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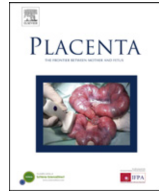

Cigarette smoke induces oxidative stress and apoptosis in normal term fetal membranes

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ABSTRACT

Cigarette smoking and bacterial infections are two major risk factors associated with preterm prelabor rupture of membranes (pPROM). We hypothesized that exposure of fetal membranes to cigarette smoke extracts might induce oxidative stress (OS) and fetal membrane apoptosis, culminating in an alternate pathway to that commonly activated by infection. To test this, we characterized the production of prostanoids and biomarkers of apoptosis in normal term human fetal membrane explant cultures. Fetal membrane explants collected at term (from cesarean deliveries, not in labor) were stimulated with cigarette smoke extract (CSE) for 24 h. Two classes of prostanoids, F2-Isoprostane (F2-IsoP), a marker of OS and PGF2α, a classical uterotonin, were measured by gas chromatography/mass spectrometry. Western blot analyses of tissue lysates were performed to quantify the anti-apoptotic protein Bcl2 and actin (as a control). Fetal membrane apoptosis was detected by immunohistochemistry for active caspase 3 and confirmed by TUNEL staining for nuclear fragmentation. CSE exposure resulted in significantly more F2-IsoP production from fetal membranes (242.8 $\pm$ 79.3 pg/ml/mg of total membrane protein) compared to unstimulated controls (131.5 $\pm$ 53.1 pg/ml/mg; $p < 0.0001$). By contrast, PGF2α was not different in CSE vs. controls (1083 $\pm$ 527 vs. 1136 $\pm$ 835 pg/ml/mg of protein; $p = 0.80$). CSE-exposed tissues demonstrated a dose-dependent decrease in Bcl2 expression and increases in active caspase 3 and nuclear fragmentation in both amnion and chorion cells compared to controls. In summary, fetal membranes exposed to CSE manifest evidence of OS and apoptosis. The differential pattern of prostanoid production observed in this study supports the hypothesis that an alternate non-inflammatory pathway mediated by OS and apoptosis in pPROM may promote proteolysis resulting in membrane weakening and rupture.

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1. Introduction

Preterm prelabor rupture of membranes (pPROM; <37 weeks of completed gestation) occurs in about 3–4% of all pregnancies including many without identifiable risk factors and is directly antecedent to ~40% of all preterm births. [1–3] Despite remarkable improvements in prenatal care over the past three decades, rates of pPROM and subsequent preterm delivery have worsened and racial and ethnic disparities have increased. [3,4] While several tests are available to confirm a diagnosis of pPROM post facto, no method to reliably predict pPROM is available.[5–7] This dilemma is mostly attributable to the fact that precise causes or pathophysiologic pathways resulting in pPROM are unknown. Proper diagnosis and management of pPROM in the future will require thorough characterization of specific exposure-induced mechanistic pathways, and identification of biomolecular markers predicting pPROM. [8] Multiple epidemiological and clinical factors have been proposed as precursors to pPROM, including maternal reproductive tract infections, behavioral stress, cigarette smoking and obstetric complications. Environmental factors and genetic predisposition also have been considered. [9] In addition, biochemical signals from the fetus, including endocrine signals that promote term parturition and fetal membrane apoptosis also have been implicated in the initiation of pPROM [3,5–8].

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Weakening of the collagen-rich extracellular matrix (ECM), connecting amnion and chorion layers of the fetal membranes, by protein degradation is one of the key events leading to rupture of membranes. [3,10,11]. Microbial infection is a major risk factor of pPROM and even aseptic inflammatory processes, with cytokine and matrix metalloproteinase [MMP] activation are well documented pathophysiologic pathways that lead to collagenolysis and rupture of membranes. [12,13] Although behavioral risk factors like cigarette smoking have been associated with pPROM, the pathophysiologic mechanisms initiated by such exposures are unclear [14–17] and paradoxically, exposure to cigarette smoke extracts (CSE) has antiinflammatory effects on fetal membranes in vitro, [18] suppressing inflammatory cytokines and MMPs that are considered integral features of pPROM. Thus we postulated that an alternate non-inflammatory pathway of pPROM might exist in cigarette smokers. Cigarette smoking is known to activate oxidative stress (OS) [19] and healthy pregnancy, as other physiological states, is characterized by a stable redox balance between reactive oxygen species (ROS) and antioxidants. [20–22] An imbalance mediated by cigarette smoking can promote OS and the production of isoprostanes (F2-IsoP), non-enzymatic byproducts of arachidonic acid (AA) oxidation. [23]

We hypothesize that cigarette smoking and potentially other environmental exposures during pregnancy induce OS and endogenous production of autotoxic lipids, accelerating fetal membrane apoptosis and proteolysis. Precise understanding of the mechanistic pathways associated with pPROM is expected to improve our knowledge of pPROM biomarkers. The objective of this study was to document cigarette smoke induced OS responses in normal term fetal membranes by measuring the OS biomarker F2-IsoP, and correlating this with fetal membrane apoptosis.

2. Methods

This study was conducted at Centennial Women's Hospital, Nashville, TN and Emory University; Atlanta, GA and was approved by the TriStar Nashville institutional review board at Centennial Medical Center, Western Institutional Review Board, Seattle, WA and the Emory University IRB in Atlanta, GA.

2.1. Subject selection and collection of fetal membranes

Fetal membranes (amniochorion) were collected from 15 subjects with uncomplicated gestations who underwent elective repeat cesarean section at term after obtaining written consent. We excluded women who had smoked prior or during pregnancy or who experienced exposure to second hand smoking within the household or at work place (information obtained by personal interview). In addition, women with prior history of pPROM or spontaneous preterm birth, obstetrical or medical complications during pregnancy, infection (subclinical, as indicated by elevated serum CRP levels or clinical, as manifested by foul smelling vaginal discharge, fever, or antibiotic treatment) were excluded.

2.2. Fetal membranes organ culture

Fetal membranes were separated from the placenta and transported to the laboratory in Hank's balanced salt solution (pH 7.4) (Sigma St. Louis, MO), containing 100 U/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin. For each culture, 6-mm-diameter tissue punches were cut using a biopsy punch (Euro-Med Cooper Surgical, Richmond, VA) were derived from the mid zone region of the reflected fetal membranes. After scraping off decidua, membranes were rinsed in phosphate-buffered saline (pH 7.4) and tissue discs were placed in an organ explant system [24,25] Representative samples from each culture were fixed in formaldehyde and H&E stained to assure that all tissue layers were present and to rule out subclinical chorioamnionitis (>5 PMNs/high power field). Organ culture was performed with minor modifications from prior reports, specifically, our conditions differed in the following ways; 4 discs/well were evaluated and an additional 50 μL of media (DMEM: F12 Ham's mixture with antibiotics described above, 15% FBS and 1% glutamine; all from Sigma) were added. This modification did not affect explant viability but doubled the number of sample replicates and media for analysis. Media used for this study contained the lowest concentration of LPS (<0.05 ng/ml) commercially available. Cultures were incubated at 37 °C in an atmosphere of 5% CO_2 and room air. Fresh media were exchanged on a daily basis and explants were maintained for up to 72 h as reported. [Fortunato SJ 1994, Menon R 1995] Fixed membrane histology revealed that none of the 15 subjects had evidence of histologic chorioamnionitis and confirmed the presence of intact amniochorion as a unit structure.

2.3. Cigarette smoke extract (CSE) preparation and stimulation of membrane explants with CSE

Cigarette smoke extracts (CSE) were prepared by bubbling smoke drawn from a single lit commercial cigarette (unfiltered Camel™, R.J. Reynolds Tobacco Co, Winston Salem, NC) through 50 ml of tissue culture medium (DMEM: F12 Ham's mixture with antimicrobial agents and FBS). Each cigarette is reported to contain 26 mg of tar and 1.7 mg of nicotine. The stock CSE was filter-sterilized using 0.25 μm Millipore filter units (Waters, Bradford, MA) and diluted 1:10, 1:50 and 1:100 in culture media as reported previously. [18] CSE was prepared freshly for each experiment.

After a pre-incubation period of 48 h, membranes were stimulated with CSE, or vehicle (regular medium) for another 24 h. In some experiments, LPS (100 ng/ml in culture media; E.coli O55:B5; Sigma) stimulation was performed to confirm consistent responsiveness of the membranes. The LPS concentration was chosen based on the mean concentration documented in amniotic fluid of women with intraamniotic infection and also based on our prior experimental reports [18]. Tissue discs and conditioned media from CSE and unstimulated control cultures (referred to as 'controls' in the rest of this paper) were retrieved from the cultures, frozen and stored at −80 °C.

2.4. Quantification of F2-IsoP and prostaglandin (PG) F2α in conditioned media by gas chromatography/mass spectrometry (GC/MS)

Media sample aliquots were transported on dry ice to the Vanderbilt University Eicosanoid Core Laboratory, where the following GC/MS protocols were conducted. Media (0.20 mL) were diluted to a volume of 10 mL with 0.01N HCl and to that solution 1.0 ng of the internal standards [$^2H_4$]-15-F_{2t}-IsoP ([$^2H_4$]-8-iso-PGF2) (Cayman Chemicals, Ann Arbor, MI) were added. The samples were vortexed to extract the lipids and then centrifuged for 10 min at 5000× *g* to remove particulates. The soluble fraction was transferred to another tube and methanol was dried under a stream of dry nitrogen. The residue was reconstituted in 10 mL of 0.01N HCl and was then applied to C-18 Sep-Pak columns (Waters, Milford, MA, USA) that had been prewashed with 5 mL methanol and 5 mL H_2O (pH 3). The columns were washed with 10 mL 0.01N HCl, followed by 10 mL heptane and prostanoids were eluted with 10 mL ethyl acetate: heptane (50:50, v/v). The eluate was evaporated under nitrogen. Compounds were converted to pentafluorobenzyl (PFB) esters by the addition of 40 μL of 10% PFB bromide and 20 μL of 10% diisopropylethanolamine in acetonitrile and incubated for 30 min at 37 °C. Samples were dried under nitrogen and the residue reconstituted in 30 μL chloroform and 20 μL methanol and chromatographed on silica TLC plates in a solvent system of ethyl acetate:methanol (98:2, v/v). A methyl ester standard of PGF2α was chromatographed on a parallel plate and visualized with 10% phosphomolybdic acid in ethanol after heating. F2-IsoP and PGF2α were removed from the TLC plates by scraping the region from the mid-point of the PGF2α-methyl ester standard. Following TLC purification, compounds were converted to trimethylsilyl (TMS) ether derivatives by addition of 20 μL *N,O*-bis (trimethylsilyl)trifluoroacetamide and 10 μL dimethylformamide. The samples are incubated at 37 °C for 10 min and then dried under nitrogen. The residue was redissolved for GC/MS analysis in 10 μL undecane that was stored over a bed of calcium hydride. GC/negative ion chemical ionization(NICI)-MS is carried out on an Agilent 5973 Inert Mass Selective Detector coupled with an Agilent 6890n Network GC system and computer. The GC was performed using a 15 m, 0.25 mm film thickness, DB-1701 fused silica capillary column (J and W Scientific, Folsom CA). The column temperature is programmed from 190° to 300 °C at 20 °C increment per minute. The major ion generated in the NICI mass spectra of the PFB ester and TMS ether derivatives of F2-IsoP and PGF2α were *m*/*z* 553 for both while the major ion generated for the internal standard was *m*/*z* 557. Levels of endogenous eicosanoids in the biological samples were calculated from the ratio of intensities of the [$^2H_0$]- and [$^2H_4$]-standard ion signals. The interday variability for each assay was <10%. The precision for each assay was ±5% while the accuracy for each assay was 95%. Concentrations were normalized to mg/ml of total membrane protein.

2.5. Polyacrylamide gel electrophoresis (PAGE) and western blot analysis of membrane proteins

Western blot analyses were performed to document the effect of CSE on Bcl2, an anti-apoptotic protein. Thromboxane receptor A2, a putative F2-IsoP receptor, and β-actin (used as an internal control) were also studied. Control and CSE stimulated fetal membrane tissues (∼400 mg each) were lysed in 0.3 mL of tissue extraction buffer (Biosource International, Inc., Camarillo, CA), centrifuged at 10,000× g for 1 min and protein concentrations in the supernatants were determined by BCA Protein Assay Kit (Thermo Scientific). Membrane lysates were mixed with loading buffer (Invitrogen, Carlsbad, CA) and subjected to PAGE using 4–12% Bis-Tris NuPAGE precast gels (Invitrogen) with each lane containing 60 μg of membrane protein. The separated proteins were transferred to PVDF membranes and blocked

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