Proteomic profile of an acute partial bladder outlet obstruction

Bader Alsaikhan, MD;[†] Richard Fahlman, MD;[§] Jie Ding, MD;[†] Edward Tredget, MD;[¥] Peter D. Metcalfe, MD, MSc, FRCSC[*]

[†]Division of Experimental Surgery, Department of Surgery, University of Alberta, Edmonton, AB; [§]Department of Biochemistry, University of Alberta, Edmonton, AB; [¥]Division of Plastic Surgery, Department of Surgery, University of Alberta, Edmonton, AB; [*]Division of Pediatric Surgery, Department of Surgery, University of Alberta, Edmonton, AB

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Abstract

Introduction: Partial bladder outlet obstruction (pBOO) is a ubiquitous problem in urology. From posterior urethral valves to prostatic hypertrophy, pBOO results in significant morbidity and mortality. However, the pathophysiology is not completely understood. Proteomics uses mass spectrometry to accurately quantify change in tissue protein concentration. Therefore, we have applied proteomic analysis to a rodent model to assess for protein changes after a surgically induced pBOO. We hypothesize that proteomic analysis after an acute obstruction will determine the most prevalent initial protein response and, potentially, novel molecular pathways.
Methods: Sprague Dawley rats underwent a surgically induced pBOO (n = 3 per group) for 3, 7, or 14 days. Bladders were assessed for weight and urodynamic parameters. Proteomics used liquid-chromatography based mass spectrometry. Polymerase chain reaction (PCR) was performed on tissue samples to confirm increased mRNA transcription.
Results: Bladder weight and capacity increased over the experimental period, but no changes were seen in bladder pressure. Statistically significant increases in protein quantities were seen in 3 proteins related to endoplasmic reticulum stress: GRP-78 (3.66-fold), RhoA (1.90-fold), and RhoA-GDP (1.95-fold), and 2 cytoskeleton molecules: actin (1.7-fold) and tubulin a/b (3.01-fold). Decorin and lumican, members of the small leucine rich proteoglycan (SLRP) family, were also elevated (0.35- and 0.34-fold, respectively). Real-time PCR data confirmed protein elevation.
Conclusion: Our experiment confirms that molecular changes occur very soon after the initiation of pBOO, and implicates several molecular pathways. We believe these insights may provide insight into novel prevention and treatment strategies targeted at the pathophysiology of pBOO.

Introduction

Partial bladder outlet obstruction (pBOO) is a ubiquitous problem in urology, affecting the fetus with posterior urethral valves, the child with spina bifida, the young adult with a urethral stricture, and the elderly male with prostatic hypertrophy. However, despite significant medical and surgical advancements, pBOO still results in significant morbidity and mortality. Fundamentally, the deleterious consequences are not from the obstruction, as this is easily relieved, but from the secondary deterioration of the bladder. Multiple factors have been linked to this pathology, including inflammation, hypoxia, and remodeling of extracellular matrix (ECM) components.[1,2] We have previously published results from our animal model that demonstrates a programmed, progressive series of molecular and physical changes after pBOO. We described an initial period of inflammation, progressing to smooth muscle hypertrophy, and this eventually deteriorated into a poorly compliant, fibrotic bladder.[3] We believe that our model reasonably replicates the clinical scenarios seen with valve bladder syndrome and other obstructive states.

Mass spectrometry-based proteomic studies are used to identify and quantify the entire protein content of a cell, tissue, or organ.[4] This is considered more sensitive and robust than genomics, due to the dynamic state of protein expression, depending on the physiological state of the biologic system. Because the amount of information can be overwhelming, most authors apply this technology to monitor changes in protein levels.

Herein, we explored the changes in protein levels of the urinary bladder over the first 14 days after pBOO, aiming to identify major changes in protein expression. This is an earlier time point than our previous experiments; therefore, we are examining the initial tissue response to the stress outlet obstruction. We hypothesize that proteomic analysis after an acute obstruction will determine the most prevalent protein response and, potentially, novel molecular pathways.

Methods

Approval from the University of Alberta Animal Care and Use Committee was obtained. Three un-obstructed Sprague Dawley rats were used as controls, and surgical induction of

pBOO was performed as previously described[5] for 3, 7 and 14 days with 3 animals per time point. Main outcomes were bladder weight, urodynamic studies, and bladder proteomic analysis. Real-time polymerase chain reaction (RT-PCR) was performed to determine gene transcription.

Surgical induction of pBOO

Isoflurane was used for anesthesia, and an 18-gauge angiocatheter was introduced into the bladder dome, and advanced into the urethra to serve as a stent for calibration. A 2-0 silk suture was tied outside the urethra, with the angiocatheter in place, to prevent complete obstruction. (Fig. 1).

Urodynamics and bladder weight

After the pre-determined time point, urodynamics were performed via cystotomy and the bladder filled with saline at 0.1 mL/minute. Bladder capacity and pressure (end-fill pressure) were recorded once urinary leakage was observed. Bladders were excised, dried, weighed, and fixed in 4% paraformaldehyde.

Protein extraction

In total, 200 mg of bladder was submerged in liquid nitrogen, homogenized, and suspended in a cell lysis buffer. Supernatant was obtained and protein concentration was measured using the Bradford Bio-Rad protein assay (Bio-Rad, Hercules, CA) to yield a final concentration of 2 mg/mL for each sample. Bovine serum albumin was used as a standard.

Gel electrophoresis and staining

SDS-PAGE, 12% BisTris Criterion precast gels (Bio-Rad, Hercules, CA) were used. Molecular weight ladder was loaded into the first gel lane and 40 µL of each sample was loaded into the remaining lanes. Gels were run at 150V for 10 minutes, followed by 200V until the dye was seen at the bottom of the gel.

Liquid chromatography-mass spectrometry analysis

Each gel lane was cut into 10 regions, digested, and analyzed using liquid chromatography-mass spectrometry (LC-MS/MS) at the University of Alberta's Institute of Biomolecular Design (IBD). The excised gels were de-stained twice in 100 mM ammonium bicarbonate (AmBic)/acetonitrile (ACN) (50:50). Samples were then reduced (10 mM β-mercaptoethanol 100 mM AmBic) and alkylated (55 mM iodoacetamide in 100 mM AmBic). After dehydration, trypsin (6 ng/uL) was added and digestion occurred overnight (about 16 hours) at room temperature. Tryptic peptides were extracted using 97% water/2% ACN/1% formic acid followed by a 1:1 mixture of extraction buffer and acetonitrile.

Fractions containing tryptic peptides were dissolved in aqueous 25% v/v ACN and 1% v/v formic acid and these were resolved and ionized by using nanoflow HPLC (Easy-nLC II, Thermo Scientific, Waltham, MA) coupled to the LTQ XL-Orbitrap hybrid mass spectrometer (Thermo Scientific). Peptide mixtures were injected onto the column at a flow rate of 3000 nL/min and resolved at 500 nL/min using 60-min linear ACN gradients from 0 to 45% v/v aqueous ACN in 0.2% v/v formic acid. The mass spectrometer was operated in data-dependent acquisition mode, recording high-accuracy and high-resolution survey Orbitrap spectra using external mass calibration, with a resolution of 60 000 and m/z range of 400 to 2000.

The 10 most intense multiply-charged ions were sequentially fragmented using collision-induced dissociation, and recorded in the linear ion trap. Data were processed using Proteome Discoverer 1.3 (Thermo Scientific) and the Rattus norvegicus protein database was searched using SEQUEST (Thermo Scientific). Search parameters included a precursor

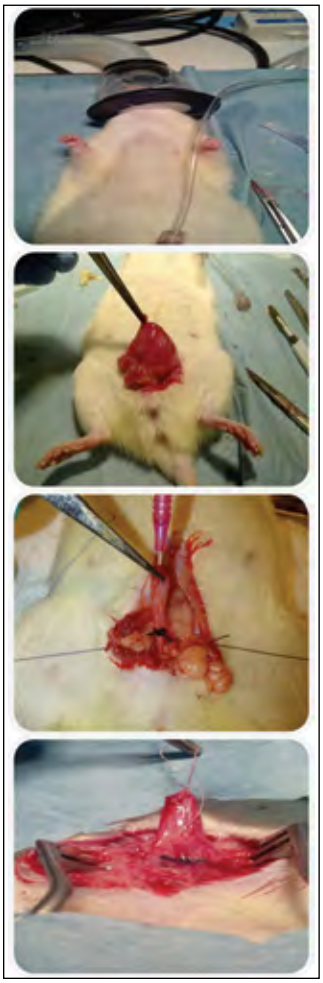

Fig. 1. Photographs of surgical induction of partial bladder outlet obstruction. Midline laparotomy is used to expose the bladder, then an 18-g angiocatheter is advance antegrade through the urethra, and silk suture tied around the urethra. The angiocatheter is used to prevent too much obstruction, and to ensure a reproducible degree of resistance.

mass tolerance of 10 ppm and a fragment mass tolerance of 0.8 Da, and spectral counting was used for quantification.[6,7]

Quantitative real-time polymerase chain reaction

RNA extraction was performed after homogenization and suspension in Trizol solution, using RNeasy spin columns (Qiagen, Mississauga, ON). DNA was digested for 60 minutes, and random primers were used to synthesize first cDNA strand (Sigma, Oakville, ON). RT-PCR was then performed (Power SYBR Green PCR Master Mix, ABI, Foster City, CA). Hypoxanthine-guanine phosphoribosyltransferase (HPRT) gene was used as control. Amplifications were done using an ABI 7300 real-time system (Applied Biosystems, Foster City, CA). Table 1 outlines primer sequences.

Statistical analysis

Only proteins that were present in all samples were used for statistical analysis. Data were reported as mean-fold change from normal control ± standard error, and analysis of variance (ANOVA) was performed (STATA Version 12.1). Statistical significance was set at $p \leq 0.05$.

Results

All animals remained healthy, and there was no weight loss. pBOO resulted in an increase in bladder weight compared to unobstructed controls, with significant increases seen at 3 days (303.3 ± 24.0 mg vs. 200.7 ± 4.6 mg, $p = 0.01$), and 14 days (535.0 ± 82.5 mg vs. 200.7 ± 4.6 mg, $p = 0.02$).

Urodynamics

The bladder pressures recorded at the time of urethral leakage (end-fill pressures) did not significantly change (Fig. 2). However, bladder capacity was significantly increased after 14 days (2.2 ± 0.3 vs. 0.9 ± 0.2 mL, $p = 0.02$) (Fig. 3).

Proteomic analysis

An average of 613 proteins per sample was identified initially, and 140 proteins were detected in all samples. Table 2 outlines proteins with significant change from un-obstructed bladder tissue, and their fold-change from baseline in all groups. A total of 19 proteins had a statistically significant change from their baseline values. Several of these, including the cofilins, annexin, C9, serping1, Arhgdia, serpina3n, mdh1, Igh-1a, ppia, and csrp1, are of unknown metabolic significance.

We grouped the remaining molecules, which were felt to have relevant biologic roles, into markers of cellular stress, cytoskeleton molecules, and small leucine-rich proteoglycans (SLRPs).

Markers of endoplasmic reticulum stress, GRP78 (3.66-fold increase), RhoA (1.90-fold increase), and RhoA-GDP (1.95-fold increase) increased after 14 days of pBOO (all $p < 0.05$). Cytoskeleton molecules, actin (1.7-fold) and tubulin-α/β (3.01-fold), also showed a statistically significant increase after 14 days. SLRPs decorin (0.35-fold) and lumican (0.34-fold) decreased after 14 days.

RT-PCR

GRP78 mRNA was reduced after 7 days (0.56-fold), and then increased after 14 days (2.57-fold) (Fig. 4, part A). RhoA mRNA decreased in the 3-day group (0.27-fold) and then increased significantly after 14 days of pBOO (1.93-fold) (Fig. 4, part B) (all $p < 0.05$).

Down-regulation of decorin mRNA was significant in the 3-day group (0.20-fold) (Fig. 4, part C). Lumican mRNA changes, however, did not achieve statistical significance (Fig. 4, part D).

Discussion

The pathophysiologic progression from pBOO to bladder fibrosis remains a significant problem in urology, as these high-pressure, poorly compliant bladders result in incontinence, hydronephrosis, and eventually renal failure. Several causative factors have been identified, including mechanical strain, bladder wall hypoxia, and inflammation.[1] Multiple cytokines and pathways have been related to the aforementioned stressors, including TGFβ, connecting tissue growth factor, HIF1α, and ECM proteoglycans.[1,2] However, the exact mechanisms and interplay have not been fully elucidated.

Table 1. List of PCR primer sequences

Gene	Forward Sequence	Reverse Sequence
GRP78	5′-CCAGCTTACTTCAATGATGCAC-3′	5′-CTTCTCTCCCTCTCTCTTATCC-3′
RhoA	5′-AGGACCAGTTCCCAGAGGTT-3′	5′-ACTATCAGGGCTGTCGATGG-3′
Decorin	5′-ACACCAACATAACTGCTATTCC-3′	5′-AGACTGCCATTTTCCACAAC-3′
Lumican	5′-CACCAGAATGTAACTGTCCC-3′	5′-TCAGCTTAGAGAAGACCTTTCC-3′
TGFβ1	5′-GAGGTGACCTGGGCACCAT-3′	5′-GGCCATGAGGAGCAGGAA-3′
HIF1α	5′-TGCTTGGTGCTGATTTGTGA-3′	5′-GGTCAGATGATCAGAGTCCA-3′

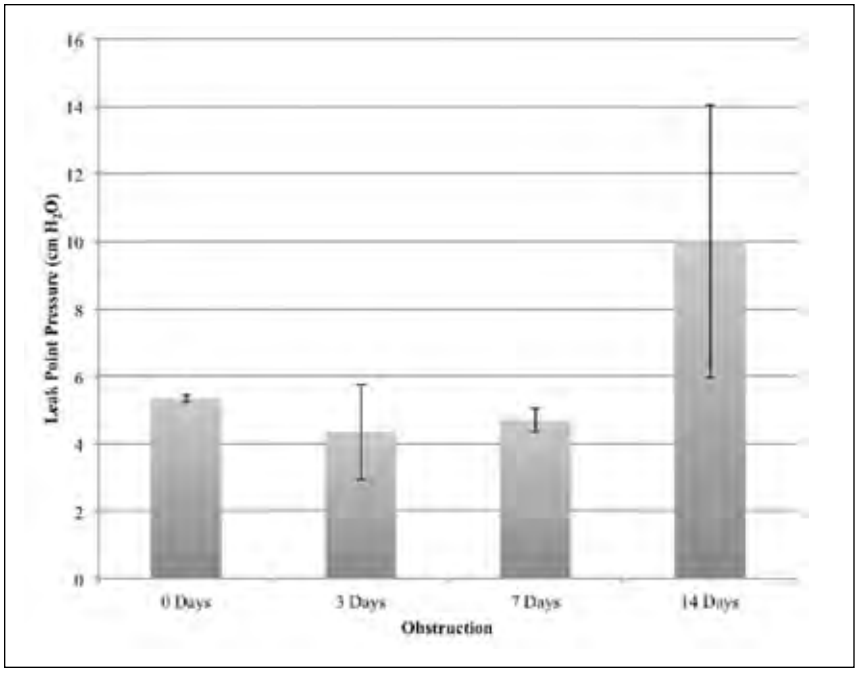

Fig. 2. Bladder pressure at end of filling, after 3,7, and 14 days of partial bladder outlet obstruction. There were no significant differences found between any of the bladder pressures.

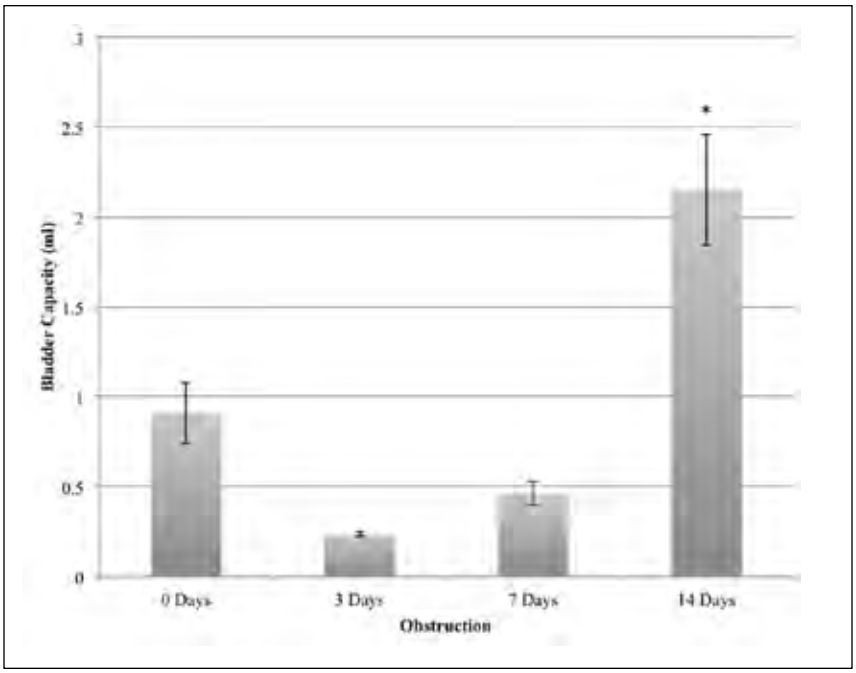

Fig. 3. Bladder capacity for controls, and after 3,7, and 14 days of partial bladder outlet obstruction (pBOO). Rat baldders were significantly bigger after 14 days of pBOO. * *p* value <0.05 vs. unobstructed.

Table 2. Protein identification and quantification results, representing the ratio of obstructed to normal unobstructed groups and their fold change from normal unobstructed bladder tissue

Accession	Gene	Protein	Ratio (O/N)1		
			3 Days	7 Days	14 Days
Up-regulated					
P06761	Hspa5	78 kDa glucose-regulated protein	2.04	2.15	3.66*
P69897	Tubb5	Tubulin β-5 chain	2	2.1	3.01*
P45592	Cfl1	Cofilin-1	0.55	0.16	2.65*
P20761	Igh-1a	Ig γ-2B chain C region	1.82	1.01	2.63*
Q6P9V9	Tuba1b	Tubulin α-1B chain	1.75	1.73	2.49*
P10111	Ppia	Peptidyl-prolyl cis-trans isomerase A	0.68	0.3	2.34*
P63018	Hspa8	Heat shock cognate 71 kDa protein	0.94	1.13	2.16*
Q07936	Anxa2	Annexin A2	1.49	0.79	2.16*
O88989	Mdh1	Malate Dehydrogenase, Cytoplasmic	1.04	0.61	2.14*
P09006	Serpina3n	Serine protease inhibitor A3N	4.93*	1.22	2.02
Q5XI73	Arhgdia	Rho GDP-dissociation inhibitor 1	0.87	0.88	1.95*
P61589	Rhoa	RhoA transforming protein	0.8	1.3	1.9*
P60711	Actb	Actin, cytoplasmic 1	0.94	0.41	1.7*
Q62930	C9	Complement component C9	2.17*	0.98	0.91
Q6P734	Serping1	Plasma protease C1 inhibitor	2.74*	2.18*	0.89
Down-regulated					
P47875	Csrp1	Cysteine and glycine-rich protein 1	0.34	0.21*	1.09
P31232	Tagln	Transgelin	0.50	0.16*	1.06
Q01129	Dcn	Decorin	0.59	0.59	0.35*
P51886	Lum	Lumican	0.75	0.58	0.34*

*p value <0.05 vs. normal unobstructed group.

Mass spectrometry is an extremely powerful tool able to identify proteins and quantitate changes in concentrations. The proteins identified in this study can be grouped and related to cellular structure, inflammation, hypoxia and oxidative stress.

GRP78 and endoplasmic reticulum stress

GRP78 is a 70kDa heat shock protein, which is involved in protein folding in the endoplasmic reticulum (ER).[1,2,8] During states of stress, unfolded and misfolded proteins accumulate in the ER resulting in endoplasmic reticulum stress (ERS). This activates a quality control process known as the unfolded protein response (UPR),[4,8] which is intended to restore the proper folding of proteins, but may also trigger apoptosis.[1,8] Our findings confirm the presence of ERS after 14 days of pBOO, evident by the increase in GRP78 protein levels.

ERS and UPR activation is seen in several fibrotic conditions, including heart failure, cystic fibrosis, renal and interstitial lung fibrosis.[9-12] This has also been associated with renal tubular apoptosis,[13] and increased levels have been used as a biomarker of cellular stress.[5,14] In a rat model of myocardial infarction, atorvastatin resulted in lower GRP78 and caspase-12 levels,[15] and candesartan alleviated the ERS response after ureteric obstruction.[16] These findings suggest an important role of ERS after pBOO. Further work confirming a causative role may lead to novel medical preventive strategies.

RhoA/RhoA kinase pathway

RhoA (Ras homolog transforming protein member A) increased significantly after 14 days of pBOO. Activation of RhoA results in increased contractility and cytoskeletal modulation.[17] The RhoA/ROCK (RhoA Kinase) pathway is involved in bladder smooth muscle contractility.[18-20]

Hypoxia and mechanical stress, two primary factors after pBOO, have been shown to activate RhoA/ROCK.[21,22] Several studies have found that the increased contractility after pBOO[23,24] can be improved with ROCK inhibition.[23,25]

Moreover, activation of RhoA/ROCK may further increase pathologic changes by reducing eNOS gene expression in vascular endothelium, reducing perfusion which will worsen hypoxia.[26]

Small leucine-rich proteoglycans

Small leucine-rich proteoglycans (SLRPs) are ECM proteoglycans required for collagen fibrillogenesis.[2,27] We detected a significant reduction in decorin and lumican after 2 weeks of pBOO, consistent with a pro-fibrotic environment and deregulated collagen assembly.[27]

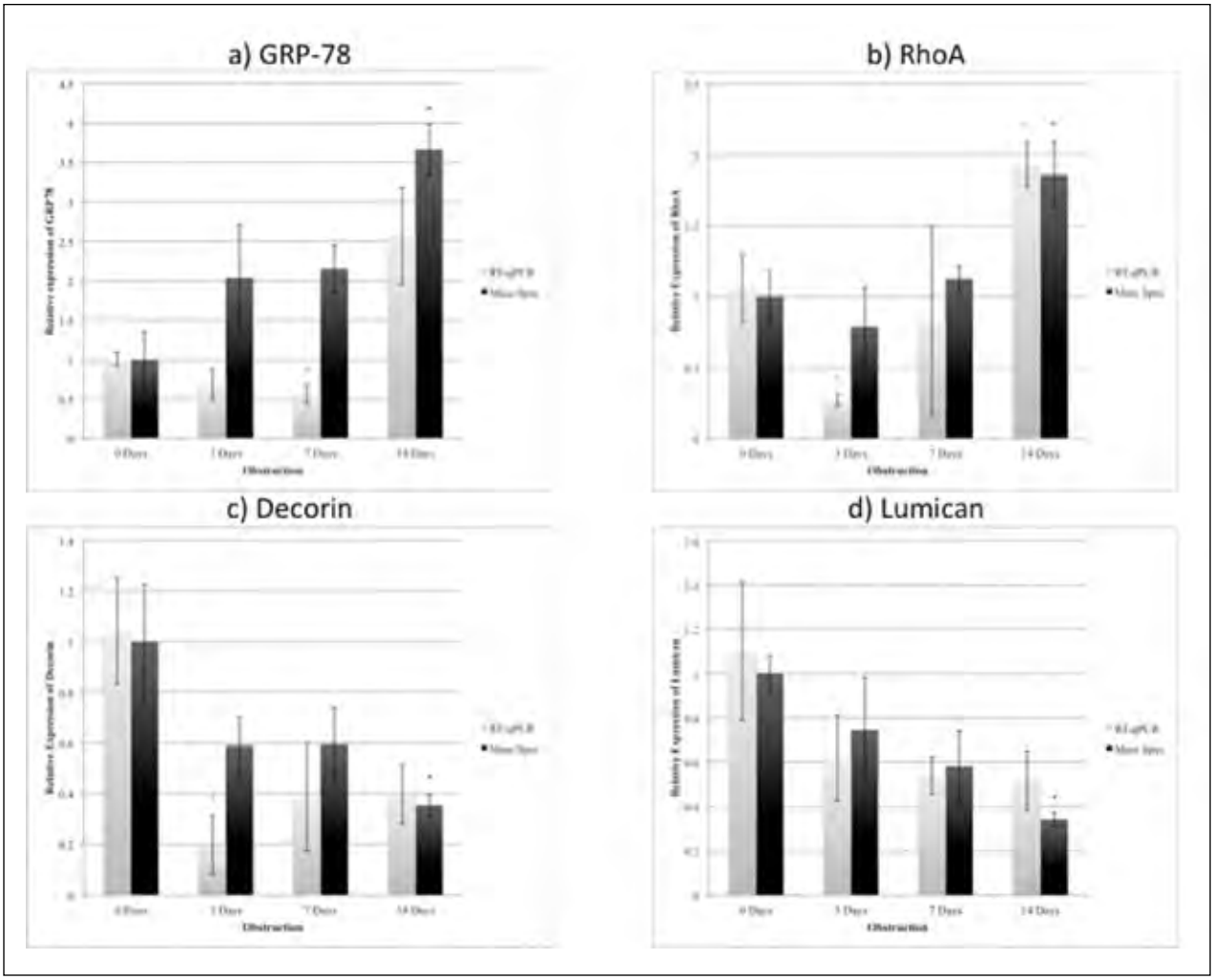

Fig. 4. A: Protein and mRNA expression of ER stress marker GRP78. B: Protein and mRNA expression of RhoA. C: Protein and mRNA expression of decorin. D: Protein and mRNA expression of lumican. **p* value <0.05 vs. unobstructed for mass spectrometry data. #*p* value <0.05 vs. unobstructed for RT-qPCR data.

Figure 5 summarizes the changes in protein expression detected in this study, and their possible inducers, downstream effects, and interactions after pBOO.

Although a very powerful tool, mass spectrometry-based proteomic analysis has its limitations, including extremes of molecular weight, protein length, peptide characteristics and the database selection. Furthermore, our technique relies upon several-fold increases in protein levels, and many molecules exert their effect via post-translational changes and downstream mediators. Therefore, a small increase in an important upstream cytokine may not be detected, but the effects may be critical. Finally, the very acute time frame we studied in this manuscript may not reflect molecular pathways responsible for chronic changes.

Another fundamental concern is the question regarding the primary versus reactionary role of the proteins. We have assumed that the physical stress of the acute pBOO creates a deliberate increase in the aforementioned protein levels, which results in downstream activation of the pathways. However, the increased concentrations may all be secondary to an undetected mechanism.

Conclusion

The bladder damage secondary to pBOO is not completely understood. We have developed an animal model to further elucidate the primary factors involved, and this study uses proteomics to study the protein response very early after pBOO. Endoplasmic reticular stress (GRP-78), mechanical strain (Rho A/ ROCK), and inflammation (TGF-B) appear to be early, primary factors. We have confirmed that cellular and extracellular stress occurs very early after the initia-

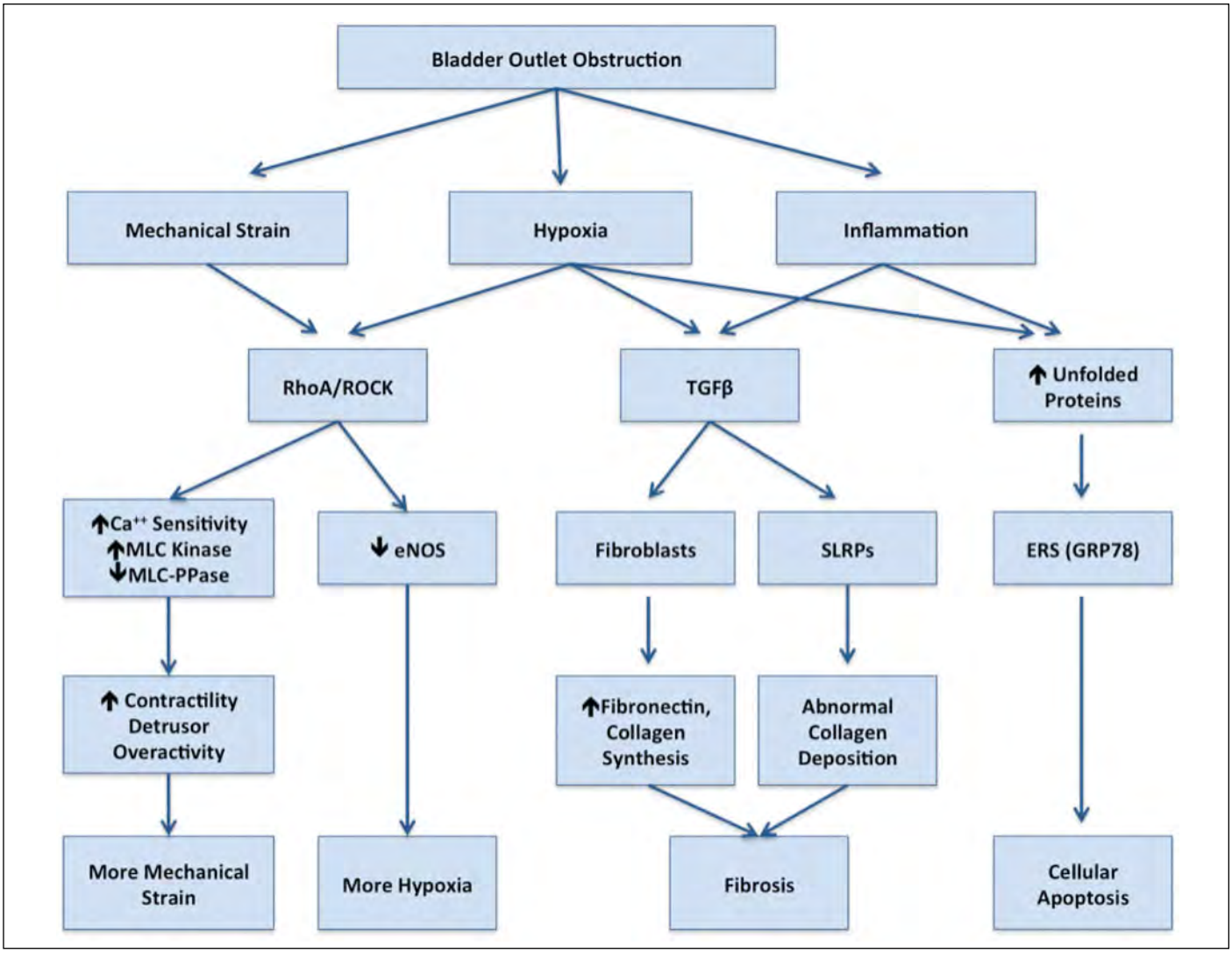

Fig. 5. Summary of detected proteins and their possible effects & interactions reported in the literature.

tion of pBOO and that multiple interrelated pathways are involved. This work may lead to novel pharmacologic interventions and reaffirms the need for early and aggressive care for our patients.

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Competing interests: Authors declare no competing financial or personal interests.

This paper has been peer-reviewed.

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Correspondence: Dr. Peter D. Metcalfe, Associate Professor, University of Alberta, Stollery Children's Hospital, 2C3.79 WMC 8440-112 Street, Edmonton, AB T6G 2B7; pmetcalf@ualberta.ca