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Smoking alters hydroxyprostaglandin dehydrogenase expression in fetal membranes

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ABSTRACT

Introduction: The way in which tobacco smoking increases the risk of preterm labor remains uncertain. Altered prostaglandin metabolism is one potential mechanism.
Methods: Proteins in fetal membrane samples (amniochoriodecidua) from 20 women were relatively quantified using Tandem Mass Tagging nano-liquid chromatography mass spectrometry.
Results: Prostaglandin synthases and two enzymes involved in prostaglandin degradation, hydroxyprostaglandin dehydrogenase (HPGD) and CBR1, were detected by the mass spectrometer. The expression of HPGD was significantly lower in smokers relative to non-smokers (0.43 fold, $p = 0.016$). There was no effect of labor, inflammatory status or gestational age on the HPGD levels.
Discussion: We describe for the first time an association between maternal smoking and HPGD expression. We propose that reduced expression of HPGD is one mechanism through which smoking may contribute to preterm labor. Lower levels of this enzyme, key to metabolising prostaglandins, may result in higher levels of prostaglandins and therefore precipitate labor prematurely.

1. Introduction

Smoking is known to increase the risk of both spontaneous and iatrogenic preterm birth, but the association with spontaneous preterm birth is stronger [1]. There are many potential pathways through which smoking may cause premature labor [2]. These include nicotine-induced vasoconstriction [3,4], carbon monoxide-induced fetal hypoxia [5,6], cadmium disruption of calcium signalling [7,8], altered steroid hormone production [9,10], changed responses to oxytocin [11,12] and altered prostaglandin (PG) production and metabolism [13].

The use of animal models to investigate potential mechanisms from preterm birth has shifted from endocrine pathways (e.g. cortisol induced placental progesterone withdrawal in sheep; luteolysis in rats) to inflammatory models e.g. lipopolysaccharide (LPS) induced proinflammatory response in mice [14]. The induction of premature labor by LPS in mice has highlighted the importance of genes involved in the regulation of connective tissue remodelling and tensile strength such as tenascin (*Tnc*) and thrombospondin (*Thhbs2*) as well as the activity of several matrix metalloproteinases [15]. These advances have contributed to our understanding of cervical ripening and dilatation mechanisms associated with spontaneous preterm labor.

Amnion choriodecidual (ACD) cells, especially tissue macrophages, may have a role in spontaneous preterm labor by promoting the release of prostaglandins and proinflammatory cytokines [16]. Whilst the amnion produces large quantities of PGE2, the chorion produces both PGE2 and PGF2α as well as an important modulator of PG activity, 15-hydroxy PG dehydrogenase (HPGD) [17].

Proinflamatory cytokines (e.g. interleukin 1, tumour necrosis factor-α) promote upregulation of prostaglandin synthase enzymes in the fetal membranes and decidua [18]. Inflammation of the fetal membranes is much more common in preterm deliveries than at term: Acute chorioamnionitis has been detected in 30% of preterm pregnancies but only

Abbreviations: PGF2α, prostaglandin F2α; PGE2, prostaglandin E2; PGD2, prostaglandin D2; HPGD, 15-hydroxyprostaglandin dehydrogenase

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in 5% of term pregnancies [19,20]. Chorioamnionitis causes early preterm labor by increasing prostaglandin production in ACD [21]; however the mechanism of labor in spontaneous preterm deliveries without signs of infection or inflammation remains unexplained. There is a complex interaction among maternal and fetal factors at the ACD interface; these involve prostaglandins, cytokines, growth factors and reactive oxygen species. These pathways are superimposed on genetic predisposition and immune defence mechanisms involved in the regulation of human labor [22]. The breakdown of maternal-fetal tolerance at the decidua/fetal membrane interface can disrupt the production of chemokines and cytokines (IL-1α, TNFα), as well as prostaglandins, reactive oxygen radicals and proteases. These products can initiate uterine contractions directly and/or provoke cervical changes and premature rupture of the membranes [23].

Whilst smoking has been shown to affect the levels of prostaglandins, such as PGF2α, PGE2, prostaglandin D2 and the prostacyclin metabolite 6-ketoprostaglandin F1α [13] in amniotic fluid, the effect of smoking on protein expression within the fetal membranes and decidua has not previously been considered.

Here we present proteomic data using gel-free separation methods that allow the identification and measurement of relative changes of thousands of proteins simultaneously. The results show a very significant effect of smoking on the amnion/chorion/decidual proteome; enormous disruption caused by inflammatory infiltration and changes specifically associated with spontaneous term and preterm labor.

2. Materials and methods

2.1. Tissue collection

Fetal membrane tissue (combined amnion-chorion-decidua parietalis, ACD) was obtained immediately after delivery from women who were not in labor (NL; no uterine activity or cervical change) and were undergoing planned Caesarean section with the following indications: previous Caesarean section, previous obstetric anal sphincter injury or pelvic outlet obstruction. ACD samples were also obtained from women after vaginal deliveries following spontaneous labor (L). Cases were further divided into either term (TL or TNL; 37–42 weeks gestational age) or preterm (PTL; less than 35 weeks of gestation). PTL cases were further subdivided into those with evidence of inflammation/infection (PTLi) and those without (PTLn), based on clinical features of the women (pyrexia, offensive liquor or uterine tenderness) or histological determination of the presence of leucocyte infiltration in the fetal membranes (chorioamnionitis), decidua (deciduitis) or placenta (intervillositis), with or without maternal pyrexia or uterine tenderness. Smoking was determined by self-report. Tissues were snap-frozen in liquid nitrogen and stored at −80 °C. This study was approved by the NHS South West Research Ethics Committee. All patients gave written informed consent.

2.2. Paraffin-embedded sections for histopathology

Tissues were rinsed in sterile saline and fixed in 4% phosphate buffered formaldehyde. After 24 h fixation, tissues were washed with sterile saline and processed using Leica JUNG TP 1050 Tissue processor into paraffin wax and sectioned into 3 μm thickness.

2.3. Tissue homogenisation

Frozen tissue samples were homogenised in RIPA (Radio-ImmunoPrecipitation Assay) lysis buffer (50 mM Tris pH 7.5, 150 mM sodium chloride, 1% NP-40, 0.1% SDS, 0.5% sodium deoxycholate, 1 x Complete protease inhibitor cocktail (Roche), 1 x PhosSTOP protease inhibitor cocktail (Roche)) at ~200 mg/ml using a Polytron homogeniser at room temperature. Homogenates were immediately cleared by centrifugation at 16 000 *g* for 15 min at 4 °C. Protein concentration was determined using the Pierce™ BCA assay kit (ThermoFisher Scientific, Loughborough, UK). The supernatants were adjusted by addition of further RIPA buffer to make each sample 175 μl at 2 mg/ml.

2.4. Tandem mass tag (TMT) labelling and cation exchange chromatography

Aliquots of 100 μg of six samples per experiment were digested with trypsin (2.5 μg trypsin per 100 μg protein; 37 °C, overnight), labelled with Tandem Mass Tag (TMT) sixplex reagents according to the manufacturer's protocol (Thermo Fisher Scientific, Loughborough, UK) and the labelled samples pooled.

A 50 μg aliquot of the pooled sample was used, evaporated to dryness and re-suspended in Buffer A (10 mM KH_2PO_4, 25% MeCN, pH3) prior to fractionation by strong cation exchange using an Ettan LC system (GE Healthcare). In brief, the sample was loaded onto a PolysulphoethylA column (100 x 2.1 mm, 5 μm, 200 A; PolyLC Inc.) in buffer A and peptides eluted with an increasing gradient of buffer B (10 mM KH_2PO_4, 25% MeCN, 1 M KCl, pH3) from 0 to 100% over 30 min. The resulting fractions were evaporated to dryness, re-suspended in 5% (v/v) formic acid and then desalted using SepPak cartridges according to the manufacturer's instructions (Waters, Milford, Massachusetts, USA). Eluate from the SepPak cartridge was again evaporated to dryness and re-suspended in 1% formic acid prior to analysis by nano-LC MSMS using an Orbitrap Fusion Tribrid Mass Spectrometer.

2.5. Nano-liquid chromatography mass spectrometry (Nano-LC MS)

The cation exchange fractions were further fractionated using an Ultimate 3000 nanoHPLC system in line with an Orbitrap Fusion Tribrid mass spectrometer (Thermo Scientific). In brief, peptides in 1% (v/v) formic acid were injected onto an Acclaim PepMap C18 nano-trap column (Thermo Scientific). After washing with 0.5% (v/v) acetonitrile 0.1% (v/v) formic acid, peptides were resolved on a 250 mm × 75 μm Acclaim PepMap C18 reverse phase analytical column (Thermo Scientific) over a 150 min organic gradient, using 7 gradient segments (1–6% solvent B over 1 min, 6–15% B over 58 min, 15–32% B over 58 min, 32–40% B over 5 min, 40–90% B over 1 min, held at 90% B for 6 min and then reduced to 1% B over 1 min) with a flow rate of 300 nl/minute. Solvent A was 0.1% formic acid and Solvent B was aqueous 80% acetonitrile in 0.1% formic acid. Peptides were ionised by nano-electrospray ionisation at 2 kV using a stainless-steel emitter with an internal diameter of 30 μm (Thermo Scientific) and a capillary temperature of 275 °C.

All spectra were acquired using an Orbitrap Fusion Tribrid mass spectrometer controlled by Xcalibur 2.0 software (Thermo Scientific) and operated in data-dependent acquisition mode using an SPS-MS3 workflow. FTMS1 spectra were collected at a resolution of 120 000, with an automatic gain control (AGC) target of 400 000 and a maximum injection time of 100 ms. The top ten most intense ions were selected for MS/MS. Precursors were filtered according to charge state (to include charge states 2–6) and with monoisotopic precursor selection. Previously interrogated precursors were excluded using a dynamic window (40 s +/-10 ppm). The MS2 precursors were isolated with a quadrupole mass filter set to a width of 1.2 *m/z*. ITMS2 spectra were collected with an AGC target of 5000, max injection time of 70 ms and CID collision energy of 35%.

For FTMS3 analysis, the Orbitrap was operated at 30 000 resolution with an AGC target of 50 000 and a max injection time of 105 ms. Precursors were fragmented by high energy collision dissociation (HCD) at a normalised collision energy of 55% to ensure maximal TMT reporter ion yield. Synchronous Precursor Selection (SPS) was enabled to include up to 10 MS2 fragment ions in the FTMS3 scan. The method allowed us to analyse the full proteome for each sample of ACD.