Preterm and infection-driven preterm labor: the role of peroxisome proliferator-activated receptors and retinoid X receptor

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Abstract

Approximately 8% of births are complicated by preterm delivery. To improve neonatal outcomes, a greater understanding of the mechanisms surrounding preterm parturition is required. Peroxisome proliferator-activated receptors (PPARs) have been implicated in the regulation of labor at term where they exhibit anti-inflammatory properties. Thus, we hypothesize that dysregulation of PPAR expression and activity may be associated with preterm labor and infection-associated preterm labor. The aim of this study was to compare the expression and activity of PPARs and the expression of retinoid X-receptor α (RXRA) in gestational tissues from term and preterm deliveries, and from infection-associated preterm deliveries. Quantitative RT-PCR, western blotting and activity ELISA were used to study expression and DNA binding profiles. Compared with term, preterm parturition was associated with an increased expression of PPAR δ (PPARD; mRNA and protein), PPAR γ (PPARG; protein) and RXRA (protein) in the placenta and PPARD (mRNA and protein) and RXRA (mRNA) in the choriodecidua. There was, however, no change in preterm PPAR DNA binding activity compared with term. Preterm chorioamnionitis (CAM) demonstrated protein degradation in the choriodecidua and was associated with a decline in the mRNA expression of PPAR α (PPARA) and RXRA compared with uninfected preterm cases. PPAR DNA binding activity increased in the placenta (PPARD and PPARG) and decreased in the amnion (PPARA and PPARG) in association with preterm CAM. In conclusion, idiopathic preterm deliveries were associated with an increase in PPAR:RXR expression and preterm CAM was associated with a decrease in PPAR:RXR expression and tissue-specific alterations in transcriptional activity. The reasons for such dysregulation remain to be determined; however, the data are consistent with the hypothesis that PPARs may play a role in preterm labor and infection-complicated preterm deliveries.

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Introduction

Approximately 8% of births occur before 37 weeks complete gestation, with the majority of preterm births occurring between 32 and 36 weeks (Laws *et al.* 2007, Saigal & Doyle 2008). Spontaneous premature delivery is the leading cause of neonatal mortality and is responsible for neonatal morbidity including respiratory distress, temperature instabilities, and feeding complications (Laws *et al.* 2007, Saigal & Doyle 2008). Neurodevelopmental, respiratory, and gastrointestinal problems are common into early childhood (Goldenberg *et al.* 2008, Saigal & Doyle 2008). The causes of preterm labor remain unresolved; possibly representing the activation of normal labor mechanisms, albeit too early, or resulting from adverse pathological complications (Lamont 2001, Goldenberg *et al.* 2002, Romero *et al.* 2006). To improve preterm morbidity and mortality,

a greater understanding of the processes associated with preterm labor are required.

Although a large degree of preterm births are idiopathic, intra-amniotic infection has been implicated in reduced gestational duration, especially in earlier deliveries <30 weeks (Goldenberg *et al.* 2002, Romero *et al.* 2006, Gargano *et al.* 2008). Chorioamnionitis (CAM), inflammation of the amniochorion, is caused by ascending bacterial infection from the lower genital tract (Fahey 2008). Organisms colonize the chorioamniotic surface and sequential inflammatory responses are activated in the chorionic plate (acute subchorionitis; Stage 1); the membranous chorion (acute chorionitis; also Stage 1); the connective tissue of the chorion and amnion (acute CAM; Stage 2); and lastly causing amnion epithelial cell necrosis (necrotizing CAM; Stage 3; Redline 2006).

Peroxisome proliferator-activated receptors (PPARs) have been implicated in several aspects of early pregnancy development including implantation, placental, and trophoblast differentiation (Barak *et al.* 1999, Lim & Dey 2000, Tarrade *et al.* 2001). We and others have presented data implicating PPARs in the inflammatory events that contribute to normal labor at term (Lim & Dey 2000, Lappas *et al.* 2002*b*, 2004, Berry *et al.* 2003, Dunn-Albanese *et al.* 2004, Ackerman *et al.* 2005, Lindstrom & Bennett 2005, Froment *et al.* 2006, Schaiff *et al.* 2006, Fournier *et al.* 2007, Holdsworth-Carson *et al.* 2009). PPARs exhibit anti-inflammatory properties (Jiang *et al.* 1998, Ricote *et al.* 1998, Delerive *et al.* 2001, Daynes & Jones 2002, Lappas *et al.* 2002*b*, Kielian *et al.* 2008, Perez *et al.* 2008) and are hypothesized to promote pregnancy quiescence and initiate labor (Dunn-Albanese *et al.* 2004, Froment *et al.* 2006, Schaiff *et al.* 2006, Wieser *et al.* 2008). There are three isoforms of PPAR nuclear receptors (α (PPARA), δ (PPARD), and γ (PPARG)) and they act as ligand-dependent transcription factors (Issemann & Green 1990, Dreyer *et al.* 1992). Activation by binding of ligand results in nuclear translocation where PPAR binds to specific sequences within the promoter regions of target genes (peroxisome proliferator responsive elements (PPRE)). Once bound to the PPRE site, PPAR will either activate or inhibit gene expression. Co-operative heterodimerization of PPAR to retinoid X receptor (RXR) is obligatory for PPAR-dependent gene transcription (Mangelsdorf & Evans 1995).

PPAR isoforms and RXRA have been identified in human gestational tissues (Berry *et al.* 2003, Dunn-Albanese *et al.* 2004, Borel *et al.* 2008, Holdsworth-Carson *et al.* 2009). Research has inferred a role for PPAR in preterm labor (Schaiff *et al.* 2006, Borel *et al.* 2008, Wieser *et al.* 2008) and carriers of a *PPARG* genetic polymorphism have been associated with being born prematurely (Meirhaeghe *et al.* 2007). PPAR-associated inflammatory mediator suppression suggests that a breakdown in PPAR expression or activity may be apparent during periods of preterm intra-amniotic infection, as seen in non-gestational tissue infections (Kielian *et al.* 2008, Perez *et al.* 2008). While some groups have investigated PPAR expression in first (Tarrade *et al.* 2001, Capparuccia *et al.* 2002, Rodie *et al.* 2005) and second trimester placenta (Waite *et al.* 2000, Rodie *et al.* 2005) and serum samples throughout the course of pregnancy (Wieser *et al.* 2008), data are lacking regarding the expression and activity of PPAR isoforms in association with preterm delivery, with and without infection. The aim of this study was to compare the expression and DNA binding activity patterns of PPAR isoforms and the expression of RXRA in human gestational tissues from term and preterm vaginal deliveries, and to determine the effect of infection-associated preterm delivery on PPAR isoforms and RXRA expression. We propose that downregulation in PPAR

expression and/or DNA binding activity is apparent during preterm labor, more so in infection-complicated preterm deliveries, thereby promoting inflammatory mediated pro-labor mechanisms.

Results

Clinical characteristics of the patients

Table 1 presents the clinical characteristics for the patients included in this study. The term and preterm women (without infection) all went into spontaneous labor and delivered vaginally. The term women all had spontaneous rupture of the fetal membranes with labor, while most of the preterm women (83%) had pre-labor rupture of membranes (PROM; McParland & Bell 2004). The duration of fetal membrane rupture was significantly less in the term group compared with the preterm group. It should be noted that one patient in the preterm group had PROM 14 days prior to spontaneous labor onset and delivery. Gestational age and fetal weight were significantly lower in the preterm group. Although maternal body mass index at ~12 weeks gestation was significantly higher for women who delivered at term, the mean value was still within the healthy range.

Table 1 Clinical details of tissues collected from term, preterm and preterm chorioamnionitis deliveries (mean $\pm$ S.E.M.).

Clinical feature	Term (n=6)	Preterm (n=6)	Chorioamnionitis (n=8)
Weeks gestation	40.2 $\pm$ 0.4*	34.7 $\pm$ 0.4	31.2 $\pm$ 1.0†
Labor status			
Spontaneous	100% (6/6)	100% (6/6)	87.5% (7/8)
Induced	–	–	12.5% (1/8)
Delivery mode			
Vaginal	100% (6/6)	100% (6/6)	37.5% (3/8)
Caesarean section	–	–	62.5% (5/8)
Membrane rupture			
SROM	100% (6/6)	17% (1/6)	–
PROM	–	83% (5/6)	50% (4/8)
ARM	–	–	50% (4/8)
Rupture duration	5 h 25 min $\pm$ 1 h 2 min*	14 h 8 min $\pm$ 4 h 2 min	> 24 h (PROM)
Histopathology			
Chorioamnionitis Stage 1	–	–	50% (4/8)
Chorioamnionitis Stage 2	–	–	50% (4/8)
Fetal weight (g)	3645.8 $\pm$ 92.7*	2366.3 $\pm$ 129.2	1753.9 $\pm$ 166.9†
Fetal sex (% female)	33% (2/6)	33% (2/6)	25% (2/8)
Apgar 5 min	9.0 $\pm$ 0.0	8.8 $\pm$ 0.5	8.5 $\pm$ 0.6
Maternal age (years)	31.0 $\pm$ 1.6	32.2 $\pm$ 1.3	28.5 $\pm$ 1.6
Twelve week BMI (kg/m ²)	24.2 $\pm$ 0.7*	20.6 $\pm$ 1.1	21.0 $\pm$ 1.0

SROM, spontaneous rupture of membranes with labor; PROM, pre-labor rupture of membranes) and ARM, artificial rupture of membranes, either during labor or at Caesarean section. *Denotes significant difference between term and preterm ($P < 0.05$). †Denotes significant difference between preterm and chorioamnionitis ($P < 0.05$).

Compared to preterm patients without infection, women with histopathologically diagnosed CAM displayed variability with respect to labor type (spontaneous versus induced labor) and mode of delivery (vaginal versus Caesarean section). This reflects the unpredictability of CAM infection and the degree of medical intervention involved in treatment, where prompt delivery of the infant is often the safest option for mother and baby (Fahey 2008). In comparison with the non-infected preterm women, CAM women delivered infants with significantly lower gestational age and fetal weight.

The effect of term and idiopathic preterm gestations on PPAR and RXRA mRNA expression, protein expression, and DNA binding activity in spontaneous vaginal deliveries

mRNA and protein expression

Quantitative RT-PCR (qRT-PCR) and western blotting was used to determine PPARA, -D and -G, and RXRA mRNA and nuclear protein expression between term and

preterm deliveries, in placenta, amnion, and choriodecidea (Fig. 1). There was no difference in PPARA mRNA or nuclear protein expression between the term and preterm groups for placenta (Fig. 1a, b and j). PPARG mRNA expression also remained unchanged between the two gestational groups for placenta. By contrast, mRNA expression for PPARD and nuclear protein expression of PPARD, PPARG, and RXRA were all significantly higher at preterm when compared with term placental samples ($P < 0.05$; Fig. 1a, b and j).

For amnion, mRNA and protein expression was unchanged between the term and preterm deliveries for all PPARs and RXRA (Fig. 1d, e and k). As previously published, PPARA protein was undetectable in amnion nuclear fractions (Holdsworth-Carson *et al.* 2009). For choriodecidea, PPARD nuclear protein and PPARD and RXRA mRNA expression was significantly higher in the preterm group compared with the term group ($P < 0.05$; Fig. 1g, h and l). There was no difference in PPARA and PPARG mRNA expression, and PPARA, PPARG and RXRA nuclear protein expression between preterm and term groups for choriodecidea.

Figure 1 PPAR isoform and RXRA mRNA expression, protein expression and DNA binding activity in idiopathic preterm and term spontaneous vaginal deliveries. qRT-PCR, western blotting, and activity ELISA were performed on preterm (PT) and term (T) (a–c) placenta, (d–f) amnion, and (g–i) choriodecidea. PT mRNA, nuclear protein and DNA binding activity were compared with T by two-sample comparison (Students *t*-test). Expression is displayed as the mean fold change ratio $\pm$ S.E.M. and DNA binding activity is displayed as the mean optical density (at 450 nm) minus the blank $\pm$ S.E.M. with *P* values < 0.05 denoted with *. Representative western blot images for PPARA, -D, -G, and RXRA are displayed in (j) placenta, (k) amnion and (l) choriodecidea. Note that PPARA nuclear protein could not be detected.

Figure 2 Correlation between *PPARD* mRNA expression and fetal membrane rupture duration in term and idiopathic preterm placenta. Correlation analyses (linear) were performed on fetal membrane rupture duration and qRT-PCR data from five term women and five preterm women ($R^2 = 0.575$ and P value = 0.01).

PPAR DNA binding activity

PPAR DNA binding activity was measured from nuclear protein extracts of placenta, amnion, and choriodecidua (Fig. 1c, f, and i). No significant changes in DNA binding activity for any PPAR isoform were detected in placenta, amnion or choriodecidua when comparing preterm gestations to term gestations.

Effect of duration of fetal membrane rupture on PPAR isoforms and RXRA

Correlation analysis was performed between the duration of fetal membrane rupture (Table 1) and the mRNA and protein expression of PPARs and RXRA and the DNA binding activities of PPAR isoforms (Fig. 1). Analysis revealed that *PPARD* mRNA expression in the placenta is positively associated with rupture duration ($P < 0.01$; Fig. 2). None of the other variables tested shared a significant relationship (data not shown).

The effect of CAM on PPAR and RXRA mRNA expression, protein expression, and DNA binding activity in preterm deliveries

qRT-PCR and western blotting were used to demonstrate differences in expression of *PPARA*, -D, and -G and *RXRA* between preterm deliveries, with and without histological CAM, in placenta, amnion, and choriodecidua (Fig. 3). Patients with CAM were stratified according to degree of infection; Stage 1 or Stage 2 CAM (Redline *et al.* 2003, Redline 2006), and compared individually to preterm patients without infection. Stage 1 and 2 CAM were also combined (CAM (1+2)) and compared separately to preterm patients without infection.

mRNA expression

There was no significant effect of CAM on placental and amnion mRNA expression of all PPAR isoforms and *RXRA* (Fig. 3a and d). Furthermore, choriodecidual *PPARD* and -G mRNA expression was also unchanged

between the preterm groups with and without infection (Fig. 3g). By contrast, *PPARA* mRNA from the CAM (1+2) group was significantly lower than the no-infection preterm group for choriodecidua ($P < 0.05$; Fig. 3g). In addition, *RXRA* mRNA in the choriodecidua was significantly reduced in the Stage 1 CAM group compared with the non-infected preterm group ($P < 0.05$; Fig. 3g).

Protein expression

Analysis of PPAR protein expression was based on whole cell protein extracts as nuclear protein extracts displayed severe CAM-associated protein degradation (data not shown). Stage 2 CAM was associated with protein degradation in all of the choriodecidua samples (Fig. 3h and j). It is likely that protein degradation was a result of loss of tissue integrity. Previously published examples of this include: chorion layer loss in preterm CAM membranes (Premyslova *et al.* 2003); extensive chorion, amniotic epithelium and decidual stromal cell integrity loss with Stage 3 preterm CAM (Van Meir *et al.* 1996, 1997); and destruction of chorion trophoblasts with CAM in rhesus monkey fetal membranes (Giannoulis *et al.* 2005). Stage 1 CAM choriodecidua samples were also associated with some loss of protein (see Fig. 3j). Both tubulin and β -actin clearly demonstrate the protein degradation in lanes 2, 3, 6, and 9. Thus, the effect of CAM Stage 2 on choriodecidual PPAR isoform and *RXRA* protein expression could not be performed. When compared with Stage 1 CAM, there was no significant difference in PPAR and *RXRA* protein expression in preterm choriodecidua samples (Fig. 3h).

There were no infection-associated changes in PPAR isoform and *RXRA* protein expression in the placenta (Fig. 3b) and amnion (Fig. 3e). It should be noted that in amnion, one patient showed protein degradation associated with Stage 2 CAM, this patient was therefore excluded from the amnion analysis.

PPAR DNA binding activity

PPAR DNA binding activity was measured in idiopathic preterm and preterm CAM-infected pregnancies using nuclear extracts of placenta, amnion, and choriodecidua, see Fig. 3c, f, and i. Patients with CAM were stratified as above and compared individually with preterm patients without infection. Stage 1 and 2 CAM were also combined (CAM (1+2)) and compared separately with idiopathic preterm patients.

PPAR DNA binding activity did not differ between Stage 1 CAM and uninfected preterm deliveries in choriodecidua (Fig. 3i). Since Stage 2 CAM was associated with protein loss, analysis of Stage 2 CAM in choriodecidua could not be performed. *PPARA* DNA binding activity was unchanged in placental tissue between preterm and infected groups (Fig. 3c). Both *PPARD* and *PPARG* isoforms, however, demonstrated an

Figure 3 PPAR isoform and RXRA mRNA expression, protein expression, and DNA binding activity in idiopathic preterm and chorioamnionitis preterm deliveries. qRT-PCR, western blotting, and activity ELISA were performed on PT (a–c) placenta, (d–f) amnion, and (g–i) choriodecidua, with and without CAM infection. CAM infection is categorized according to stage of infection; Stage 1 CAM, Stage 2 CAM, and combined as CAM (1 + 2). Expression is displayed as the mean fold change ratio $\pm$ s.e.m. and DNA binding activity is displayed as the mean optical density (at 450 nm) minus the blank $\pm$ s.e.m. Preterm non-infected expression was compared with CAM expression by two-sample comparison (Students *t*-test). Significant difference (P value < 0.05) as compared with PT no infection is denoted with a *. Representative choriodecidua western blot images for PPARA, PPARG, RXRA, tubulin, and β -actin are displayed in (j). Lanes 1, 4, and 7 are PT non-infected. Lanes 2, 5, and 8 are PT CAM (Stage 1). Lanes 3, 6, and 9 are PT CAM (Stage 2).

increase in DNA binding activity in association with CAM. Stage 1 and Stage 2 CAM all displayed significantly higher activity in placenta compared with preterm patients without infection ($P < 0.05$). In amnion, PPARA and PPARG DNA binding activity was significantly lower in association with Stage 2 CAM and CAM (1 + 2) compared with idiopathic preterm patients (Fig. 3f). PPARG DNA binding activity did not differ when comparing infected and non-infected patients in the amnion.

Discussion

The present study reports unique observations comparing mid-third trimester to late-third trimester gestations and the expression and activity of PPAR isoforms and expression of RXRA. Specifically, we have shown that compared with term, preterm parturition at ~ 34 weeks gestation is associated with increased

expression of PPARG, and RXRA in the placenta and PPARG and RXRA in the choriodecidua. Preterm CAM, while demonstrating protein degradation in the choriodecidua, was found to be associated with lower expression of choriodecidua PPARA and RXRA mRNA, lower PPARA and PPARG DNA binding activity in the amnion and increased PPARG activity in the placenta when compared with uninfected preterm cases.

In this study, PPARA (mRNA, nuclear protein and activity) and PPARG mRNA expression and activity in placenta, amnion, and choriodecidua were unaltered between preterm and term gestations. PPARG nuclear protein expression in amnion and choriodecidua was also unchanged, indicating that PPARA and PPARG (in the fetal membranes) are not affected by preterm parturition. In support of this, PPARA protein has previously been described as being undetectable in first and second trimester placenta (Waite *et al.* 2000),

while no changes in placental PPARG protein expression between first and third trimester placentas have been reported (Capparuccia *et al.* 2002).

We observed an increase in the expression of PPARG nuclear protein in the preterm placenta which is in contrast to our observed *PPARG* mRNA expression and the findings of Capparuccia *et al.* (2002). This indicates tissue-specific expression patterns and a post-transcriptional modification in PPARG processing. The discrepancy between protein findings are probably due to different protein preparation techniques, for example we used nuclear fractions to show nuclear receptor localization compared with the whole cell lysates used by Capparuccia *et al.* (2002). RXRA expression was also observed to be increased in the placenta (mRNA and protein) and choriodecidua (mRNA) at preterm compared with term, demonstrating the co-operative partnership between PPARs and RXRA. By contrast, Wang *et al.* (2002) reported increased placental RXRA mRNA expression with increasing gestational age in rats.

PPARD mRNA and protein expression in the placenta and choriodecidua are increased during the mid-third trimester and then decline significantly at term. Like PPARG and PPARG, however, we saw no change in PPARD DNA binding activity. Therefore, all PPAR isoform DNA binding transcriptional activities are unaffected by gestational age (comparing mid- to late-third trimester). Suggesting that PPARs may be influencing gene expression of non-PPRE targets in a non-DNA binding capacity, including by post-translational modifications, transrepression or sequestering of co-activator proteins (Bensinger & Tontonoz 2008). Increased expression of *PPARD* mRNA was found to be significantly related to longer fetal membrane rupture duration, a characteristic significantly associated to prematurity. By contrast, others have reported that PPARD mRNA and protein in the placenta either increase with increasing gestational age or remain unchanged (Wang *et al.* 2002, Rodie *et al.* 2005). The study by Rodie *et al.* (2005) compared first trimester to term placenta, whereas we examined mid- to late-third trimester gestational tissues, thus identifying a novel change in PPARD expression.

We suggest that the higher expression of PPARD, PPARG and RXRA seen in our preterm groups may represent a PROM-related mechanism opposed to spontaneous fetal membrane rupture with labor. The women included in our investigation all went into spontaneous labor (collected post-labor), therefore we can conclude that the changes observed are not labor-associated. Clinically, the main differences between the preterm and term women were PROM and consequently longer fetal membrane rupture duration in the preterm group. PROM is a complication of pregnancy that can occur at any gestation; however, it is associated with 20–50% of preterm births (French & McGregor 1996). The causes of PROM are numerous, and in part are

caused by apoptosis and inflammatory cytokines (Fortunato & Menon 2001, Menon & Fortunato 2004, Reti *et al.* 2007). Increased expression or activation of PPARs have been associated with apoptosis (Keelan *et al.* 2001, Yang & Frucht 2001, Wieser *et al.* 2008) and pro-inflammatory cytokines (Ackerman *et al.* 2005).

Preterm labor often results from CAM (Fahey 2008). Bacterial infections evoke an acute inflammatory response, which in turn is modulated by PPAR (Deleive *et al.* 2001, Daynes & Jones 2002). CAM infection is confined to the fetal membranes, either chorion alone (Stage 1) or both the amnion and chorion (Stage 2), therefore the lack of PPAR:RXR expression response observed in the placenta is not surprising. In contrast to our original hypothesis, PPARG and PPARG DNA binding activity increased in association with CAM in the placenta. It is possible that the increased PPAR transcriptional activity may be a compensatory mechanism, whereby inflammatory products released from the fetal membranes may be acting in a paracrine fashion to assist in controlling the inflammatory insult. In support of this hypothesis, we have previously shown that PPAR and RXRA expression is increased at term during active labor, at the height of parturition-related inflammatory activity, compared with before labor onset and post-labor (Holdsworth-Carson *et al.* 2009). Furthermore, in a non-gestational tissue model, PPAR expression was enhanced in response to infection with *Chlamydia pneumoniae* (Kim *et al.* 2008). Therefore, PPAR DNA binding activity does not necessarily correlate to receptor expression, indicating that regulation of PPAR activity is controlled independent of transcription factor bioavailability.

Stage 2 preterm CAM was associated with a decrease in PPARG and PPARG DNA binding activity in amnion. Furthermore, choriodecidual CAM (1+2) samples were associated with lower expression of PPARG and RXRA mRNA. Similarly, investigations in mouse adipose and kidney tissues with bacterial endotoxin exposure have demonstrated reduced PPARG, PPARG, and RXRA mRNA expression (Hill *et al.* 1997, Feingold *et al.* 2008). Although DNA binding activity remained unaltered, the CAM-induced decrease in mRNA expression suggests that the anti-inflammatory properties of the PPAR and RXR may also be diminished in the choriodecidua. We propose that the decrease in PPAR in fetal membranes with CAM may activate the pro-inflammatory mechanisms associated with acute inflammation, thus lending supports our hypothesis that dysregulation of PPAR expression could be associated with infection-complicated preterm deliveries.

In summary, the data obtained are consistent with the hypothesis that the expression of PPARG, PPARG, and RXRA in placenta and choriodecidua are modified in association with preterm spontaneous vaginal delivery. This heightened expression may be a consequence of PROM. CAM-associated preterm

cases, although associated with protein degradation in choriodecidua, demonstrated decreased *PPARA* and *RXRA* mRNA. PPAR DNA binding activity was found to be increased in the placenta and decreased in the amnion in association with preterm CAM. These data demonstrate potential divergent roles for PPARs and RXRA in preterm parturition and associated complications, with tissue-specific and isoform-specific mechanisms involved. Further studies are, however, required to uncover the exact role of PPAR in human preterm labor, with and without histological CAM.

Materials and Methods

Reagents

Mouse monoclonal β -actin antibody (A5316) and TRI reagent were supplied by Sigma. Pancreatic deoxyribonuclease (DNase I) was supplied by GE Healthcare (Rydalmore, NSW, Australia). The following antibodies and reagents were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA) ponceau-S, western blotting luminol reagent, rabbit polyclonal *PPARA* sc-9000, rabbit polyclonal *PPARD* (*PPAR*- $\beta(\delta)$) sc-7197, rabbit polyclonal *PPARG* sc-7196, rabbit polyclonal *RXRA* sc-553, mouse monoclonal β -tubulin sc-5274, goat anti-rabbit IgG-HRP sc-2004 and goat anti-mouse IgG-HRP sc-2005. iScript cDNA synthesis kit was purchased from Bio-Rad Laboratories. Predesigned bioinformatically validated QuantiTect primer assays for 18s QT00199367, β -actin QT00095431, *PPARA* QT00017451, *PPARD* QT00078064, *PPARG* QT00029841, and *RXRA* QT00005726 were purchased from Qiagen. SensiMixPlus SYBR and fluorescein was purchased from Quantace (Alexandria, NSW, Australia). Coomassie Plus Protein Assay and BCA Protein Assay reagents were from Pierce (Rockford, IL, USA). *PPARA*, -D, -G complete transcription factor assay kit was purchased from Cayman Chemical (Ann Arbor, MI, USA).

Tissue collection and preparation

Human placentae and attached fetal membranes ($n=14$) were obtained (with the Research Ethics Committee of Mercy Health and Aged Care approval) from consenting women who delivered singleton infants at preterm (<37 weeks gestation) with and without CAM. Term placentae and fetal membranes ($n=6$) were collected from women with uncomplicated pregnancies, who went into natural labor, with spontaneous rupture of the fetal membranes and delivered vaginally. Term patients exhibited no clinical signs of infection. Refer to Table 1 for the clinical details of the patients.

Tissues were obtained within 10 min of delivery. A placental lobule was removed from the central region of the placenta. The basal plate and chorionic surface were removed and villous tissue was obtained from the middle cross-section. Placental tissue was bluntly dissected to remove visible connective tissue and calcium deposits. Amnion and choriodecidua were systematically obtained 2 cm away from the peri-placental edge. After several washes in chilled sterile PBS, tissues were snap frozen and stored at -80°C until ready for

mRNA and protein extraction. Before dissection, preterm placenta and fetal membranes were swabbed for microbiological culture investigations. The preterm placentas were then assessed for histopathological evidence of infection. Of the preterm placentae collected ($n=14$), eight had confirmed CAM and the remaining six were classified with normal pathology (no evidence of infection or pathological microflora). Placental inflammatory response stages were classified according to Redline ($n=4$ Stage 1 CAM and $n=4$ Stage 2 CAM; Redline *et al.* 2003, Redline 2006).

RNA extraction and qRT-PCR

Total RNA was extracted from 200 mg frozen tissue using TRI reagent as per manufacturers' instructions and as described previously (Holdsworth-Carson *et al.* 2009). qRT-PCR was performed by the two-step method. RNA ($1\text{ }\mu\text{g}$ DNase I-treated nucleic acid) was converted to cDNA using iScript cDNA synthesis kit. qRT-PCR reactions (final volume of $25\text{ }\mu\text{l}$) consisted of Qiagen QuantiTect primer ($1\text{ }\mu\text{l}$ for placenta and choriodecidua and $2.5\text{ }\mu\text{l}$ for amnion), cDNA (diluted 1:5; $5\text{ }\mu\text{l}$ for placenta and choriodecidua and $10\text{ }\mu\text{l}$ for amnion) and $12.5\text{ }\mu\text{l}$ SensiMixPlus SYBR and fluorescein. An internal β -actin control was included in each reaction plate. 18s ribosomal RNA primers were used for normalization of the data. The cycling conditions for qRT-PCR were as described previously (Holdsworth-Carson *et al.* 2009). The qRT-PCR was performed using a MiniOpticon 2-colour real-time PCR system (Bio-Rad Laboratories). Opticon Monitor software (Version 3.1.32; MJ Geneworks Inc., and Bio-Rad Laboratories) was used for C_T measurements and melt-curve analysis. Relative mean fold change expression ratios were calculated using the $2^{-\Delta\Delta C_T}$ method using the term group or non-infected preterm group as the calibrators respectively (Livak & Schmittgen 2001). For CAM amnion analysis, one patient was excluded because the values were determined to be outliers (>20-fold excess of the mean), therefore the n value for the Stage 2 CAM group was 3.

Protein extraction and western blotting

Nuclear proteins for term versus preterm western blotting analysis were extracted as described previously (Lappas *et al.* 2002a). Nuclear protein concentrations were determined using the Coomassie Plus Protein Assay. Whole cell protein extraction for preterm infection studies were prepared as described previously (Lappas *et al.* 2007, Reti *et al.* 2007). Whole cell protein concentrations were determined using the BCA Protein Assay. Both nuclear and whole-cell protein lysates were prepared in the presence of protease inhibitors ($10\text{ }\mu\text{g/ml}$ aprotinin, $5\text{ }\mu\text{g/ml}$ leupeptin, 1 mM AEBSEF, 1 mM Na_3VO_4 and 1 mM NaF).

Fifty micrograms of protein were resolved in 10% SDS-PAGE gels at 200 V for 1 h and transferred onto PVDF membrane (Millipore Corporation, Billerica, MA, USA) at 105 mAmps for 1 h . The membrane was blocked with 5% (w/v) skim milk in TBST for 1 h at room temperature. Primary antibody incubations occurred for between 2 h at room temperature to 48 h at 4°C , depending on tissue type and antigen. Dilution of

primary antibody was made in 5% (w/v) skim milk in TBST. All primary antibody dilutions were 1:250. HRP-conjugated secondary antibody diluted between 1:2500 and 1:5000 was incubated for 30 min at room temperature. Western blot luminal was used to detect the chemiluminescent signal. Nuclear western blots were normalized using reversible total protein stain, ponceau-S. Whole cell immunoblots were normalized using β -actin. Densitometry values were measured using quantity one software (Version 4.6.5; Bio-Rad Laboratories). Expression of proteins was calculated as a ratio, with the term group or non-infection group serving as the reference value.

PPAR transcription factor DNA binding activity assay

Nuclear protein was prepared as described previously (Lappas *et al.* 2002a) and protein content was determined by Coomassie Plus Protein Assay (Pierce). Transcription factor DNA binding activity was measured using the commercially available PPARA, -D, -G complete transcription factor assay kit following the manufacturers' instructions (Cayman Chemical). Briefly, a dsDNA sequence containing the PPRE is linked onto the bottom of wells (96-well plate). PPARs within the nuclear fraction bind specifically to this sequence and isoforms are detected using primary antibodies directed against the individual PPARs. Clarified cell lysates were supplied for each PPAR isoform and acted as effective positive controls for PPAR DNA binding. Specificity of binding was also demonstrated using wells with no nuclear protein added. In these wells, no binding was detected (data not shown). Binding activity was measured at 450 nm (minus the blank).

Statistical analysis

Statistical analyses were performed using a commercially available statistical software package, Statgraphics Plus (Version 3.1; Statistical Graphics Corp., Rockville, MD, USA). Patient clinical information, qRT-PCR, western blot densitometry and PPAR DNA binding activity were analyzed by two-sample comparison and t-test to compare the means. Linear regression analyses were used to evaluate the relationship between fetal membrane rupture duration and expression studies (95% confidence). Data are expressed as mean $\pm$ s.e.m. Statistical difference was indicated by a *P* value of <0.05 .

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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