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Mechanical strain induced phospho-proteomic signaling in uterine smooth muscle cells

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ABSTRACT

Mechanical strain associated with the expanding uterus correlates with increased preterm birth rates. Mechanical signals result in a cascading network of protein phosphorylation events. These signals direct cellular activities and may lead to changes in contractile phenotype and calcium signaling. In this study, the complete phospho-proteome of uterine smooth muscle cells subjected to mechanical strain for 5 min was compared to un-strained controls. Statistically significant, differential phosphorylation events were annotated by Ingenuity Pathway Analysis to elucidate mechanically induced phosphorylation networks. Mechanical strain leads to the direct activation of ERK1/2, HSPB1, and MYL9, in addition to phosphorylation of PAK2, vimentin, DOCK1, PPP1R12A, and PTPN11 at previously unannotated sites. These results suggest a novel network reaction to mechanical strain and reveal proteins that participate in the activation of contractile mechanisms leading to preterm labor.

1. Introduction

During pregnancy, the uterus increases in size to accommodate the growing fetus, placenta, and amniotic fluid. Despite the associated increases in intra-uterine pressure, the normal myometrium adapts to prevent increased tension and remain in a quiescent state until term (Sivarajasingam et al., 2016; Sokolowski et al., 2010). Abnormal distension has been associated with early birth in twin (Gardner et al., 1995; Mercer et al., 1996), as well as approximately 10% of singleton (Lockwood and Kuczynski, 2001) preterm deliveries. Increasing uterine volume via a balloon catheter initiates prostaglandin secretion, myometrial contractions, and labor in women (Delaney et al., 2010; Manabe et al., 1984), and this process appears to occur independently of fetal signals (Yoshida and Manabe, 1988). These data suggest failure of the uterine myometrium to adapt to the continued expansion required during pregnancy may increase the risk of preterm birth (Waldorf et al., 2015).

In various animal models of uterine strain, changes in expression of chemokine, oxytocin receptor, connexin-43, and matrix metalloproteinases genes have been reported (Hua et al., 2012; Lee et al., 2015; Nguyen et al., 2016; Parry and Bathgate, 2000; Shynlova et al., 2013; Waldorf et al., 2015; Yulia et al., 2016). Fewer data are available regarding signal transduction pathways activated by uterine stress, but ERK1/2 pathway activation is a well-established strain response in pregnant myometrial cells and tissue (Li et al., 2009). In isolated rat myocytes biaxial strain is followed by phosphorylation of the kinases ERK1/2, JNK, and p38 (Oldenhof et al., 2002). Over all, protein phosphorylation is critically important for cell signaling response pathways and mediates many important functions such as protein interactions, localization, and activity (Sharma et al., 2014). *In vitro* work suggests MAPK pathways activate transcription factors such as c-fos, resulting in increased expression of contractile associated proteins to drive the onset of labor (Oldenhof et al., 2002).

We hypothesized specific phosphorylation signaling events related to the strain-induced integrin response would be upregulated in a telomerized cell culture model of human uterine smooth muscle cells. To test this hypothesis, we performed mechanical strain experiments on immortalized human uterine smooth muscle cells followed by phospho-peptide enrichment, identification, and quantitation.

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2. Materials and methods

2.1. Cell culture

Previously our lab developed telomerized pregnant human uterine smooth muscle cells (PHUSMC-hTRT) as a model of pregnant myometrium; in brief, cells were grown through passage 5 in primary culture and combined in equal numbers from three 24–29 year old Caucasian donors at full term and telomerized as described in Heyman et al. (2013). These PHUSMC-hTRT were used at passage 27 and plated on collagen coated BioFlex® plates (Flexcell® INC, Burlington, NC) and grown in HyClone™ Dulbecco's Modified Eagle Medium (DMEM; Thermo Scientific, Pittsburg, PA) supplemented with 0.11 mg/mL Sodium Pyruvate, 10% Fetal Bovine Serum (FBS, Atlanta Biologicals, Flowery Branch, GA), 100 U/mL penicillin 100 μg/mL streptomycin (Pen/Strep; Thermo Scientific, Pittsburgh, PA), 636 nM progesterone (P4), and 55 nM estradiol (E4). Media was refreshed every 3 days and cells were grown to confluence. At confluence, cells were placed in DMEM supplemented with 0.1% Insulin-Transferrin-Selenium-Ethanolamine (ITS-X) supplement (Thermo Scientific, Pittsburgh, PA) in the absence of FBS for 7 days to allow for growth arrest and differentiation.

2.2. Strain experiment

Growth-arrested PHUSMC-hTRT on BioFlex® collagen plates were subjected to 18% biaxial strain for 5 min on a Flexcell® FX-5000 tension system (Flexcell® INC, Burlington, NC). PHUSMC-HTRT cells grown on BioFlex® plates not subjected to mechanical strain served as controls. Cells were lysed in 300 μL/well MAPK buffer (60 mM Tris-HCl [pH 6.8], 2% SDS, 10% glycerol, 0.1 mM EGTA) (Singer et al., 2003) supplemented with Halt protease and phosphatase inhibitor cocktail (Thermo Scientific, Pittsburgh, PA). Strained and unstrained samples were grown on 6 well plates and randomly divided into triplicates. Lysates were probe sonicated (Qsonica, Newtown, CT) and centrifuged at 4000*g* for 15 min. Pellets were re-suspended in MAPK buffer, precipitated in 4× volume of 100% acetone and washed three times in 4× volume of 70% acetone. Precipitates were re-suspended in 8 M urea/50 mM Tris-HCl (pH 8), reduced in 5 mM DTT at 37 °C for 20 min in the dark, and alkylated in 10 mM iodoacetamide (BioRad, Hercules, Ca) for 20 min in the dark. Samples were re-precipitated with acetone as described above and re-suspended, using sonication, in 50 mM ammonium bicarbonate. Protein samples were digested with Trypsin/Lys C mix (Promega, Madison, WI) at a 75:1 protein:protease mix overnight at 37 °C, acidified in 0.1% formic acid, and desalted using Sep-Pak C18 cartridges (Waters, Milford, MA).

2.3. Phospho-peptide enrichment and LC-MS^2

Samples were enriched on IMAC columns containing nickel-nitrilotriacetic acid (Ni-NTA) agarose beads charged with TO_2 and labeled with 6-plex tandem mass tags (TMT) (Thermo Scientific, Pittsburgh, PA) according to the manufacturer protocols. Samples were pooled and then separated by hydrophilic interaction liquid chromatography fractionation as described previously (Albuquerque et al., 2008) for 60 min with a gradient change in solvent A (900 mL ACN + 100 mL ddH2O + 500 μL 10% TFA) from 0% solvent B (1 L ddH2O + 500 μL 10% TFA) to 90% at 45 min. 40 fractions were re-suspended in 100 μL 5% acetonitrile 0.1% formic acid for mass spectrometry analysis.

Samples were analyzed using liquid chromatography tandem mass spectrometry (LC-MS^2) at the Mick Hitchcock, Ph.D. Nevada Proteomics Center (University of Nevada, Reno). Peptides were separated and analyzed using a Michrom Paradigm Multi-Dimensional Liquid Chromatography instrument (Michrom Bioresources Inc., Auburn, CA) coupled with a Thermo LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific, San Jose, CA). Samples were dissolved in 100 μL of 0.1% formic acid were loaded onto an Acclaim Pepmap 100 C18 LC column (100 μm × 2 cm, C18 5 μm, 100 Å, Thermo Fisher Scientific, San Jose, CA), eluted, and then separated by reverse phase New Objective (New Objective Inc, Woburn, MA) ReproSil-Pur C18-AQ column (3 μm, 120 Å, 0.075 × 105 mm) with a gradient elution using solvent A (0.1% formic acid) and solvent B (0.1% formic acid in acetonitrile) at a flow rate of 300 nL/min. The gradient was set from 5% to 40% solvent B for 90 min, increased to 80% solvent B in 10 s and held at 80% solvent B for 1 min. MS spectra were recorded over the mass range of *m*/*z* 400–1600 with resolution of 60,000. The three most intense ions were isolated for fragmentation in the linear ion trap using collision induced dissociation (CID) with minimal signal of 500% and collision energy of 35.0% or using higher-energy collision dissociation (HCD) with minimal signal of 1000%, Collision energy of 55.0%, and an activation time of 30 ms. Dynamic exclusion was imple-

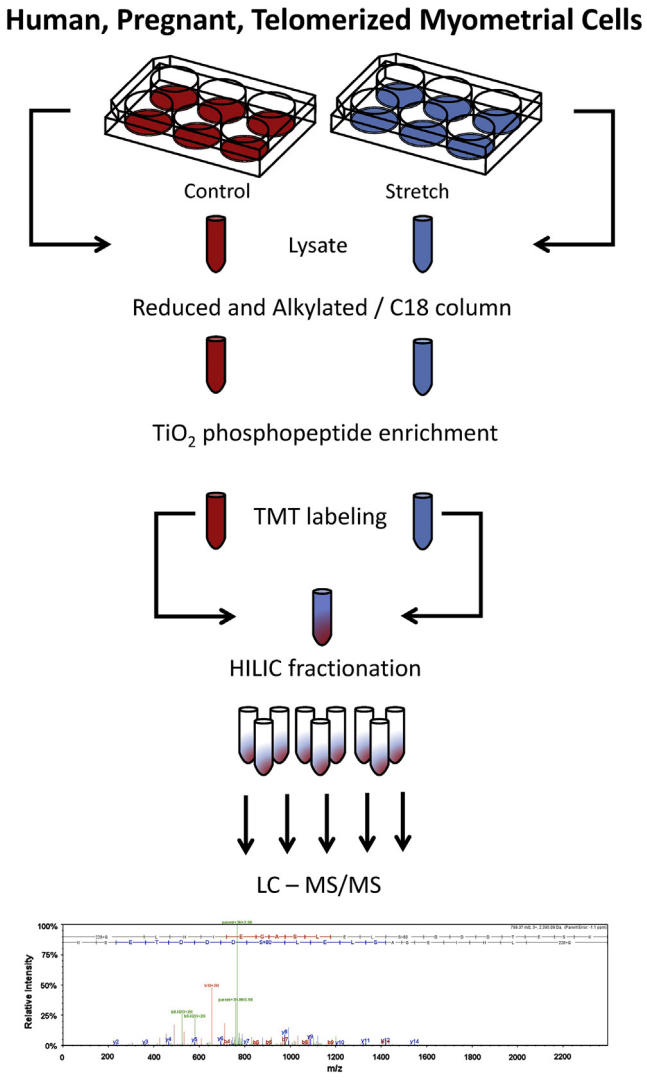

Fig. 1. Myometrial cell mechanical strain experimental design. Growth arrested PHUSMC-hTRT on BioFlex® plates were subjected to 18% biaxial mechanical strain for 5 min. Annotated SEQUEST peptide results were analyzed using IPA for pathway associations. Specific phospho-site annotations were taken from the Phosphosite.org database.