Nuclear factor-κB mediates placental growth factor induced pro-labour mediators in human placenta

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ABSTRACT: Prostaglandins, pro-inflammatory cytokines, extracellular matrix remodelling enzymes and nuclear factor-kappa B (NF-κB) are involved in the mechanisms of term and preterm parturition. Recent studies have reported an increase in angiogenesis-related genes during term and preterm labour, including placental growth factor (PLGF). In non-gestational tissues, PLGF induces inflammation via NF-κB. The aim of this study was to determine the effect of PLGF on the gene expression and release of pro-labour mediators in human placenta. Samples were obtained from normal pregnancies at the time of Caesarean section. Human placenta was incubated in the absence (basal control) or presence of a 10 ng/ml PLGF for 24 h. Inflammatory gene expression was analysed by quantitative RT–PCR, concentration of pro-inflammatory cytokines and prostaglandins was quantified by ELISA, and secretory matrix metalloproteinases (MMPs) activity by zymography. NF-κB DNA-binding activity and IκB-α (inhibitor of NF-κB) protein degradation were analysed by ELISA and Western blotting, respectively. PLGF significantly increased interleukin (IL)-6 and IL-8 gene expression and secretion, cyclooxygenase-2 expression and resultant prostaglandin (PG) E2 and PGF2α release, and MMP-9 gene expression and enzyme production. PLGF induced the degradation of IκB-α whilst increasing NF-κB p65 DNA-binding activity. The PLGF-induced pro-labour responses were abrogated by co-treatment with the NF-κB inhibitor BAY 11-7082. In summary, the pro-inflammatory and pro-labour effects of PLGF in human placenta are mediated by NF-κB.

Key words: PLGF / placenta / cytokine / prostaglandin / NF-κB

Introduction

Approximately 13 million babies, or about 10% of total worldwide births, are born prematurely each year (Beck et al., 2010). Spontaneous premature delivery is the leading cause of neonatal mortality and morbidity; with morbidity continuing into early childhood (Goldenberg et al., 2008; Saigal and Doyle, 2008). Not only is this an enormous financial and emotional burden on the family, but it also presents a significant and growing challenge for health systems and society (Moster et al., 2008). To develop clinically useful interventions and improve neonatal outcome, elucidation of the mechanisms involved in the initiation and progression of labour, both at term and preterm, is essential.

What triggers human labour and delivery at term or preterm is not known. Of the mechanisms studied to date, however, a strong case can be made for the involvement of eicosanoids (e.g. prostaglandins), inflammatory cytokines and extracellular matrix (ECM) remodelling enzymes (Fata et al., 2000; Rice, 2001; Bowen et al., 2002; Olson, 2003; Lappas and Rice, 2004; Christaens et al., 2008). To identify critical sites of intervention within these pathways, it is essential to first identify regulators of their expression and to evaluate the effects of inhibiting their activity on labour and delivery. Studies over the last 10 years have implicated the nuclear transcription factor signalling pathway involving nuclear factor-kappa B (NF-κB) as a common, central pathway involved in promoting the formation of pro-inflammatory and pro-labour mediators (Lindstrom and Bennett, 2005; Lappas and Rice, 2007, 2009).

A few recent studies have shown an increase in angiogenesis-related genes in the uterine cervix after spontaneous term parturition in humans (Hassan et al., 2007) and mice (Haddad et al., 2008). These genes include members of the vascular endothelial growth factor (VEGF) family such as placental growth factor (PLGF). There is now increasing evidence that VEGF family members, in addition to their well-established role in angiogenesis and vascular biology (Senger et al., 1983; Leung et al., 1989; Kim et al., 2001; Odoriso et al., 2002; Khurana et al., 2005) also play a significant role in inflammation (Carmeliet et al., 2001; Oura et al., 2003; Perelman et al., 2003; Pipp et al., 2003; Reinders et al., 2003; Selvaraj et al., 2003; Khurana et al., 2005; Pilarczyk et al., 2008; Yoo et al., 2009; Ding et al., 2010; Roncal et al., 2010; Van Steenkiste et al., 2011). As such, PLGF has been implicated as a pathophysiological mediator in several human disease states, including rheumatoid arthritis, cancer and inflammatory bowel disease.
PLGF gene expression is increased in human uterine cervix at term and mouse uterus after labour (Hassan et al., 2007; Haddad et al., 2008). However, the effect of human labour on PLGF expression has yet to be elucidated. Thus, an aim of this study was to determine the effect of human labour on PLGF expression in human gestational tissues. In non-gestational tissues, PLGF regulates inflammation. Whether PLGF also regulates pro-inflammatory and pro-labour mediators in human gestational tissues is not known. An additional aim of this study was to investigate the effect of PLGF on pro-inflammatory cytokines [interleukin (IL)-6 and IL-8 gene expression and release]; phospholipid metabolism [cyclooxygenase (COX) expression and subsequent prostaglandin release]; proteases [matrix metalloprotease (MMP)-2 and MMP-9 expression and activity]; and NF-κB signaling. The hypotheses to be tested is that (i) human labour is associated with increased PLGF expression in human gestational tissues; (ii) PLGF exerts pro-inflammatory and pro-labour properties in human placenta via activation of the NF-κB pathway and (iii) inhibition of NF-κB signaling using BAY 11-7082 will abrogate the pro-inflammatory and pro-labour actions of PLGF. BAY 11-7082 was chosen as previous studies have shown that it inhibits NF-κB p65 DNA-binding activity in human placenta (Coughlan et al., 2004) and placental JEG-3 cells (Lappas et al., 2006).

Materials and Methods

Tissue collection

Approval for this study was obtained from the Mercy Hospital for Women's Research and Ethics Committee and informed consent was obtained from all participating subjects. Human placentae and attached fetal membranes were obtained from women who delivered healthy, singleton infants at term (≥37 weeks gestation) (i) before labour undergoing elective Caesarean section (indications for Caesarean section were breech presentation and/or previous Caesarean section); and (ii) after spontaneous labour and normal vaginal delivery. Women with any adverse underlying medical condition (i.e. including asthma, clinical signs of infection, diabetes and pre-eclampsia) were excluded from this study.

Placental explant incubation

Placental lobules (cotyledons) were obtained from various locations of the placenta; the basal plate and chorionic surface were removed from the cotyledon, and villous tissue was obtained from the middle cross-section. Placental tissue was blunt dissected to remove visible connective tissue and calcium deposits. The tissue was placed in Dulbecco’s modified Eagle’s medium (containing 5 mM glucose, 100 U/ml penicillin G and 100 μg/ml streptomycin) at 37°C in a humidified atmosphere of 5% CO₂, and 8% O₂ for 2 h. Explants were blotted dry on sterile filter paper and transferred to 24-well tissue culture plates (200 mg tissue per 2 ml media). Human recombinant PLGF-1 was obtained from ProSpec-Tany TechnoGene Ltd. (Rehovot, Israel). An initial dose–response experiment was performed whereby placenta was incubated in the absence or presence of 2.5, 5, 10 and 20 ng/ml PLGF (n = 3 patients). The concentration of PLGF was based on our previously published studies (Arroyo et al., 2004). PLGF concentrations higher than 5 ng/ml significantly increased cytokine release. All subsequent experiments were thus performed using 10 ng/ml PLGF (n = 4 patients). Additional explants were performed in which placental explants were also co-treated with 50 μM BAY 11-7082 (n = 4 patients). The concentration of BAY 11-7082 was based on our previously published studies (Lappas et al., 2006). Each treatment was performed in duplicate or triplicate; the average of the duplicate/triplicate was used for final data analysis. After 24 h incubation, medium and tissue was collected separately and stored at −80°C until assayed as detailed below. Placenta was also incubated with PLGF for 45 min for analysis of IkB-α protein by western blotting (n = 4 patients).

Cytokine and prostaglandin immunoassays

After 24 h incubation, the explant incubation medium was collected and the release of IL-6 and IL-8 was performed by sandwich ELISA according to the manufacturer’s instructions (Invitrogen, Carlsbad, CA, USA). The release of PGE₂ and PGF₂α into the incubation medium was assayed using a commercially available competitive enzyme immunoassay kit according to the manufacturer’s specifications (Kookaburra Kits from Saphire Bioscience, Redfern, NSW, Australia). All data were corrected for total protein and expressed as nanogram per milligram protein. The protein content of tissue homogenates was determined using BCA protein assay (Pierce, Rockford, USA), using bovine serum albumin as a reference standard, as we have previously described (Lappas et al., 2002, 2003, 2004). The calculated inter-assay and intra-assay coefficients of variation were all < 10%.

Gelatin zymography

Pro-MMP-2 and MMP-9 enzyme activity in conditioned media was analysed by gelatin zymography as previously described. Gels were viewed and analysed using the Chemi-Doc system (Bio-Rad). Quantitative analysis of the relative density was performed using Quantity One 4.2.1 image analysis software (Bio-Rad). Data were corrected for background, and expressed as optical density (OD/mm²).

Western blotting

Assessment of tissue protein expression was analysed by western blotting as previously described. Blots were incubated with 1 μg/ml rabbit polyclonal anti-IkB-α (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted in blocking buffer [5% skim milk/TBS-T (0.05%)] for 24 h at 4°C. Membranes were viewed and analysed using the Chemi-Doc system (Bio-Rad). Quantitative analysis of the relative density of the bands in western blots was performed using Quantity One 4.2.1 image analysis software (Bio-Rad). Data were corrected for background, and expressed as OD (mm²).

Assessment of NF-κB p65 DNA-binding activity

Nuclear protein was extracted as previously described. NF-κB DNA binding in nuclear protein extracts was assessed using a commercially available NF-κB p65 Transcription Factor ELISA according to manufacturer’s instructions (Cayman Chemical, Ann Arbor, MI, USA). Specificity of NF-κB binding was assessed using wild-type and mutated consensus oligonucleotides. A BioRad xMark microplate absorbance spectrophotometer was used to read the sample absorbance, with data expressed as OD at 450 nm.

RNA extraction and RT–PCR

Total RNA was extracted from ~100 mg of tissue using Tri Reagent according to manufacturer’s instructions (Sigma-Aldrich, Saint Louis Missouri). RNA concentrations were quantified using a spectrophotometer (Smart Spec, Bio-Rad). The quantification and estimation of RNA purity was assessed using a NanoDrop spectrophotometer (ThermoFisher Scientific, Waltham, MA, USA). One microgram of RNA was converted to cDNA using the SuperScript® VILO™ cDNA synthesis kit (Invitrogen), the cDNA was diluted 10-fold, and 0.8 μl of cDNA was used for each
reaction. RT–PCR was performed on the CFX384 real-time PCR detection system (BioRad) using Sensimix Plus SYBR green (Quantace, Alexandria, NSW, Australia) and 100 nM of QuantiTect Primer Assays (Qiagen, Germantown, MD, USA). Average gene Ct values were normalized to the average GAPDH Ct values of the same cDNA sample. Of note, there was no effect of experimental treatment on GAPDH gene expression. There was equal efficiency of PCR amplification of target and reference mRNA. The specificity of the product was assessed from melting curve analysis. RNA without reverse transcriptase during cDNA synthesis as well as PCR reactions using water instead of template showed no amplification. Variations in gene expression were calculated by the comparative Ct method.

**Statistical analysis**

Statistical analyses were performed using a commercially available statistical software package (Statgraphics Plus version 3.1, Statistical Graphics Corp., Rockville, MD, USA). The effect of human labour on PLGF expression was assessed by Student’s t-test. To determine the effect of PLGF on pro-labour mediators, two sample comparisons were analysed by a paired sample comparison. For all other comparisons, analysis was performed using a one-way analysis of variance; homogeneity of data were assessed by Bartlett’s test, and when significant, data were logarithmically transformed before further analysis. After log transforming, all data were normally distributed. Statistical difference was indicated by a P-value of <0.05. Data are expressed as mean ± standard error of the mean (SEM).

**Results**

**Effect of term spontaneous labour on PLGF expression in placenta and choriodecidua**

To determine the effect of human labour on PLGF expression, qRT–PCR was performed on placenta and choriodecidua obtained before and after term labour. Placenta and choriodecidua (n = 6) were obtained (i) at Caesarean section before the onset of labour (no labour) and (ii) after spontaneous labour and membrane rupture (after labour). There was no difference in PLGF mRNA expression between non-labouring and after labour choriodecidua (Fig. 1). On the other hand, when compared with non-labouring placenta, PLGF expression was significantly higher after spontaneous labour at term (Fig. 1). Given that human term labour was associated with increased PLGF in placenta; all further experiments described below were carried out only in placenta.

**PLGF induces the expression and release of pro-labour mediators from human placenta**

An initial dose–response was performed to determine the optimal PLGF concentration. Human placenta was incubated with 0 (basal), 2.5, 5, 10 and 20 ng/ml PLGF for 24 h. IL-6 and IL-8 cytokine concentration in the incubation medium was assayed by ELISA. As shown in Fig. 2, PLGF at 5, 10 and 20 ng/ml significantly increased IL-8 concentration; there was no effect of 2.5 ng/ml PLGF. Of note, there was no difference in response between 10 and 20 ng/ml PLGF, and both 10 and 20 ng/ml induced significantly greater IL-8 release than 5 ng/ml. Similar results were obtained for IL-6 (data not shown). For all subsequent experiments, human placenta was incubated in the absence (basal) or presence of 10 ng/ml PLGF for 24 h.

The effect of 10 ng/ml PLGF on cytokine and prostaglandin release from human placenta is demonstrated in Fig. 3. Compared with control, tissues incubated in the presence of PLGF caused a significant increase in IL-6 and IL-8 release (Fig. 3A). Similarly, PLGF significantly increased PGE$_2$ and PGF$_{2\alpha}$ release (Fig. 3B). The effect of PLGF treatment on MMP activity from human placental explants is shown in Fig. 4. PLGF significantly increased the secretion of pro-MMP-9; however, there was no effect on pro-MMP-2 activity.

**PLGF regulates pro-labour mediators via the NF-κB pathway**

The next aim was to determine if PLGF regulates pro-labour mediators via the NF-κB pathway. In order to do this, we first determined the effect of PLGF on the NF-κB pathway. Human placenta was incubated in the absence (basal) or presence of 10 ng/ml PLGF for 45 min, and cytoplasmic IκBα protein expression was analysed by western blotting. Nuclear NF-κB p65 DNA-binding activity, as assessed by ELISA, was determined after incubation of placenta with...
PLGF for 24 h. As shown in Fig. 5A, IκB-α protein expression was lower in the presence of PLGF, which is indicative of increased protein degradation. Additionally, PLGF significantly increased NF-κB p65 DNA-binding activity (Fig. 5B).

Having shown that PLGF increases NF-κB activity, the final aim was to determine if NF-κB is involved in PLGF-induced pro-labor mediators in human placenta. Placental tissue was incubated with 10 ng/ml PLGF with and without 50 μM BAY 11-7082 for 24 h. Cytokine and prostaglandin concentration in the incubation medium was assayed by ELISA; gelatin substrate gels were used to determine the release of protease activity; and cytokine and COX gene expression was evaluated by qRT–PCR.
As shown in Fig. 6, in addition to significantly increasing cytokine release, PLGF also significantly increased cytokine gene expression. Treatment of placental explants with BAY 11-7082 significantly inhibited PLGF-induced cytokine mRNA expression (Fig. 6A) and release into incubation medium (Fig. 6B). In addition to this, BAY 11-7082 significantly decreased PLGF-induced COX-2 expression (Fig. 6C) and subsequent PGE\textsubscript{2} and PGF\textsubscript{2\alpha} release (Fig. 6D). Of note, there was no effect of PLGF with or without BAY 11-7082 on treatment COX-1 mRNA expression (Fig. 6D). BAY 11-0782 also significantly attenuated effect of PLGF with or without BAY 11-7082 on treatment pro-MMP-2 mRNA expression (Fig. 6E) or release (Fig. 6F).

**Discussion**

It has been recently reported that PLGF increases in the uterine cervix after spontaneous term parturition in humans (Hassan et al., 2007) and mice (Haddad et al., 2008). Similarly, in this study, increased PLGF gene expression was observed in placenta after spontaneous term labour and delivery. In this study, a well-characterized in vitro human tissue explant system was used to elucidate the effects of PLGF in human placenta. The data show that PLGF induces the expression and secretion of the pro-inflammatory cytokine IL-6 and the chemokine IL-8; COX-2 gene expression and subsequent PGE\textsubscript{2} and PGF\textsubscript{2\alpha} release; expression and activity of MMP-9; and induced the degradation of I\textkappa{B-\alpha} whilst increasing NF-\kappa{B} p65 activity. These actions of PLGF were abrogated by co-treatment with the NF-\kappa{B} inhibitor BAY 11-0782. Collectively, this data supports the hypothesis that NF-\kappa{B} mediates the pro-inflammatory and pro-labour of PLGF in human placenta.

Cytokines, prostaglandins and MMPs released from the placenta may act in an autocrine or paracrine manner to control the processes of human labour. For example, PGE\textsubscript{2} stimulates the placental release of IL-8 (Denison et al., 1998), PGF\textsubscript{2\alpha} acts on the decidua to increase production of MMP-9 (Ulug et al., 2001), cells circulating in the placental blood are producing mediators capable of conditioning a microenvironment of collagenolysis in fetal membranes (Estrada-Gutierrez et al., 2005), and stimulation of placental blood with *Ureaplasma urealyticum* increases IL-1\beta and PGE\textsubscript{2}, which can induce an additional inflammatory response by the amniochorion (Estrada-Gutierrez et al., 2010). In non-gestational tissues, MMP-9 plays an important role regulating the cytokine, growth factor and caspase expression (Olle et al., 2006).

The data in this study are supportive of previous findings in non-gestational tissues demonstrating a regulatory role for PLGF in inflammation. It plays a fundamental role in activating and recruiting myelomonocytic cells in sickle cell disease (Selvaraj et al., 2005), and stimulation of placental blood with *Urea* increases IL-1\beta and PGE\textsubscript{2}, which can induce an additional inflammatory response by the amniochorion (Estrada-Gutierrez et al., 2010). In non-gestational tissues, MMP-9 plays an important role regulating the cytokine, growth factor and caspase expression (Olle et al., 2006).

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The pro-inflammatory and pro-labour actions of PLGF in human placenta are mediated by NF-\kappa{B}. I\kappa{B}-\alpha binds to NF-\kappa{B} dimers keeping them sequestered in an inactive state in the cytoplasm. Upon activation, I\kappa{B}-\alpha is ubiquitinated and degraded, allowing free NF-\kappa{B} dimers to translocate to the nucleus where it binds to the \kappa{B} motif in the promoter region of target genes to initiate transcription. In this study, PLGF induced the degradation of I\kappa{B}-\alpha concomitant with increased NF-\kappa{B} p65 binding activity. Furthermore, PLGF-induced pro-inflammatory and pro-labour mediators were suppressed by co-treatment with the NF-\kappa{B} inhibitor BAY 11-0782. This data are supportive of the previous studies that have shown an important role for NF-\kappa{B} in the regulation of cytokines, prostaglandins and proteases (Lappas et al., 2002, 2003, 2004, 2006; Lindstrom and Bennett, 2005; Lappas and Rice, 2007, 2009). In human trophoblast cells, PLGF induces activation of the mitogen-activated protein kinase (MAPK) proteins c-Jun-N-terminal kinase (JNK) and p38 MAPK (Desai et al., 1999; Arroyo et al., 2004). These proteins have previously been shown to regulate pro-inflammatory cytokines and prostaglandins in human gestational tissues (Lappas et al., 2007).

Preterm labour may represent early idiopathic activation of the normal labour process or the result of pathological insults such as infection and/or inflammation (Romero et al., 2007; Christaens et al., 2008). These processes stimulate the formation of pro-inflammatory cytokines [such as IL-1\beta and tumour necrosis factor (TNF-\alpha)], which play important roles in the cascade of inflammatory events associated with the onset of preterm labour (Bowen et al., 2002; Keelan et al., 2003). Studies support the notion that PLGF is a late marker of sepsis, which lies downstream of early response cytokines (Yano et al., 2006). Thus, in the context of preterm labour, TNF-\alpha and IL-1\beta may induce PLGF transcription. This may occur via activation of the NF-\kappa{B} pathway as PLGF has several putative binding sites for NF-\kappa{B} in its promoter/enhancer region (Cramer et al., 2005; Chaballe et al., 2011). PLGF, as demonstrated in this study, can then also activate NF-\kappa{B} signalling and its downstream effector pathways. Downstream actions of NF-\kappa{B} include stimulating the production of prostaglandins and MMPs; prostaglandins induce uterine contractions and act in concert with collagenases to cause cervical ripening and fetal membrane remodelling (Lappas et al., 2002, 2003, 2004, 2006; Lindstrom and Bennett, 2005; Lappas and Rice, 2007, 2009). The effect of preterm labour on PLGF expression in human gestational tissues is not known. However, there is no effect of intra-amniotic infection in the amniotic fluid concentration of PLGF (Seubert et al., 2000), and maternal infection during pregnancy, such as acute pyelonephritis, is associated with lower plasma PLGF concentrations (Chaiworapongsa et al., 2010). Not with standing, the data presented in this study demonstrate an increase in placental PLGF gene expression after labour. Future studies are required to determine the effect of preterm labour and pro-inflammatory mediators of labour on PLGF expression in human gestational tissues.

It is possible that PLGF can activate NF-\kappa{B} through an intermediary TNF-\alpha step. In myelomonocytic cells, TNF-\alpha is significantly up-regulated by PLGF which in turn elicits the PLGF-induced myelomonocytic cell recruitment (Ding et al., 2010). The use of
Figure 6: Effect of BAY 11-0782 on PLGF-induced inflammation and pro-labour mediators. Human placenta was incubated in the absence or presence of 10 ng/ml PLGF with or without 50 μM BAY 11-0782 for 24 h (n = 4). (A) IL-6 and IL-8 mRNA expression; (B) IL-6 and IL-8 release; (C) COX-1 and COX-2 gene expression; (D) PGE₂ and PGF₂α release; (E) MMP-2 and MMP-9 gene expression; and (F) secreted pro-MMP-2 and pro-MMP-9 enzyme activity. All data represent the mean ± SEM. *P < 0.05 versus PLGF.
TNF-α-neutralizing antibodies to block extracellular TNF-α or inhibition of TNF-α expression by siRNA will help to elucidate whether or not PLGF is increasing NF-κB induced gene transcription via TNF-α. However, in these studies, TNF-α gene expression or concentration in the incubation medium was undetectable (data not shown). Additionally, future studies are required to elucidate the role of the PLGF receptor, Flt-1 [or VEGF receptor (VEGFR)-1], in PLGF mediated pro-labour actions.

Concluding remarks
If we are to provide the best possible start to life it is essential to understand the processes that regulate normal, spontaneous-onset of labour at term and how these processes are subverted in association with preterm birth. The present study is the first to report the regulation of pro-inflammatory cytokines, prostaglandins and proteases by PLGF in human placenta. Of note, PLGF-induced pro-inflammatory and pro-labour actions in human placenta via activation of NF-κB. Potentially, this has important clinical implications as preterm labour remains a major obstetric concern, associated with increased perinatal mortality and morbidity (Goldenberg et al., 2008; Saigal and Doyle, 2008).

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Author’s role
M.L. conceived the idea, designed the study, performed the experiments, analysed the data and wrote the manuscript.

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Conflict of interest
The author has nothing to declare.

References


