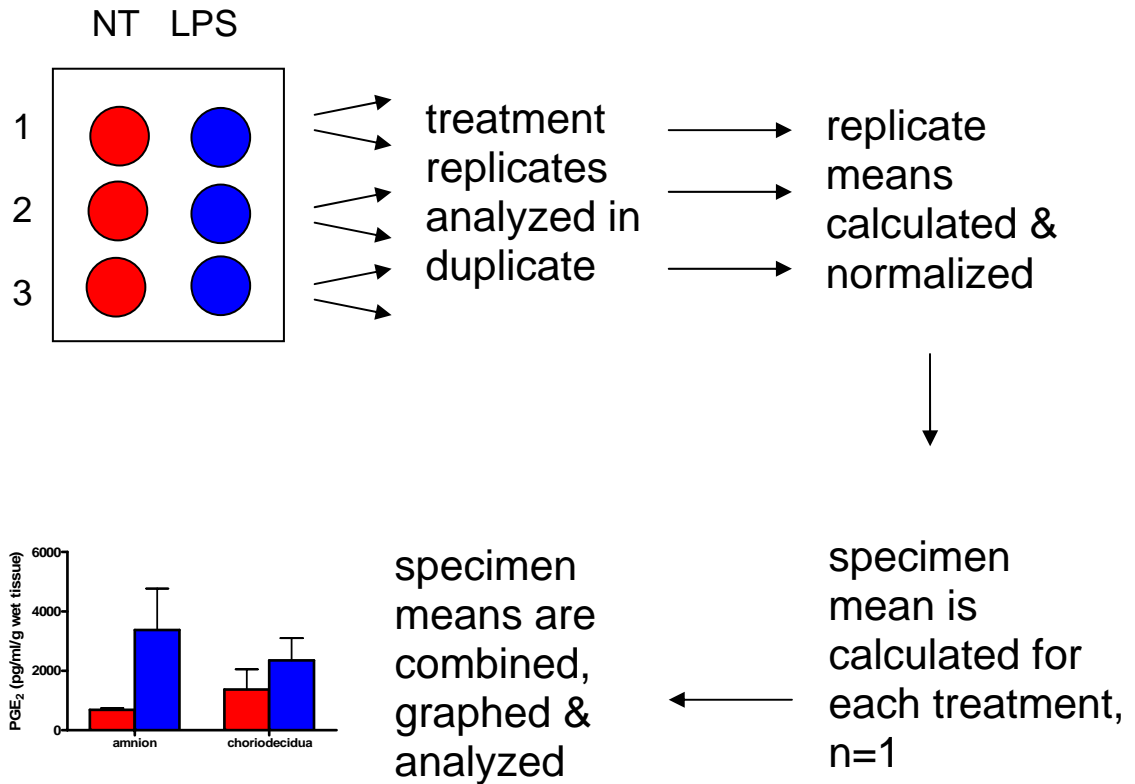
Figure 2.5. Diagram of Transwell analysis scheme. From each subject, three pieces of gestational membrane were exposed to each treatment. In the simplified design shown here, there were two treatment groups: non-treated control (NT) and LPS-exposed. Prostaglandin values in medium from the amnion and choriodecidual chambers of the cultures for each treatment replicate were analyzed separately by enzyme immunoassay in duplicate. Means from analysis duplicates were averaged, and then treatment replicate means for each specimen/subject were calculated. Means from each specimen/subject were combined into one value making the unit of analysis the specimen/subject.

Figure 2.6. Comparison of non-treated control- (NT) and lipopolysaccharide- (LPS) stimulated PGE$_2$ graphs after different methods of data processing. A) PGE$_2$ concentrations (pg/ml) measured in from culture medium on amnion or choriodecidual sides of gestational membrane. Mean concentrations from 5 subjects ± standard error of the mean are graphed. B) Graph of amnion or choriodecidual PGE$_2$ concentrations per sample divided by wet tissue weight of that sample (pg/ml/g wet tissue). Treatment or control mean concentrations per gram wet tissue were calculated for each subject and combined with data from other subjects to graph mean results from 5 subjects ± standard error of the mean. C) PGE$_2$ concentrations per gram wet tissue from each subject were normalized to percent amnion non-treated control (% A-NT) from the same subject and combined with values from other subjects. Values are expressed as mean % A-NT for 5 subjects ± standard error of the mean.
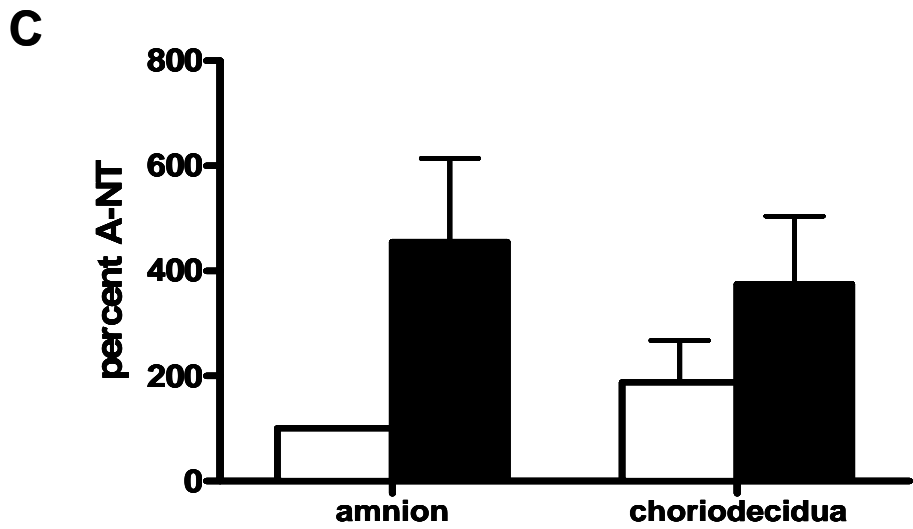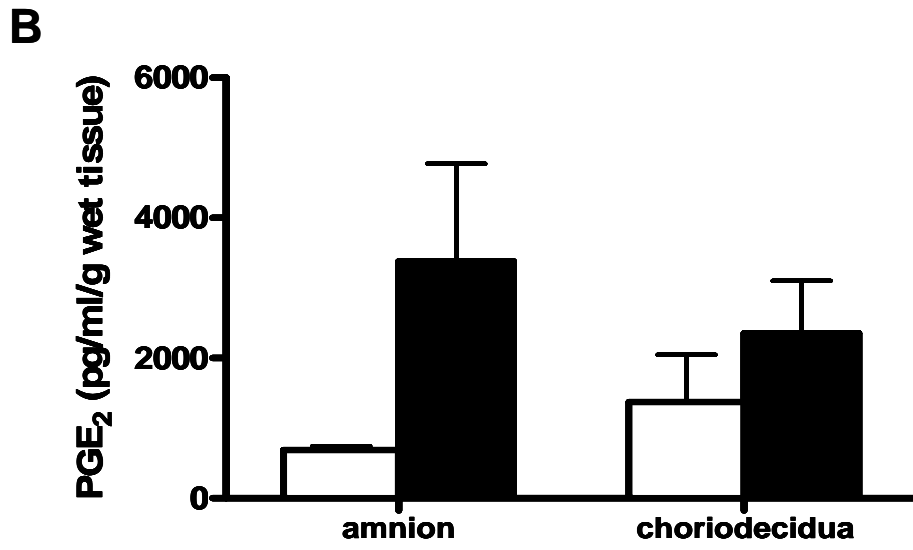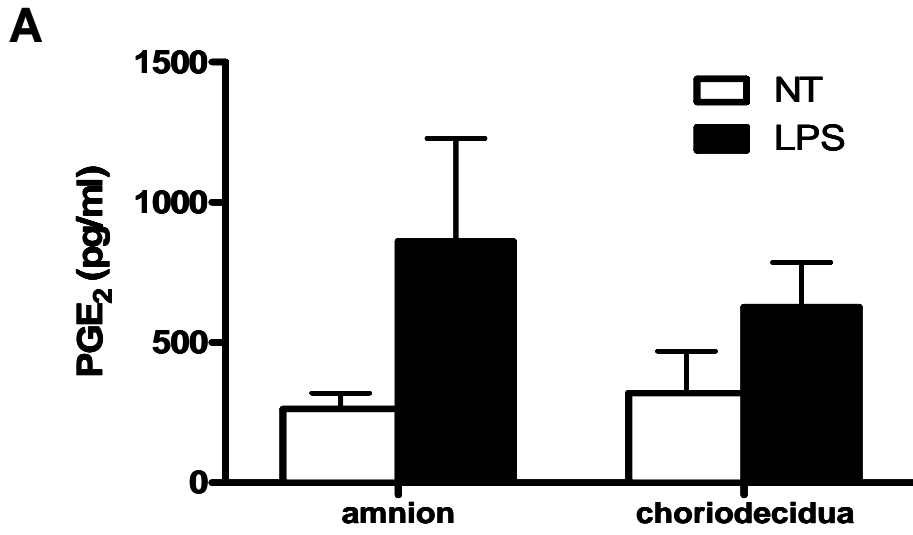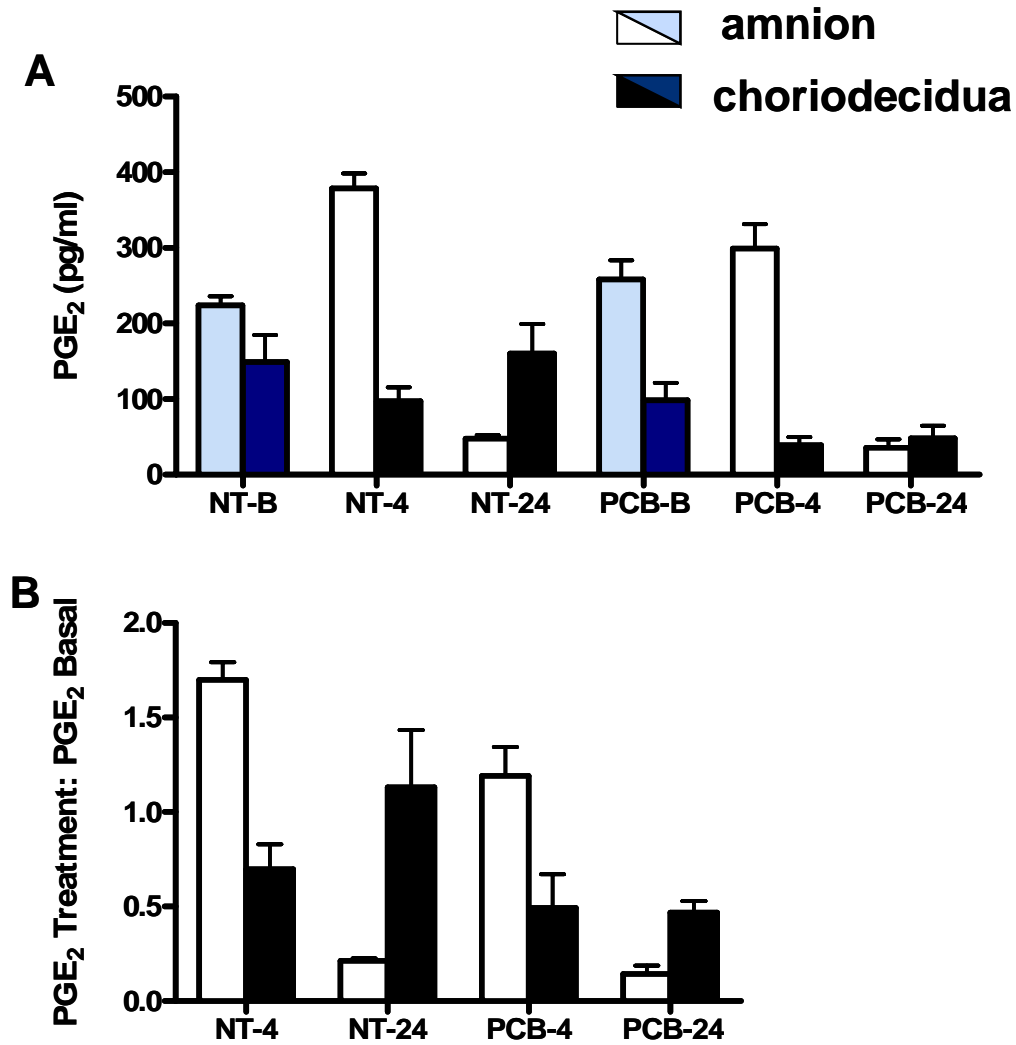
Figure 2.7. Comparison of raw PGE$_2$ concentrations and data expressed as a ratio of treatment to basal PGE$_2$ levels. A) Mean PGE$_2$ concentrations (pg/ml) from amnion or choriodecidual chambers during 2 h basal period (dark and light blue) and 4 and 24 h non-treated control or PCB 153 treatment periods (black and white). B) 4- and 24-h treatment PGE$_2$ concentrations normalized to basal PGE$_2$ concentrations from the same gestational membrane tissue sample. These are data from one subject.
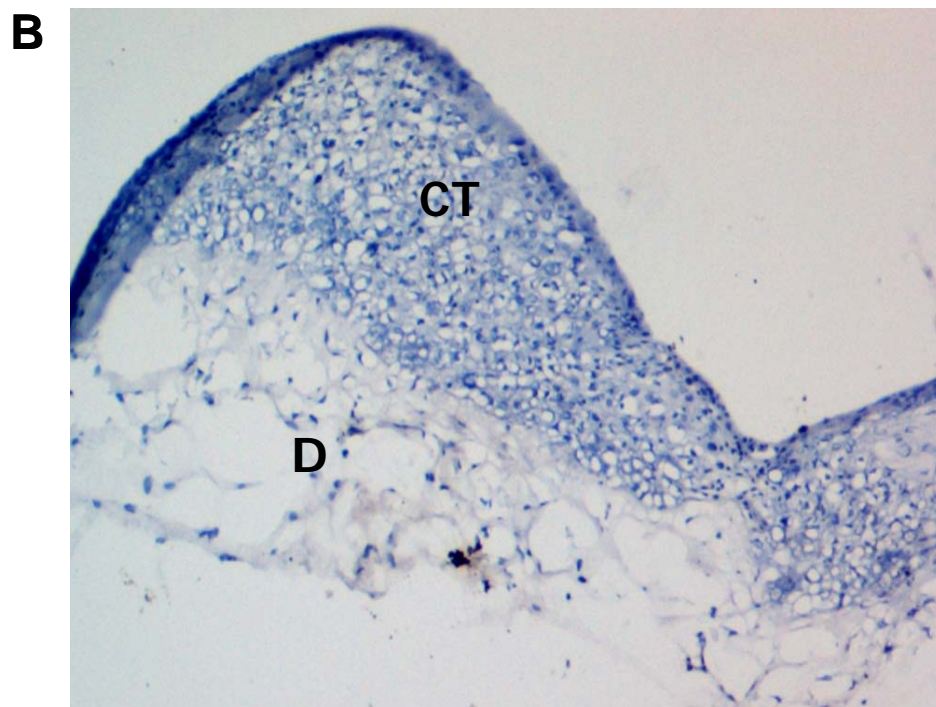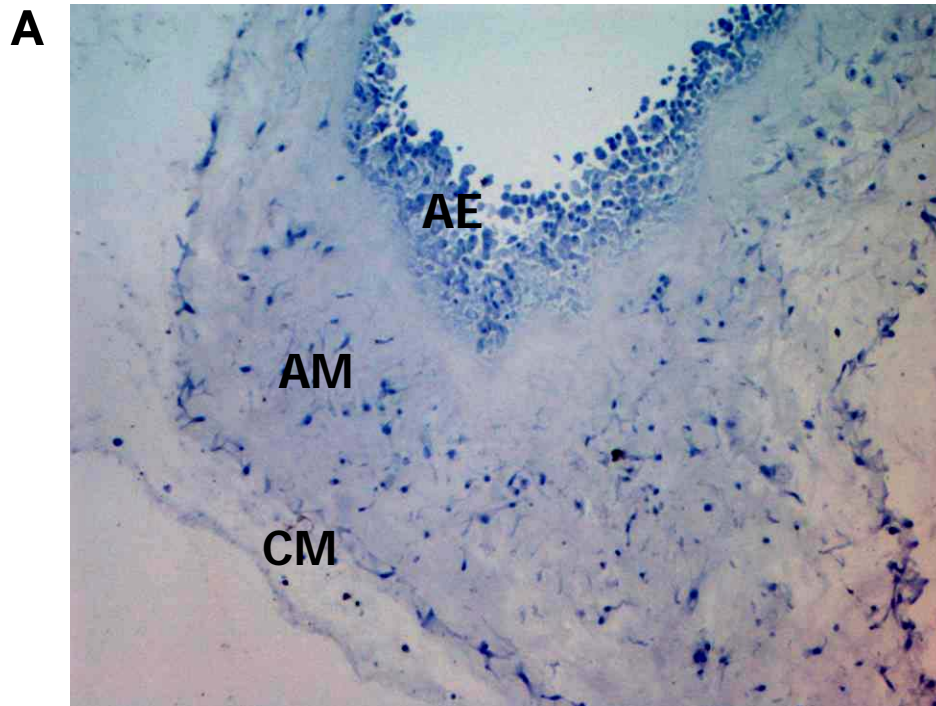
Figure 2.8. Hematoxylin stained cryosections of amnion and choriodecidua after blunt dissection of gestational membrane tissue. A) Amnion epithelium (AE) was degraded after blunt dissection. Amnion mesenchyme (AM) and chorion mesenchyme (CM) partition with amnion layer. B) After blunt dissection of gestational membranes the choriodecidual layer contains chorion trophoblast (CT) cells and decidual (D) cells.
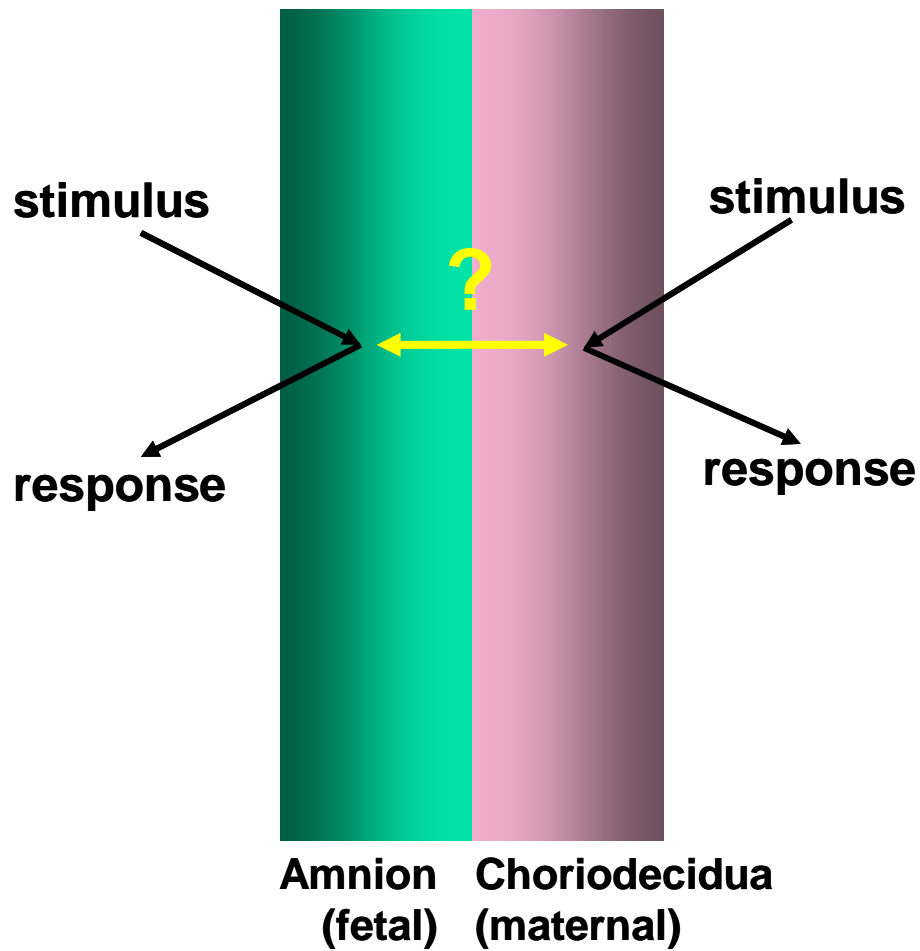
Figure 2.9. Model of the gestational membrane Transwell system. Mounting gestational membranes for tissue culture allows the researcher to stimulate the amnion or choriodecidual sides of the membrane independently. It also allows independent measurement of the response on either side of the membrane.

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CHAPTER 3

LIPOPOLYSACCHARIDE-INDUCED PROSTAGLANDIN SECRETION FROM HUMAN GESTATIONAL MEMBRANE TRANSWELL CULTURES

ABSTRACT

In women, uterotonic prostaglandins from gestational membranes play an important role in uterine contractility and cervical effacement during parturition. Prior to term, exogenous inflammatory conditions such as infection and the subsequent increased production of prostaglandins may cause contractions and lead to preterm delivery of the baby, a significant public health concern. The purpose of this research is to investigate the side-specific prostaglandin secretion from the amnion (fetal) and choriodecidual (maternal) sides of intact human gestational membranes after exposure to the inflammatory stimulus lipopolysaccharide (LPS), a component of bacteria cell membranes. Pieces of gestational membranes from healthy, non-laboring cesarean deliveries were mounted on Transwell frames and exposed to LPS on one or both sides of the membrane. Lipopolysaccharide-stimulated prostaglandin E_2 (PGE_2) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) were measured in the medium of the amnion and choriodecidual sides of the membranes. More PGE_2 was produced on the amnion side whereas $PGF_{2\alpha}$ was secreted to a similar extent from both sides of the gestational membranes. Side-specific differences for LPS-stimulated concentration-response and time-course release of prostaglandins indicated relative independence of the amnion and choriodecidua responses to LPS stimulation. Immunohistochemistry revealed LPS-

dependent and side-specific expression of the rate-limiting enzyme in prostaglandin synthesis, prostaglandin endoperoxide H synthase-2 (PGHS-2). Induction of PGHS-2 was seen on the side of the tissue exposed to LPS treatment. LPS exposure to the amnion side caused PGHS-2 induction in cells of the sub-amniotic mesenchymal layer, and LPS exposure to the choriodecidual side caused PGHS-2 induction in decidual cells. When both sides of the membranes were exposed to LPS, PGHS-2 induction was seen in the mesenchymal and decidual layers of the tissue. Results presented here suggest that LPS induction of PGHS-2 leads to increased prostaglandin secretion from both sides of the gestational membranes.

INTRODUCTION

Extraplacental human gestational membranes are comprised of amnion, chorion and decidua. In situ, the amnion faces the fetal compartment while the decidua is interdigitated with the uterine wall. The gestational membranes make prostaglandins during human pregnancy. Close proximity to the myometrium may allow prostaglandins from gestational membranes to play an important role in parturition. The uterotonic prostaglandins, PGE_2 and $PGF_{2\alpha,}$ are of particular interest because they are potent stimulators of myometrial contractility and are elevated in women experiencing preterm and term labor (Munns, King et al. 1999).

In contractility experiments with pregnant rat uterine strips, we found that an intact uterine lining was required to elicit increased force and frequency of contractions in response to 2,2',4,6-tetrachlorobiphenyl (PCB 50) (Brant and Caruso 2006). This finding led us to speculate that prostaglandin synthesis in the uterine lining (decidua in human pregnancies) may be responsible for increased myometrial contractility during

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exposure to toxicants and inflammatory stimuli. Previous studies demonstrated increased prostaglandin secretion from human gestational membranes obtained after normal vaginal delivery compared to scheduled, non-laboring Cesarean section delivery (Mijovic, Zakar et al. 1997). Researchers hypothesize that preterm uterine contractility and parturition are caused by preterm gestational membrane prostaglandin production due to exogenous inflammatory stimuli (Van Meir, Sangha et al. 1996).

Prostaglandins are derived from arachidonic acid, which is released from phospholipids in the cell membrane by phospholipases. Arachidonic acid is converted to prostaglandin H by prostaglandin endoperoxide H synthase (PGHS). Prostaglandin H synthase-2, also known as cyclooxygenase-2 or COX-2, is the inducible isoform of the enzyme. It is induced in human gestational tissues at the time of parturition, and is responsible for the increased prostaglandin formation seen near term (Gibb and Sun 1996). Prostaglandin endoperoxide H synthase-2 is also induced in response to inflammatory agents. Prostaglandin H is converted to PGE_2 or $PGF_{2\alpha}$ by prostaglandin E synthase or prostaglandin F synthase, respectively (Sales and Jabbour 2003). Cell and tissue specific localization of synthase enzymes may determine which prostaglandin species are produced. Traditionally, PGE_2 synthesis has been thought to occur throughout the extraplacental gestational membranes with high levels of synthase activity in the amnion while $PGF_{2\alpha}$ synthesis is located in the decidua (Okazaki, Casey et al. 1981). The enzymes in the prostaglandin pathway are subject to a variety of regulatory mechanisms from hormones, cytokines, and growth factors.

Prostaglandin catabolism is rapid and prostaglandin isomers are inactivated by 15-hydroxy-prostaglandin dehydrogenase (PGDH). Some reports indicate that PGDH

activity is reduced in term or preterm labor, and researchers hypothesize that the localization of PGDH in chorionic trophoblasts creates a metabolic barrier preventing passage of prostaglandins from the amnion to the underlying myometrium (Challis, Lye et al. 2001).

Intrauterine infection is thought to be the single most causative factor in the etiology of preterm labor and birth in humans, accounting for as many as one-third of all preterm deliveries (Goldenberg, Culhane et al. 2008). Lipopolysaccharide (LPS) is a cell membrane component from gram negative bacteria that elicits a strong immune response and may be an important signaling molecule in these cases of intrauterine infection and preterm birth. It is commonly used as a model inflammatory agent. In mouse models of preterm labor and birth, injection of LPS into the cervix causes preterm expulsion of the fetuses (Reznikov, Fantuzzi et al. 1999).

Lipopolysaccharide induces prostaglandin synthesis by inducing enzymes of the prostaglandin synthetic pathway, particularly PGHS-2 (Brown, Alvi et al. 1998). It may also indirectly affect prostaglandin production by inducing production of inflammatory cytokines such as tumor necrosis factor-α (Sato, Keelan et al. 2003).

The goal of this research was to investigate the side-specific prostaglandin secretion from intact gestational membranes exposed to the inflammatory stimulus, LPS. This was modeled in our laboratory by LPS treatment of membranes mounted on Transwell devices followed by measurement of prostaglandins in amnion or choriodecidual culture medium and immunohistochemical analysis of PGHS-2 induction. Better knowledge of prostaglandin production in non-laboring gestational membranes will enhance our understanding of preterm labor or other complications of parturition.

Exposure to exogenous stimuli such as LPS may activate prostaglandin signaling prematurely.

MATERIALS AND METHODS

Chemicals, Reagents and Antibodies

Tissue culture reagents including high glucose Dulbecco's Modified Eagle's Medium (DMEM) with no phenol red, penicillin/streptomycin antibiotic, and heat-inactivated fetal bovine serum were purchased from Invitrogen (Carlsbad, CA). Lipopolysaccharide (LPS) from Salmonella Typhinurium (Lot #225) was purchased from List Biological Laboratory (Campbell, CA). The Vectastain Elite ABC immunohistochemical staining kit, DAB Substrate Kit (3,3'-diaminobenzidine) and hematoxylin counterstain were purchased from Vector Laboratories (Burlingame, CA). Prostaglandin E_2 and prostaglandin $F_{2\alpha}$ enzyme immunoassay kits and anti-cyclooxygenase monoclonal antibody were purchased from Cayman Chemical Co. (Ann Arbor, MI). Transwell frames with no micropore membrane insert were a gift from Corning Corporation (Corning, NY).

Tissue Collection

Methods pertaining to human tissue were reviewed and approved by the local Institutional Review Board prior to initiation of experiments; review and approval were updated yearly. Full-thickness gestational membranes comprised of amnion, chorion and decidua were collected at 37-39 weeks from non-laboring elective Cesarean sections following healthy pregnancies. Exclusion criteria included smoking, multiple fetal gestation, complications of pregnancy such as gestational diabetes or hypertension, use of drugs that may have an effect on arachidonic acid metabolism (e.g., aspirin, Singulair®),

or any other condition which would require the tissue to be sent to Pathology. Within 1-60 min after delivery, full-thickness extraplacental membranes were sharp dissected from the placenta, placed in phosphate buffered saline solution and transported to the laboratory. Alternatively, the entire placenta and attached membranes were transported to the laboratory.

Tissue Culture

In the laboratory, the integrity of the membranes was examined under sterile conditions to verify that the amnion and choriodecidua remained attached to one another and that the collagen compact layer between them was not degraded. The membranes were rinsed with culture medium (DMEM supplemented with 1% heat inactivated-fetal bovine serum and 100 units penicillin/100 μg streptomycin per milliliter) to remove excess blood clots. The membranes were cut into approximately 2 x 2 cm^2 pieces and affixed by latex rubber bands onto ethylene oxide sterilized Transwell frames without a micropore membrane insert (Zaga, Estrada-Gutierrez et al. 2004). The choriodecidual side of the tissue faced the inner chamber of the device. Extra tissue was removed with a scalpel. Each Transwell frame with attached tissue was placed in a single well of a 12-well plate with culture medium on both sides of the membranes, creating two distinct chambers on either side of the membrane. Mounted membranes were equilibrated in a 5% CO_2 tissue culture incubator. To maintain optimal culture conditions and health of the tissue, membranes in culture medium were equilibrated for 18-24 h with a medium change after 2-4 h. Prior to beginning the treatment protocol, the overnight culture medium was removed and the membranes were pretreated with fresh culture medium for 1-2 h.

Treatment

The LPS was diluted in water to make a primary stock of 100 μg/ml. Just prior to the experiment, the LPS primary stock was further diluted with culture medium (DMEM supplemented with 1% heat inactivated-fetal bovine serum and 100 units penicillin/100 μg streptomycin per milliliter) to achieve the experimental concentration (1-1000 ng/ml, as indicated), and added to one or both chambers of the culture. In cases where only one side of the membranes was treated with LPS, the opposite side was cultured in culture medium. Mounted full-thickness gestational membrane pieces collected from different regions of the membranes were assigned randomly to treatment groups. Treatments were conducted in triplicate, i.e., three pieces of tissue were included in each treatment. Membranes were incubated with the treatments for 1-24 h. After the specified treatment time, treatment medium was recovered from each side of the membrane and stored at -20°C or -80°C for subsequent enzyme immunoassay analysis of PGE_2 and $PGF_{2\alpha}$. The tissue was removed from the Transwell device, weighed, embedded in freezing medium and frozen in tissue molds for subsequent immunohistochemistry analysis.

Enzyme Immunoassay

Prostaglandin E_2 and $PGF_{2\alpha}$ in the culture medium were assayed by specific enzyme immunoassays according to the manufacturer's protocol (Cayman Chemical Co., Ann Arbor, MI). Prior to the assay, samples were thawed and diluted to fall within the range of the standard curve. Each medium sample was assayed in duplicate. PGE_2 concentrations were calculated relative to the values from the standard curve. Prostaglandin concentrations were divided by the wet weight of the full-thickness gestational membrane tissue taken after the experiment, and the mean of the treatment

replicates was calculated. An overall mean for each specimen was calculated and combined with results from other specimens. Mean results and standard error of the mean were calculated from multiple specimens and graphed with n referring to the number of specimens (subjects) in each experiment.

Immunohistochemistry

After tissue was removed from the Transwell frames, it was rolled, embedded in freeze medium, frozen in liquid nitrogen-cooled isopentane and stored at -80° C. The tissue was sliced with a cryotome into 8 μm sections, the cryosections were mounted on slides and stored at -80° C. On the day of staining, slides were warmed to room temperature, fixed with 4% paraformaldehyde and stained using the Vectastain Elite ABC Kit. Sections were incubated with primary antibody (anti-PGHS-2 monoclonal antibody), labeled with a biotinylated secondary antibody and an avidin:biotinylated enzyme complex, developed with 3,3-diaminobenzidine and counterstained with hematoxylin. Stained sections were visualized with an upright light microscope and recorded digitally.

Statistical Analyses

The prostaglandin enzyme immunoassay results were analyzed by one-way or two-way analysis of variance (ANOVA) using Sigma Stat v 3.5 software (Systat Software, Inc., Richmond, CA). Prior to analysis, the data were transformed using log normal transformation to correct for non-normality. Post hoc pair-wise comparisons of means were performed by the Student-Newman-Keuls methods. A p-value <0.05 was considered statistically significant.

RESULTS

Concentration-response of LPS-stimulated prostaglandin E_2 release

To study LPS-dependent concentration effects on PGE_2 release from gestational membrane Transwell cultures, PGE_2 secretion was measured in amnion and choriodecidua culture media after an 8-h exposure to 1, 10, 100 or 1000 ng/ml LPS or non-treated control (NT) (Fig. 3.1). The basal concentration of PGE_2 in the amnion NT media was 1601 pg/ml/g after 8 h of culture. In the amnion medium, near maximal PGE_2 concentration (13507 pg/ml/g) was observed with 1 ng/ml LPS exposure, the lowest concentration of LPS tested. Amnion PGE_2 responses to 10, 100 and 1000 ng/ml LPS were similar to the 1 ng/ml response with concentrations of 12495, 11660, and 11229 pg/ml/g, respectively. High variability in the amnion response precluded a statistically significant difference between LPS treatments and controls.

The choriodecidual non-treated control PGE_2 concentration after 8 h of culture was 509 pg/ml/g. In the choriodecidual culture medium, concentrations of PGE_2 increased modestly with increasing LPS treatment concentration, such that PGE_2 concentrations of 3717, 4699, 5200 and 5863 pg/ml/g were observed for 1, 10, 100 and 1000 ng/ml LPS treatment, respectively. All choriodecidual LPS-stimulated PGE_2 concentrations were significantly increased compared with non-treated control samples ($p<0.05$), but were not significantly different from each other.

The concentration-response experiment indicated that LPS stimulated PGE_2 release at 1 ng LPS/ml, the lowest concentration tested. There was no significant additional PGE_2 response with increasing LPS treatment concentration for either the amnion or choriodecidual side, suggesting a threshold effect.

Time-course of LPS-stimulated prostaglandin E₂ release

Tissues were incubated for 1-24 h to analyze the dynamics of gestational

membrane PGE_2 concentrations in media in response to LPS. Secreted PGE_2 was

measured by enzyme immunoassay in culture medium taken from the amnion (Fig. 3.2A)

or choriodecidual sides (Fig. 3.2B) of the gestational membrane Transwell culture after 1,

4, 8 or 24 h exposure to 100 ng/ml LPS.

Prostaglandin E_2 concentrations in amnion medium from NT samples increased

slightly over 1 to 8 h of exposure with mean concentrations of 261, 815 and 1051 pg/ml/g

tissue for 1, 4 and 8 h, respectively (Fig. 3.2A, left side). Mean PGE_2 concentrations

from 4- and 8 h- amnion NT culture media were significantly higher than 1-h levels

($p<0.05$). Prostaglandin E_2 concentrations decreased in 24-h culture medium to 441

pg/ml/g, which was not statistically significantly different from concentrations observed

at 1 h.

LPS-stimulated prostaglandin E_2 production increased over time in the amnion

medium. Mean concentrations of 1461, 4157 and 10029 pg/ml/g tissue for 1, 4 and 8 h

exposures, respectively, were significantly increased compared with non-treated controls

of the same exposure duration (Fig. 3.2A, right side; $p<0.05$). Little PGE_2 was measured

in the amnion medium after 24 h of LPS exposure (575 pg/ml/g), and the mean PGE_2

concentration from 24 h LPS exposed tissue was significantly different from 8 h levels

($p<0.05$).

Concentrations of PGE_2 in the choriodecidual culture medium of controls (NT)

were not statistically significantly different from each other at 1, 4, 8 and 24 h of culture,

respectively (Fig. 3.2B, left side). LPS stimulated PGE_2 release from the choriodecidual

side of the membranes, with values of 2956, 2744, and 3105 pg/ml/g measured at the 1, 4 and 8 h LPS-treatment time points, respectively (Fig. 3.2B, right side). Mean PGE_2 concentrations from 1, 4 and 8 h LPS-treated samples were significantly greater than the mean PGE_2 concentrations from the 24-h time point (333 pg/ml/g) ($p < 0.05$).

The rate of PGE_2 response to LPS stimulation is different between the amnion and choriodecidual sides of the membranes. The peak PGE_2 response is observed later in the amnion (8 h) compared to choriodecidua. In both amnion and choriodecidual culture media, little PGE_2 was recovered in the non-treated control or LPS-treated samples after 24 h of exposure, indicating that PGE_2 ceases to be synthesized beyond 8 h of stimulation and suggesting that PGE_2 from earlier in the exposure may be degraded in the culture medium or taken up by the tissue. Future experiments were conducted with an 8-h LPS exposure time to capture peak LPS-stimulated prostaglandin production.

PGE_2 and $PGF_{2\alpha}$ response to side-specific LPS treatment of gestational membranes

Prostaglandin release was assessed from the amnion and choriodecidual sides of intact gestational membranes cultured on Transwell devices treated with LPS in a side-specific manner. Membranes were treated for 8 h with 100 ng/ml LPS on only the amnion side of the membranes, only the choriodecidual side of the membranes, both sides of the membranes or neither side of the membranes (non-treated control). In cases where only one side of the membranes was treated with LPS, the other side was incubated with culture medium (DMEM plus 1% FBS and antibiotics). Prostaglandin E_2 (Fig. 3.3A) and $PGF_{2\alpha}$ (Fig. 3.3B) were measured in the culture media on both the amnion and choriodecidual sides of the membrane after an 8-h exposure.

All forms of LPS treatment resulted in statistically significant amniotic PGE_2 response compared to non-treated control samples (p<0.05; Fig. 3.3A). Moreover, the amnion produced significantly more PGE_2 than the choriodecidua side of the membranes under all LPS-treated conditions. When only the amnion side was treated with LPS, there was a robust PGE_2 response on the amnion side (13370 pg/ml/g; p<0.05) but not the choriodecidual side (609 pg/ml/g). However, when only the choriodecidual side was treated, the amnion PGE_2 response remained significantly greater than the choriodecidua response (7582 pg/ml/g vs.1398 pg/ml/g; p<0.05). Similarly, when both sides of the membrane were treated with LPS, the mean amnion PGE_2 response was greater than the choriodecidual response (13040 vs. 4773 pg/ml/g; p<0.05), indicating that no additional increase in amnion PGE_2 response was gained from treating both sides of the membrane.

Though choriodecidua PGE_2 concentrations were lower overall compared to the amnion, there remained a statistically significant increase in choriodecidual PGE_2 concentrations from samples treated with LPS on the choriodecidual side only (1398 pg/ml/g) and samples treated with LPS on both sides (4173 pg/ml/g) compared to non-treated control samples (358 pg/ml/g) (p<0.05). PGE_2 from choriodecidual samples treated with LPS on both sides of the membrane was significantly higher than from samples treated on only the amnion side of the membrane, also (p<0.05).

In contrast to the side-specific PGE_2 results, $PGF_{2\alpha}$ response to LPS treatment was comparable in medium from both sides of the membranes (Fig. 3B). Mean $PGF_{2\alpha}$ concentrations in amnion culture medium from samples treated on the amnion, choriodecidual or both sides of the membranes were 3789, 2663, and 5604 pg/ml/g, respectively, and were significantly increased relative to non-treated control samples (525

pg/ml/g) (p<0.05). In the choriodecidual culture medium, mean $PGF_{2\alpha}$ concentrations were increased in samples treated on the choriodecidual or both sides of the membrane (7547 and 10698 pg/ml/g) compared to non-treated controls (1587 pg/ml/g) (p<0.05).

Gestational membrane prostaglandin endoperoxide H synthase-2 immunohistochemistry from side-specific LPS treatments

Immunohistochemistry was used to investigate whether LPS-induced changes in prostaglandin secretion from gestational membranes were related to changes in expression of prostaglandin H synthase 2 (PGHS-2). After medium was collected for the LPS-stimulated prostaglandin release experiments described above, the gestational membranes were removed from the Transwell devices, fixed, cryosectioned and immunostained for PGHS-2 (Fig. 3.4).

No positive PGHS-2 staining was seen in amnion, chorion or decidua of the non-treated control samples (Fig. 3.4A and B). In tissue treated with LPS on the amnion side only, trophoblast cells in the amnion and chorion mesenchymal region stained positive for PGHS-2 (Fig 3.4C), but no positive PGHS-2 staining was seen in the decidua (Fig. 3.4D). In tissue treated with LPS on the choriodecidual side only, PGHS-2 was detected in cells in the decidua (Fig. 3.4F), but cells in the amnion and chorion did not stain positive for PGHS-2 (Fig. 3.4E). In tissue treated with LPS on both sides of the membranes, cells in both the amnion and chorion mesenchymal region and the decidua stained positive for PGHS-2 (Fig. 3.4G and H).

DISCUSSION

The Transwell experimental model used in the present study allowed side-specific LPS stimulation of intact gestational membranes and side-specific measurement of

prostaglandin response. Differentiating the sources of prostaglandins released in response to LPS in non-laboring gestational membranes may help clarify the role of inflammation- or infection-stimulated prostaglandins in events related to preterm parturition.

Side-specific LPS exposures revealed an increase in prostaglandin release on the amnion side but not the choriodecidual side of the membranes when only the amnion side was exposed to LPS. In tissue from these experiments, detection of immunoreactive PGHS-2 in the mesenchymal fibroblasts suggested that mesenchymal PGHS-2 induction may be responsible for amniotic prostaglandin response. When only the choriodecidual side was exposed to LPS, PGE_2 and $PGF_{2\alpha}$ were secreted into the choriodecidual culture medium correlating with appearance of immunoreactive PGHS-2 in the decidua. The amniontic PGE_2 response observed when only the choriodecidual side was exposed to LPS could be due to passage of an inflammatory signal from the choriodecidua to initiate prostaglandin synthesis within the amnion. However, absence of positive PGHS-2 immunostaining in the amnion when only the choriodecidua was treated suggest that the PGE_2 originated in the choriodecidua.

The concentration-response experiments indicated a threshold effect on PGE_2 response to LPS stimulation. No increase of PGE_2 concentrations was seen in the amnion or choriodecidua medium with LPS treatment concentrations ranging from 1 to 1000 ng/ml. Although not examined in this work, possible explanations for this threshold of response may be saturation of its co-receptors, CD-14 and toll-like receptor 4, or some other aspect of the signal transduction pathway.

Gestational membranes are sensitive to LPS concentrations as low as 1 ng/ml. Other researchers generally use between 100-1000 ng/ml LPS in cell and tissue culture experiments. Of particular interest, Garcia-Lopez et al. (Garcia-Lopez, Vadillo-Ortega et al. 2007) observed degradation of the collagen in the mesenchymal layer of gestational membranes exposed to 500 ng/ml LPS for 24 h in Transwell cultures. In the latter study, the effects on the compact collagen layer were proposed as a mechanism for preterm premature rupture of the membranes. However, in the present study, gestational membranes treated with 100 ng/ml LPS treatment showed no evidence of compact layer degradation or other histopathology. We conclude that there is little value in using excessively high LPS concentrations in gestational membrane experiments. At least in the case of PGE_2, a threshold response is reached at much lower LPS treatment concentrations without evidence of overt tissue damage or cytotoxicity. Lipopolysaccharide concentrations above 10 ng/ml are outside of the range of meaningful physiologic concentrations and may activate non-physiological pathways other than toll-like receptor/CD-14 pathways (Levin, Pollack et al. 1998).

Secretion of PGE_2 into the amnion culture medium increased between 1 and 8 h of exposure to 100 ng/ml LPS, suggesting a steady rate of synthesis over that time period. The cellular and tissue responses to LPS occurred rapidly with increased PGE_2 secretion after only 1 h of LPS exposure (Fig. 2A). These results are in agreement with those of Brown et al. (Brown, Alvi et al. 1998), who observed a similar time-course with increases in PGE_2 concentrations from floating intact gestational membrane cultures exposed to LPS for 1-8 h, and no further increase of PGE_2 concentrations after 8 h. Rajasingam et al. also reported significant increases in amniotic PGE_2 production after 6 h of LPS

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treatment in a dual-chambered culture system (Rajasingam, Bennett et al. 1998).

However, they reported significant increases in choriodecidual PGE_2 after 30 min of

treatment (Rajasingam, Bennett et al. 1998), whereas our experiments failed to

demonstrate significant increases of choriodecidual PGE_2 release after 1, 4 or 8 h of LPS

exposure (Fig. 2B). The report by Rajasingam et al. suggested that the rapid PGE_2

increase observed in that study was due to activation of phospholipase A_2, whereas

responses requiring longer exposure durations could be due to increases in prostaglandin

synthesis enzymes levels (Rajasingam, Bennett et al. 1998). However, the latter

explanation does not account for their observed differences between PGE_2 and $PGF_{2\alpha}$

release. The activity of upstream enzymes, such as phospholipase A_2, would presumably

affect PGE_2 and $PGF_{2\alpha}$ synthesis equally.

The leveling off of PGE_2 accumulation seen in the choriodecidual medium of the

Transwell system after 1 h of exposure may be due to a number of factors. The shortened

LPS-stimulated response observed in choriodecidua could be explained by a shorter

duration of prostaglandin synthetic enzyme activation or by prostaglandin reuptake and

catabolism. Additionally, PGDH in the chorion trophoblast cells may metabolize

prostaglandins in the choriodecidual tissue, thereby preventing additive accumulation of

choriodecidual PGE_2 during the 4 and 8 h treatments. Alternatively, results presented in

chapter 4 show that LPS exposure caused choriodecidual side-specific increase in IL-10,

an anti-inflammatory cytokine. Attenuation of the LPS inflammatory response by a

compensatory anti-inflammatory cytokines could explain the patterns of time-dependent

choriodecidual PGE_2 release observed in this study.

Much effort has been focused on determining the ability of amniotic PGE_2 to traverse the gestational membranes and reach the myometrium (Challis, Patel et al. 1999). Chorion trophoblast cells are thought to have high PGDH activity and, as such, to be able to rapidly catabolize prostaglandins. In one popular model, chorionic PGDH is proposed to serve as an effective metabolic barrier to the passage of prostaglandins from the amnion to the decidua and myometrium (Challis, Patel et al. 1999). The presence of PGDH in gestational membrane tissues could explain the lack of choriodecidual prostaglandin release when only the amnion side was stimulated, as well as the absence of prostaglandins in choriodecidual medium at the 24 h time point. Aside from this possibility, the results presented here indicate that LPS caused release of PGE_2 and $PGF_{2\alpha}$ from the choriodecidua regardless of amniotic involvement. Therefore, discussions about whether amniotic epithelium prostaglandins cross the gestational membranes to reach the myometrium may have limited relevance to the in vivo situation. However, the contrary case whereby choriodecidual inflammatory exposure causes amniotic prostaglandin release without evidence of mesenchymal PGHS-2 induction suggests passage of prostaglandins from the choriodecidua to the amnion. This scenario may have clinical relevance because intra-amniotic prostaglandin release from choriodecidual response to ascending infection may harm the fetus.

In recent years the existence of prostaglandin transporters has become known (Kanai, Lu et al. 1995), but no prostaglandin transporters have been identified in human gestational membranes to date. Prostaglandin transporters facilitate passage of prostaglandins into cells in conjunction with lactate exchange (Chan, Endo et al. 2002). Characterization of prostaglandin transporters in gestational membranes may further

explain the source and signaling dynamics of prostaglandins in gestational membranes and myometrium.

In vivo, the presence of bacteria, LPS and other bacterial products may augment the inflammatory response and change the dynamics of prostaglandin secretion in the membranes. Therefore, lipopolysaccharide alone may not fully elicit effects on membrane integrity or passage of the inflammatory signal from one side of the membranes to the other. Presumably, the inflammatory response would be prolonged and augmented by mobilization of inflammatory cells to the gestational membranes. However, in the ex vivo experimental model utilized here, the membranes are isolated from such body-wide immunological responses.

In conclusion, the present study demonstrated that LPS caused secretion of uterotonic prostaglandins from both the amnion and choriodecidual sides of intact, non-laboring human gestational membranes in vitro. Immunohistochemistry revealed a regional induction of PGHS-2 that correlated with secreted prostaglandins in side-specific exposure experiments. Lipopolysaccharide stimulation of uterotonic prostaglandins from the choriodecidua of non-laboring gestational membranes may demonstrate a pathological induction of PGHS-2 and prostaglandin synthesis that could explain some mechanisms of preterm parturition. However, it remains possible that gestational membranes may respond differently at earlier gestational ages compared with the term tissues used in the present experiments.
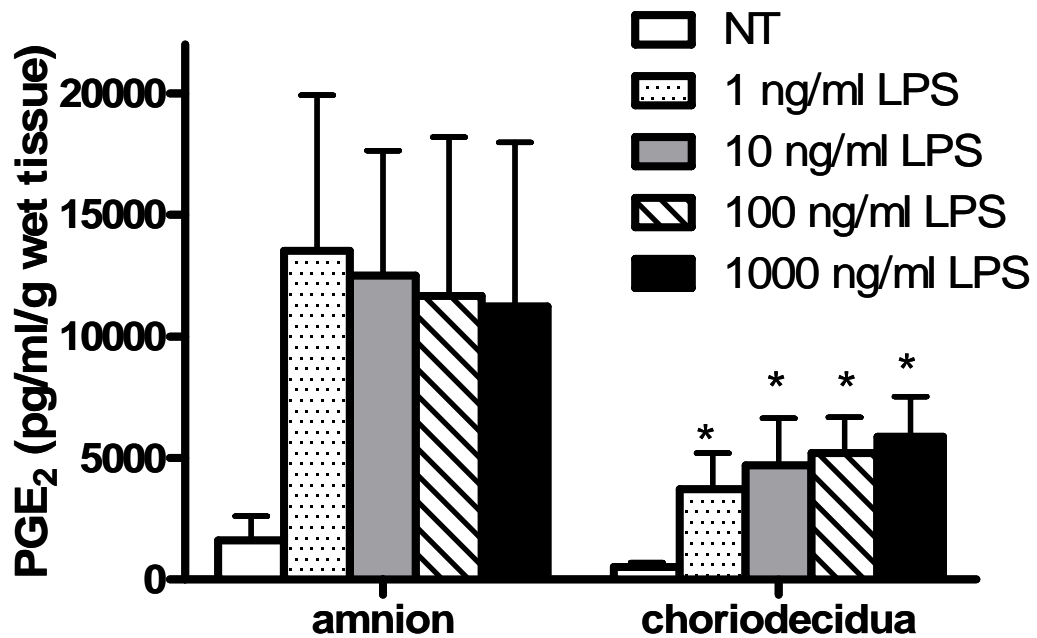
Figure 3.1. Concentration-dependent stimulation of PGE$_2$ release from amnion or choriodecidual side of human full-thickness gestational membranes in Transwell tissue culture following an 8-h LPS exposure. The x-axis shows the side of the gestational membranes assayed. The legend indicates the side of the gestational membranes exposed to LPS or not-treated (NT; controls). Values are expressed as mean ± SEM (n= 6 specimens) *Significantly different from choriodecidua non-treated control (NT; $p < 0.05$).
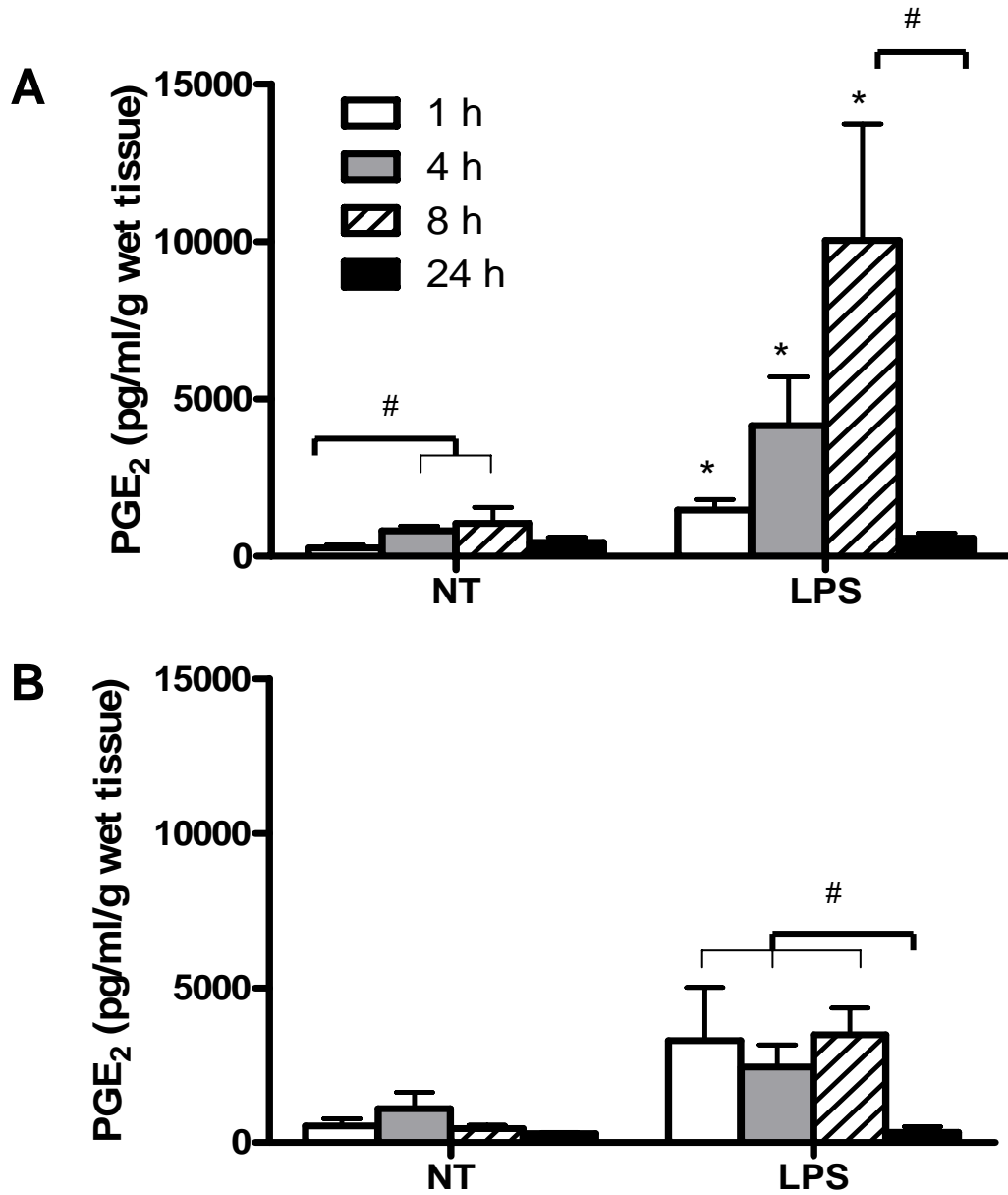
Figure 3.2. Time-dependent simulation of PGE_2 release from full-thickness human gestational membranes in Transwell cultures of non-treated controls (NT) or following exposure to 100 ng/ml LPS . A) PGE_2 concentrations in amnion chamber medium. B) PGE_2 concentrations in choriodecidua chamber medium. Values are expressed as mean ± SEM (1 h n= 6, 4 h n=8, 8 h n=11, 24 h n=4). [#]Significantly different from time points indicated within treatment group. *Significantly different from non-treated controls (NT) of same exposure time (p< 0.05).
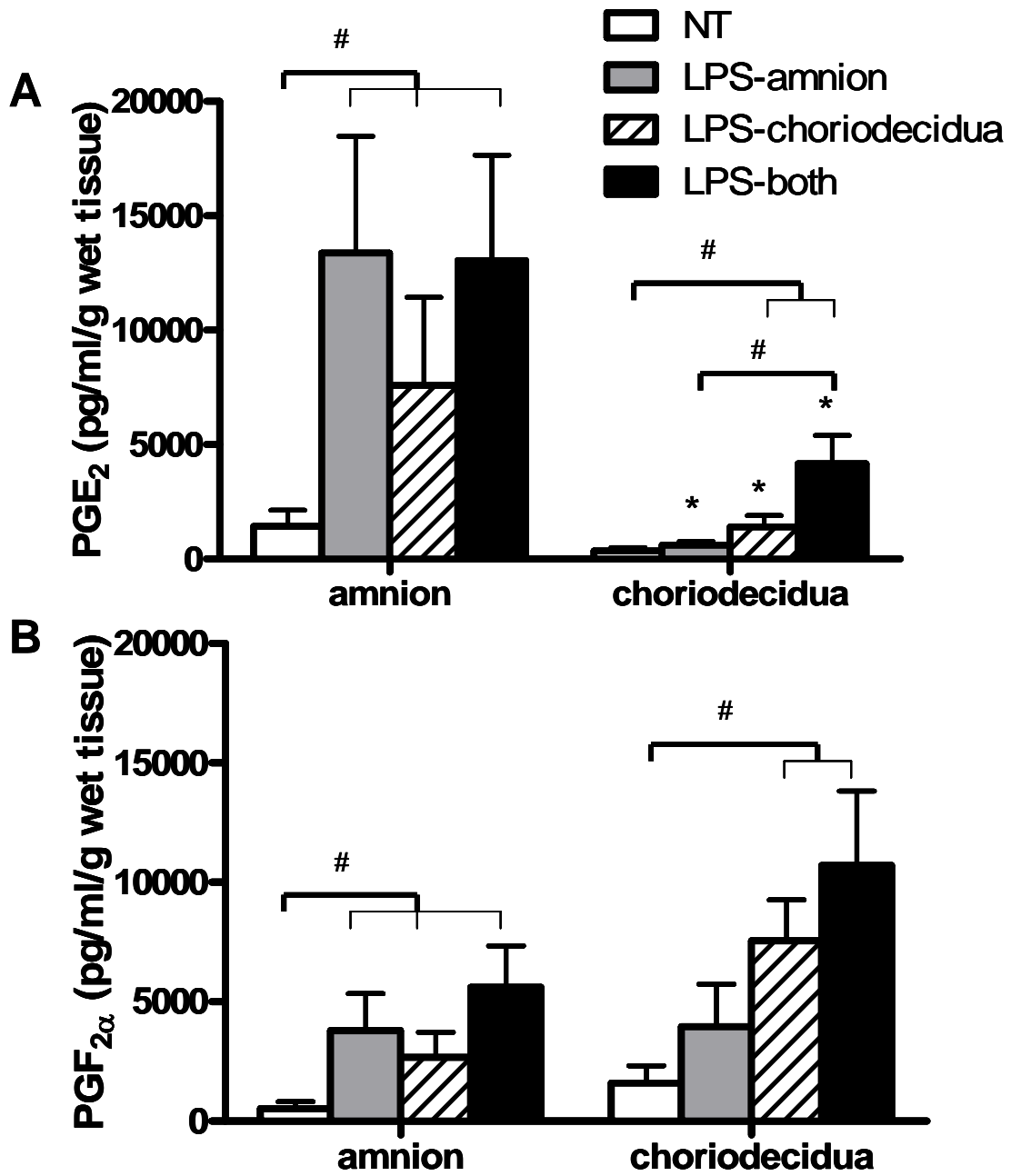
Figure 3.3. Side-specific stimulation of PGE$_2$ and PGF$_{2\alpha}$ release from human full-thickness gestational membranes following an 8-h side-specific LPS exposure (100 ng/ml). A) PGE$_2$ concentrations in culture medium. B) PGF$_{2\alpha}$ concentrations in culture medium. The x-axis shows the side of the gestational membranes assayed. The legend indicates the side of the gestational membranes exposed to LPS or not-treated (NT; controls). Values are expressed as mean ± SEM (n=6). [#]Significantly different from treatments or controls indicated. *Significantly different from amnion of the same treatment (p< 0.05).

Figure 3.4. Prostaglandin endoperoxide H synthase-2 (PGHS-2) immunostaining in gestational membrane sections following side-specific LPS exposure. There is no evidence of PGHS-2 in non-treated control samples (A, B). In tissues treated with LPS on the amnion side only, amnion & chorion mesenchymal cells stain positive (brown) for PGHS-2 (C), but not decidual cells (D). In tissues treated with LPS on the choriodecidual side only, decidual cells stain positive for PGHS-2 (F), but not amnion or chorion cells (E). In tissues treated with LPS on both sides of the membrane, positive PGHS-2 staining is seen in amnion and chorion mesenchymal cells (G) and decidual cells (H). Arrows indicate IL-6 staining (brown).
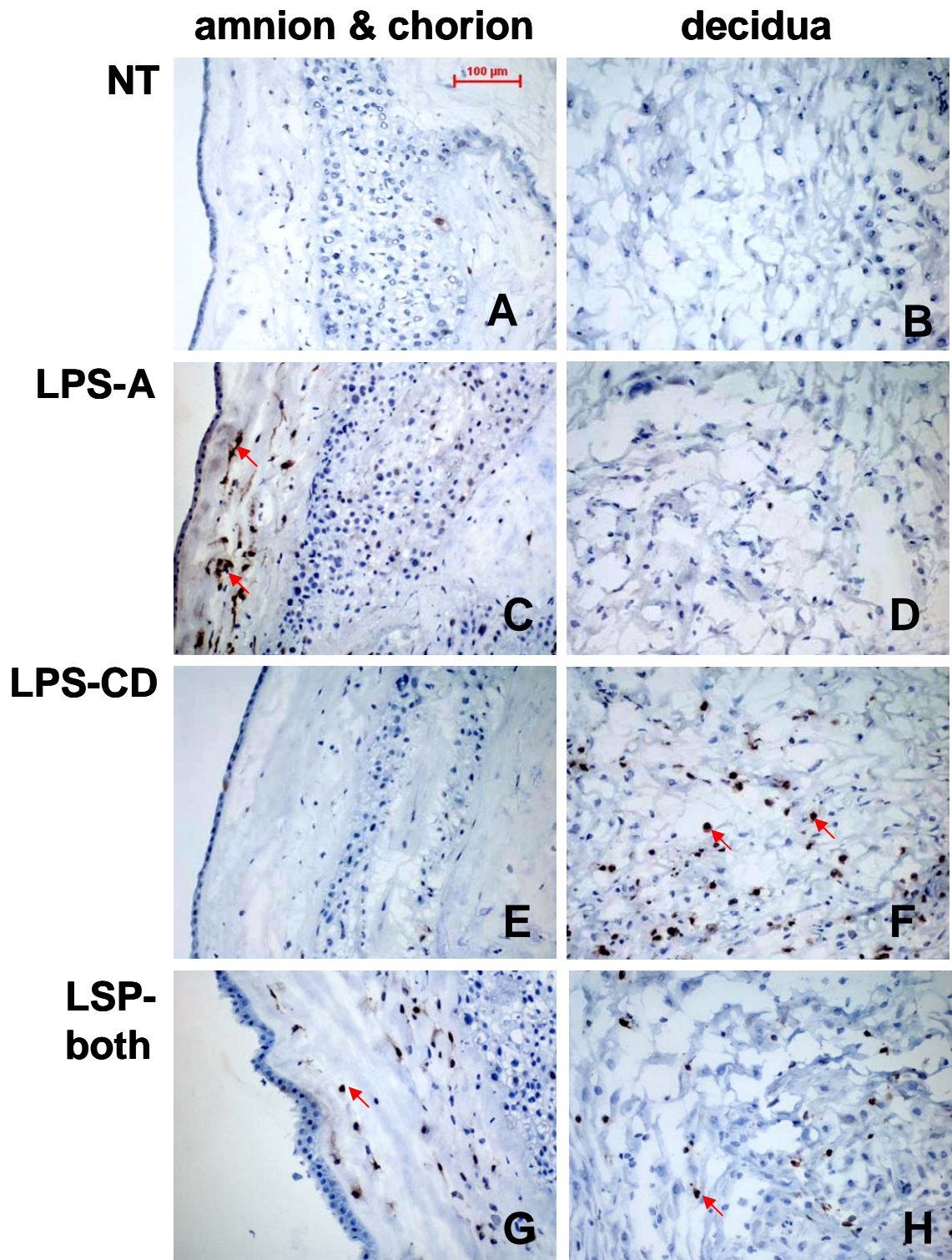
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CHAPTER 4

LIPOPOLYSACCHARIDE STIMULATES SIDE-SPECIFIC RELEASE OF INFLAMMATORY CYTOKINES IN HUMAN GESTATIONAL MEMBRANES

ABSTRACT

The objective of this study is to utilize lipopolysaccharide (LPS) as a model inflammatory agent to study the side-specific dynamics of cytokine responses in non-laboring gestational membranes. Non-labored gestational membranes were collected from healthy women undergoing elective Cesarean section deliveries at 37-39 weeks gestation. Intact gestational membranes comprised of amnion, chorion and decidua were cultured on Transwell frames to create distinct chambers for measuring cytokines secreted into the amnion and choriodecidual culture media. Concentration dependent release of tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), interleukin-4 (IL-4), interluekin-6 (IL-6), interluekin-8 (IL-8), interleukin-10 (IL-10) and transforming growth factor-β (TGF-β) were measured in Transwell culture medium after an 8-h exposure to LPS on both sides, over concentrations from 1-1000 ng/ml LPS. Side-specific cytokine responses were measured following stimulation with 100 ng/ml LPS on the amnion side only, the choriodecidual side only or both sides of the membranes. Distinct patterns of response were seen for individual cytokines. In particular, TNF-α, IL-1β and IL-6 concentrations were significantly increased on both the amnion and choriodecidual side of the membranes after an 8-h LPS exposure. In contrast, IL-8 and IL-10 were increased only on the choriodecidual side of the membranes. LPS stimulation did not affect

gestational membrane secretion of IL-4 or TGF-β. The results provide evidence for inflammatory signaling across the gestational membranes. The ex vivo side-specific cytokine responses to LPS seen here are consistent with the tissue specificity of cytokine production reported in vivo under term and preterm laboring conditions.

INTRODUCTION

Although cytokines have traditionally been thought to be immune cell messengers, understanding of their functions has expanded in recent years. Cytokines have roles in placental hormone production, prostaglandin production, cervical ripening and membrane remodeling and rupture (Bowen, Chamley et al. 2002). Interleukin-6, IL-8 and TNF-α are detected in cervico-vaginal fluids in the third trimester of pregnancy before the onset of labor and increase after the onset of labor. In amniotic fluid, IL-1β, IL-6, IL-8 and TNF-α concentrations are elevated after the onset of labor. Additionally, IL-1β, IL-6, IL-8 and TNF-α expression are increased in cells or tissues from gestational membranes after the onset of labor, although reports are inconsistent (reviewed in (Bowen, Chamley et al. 2002).

Because cytokine patterns are similar in preterm and term birth, it has been suggested that pathologic activation of the inflammatory pathway may be a mechanism of preterm birth due to preterm premature rupture of the membranes or spontaneous preterm contractions. The objective of the present study was to use LPS as a model inflammatory agent to study the side-specific dynamics of TNF-α, IL-1β, Il-4, IL-6, IL-8, IL-10 and TGF-β responses in non-laboring gestational membranes.

MATERIALS AND METHODS

Chemicals, Reagents and Antibodies

Tissue culture reagents including high glucose Dulbecco's Modified Eagle's Medium (DMEM) with no phenol red, penicillin/streptomycin antibiotic, and heat-inactivated fetal bovine serum were purchased from Invitrogen (Carlsbad, CA). Lipopolysaccharide (LPS) from Salmonella Typhinurium (Lot #225) was purchased from List Biological Laboratory (Campbell, CA). Transwell frames with no micropore membrane insert were a gift from Corning Corporation (Corning, NY).

Tissue Collection

Methods pertaining to human tissue were reviewed and approved by the local Institutional Review Board (IRB) prior to initiation of experiments; review and approval were updated yearly. Full-thickness gestational membranes comprised of amnion, chorion and decidua were collected at 37-39 weeks from non-laboring elective Cesarean sections following healthy pregnancies. Exclusion criteria included smoking, multi-fetal gestation, complications of pregnancy such as gestational diabetes or hypertension, use of anti-inflammatory drugs, or any other condition which would require the tissue to be sent to pathology. Within 1-30 min after delivery, full-thickness extraplacental membranes were sharp dissected from the placenta, placed in phosphate buffered saline solution and transported to the laboratory.

Tissue Culture

In the laboratory, membranes were checked for integrity under sterile conditions to ensure that the amnion and choriodecidua remained attached to one another and that the collagen compact layer between the amnion and the choriodecidua was not degraded.

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The membranes were rinsed with culture medium (DMEM supplemented with 1% heat inactivated-fetal bovine serum and 100 units penicillan/100 μg streptomycin per milliliter) to remove excess blood clots. The membranes were cut into approximately 2 x 2 cm^2 pieces and affixed by latex bands onto ethylene oxide sterilized Transwell frames (without a micropore membrane) to create two distinct chambers on either side of the mounted membranes (Zaga, Estrada-Gutierrez et al. 2004). The choriodecidual side of the tissue faced the inner chamber of the device. Extra tissue was removed with a scalpel. Each Transwell frame with attached tissue was placed in a single well of a 12-well plate with culture medium on both sides of the membranes. Mounted membranes were equilibrated in a 5% CO_2 tissue culture incubator. To maintain optimal culture conditions and health of the tissue, membranes in culture media were equilibrated for 18-24 h with a medium change after the first 2-4 h. Prior to beginning the treatment protocol, the overnight culture medium was removed and the membranes were pretreated with fresh culture medium for 1-2 h.

Treatment

Lipopolysaccharide was diluted in sterile, deionized water to make a primary stock of 100 μg/ml. Just prior to the experiment, the LPS primary stock was further diluted with culture medium (DMEM plus 1% FBS and antibiotics) to achieve the desired experimental concentration (1-1000 ng/ml, as indicated), and added to one or both chambers of the Transwell culture. In cases where only one side of the membranes was treated with LPS, the opposite side was cultured in culture medium. Mounted intact gestational membrane pieces were assigned randomly to treatment groups with three pieces of tissue included in each treatment. After the 8-h treatment, the medium was

recovered from each side of the membranes and stored at -20°C or -80°C for subsequent

enzyme immunoassay analysis of cytokines. After collection of medium, the tissue was

removed from the Transwell device and weighed.

Cytokine Enzyme-linked Immunosorbant Assays (ELISA)

Cytokine sandwich ELISAs for TNF-α, IL-1β, IL-4, IL-6, IL-8, IL-10, and TGF-

β were conducted by the University of Michigan Cellular Immunology Core Facility.

DuoSet ELISA Development Systems assay kits specific for each antigen were

performed according to the manufacturer's protocol (R & D Systems, Minneapolis, MN).

The assay buffer used was 0.2% casein in tris-buffered saline.

Cytokine concentrations (pg/ml) were divided by the wet weight of the intact

gestational membrane tissue taken after the experiment and the mean of the treatment

replicates per gram of tissue was calculated. This overall mean for each specimen/subject

was calculated and combined with results from other subjects. Mean results and standard

error of the mean were calculated from multiple subjects and graphed with *n* referring to

the number of subjects in each experiment.

Statistical Analyses

Before analysis, cytokine concentrations below the limit of detection for the assay

were given a value of 0.01 pg/ml. The cytokine enzyme immunoassay results were

analyzed by one-way or two-way analysis of variance (ANOVA) using SigmaStat v 3.5

software (Systat Software, Inc., Richmond, CA). Prior to analysis, the data were

transformed using log normal transformation to correct for non-normality. Post hoc pair-

wise comparisons of means were performed by the Student-Newman-Keuls methods. A

p-value < 0.05 was considered statistically significant.

RESULTS

Gestational membrane cytokine responses to LPS were assessed using membranes cultured on Transwell frames and exposed to 100 ng LPS/ml for 8 h. Cytokines released by the amnion side or choriodecidua side of the gestational membranes were measured in the medium of each chamber of the Transwell culture. For each cytokine, concentration-response and side-specific exposure/response experiments were performed.

TNF-α

TNF-α was not detected in the medium of control gestational membrane cultures, yet LPS strongly stimulated TNF-α release from both the amnion and choriodecidual sides of the membranes (Fig. 4.1). The LPS-stimulated TNF-α levels were at least 10,000-fold higher than those of the control cultures.

TNF-α release was not concentration-dependent when LPS was added to both sides of the gestational membranes in Transwell cultures. Instead, TNF-α release reached a plateau at low concentrations of LPS with no further significant increases at higher LPS concentrations (Fig. 4.1A). Statistically significant levels of TNF-α were measured in the amnion-side culture medium at all concentrations of LPS treatment (1-1000 ng/ml) compared with controls ($p < 0.05$). On the choriodecidual side, TNF-α concentrations from cultures treated with 10 or 100 ng/ml LPS were significantly different from controls ($p < 0.05$).

Side-specific LPS exposures revealed dynamics of TNF-α response in the gestational membranes. Regardless of whether LPS treatment was on the amnion side only, choriodecidual side only or both sides simultaneously, 100 ng/ml LPS stimulated significant release from both the amnion and the choriodecidua sides of the membranes

relative to controls (Fig. 4.1B; p<0.05). Nevertheless, the TNF-α response was higher on the LPS-treated side of the membranes. Specifically, TNF-α concentrations were higher in medium from the amnion side of the culture after LPS treatment of the amnion side compared with LPS treatment of the choriodecidual side of the membranes (Fig. 4.1B, left side; p < 0.05). Likewise, LPS treatment of the choriodecidua side of the membranes elicited a greater response from the choriodecidua side of the membranes compared with the amnion side of the membranes (Fig. 4.1B, right side; p<0.05). In addition, more TNF-α was secreted into the amnion culture medium when only the amnion side was treated compared to when only the chorion side was treated (p<0.05). The same pattern occurred for TNF-α concentrations in the medium from the choriodecidua side, with more TNF-α detected in the choriodecidua culture medium when only the choriodecidua side was treated compared to when only the amnion side was treated (p<0.05). These side-specific exposure/response results indicate that TNF-α or a TNF-α releasing signal passed from one side of the membranes to the other.

IL-1β

Gestational membranes released minimal IL-1β under control culture conditions and a modest amount of IL-1β in response to LPS stimulation (Fig. 4.2). The IL-1β concentrations after treatment with LPS on both the amnion and choriodecidual sides of the membranes did not vary in a concentration-dependent manner (Fig. 4.2A). There was a significant increase of IL-1β in amnion-side medium with 10 ng/ml LPS treatment and a significant increase in IL-1β in choriodecidual-side medium with 10 and 100 ng/ml LPS treatment (Fig. 4.2A; p<0.05). No statistically significant IL-1β responses were detected side-specific exposure to 100 ng/ml LPS (Fig. 4.2B).

IL-6

IL-6 release was not concentration-dependent when LPS was added to both sides of the gestational membranes in Transwell cultures. The increased amounts of IL-6 released into the medium were statistically significant compared with controls only for the choriodecidual side after treatment on both sides of the membranes with 10 ng/ml LPS (Fig. 4.3A; $p < 0.05$,).

In the side-specific exposure experiments, 100 ng/ml LPS, stimulated increased IL-6 release from the amnion side of the membranes with LPS treatment to the amnion side only or to both the amnion and choriodecidua sides ($p < 0.05$, relative to controls), but not when LPS treatment was restricted to the choriodecidual side only (Fig. 4.3B, left side). Measurements of the choriodecidual IL-6 response indicated that LPS stimulated significantly more IL-6 release than control conditions, regardless of whether the LPS was added to the amnion side only, choriodecidua side only, or both sides simultaneously (Fig. 4.3B, right side; $p < 0.05$). Thus, LPS treatment of the amnion side only increased choriodecidual IL-6 response, whereas LPS treatment of the choriodecidual side only did not increase the IL-6 response on the amnion side of the membranes.

IL-8

Both the amnion and choriodecidua sides of the gestational membranes released substantial amounts of IL-8 in the Transwell cultures under control conditions (Fig. 4.4). However, only the choriodecidua responded to LPS treatment with increased production of IL-8. Exposure to 10 or 100 ng/ml LPS on both sides of the membranes significantly increased IL-8 production from the choriodecidual side compared to the same exposure concentrations on the amnion side (Fig. 4.4A; $p < 0.05$). With side-specific exposures,

LPS treatment (100 ng/ml) on the choriodecidual side of the membranes or both sides of the membranes caused increased IL-8 release from the choriodecidual side of the membranes compared to the amnion side of the membranes exposed to the same LPS concentration (Fig. 4.4B; $p < 0.05$).

IL-10

Treatment with 100 ng/ml LPS failed to stimulate significant release of IL-10 from the amnion side of the membranes (Fig. 4.5). In contrast, there was a significant increase in IL-10 release on the choriodecidual side compared to controls, regardless of whether the gestational membranes were treated with LPS on the amnion, choriodecidual or both sides of the membranes ($p<0.05$). When both sides of the membranes were treated with LPS, the choriodecidual response was approximately 10-fold higher than control levels. In addition, the IL-10 concentrations were significantly higher on the choriodecidual side compared to the amnion side for cultures treated with LPS on the choriodecidual side only or on both the amnion and choriodecidual sides (comparing right and left sides of Fig. 4.5; $p<0.05$). These data indicate that the choriodecidua is the dominant producer of IL-10 in human gestational membranes in this system.

TGF-β and IL-4

No statistically significant changes were observed for TGF-β or IL-4 release by the amnion or choriodecidual side of the membranes in response to treatment with 100 ng/ml LPS (data not shown).

DISCUSSION

In this study, gestational membranes were cultured on Transwell frames enabling measurement of side-specific cytokine secretion after LPS treatment. Tumor necrosis

factor-α, IL-1β and IL-6 concentrations were significantly increased on both the amnion and choriodecidual sides of the membranes after an 8-h LPS exposure. In contrast, IL-8 and IL-10 were increased only on the choriodecidual side of the membranes. LPS stimulation did not affect secretion of IL-4 or TGF-β. This is the first report of LPS-stimulated side-specific release of IL-4 IL-6, IL-8, IL-10, and TGF-β from gestational membranes.

TNF-α responded dramatically to LPS treatment. Both amnion and choriodecidual sides of the membrane exhibited robust TNF-α responses to LPS treatment in the range of 10,000 to 20,000 times the control levels. When LPS was applied to only one side of the membrane, a TNF-α response was seen on the opposite side. Although the opposite-side responses were attenuated compared to same-side responses, these results indicated the passage of an inflammatory signal or the cytokine itself from one side of the membrane to the other. In a similar experiment, Zaga et al. reported that the choriodecidual TNF-α response predominated over the amnion response when membranes were treated in a side-specific manner with 500 ng/ml LPS for 24 h, though no statistics substantiated this claim (Zaga, Estrada-Gutierrez et al. 2004). Research by the same group reported a TNF-α response to side-specific *Escherichia coli* infection only when both sides of the membranes were treated (Zaga-Clavellina, Garcia-Lopez et al. 2007).

Increases in TNF-α in the amniotic fluid are associated with the onset of labor at term (Bowen, Chamley et al. 2002) and before 37 weeks gestation (Figueroa, Garry et al. 2005) . Evidence exists for TNF-α as a causal mediator of term and preterm birth. It is known to stimulate prostaglandin $F_{2\alpha}$ production from isolated term decidua cells

(Norwitz, Lopez Bernal et al. 1992). Intra-amniotic injection of TNF-α into pregnant rhesus monkeys caused onset of preterm uterine contractions, as well as elevations of other amniotic fluid cytokines, prostaglandins and matrix metalloproteinase 9 (Sadowsky, Adams et al. 2006).

In the present experiment, the IL-1β response was modest with low overall concentrations measured in response to LPS treatments. Because of the low concentrations in the sample, we were not able to obtain statistical significance with our sample size ($n = 5$ subjects) in the side-specific experiments. In contrast, Zaga et al. (Zaga, Estrada-Gutierrez et al. 2004) saw a robust IL-1β response on both the amnion and choriodecidua sides of the membranes when tissues were treated with 500 ng/ml LPS for 24 h. Differences between our results and those of Zaga et al. (Zaga, Estrada-Gutierrez et al. 2004) may be due to different LPS concentrations or lengths of exposure used in the studies. When membranes in Transwell culture were infected with *E. coli* for 24 h, the primary response was from the choriodecidual side (Zaga-Clavellina, Garcia-Lopez et al. 2007).

Concentrations of IL-1β measured in the Transwell culture system may be low due to inability of leukocytes to accumulate in gestational tissues in response to inflammatory stimulus. Evidence exists that IL-1β is a potent activator of uterine contraction, because intra-amniotic infusions of pregnant rhesus monkeys causes powerful preterm contractions (Sadowsky, Adams et al. 2006).

High levels of IL-6 were secreted from both sides of the gestational membranes under LPS-stimulated or control conditions. Lipopolysaccharide-stimulated IL-6 concentrations were approximately 4-fold higher than non-stimulated levels. In side-

specific exposure experiments, increased IL-6 was always detected on the side of the membrane exposed to the stimulus. In addition, some IL-6 was measured on the choriodecidual side of the membrane when only the amnion side was treated. The latter finding suggests that transfer of IL-6 or an inflammatory mediator occurs from the amnion to the choriodecidua. Similar IL-6 secretion patterns are seen in response to side-specific *E. coli* exposure (Zaga-Clavellina, Garcia-Lopez et al. 2007). Production of IL-6 under basal conditions reported here conflicts with a previous report by Dudley et al. (1996) that showed little IL-6 mRNA is detected in non-laboring membranes (Dudley, Collmer et al. 1996). Secretion of IL-6 in ex vivo culture may be a result of an inflammatory reaction to tissue preparation procedures or culture conditions.

High levels of IL-6 are measured in the amniotic fluid during term and preterm parturition, and are associated with neonatal morbidities such as bronchopulmonary dysplasia and cerebral palsy (Hagberg, Mallard et al. 2005). However, IL-6 may be a marker of inflammation rather than a causal agent for preterm birth, because intra-amniotic infusions into pregnant rhesus monkeys fail to elicit preterm labor or birth (Sadowsky, Adams et al. 2006).

The IL-8 response was striking in that LPS stimulated no excess IL-8 from the amnion side of the membranes. The side-specific nature of the IL-8 response may indicate a role in fighting off ascending infection. Though amnion makes IL-8 under basal conditions, no additional IL-8 was released by the amnion in response to LPS stimulation. Intra-amniotic infusion of IL-8 to pregnant rhesus monkeys does not cause preterm labor (Sadowsky, Adams et al. 2006). It is possible that IL-8 response and effects are significant only on the choriodecidual side of the membrane.

Similar to IL-6, basal secretion of IL-8 was high on both sides of the membranes. LPS-stimulated concentrations were at most 2-fold higher than control concentrations on the choriodecidual side of the membranes only. The absence of LPS-stimulated IL-8 release on the amniotic side of the membranes is in contrast to earlier reports showing LPS increases IL-8 secretion from isolated amnion cells as well as from decidual cells (Makhlouf and Simhan 2006). Transwell cultures treated with *E. coli* had lower basal secretions of IL-8 compared to our system, with responses to treatment seen on both sides of the membranes (Zaga-Clavellina, Garcia-Lopez et al. 2007).

The choriodecidua exhibited a marked IL-10 response to LPS exposures. When both sides were treated with LPS, IL-10 concentrations were approximately 10-fold higher than control levels. Like IL-8, there was no IL-10 response to LPS stimulation on the amnion side of the membranes. However, there was very little amnion-side secretion of IL-10 in general, suggesting that the amnion side may have limited capacity for producing and/or secreting IL-10.

The anti-inflammatory properties of IL-10 could limit the inflammatory cytokine responses seen here and the prostaglandin responses reported in Chapter 3. IL-10 null mutant mice were more susceptible to LPS-induced fetal loss (a model for preterm delivery) than wild-type mice. Exogenous IL-10 protected both IL-10 knockout mice and wild-type mice from fetal loss, suggesting that up-regulation of IL-10 and subsequent anti-inflammatory effects are important for preventing preterm labor due to inflammatory stimuli (Robertson, Skinner et al. 2006).

The side-specific cytokine responses seen with ex vivo tissue exposure to LPS are consistent with tissue specificity of cytokine production observed in gestational

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membranes from term and preterm labor. We detected LPS-stimulated increase of TNF-α, IL-1β and IL-6 in medium from both sides of the membrane culture. These results correspond with detection of TNF-α, IL-1β and IL-6 in amniotic fluid from cases of preterm and term labor (Bowen, Chamley et al. 2002). Similarities seen here may be due to inherent abilities in the amnion to initiate an inflammatory response. Likewise, IL-1β, IL-6 and IL-8 are detected in cervico-vaginal fluids in cases of spontaneous term parturition and preterm labor (Bowen, Chamley et al. 2002). We measured these cytokines in medium from the choriodecidual sides of the membrane (in addition to TNF-α and IL-10), indicating that the LPS-stimulated choriodecidual response of the ex vivo gestational membranes is similar to in vivo laboring conditions.

Inflammatory cytokine responses are elevated in preterm birth cases associated with infection. In vivo, intrauterine infection is characterized by influx of immune cells to the uterus. Therefore, the origin of cytokines in amniotic fluid or gestational tissues in cases of preterm birth may be due to expression from mobilized immune cells. Here we see cytokine responses from tissues cultured ex vivo that are isolated from a systemic immune response, suggesting that membranes themselves, rather than infiltrating immune cells, are a source of inflammatory cytokines.

In summary, utilization of the Transwell tissue culture system allowed characterization of distinct patterns of cytokine release from human gestational membranes. Although IL-1β, IL-6, IL-8 and TNF-α are acute phase cytokines, each responds differently to LPS stimulation. Interleukin-6 and IL-8 have high basal levels of secretion from non-laboring term gestational membranes and modest response to LPS stimulation, though no additional IL-8 is made on the amnion side. Interleukin-1β and

TNF-α had low levels of basal secretion, but only TNF-α responded significantly to stimulus. Although IL-4, IL-10 and TGF-β are anti-inflammatory cytokines that are important for controlling response to stimulus, only IL-10 was made in response to LPS. Together, these results point to a complicated network of intra-gestational membrane signaling and response to an inflammatory stimulus. More work is needed to clarify the causal or associative roles each cytokine plays in the inflammatory response in these tissues leading to preterm birth.
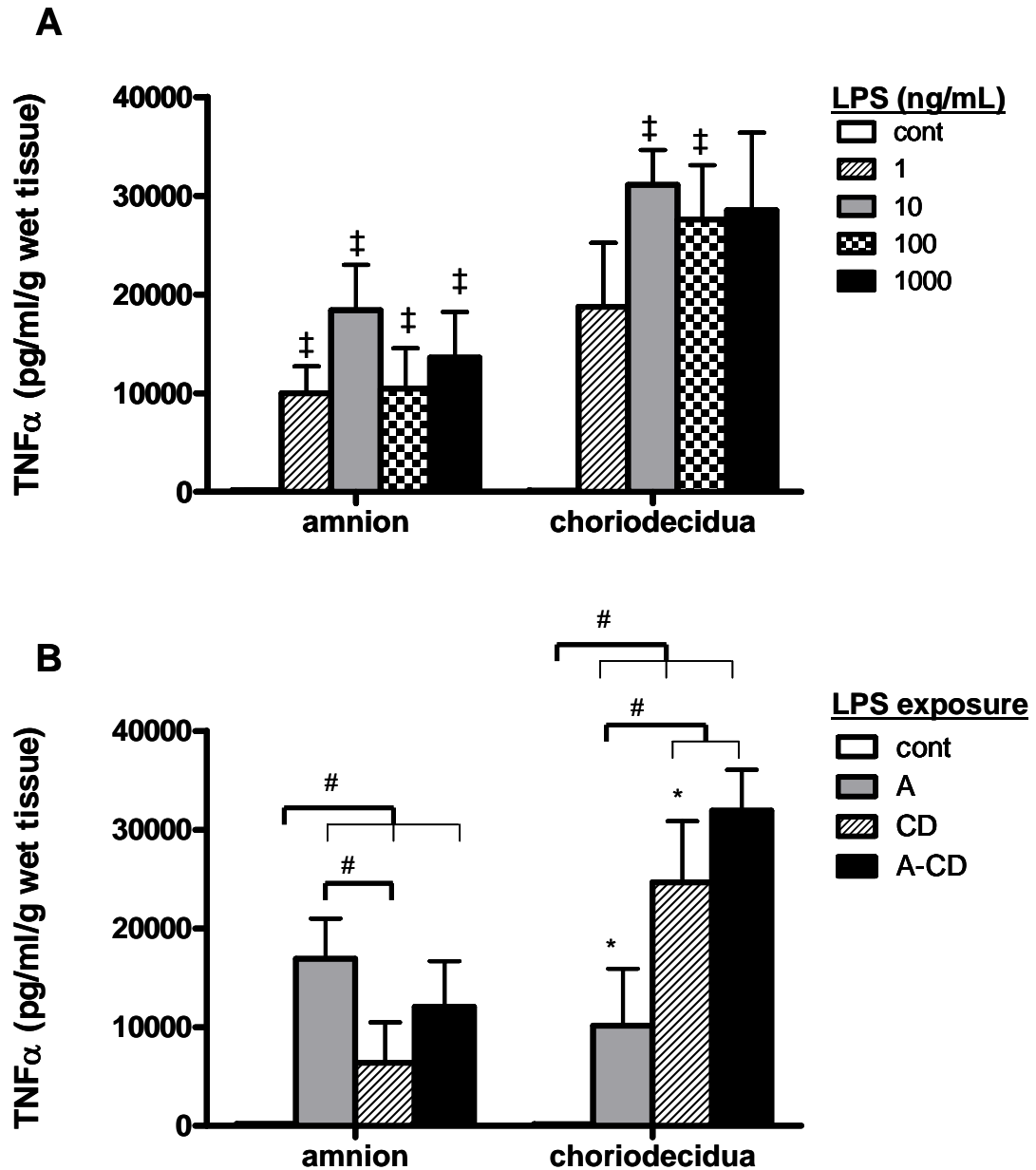
Figure 4.1. **Side-specific LPS-stimulated release of TNF-α from intact human gestational membranes in Transwell cultures.** TNF-α concentrations were measured in medium from the amnion or choriodecidual side of the Transwell cultures. A) Concentration-response of TNF-α release after an 8-h exposure to 1, 10, 100 or 1000 ng/ml LPS (n = 7 subjectss). B) TNF-α release after an 8-h exposure to 100 ng/ml LPS on the amnion side only (A), choriodecidua side only (CD) or both sides of the membranes (A-CD) (n = 5 subjects). Controls (cont) were exposed to culture medium only. The very low means of controls are nearly contiguous with the x-axis. Values are expressed as mean ± SEM. ‡Significantly different from controls (cont) within tissue (p < 0.05). #Significantly different from designated treatment groups within tissue (p < 0.05). *Significantly different from amnion values of the same treatment (p < 0.05).
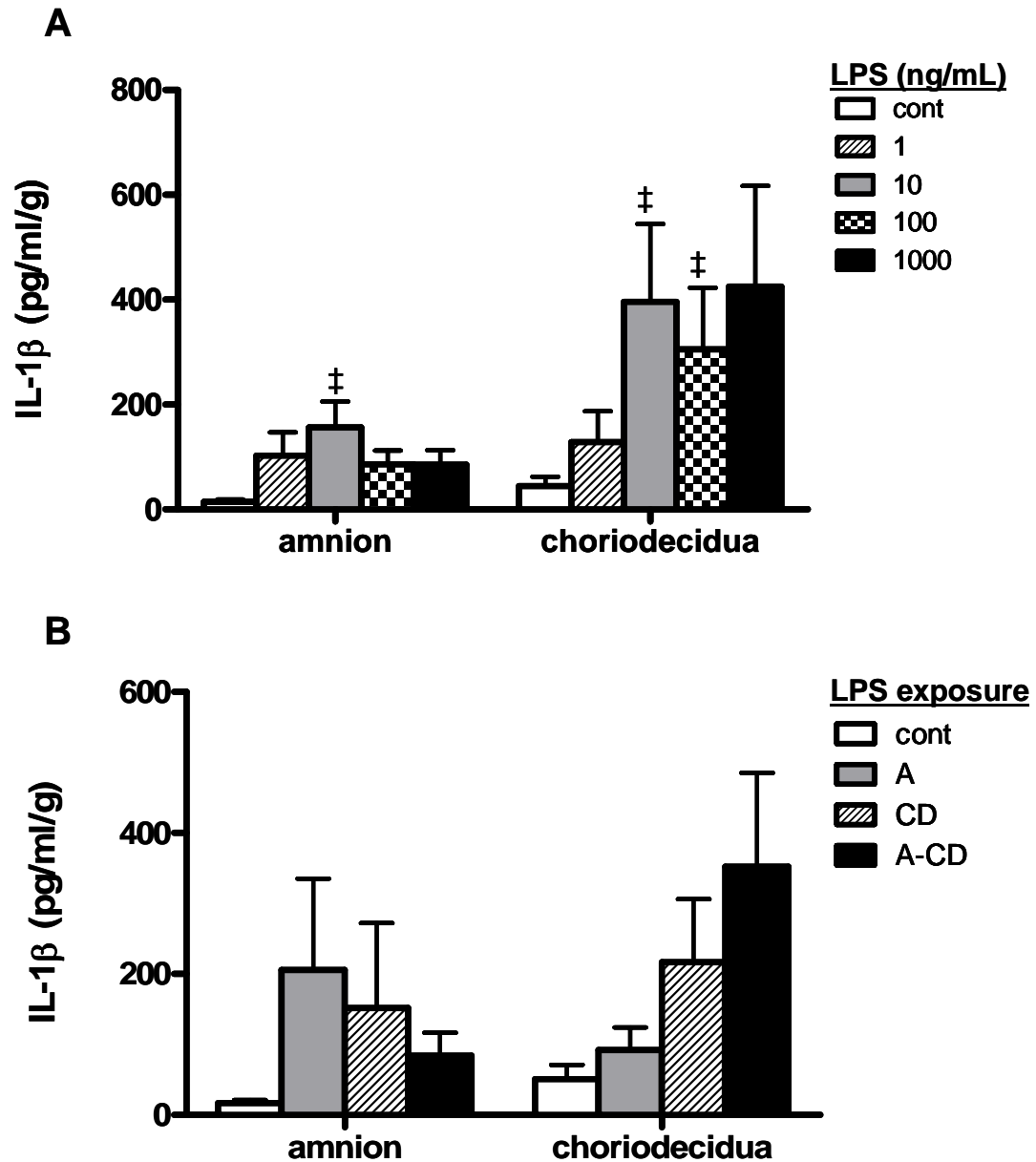
Figure 4.2. **Side-specific LPS-stimulated release of IL-1β from intact human gestational membranes in Transwell cultures.** Medium from the amnion or choriodecidual side of the Transwell cultures was sampled and IL-1β concentrations were analyzed by ELISA. A) Concentration-response of IL-1β release from membranes exposed for 8 h to 1, 10, 100, or 1000 ng/ml LPS ($n = 7$ subjects). B) IL-1β release from membranes exposed to LPS (100 ng/ml) on the amnion side only (A), choriodecidua side only (CD) or both sides of the membranes (A-CD) for 8 h ($n = 5$ subjects). Control cultures (cont) were exposed to culture medium only. Values are expressed as mean ± SEM. ‡Significantly different from control (cont) within tissue ($p < 0.05$).

Figure 4.3. **Side-specific LPS-stimulated release of IL-6 from intact human gestational membranes in Transwell cultures.** Medium from the amnion or choriodecidual side of the Transwell cultures was sampled and IL-6 concentrations were analyzed by ELISA. A) Concentration-response of IL-6 release from membranes exposed for 8 h to 1, 10, 100, or 1000 ng/ml LPS ($n = 7$ subjects). B) IL-6 release from membranes exposed to LPS (100 ng/ml) on the amnion side only (A), choriodecidua side only (CD) or both sides of the membranes (A-CD) for 8 h ($n = 5$ subjects). Control cultures (cont) were exposed to culture medium only. Values are expressed as mean ± SEM. ‡Significantly different from control (cont) within tissue ($p < 0.05$). #Significantly different from designated treatment groups within tissue ($p < 0.05$).
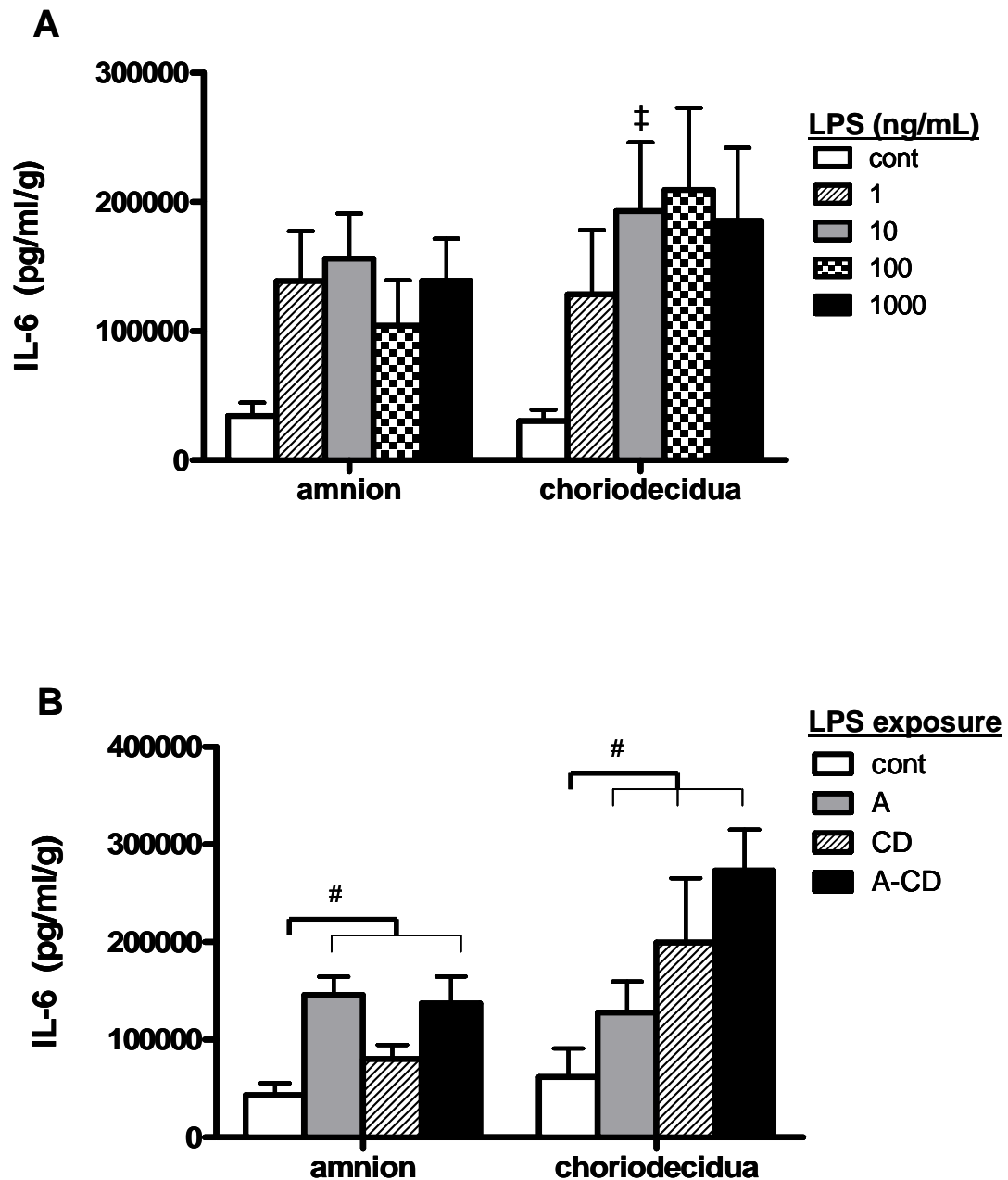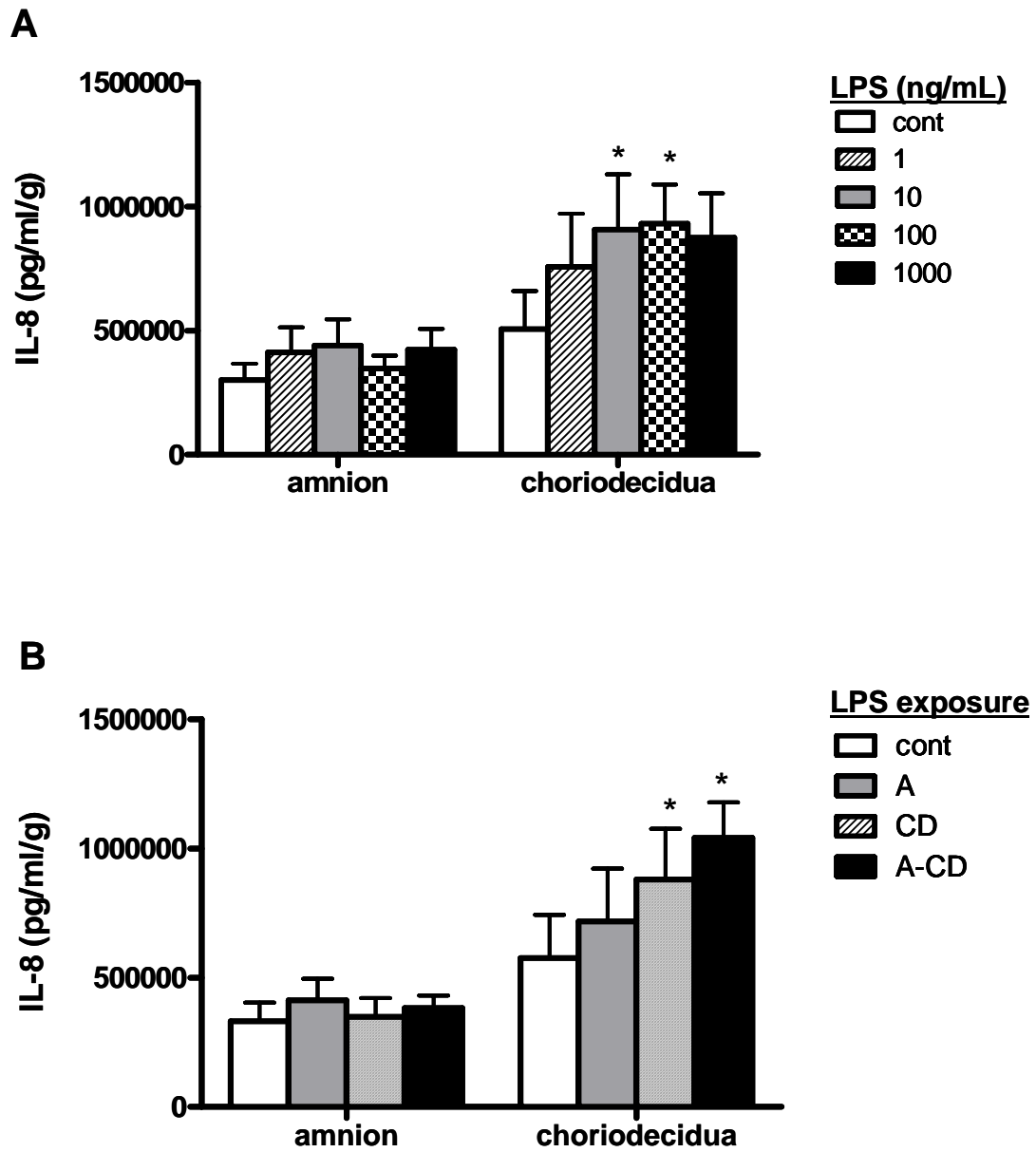
Figure 4.4. **Side-specific LPS-stimulated release of IL-8 from intact human gestational membranes in Transwell cultures.** Medium from the amnion or choriodecidual side of the Transwell cultures was sampled and IL-8 concentrations were analyzed by ELISA. A) Concentration-response of IL-8 release from membranes exposed for 8 h to 1, 10, 100, or 1000 ng/ml LPS ($n = 7$ subjects). B) IL-8 release from membranes exposed to LPS (100 ng/ml) on the amnion side only (A), choriodecidua side only (CD) or both sides of the membranes (A-CD) for 8 h ($n = 5$ subjects). Control cultures (cont) were exposed to culture medium only. Values are expressed as mean ± SEM. *Significantly different from amnion values of the same treatment ($p < 0.05$).
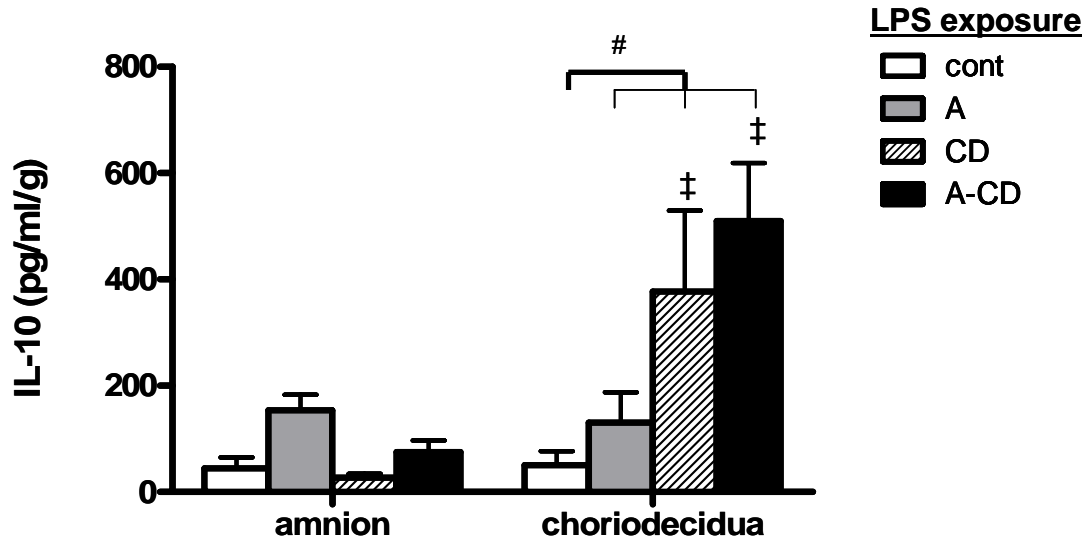
Figure 4.5. **Side-specific LPS-stimulated release of IL-10 from intact human gestational membranes in Transwell cultures.** Medium from the amnion or choriodecidual side of the Transwell cultures was sampled and IL-10 concentrations were analyzed by ELISA. IL-10 release was measured from membranes exposed for 8 h to LPS (100 ng/ml) on the amnion side only (A), choriodecidua side only (CD) or both sides of the membranes (A-CD) (n = 5 subjects). Control cultures (cont) were exposed to culture medium only. Values are expressed as mean ± SEM. ‡Significantly different from control (cont) within tissue ($p < 0.05$). #Significantly different from designated treatment groups within tissue ($p < 0.05$).

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CHAPTER 5

PROSTAGLANDIN SYNTHESIS AND CATABOLISM IN GESTATIONAL TISSUES IS NOT AFFECTED BY PCB 153 STIMULATION

ABSTRACT

Previous research has established that the environmental toxicants, polychlorinated biphenyls (PCBs) are present within the uterus during pregnancy. 2,4,5,2',4',5-hexachlorinated biphenyl (PCB 153) is a predominate congener found in the environment and induces prostaglandin endoperoxide H synthase-2 (PGHS-2), a rate-limiting enzyme in prostaglandin synthesis, and interleukin-6, (IL-6) an inflammatory cytokine. Increased prostaglandin and cytokine expression in gestational membranes are associated with the onset of term and preterm parturition. The immediacy of PCBs to sites of utero-placental prostaglandin synthesis, metabolism and action creates the potential for toxicant disruption of the concentration, distribution and activity of prostaglandins and other inflammatory mediators with implications for the disruption of parturition. Placentas and intact gestational membranes comprised of fused amnion, chorion and decidua were collected at 37-39 weeks from non-laboring elective Cesarean sections following healthy pregnancies. Placental cells were cultured from the villous placenta and intact gestational membrane tissues were cultured on Transwell frames. The effects of PCB 153 on prostaglandin synthesis in gestational membranes were evaluated because preliminary experiments in villous placental trophoblast cells indicated that PCB 153 inhibited the conversion of exogenous $PGF_{2\alpha}$ to a metabolite, suggesting an effect on

15-hydroxy prostaglandin dehydrogenase (PGDH) activity. However, after 1, 4 or 8 h of PCB treatment no effect on PGE_2, $PGF_{2\alpha}$ or IL-6 secretion from either side of gestational membranes was detected. To determine whether PCB 153 caused sensitization to lipopolysaccharide (LPS)-induced inflammation, gestational membranes from one subject were exposed to PCB 153 for 18 h followed by LPS and PCB 153 for 8 h. However, no significant increase in prostaglandin release was seen under these exposure conditions compared to controls. Experiments described here indicate the absence of an effect of PCB 153 on gestational membrane prostaglandin production.

INTRODUCTION

Polychlorinated biphenyls (PCBs), are persistent organic toxicants that are ubiquitously present in the soil, groundwater and sediment, and consequently bioaccumulate in the food chain (Patandin, Koopman-Esseboom et al. 1998). Body burdens above typical background levels are due to occupational exposures or consumption of specific food products such as Great Lakes sport fish (Stewart, Darvill et al. 1999). In humans these toxicants have long half-lives (~10 years) because they are poorly metabolized and lipophilic (Jacobson, Fein et al. 1984).

Previous research has established that PCBs are present in the placenta and uterus during pregnancy. In one study, the extracted lipids of the uterine muscle, placenta and amniotic fluid had relatively high levels of PCBs compared to maternal serum, with the highest levels being in the lipids of the amniotic fluid (Polishuk, Wassermann et al. 1977). A more contemporary study of women exposed to background levels of environmental pollutants found one in three amniotic fluid samples contained detectable levels PCBs (Foster, Chan et al. 2000). The immediacy of these toxicants to sites of

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uteroplacental prostaglandin synthesis, metabolism and action creates the potential for toxicant disruption of the concentration, distribution and activity of prostaglandins.

Known actions of PCBs suggest plausible potential mechanisms for disruption of prostaglandins during pregnancy. 2,4,5,2',4',5-Hexachlorinated biphenyl (PCB 153) (Fig. 5.1) is a predominate coplanar congener found in the environment (Dabrowska, Fisher et al. 2006). A coplanar PCB mixture that includes PCB 153 induces expression of prostaglandin endoperoxide H synthase-2 (PGHS-2) mRNA and reduces expression of interleukin-1β (IL-1β) mRNA in rabbit pre-implantation embryos (Kietz and Fischer 2003). In human mast cells, PCB 153 induces PGHS-2 and interleukin-6 mRNA by a nuclear factor-κB (NF-κB)-dependent mechanism (Kwon, Lee et al. 2002). Because PGHS-2, IL-1β, IL-6 and NF-κB are important mediators of prostaglandin production in human gestational membranes (Lindstrom and Bennett 2005), their modulation by PCBs suggests a mechanism for toxicant disruption of prostaglandin metabolism and catabolism that may be relevant to parturition.

Human gestational membranes are available from healthy, term human pregnancies. Non-labored membranes from cesarean deliveries were obtained and cultured in a Transwell tissue culture as described in Chapter 2. The objective of the present work was to determine whether PCB 153 affected PGDH activity in gestational tissues and to characterize gestational membrane prostaglandin secretion in response to PCB 153.

MATERIALS AND METHODS

Chemicals, Reagents and Antibodies

Tissue culture reagents including high glucose Dulbecco's Modified Eagle's Medium (DMEM) without phenol red, penicillin/streptomycin antibiotic, and heat-inactivated fetal bovine serum were purchased from Invitrogen (Carlsbad, CA). Lipopolysaccharide (LPS) from Salmonella Typhinurium (Lot #225) was purchased from List Biological Laboratory (Campbell, CA) and 2,2',4,4',5,5'-hexachlorobiphenyl (PCB 153) was purchased from AccuStandard, Inc. (New Haven, CT). Prostaglandin E_2 and $PGF_{2\alpha}$ enzyme immunoassay kits were purchased from Cayman Chemical Co. (Ann Arbor, MI). Transwell frames with no micropore membrane insert were a gift from Corning Corporation (Corning, NY).

Tissue Acquisition

Methods pertaining to human tissue were reviewed and approved by the local Institutional Review Board prior to initiation of experiments; review and approval were updated yearly. Intact gestational membranes comprised of fused amnion, chorion and decidua were collected at 37-39 weeks from non-laboring elective Cesarean sections following healthy pregnancies. Exclusion criteria included smoking, multi-fetal gestation, complications of pregnancy such as gestational diabetes or hypertension, use of anti-inflammatory drugs, or any other condition which would require the tissue to be sent to pathology. Within 1-60 min after delivery, full-thickness extraplacental membranes were sharp dissected from the placenta, placed in phosphate buffered saline solution and transported to the laboratory. Alternatively, the entire placenta and attached membranes were transported to the laboratory.

Tissue Culture

In the laboratory, under sterile conditions, membranes were checked for integrity to ensure that amnion and choriodecidua remained attached to one another and that the collagen compact layer between the amnion and the choriodecidua was not degraded. The membranes were rinsed with culture medium (DMEM supplemented with 1% heat inactivated-fetal bovine serum and 100 units penicillan/100 μg streptomycin per milliliter) to remove excess blood clots. The membranes were cut into approximately 2 x 2 cm^2 pieces and affixed by latex rubber bands onto ethylene oxide sterilized Transwell frames (without a micropore membrane) to create two distinct chambers on either side of the mounted membranes (Zaga, Estrada-Gutierrez et al. 2004). The choriodecidual side of the tissue faced the inner chamber of the device. Extra tissue was removed with a scalpel. Transwell frames with attached tissue were placed in 12-well plates with culture medium on both sides of the membranes. Mounted membranes were cultured in a 5% CO_2 tissue culture incubator. To maintain optimal culture conditions and health of the tissue, the medium was changed after the first equilibration period of 2-4 h and then tissues were left overnight. Prior to beginning the treatment protocol, the overnight culture medium was removed and the membranes were pretreated with fresh culture medium for 1-2 h.

Placental and Chorionic Cell Isolation and Cell Culture

Placental cells were cultured from the villous placenta and chorion. Briefly, fresh placentas were collected from the University of Michigan hospitals following non-laboring cesarean sections. Villous placenta and chorion were dissected and subjected to digestion at 37°C by a trypsin/DNase or trypsin/DNase/collagenase solution,

respectively. The digestion solution was strained through a 200 μm nylon membrane and placental cells were recovered by centrifugation. Cells were purified on a 5-70% continuous Percoll gradient, washed, and plated on 96-well plates, 24-well plates (1 million cells per well) or in 10 cm^2 dishes (10 million cells) in media (DMEM) supplemented with 10% fetal bovine serum and 100 units penicillin-100 μg streptomycin/ml. Cells were washed, and medium was replenished on day one of culture. Experiments were conducted on day three of culture.

Treatment

The PCB 153 was dissolved in dimethyl sulfoxide (DMSO) to achieve a primary stock concentration of 10 mM. The PCB 153 stock solution was diluted with culture medium at the time of the experiment to achieve the experimental concentration (10 μM). The positive control, LPS, was diluted in sterile, deionized water to make a primary stock of 100 μg/ml. Just prior to the experiment, the LPS primary stock was further diluted with culture medium to achieve the experimental concentration (100 ng/ml). To initiate exposure, medium was removed and fresh medium with PCB, LPS (positive control) or DMSO (solvent control) was added to both amnion and choriodecidual chambers of the culture dish. Mounted intact gestational membrane pieces were assigned randomly to treatment groups with three pieces of tissue included in each treatment.   Membranes were incubated with the treatments for 1, 4, 8 or 24 h. After the specified treatment time, treatment medium was recovered from each side of the membrane and stored at -20°C or -80°C for subsequent enzyme immunoassay analysis of secreted PGE_2 and $PGF_{2\alpha}$. The tissue was removed from the frame and weighed.

Enzyme Immunoassay

Prostaglandin E_2 and $PGF_{2\alpha}$ in the culture medium were assayed by enzyme immunoassay according to the manufacturer's protocol (Cayman Chemical Co., Ann Arbor, MI). Prior to the assay, samples were thawed and diluted to concentrations that fell within the range of the standard curve. Each sample was assayed in duplicate. Prostaglandin concentrations were calculated using the analysis spreadsheet provided by the manufacturer. Mean prostaglandin concentrations were divided by the wet weight of gestational membrane tissue pieces and the mean of the treatment replicates was calculated. Mean results and standard error of the mean were calculated from multiple specimens and graphed.

Statistical Analyses

The prostaglandin enzyme immunoassay results were analyzed by one-way or two-way analysis of variance (ANOVA) using Sigma Stat v 3.5 software (Systat Software, Inc., Richmond, CA). Prior to analysis, the data were transformed using log normal transformation to correct for non-normality. Post hoc pair-wise comparisons of means were performed by the Student-Newman-Keuls methods. A p-value < 0.05 was considered statistically significant.

RESULTS

Villous Trophoblast PGDH Activity Assay

Trophoblast cells were cultured from placental villous tissue collected from healthy, term Cesarean section deliveries. After isolation and equilibration in culture, cells were treated for 12 h with PCB 153, DMSO (solvent control) or TNF-α (positive control). Additional unexposed cultures served as non-treated controls (NT). Exposure

medium was removed from the cells and fresh medium containing exogenous $PGF_{2\alpha}$ and indomethacin, a PGHS-2 inhibitor, was added. To assess PGDH activity, $PGF_{2\alpha}$ conversion media was recovered after 2 h and was analyzed for the presence of 13, 14-dihydro-15 keto $PGF_{2\alpha}$ metabolite (PGFM) by enzyme immunoassay. PGFM was significantly decreased in cells treated for 12 hours with 50 µM PCB 153 or TNF-α compared to non-treated control and solvent control levels (Fig. 5.2). Data presented here are from one cell isolation from one placenta. Three wells of cells were exposed for each treatment or control condition.

PCB 153 effects on PGE_2 and $PGF_{2\alpha}$ secretion

Because isolated cell experiments indicated an effect of PCB 153 on prostaglandin catabolism by PGDH, intact gestational membrane experiments were conducted to investigate a tissue response. Intact gestational membrane pieces were mounted and cultured on Transwell devices allowing side-specific measurement of prostaglandins. Membranes were treated with 10 µM of PCB 153 for 1, 4, or 8 h and PGE_2 or PGF_{2a} was measured in amnion and choriodecidual culture medium.

After 1, 4 or 8 h of PCB treatment, there was no effect of PCB 153 on PGE_2 (Fig. 5.3) or $PGF_{2\alpha}$ secretion (Fig. 5.4) from either side of the gestational membranes. Limited experiments with higher concentrations (25 µM or 50 µM) or longer exposure times (24 h) showed no effect (data not shown).

PCB 153/LPS co-exposure experiment

Although PCB 153 exposure showed no effect on prostaglandin secretion from gestational membranes, I hypothesized that PCB 153 might sensitize tissue to other inflammatory stimuli. To investigate this hypothesis gestational membrane tissue was

treated with 25 or 50 uM PCB for 18 h followed by co-exposure with 25 or 50 uM PCB and10 or 100 pg/ml LPS stimulation for 8 h. No significant effects were seen on PGE_2 secretion by the amnion or choriodecidual side of gestational membranes under these exposure conditions.

DISCUSSION

Preliminary experiments indicated that PCB 153 inhibited the conversion of exogenous $PGF_{2\alpha}$ to PGFM, suggesting an effect on PGDH in villous placental trophoblast cells. However, cytotoxicity was not assessed in these experiments. Because death of the cells would result in failure to convert $PGF_{2\alpha}$ to PGFM, a possible explanation for the results may be overt cytotoxicity of PCB 153 on trophoblast cells.

Because the isolation procedure for villous placental and chorion trophoblast cells was time-consuming, the yields were low and culture purity was unpredictable. In subsequent experiments, intact gestational membrane Transwell cultures were used as the experimental model. I hypothesized that PCB 153 would induce PGHS-2 and inhibit PGDH causing an overall increase of gestational membrane prostaglandin release. Because PGDH is primarily located in the chorion trophoblast cells, I expected that the increased prostaglandin release would be especially high on the choriodecidual side of the gestational membranes. However, at PCB 153 concentrations up to 50 µM and exposures as long as 24 h, no effect was seen on prostaglandin release. Preliminary immunohistochemical analysis of immunoreactive PGDH in post-exposure gestational membrane cryosections indicated that PGDH protein levels were drastically reduced in PCB 153-exposed membranes (data not shown). However, these results were not reproducible. It remains possible that the individual specimens were differentially

sensitized to PCB 153, but I am unable to determine the cause of the decrease in PGDH seen with that specimen.

Limited data on cytokine release from gestational membranes exposed to 10 µM PCB 153 for 1 or 4 h showed no increase in IL-6 secretion from amnion or choriodecidual sides of gestational membranes (data not shown).

To determine whether PCB 153 caused sensitization of gestational membranes to LPS-induced inflammation, a co-exposure experiment was conducted on samples from one specimen. However, no significant prostaglandin release was seen under these exposure conditions. Because the experiment was only conducted on one specimen, it remains possible that this exposure scenario is still valid. The particular specimen on that experimental day may have been a "non-responder" to LPS-induced inflammation. In addition, LPS concentrations below a recognizable effect level were chosen, but experiments may need to be conducted with higher LPS concentrations to completely disprove the hypothesis.

The absence of an effect on prostaglandin production by PCB 153 may be because of experimental conditions. Choosing an appropriate concentration for polychlorinated biphenyl exposures is difficult. Reports indicate that the intracellular or intratissue lipophilic toxicant concentrations vary from the medium concentration based on length of exposure, volume of medium and mass of the cells or tissue (Mundy, Freudenrich et al. 2004). Based on the increased mass of the tissue relative to a corresponding cellular culture, similar medium concentrations between the two may result in quite disparate intracellular concentrations. In addition, PCBs accumulate in biological material because of their lipophilic nature, and have half-lives measured on the

order of years. Therefore, comparison of acute high concentrations in tissue culture to chronic low concentrations seen in vivo may result in erroneous conclusions.
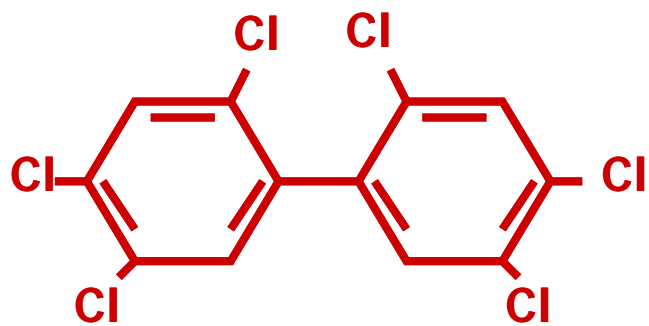
Figure 5.1. Chemical structure of 2,2',4,4',5,5'-hexachlorobiphenyl (PCB 153). The substitution of chlorine atoms in the 2 and 2' position prevents the two phenyl rings of PCB 153 from existing in a planar conformation.
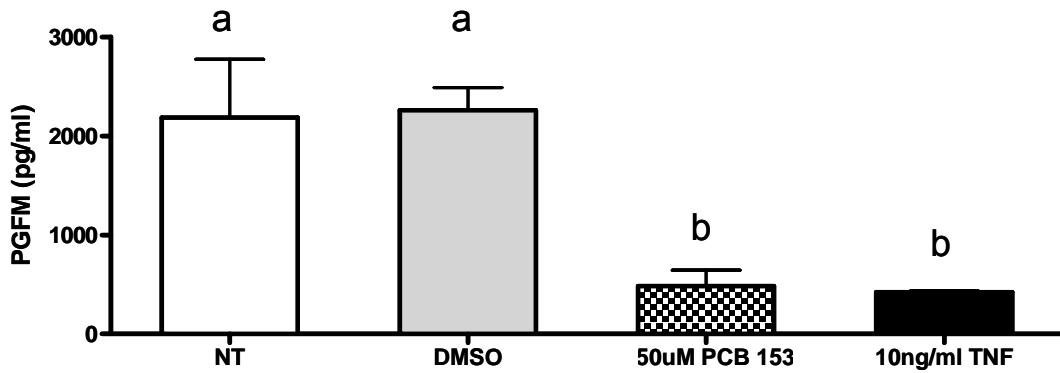
Fig. 5.2. 15-hydroxy prostaglandin dehydrogenase activity is decreased in villous placental trophoblast cells treated with 50 μM PCB 153. Isolated villous placental cells were not treated (NT, controls), or were treated with dimethyl sulfoxide (DMSO; solvent control), 50 μM PCB 153, or tumor necrosis factor-α (TNF-α; positive control) for 12 h. Following treatment, cells were incubated with exogenous $PGF_{2\alpha}$ (in the presence of indomethacin) for 2 h to measure conversion to the 13, 14-dihydro-15 keto PGF2α metabolite (PGFM), as an index of PGDH activity($n = 3$ wells).

Fig. 5.3. PCB 153-dependent stimulation of PGE$_2$ release from amnion or choriodecidual sides of intact human gestational membranes.   Prostaglandin E$_2$ (PGE$_2$) was measured in medium from Transwell gestational membrane cultures following exposure to 50 μM PCB 153 compared to non-treated controls (NT), solvent controls exposed to dimethyl sulfoxide (DMSO), or positive controls exposed to LPS . (A) 1-hour exposure, (B) 4-hour exposure, (C) 8-hour exposure. Values are expressed as mean ± SEM (n= 4 specimens).
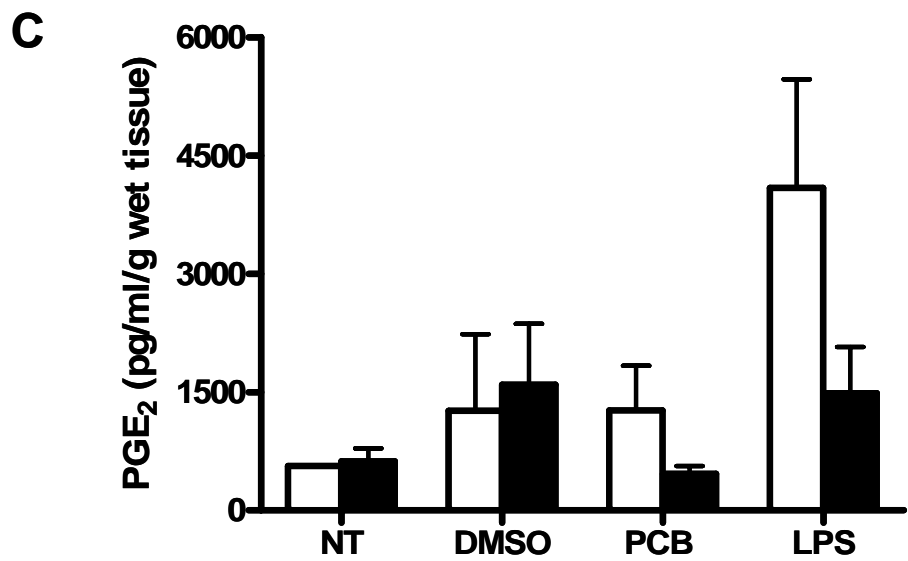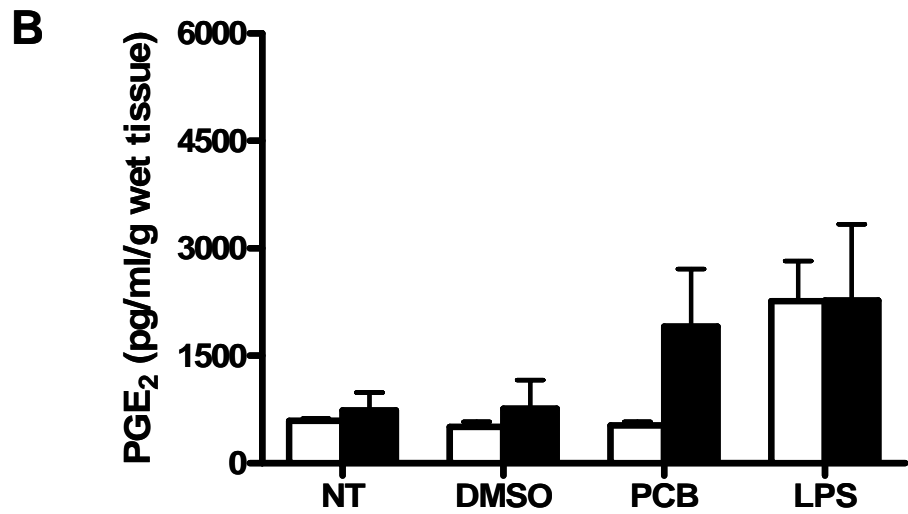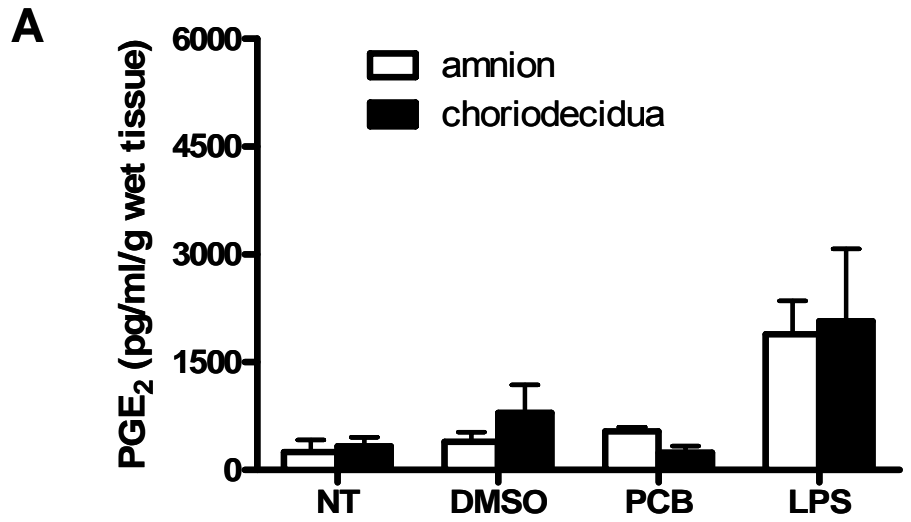
Fig. 5.4. PCB 153-dependent stimulation of PGF$_{2\alpha}$ release from amnion or choriodecidual side of intact human gestational membranes. Prostaglandin F$_{2\alpha}$ (PGF$_{2\alpha}$) was measured in medium from Transwell gestational membrane cultures following exposure to 50 µM PCB 153 compared to non-treated controls (NT), solvent controls exposed to dimethyl sulfoxide (DMSO), or positive controls exposed to LPS. A) 1-hour exposure ($n = 2$), B) 4-hour exposure ($n = 4$), C) 8-hour exposure ($n = 3$). Values are expressed as mean ± SEM.
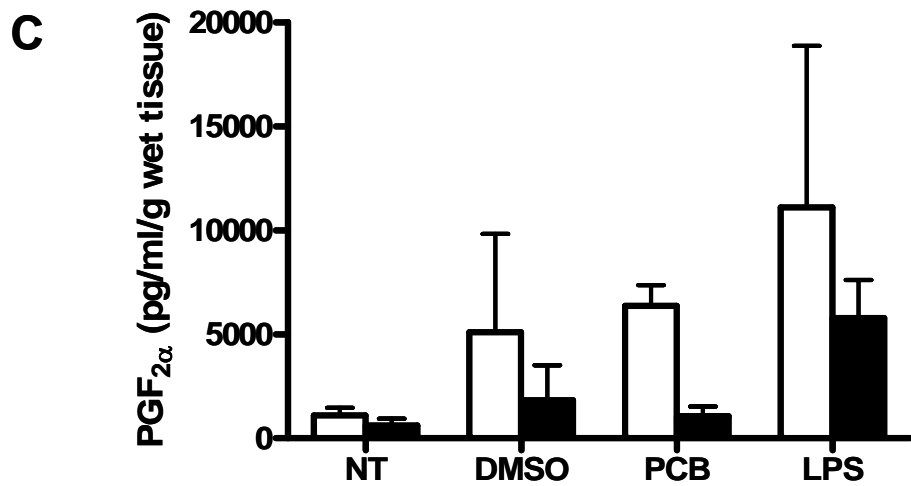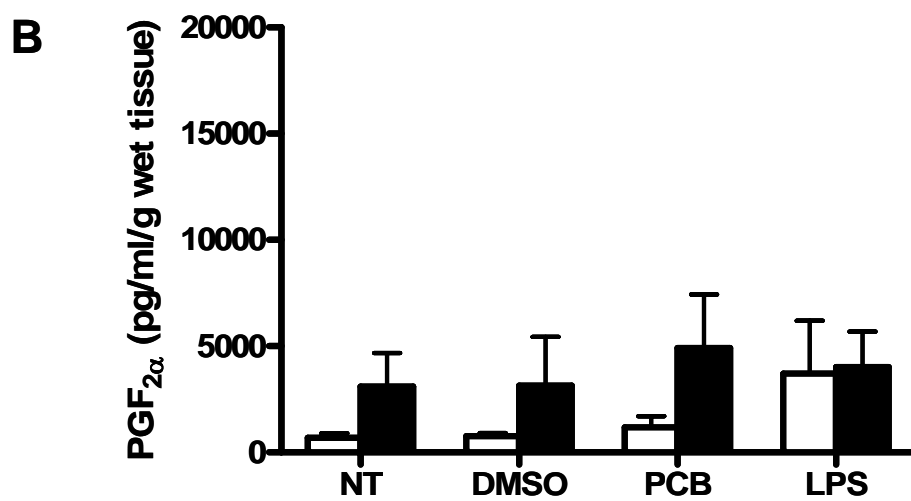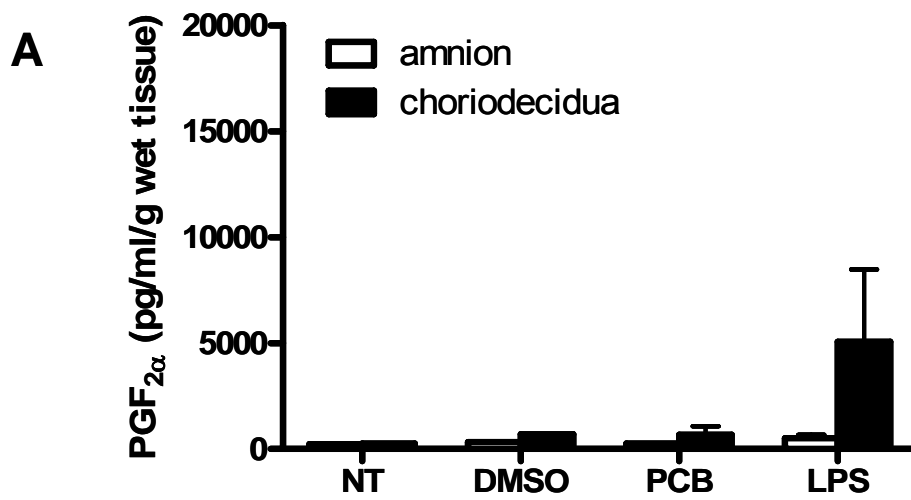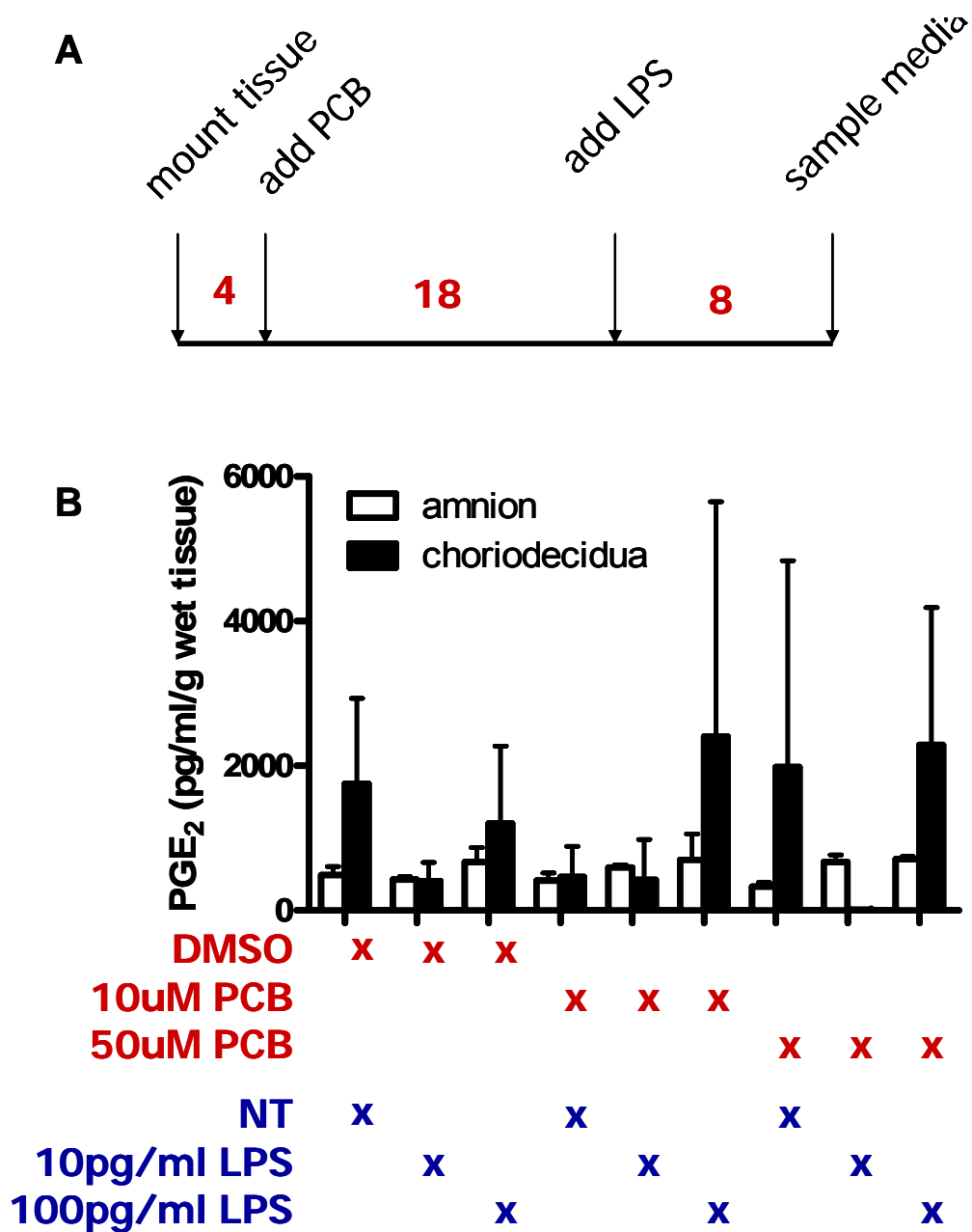
Figure 5.5. Gestational membrane PCB 153/LPS co-exposure experiment. A) Experimental design of PCB153/LPS co-exposure experiment. Intact gestational membrane tissue was mounted on Transwell frames and allowed to equilibrate in tissue culture for 4 h. Medium was removed and replaced with fresh medium containing PCB 153 or dimethyl sulfoxide (DMSO; solvent control) for 18 h. PCB 153 medium was removed and replaced with medium containing PCB 153 and LPS for 8 h. B) PGE_2 concentrations in amnion or choriodecidual medium from intact gestational membranes treated with PCB 153 and DMSO. A red x indicates PCB treatment or control (DMSO) for each tissue and a blue x indicates LPS treatment or control (NT) for each tissue.

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CHAPTER 6

DISCUSSION

The extra-placental gestational membranes may be important for signaling the onset of preterm or term parturition. Secretion of inflammatory cytokines and prostaglandins from these tissues may directly or indirectly promote myometrial contractions, cervical effacement and dilation, or rupture of the membranes (Olson 2003).

I optimized a novel system to culture intact gestational membranes from human pregnancies for the purpose of investigating side-specific prostaglandin and cytokine production caused by inflammatory stimuli (Chapter 2). No animal model completely reflects the events preceding and during human parturition. Furthermore, cell culture systems fail to incorporate important cell-to-cell and tissue-to-tissue signaling that is critical for understanding responses to inflammatory stimuli in this tissue. As such, the human gestational membrane in vitro system described in this dissertation is a valuable investigative tool for research into mechanisms of parturition.

One of the most difficult aspects of this system is the variability seen in response to experimental inflammatory stimulation. Between-subject variability may be because of sample acquisition from subjects with diverse genetic backgrounds and environmental exposures, or differences in gestational age. Within-subject variability may be related to sample location within the specimen. Although both within- and between-subject variability may be valid reflections of physiological differences caused by the above-

mentioned factors, it compromises the ability to detect differences related to experimental treatments. Therefore, improvements in specimen collection, sampling, culture methodology and assay analysis were completed in hopes of reducing extraneous variability. The objective was to be able to detect meaningful and statistically significant responses to experimental stimulus with a feasible number of gestational membrane specimens per experiment.

Prostaglandins stimulate myometrial contraction, and it is likely that prostaglandins produced by the gestational membranes, especially those in the nearby choriodecidua, promote parturition (Olson 2003). The dynamics of prostaglandin production and secretion from gestational membranes are important because preterm pathological activation of this pathway may cause preterm birth (Zakar and Hertelendy 2001). Differentiating the tissue and cellular sources of prostaglandins released in response to LPS stimulation in non-laboring gestational membranes may help clarify the role of prostaglandins in infection- and inflammation-associated preterm parturition.

A limitation of the current system is that it uses only term gestational membranes. Because preterm responses to inflammatory stimuli may be different from term responses, extrapolation of the findings for preterm birth should be made with caution. For the purposes of optimizing the system and defining the overarching phenomena, term membranes sufficed. However, it would be entirely possible and desirable for future research to include preterm membranes. IRB procedures for such experiments would be more complex, requiring informed consent from the subjects and coordination with the Pathology Department.

Results from Chapter 3 show side-specific secretion of prostaglandin E_2 (PGE_2) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), and indicate that amnion and choriodecidua respond independently to LPS. Additionally, there is evidence for passage of the inflammatory signal across the membranes, because prostaglandin release was seen on the amnion side when only the choriodecidual side was stimulated. Gestational membranes are many cell layers thick. This anatomical structure of the membranes, combined with the inability of prostaglandins to diffuse passively across cellular membranes because of their anionic nature at physiologic pH, implies that a single prostaglandin molecule most likely can not traversing the entire gestational membrane. We suggest, therefore, that transmittance of the inflammatory signal may be propagated across the tissues by a series of cytokine and prostaglandin "relays" whereby a signal is handed off from cell to cell.

Although interleukin-1β (IL-1β), interleukin-6 (IL-6), interleukin-8 (IL-8) and tumor necrosis factor-α (TNF-α) are all part of the acute inflammatory response, investigation of gestational membrane cytokine dynamics in Chapter 4 revealed distinctive cytokine secretion patterns in response to LPS stimulation. The divergence of response patterns indicates that these cytokines each have unique regulatory mechanisms and specific functions within the gestational membrane tissue. The subtle interplay among acute inflammatory cytokines is beginning to be revealed by this research. Cytokines are known modulators of prostaglandins (Bowen, Chamley et al. 2002), and knowledge of cytokine response to LPS stimulus may clarify mechanisms of increased prostaglandin secretion in exposed gestational membranes. Further research exploring cytokine regulatory mechanisms and effector functions would illuminate the roles these proteins play in gestational membrane prostaglandin regulation.

Exposure of the amnion side of the gestational membranes to LPS caused

significant levels of IL-6, TNF-α, PGE$_2$ and PGF$_{2\alpha}$ to be released by the amnion side of

the membranes into the medium (data compiled from chapters 3 and 4 in Fig. 6.1).

Lipopolysaccharide exposure of the amnion also caused IL-6, TNF-α and interleukin-10

(IL-10) to be released by the choriodecidual side by an as-yet determined mechanism

(thus, the wavy lines). It is possible that IL-6 and TNF-α traverse the membranes to be

secreted on the opposite side. However, LPS treatment of the amnion side increased IL-

10 on the choriodecidual side but not the amnion side, indicating that the inflammatory

signal propagates across the tissue. This may occur by passage of a different cytokine

such as TNF-α or IL-6 or by a theoretical "relay" type mechanism. Potential future

experiments could investigate the mechanisms of cytokine release from gestational

membranes, including study of the passage of radiolabeled cytokines such as IL-6 or

TNF-α across the membranes, analysis of cytokine mRNA or protein within the

membranes, and inhibitor studies to detect critical components of the pathway that allow

the signal to be initiated and propagated in the tissue. Considering the complexity of the

gestational membrane tissue and the relatively high number of cell membranes that a

single molecule would have to cross to get from one side of the gestational membranes to

the other, I suggest that a relay-type mechanism is most likely responsible for

propagation of the inflammatory signal from one side of the gestational membranes to the

other.

Lipopolysaccharide treatment on the choriodecidual side caused a robust

prostaglandin and cytokine response on the choriodecidual side, but only TNF-α, PGE$_2$

and PGF$_{2\alpha}$ were released in significant amounts on the amnion side (data compiled from

Chapters 3 and 4 in Fig. 6.2). The dampened response within the fetal compartment may be important for protecting the fetus from the inflammatory response. Choriodecidual-only LPS treatment simulates an ascending intrauterine infection from the vagina and may be the most clinically relevant model for considering gestational membrane prostaglandin and cytokine responses to LPS. The alternate scenario whereby the infection originates in the intra-amniotic space is thought to be relatively rare (Romero, Espinoza et al. 2007).

A comprehensive model to simulate ascending intrauterine infection was designed to incorporate prostaglandin and cytokine responses observed in medium from LPS-treated gestational membranes in response to LPS exposure on the choriodecidual side of the membranes (Fig. 6.3). I propose that PGHS-2 and TNF-α constitute the primary response to LPS and signal downstream events such as membrane rupture, cervical ripening and myometrial contractility via prostaglandin production and stimulation of additional cytokines. A by-product of the inflammatory response that spills over to the intramniotic space may be adverse fetal effects. When the inflammatory response signaled by LPS is received by the choriodecidual cells, TNF-α may cause induction of PGHS-2, the inflammatory cytokines IL-6 and IL-8, and the collagen-degrading matrix metalloproteinases (MMPs). A secondary anti-inflammatory reaction facilitated by IL-10 is proposed to down-regulate the response to control damage caused by inflammation.

Tumor necrosis factor-α is a known inducer of prostaglandin endoperoxide H synthase-2 (PGHS-2; (Perkins and Kniss 1997) and may also limit catabolism of prostaglandins by inhibiting 15-hydroxy prostaglandin dehydrogenase (PGDH; (Mitchell, Goodwin et al. 2000). Therefore, increases in TNF-α release may cause increases in

PGE_2 and $PGF_{2\alpha}$ release by inducing enzymes of the prostaglandin metabolic pathway. Correlation of TNF-α release with prostaglandin release from one or both sides of the gestational membranes may shed light on regulation of prostaglandin synthesis on the opposite side of the membranes from LPS exposure. Further research is needed to understand whether TNF-α itself or a signal for TNF-α release is transmitted across the gestational membranes after exposure to an inflammatory stimulus such as LPS. . Previously, only cytokine passage across the placenta has been studied; no reports explicitly assessed cytokine passage across gestational membranes.

The only anti-inflammatory cytokine measured in response to LPS stimulation was IL-10. Previous research shows that IL-10 inhibits PGHS-2 in preterm placenta explant cultures (Hanna, Bonifacio et al. 2006). Furthermore, IL-10 null mutant mice are more prone to preterm labor induced by LPS compared to wild-type mice (Robertson, Skinner et al. 2006). The latter reports suggest that down-regulation of PGHS-2 and subsequent decrease in prostaglandin output by IL-10 may be important for mitigating the inflammatory response and avoiding or lessening detrimental events leading preterm birth. Potential roles for IL-10 in the inflammatory prostaglandin and cytokine response in gestational membranes warrant further investigation.

Non-infectious sources of preterm inflammation leading to preterm labor are interesting to consider. Inflammatory cytokines and prostaglandins are measured in gestational tissues from preterm births not associated with infection (Romero, Espinoza et al. 2007). Potential modulators of inflammation are age of the mother and gestational age. Exaggerated inflammatory responses can be seen in mice as they age (Meador, Krzyszton et al. 2008). Researchers hypothesize that dysfunction of the IL-10 anti-

inflammatory response may explain why older mice have decreased ability to control inflammation (Meador, Krzyszton et al. 2008). Based on this, it would be interesting to research whether human gestational membrane response to inflammatory stimuli is affected by age of the mother, and whether differences in IL-10 were associated with age. Preterm amnion cells respond more robustly to inflammatory stimuli compared to term amnion cells (Jung, Yoon et al. 2005). Accounting for these non-infectious modulators of inflammation may shed light on variability of response seen from specimens collected from different women.

Inflammatory toxicants might be an important source of non-infectious inflammation and yet another mechanism for maternal age to modulate the inflammatory response. Persistent organic toxicants bioaccumulate in organisms over time because of their long half-lives. 2,4,5,2',4',5-Hexachlorinated biphenyl (PCB 153) is one such persistent organic toxicant that is reported to have inflammatory effects. Based on reports that PCB 153 caused induction of PGHS-2 and secretion of inflammatory cytokines in other cell types (Kwon, Lee et al. 2002) and that PCBs are associated with shortened gestational length in epidemiological studies (Taylor, Stelma et al. 1989), I hypothesized that PCB 153 would cause increased prostaglandin secretion by inducing PGHS-2 in gestational membranes. However, results presented in Chapter 5 indicate no effect on prostaglandin production with PCB 153 treatment concentrations up to 50 µM and exposure times between 1 and 24 h.

The absence of an effect on prostaglandin production by PCB 153 may be due to experimental conditions. Choosing an appropriate concentration for polychlorinated biphenyl exposures is difficult. Reports indicate that the intracellular or intratissue

lipophilic toxicant concentrations in experimental setups vary from the medium concentration based on length of exposure, volume of medium and mass of the cells or tissue (Mundy, Freudenrich et al. 2004; Barber, Walsh et al. 2006). Based on the increased mass of the tissue relative to a corresponding cellular culture, similar medium concentrations between the two may result in quite disparate intracellular concentrations. In addition, PCBs accumulate in biological material because of their lipophilic nature, and PCB half-lives in humans are measured on the order of years (Jacobson, Fein et al. 1984). Therefore, comparison of acute high concentrations in tissue culture to chronic low concentrations seen in vivo may result in erroneous conclusions.

Many other toxicants may cause inflammation in gestational tissues. For instance, other PCB congeners may cause inflammation and play a role in prostaglandin release from gestational membranes. Additional inflammatory toxicants to consider for research are lead (Jelliffe-Pawlowski, Miles et al. 2006), alcohol (Jaddoe, Bakker et al. 2007), air pollutants (Leem, Kaplan et al. 2006) and phthalates (Latini, Massaro et al. 2005).

In addition to acting as inflammatory agents and directly causing preterm birth, anti-inflammatory toxicants might indirectly participate in events leading to preterm birth. Toxicants that interfere with immune host defense could make it more difficult to fight off an infection. For example, an individual with a certain toxicant exposure may be particularly susceptible to intrauterine invasion of normally commensal bacteria, such as occurs in bacterial vaginosis. More work needs to be done to address the effects of immunosuppresive agents on pregnancy and parturition, including the potential for toxicants to decrease resistance to infection in gestational membranes.

Because of the increasing incidence of preterm birth and serious health consequences for mother and baby, it is important to develop research aimed at understanding pathways and signaling within gestational membranes. My research indicates cytokine and prostaglandin signaling in gestational membranes that may provide the basis for further research into causes and results of intrauterine inflammation. The gestational membrane Transwell system provides an excellent model for furthering this goal. Furthermore, the model system described herein is uniquely situated to be expanded for both epidemiologic as well as laboratory-based mechanistic research. I envision experimental designs where large numbers of specimens are collected and data analysis incorporates multiple demographic variables, as well as mechanistic studies to probe intricate details of signal transduction within the gestational membranes.
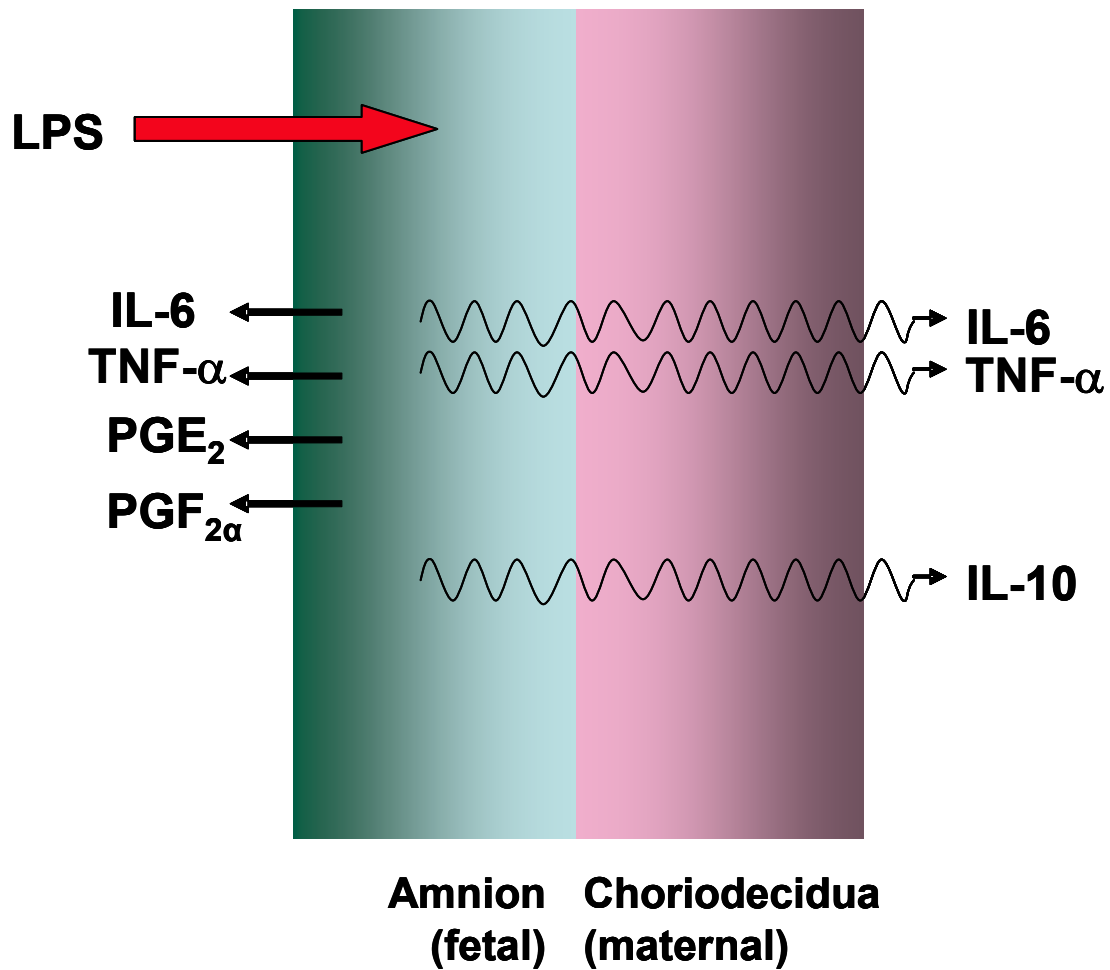
Figure 6.1. Prostaglandin and cytokine response to amnion-side lipopolysaccharide (LPS) stimulus. LPS exposure to the amnion side of gestational membranes in Transwell culture stimulated prostaglandin and cytokine release into the amnion and choriodecidual medium. Release of prostaglandins and cytokines on the amnion side may be due to direct effects in the amnion. Release of cytokines on the choriodecidual side may be due to passage of amniotic cytokines across the membranes or the inflammatory signal may be propagated in another way.
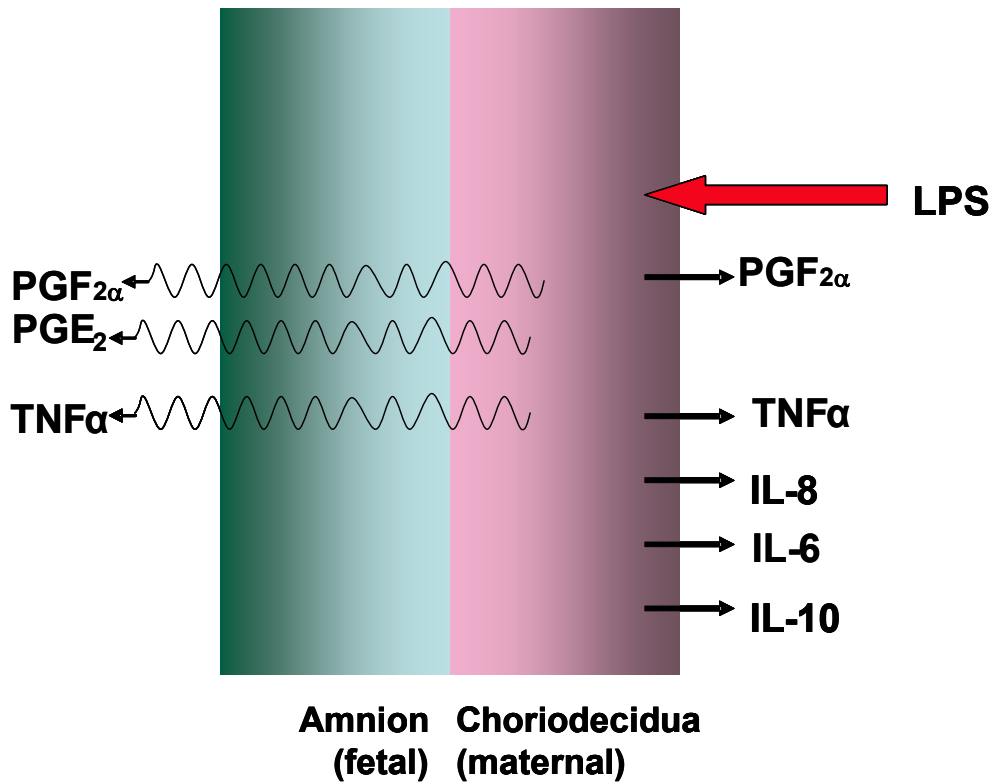
Figure 6.2. Prostaglandin and cytokine response to choriodecidual-side lipopolysaccharide (LPS) stimulus. LPS exposure to the choriodecidual side of gestational membranes in Transwell culture stimulated prostaglandin and cytokine release into the amnion and choriodecidual medium. Release of prostaglandins and cytokines on the choriodecidual side may be due to direct effects in the amnion. Release of prostaglandin and cytokines on the amnion side may be due to passage of choriodecidual cytokines (TNF-α) across the membranes or the inflammatory signal may be propagated in another way.
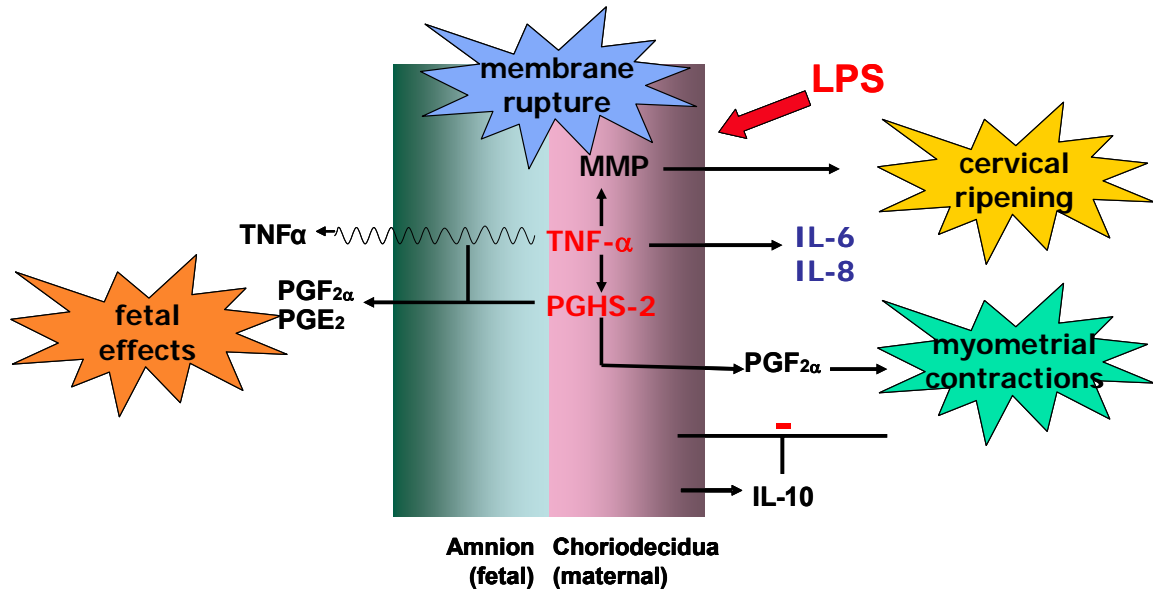
Figure 6.3. Theoretical model depicting lipopolysaccharide (LPS)-stimulated effects on cytokine and prostaglandin release in gestational membranes relating to downstream parturition-related events. Because ascending microbial infection from the vagina may be the most clinically relevant route for intrauterine infection, the model shows LPS stimulation of the choriodecidual side. In this model, the primary responses to LPS stimulation on the choriodecidual side of the gestational membranes are increases in tumor necrosis factor-α (TNF-α) and prostaglandin endoperoxide synthase-2 (PGHS-2). Induction of PGHS-2 leads to increased prostaglandin secretion (PGE$_2$ and PGF$_{2\alpha}$) from the gestational membranes. PGF$_{2\alpha}$ released from the choriodecidua causes myometrial contractions. Induction of TNF-α increases matrix metalloproteinases (MMP) that result in membrane rupture and cervical ripening. TNF-α also causes induction of other inflammatory cytokines such as interleukins-6 and 8 (IL-6 and IL-8, respectively). Secondary induction of interleukin-10 (IL-10) down-regulates the inflammatory response. Preterm delivery will depend on the ability of anti-inflammatory factors such as IL-10 to limit the severity and duration of the inflammatory response.

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