A novel role for GSK3 in the regulation of the processes of human labour

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Abstract

Preterm birth remains the largest single cause of neonatal death and morbidity. Infection and/or inflammation are strongly associated with preterm delivery. Glycogen synthase kinase 3 (GSK3) is known to be a crucial mediator of inflammation homeostasis. The aims of this study were to determine the effect of spontaneous human labour in foetal membranes and myometrium on GSK3α/β expression, and the effect of inhibition of GSK3α/β on pro-labour mediators in foetal membranes and myometrium stimulated with Toll-like receptor (TLR) ligands and pro-inflammatory cytokines. Term and preterm labour in foetal membranes was associated with significantly decreased serine phosphorylated GSK3α and β expression, and thus increased GSK3 activity. There was no effect of term labour on serine phosphorylated GSK3β expression in myometrium. The specific GSK3α/β inhibitor CHIR99021 significantly decreased lipopolysaccharide (ligand to TLR4)-stimulated pro-inflammatory cytokine gene expression and release; COX2 gene expression and prostaglandin release; and MMP9 gene expression and pro MMP9 release in foetal membranes and/or myometrium. CHIR99021 also decreased FSL1 (TLR2 ligand) and flagellin (TLR5 ligand)-induced pro-inflammatory cytokine gene expression and release and COX2 mRNA expression and prostaglandin release. GSK3β siRNA knockdown in primary myometrial cells was associated with a significant decrease in IL1β and TNFα-induced pro-inflammatory cytokine and prostaglandin release. In conclusion, GSK3α/β activity is increased in foetal membranes after term and preterm labour. Pharmacological blockade of the kinase GSK3 markedly reduced pro-inflammatory and pro-labour mediators in human foetal membranes and myometrium, providing a possible therapeutics for the management of preterm labour.


Introduction

Preterm birth is the prevalent cause of mortality and morbidity in newborn infants (McCormick 1985). Potential complications that can arise include neonatal sepsis and foetal respiratory distress (Lawn et al. 2005). The survivors of preterm birth also have increased risk of neurodevelopmental impairments and gastrointestinal and respiratory complications (Goldenberg et al. 2008). While ~ 30% of preterm deliveries are iatrogenic, 70% of preterm deliveries are spontaneous, relating to preterm labour or preterm rupture of the membranes (Goldenberg et al. 2008). As there are no effective methods of prediction or prevention (Norman & Shennan 2013), it is essential that our understanding of the mechanisms involved in the initiation and progression of labour is improved in order to develop therapeutics.

For the successful culmination of labour and delivery, foetal maturation, cervical ripening and dilatation, uterine contractions and foetal membrane rupture are necessary. These events are potentiated by the production of inflammatory mediators, such as pro-inflammatory cytokines, phospholipid metabolites, including prostaglandins, and extracellular matrix remodelling enzymes, such as matrix metalloproteinase 9 (MMP; Bowen et al. 2002, Olson 2003, Lappas & Rice 2004, Weiss et al. 2007, Christiaens et al. 2008). Preterm birth may occur due to the untimely activation of these processes, regardless of foetal development. Toll-like receptors (TLRs) are key surface molecules that play an essential role in triggering the immune response. The bacterial products which are ligands to TLRs, including the synthetic lipoprotein fibroblast-stimulating lipopeptide (FSL1; TLR2 ligand), lipopolysaccharide (LPS; TLR4 ligand) and flagellin (TLR5 ligand) are known to produce an inflammatory response in human gestational tissues (Lappas et al. 2006, Lim et al. 2014a). To develop therapeutics to stop or delay preterm labour, a greater understanding of the multiple processes that are involved in human parturition is required.

Recent studies have pointed to the pleiotropic serine threonine kinase glycogen synthase kinase 3 (GSK3) as a crucial mediator of inflammation homeostasis (Jope & Johnson 2004, Jope et al. 2007, Beurel et al. 2010).
In mammals, GSK3 exists as two isoforms: GSK3α and β which are structurally similar but encoded by distinct genes and have molecular weights of 51 and 47 kDa respectively (Woodgett 1990). Although the two isoforms of GSK have similar functions, they are not functionally redundant as deletion of GSK3β leads to embryonic lethality at E16 that cannot be rescued by GSKα (Hoeflich et al. 2000). Originally involved in insulin signalling (Woodgett 1990), GSK3, particularly GSK3β, has now been shown to be essential for a number of critical cellular functions, such as cell cycle control, apoptosis, embryonic development, cell differentiation and adhesion (Frame & Cohen 2001, Grimes & Jope 2001, Doble & Woodgett 2003, Jope & Johnson 2004, Wang et al. 2011a). As such, GSK3 has been implicated in a number of diseases, including type 2 diabetes, cancer and a number of inflammatory diseases (Jope et al. 2007, Gurrieri et al. 2010), with GSK inhibitors being actively developed as therapeutics for the treatment of these various disorders.

In vitro, GSK3α/β inhibition has been shown to suppress inflammation in response to a variety of stimuli such as tumor necrosis factor alpha (TNFα), interleukin 1 beta (IL1β), bacterial endotoxin LPS and cigarette smoke (Takada et al. 2004, Martin et al. 2005, Barton-Pai et al. 2011). Stimulation of Gsk3β−/− mouse embryonic fibroblast with LPS (Martin et al. 2005) or TNFα (Steinbrecher et al. 2005) was associated with a reduction in the production of pro-inflammatory cytokines. Of note, inhibitors of GSK3 have been reported to exert beneficial effects in many different, preclinical animal models of disease. Mice treated with GSK3 inhibitors are protected from LPS-induced septic shock (Martin et al. 2005) and have increased survival rates to Francisella tularensis infection (Zhang et al. 2009). In addition, GSK3 inhibitors reduce inflammation and tissue injury in a rat model of acute colitis (Whittle et al. 2006).

A number of mechanisms have been identified that contribute to regulating the actions of GSK3 (Grimes & Jope 2001). The most well-defined regulatory mechanism is inhibition of the activity of GSK3 by phosphorylation of a regulatory serine in either of the two isoforms of GSK3, Ser9 in GSK3β or Ser21 in GSK3α (Woodgett 1990). To our knowledge, the expression or the role of GSK3α/β has not been investigated in human foetal membranes and myometrium. We hypothesised that human labour and delivery would be associated with decreased phosphorylated GSK3α/β, and thus increased GSK3 activity, in foetal membranes and myometrium. In addition, we hypothesised that inhibition of GSK3, using a chemical inhibitor or siRNA, would be associated with decreased expression and secretion of pro-labour mediators in the presence of bacterial infection or pro-inflammatory cytokines. Thus, the aims of this study were i) to establish the effect of human labour on phosphorylated GSK3α/β expression in human foetal membranes and myometrium and ii) to determine the effect of inhibition of GSK3α/β on LPS, flagellin and FSL1-induced expression of pro-inflammatory and pro-labour mediators in human foetal membranes and myometrium.

Materials and methods

Tissue collection

The Research Ethics Committee of Mercy Hospital for Women approved this study. Informed written consent was obtained from all participating women. All tissues were obtained from women who delivered healthy, singleton infants. All tissues were brought to the research laboratory and processed within 15 min of delivery. Women with any underlying medical conditions such as diabetes, asthma, polycystic ovarian syndrome, preeclampsia and macrovascular complications were excluded. In addition, women with multiple pregnancies, obese women, foetuses with chromosomal abnormalities were excluded.

Foetal membranes were obtained from women i) at term no labour undergoing elective Caesarean section (indications for Caesarean section were breech presentation and/or previous Caesarean section; n=8 patients) and ii) term after spontaneous labour, spontaneous membrane rupture and normal vaginal delivery (n=8 patients). The foetal membranes were obtained ~2 cm from the periplacental edge. Clinical details of the patients are detailed elsewhere (Lappas et al. 2011). There was no difference in maternal age and BMI, parity or gestational age of the patients recruited. The mean duration of labour was 6.5 ± 0.7 h. The tissue samples were snap frozen in liquid nitrogen and immediately stored at −80 °C for the analysis of phosphorylated GSK3α/β protein expression by western blotting.

The foetal membranes were also obtained from women at i) preterm no labour undergoing Caesarean section (n=8 patients) and ii) after spontaneous preterm labour and normal vaginal delivery (n=8 patients). All placentas collected at preterm gestations were swabbed for microbiological culture investigations and histopathological examination. The patients with chorioamnionitis were excluded from the analyses. Women with preeclampsia, preexisting diabetes, asthma, multiple pregnancies and foetuses with chromosomal abnormalities were also excluded. Indications for preterm delivery (in the absence of labour) were placenta praevia, placental abruption, antepartum haemorrhage (APH) or Rhesus isoimmunisation. For the preterm labour study, foetal membranes from both the non-labouring and after-labour preterm groups were obtained 2 cm from the periplacental edge. Clinical details of the patients are detailed elsewhere (Lim et al. 2013a). The tissue samples were snap frozen in liquid nitrogen and immediately stored at −80 °C for the analysis of phosphorylated GSK3α/β expression by western blotting.

Myometrium was obtained from consenting women at the time of term Caesarean section (≥37 weeks’ gestation). Myometrial biopsies were collected from two groups of women: i) no labour-pregnant women undergoing elective Caesarean section in the absence of labour (n=8 patients) and ii) in labour-pregnant women who were delivered during active labour (labour was defined as the presence of regular uterine contractions (every 3–4 min) resulting in cervical effacement...
and dilation) (n=8 patients). Women were excluded from the study if they had a multiple pregnancy or evidence of an active infection. A myometrial biopsy was obtained from the upper margin of the lower uterine segment incision during the Caesarean section. Tissue samples snap frozen in liquid nitrogen and immediately stored at −80°C for the analysis of phosphorylated GSK3α/β protein expression by western blotting.

**Tissue explant culture**

Tissue explants were performed as described previously (Lappas et al. 2013, Lim et al. 2014a) to determine the effect of the GSK3α/β inhibitor CHIR99021 on pro-inflammatory and pro-labour mediators in foetal membranes and myometrium. Fresh foetal membranes and myometrium were obtained from women who delivered healthy, singleton infants at term (37–41 weeks gestation) undergoing elective Caesarean section in the absence of labour. The tissues were placed in DMEM at 37°C in a humidified atmosphere of 8% O₂ (foetal membranes) or 21% O₂ (myometrium) and 5% CO₂ for 1 h. The tissues were blotted dry on sterile filter paper and transferred to 24-well tissue culture plates (100 mg wet weight/well for foetal membranes and 50 mg wet weight/well for myometrium). The explants were incubated in 1 ml DMEM containing 100 U/ml penicillin G and 100 μg/ml streptomycin. The tissues were incubated in the presence of 10 μM CHIR99021 (Sapphire Bioscience, Waterloo, NSW, Australia) with 10 μg/ml LPS (derived from *Escherichia coli* 026:B6; Sigma–Aldrich), 250 ng/ml FSL1 (Life Research, Scoresby, VIC, Australia) and 1 μg/ml flagellin (Life Research) for 20 h. CHIR99021 was dissolved in dimethylsulfoxide (DMSO); thus, all treatments contained DMSO at a final concentration of 0.05% vol/vol. The concentration of CHIR99021 was based on past studies (Wang et al. 2011b, Balamurugan et al. 2013) and the bacterial products were based on our previously published studies (Lappas et al. 2013, Lim et al. 2014a). After incubation, tissue and media were collected separately and stored at −80°C for further analysis as detailed below. Experiments were performed on myometrium and foetal membranes from six patients.

In this study, we used the GSK3α/β inhibitor CHIR99021, an aminopyrimidine which is the most selective inhibitor of GSK3α/β reported to date, exhibiting >500-fold selectivity for GSK3 over closely related kinases, and is thought to inactivate GSK3 protein expression by western blotting as detailed below. The experiments were carried out in the myometrium obtained from five patients.

**Western blotting**

The tissue lysates and western blotting were prepared as described previously (Lappas et al. 2011). In brief, 30 mg protein were separated on polyacrylamide gels (Bio-Rad Laboratories) and transferred onto PVDF. Protein expression was identified by comparison with the mobility of protein standard. The membranes were viewed and analysed using the ChemiDoc XRS System (Bio-Rad Laboratories). Semi-quantitative analysis of the relative density of the bands in western blottings was performed using Quantity One 4.2.1 image analysis software (Bio-Rad Laboratories). For Fig. 1, the levels of phosphorylated GSK3α/β (1:1000; #9331, Cell Signalling, Beverly, MA, USA) were normalised to the levels of total GSK3α (1:1000; #9315, Cell Signalling); fold change was calculated relative to the no-labour group. For Fig. 8A, the levels of total GSK3β (1:1000; #9315, Cell Signalling) were normalised to the levels of β-actin.

**Gene silencing of GSK3β with siRNA**

Primary myometrial cells were used to investigate the effect of siRNA-mediated gene silencing of GSK3β on pro-labour mediators. Myometrium was obtained from women who delivered healthy, singleton infants at term (37–41 weeks gestation), undergoing elective Caesarean section in the absence of labour. The cells were isolated and cultured as described previously for myometrium (Lappas et al. 2013, Lim et al. 2013b). The cells at ~50% confluence were transfected using SilenceMag reagent according to the manufacturer’s guidelines (Oz Biosciences, Marseille, France) and as described previously (Lim et al. 2013a,b). GSK3β siRNA (HSC.RNAi. N001146156.12) was obtained from Integrated DNA Technologies (IDT, Coralville, IA, USA), while negative control (NC) siRNA was obtained from Sigma–Aldrich. The cells were transfected with 200 nM GSK3β or 200 nM NC siRNA in DMEM/F-12 for 48 h. The medium was then replaced with DMEM/F-12 with or without 1 ng/ml IL1β or 10 ng/ml TNFα, and the cells were incubated at 37°C for an additional 24 h. The cells were collected and stored at −80°C until assayed for mRNA expression by quantitative RT-PCR (qRT-PCR) and protein expression by western blotting as detailed below. Media were collected and stored at −80°C until assayed for cytokine release as detailed below. The cell viability was assessed by the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) proliferation assay as described previously (Lim et al. 2014b). The experiments were carried out in the myometrium obtained from five patients.

**RNA extraction and qRT-PCR**

Total RNA was extracted from tissues using TRIzol reagent according to manufacturer’s instructions (Bioline, Alexandria, NSW, Australia), as previously described (Lim et al. 2013a,b). RNA concentration and purity were measured using a NanoDrop ND1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). RNA was converted to cDNA using the Tetro cDNA Synthesis Kit (Bioline) according to the manufacturer’s instructions. The cDNA was diluted to 50-fold, and 4 μl of this was used to perform RT-PCR using the Sensimix Plus SYBR Green (Bioline) and 200 nM of pre-designed and validated QuantiTect Primers (Qiagen). The RT-PCR was performed using a CFX384 Real-Time PCR

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Detection System (Bio-Rad Laboratories). Average gene C_T values were normalised to the average GAPDH C_T values of the same cDNA sample and fold differences were determined using the comparative C_T method. For the explant studies, fold change was calculated relative to LPS, FSL1 or flagellin, which was set at 1. For the cell siRNA studies, fold change was calculated relative to IL1b- or TNFα-stimulated NC siRNA transfected cells, which was set at 1.

**Cytokine and prostaglandin assays**

Assessment of IL6, IL8 and TNFα cytokine release was performed using CytoSet sandwich ELISA, according to the manufacturer’s instructions (Life Technologies). The limit of detection of the IL6, IL8 and TNFα assays was 16, 12 and 7.2 pg/ml respectively. The release of IL1β was performed using DuoSet sandwich ELISA according to the manufacturer’s instructions (R&D Systems, Minneapolis, MN, USA). The limit of detection of the IL1β assay was 1.9 pg/ml. 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 Sapphire Bioscience). The limit of detection of the PGE2 and PGF2α assays was 16 and 60 pg/ml respectively. For all assays, the interassay and intraassay coefficients of variation were <10%.
**NfκB luciferase assay**

A luciferase assay was used to determine possible interactions between GSK3 and NfκB, as described previously (Lappas 2013, Lim et al. 2013a), with some modifications. For these studies, primary myometrial cells, prepared as described previously, at ~70% confluence were transfected with the FuGENE HD Transfection Reagent (Promega) according to the manufacturer’s guidelines. The cells were co-transfected with 0.15 μg NfκB reporter construct (Qiagen) plus 200 nM NC siRNA or GSK3β siRNA for 48 h. The medium was then replaced with DMEM/F-12 with 0.5% BSA, with or without 1 ng/ml IL1β or 10 ng/ml TNFz, and the cells were incubated at 37 °C for an additional 24 h. The cells were harvested in lysis buffer, and the lucinescence activity was measured using the Luciferase Reporter Assay Kit (Life Research) and Renilla Luciferase Flash Assay Kit (Thermo Fisher Scientific, Scoresby, VIC, Australia) as instructed. The ratio of the firefly luciferase level to the Renilla luciferase level was determined and the results are expressed as a ratio of normalised luciferase activity of IL1β- or TNFz-stimulated NfκB reporter plus NC siRNA-transfected cells, which was set as 1. The experiments were performed from the myometrium obtained from five patients.

**Statistical analyses**

Statistics was performed on the normalised data unless otherwise specified. All statistical analyses were undertaken using GraphPad Prism (GraphPad Software, La Jolla, CA, USA). For two-sample comparisons, either a paired or unpaired Student’s t-test was used to assess statistical significance between normally distributed data; otherwise, the nonparametric Mann–Whitney U (unpaired) or the Wilcoxon (matched pairs) tests were used. For all other comparisons, the homogeneity of data was assessed by the Bartlett’s test, and when significant, the data were logarithmically transformed before further analysis using a one-way ANOVA (with Fishers least significant difference (LSD) post-hoc testing to discriminate among the means). Statistical significance was ascribed to P value <0.05. Data were expressed as mean±S.E.M.

**Results**

**Effect of human term or preterm labour on phosphorylated GSK3α/β expression in foetal membranes and myometrium**

Western blotting analysis was carried out to determine the effect of human term or preterm labour on GSK3α/β activity. The inactivation of GSK3 activity can be induced by phosphorylation at one of its N-terminal serine (Ser) residues: Ser21 for GSK3α (51 kDa) and Ser9 for GSK3β (47 kDa). All data were normalised to total GSK3β expression.

The foetal membranes were obtained at term Caesarean section in the absence of labour (no labour) or after using GraphPad Prism (GraphPad Software, La Jolla, CA, USA). For two-sample comparisons, either a paired or unpaired Student’s t-test was used to assess statistical significance between normally distributed data; otherwise, the nonparametric Mann–Whitney U (unpaired) or the Wilcoxon (matched pairs) tests were used. For all other comparisons, the homogeneity of data was assessed by the Bartlett’s test, and when significant, the data were logarithmically transformed before further analysis using a one-way ANOVA (with Fishers least significant difference (LSD) post-hoc testing to discriminate among the means). Statistical significance was ascribed to P value <0.05. Data were expressed as mean±S.E.M.

**Figure 2** Effect of a GSK3 inhibitor on LPS-stimulated pro-inflammatory cytokine expression in foetal membranes. Human foetal membranes obtained from women not in labour at term Caesarean section were incubated with 10 μg/ml LPS in the absence or presence of 10 μM CHIR99021 (CHIR) for 20 h (n=6 patients). (A, B, C and D) Gene expression for TNFα, IL1β, IL6 and IL8 was analysed by qRT-PCR. Gene expression was normalised to GAPDH mRNA expression and the fold change was calculated relative to LPS. Data displayed as mean±S.E.M. *P<0.05 vs LPS (one-way ANOVA). (E, F, G and H) The incubation medium was assayed for concentration of TNFα, IL1β, IL6 and IL8 release by ELISA. Data displayed as mean±S.E.M. *P<0.05 vs LPS (one-way ANOVA).
spontaneous labour and membrane rupture (after labour) \((n=8\) patients/group). There was no change in phosphorylated serine GSK3\(\alpha\) expression in the foetal membranes after labour (Fig. 1A). On the other hand, phosphorylated GSK3\(\beta\) was significantly higher in non-labouring foetal membranes compared with labouring foetal membranes (Fig. 1B). A representative western blotting is shown in Fig. 1C.

Myometrium was obtained at term Caesarean section in the absence of labour (no labour) or during spontaneous labour onset (in labour) \((n=8\) patients/group). There was no change in phosphorylated serine GSK3\(\alpha\) (Fig. 1D) or GSK3\(\beta\) (Fig. 1E) expression. A representative western blotting is shown in Fig. 1F.

The foetal membranes were obtained from women at preterm Caesarean section in the absence of labour or after spontaneous preterm labour and normal vaginal delivery \((n=8\) patients/group). Women with preterm deliveries were matched for maternal age, BMI, gravidity, parity and gestational age. Phosphorylated serine GSK3\(\alpha\) (Fig. 1G and I) and GSK3\(\beta\) (Fig. 1H and I) expression was significantly lower after spontaneous preterm labour. Western blotting could not be performed on foetal membranes from the chorioamnionitis group due to protein degradation (Holdsworth-Carson et al. 2009).

**Figure 3** Effect of a GSK3 inhibitor on LPS-stimulated pro-inflammatory cytokine expression in myometrium. Human myometrium obtained from women not in labour at term Caesarean section was incubated with 10 \(\mu\)g/ml LPS in the absence or presence of 10 \(\mu\)M CHIR99021 (CHIR) for 20 h \((n=6\) patients). (A, B, C and D) Gene expression for TNF\(\alpha\), IL1\(\beta\), IL6 and IL8 was analysed by qRT-PCR. Gene expression was normalised to GAPDH mRNA expression and the fold change was calculated relative to LPS. Data displayed as mean\(\pm\)S.E.M. *\(P<0.05\) vs LPS (one-way ANOVA). (E, F, G and H) The incubation medium was assayed for the concentration of TNF\(\alpha\), IL1\(\beta\), IL6 and IL8 release by ELISA. Data displayed as mean\(\pm\)S.E.M. *\(P<0.05\) vs LPS (one-way ANOVA).

**Effect of GSK3 inhibitor on pro-labour mediators in human foetal membranes and myometrium in the presence of bacterial endotoxin LPS**

We next sought to determine the effect of a specific GSK3 inhibitor, CHIR99021, on the expression and secretion of pro-inflammatory and pro-labour mediators in foetal membranes and myometrium in the presence of bacterial endotoxin LPS.

As expected, treatment with LPS induced a significant increase in TNF\(\alpha\), IL1\(\beta\), IL6 and IL8 mRNA expression and secretion in both foetal membranes (Fig. 2) and myometrium (Fig. 3). In foetal membranes, co-treatment with CHIR99021 significantly attenuated LPS-induced IL1\(\beta\), IL6 and IL8 mRNA expression and secretion and TNF\(\alpha\) gene expression (Fig. 2). In myometrium, co-treatment with CHIR99021 significantly attenuated LPS-induced gene expression and secretion of TNF\(\alpha\), IL1\(\beta\), IL6 and IL8 (Fig. 3). For both foetal membranes and myometrium, there was no effect of CHIR99021 on cytokine release under basal conditions (data not shown).

Myometrium treated with LPS significantly increased COX2 mRNA expression (Fig. 4A), and the release of PGE\(_2\) (Fig. 4B) and PGF\(_{2\alpha}\) (Fig. 4C). Co-treatment with CHIR99021 was associated with a significant dampening of COX2 mRNA expression, and PGE\(_2\) and PGF\(_{2\alpha}\) release in response to LPS. There was no effect of LPS...
with or without CHIR99021 on COX1 mRNA expression (data not shown). There was also no effect of CHIR99021 on basal prostaglandin release (data not shown).

Myometrium treated with LPS significantly increased MMP9 mRNA expression (Fig. 4D) and secretory pro MMP9 expression (Fig. 4E and F). Co-treatment with CHIR99021 significantly decreased LPS-stimulated expression of MMP9 mRNA and secretory pro MMP9. There was no effect of LPS or CHIR99021 on MMP2 mRNA expression or the secretion of pro MMP2 (data not shown).

**Effect of a GSK3 inhibitor on pro-labour mediators in human foetal membranes and myometrium in the presence of the bacterial products FSL1 and flagellin**

Our previous studies have shown that bacterial products FSL1 (TLR2 ligand) and flagellin (TLR5 ligand) increase pro-labour mediators in human gestational tissues (Lim et al. 2014a). Thus, we sought to determine whether GSK3 also regulates TLR2- and TLR5-induced inflammation in foetal membranes and myometrium. As expected, treatment with FSL1 (Fig. 5A, B, C and D) and flagellin (Fig. 6A, B, C and D) induced a significant increase in IL6 and IL8 mRNA expression and secretion in foetal membranes. Co-treatment with CHIR99021 significantly attenuated FSL1- and flagellin-induced cytokine gene expression and secretion in foetal membranes. Similarly, in myometrium, treatment with FSL1 (Fig. 5E, F, G and H) or flagellin (Fig. 6E, F, G and H) induced a significant increase in IL6 and IL8 mRNA expression and secretion. Co-treatment with CHIR99021 significantly decreased FSL1- or flagellin-induced IL6 and IL8 gene expression and secretion. For both foetal membranes and myometrium, the release of TNFα and IL1β in the presence of FSL1 or flagellin was below the sensitivity of the assay.

Treatment with FSL1 significantly increased COX2 mRNA expression (Fig. 7A) and release of PGE2 (Fig. 7B) and PGF2α (Fig. 7C). Treatment with CHIR99021 significantly decreased FSL1-induced COX2 mRNA expression and release of PGE2 and PGF2α. Treatment with flagellin significantly increased COX2 mRNA expression (Fig. 7D) and release of PGE2 (Fig. 7E) and PGF2α (Fig. 7F). Co-treatment with CHIR99021 significantly decreased flagellin-induced COX2 mRNA expression, but not the release of PGE2 (P=0.06) and PGF2α (P=0.06).

**Effect of GSK3β siRNA on pro-labour mediators in primary myometrium cells**

The final aim of this study was to determine the effect of GSK3β siRNA on pro-inflammatory and pro-labour mediators. We specifically used siRNA against the β isoform as studies in non-gestational tissues have shown
that the β isoform is more important in the context of inflammation (Takada et al. 2004, Dugo et al. 2005, Martin et al. 2005, Jope et al. 2007). For these studies, we used primary myometrial cells isolated from fresh myometrial tissue. The efficacy of transfection was analysed by western blotting and a representative image is presented in Fig. 8A; protein expression of GSK3β was decreased by ~50% in GSK3β siRNA-transfected cells. MTT cell viability assay showed that when compared with NC siRNA-transfected cells, there was no significant effect of GSK3β siRNA on cell viability (absorbance at 570 nM for NC siRNA 0.27 ± 0.20 vs GSK3β siRNA 0.33 ± 0.21).

For subsequent experiments, after siRNA transfection, cells were treated with either IL1β or TNFα, as these pro-inflammatory cytokines are known to be increased in human gestational tissues with labour and can induce preterm labour in animal models. Figure 8 also describes the effects of GSK3β knockdown by siRNA on IL1β-induced secretion of pro-inflammatory cytokines and prostaglandins. Compared with basal NC siRNA, there was a significant increase in IL6 (Fig. 8B), IL8 (Fig. 8C) and PGF2α (Fig. 8F) release in NC siRNA-transfected cells treated with IL1β. This increase was significantly attenuated in GSK3β siRNA-transfected cells. Similarly, in cells treated with TNFα, the increase in IL6 (Fig. 8D) and IL8 (Fig. 8E) release was significantly reduced in GSK3β siRNA-transfected cells. The effect of GSK3β siRNA on IL1β-induced PGE2 release was unable to be detected, being below the assay reading range. Both PGE2 and PGF2α were unable to be detected in transfected cells treated with TNFα.

**Silencing of GSK3β represses NFκB p65 transcriptional activity**

To determine whether GSK3β regulates pro-labour mediators in primary myometrial cells through NFκB, a luciferase assay was used as we have described previously (Lappas 2013). As shown in Fig. 9A, in NC siRNA transfected myometrial cells, IL1β significantly increased NFκB p65 luciferase activity. However, luciferase activity was significantly decreased in GSK3β siRNA-transfected cells. Similar results were
obtained with cells treated with TNFα, where cells transfected with GSK3β siRNA decreased TNFα-stimulated NFκB luciferase activity (Fig. 9B).

Discussion

This study provides, for the first time, evidence that GSK3 is an important regulatory protein involved in inflammatory processes in foetal membranes and myometrium. In this work, we showed that GSK3 activity is increased in foetal membranes after term and preterm labour onset and delivery. GSK3 activity, however, was not significantly increased with term labour in myometrium. In this study, tissues were treated in the presence of TLR ligands and bacterial products LPS, FSL1 and flagellin, to mimic preterm labour in order to define the importance of GSK3 in the expression of pro-inflammatory cytokines, prostaglandins and proteases. We found that the chemical inhibitor of GSK3, CHIR99021, could efficiently prevent the expression and release of pro-labour mediators in foetal membranes and myometrium that were stimulated with the TLR4 ligand LPS, the TLR5 ligand flagellin and the TLR2 ligand FSL1. In addition to its role in TLR signalling, we also showed that GSK3 plays a role in IL1β and TNFα signalling. Specifically, in cells transfected with siRNA against GSK3β, there was a significant decrease in IL1β and TNFα-stimulated cytokine and prostaglandin release. Notably, a luciferase assay demonstrated a decrease in NFκB p65 transcriptional activity in cells co-transfected with GSK3β siRNA, suggesting that GSK3 may mediate its pro-inflammatory effects via the NFκB pathway.

GSK3 activity was increased in the foetal membranes obtained after spontaneous term and preterm labour compared with gestational-age matched foetal membranes obtained at Caesarean section in the absence of labour. We were unable to study the effects of preterm labour on myometrium, due to lack of suitable samples. The increase in GSK3 activity in foetal membranes may be a consequence of the labour processes. For example, sterile inflammation plays a central role in the terminal processes of normal human term labour and delivery (Keelan et al. 2003, Osman et al. 2003). Indeed, it has been shown that TNFα induces GSK3 activity in non-gestational tissues (Park et al. 2011). While term labour is due to physiological activation of the labour processes,
preterm labour is the result of pathological insults such as bacterial infection, smoking and APH (Romero et al. 2006). Thus, the increase in GSK3 activity after preterm labour may be due to the bacterial and/or pathological activation of labour processes associated with preterm birth. In support, GSK3 activity is increased with LPS (Martin et al. 2005), cigarette smoke (Cohen & Goedert 2009), and thrombin (Gushiken et al. 2004). Bacterial products such as LPS, FSL1 and flagellin have been shown to increase the expression of pro-inflammatory cytokines, prostaglandins and MMPs in foetal membranes and myometrium (Lappas 2013, Lim et al. 2014a) which participate in the terminal processes of human labour and delivery; i.e. cervical ripening, myometrial contractions and rupture of foetal membranes (Bowen et al. 2002, Olson 2003, Lappas & Rice 2004, Romero et al. 2006, Christiaens et al. 2008). Thus, to determine whether GSK3 regulates bacterial infection-induced inflammation in human gestational tissues, we used the chemical GSK3 inhibitor CHIR99021. The current study describes novel data in that the GSK3 inhibitor CHIR99021 can attenuate the effects of LPS, flagellin and FSL1-induced pro-inflammatory cytokines in foetal membranes and myometrium. Likewise, in non-gestational tissues, inhibition of GSK3 decreases LPS-stimulated TNFα and IL6 production in microglia (Yuskaitis & Jope 2009, Wang et al. 2010) and dendritic cells (Rodionova et al. 2007), and TLR-induced IL6 and IL1β production in monocytes (Martin et al. 2005).

TNFα and IL1β are two pro-inflammatory cytokines that can induce preterm labour in animal models (Sadowsky et al. 2006). They can also incite the production of other labour mediators; increased COX2 expression and prostaglandin synthesis (Molnar et al. 1993, Kniss et al. 1997), and MMPs in the myometrium (Roh et al. 2000) and foetal membranes (Vadillo-Ortega & Estrada-Gutierrez 2005). Thus, to determine if GSK3 also regulates pro-inflammatory and pro-labour mediators in the presence of TNFα or IL1β, we used siRNA against GSK3β in primary myometrial cells. We found that myometrial cells transfected with GSK3β siRNA released significantly less IL6 and IL8 when stimulated with IL1β or TNFα. GSK3β siRNA-transfected cells also displayed decreased IL1β-induced prostaglandin PGF2α release. It is worth noting, that we did not assess the effect of siRNA knockdown of the α isoform of GSK3 on pro-labour mediators. Given that GSK3α (in addition to GSK3β) was decreased in foetal membranes after preterm labour, it is possible that it may also play a role in the regulation of cytokine-induced inflammation.
Nevertheless, our data indicates that GSK3 is important, not only to TLR signalling, but also to TNFα and IL1β signalling pathways associated with preterm birth.

Our study describes for the first time that CHIR99021 can reduce LPS, FSL1 and flagellin-induced expression of COX2 mRNA expression and subsequent release of PGE2 and PGF2α in myometrium. Gene silencing of GSK3β using siRNA was also associated with a decrease in IL1β-induced PGF2α release. The COX2–prostaglandin pathway is integral in the initiation of labour, namely uterine contractions (Olson 2003). Our study also describes for the first time the effect of a GSK3 inhibitor on LPS-induced MMP9 mRNA and pro MMP9 expression in myometrium. We did not look at the effect of CHIR99021 on LPS-induced MMP9 expression in foetal membranes, as LPS does not stimulate MMP9 in our tissue explants. Increased activity of MMP9 in human myometrium is a requirement for uterine tissue remodelling during labour and the post-partum period (Roh et al. 2000).

GSK3 is necessary for the full transcriptional activity of NFκB (Hoeflich et al. 2000, Martin et al. 2005). The association between GSK3 and NFκB is of particular interest to this study given that NFκB regulates pro-inflammatory and pro-labour mediators in human gestational tissues (Lappas et al. 2002, Lappas & Rice 2007, Keelan et al. 2009). In this study, inhibition of GSK3β by siRNA in human myometrial cells is associated with decreased IL1β- or TNFα-induced NFκB transcriptional activity. Given that NFκB is a central regulator of TLR and cytokine-induced pro-inflammatory cytokines, prostaglandins and MMPs in human gestational tissues (Lappas et al. 2002, Keelan et al. 2009, Lim et al. 2014a), our data suggest that GSK3 plays a role in TLR and cytokine signalling via NFκB.

The involvement of GSK3 as a regulator of inflammation makes inhibition of GSK3 using synthetic compounds an attractive avenue of research; notably, it was shown that GSK3 inhibitors provided protection against inflammation after TLR stimulation in animal models (Martin et al. 2005). Our study demonstrates that in both foetal membranes and myometrium, CHIR99021 decreased the production of pro-inflammatory and
pro-labour mediators when stimulated with LPS, flagellin and FSL1. These results offer incentive to determine whether GSK3 inhibitors can be used in a mouse model of infection-induced preterm birth to either delay preterm labour and/or reduce maternal and foetal inflammation. In support, several animal models of inflammation have demonstrated an important role for GSKβ in the modulation of stimulus-induced production of several cytokines and the subsequent development of disease symptoms. For example, inhibition or deletion of GSK3β was protective against experimental peritonitis and arthritis (Hu et al. 2006), renal dysfunction and hepatotoxicity associated with endotoxemia (Dugo et al. 2006), colitis (Whittle et al. 2006, Hofmann et al. 2010) and endotoxin shock (Martin et al. 2005).

Conclusions

To our knowledge, this is the first study to demonstrate the expression of GSK3 in human foetal membranes and myometrium. We have shown here that GSK3 activity is increased with human spontaneous term and preterm labour and delivery in foetal membranes. Notably, the results of this study also point to GSK3 as a novel regulator of the terminal processes of human preterm labour and delivery. Specifically, the GSK3 inhibitor CHIR99021 significantly reduced pro-inflammatory and pro-labour mediators when stimulated with LPS, flagellin and FSL1. In addition, inhibition of

GSK3β by siRNA in myometrial cells decreased IL1β and TNFα-induced cytokine expression; this was associated with a decrease in NFκB transcriptional activity. These findings are of clinical relevance, particularly in the context of infection-associated preterm birth. Our results provide a rationale for including GSK3 among the potential molecular targets for the therapy of preterm birth.

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.

Funding

Associate Professor M Lappas is supported by a Career Development Fellowship from the National Health and Medical Research Council (NHMRC; grant number 1047025). Dr R Lim was, in part, supported by an early career researcher grant from the University of Melbourne. Additional funding was provided from the Medical Research Foundation for Women and Babies.

Acknowledgements

The authors gratefully acknowledge the following: Gillian Barker for her excellent technical assistance (Obstetrics, Nutrition and Endocrinology Group, Department of Obstetrics and Gynaecology, University of Melbourne, Victoria, Australia); Clinical Research Midwives Genevieve Christopher, Gabrielle Pell, Debra Jinks and Rachel Murdoch for sample collection; and the Obstetrics and Midwifery staff of the Mercy Hospital for Women for their co-operation.

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Received 21 September 2014
First decision 20 October 2014
Revised manuscript received 10 November 2014
Accepted 2 December 2014