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Human decidual cell Toll-like receptor signaling in response to endotoxin: The effect of progestins

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Abstract

Objective—To determine if progesterone (P), 17- α -hydroxyprogesterone (17OHP) and 17- α hydroxyprogesterone caproate (17OHPHC) modulate the Toll-like receptor (TLR) pathway in the response of decidua to lipopolysaccharide.

Study Design—Cultured human decidual cells were incubated under control conditions, lipopolysaccharide alone, or pretreatment with each of the three progestins. Relative expression of 113 genes in the TLR pathway was determined using microarray.

Results—We failed to demonstrate a suppression of TLR gene pathway expression in human decidual cells in response to lipopolysaccharide when pretreated with progestins. Pretreatment with each progestin prior to lipopolysaccharide resulted in a relative increase in the expression of the proapoptotic molecule, CASP8. There were no differences among the progestins.

Conclusions—Our data do not support suppression of TLR pathways as a mechanism for the benefit of 17OHPHC. Increased CASP8 gene expression raises the possibility that progestins “prime” the decidual cell to respond with a NF κ B-mediated inflammatory response.

Keywords

Toll-like receptor; decidual cell; progestin; progesterone

Preterm birth is the most important problem in modern obstetrics, accounting for 12.5% of births in 2004 and accounted for approximately 17% of all infant deaths in the US in 2002. (National Center for Health Statistics, final natality data. Retrieved February 2, 2007, from www.marchofdimes.com/peristats). Women with a history of a spontaneous preterm birth in

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a previous pregnancy are at increased risk for preterm birth in subsequent pregnancy with an overall recurrence frequency of 22.5%. If the earlier preterm birth occurred prior to 32 weeks the risk of recurrence is 30–40%.^{1–3} While there are few therapeutic or prophylactic options to reduce this increased risk, cumulative evidence over the past 15 years and a recent multi-center placebo-controlled randomized trial suggested a risk reduction of approximately 30% with the use of 17- α hydroxyprogesterone caproate (17OHP).^{2, 4}

The mechanism of action of 17OHP with respect to prematurity prevention has not been elucidated. In the classic hormone-receptor mediated gene transcription pathway, 17OHP is similar to P with respect to binding to Progesterone Receptor-A or Progesterone Receptor-B or eliciting downstream gene transcription of progesterone responsive genes (Attardi et al, *Am J Ob Gyn*, in press). Likewise, binding to glucocorticoid receptor (GR) is poor, both for P and 17OHP (Attardi et al, *Am J Ob Gyn*, in press). A more detailed exploration of effects on gestational tissues, both upper and lower genital tract, is warranted to provide insight into the elusive mechanism of action of 17OHP. While functional progesterone withdrawal has been implicated in preterm parturition⁵, it is unknown whether 17OHP exerts a therapeutic effect in this regard.

Recent data demonstrated that 17OHP does not reverse or block response to oxytocin or phenylephrine stimulation in pregnant or non-pregnant myometrium.⁶ Infection and inflammation are thought to play important etiologic roles in premature delivery. A leading hypothesis for the etiology of intrauterine infection is ascent of pathogenic microorganisms from the lower genital tract. This ascending microbial invasion may lead to infection or a host inflammatory response that, ultimately, leads to preterm labor or preterm rupture of the membranes. Infection and/or inflammation of the decidua are hypothesized to occur early in the process of ascending microbial invasion leading to prematurity. Amory and coworkers recently investigated the possibility of a systemic anti-inflammatory effect of 17OHP. They, however, failed to demonstrate a reduction in Tumor Necrosis Factor- α (TNF- α) production by LPS stimulated whole blood when pretreated with 17OHP. Indeed, they observed an augmentation of the TNF- α response.⁷

The human decidual cell has potent immune function, making it an important player in microbial invasion of the upper genital tract.^{8–11} Thus, modulation of the inflammatory response of decidua may be an early therapeutic target in prematurity prevention. Toll-like receptors (TLRs) are a highly conserved system by which host recognizes pathogen and initiates an immune response.¹² Toll-like receptors are designed to recognize specific molecular patterns of pathogens and initiate a host immune response. To date, ten of these receptors have been identified in humans. Through TLRs, decidual cells can initiate the host response to foreign proteins while still alerting the immune system to bacterial invasion. We have previously demonstrated that the human decidual cell expresses functional TLR 1, 2, 4, and 6.¹³ Using a murine model of intrauterine inflammation, Elovitz and Mrinalini demonstrated that pretreatment with medroxyprogesterone acetate decreased lipopolysaccharide-induced upregulation of TLR2 and 4 mRNA in cervix and placenta.¹⁴ The purpose of this project was to characterize the human decidual cell TLR-pathway gene expression response to lipopolysaccharide, and to evaluate the modulation of that response by three progestins: progesterone (P), 17- α hydroxyprogesterone (17OHP), and 17OHP. We hypothesized that, similar to the murine model, that progestins would reduce LPS-induced TLR upregulation in human decidual cells in culture. We further hypothesized that progestins would exert an effect on expression of genes further downstream in the TLR signaling pathway.

Material and Methods

Decidual cells were isolated from the fetal membranes of three women undergoing elective cesarean delivery without labor at term. Collection of cells was performed in an anonymous fashion without any links to personal identifiers. Exemption from consent for this collection was granted by our Institutional Review Board. The decidual cell layer was delicately scraped off the chorion with a sterile glass slide and stored on ice in Earl's salt solution with 50 µg/mL penicillin/streptomycin and 2.5 µg/mL amphotericin B for no more than 30 minutes. Cells were transferred to a fresh Earl's solution without antibiotics. Next, 0.2% collagenase Type III and 20 u/ml DNase I grade II (2000 u/mg) were added and digestion was performed at 37°C for 2 hours. The digestate was filtered, then spun at 2200 rpm for 10 minutes. The pellet was resuspended in 1X PBS with 0.5% BSA. A 2 cc layer of the suspension was placed over a discontinuous Percoll (Amersham Biosciences, Piscataway, NJ) gradient and centrifuged at 1700 rpm for 30 minutes at 4°C. The cells in the 20–30% and 30–40% interface were collected and washed with PBS-BSA. The cells were re-suspended in DMEM and plated in collagen-coated 60 mm² culture plate and incubated in the 37°C incubator with 5% CO₂. Immunofluorescence labeling for prolactin and insulin-like growth factor binding protein-1 confirmed that all of the cells in 10 high-powered fields in culture were decidual cells.

After reaching confluence, cells were treated with trypsin EDTA and suspended in 24 well plates. Cells were incubated under 8 possible conditions: (1) media for 48 hours, (2) media for 24 hours followed by *E. coli* LPS (25 ng/mL) for 24 hours, (3) progesterone (500 ng/mL) for 24 hours followed by media for 24 hours, (4) 17- α hydroxyprogesterone for 24 hours followed by media for 24 hours, (5) 17- α hydroxyprogesterone caproate for 24 hours followed by media for 24 hours, (6) progesterone (500 ng/mL) for 24 hours followed by *E. coli* LPS (25 ng/mL) for 24 hours, (7) 17- α hydroxyprogesterone (500 ng/mL) for 24 hours followed by *E. coli* LPS (25 ng/mL) for 24 hours, (8) 17- α hydroxyprogesterone caproate (500 ng/mL) for 24 hours followed by *E. coli* LPS (25 ng/mL) for 24 hours. Cellular viability was confirmed under each condition using trypan blue exclusion.

Media was then removed and cells were washed with PBS. Trypsin-EDTA (1.5 mL) and media (1.5 mL) was added, mixed, and spun at 6000 rpm for 5 minutes. The cells were then washed with 10 mL of PBS. Then, 600 µL of RLT buffer was added and vortexed for 1 minute to lyse the cells, followed by the addition of 600 µL of 70% ethanol. RNA extraction was performed using a commercially-available kit (Qiagen USA, Valencia, CA).

The Oligo GEArrays® (SuperArray Bioscience, Frederick, MD) used in this project are comprised of a nylon membrane support spotted with gene-specific 60-mer oligos for genes. The oligo probes are immobilized on a positively charged, three-dimensional nylon membrane. The Oligo GEArray® Human Toll-Like Receptor Signaling Microarray profiles the expression of 113 genes related to TLR-mediated signal transduction. Table I lists the genes profiled in this microarray, categorized by functional group. This array includes members of the TLR signaling family as well as key mediators of the TLR signaling pathway including adaptors and proteins that interact with toll-like receptors. Effectors of TLR signaling are represented. Members of the NF κ B, JNK/p38, NF/IL6, and IRF signaling pathways downstream of TLR signaling are also included on this array. All samples were run in duplicate. The array images were captured with X-ray film and scanned on a desktop scanner. Array data analysis was facilitated through the use of GEArray® Expression Analysis Suite web-based software (SuperArray Bioscience, Frederick, MD). Relative gene expression of each gene in the array was compared to the housekeeping gene Glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Relative expression values from each of the three cell cultures were pooled within each experimental condition. Statistical comparison of relative gene expression between each of the eight experimental conditions was performed utilizing Analysis of Variance. In light of

the multiple hypothesis tests performed during these analyses, a conservative α of 0.001 was established *a priori* to indicate significance in posthoc testing. Statistical analyses were performed using Stata 9 (Stata Corp, College Station, TX)

Results

Compared to control cells, LPS exposed cells exhibited a significant and dramatic increase in expression of five genes: BTK (3.5-fold increase, $p=0.003$), CCL2 (43-fold increase, $p=0.002$), CSF2 (211-fold increase, $p=0.003$), CXCL10 (3144-fold increase, $p=0.0004$), IL1B (20-fold increase, $p=0.002$), and IL8 (368-fold increase, $p=0.0003$). Compared to control cells, at our *a priori* determined level of significance, we noted no difference in gene expression in the cells treated with progesterone or 17- α hydroxyprogesterone alone. Compared with control cells, cells treated with 17- α hydroxyprogesterone caproate demonstrated a statistically significant, but modest, increase in expression of five genes: BTK (1.1-fold increase, $p=0.001$), CASP8 (2.2-fold increase, $p=0.004$), MAP3K7 (2.4-fold increase, $p=0.0007$), PELI1 (1.1-fold increase, $p=0.001$), and TNFRSF1A (2.4-fold increase, $p=0.0002$). When compared to cells pretreated with media alone, pretreatment of decidual cells with progesterone, 17- α hydroxyprogesterone, or 17- α hydroxyprogesterone caproate prior to LPS treatment, resulted in a relative increase in CASP8 expression (3.4-fold increase for progesterone, $p=0.001$, 4.8-fold increase for 17- α hydroxyprogesterone caproate, $p=0.002$, and 2.4-fold increase for 17- α hydroxyprogesterone caproate, $p=0.002$). The relative expression of CASP8 between the three progestin-pretreatment groups was not significantly different ($p=0.68$). While pretreatment with all 3 progestins prior to LPS treatment resulted in increase in expression of several genes compared to control, there were no other differences in gene expression when compared with LPS treatment alone (data not shown).

Comment

In our model of cultured human decidual cells, *E. coli* derived lipopolysaccharide elicited an upregulation of transcription of a number of genes in the Toll-like receptor signaling pathway. The majority of LPS-upregulated genes in this pathway are in downstream pathways and target gene subgroup. Four of these genes (CCL2, CSF2, IL1B, and IL8) are in the nuclear factor- κ B pathway, the transcription of which produce many proinflammatory cytokines. These genes and/or gene products are all known to be elements of the inflammatory response to lipopolysaccharide.^{15, 16} The above results confirm that the human decidual cell exhibits an immunologic response to *E. coli* LPS, with the novel findings describing the specific gene expression changes that LPS elicits in this pathway.

Contrary to our hypotheses, we failed to demonstrate an inhibition of TLR gene pathway expression in human decidual cells in response to LPS when pretreated with progestins. Thus, our data do not support this as a mechanistic explanation for the therapeutic benefit of prophylactic administration of 17OHPC. Interestingly, we noted that pretreatment with progestins increased the relative expression of CASP8. The gene product of CASP8, caspase-8, is a proapoptotic protease. While caspase-mediated apoptosis is an important process during early pregnancy trophoblast development¹⁷, its role in prematurity is unclear. Recently, caspase-8 has been demonstrated to be necessary for the activation of NF κ B in response to TLR signaling.¹⁸ Thus, our finding of increased CASP8 gene expression in decidual cells treated with progestin prior to LPS exposure raises the intriguing speculation that progestins “prime” the decidual cell to respond with a NF κ B-mediated inflammatory response.

The lack of concordance between our results and those of Elovitz and Mrinalini may be explained by three fundamental differences in experimental approach and design. First, our cell of interest is the decidual cell, whereas Elovitz and Mrinalini evaluated cervix, placenta,

and uterus (presumably myometrium). Clearly, in human preterm parturition, there are complex and synchronous molecular events that take place in all of these tissues. It is likely that the specific events that transpire and the specific tissues of interest vary depending upon the etiologic pathway contributing to preterm birth.¹⁹ We also recognize that the use of *in vitro* cell culture models may be incomplete analogs of *in vivo* systems. Investigations are needed to explore the effect of 17OHP on other gestational tissues in response to other pathologic stimuli, such as live bacterial infection, thrombin, or stretch. Secondly, we evaluated cells of human origin rather than from a murine model. Murine and other animal models of inflammation-induced preterm birth have provided invaluable insights into causes and consequences of prematurity.²⁰ Interspecies differences in immune response and the modulation of that response are undoubtedly a critical area of future exploration. Lastly, we evaluated P, 17OHP, and 17OHP in contrast to medroxyprogesterone acetate. As related members of the progestin family of sex steroids, these hormones are all related, but have very different properties with respect to biologic function. While the nature of the differential functions of progestational agents during pregnancy are incompletely understood, current understanding clearly supports dramatic differences between these agents with respect to tissue specificity, receptor binding affinity, and potency.^{21–23} Work in this arena is needed, particularly in light of published reports of benefit of vaginal P vis-a-vis prematurity prevention.²⁴

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Table I
Genes in the Toll-like receptor signaling pathway, grouped by functional category.

Toll-Like Receptors: LY64 (RP105/CD180), SIGIRR (TIR8), TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9, TLR10
Adaptors & TLR Interacting Proteins: BTK, CD14, GPC1 (SP-A), HMGB1, HRAS, HSPA1A, HSPA4, HSPA6, HSPD1, LY86 (MD-1), LY96, MAL, MAPK8IP3 (JIP3), MYD88, PELI1 (Pellino 1), PELI2 (Pellino 2), PGLYRP1, PGLYRP2, PGLYRP3, PGLYRPIbeta, RIPK2 (RIP2), SARM1, TICAM2, TIRAP, TOLLIP, TRIF (TICAM1)
Effectors: CASP8, EIF2AK2, FADD, IRAK1, IRAK2, IRAK3, IRAK4, MAP3K7, MAP3K7IP1 (TAB1), MAP3K7IP2 (TAB2), NR2C2 (TAK1), PPARA, PRKRA (PKR), SITPEC (ECSIT), TRAF6, UBE2N (Ubc13), UBE2V1 (Uev1A)
Downstream Pathways and Target Genes:
<u>NFκB Pathway:</u> CCL2 (MCP-1), CHUK (IKK-a), CSF2 (GM-CSF), CSF3 (G-CSF), IFNB1, IFNG, IKBKB (IKK-b), IKBKG (IKK-g), IL1A, IL1B, IL2, IL6, IL8, IL10, IL12A, IL12B, LTA (TNF-b), MAP3K1 (MEKK1), MAP3K14, MAP4K4 (NIK), NFKB1, NFKB2, NFKBIA (IκBa/mad3), NFKBIB (IκBb), NFKBIE, NFKBIL1, NFKBIL2, NFRKB, REL, RELA, RELB, TNF (TNFα), TNFRSF1A, TRADD
<u>JNK/p38 Pathway:</u> ELK1, FOS, JUN, MAP2K3 (MKK3), MAP2K4 (MKK4), MAP2K6 (MKK6), MAP3K1 (MEKK1), MAPK8 (JNK1), MAPK9 (JNK2), MAPK10, MAPK11 (p38bMAPK), MAPK12 (p38gMAPK), MAPK13, MAPK14 (p38 MAPK)
<u>NF/IL6 Pathway:</u> CLECSF9, PTGES, PTGS2 (Cox-2)
<u>IRF Pathway:</u> CXCL10 (IP-10), IFNB1, IFNG, IRF1, IRF3, IRF7, TBK1
Regulation of Adaptive Immunity: CD80, CD86, RIPK2 (RIP2), TRAF6.