Research Article

Oxidative stress-induced TGF-beta/TAB1-mediated p38MAPK activation in human amnion epithelial cells[†]

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Abstract

Term and preterm parturition are associated with oxidative stress (OS)-induced p38 mitogen-activated protein kinase (p38MAPK)-mediated fetal tissue (amniochorion) senescence. p38MAPK activation is a complex cell- and stimulant-dependent process. Two independent pathways of OS-induced p38MAPK activation were investigated in amnion epithelial cells (AECs) in response to cigarette smoke extract (CSE: a validated OS inducer in fetal cells): (1) the OS-mediated oxidation of apoptosis signal-regulating kinase (ASK)-1 bound Thioredoxin (Trx[SH]₂) dissociates this complex, creating free and activated ASK1-signalosome and (2) transforming growth factor-mediated activation of (TGF)-beta-activated kinase (TAK)1 and TGF-beta-activated kinase 1-binding protein (TAB)1. AECs isolated from normal term, not-in-labor fetal membranes increased p38MAPK in response to CSE and downregulated it in response to antioxidant N-acetylcysteine. In AECs, both Trx and ASK1 were localized; however, they remained dissociated and not complexed, regardless of conditions. Silencing either ASK1 or its downstream effectors (MKK3/6) did not affect OS-induced p38MAPK activation. Conversely, OS increased TGF-beta's release from AECs and increased phosphorylation of both p38MAPK and TAB1. Silencing of TAB1, but not TAK1, prevented p38MAPK activation, which is indicative of TAB1-mediated autophosphorylation of p38MAPK, an activation mechanism seldom seen. OS-induced p38MAPK activation in AECs is ASK1-Trx signalosome-independent and is mediated by the TGF-beta pathway. This knowledge will help to design strategies to reduce p38MAPK activation-associated pregnancy risks.

Summary Sentence

Oxidative stress-induced p38MAPK activation at term is dependent on the TGF-beta–TAB1 pathway.

Key words: pregnancy, stress, cell culture, cytokines, labor.

Introduction

The global preterm birth (PTB; birth before 37 weeks gestation) rate is approximately 10% of all pregnancies, contributing to 1 million neonatal deaths/year and other health complications for survivors [1–3]. The majority of PTBs are spontaneous with unknown etiologies but are pathologically highlighted by infection/inflammation and oxidative stress (OS) [4–10]. Similarly, normal term labor and delivery is also affected by sterile inflammation and OS, compared to normal term, not-in-labor cesarean deliveries [8, 9, 11, 12]. It is difficult to ascertain the cause and origin of sterile inflammation in PTB [10, 13–16]. Senescence of feto-maternal tissues, often characterized by chronic sterile inflammation, has recently been linked to term and PTB [3, 11–14]. Further, studies using human and murine fetal membranes (amniochorion) determined that OS is one of the promoters of cellular senescence that generates sterile inflammation [15–20]. Fetal membranes encounter increased OS at term due to metabolic demands that expand in response to fetal growth, heightened respiratory needs, and altered maternal metabolic supply [19, 21, 22]. Inflammatory markers from senescent cells are inherently uterotonic and capable of transitioning quiescent uterine tissues to an active contractile state (labor) at term [16, 17]. Conversely, premature senescence activation caused by various risk factors can trigger PTB [23, 24].

Mechanistically, OS-induced senescence in fetal membranes, at term and preterm, is mediated through the stress signaler p38 mitogen-activated protein kinase (p38MAPK) [15, 16, 18, 20]. p38MAPK, including all four of its isoform (α , β , γ and δ), are part of a 14-member MAPK super family containing extracellular signal-regulated kinases (ERK)1/2, ERK5, and c-Jun N-terminal kinases (JNK) are the others [25]. In general, all MAPK pathways consist of three dependent kinases activated in a sequential manner that include MAPK kinase kinases (MKKKs), MAPK kinases (MKKs), and MAPKs. Dual phosphorylation of tyrosine and threonine residues, which can be activated by one or two kinases in the activation loop of kinase subdomain, phosphorylate all 4 isoforms of MAPK [26]. p38MAPK also consists of four signaling isoforms (p38MAPK α , β , γ , and δ) that are evolutionarily conserved serine/threonine kinases whose functions differ significantly [26, 27]. The p38MAPK α isoform is the most dominant of the four in reproductive tissues; its activities are essential for normal development [26] and other physiological processes [28, 29]. Multifactorial functions of p38MAPK in reproduction [30] include preimplantation [31], embryogenesis [32, 33], placental function and development [29, 34], and decidualization [35, 36]. p38MAPK is also a mediator of cellular senescence, a mechanism of aging, which is a non-reversible process. Senescent cells can remain in the tissue environment without undergoing cell death and constantly secrete inflammatory mediators and damage-associated molecular pattern markers. In fetal membranes, OS increases p38MAPK activation (P-p38MAPK α), leading to senescence and sterile inflammation seen in normal term [16] and PTB [24, 37]. In vitro, primary cultures of fetal membrane cells (amnion epithelial cells—AECs) exposed to OS showed p38MAPK-mediated senescence and inflammation that

was reduced by p38MAPK inhibitor SB203580 [38]. Similarly, a progressive increase in p38MAPK in fetal membranes and placenta in murine pregnancy that peaked on days 15–18 of gestation also correlated with OS and senescence [15]. Exogenous signals—such as environmental pollutants [39], cigarette smoke [40], infection and inflammation (unpublished data from our lab), and cell damage-associated molecular pattern markers, such as HMGB1 [20]—were all shown to induce p38MAPK-mediated senescence and inflammation in AECs. These data support the hypothesis that p38MAPK-mediated premature senescence activation can contribute to PTB. Thus, p38MAPK plays a role in generating inflammation at term and preterm. We postulate that minimizing its activation in gestational tissues can reduce the risk of OS-associated PTB. As p38MAPK activation can occur through multitudes of pathways in a cell-type-dependent manner [26], a precise mechanism of activation in fetal membrane cells is required to design intervention strategies to minimize p38MAPK-mediated PTB risks.

OS in AECs is expected to cause two major pathways of p38MAPK activation: (1) one path is mediated by activation of apoptosis signal-regulating kinase (ASK1)-signalosome [41–43] and (2) the second is caused by activation of transforming growth factor (TGF)-beta-activated kinase 1-binding (TAB1) protein 1-mediated p38 autophosphorylation [44–46]. Briefly, the ASK1-signalosome-mediated activation involves antioxidant Thioredoxin 1 (Trx). Reduced Trx forms an inhibitory Trx(SH)₂-ASK1 complex that can attenuate ASK1-signalosome-mediated p38MAPK activation [47]. In this mechanism, Trx(SH)₂ interacts with the N-terminal domain of ASK1 and serves as a negative regulator of ASK1 and attenuator of p38MAPK activity [42, 47, 48]. The OS-mediated oxidation of ASK1-bound Trx(SH)₂ dissociates this complex, creating free and activated ASK1-signalosome and leading to p38MAPK activation [48]. TGF-beta is an OS-responding growth factor involved in embryogenesis [49] and tissue remodeling [50]. Interaction between TGF-beta-TGF receptors I and II activates TGF-activated kinase (TAK1), leading to its interaction with TAB1 [49]. TGF-beta/TAK1/TAB1 complex, either through canonical MAPKKs or TGF-beta directly through TAB1 (independent of TAK1) by autophosphorylation, results in p38MAPK activation [51–53]. We tested the mode of activation of p38MAPK in AECs through these two pathways because both ASK1 [54] and TGF-beta pathways [54] were reportedly activated in oxidatively stressed AECs. We found the existence of the ASK1-signalosome in AECs, but an absence of the ASK1-signalosome complex-mediated p38 activation; however, cigarette smoke extract (CSE)-induced OS in AECs causes TGF-beta to increase, leading to a TGF-beta–TAB1-mediated p38MAPK activation.

Methods

Institutional Review Board approval

This study protocol was approved by the Institutional Review Board (IRB) at The University of Texas Medical Branch (UTMB) at Galveston, Texas, as an exempt protocol to use discarded placenta after normal term cesarean deliveries (UTMB 11-251). No subject

Table 1. List of siRNA target sequences.

Gene Symbol	Target sequence 1	Target sequence 2	Target sequence 3	Target sequence 4
TAB1	GAUGAGCUCUCCGUCUUU	GAACAACUGCUCCUGUAU	GGAGAUUGCUGCGAUGAUU	AAAUAGGCUACACGGACA
ASK1	CAGGAAAGCUCGUAAUUUA	GGAAAGCUCGUAAUUUAUA	GAUGUUCUCUACUAUGUUA	UAAGUAGUCUUCUUGGUA
MKK3	GGUGGAGGCUGAUGACUUG	CCGCAGAGCGUAUGAGCUA	CCAAUGUCCUUAUCAACAA	GGAGAUUGCUGUGUCUAUC
MKK4	GGACAGAAGUGGAAUAUUA	GACAGAAGUGGAAUAUUA	GCGCAUCACGACAAGGAUA	UCCCAUGUAUGUCGAUUGA
MKK6	CCAAAGAACGGCCUACAUA	GAUAAAGGCCAGACAAUUC	GCUCAUGGAUACAUCACUA	CAUCAAUGCUCUCGGUCAA
TAK1	CCCAAUGGCUUAUCUACA	GGACAGCCAAGACGUAGAU	UACACUGGAUCACCAACUA	AGUGAUAAACGCGUCGGAAA
NT siRNA	UAAGGCUAUGAAGAGAUAC	AUGUAUUGGCCUGUAUUAG	AUGAACGUGAAUUGCUCAA	UGGUUUACAUGUCGACUAA

recruitment or consenting was done for this study. All placental collection methods were performed in accordance with the relevant guidelines and regulations of the IRB.

Clinical samples and cell culture

Fetal membrane samples for this study were obtained from John Sealy Hospital at UTMB at Galveston, Texas, USA. Primary AECs were isolated from reflected amnion (about 10 g), peeled from the chorion layer, and dispersed by successive treatments with 0.024% collagenase and 1.2% trypsin. This processing was completed within 2–3 h of getting the placenta. The dispersed cells were plated in a 1 : 1 mixture of Ham's F12/DMEM, supplemented with 10% heat-inactivated fetal bovine serum (FBS), 10 ng/mL EGF, 2 mM L-glutamine, 100 U/mL penicillin G, and 100 mg/mL streptomycin at a density of 3–5 million cells per T75 [38], and incubated at 37°C with 5% CO₂ for 4–5 days until the cells were ready to be passaged for treatment. Cytokeratin-18 staining and vimentin staining were conducted to determine epithelial characteristics of amnion cells in culture.

Cell culture treatments

To induce OS in AECs, we used CSE as previously described [38, 55], with slight modifications. CSE is a validated and reproducible OS inducer in our system and we do not use CSE in our experiments as a risk factor of pregnancy. Smoke from a single lit commercial cigarette (unfiltered Camel, R.J. Reynolds Tobacco Co, Winston Salem, NC) was infused into 25 mL of media, consisting of DMEM/F12 supplemented with 10% FBS (System Biosciences, Mountain View, CA). The stock CSE was sterilized using a 0.25 mm Steriflip filter unit (Millipore, Billerica, MA). CSE concentrate was diluted 1:10 in complete DMEM media prior to use. Once cells reached 70%–80% confluence, cells were passaged (p1), allowed to establish baseline characteristics for 24 h prior to performing additional treatments. Each flask was serum starved for 1 h and rinsed with sterile 1× PBS followed by treatment with media (control), CSE containing media, TGF-beta receptor 1, 2, and 3 antagonist (3 ng/mL) (R&D, Minneapolis, MN), or TGF-beta 2 ng/mL (R&D, Minneapolis, MN). Then, they were incubated at 37°C, 5% CO₂, and 95% air humidity for 1 h. The culture media were collected and stored at –80°C for evaluation by ELISA.

AEC siRNA transfections

To determine the potential role of each of the two investigated signaling pathways leading to activation of p38MAPK by CSE, we downregulated ASK1, MKK3/6, TAK1, and TAB1 in AECs using siGENOME siRNA (GE Healthcare Dharmacon) (Dharmacon, Thermo Fisher Scientific (Rockford, IL); Table 1). Briefly, AECs were cultured to nearly 50% confluence in DMEM/F12 medium supplemented with 10% FBS and antimicrobial agents

(Penicillin/Streptomycin, Amphotericin). Prior to siRNA transfection, cells were incubated with antimicrobial-free medium overnight. Next, cells were incubated for 4 h with siRNA complexes, which were freshly prepared using either 150 nM siRNA to specific genes or NT siRNA as control and 0.3% Lipofectamine RNAiMAX (Invitrogen, Eugene, OR) in Opti-MEM I Reduced Serum Medium. Cells were further incubated in growth media for 48 h. Downregulation efficiency of the target genes was validated by qRT-PCR. Gene expression was normalized to nontransfected control.

Crystal violet proliferation assay

AECs were seed in a 12-well plate (90 000 per well) and treated in replicates of six with control or CSE 1:10 media for 1 h. After 1 h, cells were washed with 1× PBS, fixed with 4% paraformaldehyde (PFA) for 15 min, washed with 1× water, and stained with 0.1% crystal violet for 20 min. After 20 min, cells are washed, allowed to dry, and 10% acetic acid was added to each well. A 1 : 4 dilution of the colored supernatant was measured at an absorbance of 590 nm.

Quantitative RT-PCR

To determine the expression levels of TAB1, ASK1, MKK3, MKK6, and MKK4 after treatments, cells were collected and lysed using lysis buffer (Qiagen, Valencia, CA, USA). RNA was extracted using an RNeasy kit (Qiagen) per the manufacturer's instructions. Total RNA (1 µg) was reverse-transcribed using a High-Capacity RNA-to-cDNA Kit (Applied Biosystems, Foster City, CA). qRT-PCR was performed on an ABI7500 Fast Real-Time PCR System (Applied Biosystems) using Fast SYBR Green Master Mix (Applied Biosystems). The amplification thermal profile was 20 s at 95°C and 3 s 95°C, followed by 30 s 60°C (40 cycles). To confirm the presence of a single amplicon, a melt curve stage was carried out: 15 s at 95°C, 1 min at 60°C, 15 s at 95°C, and 15 s at 60°C. Changes in gene expression levels were calculated by using the $\Delta\Delta C_t$ method [56]. We used predesigned qPCR assays primers from Integrated DNA Technologies (Table 2).

Protein extraction and immunoblot assay

Portions of these cells were collected using NE-PER nuclear and cytoplasmic extraction reagents (Thermo fisher #78835), while the rest of the cells were lysed with Pierce Lysis Buffer for separate ASK1 and Trx pulldown assays. Cells and tissue were lysed with RIPA lysis buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1% Triton X-100, and 1.0 mM EDTA pH 8.0, 0.1% SDS) supplemented with protease and phosphatase inhibitor cocktail and phenylmethylsulfonyl fluoride. After centrifugation at 10 000 RPM for 20 min, the supernatant was collected, and protein concentrations were determined using BCA (Pierce, Rockford, IL). The protein samples were separated using SDS-PAGE on a gradient (4%–15%) Mini-PROTEAN TGX Pre-cast Gels (Bio-Rad, Hercules, CA) and transferred to the membrane

Table 2. List of qPCR assays primers for AEC experiments.

Gene symbol	GenBank/Ref-Seq ID	Forward 5'-3'	Reverse 5'-3'
TAB1	NM_006116	TCTGCTTGGCAAACACTCAGTG	CTTGCTGCTGATGTCGGA
ASK1	NM_005923	TGTAGTTCCAGTGCACATAG	GCCAAACATCATCCTCTACT
MKK3	NM_145109	GGCTTGGACATGCAGGATA	GCAGTCCTCTAGATTAGTCTCCA
MKK4	NM_003010	TCAAACGAGGTAGACATGAGTTC	TTTGGATGTAGTAATGCGGAGT
MKK6	NM_002758	TGGAACCTGGTCTGAGGTTGT	GAAGAGAAGCAAGGCAAAGTC

Table 3. Antibodies used for the western blot.

Protein Name	Catalog Number	Company	Dilution	Method
ASK1	D11C9	Cell Signaling	1:750	Western Blot
TRX	Ab16965	Abcam	1:1000	Western Blot
TAB1	AF3578	R&D	1:1000	Western Blot
P-TAB1(Thr431)	06-1334	Milipore	1:800	Western Blot
P-p38 MAPK (T180/Y182)	9211L	Cell Signaling	1:300	Western Blot
p38 MAPK	9212S	Cell Signaling	1:1000	Western Blot
B-Actin	A5441	Sigma-Aldrich	1:20,000	Western Blot
Cytokeratin-18	Ab668	Abcam	1:300	Immunofluorescence
ASK1	Ab45178	Abcam	1:250	Immunofluorescence
TRX	Ab16965	Abcam	1:500	Immunofluorescence
P-p38 MAPK (T180/Y182)	9211L	Cell Signaling	1:300	Immunofluorescence
TAK1	Sc-7967	Santa Cruz	1:300	Immunofluorescence
TAB1	AF3578	R&D	1:300	Immunofluorescence
ASK1	D11C9	Cell Signaling	1:100	Immunoprecipitation
TRX	Ab16965	Abcam	1:100	Immunoprecipitation
GSK3B	mAb#9832	Cell Signaling	1:100	Immunoprecipitation

using iBlot Gel Transfer Device (Thermo Fisher Scientific). Membranes were blocked in 5% nonfat milk in 1× Tris buffered saline-Tween 20 or in 5% BSA buffer for a minimum of 1 h at room temperature and then probed (or reprobed) with primary antibody overnight at 4°C. The membrane was incubated with appropriate secondary antibody conjugated with horseradish peroxidase and immunoreactive proteins were visualized using Luminata Forte Western HRP substrate (Millipore, Billerica, MA). The stripping protocol followed the instructions of Restore Western Blot Stripping Buffer (Thermo Fisher). No blots were used more than three times. The following antihuman antibodies were used for western blot: ASK1 (1:750, Cell Signaling, Danvers, MA), TRX (1:1000, Abcam, Cambridge, United Kingdom), TAB1 (1:1000, R&D, Minneapolis, MN), P-TAB1 (1:800, Milipore, Thr431, Burlington, MA), P-p38MAPK (1:300, Cell Signaling, T180/Y182), p38MAPK (1:1000, Cell Signaling) (Table 3).

Immunochemical localization

For immunocytochemistry of Cytokeratin-18 (1:300, Abcam), ASK1 (1:250, Cell Signaling), TRX (1:500, Abcam), P-p38MAPK (1:300, Cell Signaling, T180/Y182), TAK1 (1:300, Santa Cruz), and TAB1 (1:300, R&D) AECs were seeded on glass coverslips at a density of 30 000 cells per slip and incubated overnight prior to treatment with control or CSE 1:10 media. After a 1-h serum starvation, cells were treated for 1 h and promptly fixed with 4% PFA, permeabilized with 0.5% Triton X, and blocked with 3% BSA in PBS prior to incubation with primary antibodies overnight at 4°C. After washing with PBS, slides were incubated in Alexa Fluor 488 or 594 conjugated secondary antibodies (Life Technologies) diluted 1:1000 in PBS for 1 hour in the dark. Slides were washed with PBS and then treated with NucBlue Live ReadyProbes Reagent (Life Technologies) and mounted using Mowiol 4–88 mounting medium (Sigma-Aldrich).

Confocal microscopy and image analysis

Images were captured using LSM 510 Meta UV confocal microscope (63×; Zeiss, Germany). Five regions of interest per image were used to determine red and green fluorescence intensity. Image modifications (brightness, contrast, and smoothing) were applied to the entire image using Image J (open source).

Pull down assay

ASK-1-Trx pull down assays were conducted based on methods previously reported by Hsieh and Papaconstantinou [57]. Varying concentrations of protein (500, 1000, and 500 µg plus 1% formalin fixed protein) coming from total cell lysates of both control and treated cells were incubated overnight with ASK1 antibody (1:100) or GSK3beta antibody (1:100) (control) rocking at 4°C (Figure 3). The next day 40 µL of protein-A agarose beads were added and incubated at 4°C for 1 h. Samples were then spun down at 6000 g, and the beads were washed three times with 1 mL of ice cold PBS. Blue dye was added, and the samples were heated for 5 min at 95°C and loaded onto a Western blot to look for ASK1 and Trx pool levels. Trx pulldowns were carried out with 500 µg of control and CSE total cell lysates and followed the same protocol as the ASK1 pulldown.

TGF-beta ELISA

AEC-conditioned media were collected from control and CSE after 48 h of treatment. Human amniotic fluid was collected at term vaginal deliveries (TL) and term-not-in-labor caesarian deliveries (TNIL), and a human/mouse TGF-beta 1 ELISA Ready-SET-Go! ELISA (second generation) (Affymetrix eBioscience) was conducted following the manufacturer's instructions. Standard curves were developed with recombinant protein samples of known quantities. Sample

concentrations were determined by correlating the samples absorbance to the standard curve by linear regression analysis.

Statistics

Statistical analyses for normally distributed data was performed using an ANOVA with the Tukey Multiple Comparisons Test ($N = 3$, $N = 5$; Figures 1 and 4), one-tailed t -test ($N = 3$) (Figure 4), and a two-tailed t -test ($N = 3$; Figures 3, 5, and 6). Statistical values were calculated using PRISM or IBM SPSS. P values of less than .05 were considered significant.

Results

Characterization of the ASK1-Signalosome in AECs

To determine the role of the ASK1-signalosome, we first studied the expression of the ASK1-Trx complex in primary AECs in culture. AECs were grown to confluence under standard cell culture conditions, and they showed expression of cytokeratin-18 (Figure 1a). AECs contain both ASK1 and Trx in total cell lysates, the two key components of the ASK1-signalosome complex, in cells grown under standard cell culture conditions (control) as well as in cells treated with OS inducer CSE (Figure 1b) (Supplementary Figure S1a). Cytotoxicity experiments showed viability of AECs after 1-h treatment with CSE (Figure 1c). As expected and shown before [38], CSE treatment induced p38MAPK phosphorylation in AECs, which was inhibited by antioxidant N-acetylcysteine (NAC) ($P < .05$) (Figure 1d and e) (Supplementary Figure S1b). These data (Figure 1b and d) confirmed that AECs contain both components of the ASK1-signalosome complex, and OS causes p38MAPK activation. To determine the existence of the ASK1-Trx signalosome as a complex in AECs, we conducted a series of pulldown assays (Figure 1f) (Supplementary Figure S1c). These assays included testing with different protein concentrations as well as strengthening the covalent bonds between the molecules using formalin. First, ASK1 was pulled down using 500 μg of total protein from both control and CSE. Western blot (Figure 1f) data showed that ASK1 was not associated with Trx in AECs. Reversing this experiment, a Trx pulldown was carried out using 500 μg of total protein from both control and CSE, revealing that Trx was not associated with ASK1. Next, we tested the existence of this complex by strengthening the bond between the molecules. To strengthen the covalent bonds connecting ASK1-Trx, total cell protein was fixed with 1% formalin, and then ASK1 was pulled down from 500 μg of protein in both groups. Western blot analysis still showed a dissociated complex, regardless of treatment. To confirm this finding and rule out issues of low amounts of proteins in our lysates, we increased the protein concentration to 1000 μg and repeated these experiments. As shown in the last column of Figure 1f, we were able to reproduce the same findings as with low protein concentration and note that ASK1 and Trx are not complexed in AECs, regardless of culture condition.

Localization of ASK1-signalosome in AECs

Immunocytochemistry followed by confocal microscopy and line graphing colocalization was used to validate western blot and pulldown findings concerning the localization of the ASK1-Trx complex in AECs. To rule out any deleterious effects of processing fetal membranes, AECs at passage number 0 (P0) and 1 (P1) were immunostained. As shown in Figure 2a, AECs in P0 and P1 under normal cell

culture conditions (control) and after CSE treatment showed ASK1 in both their cytoplasm and nuclei. Localization of ASK1 in the nucleus is rather unique and a noncanonical phenomenon. Trx was mainly localized in the cytoplasm, but staining was seen in the nucleus as well. P1 cells were further characterized since those cells were used for the rest of the experiments. Colocalization of ASK1-Trx across the total cell area was measured, and it showed low levels of association in control or CSE P1 cells (eg, P1 cells—Pearson's r coefficient 0.57 control and 0.41 CSE). Line graphs of select regions (Figure 2a, white bars) confirmed the above findings showing minimal colocalization, regardless of culture condition or treatment (Figure 2b). We further verified the existence of ASK1-Trx complex components within the nucleus of P1 cells (area within the blue line in Figure 2b) by western blot analysis. Figure 2c (Supplementary Figure S1c) revealed ASK1 and Trx in both cytoplasmic and nuclear extracts of lysed cells. Confirming immunolocalization analysis, western blots also showed ASK1 in the cytoplasm and nucleus, whereas Trx was predominantly in the cytoplasm.

Inhibition of ASK1 does not inhibit p38MAPK activation

The above experiments lead us to think that OS-induced p38MAPK activation is independent of ASK1-Trx complex dissociation, which would result in ASK1 release and canonical ASK1-MKK3/6-mediated p38MAPK activation. siRNA to ASK1 (Figure 3a and b) and MKK3/6 (Figure 3c and d) were used to test ASK1-independent activation of p38MAPK. AECs transfected with ASK1siRNA had 89.10% downregulation of the gene as determined by qRT-PCR (Figure 3a) and downregulation protein levels (Supplementary Figure S2) compared to controls (without siRNA and nontarget [NT] siRNA). This downregulation of ASK1 gene expression did not affect p38MAPK activation when AECs were simultaneously treated with siRNA to ASK1 and CSE ($P = .77$; Figure 3b and e).

Inhibition of MKK3/6 does not prevent p38MAPK activation

To verify that ASK1-independent activation of MKK3/6 was contributing p38MAPK activation, similar experiments were carried out with siRNA to MKK3/6, the upstream kinase to p38MAPK, which can be activated via phosphorylation by ASK1. Although MKK3/6 gene expression (92.40%; Figure 3c) and protein expression (Supplementary Figure S2) was knocked down compared to controls (without siRNA and NT siRNA); surprisingly, it did not affect p38MAPK activation after CSE cotreatment (Figure 3d and e; $P = .65$). These data suggested that ASK1 and MKK3/6 activations are not required for p38MAPK activation by CSE-mediated OS.

AECs increase TGF-beta production when treated with CSE

The above data suggest that p38MAPK activation in response to CSE is unlikely mediated through ASK1-Trx signalosome dissociation or downstream activation of ASK1-mediated MKK3/6. An alternate pathway of p38MAPK activation is through TGF-beta-mediated TAK1, TAB1 signaling. As shown in Figure 4a, 48-h treatment of AECs with CSE increased (70.95 pg/mL) TGF-beta release compared to control (49.23 pg/mL; $P < .05$) as determined by ELISA. The CSE-induced increase was downregulated by antioxidant NAC (20.46 pg/mL; $P < .05$), suggesting an OS effect on AEC TGF-beta production (Figure 4a). In addition, we have also shown that CSE-treated AEC-derived exosomes also carry TGF-beta signaling molecules compared to AECs grown under normal cell culture

Figure 1. ASK1-Signalsome in AECs. (a) Immunofluorescence images of Cytokeritin-18 (CK-18) positive (green) AECs. Scale bar is set to 30 μ M. (b) Western blot analysis—ASK1 (dimer 150 kDa top band, monomer 75 kDa lower band) and Trx are present in AECs, regardless of treatment. (c) Crystal violet stain (purple) was not expelled from AECs after CSE 1:10 treatment for 1 h. This was confirmed by plate reader analysis showing no difference in crystal violet intensity. (d) Cigarette smoke extract (CSE) treatment induced P-p38MAPK ($P < .05$), while NAC ($P < .05$) prevented its activation. (e) Densitometry of P-p38MAPK (1C) over total p38MAPK in CSE and CSE + NAC exposed AECs. A one-way ANOVA with the Tukey Multiple Comparisons Test was used to test statistical significance. (f) ASK1 pulldowns in columns 1, 2, 5, 6, 7, and 8 all show ASK1 present, but not Trx, in both control and CSE bead samples, while the Trx Pulldown in columns 3 and 4 show Trx present, but not ASK1, in both control and CSE bead samples.

Figure 2. Immunofluorescence staining and Western blots of ASK1 and Trx in AECs. (a) Confocal microscopy was used to analyze the ratio of ASK1 and Trx in AECs. Trx (red) and ASK1 (green) staining can be seen in the cytoplasm and nuclei of control AECs. White lines represent regions of interest to look for ASK1-Trx colocalization. Scale bar is set to 10 μ M. (b) Control and CSE graphs of ASK1-Trx showing no colocalization and ASK1 in the nucleus. (c) Western blot analysis of nuclear and cytoplasmic lysates confirms ASK1 (dimer 150 kDa top band, monomer 75 kDa lower band) in the nucleus and Trx in the cytoplasm.

conditions [54]. This is the first report to document that OS causes AECs to increase TGF-beta release. This finding rationalizes our approach to investigate OS-induced TGF-beta increase as a potential activator of p38MAPK activation. Consequently, AECs treated with TGF-beta (2 ng/mL) resulted in p38MAPK activation via phosphorylation within 1 h (two-fold change; Figure 4b) while cotreatment with a TGF receptor antagonist significantly prevented p38MAPK activation ($P = 0.04$) (Figure 4c) (Supplementary Figure S1d). Amniotic fluid ELISA analysis of TGF-beta showed that term labor samples had a higher concentration of TGF-beta (72.55 pg/mL) compared to samples from TNIL (15.40 pg/mL; $P < .05$; Figure 4d).

OS activation of the TGF-beta -signaling pathway is independent of TAK1 in AECs

To test the role of OS-induced TGF-beta signaling, its downstream effects on TAK1 and TAB1 were examined. To rule out that TAK1-TAB1 are still linked, dual immunofluorescence staining was performed for TAK1-TAB1. Data showed colocalization of TAK1-TAB1 (Figure 4e), suggesting that TAB1 is associated with TAK1 expression. Western blot analysis showed an increase in P-TAB1 after CSE treatment ($P = .047$), an effect that was prevented by NAC ($P = .057$; Figure 4f and g) (Supplementary Figure S1e). Though western blot analysis did not yield data on P-TAK1, siRNA to TAK1

was used to validate its role in TGF-beta TAK1-TAB1-mediated p38MAPK activation. AECs were transfected with siRNA to TAK1 in control and OS conditions. Control AECs transfected with siRNA to TAK1 showed (91%; $P = .003$) reduction of TAK1 gene expression, while control AECs transfected with NT siRNA did not produce any change, as expected (6%; $P = .58$; Figure 5a). Although

TAK1 gene expression and protein expression (Supplementary Figure S2) was knocked down, interestingly, it did not affect p38MAPK activation after CSE co-treatment (Figure 5b and c; $P = 1.00$). These data suggest that TAK1 activation is not required for p38MAPK activation by CSE-mediated OS in AECs.

OS induces a TGF-beta-TAB1-dependent activation of p38MAPK

To confirm the role of the TGF-beta pathway mediators in inducing p38MAPK activation under OS conditions, AECs were transfected with siRNA to TAB1 (Figure 6). TAB1 gene expression was downregulated 90.33%, as documented by qRT-PCR (Figure 6a). Protein level downregulation of TAB1 was documented by western blot analysis (Supplementary Figure S2). Surprisingly, downregulation of TAB1 decreased the phosphorylation of p38MAPK by 40.18% ($P = .03$) in CSE-treated AECs (Figure 6b and c) compared to controls (without siRNA and NT siRNA), which suggests TAB1-mediated activation of p38MAPK. As shown in other systems [51, 53], we report that p38MAPK activation in AECs under CSE-induced OS conditions are likely mediated through an autophosphorylation pathway that has not been reported previously in AECs, likely through TAB1-mediated autophosphorylation.

Discussion

Reactive oxygen species generated as a result of intrauterine OS are essential for fetal and fetal tissue (placenta and membrane) growth during pregnancy [58–60]. At term, increased OS causes telomere-dependent, p38MAPK-mediated senescence of fetal membrane tissues, elevating an intrauterine inflammatory load capable of promoting labor and delivery [15, 37, 40]. Similar mechanistic events of senescence have been reported in placenta and other uterine tissues, suggesting fetal tissue aging as a physiologic contributor of parturition [12, 15, 61, 62]. Premature activation of senescence can occur when tissues are exposed to OS-inducing risk factors. Premature senescence and untimely activation of an uterotonic inflammatory cascade are associated with PTB, especially when complicated by preterm premature rupture of fetal membranes [3, 24]. Therefore, reducing PTB risk requires a much better understanding of molecular signifiers of OS-associated events. p38MAPK is implicated as a central regulator of the senescence process, and preventing its activation can be one strategy [40, 63, 64]. Understanding novel mechanisms and molecular targets that cause p38MAPK activation are needed to stop the p38MAPK effect. As currently tested, p38MAPK inhibitors are found to be unsuitable for clinical use [63].

Figure 3. Inhibition of ASK1 and MKK3/6 does not inhibit p38MAPK activation. (a) mRNA levels of ASK1 showed siRNA to ASK1 decreases its expression by 89% ($P < .0001$) compared to NT siRNA. A two-tailed t -test was used to test statistical significance. (b) AECs treated with CSE induced P-p38MAPK, but treatment with siRNA to ASK1 and CSE did not reduce p38MAPK phosphorylation. (c) mRNA levels of MKK3/6 showed siRNA to MKK3/6 decreases its expression by 90% ($P < .0002$) compared to NT siRNA. A two-tailed t -test was used to test statistical significance. (d) AECs treated with CSE induced P-p38mapk, as seen previously, but treatment with siRNA to MKK3/6 and CSE did not reduce P38MAPK phosphorylation. (e) Quantitative densitometry (b and d) of P-p38MAPK over total p38MAPK shows a significant increase of activated p38MAPK when treated with CSE ($P < .003$); however, its phosphorylation is not inhibited when treated with siRNA to ASK1 or MKK3/6 and CSE.

Figure 4. Production and function of TGF- β in AECs. (a) ELISA for TGF- β conducted on AEC supernatant that had been stimulated with CSE for 48 h. AECs showed a significantly increased production of TGF- β when stimulated with CSE ($P < .05$) and NAC can inhibit this production ($P < .05$). A one-way ANOVA with the Tukey Multiple Comparisons Test was used to test statistical significance. (b) AECs treated with 2 ng/mL of TGF- β for up to 1 h caused activation of p38MAPK (two-fold) compared to control treated AECs. (c) AECs treated with CSE for 1 h significantly induced p38MAPK activation ($P < 0.0001$) while TGF receptor antagonist prevented p38MAPK activation ($P = 0.03$). (d) ELISA for TGF- β conducted on amniotic fluid of TNIL or TL deliveries showed significant expression of TGF- β at term ($P < .05$). A one-tailed t -test was used to test statistical significance. (e) Immunofluorescence colocalized TAK (red) and TAB1 (green) inside control treated AECs. White lines represent regions of interest to look for TAK1-TAB1 colocalization. Both cells showed overlapping line graphs of TAK1-TAB1 documenting colocalization within the cytoplasm. Scale bar is set to 30 μ m. (f) Western blot analysis confirmed that TAB1 can be significantly ($P = .047$) activated by CSE and antioxidant NAC can significantly prevent this activation ($P < .057$). (g) Quantitative densitometry of P-TAB1 over actin for panel f showing statistical significance. A one-way ANOVA with the Tukey Multiple Comparisons Test was used to test statistical significance.

Figure 5. Inhibition of TAK1 does not inhibit p38MAPK activation. (a) mRNA levels of TAK1 showed siRNA to TAK1 decrease its expression by 91% ($P = .003$) but NT siRNA does not decrease TAK1 gene expression 6% ($P = .58$). A two-tailed t -test was used to test statistical significance. (b) AECs treated with CSE and siRNA to TAK1 did not significantly reduce p38MAPK phosphorylation. (c) Quantitative densitometry (b) of P-p38MAPK over total p38MAPK showed significant increase of p38MAPK phosphorylation in AECs when treated with CSE ($P = .001$) and NT siRNA ($P = .001$); however, there was no significant difference of activated p38MAPK phosphorylation when treated with siRNA to TAK1 + CSE ($P = 1.00$). Surprisingly, NT siRNA + CSE also significantly upregulated p38MAPK activation compared to CSE alone ($P = .006$). A two-tailed t -test was used to test statistical significance.

Our study tested two major mechanisms of p38MAPK activation in AECs in response to CSE-induced OS. Our findings suggest that OS-induced canonical p38MAPK activation pathway involving ASK1-Trx signalosome and MMK3/MMK6 does not exist in AECs. However, OS causes a TGF- β increase in AECs and activates its downstream effector TAB1, which can lead to autophosphorylation and, thus, activation of p38MAPK.

p38MAPK activation can occur through multitudes of signalers that can include, but are not limited to, bone morphogenetic protein receptor, G-protein-coupled receptors, and tyrosine kinase receptor 1, all of which can be activated by various stimulants (eg, OS, genotoxic and teratogenic agents, infection and inflammation, heat shock, etc.). ASK1, a member of the MAPKKK family, activates p38MAPK in response to an array of stresses, such as OS, mitochondrial, and endoplasmic reticulum stress [65]. ASK1 maintains its inactive status by complexing with Trx. Oxidation of Trx liberates ASK1 from its inhibitory state to become active, leading to the activation of the classical pathway. ASK1-mediated sequential activation of MAPKs eventually leads to the activation of MKK3/6 and/or MKK4, which are immediate precursors of p38MAPK. Screening of senescence-

Figure 6. OS induces a TGF- β -TAB1-dependent activation of p38MAPK. (a) mRNA levels of TAB1 showed siRNA to TAB1 decrease its expression by 91% ($P < .0001$) compared to NT siRNA. (b) AECs treated with CSE and siRNA to TAB1 significantly reduce p38MAPK phosphorylation ($P = .0295$). A two-tailed t -test was used to test statistical significance. (c) Quantitative densitometry (b) of P-p38MAPK over total p38MAPK shows a significant decrease of activated p38MAPK phosphorylation when treated with siRNA to TAB1 and CSE ($P = .0295$). A two-tailed t -test was used to test statistical significance.

related proteins showed phosphorylated ASK1 in AECs [38], which led us to investigate ASK1-mediated p38MAPK activation since this can be a good target for reducing OS-induced senescence. In AECs, under normal and OS conditions, Trx and ASK1 exist in both their cytoplasm and nuclei; however, they are not complexed, regardless of redox status. Reducing ASK1 or its downstream effector MKK3/6 did not affect p38MAPK, suggesting an ASK1-independent mechanism of p38MAPK activation under CSE-induced OS. Localization of ASK1 in AEC nuclear extracts was a unique finding. Although the exact role of this localization is unclear, we speculate that ASK1 is being sequestered to the nuclei of AECs during early stages of gestation to protect cells from senescence when high levels of OS are experienced during fetoplacental growth, specifically during the second trimester [60, 66]. So even with all upstream elements being present in AECs, p38MAPK activation through the traditional cascade is somewhat suppressed. It is likely that

activation of the ASK1-signalosome or existence of the canonical pathway can be detrimental to the growth and survival of the fetus by causing premature aging of fetal tissues, leading to a dysfunctional status.

TGF- β , a growth factor and OS responder, produces its effects through the TGF- β /TGF receptor pathway that involves MAPKKK family member TAK1 and scaffold protein TAB1 [67]. OS in AECs increased TGF- β and led to p38MAPK activation, an effect that was prevented by TAB1 silencing. The TGF- β -TGF β R-TAB1 axis causing p38MAPK activation through allosteric autophosphorylation has been reported in many other systems under specific conditions [45, 68, 69]. TAB1 has been shown to activate TAK1, which can cause activation of MKK3 and 6, precursors of p38MAPK activation through classic pathways [67]. Silencing of ASK1, MKK3/6, and TAK1 did not reduce p38MAPK activation in AECs, suggesting that p38MAPK activation in AECs is independent of canonical elements. Silencing of TAB1 prevented this effect, supporting that p38MAPK activation in AECs is likely an autophosphorylation by TAB1, an alternate pathway. As pointed out by DeNicola et al., since p38MAPK is a serine/threonine kinase, it is theoretically difficult to accept the autophosphorylation of Thr180 and Tyr182 within the activation loop; however, the existence of such an autophosphorylation mechanism has been reported to cause p38MAPK activation [70]. It is also to be noted that TAB1-dependent autophosphorylation is limited to p38MAPK α and not the other three isoforms. It is likely that the classic pathway may still be operational for activation p38MAPK β , the other form seen in fetal cells.

The biologic relevance of TAB1-dependent activation of p38MAPK in AECs is unclear. The existence of other upstream activators and their increased expression under OS does not rule out redundancy in p38MAPK activation. Involvement of other activators and activation of the classical pathway are likely stimulant-dependent and required to maintain base level activation of p38MAPK required for various other cellular function. As murine pregnancy models indicate, p38MAPK is a constitutively expressed protein in fetal membranes from day 9 of gestation, and it peaks at day 15. Senescence is not observed until day 15, suggesting other functional roles for p38MAPK in tissue remodeling. Early indications from our ongoing studies suggest that p38MAPK activation is required for collagenolytic processes and replenishment of cells shed from the fetal membranes to maintain membrane homeostasis. Therefore, it is likely that redox changes in fetal tissues can activate p38MAPK through canonical pathways for tissue remodeling. We have already reported the roles of lysyl oxidase (LOX) and lysyl oxidase like (LOXL) enzyme in OS-induced tissue remodeling [71] suggesting that besides p38MAPK, other enzymes may also contribute to ECM remodeling. Overwhelming OS experienced at term, or untimely OS in response to pregnancy risk factors as seen in PTB, may cause alternative pathway activation and p38MAPK autophosphorylation to facilitate parturition to avoid a hostile environment for the growing fetus.

In summary, we report, for the first time, an alternate mechanism of activation of p38MAPK in fetal cells exposed to OS. An OS-mediated TGF- β increase can lead to TAB1-mediated activation of p38MAPK. Since many pregnancy complications are associated with increased OS and OS-associated damages that can activate p38MAPK, identification of this pathway is relevant in translational medicine in designing interventional strategies to prevent p38MAPK-mediated deleterious effects on cells and adverse events during pregnancy.

Supplementary data

Supplementary data are available at [BIOLRE](https://doi.org/10.1002/biol.201800000) online.

Supplemental Figure 1. Original western blot gel images. (a) Two identical western blot gels were probed for ASK-1 or Trx and exposed at the same time. Short exposure image on top and longer exposure image on bottom. Cropped images (yellow) were used to create Figure 1b. (b) The same western blot gel was probed for both P-p38MAPK (yellow) and total p38MAPK (red) and exposed for the same time. Cropped images were used to create Figure 1d. (c) Three individual gels comprise this image and contain components of both Figures 1f and 2c. Gel 1 contains columns 1 and 2 from Figure 1f (yellow). Gel 2 contains columns 3 and 4 for Figure 1f (yellow) and all of Figure 2c (red), while gel 3 contains columns 5–8 from Figure 1f (yellow). Short exposure images on top and longer exposure images on bottom. Each gel was cut at the 37 MW line and the top half was probed for ASK-1 and the bottom half was probed for Trx. The top and bottom of each gel was then developed together with uniform exposure. (d) The same western blot gel was probed for both P-p38MAPK (yellow; right side) and total p38MAPK (yellow; left side) and exposed for the same time. Cropped images were used to create Figure 4b. (e) The same western blot gel was probed for both P-TAB1 (yellow) and actin (red) and exposed for the same time. Cropped images were used to create Figure 4f. Uniform contrast/brightness were adjusted with ImageJ for all western blot images.

Supplemental Figure 2. Representative western blot showing down-regulation of TAK1, ASK1, MKK3/6, and TAB1 in response to cotreatment of respective siRNA with CSE.

Author contributions: LSR designed and conducted experiments, performed data analysis, and drafted the manuscript. CLD helped with experiments and data interpretation. LAA helped with siRNA experiments. RM conceived the project, designed experiments, helped with data analysis and interpretation, and prepared manuscript.

Conflict of Interest: The authors have declared that no conflict of interest.

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