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Expression profiles of fetal membrane nicotinamide adenine dinucleotide phosphate oxidases (NOX) 2 and 3 differentiates spontaneous preterm birth and pPROM pathophysiologies

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ABSTRACT

Introduction: Nicotinamide adenine dinucleotide phosphate oxidases (NOX 1–5) are enzymes that generate cellular reactive oxygen species (ROS) besides mitochondria and might be important ROS sources associated with pregnancy complications, particularly preterm premature rupture of membranes (pPROM), that has been related to ROS.
Objective: To characterize NOX enzymes expression in human fetal membranes.
Methods: Differential expression and localization of NOX isoforms in human fetal membranes collected from women with uncomplicated pregnancies at term, preterm birth (PTB) or pPROM and *in vitro* in normal term membranes maintained in an organ explant system stimulated with water-soluble cigarette smoke extract (wsCSE) were documented by real time PCR and immunohistochemistry.
Results: Fetal membranes from term deliveries, PTB and pPROM expressed NOX 2, 3 and 4 mRNAs whereas NOX 1 and 5 were not detected. NOX 2 expression was 2.3-fold higher in PTB than pPROM ($p = 0.005$) whereas NOX 3 was 2.2-fold higher in pPROM compared to PTB ($p = 0.04$). NOX 2 and 3 expressions at term mimicked pPROM and PTB, respectively. No difference in NOX 4 expression was observed among the studied groups. NOX 2, 3 and 4 were localized to both amniotic and chorionic cells. Expression of NOX 2, 3 and 4 were not significant in wsCSE-stimulated membranes compared to untreated controls.
Discussion/conclusions: NOX enzymes are present in the fetal membranes and are differentially expressed in PTB and pPROM. Absence of any changes in NOXs expression after wsCSE stimulation suggests ROS generation in the membranes does not always correlate with NOX expression.

1. Introduction

Aerobic processes in a mammalian cell generate reactive oxygen species (ROS) that modulate several cellular physiologic functions, including: differentiation, proliferation, migration, and vasodilatation [1]. One of the main ROS function is in innate immunity where it exerts microbicidal effects on pathogens [2]. An overproduction of ROS and/or a decrease in cellular antioxidant capacity generates oxidative stress, a deleterious process that induces oxidative damage to cell structure and functions [3]. ROS can be produced in a cell by multiple enzymatic and non-enzymatic processes. Mitochondrial electron transport chain is the main intracellular source while the enzymatic pathways like nitric oxide synthase, xanthine oxidase and nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (NOX) are the most important extra-mitochondrial ROS sources [4,5]. Recent attention has been focused on NOXs, since, unlike other oxidoreductases, ROS production is their primary function [6].

NOXs are complex ROS-producing enzymes, first described in phagocytes and termed gp91phox, now referred to as NOX 2 [7]. NOX activity has recently been described in non-phagocytic cells and this has led to the discovery of additional isoforms [6]. Seven

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members now constitute the NOX family – NOX 1–5, DUOX 1 and DUOX 2 – and all are closely related, since they share a common 6-transmembrane domain structure, and the major catalytic subunit (gp91phox) [4]. Despite this, NOX enzymes are classified into three main groups (NOX 1-4, NOX 5 and DUOX 1 and 2), based on the presence of specific domains, i.e., regulatory subunits in addition to that of the gp91phox [6], and also based on the activation signals [8]. Thus, the expression, activity of NOX and the amount of ROS production are tissue-specific and differ based on the type of stimulus [8].

Pregnancy is characterized by redox balance where the functions of highly reactive ROS are tightly regulated by an array of antioxidant systems, thus controlling their activity and maintaining a homoeostatic environment [9]. Redox imbalance is associated with several pregnancy complications and NOXs are contributors to these conditions [10,11]. Recent reports suggest that increased collagenolysis associated with preterm premature rupture of the membranes (pPROM) compared to spontaneous preterm birth with intact membranes (PTB) may be mediated by increased ROS formation and/or antioxidant depletion [12–15]. Supporting this, higher concentrations of ROS markers were found in tissue from pPROM than gestational age matched PTB [16–18]. The exact mechanisms by which ROS participate in divergent pPROM and PTB pathways are unknown, but evidence suggests that ROS causes lipid, protein and nucleic acid peroxidation resulting in cellular dysfunctions [15]. At term, gradual increases (from no labor to after labor) in oxidative stress at fetal membranes overlying the cervix also support the role of ROS in membrane susceptibility to rupture [12]. Characterization of ROS and ROS inducing enzymes in fetal membranes is important to better understand the biomolecular, structural and mechanical changes in the fetal membranes associated with pPROM and PTB. The expression of NOX has already been reported in placental tissue [10,11,19], but its presence, expression, and site of production is not well understood in fetal membranes (amniochorion). Therefore, we investigated the differential expression and site production of all 5 NOX (1–5) enzymes in human fetal membranes from clinical specimens obtained from normal term birth, PTB and pPROM. Additionally, we examined the role of ROS producing risk factors by inducing NOX enzymes in normal term fetal membranes.

2. Materials and methods

2.1. Clinical samples collection

Clinical samples used in this study were collected at the Centennial Medical Center (Nashville, TN) and the study protocol was approved by Western Institutional Review Board, Seattle, WA, for Tristar Medical system. Informed, written consents were obtained from subjects prior to sample collection. Enrollment occurred at the time of admission for delivery. Samples for *in vitro* experiments were collected from The University of Texas Medical Branch (UTMB), John Sealy Hospital, Galveston, TX, and the study is approved by the *Institutional Review Board* at UTMB.

Placentas were collected from women with preterm birth and intact fetal membranes (PTB) ($n = 8$), and with preterm premature rupture of membranes (pPROM) ($n = 8$). PTB defined as having contractions (presence of 2 contractions/10 min) leading to delivery with intact membranes (between $24^{0/7}$ weeks and $34^{0/7}$ weeks) were also included as cases. pPROM was confirmed by Amnisure® testing and subjects delivering preterm prior to 34 weeks gestation. In both groups, all pregnant women had vaginal preterm delivery. The controls consisted of women at full-term with spontaneous labor and vaginal delivery ($>37^{0/7}$ weeks) and no pregnancy related complications or prior history of PTB or pPROM. Fetal membranes were dissected from the mid-zone portion, away from the placental bed and those overlaying the cervix, cleansed off all blood clots and decidua, and stored in RNA stabilization reagents at −80 °C. Additional samples collected from adjacent area were fixed in 10% formalin, embedded in paraffin, sectioned for histological evaluation and NOX 1–5 immunolocalization by immunohistochemistry.

2.2. wsCSE preparation and stimulation of the fetal membranes

Fetal membranes from placentas involving elective repeat cesarean sections and uncomplicated pregnancies at term (not in labor) ($n = 6$), were cultured and stimulated with water-soluble cigarette smoke extract (wsCSE) (1:10 dilution) as previously described [20,21]. After a preincubation period of 48 h at 37 °C in an atmosphere of 5% CO_2, membranes were stimulated with wsCSE for an additional 24 h. Tissue samples from wsCSE-stimulated cultures and unstimulated control were collected, and stored at −80 °C.

2.3. RNA isolation, cDNA preparation, and reverse transcription PCR

Fetal membranes were disrupted with a Polytron homogenizer (Next Advanced Inc Bullet Blender, Next Advanced Inc, NY, USA) using 1.0-mm ZrSiO beads (Next Advanced Inc) and Trizol reagent (Life Technologies, CA, USA). RNA was extracted from tissues by using the Direct-zol RNA Mini Prep (Zymo-Research, CA, USA), according to the manufacturer's instructions. Genomic DNA contamination was removed with DNaseI treatment during RNA extraction. The quality and concentration of extracted total RNA were measured by using Gen 5 Software, version 2.1 (Biotek Synergy H4 Hybrid Reader, Winooski, VT). RNA samples (0.1 mg/mL) were subjected to reverse transcription by using the High-Capacity cDNA Archive Kit (Applied Biosystems, CA, USA), in accordance with the manufacturer's instructions.

SYBR Green real-time PCR was performed by using an ABI 7500 Fast Real Time PCR System (Applied Biosystems). The sequences of the primers utilized for the quantification of members of the NADPH oxidase family genes were obtained from Primer-BLAST: NOX 1 (forward) 5′-GTT TTA CCG CTC CCA GCA GAA-3′ and (reverse) 5′- GGA TGC CAT TCC AGG AGA GAG-3′; NOX 2 (forward) 5′-TCC GCA TCG TTG GGG ACT GGA-3′ and (reverse) 5′-CCA AAG GGC CCA TCA ACC GCT-3′; NOX 3 (forward) 5′-CGG ATT GTT CGA GGC CAA AC-3′ and (reverse) 5′-GCC AGA AAA TTG AGG CAC GG-3′; NOX 4 (forward) 5′-CCC GGA AAA CCC GGC TCT GG-3′ and (reverse) 5′-GAG TGG AAG CCC GAA GGC CC-3′; NOX 5 (forward) 5′- TAT CAT GTA CAG GCA CCA GA-3′and (reverse) 5′ CCA TCT CCA GTT TAG TCA GC-3′. We tested primer specificity by reverse transcription PCR (RT-PCR) and confirmed it by melting (dissociation) curve analysis. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a housekeeping gene, was used as an internal control (at 72 h, no difference in GAPDH expression was seen). Amplification was performed under the following conditions: denaturation for 30 s at 95 °C followed by 40 cycles of denaturation for 15 s at 95 °C, and annealing/extension for 1 min at 60 °C. All reactions were performed in duplicate and no template controls were included in each run. The comparative $2^{-\Delta\Delta Ct}$ method was used to calculate relative quantification of gene expression [22] and data is presented as fold changes between groups.

2.4. Immunohistochemistry

Fetal membranes samples collected in 10% formalin were submitted to immunohistochemistry performed at the Histopathology Special Procedures Laboratory of the UTMB Pathology Department, following a standard protocol. Briefly, 3- to 5-micron-thick sections from formalin-fixed, paraffin-embedded tissue blocks mounted on positively charged slides were de-paraffinized, and endogenous peroxides were twice quenched in 3% methanol H_2O_2 for 5 min. Avidin and biotin (Avidin Biotin blocking kit, Vector Laboratories, Burlingame, CA, USA) were diluted in Antibody Diluent (Dako, Glostrup, DEN) at a ratio of 1:5. Primary antibodies were diluted in the biotin solution and applied for 1 h (Dilutions: NOX 2 and 4: 1/100; NOX3: 1/200; Novus Biologicals, Littleton, CO, USA). Sections were incubated in LSAB2, universal secondary antibody (Dako), for 15 min and in chromagen liquid DAB (3,30-diaminobenzidine tetrahydrochloride) (Dako) for 5 min. The slides were counterstained with Harris Hematoxylin (Fisher Scientific, Waltham, MA, USA). Sections were examined and photographed by using an Olympus light microscope -BX43 with a URFL-T digital camera, and the software QCapture Pro (Micropublisher

Table 1
Demographic and gestational characteristics of studied patients.

Characteristic	TERM ($n = 8$)	PTB ($n = 8$)	pPROM ($n = 8$)	p
Maternal age (y)[a]	31.8 ± 5.7	28.8 ± 5.4	27.1 ± 7.7	0.35
Marital status[b]				
Single	3 (37.5)	0	3 (37.5)	0.14
Marriage	5 (62.5)	8 (100.0)	5 (62.5)	
Ethnicity[b]				
White	5 (62.5)	5 (83.3)	5 (62.5)	0.53
Black	3 (37.5)	1 (16.7)	3 (37.5)	
Smoking	0	1 (14.3)	0	0.30
Gestational age at birth (weeks/days)[a]	39w 1d ± 4d	33w 5d ± 2w4d	31.5 ± 3w 6d	<0.0001
Chorioamnionitis	0	0	2 (25.0)	0.12
Gravidity[b]				
Primiparous	1	3	3	0.38
Multiparous	7	4	5	

[a] Mean ± standard error; Anova (Tukey's multiple comparisons test).
[b] n (%); Fisher's exact test. Ethnicity from 2 cases in PTB group was not reported.