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Stretch and inflammation-induced Pre-B cell colony-enhancing factor (PBEF/Visfatin) and Interleukin-8 in Amniotic epithelial cells

Claire E. Kendal-Wright, Ph.D^{1,*}, Daniela Hubbard¹, Jessica Gowin-Brown¹, and Gillian D. Bryant-Greenwood, Ph.D.²

¹Department of Obstetrics, Gynecology and Women's Health, University of Hawai'i, Honolulu, HI, 96813

²Department of Cell and Molecular Biology, John A Burns School of Medicine, University of Hawai'i, Honolulu, HI, 96813

Abstract

Preterm birth continues to be a growing problem in the USA. Although approximately half of preterm births are caused by intrauterine infection, uterine over-distension is also a cause. In this study we have compared the effects of static stretch, cyclic stretch/release and an inflammatory stimulus alone and in combination on the expression of Pre-B cell colony-enhancing factor (PBEF) and IL-8 in primary amniotic epithelial cells (AEC). We then sought to identify some of the mechanism(s) by which these cells respond to stretching stimuli. We show that cyclic stretch/release is a more robust stimulus for both *PBEF* and *IL-8* than static stretch. Cyclic stretch/release increased both intracellular and secreted PBEF and a combination of both types of stretch was a more robust stimulus to *PBEF* than *IL-8*. However, when an inflammatory stimulus (IL-1 β) was added to either kind of stretch, the effect on *IL-8* was much greater than that on *PBEF*. Thus, different kinds of stretch affect the expression of these two cytokines from AEC, but inflammation is a much stronger stimulus of *IL-8* than *PBEF*, agreeing with its primary role as a chemokine. Although the AEC showed morphological signs of increased cellular stress during stretching, blocking reactive oxygen species (ROS) had little effect. However, blocking integrin binding to fibronectin significantly reduced the responses of both *PBEF* and *IL-8* to cyclic stretch/release. The increased PBEF, both intracellularly and secreted, suggests that it functions both to increase the metabolism of the cells, at the same time as stimulating further the cytokine cascade leading to parturition.

Keywords

Pre-B-cell colony-enhancing factor; IL-8; stretch; inflammation; fetal membranes

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Address correspondence to: Dr. C. E. Kendal-Wright, Developmental and Reproductive Biology, University of Hawaii, 651 Ilalo Street, BSB 211-13, John A. Burns School of Medicine, Honolulu, Hawaii, USA 96813. kendal@pbrc.hawaii.edu.

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1. Introduction

Preterm birth continues to be a growing problem as its rate has risen by 20% in the USA in the last ten years [1]. Approximately 50% of premature births are associated with intrauterine infection/inflammation [2], which triggers an immune response involving the proinflammatory cytokines, which then cause the synthesis and release of uterotonic prostaglandins [3]. However, normal term parturition also involves local cytokine signaling [4]. During normal pregnancy, the fetal membranes are subjected to a combination of chronic distension, allowing their accommodation to the growing fetus [5] and acute cyclical stretch/release both during Braxton-Hicks contractions, and during the major uterine contractions of labor. The stretching of the fetal membranes is known to increase a number of cytokines [6] which are associated with preterm birth [7]. In addition, inflammation caused by local infection or sterile inflammatory mediators such as thrombin also induce localized cytokine production. Therefore, in this study we attempted to mimic the *in vivo* situation where different stretch stimuli can occur simultaneously with inflammatory stimuli.

Pre-B-cell colony-enhancing factor (PBEF/visfatin) is a cytokine involved in the events of parturition [8,9]. It is expressed in all cellular layers of the fetal membranes [10] and both labor and sterile distension cause its increased expression [8,9]. Indeed, a number of stimuli associated with inflammation and infection; LPS, TNF α , IL-1 β and IL-6 all up-regulate its expression [10] while in severe chorioamnionitis, it is produced by both the endogenous cells of the fetal membranes as well as the infiltrating neutrophils [9]. Although originally identified as a cytokine [12], more recently it was shown to be an adipokine and re-named Visfatin [13]. Although PBEF lacks a classical secretion sequence, its secretion has been demonstrated [12,14,15,13], although the mechanism is currently unknown [16]. The treatment of several different cell types with PBEF increases production of pro-inflammatory cytokines and matrix metalloproteinases (MMPs) [17,18,19,20], showing it to be an important modulator of the immune response [21], [15]. However, PBEF also functions intracellularly as the enzyme nicotinamide phosphoribosyltransferase (Nampt) and increases the amount of NAD⁺ available for metabolism [22]. Thus, the static stretch-induced increased production of PBEF [8,23] may be a mechanism for providing the extra energy needed for the cell to successfully alter its cytoskeleton and gene expression profile required for adaptation to the stimulus and accommodation for the extra work required during labor. At the same time, its action to induce some key cytokines, such as TNF α [24] clearly involved in the parturition process [2], would further assist in the progression of labor.

Distension of the fetal membranes also increases expression of the IL-8 gene [8,23,25]. IL-8 has potent chemotactic and neutrophil activation properties [26]. It is constitutively expressed by the endogenous cells of the fetal membranes [27] and its expression is increased during normal gestation, resulting in accumulation in amniotic fluid during the third trimester [7]. However, its expression is increased in acute infection [28] and IL-8 is therefore involved in both the initiation of normal term parturition and in infection-induced preterm birth.

The aims of this study were (1) to compare the effects of static stretch, cyclic stretch/release, and inflammation (alone and in combination) on the expression of PBEF and IL-8 in primary amniotic epithelial cells (AEC). (2) To show which pathways are activated and cause their up-regulation, focusing on the roles of reactive oxygen species (ROS) and integrins.

2. Methods and Materials

2.1. Tissue collection and amniotic epithelial cell culture

Fetal membranes (n=33) were collected from patients having elective Cesarean sections before labor (38-40 weeks gestation) at Kapiolani Medical Center for Women and Children (Honolulu, HI, USA) with approval from the University Committee on Human Experimentation and the Hospital Institutional Review Board. All tissues were examined by a pathologist for histological evidence of infection, and if positive were excluded.

Primary AEC were isolated as previously described [29] and as used in our prior studies [30]. In brief, the amnion was stripped from adjacent choriodecidua and the epithelial cells isolated by consecutive trypsin (0.2%) digestion (Sigma, St. Louis, MO). The purity of epithelial cells obtained from each patient was similar to that previously reported [30]. The cells were utilized without passage and were seeded at a density of 2 million per well in a 6 cm culture plate in DMEM:F12 supplemented with heat inactivated 10% FBS (Invitrogen), penicillin (50U/ml)-streptomycin (50µg/ml) and incubated at 37°C in 95% air/5% CO₂ for 4 days. Media was changed every 3 days until the cells were 70-80% confluent (7-10 days).

2.2. Culture of amniotic epithelial-like cells (WISH)

Human amnion-derived WISH cell (ATCC CCL25) were obtained from the American type Culture Collection (ATCC, Manassas, VA) and grown in Dulbecco's Modified Eagle Medium: Ham F-12 (DMEM:F12) (1:1) supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Life technologies, Carlsbad, CA), penicillin (50U/ml)-streptomycin (50µg/ml) and incubated at 37°C in 95% air/5% CO₂. When confluent, cells were plated onto Flexcell collagen IV (Flexcell International Corp, Hillsborough, NC) coated plates, at a density of 500,000 per well in DMEM:F12 medium containing 10% FBS and always used between passages 5-9.

2.3. Cell stretching experiments

When primary AEC (or WISH cells) reached sub-confluency, the medium was replaced with 0.5% FBS DMEM/F12 for 12-16 h prior to their use. In addition, fresh DMEM:F12 containing 0.5% FBS was added before exposure to any stimulation. Stretching was performed using a Flexcell instrument (Flexcell tension PlusT, FX-4000T) that simulates biological strain conditions using a vacuum to deform the cells cultured on a silastic flexible growth surface. This was set up and used based on the parameters designed by the manufacturer. The 20% static stretching of these cells was performed as described in our previous studies [23] and represents the maximum amount of stretch achievable with this instrument. Cyclic stretch/release was performed using repetition of 23 secs of 20% stretch followed by 7 secs of release to 0% stretch. In order to block integrin binding, the RGD peptide GRGDNP, 50 µM (Biomol International, Plymouth Meeting, PA) was pre-incubated with the cells for 2 h before stretching. Integrins bind to ligands with an exposed arginine-glycine-aspartate (RGD) sequence. Therefore, RGD peptides mimic this adhesion and can be used to block this interaction of the integrin with its usual extracellular matrix substrate. In order to inhibit reactive oxygen species, N-acetyl-L-cysteine (NAC, 30µM) (Sigma) was added to the cells 2 h before stretching.

2.4. RNA isolation and Quantitative Real-time PCR

Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Valencia, CA) and DNase (Ambion, Austin, TX) treated, in accordance with the manufacturer's instructions. Reverse transcription was performed with random hexamers. The resulting complementary DNA was used as a template for quantitative real-time PCR. Fluorescence resonance energy transfer (FRET) probes and primers for *PBEF*, *IL-8* and *I8S*, were purchased from Applied

Biosystems Taq Man Assays on Demand (ABI, Foster City, CA) and the ABI protocol used; one cycle for 10 mins at 95°C and 40 cycles of 15 secs at 95°C and 1 min at 60°C. Each 96-well plate included a water blank and a reverse transcriptase blank. cDNA dilutions were used to generate standard curves for each gene. Real-time PCR was carried out on a MJ Research Opticon 2 Continuous Fluorescence Detector (MJ Research, Waltham, MA). The contents of the PCR mix were; primers (200 nmol/L), FRET probe (100 nmol/L), magnesium (5.5 mmol/L) and Amplitaq Gold (0.025 U/μl). Each reaction was performed in triplicate and the results normalized to the expression of *18S* in each sample.

2.5. Immunofluorescence localization of PBEF and the integrin β1 subunit

After stretching, the cells were fixed in 3.7 % formaldehyde in 1X PBS followed by three washes in 1X PBS for 10 mins total. Non-specific binding was blocked with 1 % BSA (Sigma) in 1X PBS for 45 min. The cells were again washed and PBEF antibody at 1/500, (Phoenix Pharmaceuticals) or Integrin β1 subunit antibody at 1/500 (Chemicon) added to each well. After incubation for 1h, the cells were again washed and secondary antibody; anti-rabbit or anti-mouse alexafluor 546 at 1/10,000 (Molecular Probes, Eugene, OR), was added for 45 min. The cells were washed again and Flexcell membranes, with cells still attached, were cut around the edges with a scalpel to release them from the plastic tissue culture plate. These were then mounted in Mowiol/glycerol mounting media (Sigma) containing 1,4-Diazabicyclo[2.2.2]octane (DABCO) (Sigma) and viewed under fluorescence microscopy.

2.6. Phalloidin-TRITC staining for Actin cytoskeleton

After stretching, cells adherent to the Flexcell plates were fixed in 4% formaldehyde in 1X PBS containing calcium (0.901mM) and magnesium (0.493) followed by three washes in 1X PBS for 10 mins total. The cells were then permeabilized in 0.2% Triton x-100 in 1X PBS for 5 mins and washed three times with 1X PBS followed by blocking in 10% FBS (Invitrogen) in 1X PBS for 40 mins. The cells were washed again and then stained with Phalloidin-TRITC (Sigma) at 1/2500 in 1X PBS for 1 h. The cells were washed again before the Flexcell membranes were cut around the edges with a scalpel to release the membrane with the adherent cells. These were then mounted in Mowiol/glycerol mounting media (Sigma) containing 1,4-Diazabicyclo[2.2.2]octane (DABCO) (Sigma) and viewed under fluorescence microscopy.

2.7. Western blotting for PBEF, phosphorylated FAK and paxillin

Cell lysates (20 μl) were boiled for 5 min in 4X SDS loading buffer with 5mM DTT and loaded into a 10% SDS polyacrylamide gel. The electrophoretically separated proteins were then transferred onto nitrocellulose membranes. Immunoblotting was performed by overnight blocking with 5% non-fat milk (BioRad) 0.1% Tween-20 (Fisher) in 1X PBS. The membranes were then incubated with primary antibodies for 1h in blocking solution. Antibodies were used at; 1/1000 PBEF (Phoenix Pharmaceuticals, Belmont, CA), 1/300 phosphorylated FAK (Try 576), 1/500 phosphorylated Paxillin (Try 118) (Upstate). After washing, the membranes were incubated with secondary anti-rabbit antibody conjugated to horseradish peroxidase at 1/3000 (BioRad) for 45 min. The membranes were again washed and developed using an enhanced chemiluminescence kit (Amersham Inc, Piscataway, NJ). The blots were immediately exposed to hyperfilm-enhanced chemiluminescence (Amersham) and the signals quantitated with a densitometer (Kodak EDAS290 System: Eastman Kodak Co, Rochester, NY). The membranes were washed overnight with PBS 0.1% tween and then re-probed with GAPDH antibody (Abcam, Cambridge, MA) conjugated to HRP at 1/1000 at room temperature for 1h as a protein loading control. The membranes were again washed and developed using an enhanced chemiluminescence kit,

the blots exposed and the signals quantitated with a densitometer, the relative levels of each protein were normalized to the amount of GAPDH in each sample.

2.8. Statistical analysis

The Mann-Whitney U test was performed and only p values <0.05 were considered significant. All statistics were calculated with GraphPad software (GraphPad Software Inc, San Diego, CA).

3. Results

3.1. PBEF and IL-8 are increased by cyclic stretch/release in primary AEC

The effect of cyclic stretch/release has not previously been investigated using cells of the fetal membranes. Preliminary experiments were therefore performed with amniotic epithelial-like cells (WISH) (data not shown) and the Flexcell instrument using a protocol previously devised to simulate the dynamic effects of stretching myometrial cells [31]. Once the technique was established, primary AEC were then used to show the *PBEF* and *IL-8* gene responses. *PBEF* gene expression (Fig. 1A) was significantly ($p<0.05$) increased by 60% after 4 h of cyclic stretch/release. *IL-8* gene expression (Fig. 1B) increased more rapidly, reaching significance after only 20 mins of stimulation ($p<0.05$) and continued to rise over the 4 h, at which time, it was significantly increased ($p<0.05$) representing a 15-fold increase compared to the control. After 24 h of stretch/release stimulation, media were collected and the secreted PBEF and IL-8 proteins measured to confirm that the changes in gene expression were translated into protein. Secreted PBEF (Fig. 1C) was significantly increased ($p<0.005$) and IL-8 was significantly increased ($p<0.05$) (Fig. 1D).

PBEF is also active intracellularly and the effects of stretch/release on its intracellular pool were investigated. Immunofluorescence for intracellular PBEF in AEC showed increases in PBEF during 4 h of stretch/release stimulation (Fig. 2A-D). After 1 h, areas of very high density PBEF immunofluorescence were evident (Fig. 2C). The reason(s) for this are currently unknown. After 4 h of stretch/release, cytoplasmic staining of PBEF was generally more intense, and diffuse than at 1 h, although still punctate in its distribution. This PBEF increase was confirmed by quantitative Western blotting using cell lysates. An example Western blot is shown (Fig. 2E) showing the characteristic two PBEF bands [23] and the GAPDH protein loading control below. Quantitation ($n=4$) showed a significant increase ($p<0.05$) in intracellular PBEF after 4 h of cyclic stretch/release. These results show that both intracellular and secreted PBEF were increased by the cyclic stretch/release regimen.

3.2. Comparison of the effects of cyclic and static stretch on PBEF and IL-8 expression

The fetal membranes are subjected to different patterns of distension/stretching in the third trimester of pregnancy and different *in vitro* stretch protocols have been shown to cause differential effects on stretch-responsive genes [32,33]. We therefore compared the effects of different types of stretch on *PBEF* and *IL-8* gene expression. First, AEC were subjected to 2 and 4 h of 20% static stretch or 20% cyclic stretch/release, allowing a direct comparison of their effects in cells isolated from the same patients. Both static and cyclic stretch significantly up-regulated *PBEF* gene expression ($p<0.05$) compared to the unstretched controls (Fig. 3A). The cyclic stretch/release caused a significantly greater response ($p<0.05$) compared to static stretch at 2 h but not after 4 h (Fig. 3A). However, when the cells were subjected to a protocol of 2 h of static stretch followed by 2 h of cyclic stretch/release, the response was significantly greater ($p<0.05$) than for either type of stretch alone for 4 h (Fig. 3A). Expression of the *IL-8* gene was also measured (Fig. 3B). After 2 h both stretch protocols caused a significant increase in *IL-8* gene expression ($p<0.05$) compared to the unstretched controls (Fig. 3B). After 4 h the static stretch response had declined to

control levels (Fig. 3B) confirming our previous observation [23]. However, the cyclic stretch protocol caused a further significant increase in *IL-8* ($p<0.05$). The 2 h static stretch followed by 2 h of cyclic stretch also caused a significant increase in *IL-8* expression ($p<0.05$), but this was not as robust as that caused by 4 h of cyclic stretch/release alone (Fig. 3B). These data suggest that cyclic stretch/release is more robust at stimulating both *PBEF* and *IL-8* gene expression than static stretch and that the response of *IL-8* was more attenuated than that of *PBEF*. In addition, *PBEF* was more responsive to the consecutive effects of static stretch and cyclic stretch/release than *IL-8*.

3.3. The combined effect of stretch and inflammation on PBEF and IL-8 expression

Inflammation often occurs simultaneously with stretching in the fetal membranes. Therefore, we determined if the effect(s) of these stimuli were additive. Cells were stretched by either 20% static stretch or 20% cyclic stretch/release for 2 h, with or without the addition of IL-1 β (1ng/ml) to mimic a mild inflammatory response. 2 h was chosen as both the *PBEF* and *IL-8* genes were shown to be responsive to both static and cyclic stretch/release at this time. IL-1 β alone caused a significant increase in *PBEF* gene expression ($p<0.05$) (Fig. 4A). However, the response of *PBEF* in cells either statically stretched or exposed to cyclic stretch/release for 2 h was significantly greater ($p<0.05$) when IL-1 β was present during these stretching protocols (Fig. 4A). This was also the case for *IL-8* (Fig. 4B) although the magnitude of its response to IL-1 β was two orders more than that caused by either type of stretching alone (Fig. 4B). Thus, although both the *PBEF* and *IL-8* genes were responsive to stretch, an inflammatory stimulus (IL-1 β) caused a much more robust response in *IL-8* than in *PBEF*, and this was markedly greater when combined with static stretch.

3.4. The effect of reactive oxygen species (ROS) on the expression of PBEF and IL-8 after stretching

A morphological study of the effect of static stretching on the AEC was carried out. These were grown and statically stretched for up to 4 h and labeled for actin, in order to examine the cytoskeleton morphology. This demonstrated the formation of stress fibers after only 15 mins of static stretching (Fig. 5A). These fibers were visualized throughout static stretching for up to 4 h (Fig. 5A). By 4 h the cells also produced increased numbers of filopodia, indicating their increased levels of cellular stress. One of the typical consequences of this is the production of reactive oxygen species (ROS), known to be produced by cellular stretching [34] and previously shown to be involved in the production of some other cytokines [35]. Therefore, we blocked the production of ROS by the addition of N-acetyl-L-cysteine (NAC). WISH cells were used to avoid the patient variability intrinsic to AECs, these were pre-incubated with NAC (30 μ M) for 3 h before stretching. Again there were significant increases in *PBEF* and *IL-8* ($p<0.05$) gene expression after both static and cyclic stretch/release (Fig. 5B and C). However, there were no significant effects on either gene when NAC was added to either stretch protocol (Fig. 5 B-C). Therefore, taken together with the morphological changes, it is likely that ROS is produced during stretching of AEC but from these results it does not appear to be of major importance for the response of *PBEF* or *IL-8* to stretching.

3.5. The effect of stretch on integrin clustering and signaling

Integrins are transmembrane molecules that mediate cell attachment and transduce mechanical forces. Therefore, we investigated the effect of stretch on their activation in AEC. As integrins change in number, location or strength of adhesive contacts to alter cell harmonics, we investigated their expression pattern by immunofluorescence localization. The β 1 integrin subunit is present at the basal surface of AEC [36] and has been shown to cluster in keratinocytes facilitating adhesion after mechanical stretch [37]. Therefore, primary AEC were stretched using both stretch protocols for up to 4 h and the cells then

labeled with anti- $\beta 1$ antibody. Clustering of the $\beta 1$ integrin subunit was visualized on the basal surface of the AEC after 15 mins of cyclic stretch/release and remained over the 4 h tested (Fig. 6A-D). In Fig. 5A the normal cell surface expression of $\beta 1$ integrin expression was localized between cells at their membranous interface. However, after stretching for 15 mins and longer, $\beta 1$ clustering was also visible at the basal surface of the AEC where these cells adhere to the Flexcell plate (Fig. 6B-D). This clustering was not evident after static stretching (data not shown). Integrin receptors are known to function through the activation of a number of signaling molecules [38], we therefore showed that the stretch/release of these cells also caused the activation of the integrins by examining two of the signaling molecules involved; focal adhesion kinase (FAK) and paxillin. Primary AEC were cyclically stretch/released for 5, 10, 30 and 60 mins, cell lysates prepared and Western blots performed. Example Western blots for phosphorylated FAK and Paxillin are shown with GAPDH protein loading controls below each blot (Fig. 6E and F). Quantitation of Western blots (n=4) for both phosphorylated FAK (Fig. 6E) and phosphorylated paxillin (Fig. 6F), showed significantly increased levels of phosphorylation after 5 and 5-10 mins respectively ($p < 0.05$), confirming integrin signaling events occurred subsequent to the cyclic stretch/release stimulus.

3.6. The effect of integrin binding on PBEF and IL-8 gene expression after stretching in WISH cells

In order to determine if the activation of integrins is important for the increases in both *PBEF* and *IL-8* gene expression after stretching, WISH cells were again used. These were pre-incubated with an RGD peptide (50 μ M) for 3 h before stretching, in order to inhibit the binding of integrin subunits to fibronectin [33] and then stretched for 2 h. We also established that the RGD peptide alone had no effect on the expression of either *PBEF* or *IL-8* in the absence of stretching (Fig. 7A and 6B respectively). Static stretching and cyclic stretch/release both significantly ($p < 0.05$) increased the expression of both *PBEF* (Fig. 7A and B) and *IL-8* (Fig. 7C and D respectively), as shown previously for primary AEC (Fig. 3A and B). The RGD peptide marginally but not significantly reduced the expression of *PBEF* and *IL-8* during static stretch (Fig. 7A and C). However, blocking integrin binding to fibronectin significantly ($p < 0.05$) inhibited the up-regulation of both *PBEF* and *IL-8* caused by cyclic stretch/release (Fig. 7B and D). These results were confirmed with primary AEC, but because of large inter-patient variability in the responses of these cells, results with cells from one patient are shown for *PBEF* and *IL-8* respectively (Fig. 7E and F). The RGD peptide only reduced the expression of *PBEF* (Fig. 7E) and *IL-8* (Fig. 7F) when the cyclic stretch/release protocol was used.

4. Discussion

In this study we have compared the effects of static stretch, cyclic stretch/release and an inflammatory stimulus alone, and in combination, on the expression of *PBEF* and *IL-8* in primary AEC. We then sought to identify the mechanism(s) by which these cells responded to the stretching stimuli. Thus, we hoped to gain insights into how cellular stretching affects these two cytokines and how concurrent stretching and inflammation may alter their responses.

We have shown that cyclic stretch/release of AEC up-regulates both the *PBEF* and *IL-8* genes and that these were translated into increases in their secreted proteins. We used the maximum amount of stretch (20%) possible with the Flexcell system as fetal membranes are stretched to as much as 70% *in vivo* at term [5]. *PBEF* increased by almost 400% compared to an increase of approximately 50% for *IL-8*. Thus, although *IL-8* gene expression was robustly increased, this was not translated into high levels of secreted *IL-8*. This is similar to our previously published data where we showed that transiently increased *IL-8* gene

expression following static stretch was not translated into protein [23]. Indeed, it may be that the modest increase in secreted IL-8 caused by stretching is merely a mechanism to assure its constitutive expression during gestation [27]. This is the first time that the effects of cyclic stretch/release have been shown for *PBEF* expression, although it has been shown to increase *IL-8* expression in smooth muscle cells [39], cervical fibroblasts [40] and myometrial cells [41]. As *PBEF* is an intracellular enzyme as well as a secreted cytokine, we also measured its intracellular changes after cyclic stretch/release. We show that this rapidly increased after only 15 min, and was maintained over the 4 h tested. Some of this intracellular pool was likely to be stored *PBEF* but it probably also represents *de novo* production. *PBEF* in its intracellular form is identical to that of its secreted form [42] and functions as the metabolic enzyme 'Nampt' which sequesters NAD^+ by its salvage pathway [22]. Thus, by cyclically stretching these cells, their increased metabolic needs may also be met.

We have compared the effects of cyclic stretch/release with static stretching and shown that the former stimulated these genes more than static stretch. In addition, we showed that if these two types of stretch were applied consecutively, they had a marked additive effect on the expression of *PBEF*. *PBEF* is responsive to static stretching over longer periods of time [23], thus it is likely that in this case the static stretch 'primed' the cells for the subsequent response to cyclic stretch/release, resulting in a greatly enhanced up-regulation of *PBEF* compared to either stretch stimulus alone for the same amount of time. This is important because the cells of the fetal membranes *in utero* are in a state of constant stretch, as they accommodate the growing fetus, but as the pregnancy progresses they are also subjected to fetal movements and Braxton Hicks contractions. Therefore our data suggests that static stretching, followed by cyclic stretch/release *in vivo* would cause a robust up-regulation of *PBEF*.

The effect(s) of an inflammatory stimulus, at the same time as stretching was also studied, because *in vivo* these tissues are often subjected to both stimuli together. IL-1 β is often used to induce a milder (non-infection driven) inflammatory response compared to the addition of LPS, which is used to mimic bacterial infection. IL-1 β is able to induce endogenous expression of other inflammatory cytokines and is an acceptable way to mimic non-infection driven inflammation. We showed that IL-1 β had a marked additive effect on the expression of *PBEF* and *IL-8* when used with either stretching protocol. However, this effect was much greater for *IL-8*, than *PBEF*.

During static stretching, actin stress fibers and filapodia became visible suggesting an increased production of ROS due to cellular stress. However, blocking the production of ROS with NAC failed to significantly alter the stretch-induced increases in *PBEF* and *IL-8*. Although NAC has anti-oxidant properties and functions as a free radical scavenger blocking ROS and decreasing cytokine production [35] the lack of any effects here suggests that it contributes little to the effects of stretch on these genes. However, other ROS inhibitors need to be investigated for further clarification of the importance of ROS in the production of other cytokines after stretch.

The role of integrins in the stretch-induced increases in *PBEF* and *IL-8* was also investigated, as the $\alpha\text{v}\beta\text{6}$ integrin has been localized to the amnion of the fetal membranes and shown to increase near term [44]. We confirmed their activation by clustering and downstream signaling by FAK and paxillin phosphorylation after cyclic stretch/release. When the binding of integrins to their fibronectin substrate was blocked and the cells stretched, the increases in *PBEF* or *IL-8* after cyclic stretch/release was prevented. To do this a previously successful strategy in which we pre-treated our cells with an RGD peptide was used [33]. Although, this inhibited the increases in *PBEF* and *IL-8* by cyclic stretch/

release, it had no effect on their increased expression after static stretching. Integrins are mechanoreceptors sensing changes between the cell surface and the surrounding extracellular matrix. Thus during the static stretch stimulus, only a single change upon the initial stretching stimulus would be registered. However, during cyclic stretch/release, repeated changes between the cell and the extracellular matrix upon each stretch/release cycle would be registered. We conclude that integrin activation is central to the increases in these two cytokine genes caused by cyclic stretch/release. Endogenous changes in integrin expression in the fetal membranes from patients in preterm labor, with or without PPROM, have not been studied to date, but would be of interest in light of these results.

In conclusion, we have shown that cyclic stretch/release is a more robust stimulus for both *PBEF* and *IL-8* than static stretch. Cyclic stretch/release increased both intracellular and secreted PBEF, suggesting that its dual functions as an enzyme and secreted cytokine are important. A combination of static stretch followed by cyclic stretch/release was a more robust stimulus for *PBEF* than for *IL-8*. However, when IL-1 β was added to either kind of stretch, the effect on *IL-8* was very much greater than for *PBEF*, in line with its major role as a chemokine [26]. Blocking the integrin binding to fibronectin significantly reduced the responses of both *PBEF* and *IL-8* to cyclic stretch/release. Therefore, we have shown that different kinds of stretching affect the expression of these two cytokines produced by AEC and although inflammation was a much stronger stimulus for *IL-8* than PBEF, had an additive effect on the expression of both cytokines.

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Fig. 1.

Effects of cyclic stretch/release on expression of *PBEF* and *IL-8* in primary AEC (n=4 cells from different patients). (A) *PBEF* and (B) *IL-8* gene expression during stimulation by cyclic stretch/release for 4 h. (C) *PBEF* and (D) *IL-8* proteins secreted into medium after 24 h of cyclic stretch/release. Values are mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.005$ compared to control (no cyclic stretch/release).

Fig. 2. Effects of cyclic stretch/release on expression of intracellular PBEF protein. Immunofluorescence labeling of PBEF in AEC during 4 h of cyclic stretch/release stimulation. (A) T0 shows diffuse cytoplasmic labeling of PBEF, (B) at 15 mins there was increased intensity of PBEF labeling which continued during (C) 1 h and (D) 4 h of stimulation. (E) An example of a Western blot of PBEF with GAPDH as control using AEC lysates. Below, quantitation of Western data from n=4 (cells from different patients). Values are mean $\pm$ SEM. *p<0.05 compared to control (no cyclic stretch/release).

Fig. 3. Effects of static stretch and cyclic stretch/release on *PBEF* and *IL-8* gene expression in AEC (n=4) from different patients. (A) *PBEF* (B) *IL-8*. Values are mean $\pm$ SEM. $P < 0.05$ for dissimilar letters.

Fig. 4.

The combined effect(s) of stretch and an inflammatory stimulus (IL-1 β (1ng/ml)) on *PBEF* and *IL-8* gene expression in AEC. (A) *PBEF* (B) *IL-8*. Values are mean $\pm$ SEM. $P < 0.05$ for dissimilar letters.

Fig. 5.

The role of reactive oxygen species (ROS) in the responses of *PBEF* and *IL-8* to stretching was shown by the specific blocking of ROS by NAC (30 μ M). In AEC the localization of stress fibers (white arrows) and filopodia (green arrows) in primary AEC during 4 h of static stretching. In WISH cells (n=6) static and cyclic stretch/release increased both *PBEF* (B) and *IL-8* (C) gene expression. Values are mean $\pm$ SEM.

Fig. 6.

Integrins were activated in AEC by the cyclic stretch/release stimulus, shown by immunofluorescent localization of integrin $\beta 1$ subunit (A-D). Clustering on the basal surface of the AEC (white arrows) can be seen after 15 mins of stimulation, maintained at both 1 h and 4 h of continued cyclic stretch/release. (E) Western blot of p-FAK with AEC lysates during 1 h of cyclic stretch/release, with GAPDH as control and quantitation (n=4) cells from different patients shown below. (F) Western blot of p-Paxillin of the same cell lysates shown during 1 h of cyclic stretch/release, with GAPDH as control and quantitation (n=4) shown below. Values are mean $\pm$ SEM. * $p < 0.05$ compared to no stretch control.

Fig. 7.

The role of integrins in *PBEF* and *IL-8* responses to stretching was shown by addition of a blocking peptide (RGD) to WISH cells during static and cyclic stretch/release (n=5). (A) *PBEF* (C) *IL-8* gene expression with static stretch and (B) *PBEF* and (D) *IL-8* with 2 h of cyclic stretch/release. An example experiment completed with primary isolated AEC from one patient showing gene expression for (E) *PBEF* and (F) *IL-8*. Values are mean $\pm$ SEM. $P < 0.05$ for dissimilar letters.