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Proteomic analysis of rat proximal tubule cells following stretch-induced apoptosis in an *in vitro* model of kidney obstruction☆

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ABSTRACT

Urinary tract obstruction (UTO) is a commonly noted disorder on prenatal ultrasound that has the potential to lead to permanent loss of renal function. To study the molecular processes of the disease, an *in vitro* model has been developed which involves mechanical stretch of proximal tubule cells grown on flexible plates which mimics the physiological conditions during UTO. This study employs a one dimensional SDS-PAGE fractionation procedure, followed by in-gel digest and LC-MS/MS analysis in a semi-quantitative experiment using spectral counting to relatively quantify changes in protein expression following the established model of UTO. Quantitative analysis shows 317 of the 1630 identified proteins express altered abundance, with 135 increased and 182 decreased in abundance as a result of stretch. Gene ontology (GO) and KEGG annotations implicate a number of physiological processes that have been previously shown in addition to some potentially novel processes in UTO. The quantitative proteomic analysis we performed here provides a more complete characterization of changes in protein abundance as a result of stretch than previous studies, and provides a number of previously undescribed proteins in proximal tubule cells that may play a role in UTO.

Biological significance
Urinary tract obstruction (UTO) is a commonly noted abnormality on prenatal ultrasound that can either resolve spontaneously or require surgical intervention to prevent permanent renal damage or loss of function. While targeted studies of UTO have shown a number of pathological responses in proximal tubule cells, there are currently no large-scale quantitative studies that aim to elucidate a global cellular response. This study uses a semi-quantitative approach and applies a well characterized model of UTO to determine a number of cellular processes affected by UTO simulation and identifies a number of proteins with altered abundance that have not been noted previously in UTO.
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1. Introduction

Urinary tract obstruction (UTO) is a common congenital anomaly that leads to morphological changes within the kidney including dilation, hydronephrosis, renal tissue fibrosis, and tubular cell apoptosis [1]. Noted in up to 1% of pregnancies on prenatal ultrasound, many cases may resolve spontaneously, however some children with UTO require surgical intervention to prevent permanent loss of kidney function [2]. Postnatal management therefore requires lengthy surveillance of renal function with invasive testing to determine which children will require surgical intervention [3]. To improve care of children experiencing UTO, a more complete understanding of the molecular processes is required.

Previous studies have demonstrated the role of oxidative stress [4–6], renal metabolism [7,8], and tissue fibrosis [9,10] in UTO. These studies suggest renal interstitial fibrosis and tubular cell apoptosis to be the major effectors leading to loss of kidney function. To study the pathophysiology of UTO, an *in vitro* model of UTO was developed [11]. The model employs a mechanical stretch stimulus applied to proximal tubule cells grown on a flexible membrane which mimics the physiological characteristics experienced by these cells during UTO [9,11–13], Application of this model has demonstrated many effectors in tubular cell damage and fibrosis on proximal tubule cells, including transforming growth factor β (TGF-β) [9], nitric oxide [12], and apoptosis [13]. While these studies characterize specific effectors in disease progression during UTO, this study applies a quantitative proteomic analysis of the rat proximal tubule cell line NRK-52E to elucidate global changes in protein abundance as a result of UTO simulation. We used a gel-based method coupled to spectral counting as a label-free method to quantify changes in protein abundance as a result of UTO simulation.

Proteins altered in abundance were assessed by gene ontology (GO) and KEGG classifications to demonstrate specific pathways and subcellular processes that are affected by simulation of UTO. Furthermore, we discuss the specific protein alterations resulting from stretch that have been described to play critical roles in the pathophysiology of other models of renal injury. The aim of this study is therefore to characterize the effects that mechanical stretch has on proximal tubule cells and to identify novel effector pathways that are important in UTO.

2. Materials and methods

2.1. Materials

NRK-52E cells were obtained from the American Type Culture Collection (ATCC; Burlington, Canada) with cell culture growth and maintenance materials: Dulbecco's Modified Eagle Medium (DMEM; 11965-092), newborn calf serum (NCS; 16010-159), phosphate buffered saline (PBS; 10010-023), sodium bicarbonate (25080-094), and trypsin (25300-062) obtained from Gibco (Carlsbad, CA). Protease inhibitor (P2714), trypsin (T802), ammonium bicarbonate (ABC; A6141), acrylamide (A3699), along with formic acid (94318), and trifluoroacetic acid (TFA; T6508) were from Sigma (Oakville, Canada). Tris (161-0719), iodoacetamide (163-2109), dithiothreitol (DTT; 161-0611), sodium dodecyl sulfate (SDS; 161-0302) were from Bio-Rad (Hercules, CA). Water was purified to 18.2 MΩ·cm and solvents were of HPLC grade and from Fisher Scientific (Ottawa, Canada).

2.2. Experimental workflow

The experimental workflow was as summarized in Fig. 1. Briefly, NRK-52E cells were grown on flexible, 6-well BioFlex™ plates. Cells were then either exposed to cyclic mechanical stretch or incubated without stretch for 24 h. This experiment was conducted over three consecutive passages of cells. Following the stretch or control conditions, cells were collected by scraping, lysed, and fractionated into cytoplasmic and membrane enriched protein samples *via* stepwise solubilization and ultracentrifugation. Each protein fraction was separated by SDS-PAGE, processed into 15 gel slices, and analyzed by GeLC-MS/MS. Data was searched against a database by SEQUEST, and protein-level spectral count data was analyzed by the statistical method QuasiTel, followed by classification using gene ontology (GO) and KEGG pathway analysis of significantly altered proteins.

2.3. Cell growth and stretch conditions

NRK-52E cells were maintained at 37 °C (5% CO_2) in 'growth media' (DMEM supplemented with 10% NCS, 2% sodium bicarbonate (v/v)). UTO was simulated by mechanical stretch as described previously [11], with minor modifications. Cells were grown on six-well elastomer culture plates coated with collagen type I (Bioflex; Flexcell, Hillsborough, N.C., USA). Cells were grown to 75–80% confluence and rendered quiescent by incubation for 12 h in 'starvation media' (DMEM with 1% NCS, 2% sodium bicarbonate). Cells were then subjected to continuous cycles of stretch-relaxation ('stretched cells') in an FX-4000 Flexercell Strain Unit (Flexcell), or alternatively incubated under identical atmospheric conditions without stretch ('control cells') for 24 h. Each stretch/relaxation cycle consisted of 2 s of stretch according to a sinusoidal half wave pattern and 2 s of zero stretch relaxation. Stretch distribution and orientation on the membrane was 25% maximal biaxial stretch in the membrane center region changing gradually to 25% maximal radial stretch at the membrane periphery [14]. Immediately following stretch, cells were visualized from random regions of the membrane with a Leica DMIRB inverted stage microscope.

2.4. Cell lysis and protein isolation

Following stretch, two wells from each test condition were sacrificed for cell death ELISA according to manufacturer's instructions (1 544 675; Roche, Indianapolis, Ind., USA). In this ELISA, the cell membranes are lysed gently without disrupting the nuclear membrane, and insoluble material is pelleted. The supernatant is analyzed for the presence of DNA histone complexes, whose formation is indicative of an apoptotic response. In each test, the relative levels of apoptosis were normalized to the basal level of apoptosis noted in the controls. Remaining cells were washed with PBS and pelleted by centrifugation at 300 ×*g* for 5 min. Isolation of cell protein was accomplished in a two-step fractionation procedure, similar to described previously [15]. Briefly, cell pellets were