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Eric James Gonzalez

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A ROLE FOR TRANSFORMING GROWTH FACTOR-BETA IN URINARY BLADDER DYSFUNCTION WITH CYCLOPHOSPHAMIDE-INDUCED CYSTITIS

A Dissertation Presented

by

Eric James Gonzalez

to

The Faculty of the Graduate College

of

The University of Vermont

In Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy
Specializing in Neuroscience

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ABSTRACT

Bladder pain syndrome (BPS)/interstitial cystitis (IC) is a chronic pain disorder characterized by at least six weeks of lower urinary tract symptoms and unpleasant sensations (pain, pressure and discomfort) thought to be related to the urinary bladder and not meeting exclusion criteria. While the etiology is not known, BPS/IC may involve a “vicious circle” of uroepithelial dysfunction, inflammation and peripheral and central sensitization. We propose that the urinary bladder inflammatory insult partly mediates voiding dysfunction and visceral neurogenic pain characteristic of BPS/IC. Several studies from our laboratory have already demonstrated the role(s) of cytokines and their downstream targets in the functional alterations in micturition reflex pathways following chemically (cyclophosphamide, CYP)-induced cystitis. More recently, the pleiotropic protein, TGF-β, has been implicated in the pathogenesis of CYP-induced cystitis.

TGF-β is activated locally at the initial site of injury by protease-dependent or protease-independent mechanisms to initiate a proinflammatory milieu. Depending on its contextual cues, TGF-β may then aid in resolving the primary immune response and support tissue repair. Though TGF-β is necessary to maintain normal immunological function, its aberrant expression and activation may have detrimental effects on responding tissues and cell types. A sustained increase in peripheral TGF-β reactivity, such as what may be observed in chronic inflammatory bladder conditions, may influence bladder afferent excitability to amplify nociceptive transmission and CNS input. The subsequent sensitization of peripheral afferent nociceptors at the level of the DRG or urothelium may promote spinal cord ‘wind-up’ and cascade into visceral hyperalgesia and allodynia.

In the first aim of this dissertation we investigated the functional profile of TGF-β isoforms and receptor (TβR) variants in the normal and inflamed (CYP-induced cystitis) urinary bladder with qRT-PCR, ELISA, IHC and in vivo cystometry. Our studies determined (i) the involvement of TGF-β in lower urinary tract neuroplasticity following urinary bladder inflammation, (ii) a functional role for TGF-β signaling in the afferent limb of the micturition reflex and (iii) urinary bladder TβR-1 as a viable target to reduce voiding frequency with cystitis. In the second aim of this dissertation we investigated the sensory components of the urinary bladder that may underlie the pathophysiology of aberrant TGF-β activation with bladder-pelvic nerve electrophysiology and luciferin-luciferase assays for ATP measurement. Our studies determined that TGF-β1 increased bladder afferent nerve excitability by stimulating ATP release from the urothelium via vesicular exocytosis mechanisms with minimal contribution from pannexin-1 channels. Furthermore, blocking aberrant TGF-β signaling in CYP-induced cystitis with TβR-1 inhibition decreased afferent nerve excitability with an equivalent decrease in ATP release.

Taken together, these results establish a causal link between an inflammatory mediator, TGF-β, and intrinsic signaling mechanisms of the urothelium that may contribute to the altered sensory processing of bladder filling to facilitate increased voiding frequency. The distinct interactions of multiple mediators underscore the challenges for single target therapies and support the development of combinatory therapeutics for bladder dysfunction. Ultimately, these studies have increased our understanding of functional disorders and visceral pain and have the potential to improve the health of those suffering from inflammation-associated bladder syndromes.
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CHAPTER 1: COMPREHENSIVE LITERATURE REVIEW

1.1. Lower Urinary Tract

1.1.1. Anatomy

The lower urinary tract (LUT, bladder and urethra) is a division of the renal system that functions to passively store kidney byproducts until it is appropriate to void. To accomplish this, the urinary bladder is a muscular and membranous organ whose structure embodies its reservoir function. Its external features are organized into an apex, fundus, body and neck. The apex, or vertex, is on the anterior surface of the urinary bladder and is associated with ligament remnants attaching to the umbilicus (Tank, 2009). The posterior surface is the fundus and its most inferior aspect is termed the base of the urinary bladder (Tank, 2009). The body typically represents the area between the apex and the fundus and the bladder neck is the most caudal aspect of the inferior bladder surface that is perforated by the internal urethral orifice (Tank, 2009).

The urinary bladder wall is divided into three layers: tunica mucosa, tunica muscularis propria and tunica serosa/adventitia (Figure 1). The tunica mucosa consists of transitional epithelium and a lamina propria (Figure 1). Transitional epithelial cells in the urinary bladder are termed the urothelium and are arranged in basal, intermediate and apical cell layers. Basal cells are monolayers directly attached to the basement membrane (Lewis, 2000). Intermediate cells are generally larger in diameter than basal cells and range from one to multiple cell layers depending on the species (Lewis, 2000). The apical, or umbrella, cells are hexagonal in shape and range from 25-250 μm depending on urinary bladder distention (L. A. Birder & K. E. Andersson, 2013; Lewis, 2000).
Several distinct features of the luminal surface of umbrella cells establish antiadherence and an impermeable barrier characteristic of the urinary bladder mucosa. First, tight junction complexes comprised of occludin and claudin proteins regulate paracellular transport between adjacent umbrella cells (L. A. Birder & K. E. Andersson, 2013). The apical membrane is also occupied by uroplakin, a crystalline plaque cell surface protein that forms an asymmetric unit membrane to maintain impermeability during bladder expansion (X. R. Wu, Kong, Pellicer, Kreibich, & Sun, 2009). Lastly, a layer of proteoglycans on the mucosal surface of umbrella cells serves as an antiadherence factor and provides yet another physical barrier between urinary constituents and the lamina propria (Figure 1) (Janssen et al., 2013).

The extracellular matrix of the lamina propria is deep to the basement membrane of the urothelium and contains a diverse array of interstitial cells, nerve terminals and vasculature (Figure 1) (Andersson & McCloskey, 2013; L. A. Birder & K. E. Andersson, 2013). It has been suggested that the lamina propria may have an important role in integrating epithelial and smooth muscle function due to its innervations and proximity to the urothelium and tunica muscularis propria (Andersson & McCloskey, 2013). The tunica muscularis propria consists of three smooth muscle layers termed the detrusor (Figure 1). The internal and external layers are arranged longitudinally, whereas those in the middle are circular (Andersson & Arner, 2004; Krstic, 2004). The smooth muscle cells in the muscularis propria retain their classic spindle shape and are bundled together by collagen-rich connective tissue (Andersson & Arner, 2004). External to the muscularis propria, the tunica serosa surrounds the superior and lateral surfaces of the urinary
bladder wall, whereas, the retroperitoneal aspects contain a vascular, loose connective tissue termed the tunica adventitia (Figure 1) (Krstic, 2004).

Caudal to the inferior surface of the urinary bladder is the urethra. Similar to the urinary bladder wall, the urethral wall is composed of a tunica mucosa, tunica muscularis propria and tunica adventitia. The tunica mucosa consists of transitional epithelium proximal to the urinary bladder followed by nonkeratinized, stratified squamous epithelium distally (Krstic, 2004; Yucel & Baskin, 2004). The tunica muscularis propria is composed of inner and outer smooth muscle arranged longitudinally and circularly, respectively (Krstic, 2004). In the male urethra, the circular smooth muscle fascicles join with urinary bladder smooth muscle at the urethrovescical junction to form the internal urethral sphincter (Krstic, 2004; Yucel & Baskin, 2004). The smooth muscle fascicles along the proximal female urethra, however, do not appear to anatomically arrange into a sphincter (Yucel & Baskin, 2004). Skeletal muscle of the urethral wall forms the external urethral sphincter and extends along the membranous urethra in males to generate voluntary pressure during bladder filling (Fowler, Griffiths, & de Groat, 2008). The skeletal muscle fibers in the female urethra join to form an ‘external’ urethral sphincter comprised of a sphincter urethrae, compressor urethrae and sphincter urethrovaginalis to provide urinary continence through urethral and vaginal closure (Ashton-Miller & DeLancey, 2007).
Figure 1: Urinary bladder wall anatomy

Left: Stained human urinary bladder. Right: Tissues and cell types within the bladder wall. The mucosa layer is comprised of the urothelium and the lamina propria that contains vasculature, lymphatics, nerves and smooth muscle cells. The muscularis propria is comprised of detrusor smooth muscle cells and surrounded by outer connective tissue layer termed the adventitia or serosa. Reprinted from BJU International, 96(4), Neuhaus, J., Pfeiffer, F., Wolburg, H., Horn, L., Dorschner, W., Alterations in connexin expression in the bladder of patients with urge symptoms, 670-676, Copyright (2005), with permission from John Wiley and Sons. Reprinted from Physiological Review, 93(2), Birder, L., Andersson, K.E., Urothelial signaling, 653-680, Copyright (2013), with permission from The American Physiological Society.

1.1.2. Neural Control

The LUT is regulated by supraspinal, spinal and peripheral nervous system (PNS) input to maintain “switch-like” patterns of storage and elimination activity and has been previously reviewed in greater detail (Figure 2) (Fowler et al., 2008). Briefly, bladder wall mechanoreceptors initiate visceral afferent (Aδ fibers) activity during the storage phase that synapse on spinal interneurons (de Groat & Wickens, 2013; Fowler et al., 2008). Spinal reflex pathways then facilitate storage by directly enhancing thoracolumbar sympathetic outflow and somatomotor discharge or ascending, in some species, to metencephalic integration centers (Figure 2) (de Groat & Wickens, 2013; Fowler et al., 2008).
Spinal interneurons activate preganglionic sympathetic fibers from the intermediolateral cell column of the lower thoracic (T10) through upper lumbar (L2) spinal cord that form thoracic and lumbar splanchnic nerves (Beckel & Holstege, 2011; Ochodnicky, Uvelius, Andersson, & Michel, 2013). The preganglionic fibers then synapse on the prevertebral inferior mesenteric ganglia or paravertebral ganglia and travel along the hypogastric and pelvic nerves, respectively (Fowler et al., 2008). Sympathetic adrenergic neurotransmission on the urinary bladder smooth muscle β-adrenergic receptors promotes bladder wall relaxation and accommodation (Ochodnicky et al., 2013). Bladder filling is also facilitated by the activation of α-adrenergic receptors on the internal urethral sphincter resulting in contraction of the urethral outlet (Ochodnicky et al., 2013). Spinal reflex pathways not only enhance sympathetic outflow but also α-motoneuron discharge from Onuf’s nucleus in the ventrolateral horn of the sacral (S2-S4) spinal cord (Figure 2) (de Groat & Wickens, 2013). Propagation of this signal along the pudendal nerve to the external urethral sphincter elicits skeletal muscle contraction by activating nicotinic acetylcholine receptors to provide voluntary control over urinary continence (Ochodnicky et al., 2013).

Upon reaching the tension threshold, bladder afferents (Aδ fibers) bypass local spinal reflexes and ascend to the mesencephalic periaqueductal gray (PAG) (Figure 2). Unlike the reflexes underlying the storage phase, the elimination phase relies on supraspinal circuitry as evidenced by voiding dysfunction following lower thoracic spinal cord injury (Beckel & Holstege, 2011; de Groat & Yoshimura, 2006). After cortical processing, the PAG sends excitatory input to a region in the dorsolateral pontine
tegmentum termed the pontine micturition center (PMC) (Griffiths, 2002). The PMC then sends descending cortical projections that synapse on preganglionic parasympathetic neurons and inhibitory interneurons in the sacral spinal cord (Figure 2) (Beckel & Holstege, 2011; Griffiths, 2002).

The preganglionic parasympathetic fibers arise from the intermediolateral cell column of the human sacral (S2-S4) spinal cord to form pelvic nerves. Upon coursing through and exiting the hypogastric and pelvic plexus, the fibers join the pelvic and pudendal nerves to synapse on terminal ganglia and innervate the detrusor smooth muscle and urethra (de Groat & Wickens, 2013; Ochodnicky et al., 2013). Cholinergic and non-adrenergic/non-cholinergic neurotransmission on the urinary bladder smooth muscle promotes bladder wall contraction by activating muscarinic acetylcholine receptors and purinergic receptors, respectively (Beckel & Holstege, 2011). Elimination of urine is also facilitated by nitric oxide release onto the internal urethral sphincter resulting in a relaxation of the urethral outlet (Beckel & Holstege, 2011). The PMC not only augments parasympathetic outflow but also attenuates preganglionic sympathetic and α-motoneuron discharge to the LUT (Figure 2) (Griffiths, 2002). The descending cortical projections terminating on inhibitory interneurons in the sacral spinal cord prevents excitatory input into the urethral sphincters resulting in dilation of the urethral orifice and continuous flow of urine. As distention of the urinary bladder decreases during the elimination phase, ascending excitation to the dorsolateral metencephalon is diminished and the storage phase is once again switched on.
Figure 2: Neural control of continence and micturition

a: Storage reflex. Bladder wall mechanoreceptors initiate visceral afferent activity during the storage phase. Spinal reflex pathways then facilitate storage by enhancing sympathetic discharge in the hypogastric nerve and somatomotor discharge in the pudendal nerve. In some species, the pontine storage center may also promote urethral contraction. Together, this has a net effect of contracting the urethra and relaxing the detrusor smooth muscle to accommodate bladder filling. b: Micturition reflex. Upon reaching the tension threshold, afferent nerve discharge in the pelvic nerve ascends to the pontine micturition center and/or the periaqueductal gray (PAG). Descending cortical projections then activate parasympathetic discharge and inhibit sympathetic and somatomotor discharge. This has a net effect of promoting bladder wall contraction, relaxing the urethra and facilitating the elimination of urine. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Neuroscience, Fowler C.J., Griffiths D., de Groat W.C.. The neural control of micturition. 9(6):453-466, copyright (2008).

1.1.3. Symptoms and Dysfunction

The terminology used in the following section is consistent with the standardization report of LUT symptoms and function by the International Continence Society and will refer to their definitions when appropriate (Abrams et al., 2002). Similar to other clinical indications, LUT symptoms are the patient’s qualitative representation of a purported condition. These symptoms, in particular, refer to a spectrum of LUT functions that include storage, elimination and post micturition disturbances.
Symptoms associated with the storage phase include, but are not limited to, “increased frequency, urgency and incontinence” (Abrams et al., 2002). The complaint of increased urinary frequency is prevalent among both men and women with LUT dysfunction and has been suggested to affect an individual’s quality of life as demonstrated by a strong correlation between frequency and bothersome endorsements (Coyne et al., 2009). Increased urgency is a complaint of the “sudden compelling desire to pass urine” that may be accompanied by pain, pressure or discomfort associated with the LUT (Abrams et al., 2002). Lastly, urinary incontinence includes a complaint of the “involuntary leakage of urine” and may manifest in various forms and severities (Abrams et al., 2002). It is important to note that incontinence is not representative of one particular LUT dysfunction but rather can arise from multiple sources including stress, comorbid disorders and congenital abnormalities (Cameron, Heidelbaugh, & Jimbo, 2013).

Symptoms associated with the elimination phase include “hesitancy, slow or intermittent stream, straining and terminal dribble” (Abrams et al., 2002). These symptoms generally involve complaints of the initiation and continuation of voiding and alterations to their urine stream and appear to be more prevalent in men compared to women (Abrams et al., 2002; Coyne et al., 2009). Symptoms associated with the post micturition phase occur after voiding and include “incomplete emptying and post micturition dribble” (Abrams et al., 2002). Although equally bothersome, post micturition dribble may be more prevalent in men, whereas in women, incomplete emptying may be more prevalent (Coyne et al., 2009). As briefly mentioned above, LUT symptoms are not
confined to urodynamic disturbances, but may also include unpleasant sensations of pain or discomfort during storage or elimination. These sensations are generally perceived to emanate from the urogenital organs and may exacerbate storage and elimination symptoms (Le & Schaeffer, 2009).

1.2. Bladder Pain Syndrome/Interstitial Cystitis

1.2.1. Background

LUT signs and symptoms resembling what is currently termed bladder pain syndrome (BPS)/interstitial cystitis (IC) have been documented throughout history and its perspective has been previously reviewed in detail (Hanno, 2011). Briefly, Drs. Philip Syng Physick and Joseph Parish first recognized an inflammatory condition called tic doloureux of the bladder whose symptoms included chronic urinary frequency, urgency and pelvic pain (J. K. Parsons & Parsons, 2004). Skene (Skene, 1887) expanded the cystoscopic features of this concept in the late 19th century and introduced the term IC which included ulceration of the mucous membrane and inflammation within the bladder wall. Focal, ulcerative bleeding in the urinary bladder wall remained a hallmark of IC due, in part, to the work of Hunner (Hunner, 1915) in the early 20th century (Hanno, 2011). Many patients, however, were misdiagnosed as current estimates suggest only 5-7% of those with BPS/IC present with bladder ulcerations (Hanno, 2011; Jones & Nyberg, 1997).

In the absence of a formal classification for IC, the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) attempted to standardize its research definition in 1987 by establishing a diagnostic criteria (Hanno, Staskin, Krane, & Wein,
The criteria included the presence of “glomerulations on cystoscopic examination or a classic Hunner ulcer, pain associated with the bladder, urinary urgency” and eighteen exclusion conditions (Hanno, 2011; Hanno et al., 1990). After several iterations and international consultations, the term IC was expanded to include BPS (Hanno et al., 2011; van de Merwe et al., 2008). The patient selection for BPS is based on “chronic pelvic pain, pressure or discomfort perceived to be related to the urinary bladder accompanied by at least one other urinary symptom such as persistent urge to void or frequency”, whereas, IC is “reserved for cystoscopic and histological features” (Abrams et al., 2002; van de Merwe et al., 2008). At this time, the terms BPS and BPS/IC are analogous and is defined by the American Urological Association Interstitial Cystitis Guidelines Panel as at least six weeks of LUT symptoms and unpleasant sensations perceived to be related to the urinary bladder and with no other clinically identifiable sources (Hanno et al., 2011).

1.2.2. Epidemiology

The epidemiology of BPS/IC is limited due to the absence of standardized definitions, markers and examinations (Berry et al., 2011; Hanno, 2011; Jones & Nyberg, 1997; Sant & Hanno, 2001). Taking into account this variability, it is estimated that there is a 5:1 female-to-male ratio among BPS/IC patients (Hanno, 2011; Jones & Nyberg, 1997). It is estimated that 300 per 100,000 women worldwide suffer from BPS/IC (Hanno, 2011). In the United States alone, 3.3 to 7.9 million women are estimated to meet the criteria for BPS/IC (Berry et al., 2011). As expected, BPS/IC puts an enormous financial burden on the individual and economy as a whole. Health care costs for an individual with BPS/IC ranges from 4 to 7 thousand dollars per year, while the economic
burden approaches 500 million dollars per year in lost productivity and therapeutics (Anger et al., 2011; Hanno, 2011).

1.2.3. Pathophysiology

While the primary insult underlying BPS/IC is not known, it has been suggested that the pathophysiology is a “vicious circle” involving uroepithelial dysfunction, inflammation, afferent nerve hyperexcitability and visceral hyperalgesia and allodynia (Figure 3) (Sant, Kempuraj, Marchand, & Theoharides, 2007). This section will explore said mechanisms that have been proposed to feed-forward to promote the chronicity of LUT symptoms observed in BPS/IC (Sant et al., 2007).

The urothelium is a specialized, stratified epithelium that when intact provides a non-adherent, passive barrier through tight junction proteins, plaque proteins and surface proteoglycans (Lewis, 2000). Any perturbation to the components of this permeability barrier may lead to increased infiltration into the bladder wall and exposure of the interstitium to urinary constituents (Hurst et al., 1996; C. L. Parsons, 2011; C. L. Parsons, Greene, Chung, Stanford, & Singh, 2005; C. L. Parsons & Hurst, 1990). The diffusion of urinary constituents like potassium into the bladder interstitium may depolarize muscle and nerve cells, inflame tissues, degranulate mast cells and cascade to the development of LUT symptoms (Figure 3) (C. L. Parsons, 2011). Uroepithelial dysfunction specific to BPS/IC, however, remains controversial. For example, Chelsky et al. (Chelsky et al., 1994) demonstrated that the permeability in IC was comparable to the variation seen in symptom-free controls, whereas, Parsons et al. (C. L. Parsons et al., 2005) demonstrated abnormal permeability and potassium absorption in those with IC (Hanno, 2011). The
abundance of studies for or against uroepithelial dysfunction in BPS/IC suggest it may not be a primary insult but rather may occur in a subset of patients to exacerbate LUT symptoms (Hanno, 2011).

In addition to the uroepithelial disruption, visceral inflammation also remains a central pathological process in BPS/IC and has been suggested to underlie the development of LUT symptoms (Figure 3). Inflammation within the urinary bladder viscera is characterized by increased vasculature, mucosal irritation that may result in barrier dysfunction and infiltration of inflammatory mediators (Erickson, Belchis, & Dabbs, 1997; Grover, Srivastava, Lee, Tewari, & Te, 2011). The proliferation and activation of mast cells, in particular, has received considerable attention in the urinary bladder immune response (Sant et al., 2007). Mast cells secrete vasoactive chemicals to promote innate and auto-immunity and their increased activity has been widely demonstrated in BPS/IC (W. Boucher, el-Mansoury, Pang, Sant, & Theoharides, 1995; Enerback, Fall, & Aldenborg, 1989; Kastrup, Hald, Larsen, & Nielsen, 1983; Theoharides et al., 1995). The subsequent exposure in the bladder interstitium to vasoactive chemicals, inflammatory mediators and neuropeptides from visceral inflammation may lead to afferent nerve hyperexcitability and neurogenic inflammation (Figure 3) (Vizzard, 2001; Yoshimura & de Groat, 1999; Y. Yu & de Groat, 2008).

The loss of inhibition on peripheral afferents (Aδ and C fibers) increases input into the spinal cord and may eventually promote central sensitization (Sant et al., 2007). An unregulated state of central and peripheral reactivity causes ‘wind-up’ which is observed clinically as hyperalgesia and allodynia (Figure 3). In BPS/IC, hyperalgesia and
allodynia are characterized by an elevated state of urinary bladder sensation that may cause pain, pressure or discomfort and may result in increased urinary frequency and urgency (Grover et al., 2011). The “vicious circle” continues as mast cell degranulation and infiltration of mediators from uroepithelial dysfunction and/or visceral inflammation sustain peripheral and central sensitization to establish visceral hyperalgesia/allodynia and chronic LUT symptoms (Figure 3) (Sant et al., 2007).

**Figure 3: Potential etiologic cascade and pathogenesis underlying BPS/IC**

It is likely that BPS/IC has a multifactorial etiology that may act predominantly through one or more pathways resulting in the typical symptom-complex. There is a lack of consensus regarding the etiology or pathogenesis of BPS/IC but a number of proposals include a “leaky epithelium,” release of neuroactive compounds at the level of the urinary bladder with mast cell activation, “awakening” of C-fiber bladder afferents, and upregulation of inflammatory mediators including cytokines and chemotactic cytokines (chemokines). Inflammatory mediators can affect CNS and PNS neural circuitry including central “wind-up” and nociceptor sensitization resulting in chronic bladder pain and voiding dysfunction. BPS/IC is associated with diseases affecting other viscera and pelvic floors. Reprinted from Urology, 69(4 Suppl), Sant, G.R., Kempuraj, D., Marchand, J.E., Theoharides, T.C., The mast cell in interstitial cystitis: Role in pathophysiology and pathogenesis, S34-40, Copyright (2007), with permission from Elsevier. Reprinted from BioMed Research International, 2014, Gonzalez, E.J., Arms, L., Vizzard, M.A., The role(s) of cytokines/chemokines in urinary bladder inflammation and dysfunction, 1-17, Copyright (2014), with permission from Hindawi Publishing Corporation.
1.2.4. Animal Models

Numerous animal models have been implemented to determine the onset and chronicity of LUT dysfunctions like BPS/IC. While one model cannot currently account for the constellation of symptoms in BPS/IC, they each aid in identifying distinct mechanisms underlying part of its pathophysiology. This section will explore a naturally occurring cystitis model in felines and focus its review on experimental models of cystitis induced chemically. It is important to note that models of BPS/IC are not limited to what will be discussed in this section and exhaustive reviews have been previously published (Bjorling, Wang, & Bushman, 2011; Fry et al., 2010; Westropp & Buffington, 2002).

The natural development of spontaneous LUT symptoms has been documented in cats for several decades and is termed feline interstitial cystitis (FIC) (Bjorling et al., 2011; Lyngset, 1972). Though the primary insult for FIC is not known, the pathophysiology has marked similarities to BPS/IC including uroepithelial dysfunction and visceral inflammation. Cats with FIC have been shown to have a disruption to the epithelial cytoarchitecture that increased diffusion and infiltration of urinary constituents (Gao, Buffington, & Au, 1994; Lavelle et al., 2000). Uroepithelial dysfunction in FIC further leads to a peripheral upregulation of neuropeptides and inflammatory mediators that alter bladder afferent soma size and increase input to the central nervous system (CNS) (Birder et al., 2010). As previously discussed, the alterations to central and peripheral reactivity following uroepithelial dysfunction and/or visceral inflammation may promote the development of LUT symptoms that is observed in FIC and, by extension, BPS/IC (Birder et al., 2010; Grover et al., 2011; Sant et al., 2007).
Despite these pathophysiological similarities, FIC as a model for BPS/IC is limited due to its spontaneity and epidemiology. Investigators are practically and financially restricted to structural and functional alterations following its spontaneous induction and thus inadequately define insults preceding the development of FIC (Westropp & Buffington, 2002). Furthermore, unlike BPS/IC, FIC occurs irrespective of biological sex (Westropp & Buffington, 2002). While this may be due to a misdiagnosis of BPS/IC in males, one cannot discount hormonal differences that may affect LUT symptoms in humans (Robinson & Cardozo, 2003; Rohrmann et al., 2007).

LUT symptoms have also been induced by an assortment of chemical irritants including, but not limited to: hydrochloric acid, acetic acid, protamine sulfate (PS) and cyclophosphamide (CYP). The inflammation induced by intravesical instillation of irritants like hydrochloric acid and acetic acid help reveal the anatomical, organizational and functional alterations attributable to the visceral immune response (Bjorling et al., 2011). Specifically, the functional and histological features following acid instillation are similar to a BPS/IC subset and include urothelial hyperplasia, bladder ulceration, mucosal edema, inflammatory cell infiltration and the development of LUT symptoms (Osorio, Simckes, & Hellerstein, 1996; Yoshida, Kageyama, Fujino, Nozawa, & Yamada, 2010). Though acid instillation allows for a more controlled environment than FIC, the studies must be interpreted cautiously as the degree of inflammation resulting from exogenous irritants may not be representative of the naturally occurring BPS/IC (Bjorling et al., 2011).
Unlike acid instillation, PS lacks a pervasive inflammatory element but rather disrupts uroepithelial barrier function by targeting bladder surface proteoglycans (C. L. Parsons, Stauffer, & Schmidt, 1988). Similar to the uroepithelial dysfunction observed in FIC, PS instillation is sufficient to induce LUT symptoms (Soler et al., 2008). More recently, PS has been used in conjunction with bacterial induced cystitis. Instillation of both PS and E. coli lipopolysaccharide to respectively damage the urothelium and induce a visceral inflammatory cascade may help clarify the interaction(s) of multiple processes underlying LUT symptoms in BPS/IC (Bjorling et al., 2011; Stein, Pham, Ito, & Parsons, 1996).

CYP is an antineoplastic prodrug that requires enzymatic activation to release phosphoramidate mustard and the byproduct acrolein (Emadi, Jones, & Brodsky, 2009; Powers & Sladek, 1983). A known adverse toxicity following systemic CYP administration is hemorrhagic cystitis (Emadi et al., 2009). Hemorrhagic cystitis arises from the bladder mucosal walls contact with acrolein, which increases vascular permeability and results in bladder ulceration and hypertrophy (Batista et al., 2006). In addition to hemorrhagic cystitis, systemic CYP treatment causes functional and histological changes similar to BPS/IC including: mucosal edema, uroepithelial dysfunction, inflammatory cell infiltration, afferent nerve hyperexcitability and the development of LUT symptoms (M. Boucher et al., 2000; Eichel et al., 2001; Malley & Vizzard, 2002; Stewart, 1986; Yoshimura & de Groat, 1999). CYP administration also produces behavioral alterations consistent with the development of viscerosomatic pain including decreased breathing rate, closing of the eyes and rounded back postures (M.
Boucher et al., 2000). While the urinary bladder inflammatory response following systemic CYP administration is greater than that observed in BPS/IC, this experimental model of cystitis is appealing because of its route of administration (intraperitoneal) and the chronicity and reproducibility of histopathological and functional alterations (Bjorling et al., 2011).

1.3. Transforming Growth Factor-beta

1.3.1. Background

The transforming growth factor-beta (TGF-β) superfamily is comprised of at least 35 pleiotropic proteins belonging to four subfamilies grouped by their sequence homology—decapentaplegic-Vg-related (DVR), activin/inhibin, TGF-β sensu stricto and other divergent members (Herpin, Lelong, & Favrel, 2004). Even though TGF-β superfamily members have distinct expression patterns and regulate a variety of functions, they are each translated as a pre-pro-protein that contains a peptide sequence signaling to the endoplasmic reticulum, a N-terminal prodomain and a C-terminal mature protein (Annes, Munger, & Rifkin, 2003; Herpin et al., 2004). After proteolytic processing and posttranslational modifications, the C-terminal fragment is either secreted as a mature protein dimer or forms a latent complex by maintaining a noncovalent bond to the prodomain (Annes et al., 2003; Herpin et al., 2004).

The canonical members of TGF-β sensu stricto are one such proprotein to form a latent complex. The interactions between the N-terminal prodomain, termed latency associated peptide (LAP), and the mature TGF-β dimer is sufficient to sequester its extracellular activity (Shi et al., 2011). Additionally, LAP associates with a latent TGF-β
binding protein (LTBP) that regulates TGF-β bioavailability by chaperoning the complex to the extracellular matrix (Todorovic et al., 2005). The subsequent activation of latent TGF-β in the extracellular matrix via LAP cleavage occurs by protease-dependent or protease-independent (protons, integrins, reactive oxygen species, etc.) mechanisms (Annes et al., 2003; Barcellos-Hoff & Dix, 1996; Lyons, Keski-Oja, & Moses, 1988; Murphy-Ullrich & Poczatek, 2000; van der Flier & Sonnenberg, 2001).

After its secretion, the mature or activated protein dimers transduce a signal through transmembrane Ser-Thr receptor kinases (Herpin et al., 2004). The TGF-β family of receptors is comprised of type I and type II receptors. Type II receptors selectively bind their respective ligands to define part of the specificity of signal transduction (Herpin et al., 2004). Ligand binding can either be “sequential” or “cooperative” and may involve an accessory receptor (type III) to enhance ligand presentation (Massague, 1998). Following receptor-ligand interaction, the type II receptor forms a heterotetrameric complex with the type I receptor to transphosphorylate residues of the Gly-Ser (GS) box (Massague, 1998). The activated type I receptors then phosphorylate Smad-dependent or Smad-independent substrates to regulate the transcription of target genes (Derynck & Zhang, 2003).

Smad proteins exist in three families: receptor-activated, common mediator and inhibitory. Receptor-activated (R-) Smads dock onto type I receptors and are phosphorylated on distal serine residues following receptor activation (Derynck & Zhang, 2003). Phosphorylated R-Smads dissociate from the receptor and interact with common mediator Smad4 (Derynck & Zhang, 2003). The oligomeric R-Smad/Smad4 complex
then translocates to the nucleus where it alters the transcription of target genes (Derynck & Zhang, 2003). Type I receptors not only function through Smad signaling but may also directly activate Smad-independent pathways such as TGF-β-activated kinase 1 (TAK1), Ras, nuclear factor-κB (NF-κB) and the mitogen-activated protein kinase (MAPK) subfamily members (Engel, McDonnell, Law, & Moses, 1999; Freudlsperger et al., 2013; M. K. Lee et al., 2007; Moustakas & Heldin, 2005; Yamaguchi et al., 1995; L. Yu, Hebert, & Zhang, 2002). The variety of direct and context-dependent downstream signaling pathways preserves the multifunctional role(s) of TGF-β superfamily ligands while providing the specificity required to control distinct target genes.

### 1.3.2. Immune Response

The canonical members of TGF-β sensu stricto maintain immunological function by regulating the initiation and resolution of the immune response and a comprehensive review has been previously published (M. O. Li, Wan, Sanjabi, Robertson, & Flavell, 2006). Briefly, activated TGF-β at the site of injury may initiate a proinflammatory milieu characterized by matrix remodeling and the recruitment and activation of leukocytes (Ashcroft, 1999; M. O. Li et al., 2006). TGF-β may then aid in resolving the primary immune response and support a milieu for tissue repair and immunological memory to progress by suppressing the proliferation, differentiation and survival of a subset of lymphocytes (M. O. Li et al., 2006).

To initiate an immune response, TGF-β may mobilize monocytes, mast cells and granulocytes to the site of injury and influence their adhesion to the extracellular matrix (Gruber, Marchese, & Kew, 1994; M. O. Li et al., 2006; Reibman et al., 1991; van Royen
et al., 2002). While TGF-β may also recruit monocyte-derived macrophages, their activation and function is typically inhibited to help resolve the immune response (Ashcroft, 1999; S. Chen et al., 2005; Werner et al., 2000). Since immune cells continue to infiltrate the site of injury, the extracellular matrix undergoes pathological remodeling characterized by protease secretion and matrix degradation (Sorokin, 2010). TGF-β supports the remediation and repair of these tissues by increasing the deposition of matrix proteins and inhibiting protease activation (Leask & Abraham, 2004).

To sustain the resolution of the immune response, TGF-β may regulate T cell proliferation, differentiation and survival (Gorelik & Flavell, 2002). TGF-β promotes T cell growth arrest by suppressing interleukin 2 in areas of subthreshold antigen presentation (Kehrl et al., 1986; M. O. Li et al., 2006). During the polarizing conditions of the immune response, TGF-β maintains peripheral immunological tolerance by inducing the transcription factor FoxP3 to promote CD4+CD25+ T cell differentiation to regulatory T cells (M. O. Li et al., 2006; Marie, Letterio, Gavin, & Rudensky, 2005). CD4+ T cell differentiation to the T helper (Th) 1 and Th2 cell lineages, however, is inhibited by TGF-β mediated repression of the transcription factors T-bet and GATA-3, respectively (Gorelik, Fields, & Flavell, 2000; Gorelik & Flavell, 2002). In addition to its effects on CD4+ T cells, TGF-β may also attenuate the cytotoxicity of CD8+ T cells by inhibiting its cytolytic genes (Thomas & Massague, 2005).

TGF-β not only stabilizes T cell expression and function to resolve the immune response, but also regulates B cell proliferation, survival and development (Lebman & Edmiston, 1999). TGF-β inhibits both the proliferation and cell cycle progression of B
cells through Smad-dependent or Smad-independent pathways (Heldin, Landstrom, & Moustakas, 2009; Kee, Rivera, & Murre, 2001; M. O. Li et al., 2006; Smeland et al., 1987). TGF-β utilizes comparable B cell growth arrest pathways, as well as a distinct Smad-independent pathway, to induce the apoptosis of B cells (M. O. Li et al., 2006; Perlman, Schiemann, Brooks, Lodish, & Weinberg, 2001). Lastly, TGF-β may regulate the maturation and activation of B cells through its induction of isotype switching, suppression of B cell antigen receptor signaling and inhibition of immunoglobulin secretion (Cazac & Roes, 2000; Kehrl, Thevenin, Rieckmann, & Fauci, 1991; M. O. Li et al., 2006).

1.3.3. Nociception

The members of TGF-β sensu stricto contribute to both the peripheral and central processing of noxious stimuli. TGF-β1 and TGF-β2 have been demonstrated to increase de novo neuropeptide synthesis in the DRG that may directly sensitize primary afferent nociceptors (Chalazonitis, Kalberg, Twardzik, Morrison, & Kessler, 1992; Levine, Fields, & Basbaum, 1993). TGF-β may also influence DRG excitability by regulating several ion channels including the voltage-gated potassium (Kv) channel and TRPV-1. Application of recombinant TGF-β1 in vitro has been demonstrated to downregulate KCNA4 gene expression and decrease A-type Kv currents in primary DRG cultures (Zhu et al., 2012). Additionally, TGF-β1 Smad-independent signaling may phosphorylate TRPV-1 on Thr residues and potentiate capsaicin-evoked calcium influx in the DRG (Utreras et al., 2012; Utreras et al., 2013). The subsequent prolonged
depolarization and an impaired repolarization may lead to an amplification of nociceptive transmission and CNS input.

Unlike its role in the periphery, TGF-β in the CNS appears to be neuroprotective by regulating neuronal and nonneuronal response to inflammatory injury (Lantero, Tramullas, Diaz, & Hurle, 2012). Nonneuronal glial cells have recently been recognized to enhance the proinflammatory milieu and facilitate the central processing of nociception (O'Callaghan & Miller, 2010). Activated TGF-β in the CNS may inhibit the proliferation and activation of these spinal glial cells to attenuate the induction of neuropathic pain (Abutbul et al., 2012; Echeverry et al., 2009; Xiao, Bai, Zhang, & Link, 1997). TGF-β may further reduce excitatory synaptic transmission of second-order neurons by directly suppressing the proinflammatory milieu in the spinal cord (Echeverry et al., 2009). As a result of its biphasic and modulatory role in the peripheral and central transmission of nociception, TGF-β appears to have a profound impact on the perception of pain and may initiate, in part, pathological pain syndromes.

### 1.3.4. Role(s) in Cystitis

TGF-β ligands and its cognate receptors are expressed at low, basal levels in rat urinary bladder tissues (Gonzalez, Girard, & Vizzard, 2013). Following chemically (CYP)-induced cystitis of varying durations, TGF-β ligand and receptor expression appears to display a time- and tissue-dependent regulation. TGF-β exhibits a delayed, but sustained, increase in urinary bladder gene and protein expression 8-48 h after CYP treatment (Gonzalez et al., 2013; Tyagi et al., 2009; Zhang & Qiao, 2012). Furthermore, urinary excretion of active and latent TGF-β1 is increased up to 100-fold 24 h after acute
CYP treatment (Tyagi et al., 2009). The aforementioned regulation of TGF-β gene and protein expression has been suggested to be more pronounced in the afferent limb of the micturition reflex suggesting a possible role in the development of LUT symptoms (Gonzalez et al., 2013). Its role in micturition reflex dysfunction was confirmed with cystitis and the pharmacological inhibition of aberrant TGF-β signaling. SB505124, a potent inhibitor of TGF-β type I receptor kinase activity, decreased urinary frequency and increased bladder capacity, void volume and intercontraction intervals 48 h following CYP-induced cystitis (Gonzalez et al., 2013). These studies raise the possibility of targeting TGF-β at the level of the urinary bladder to alleviate voiding dysfunction with cystitis.

1.4. ATP and Purinergic Receptor/Signaling

1.4.1. Background

The urothelium releases neuroactive transmitters, in particular ATP, from its apical and basolateral surfaces in response to physical and chemical stimuli (L. Birder & K. E. Andersson, 2013; Burnstock, 2014). ATP and other nucleotides derived from the urothelium can be released through several mechanisms that include transporters, ion channels or vesicular exocytosis (Bodin & Burnstock, 2001; Wang et al., 2005). Extracellular ATP that is not degraded by ectonucleotidases or exonucleotidases is then able to stimulate autocrine or paracrine pathways that may aid in the sensory transduction to the CNS (Schwiebert & Zsembery, 2003). The transduction pathways within the urinary bladder are affected by receptor subtype expression and their proximity to the urothelium. The tissues and cell types that may contribute to purinergic or pyrimidinergic
signaling include the urothelium (Dunning-Davies, Fry, Mansour, & Ferguson, 2013; W. Yu, Zacharia, Jackson, & Apodaca, 2006), ICC (Y. Li et al., 2013), smooth muscle cells (Gopalakrishnan et al., 2002; Yang et al., 2000) and suburothelial nerve fibers (L. Birder & K. E. Andersson, 2013; Gonzalez, Merrill, & Vizzard, 2014).

1.4.2. P2X and P2Y Receptor Expression and Function in the LUT

P2-purinoceptors are classified as ligand-gated ion channels, P2X, or G-protein coupled receptors, P2Y (Fredholm et al., 1994). There are currently seven P2X subunits (P2X1-7) that may arrange as heteromeric or homomeric ligand-gated ion channels and eight metabotropic P2Y subunits (P2Y1, P2Y2, P2Y4, P2Y6, P2Y11, P2Y12, P2Y13, P2Y14) that may couple to Gs, Gi or Gq (Abbracchio et al., 2006; North, 2002). While the kinetics and tissue distribution of each P2-purinoceptor differs, there is substantial evidence that many of these subunits are functionally expressed throughout the urinary bladder urothelium, lamina propria and detrusor smooth muscle.

The distribution of P2X and P2Y receptors in the urothelium has been described in multiple species, including rodents, felines and humans. P2X2 and P2X4-7 receptor immunoreactivity (IR) was detected in the rodent urothelium, whereas, positive IR was detected for P2X1-7 in the feline urothelium (Table 1) (Birder et al., 2004; H. Y. Lee, Bardini, & Burnstock, 2000; Vial & Evans, 2000). In the human bladder urothelium, glycosylated P2X2 and P2X3 transcript and protein expression has also been detected (Tempest et al., 2004). There appears to be less diversity in the uroepithelial distribution of P2Y receptors where P2Y2 and P2Y4 transcript and protein expression has been demonstrated in cultured rat uroepithelial cells (Chopra et al., 2008). Additionally, in the
human uroepithelial cell line, UROtsa, P2Y1, P2Y2 and P2Y11 transcript expression was detected (Save & Persson, 2010).

Nucleotides and their metabolites seem to have an intricate role in ATP release and P2 receptor signaling in the urothelium. More specifically, adenosine diphosphate (ADP) has been demonstrated to evoke ATP release from the urothelium, whereas adenosine exerts inhibition on ATP release via A1 receptors (Dunning-Davies et al., 2013; Mansfield & Hughes, 2014). The diverse expression of purine and pyrimidine receptor subtypes in the urothelium also allows for distinct signal transduction (Chopra et al., 2008; H. Y. Lee et al., 2000; Tempest et al., 2004). The activation of P2Y, but not P2X, receptors on the urothelium evokes ATP release suggesting urothelial P2Y receptors may contribute to purinergic neurotransmission (Chopra et al., 2008; G. Sui et al., 2014).

The urinary bladder lamina propria is deep to the mucosal basement membrane and includes loose connective tissue, vasculature, lymphatics, nerves and interstitial cells (Andersson & McCloskey, 2013). A population of interstitial cells in the lamina propria (ICC-LP) also express P2X3, P2Y2, P2Y4 and P2Y6 receptors and are proposed to form a functional syncytium with smooth muscle cells (Table 1) (Drumm, Koh, Andersson, & Ward, 2014; G. P. Sui, Wu, & Fry, 2006). In response to ATP, ICC-LP generate P2Y-dependent intracellular calcium transients followed by inward currents (C. Wu, Sui, & Fry, 2004). These ATP-generated transients may then propagate to smooth muscle cells via gap junctions to alter contractility (Drumm et al., 2014). Although the mechanism coupling ICC-LP to sensory activity is not yet known, the localization of ICC-LP and its
responsiveness to ATP suggest they may have a regulatory role in the afferent limb of the micturition reflex (C. Wu et al., 2004). In addition to the functional syncytium with ICC-LP, urinary bladder smooth muscle cells express P2X1–P2X6 and P2Y receptors (Dutton, Hansen, Balcar, Barden, & Bennett, 1999). P2X1 receptors have received much of the focus on bladder contractility due to their contributions to nerve evoked bladder contractions (Vial & Evans, 2000). More recently, P2Y6 receptors have also been demonstrated to modulate bladder smooth muscle tone and augment P2X-mediated bladder contraction (W. Yu, Sun, Robson, & Hill, 2013).

Afferent (and efferent) nerves terminating in the urothelium, lamina propria and detrusor smooth muscle have received much of the attention in characterizing P2-purinoceptor distribution (Table 1). While the transcript and protein expression of all seven P2X subunits (P2X1-7) have been detected in the rodent DRG, there appears to be a differential distribution of P2X2 and P2X3 depending on spinal cord level (X. Chen & Gebhart, 2010; Ruan et al., 2005; Xiang, Bo, & Burnstock, 1998). P2X2 mRNA has been detected in both thoracolumbar and lumbosacral urinary bladder afferent neurons, but transcripts at the thoracolumbar level appear to be co-expressed with P2X3 (X. Chen & Gebhart, 2010). P2X3 transcripts, on the other hand, appear to be restricted to small- and medium-diameter afferent neurons and have a greater frequency of expression in thoracolumbar than lumbosacral neurons (Bradbury, Burnstock, & McMahon, 1998; X. Chen & Gebhart, 2010; Ruan et al., 2005). Similar to the urothelium, there appears to be less diversity in the distribution of P2Y receptors in DRG neurons. P2Y1, P2Y2 and P2Y4 transcript expression has been detected in rodent DRG neurons with P2Y1
restricted to small-diameter neurons and P2Y4 to medium- and large-diameter neurons (Ruan & Burnstock, 2003). P2Y2 and P2Y4, in particular, have been detected in thoracolumbar and lumbosacral bladder afferent neurons by retrograde labeling (X. Chen, Molliver, & Gebhart, 2010).

Although not yet conclusive, the proximity of afferent nerve terminals to the urothelium indicates a role in purinergic sensory transduction. Previous studies have established the functional contributions of P2X2 and P2X3 receptors to the afferent limb of the micturition reflex (Kaan et al., 2010). P2X3 receptor-mediated mechanisms have been demonstrated to contribute to both nociceptive and non-nociceptive mechanosensory transduction in the bladder (Rong, Spyer, & Burnstock, 2002). Furthermore, mice lacking P2X2/P2X3 receptor subunits exhibit decreased urinary bladder reflexes and decreased pelvic afferent nerve activity in response to bladder distention (Cockayne et al., 2005). The functional contributions to bladder sensory neuron sensitization have also been demonstrated with P2Y2 receptors (X. Chen et al., 2010). P2Y2 receptor activation was able to facilitate P2X2 and P2X3 evoked currents and increase bladder neuron excitability (X. Chen et al., 2010).

Table 1: Purinergic receptors/channels in the LUT

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<th>Purinergic receptors/channels in the LUT</th>
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<td>Purinergic receptors are functionally distributed throughout the tissues and cell types of the urinary bladder. Abbreviations: ADP, adenosine diphosphate; ATP, adenosine triphosphate; BAN, bladder afferent nerve; Det, detrusor; ICC, interstitial cell of Cajal; P, purinergic; UC, urothelial cell; UDP, uridine diphosphate; UTP, uridine triphosphate. *Species differences. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Urology, Merrill, L., Gonzalez, E.J., Girard, B.M., Vizzard, M.A.. TRP channels and purinergic signaling involvement in the urothelial sensory system in the urinary bladder. In press, copyright (2016).</td>
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<td>Purinergic receptors/channels</td>
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### 1.4.3. Role(s) in Cystitis

Of the seven P2X subunits, P2X2 and P2X3 have been suggested to be involved in the sensitization of urinary bladder sensory transduction with cystitis. Specifically, P2X2 and P2X3 protein expression in the human bladder urothelium has been demonstrated to increase in IC (Tempest et al., 2004). Likewise, P2X2 transcript expression was increased in mouse thoracolumbar DRG neurons with cyclophosphamide (CYP)-induced cystitis (X. Chen & Gebhart, 2010). Inhibition of P2X3 or P2X2/3 receptors with A-317491 following CYP-induced cystitis reduced non-voiding contractions and residual urine volume and increased intermicturition intervals suggesting a role for purinergic signaling in bladder hyperreflexia with cystitis (Ito, Iwami, Katsura, & Ikeda, 2008).

CYP-induced cystitis has also been demonstrated to increase peak urinary bladder afferent nerve activity that was significantly decreased following P2X receptor antagonist (TNP-ATP or PPADS) instillation (Y. Yu & de Groat, 2008). The significance of P2X3 specifically in bladder afferent nerve sensitization was demonstrated with P2X3-null mice and its attenuation of P2X agonist (ATP or α,β-meATP) induced afferent nerve excitation (Vlaskovska et al., 2001). Additionally, following CYP-induced cystitis,
thoracolumbar rat DRG neurons increased homomeric P2X3 current, whereas, lumbosacral rat DRG neurons increased heteromeric P2X2/3 current (Dang, Lamb, Cohen, Bielefeldt, & Gebhart, 2008). While CYP-treated urinary bladder neurons exhibit increased responsiveness to purinergic agonist application, the changes in P2X subtypes do not appear to be conserved across species (X. Chen & Gebhart, 2010; Dang et al., 2008). For instance, homomeric P2X2 currents not seen in rat DRG neurons increased in mouse lumbosacral DRG neurons (X. Chen & Gebhart, 2010).

Although the role of P2Y receptors in urinary bladder sensory transduction is still being resolved, P2Y2 has been implicated in bladder afferent hyperexcitability. UTP, a P2Y2 and P2Y4 agonist, application depolarized the resting membrane potential and increased action potential frequency in bladder afferent neurons (X. Chen et al., 2010). UTP application also potentiated homomeric P2X2 current in wildtype (WT) but not P2Y2-null mice suggesting P2Y2 may contribute to P2X-mediated afferent nerve sensitization that was observed with cystitis (X. Chen et al., 2010).

1.4.4. Role(s) in Human Bladder Dysfunction

Increased levels of urinary ATP have been demonstrated in patients with IC (Sun, Keay, De Deyne, & Chai, 2001), OAB (Silva-Ramos et al., 2013) and other functional disorders of the urinary bladder (Burnstock, 2014). Primary bladder urothelial cells taken from these patients also exhibit increased ATP release in response to mechanical stretch (Sun et al., 2001), hypotonic stress (Sun & Chai, 2006) or electrical field stimulation (Kumar, Chapple, Rosario, Tophill, & Chess-Williams, 2010). In addition to increased urothelial-derived ATP release, changes in P2X receptor subtype
expression within various bladder tissues have also been demonstrated in patients with IC (Tempest et al., 2004), detrusor instability (O'Reilly et al., 2002) or bladder outlet obstruction (O'Reilly et al., 2001). The established role(s) of purinergic signaling in bladder sensation suggest that these neurochemical alterations may contribute to the development of LUT symptoms in these patients. Thus, an alternative therapeutic approach to improve bladder function may be to target purinergic neurotransmission within sensory components of the micturition reflex (Burnstock, 2011).

1.5. Project Goals and Hypotheses

BPS/IC is a major unresolved health concern in the United States with an economic burden approaching $500 million per year in lost productivity and health care. BPS/IC is defined by at least six weeks of dysuric symptoms and unpleasant sensations (visceral pain and discomfort) perceived to be related to the urinary bladder and in the absence of other clinically identifiable sources. Despite advancements in our understanding of the biological mechanisms of these pain syndromes, current pharmaceutical and behavioral therapies are ineffective and fail to completely resolve pathological symptoms. There is, therefore, a critical need to understand mechanistically the neurochemical, functional and organizational alterations underlying BPS/IC and how this translates into the constellation of clinical symptoms. In the absence of such knowledge and effective management, BPS/IC continues to negatively impact the economy and those suffering from BPS/IC continue to have physical and psychological health dysfunction.
Our long-term research goal is to develop successful therapeutic interventions in the treatment of BPS/IC. The objective of this dissertation is to identify and characterize a novel neurochemical alteration in the micturition reflex pathway and to determine its functional role in modulating micturition reflexes. Our central hypothesis is that the pleiotropic protein, TGF-β, and its cognate receptors contribute to afferent nerve hyperexcitability that may facilitate increased voiding frequency in an experimental cystitis model of BPS/IC. Identifying novel components of the urinary bladder inflammatory response underlying dysfunction provides a basis for eventual preclinical and clinical trials.

In Chapter 2 of this dissertation we explored the functional expression of TGF-β ligands and receptors in the inflamed urinary bladder. We hypothesized that aberrant expression will compartmentalize to distinct tissues to reflect the inflammatory state of the urinary bladder and contribute to bladder dysfunction. In Chapter 3 of this dissertation we explored the sensory components of the urinary bladder that may underlie the pathophysiology of aberrant TGF-β activation. We hypothesized that TGF-β1 will contribute to bladder afferent nerve excitability through enhanced ATP release from the urothelium and purinoceptor activation. At the conclusion of these studies, we will have characterized TGF-β ligand and receptor lower urinary tract expression and provided evidence for a role of TGF-β signaling in bladder dysfunction. These studies will not only increase our understanding of functional disorders and visceral pain, but also have the potential to improve the health of those suffering from inflammation-associated bladder syndromes.
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CHAPTER 2: EXPRESSION AND FUNCTION OF TRANSFORMING GROWTH FACTOR-BETA ISOFORMS AND COGNATE RECEPTORS IN RAT URINARY BLADDER FOLLOWING CYCLOPHOSPHAMIDE-INDUCED CYSTITIS

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Abstract

Numerous proinflammatory cytokines have been implicated in the reorganization of lower urinary tract function following cyclophosphamide (CYP)-induced cystitis. The present study investigated the functional profile of three pleiotropic transforming growth factor-beta (TGF-β) isoforms and receptor (TβR) variants in the normal and inflamed (CYP-induced cystitis) rat urinary bladder. Our findings indicate that TGF-β (1, 2 and 3) and TβR (1, 2 and 3) transcript and protein expression were regulated to varying degrees in the urothelium or detrusor smooth muscle following intermediate (48 h; 150 mg/kg, i.p.) or chronic (75 mg/kg, i.p.; once every three days for ten days), but not acute (4 h; 150 mg/kg, i.p.), CYP-induced cystitis. Conscious, open outlet cystometry was performed to determine whether aberrant TGF-β signaling contributes to urinary bladder dysfunction following intermediate (48 h) CYP-induced cystitis. TβR-1 inhibition with SB505124 (5 µM) significantly (p ≤ 0.001) decreased voiding frequency and increased bladder capacity (2.5-fold), void volume (2.6-fold) and intercontraction intervals (2.5-fold) in CYP-treated (48 h) rats. Taken together, these results provide evidence for: (i) the involvement of TGF-β in lower urinary tract neuroplasticity following urinary bladder inflammation, (ii) a functional role of TGF-β signaling in the afferent limb of the micturition reflex and (iii) urinary bladder TβR-1 as a viable target to reduce voiding frequency with cystitis.

Keywords: inflammation, transforming growth factor-β, urothelium, detrusor smooth muscle, cyclophosphamide
Introduction

Interstitial Cystitis/Bladder Pain Syndrome (IC/BPS) is defined by the presence of dysuric symptoms and unpleasant sensations (visceral pain and discomfort) perceived to be related to the urinary bladder lasting longer than 6 weeks and in the absence of other clinically identifiable sources (25). While the etiology remains unknown, overlapping clinical signs and symptoms suggest an involvement of microbial infection (14), submucosal immune cell infiltration (10), disrupted urothelial cell permeability (34) or inflammation (44) (for review 22). We propose that micturition dysfunction and visceral neurogenic pain associated with IC/BPS are partly mediated by the urinary bladder inflammatory response (18, 21, 41). Several studies have already established the role of proinflammatory cytokines and their downstream targets in the organizational (19, 31) and functional (3, 29) alterations in micturition reflex pathways following chemically (cyclophosphamide; CYP)-induced cystitis. Most recently, the inflammatory mediator, TGF-β, has been implicated in the pathogenesis of CYP-induced cystitis (45, 55).

The TGF-β superfamily is comprised of at least 35 structurally related pleiotropic proteins (28). In addition to classical TGF-β, the superfamily can be subdivided into bone morphogenetic proteins (BMPs), activins/inhibins, growth and differentiation factors (GDFs), anti-müllerian hormone (AMH) and nodal (for review 30). In mammals, the prototypic TGF-β cytokine exists in three isoforms (TGF-β1, TGF-β2 and TGF-β3) and is synthesized as a 75 kDa homodimeric propeptide, in which the mature growth factor is non-covalently bound to a pro-domain (latency-associated protein, LAP) (1). During secretion, covalent interactions between LAP and a
glycoprotein, latent TGF-β Binding Protein (LTBP), translocate the latent complex to the extracellular matrix where it can be activated through protease-dependent (54) or protease-independent (thrombospondins, integrins, reactive oxygen species and protons (6, 35, 38, 47)) mechanisms (for review 1). The destabilization of TGF-β/LAP and LAP/LTBP releases an activated TGF-β dimer that forms a heterotetrameric receptor complex comprised of transforming growth factor-β type I receptor (TβR-1) and TβR-2. Ligand binding can also be enhanced through a third co-receptor (TβR-3) that lacks an intracellular signaling domain (52). To propagate an intracellular signal, intrinsic serine/threonine kinase activity of TβR-2 transphosphorylates TβR-1 to initiate receptor regulated SMAD-dependent or SMAD-independent transduction pathways (33).

The maintenance and modulation of TGF-β signaling is essential for epithelial cell cycle progression, proliferation and apoptosis (26), as well as immune cell function and inflammation (7). The present study examined the transcriptional and translational regulation of TGF-β (1, 2 and 3) and TβR (1, 2 and 3) in the rat urinary bladder inflammatory response and the role for TGF-β signaling in urinary bladder dysfunction following CYP-induced cystitis. Cytotoxicity in the urinary bladder accompanying CYP metabolism increases urinary bladder permeability (16), urothelial hyperplasia and voiding frequency (43), in addition to altering somatic sensitivity (23, 24), neurochemical (48, 50) and electrophysiological (53) properties of micturition reflex function. Therefore, CYP-induced cystitis permits a controlled, systematic identification of inflammatory mediators underlying urinary bladder dysfunction that would otherwise be inaccessible with a clinical population (18). With this in mind, we determined (i) the
temporal and tissue (urothelium and detrusor smooth muscle) expression of TGF-β (1, 2 and 3) and TβR (1, 2 and 3) urinary bladder transcripts following CYP-induced cystitis (acute, intermediate or chronic); (ii) the temporal expression of TGF-β (1, 2 and 3) urinary bladder protein following CYP-induced cystitis; (iii) the temporal and tissue (urothelium and detrusor smooth muscle) expression of TGF-β1 and TβR (1, 2 and 3) using immunohistochemistry following CYP-induced cystitis and (iv) the functional effects of pharmacological TβR-1 inhibition using conscious, open outlet cystometry following intermediate (48 h) CYP-induced cystitis.
Materials and Methods

Animals

Naïve adult female Wistar rats (200-375 grams) purchased from Charles River Laboratories (Wilmington, MA) were housed two per cage and maintained in standard laboratory conditions with food and water available ad libitum. Estrous cycles were not determined at any point in these studies. Experimental procedures were approved by the University of Vermont Institutional Animal Care and Use Committee (protocol 08-085) and experimentation was in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (8th edition).

Induction of CYP-induced cystitis

Female Wistar rats (n=4-8 per condition) received either no treatment, acute (150 mg/kg), intermediate (150 mg/kg) or chronic (75 mg/kg) intraperitoneal (i.p.) injections of cyclophosphamide (CYP; Sigma Aldrich, St. Louis, MO). Following CYP (150 mg/kg, i.p.) treatment, rats were harvested either 4 (acute) or 48 (intermediate) hours (h) post injection (5, 11, 31). For chronic CYP (75 mg/kg, i.p.) treatment, rats received injections every third day for ten days and tissues were harvested on the tenth day (5, 11, 31).

Antibodies

Polyclonal primary antibodies were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX): rabbit anti-TGF-β1 (sc-146, 1:1000), rabbit anti-TβR-1 (sc-398, 1:5000), rabbit anti-TβR-2 (sc-220, 1:2000), goat anti-TβR-3 (sc-6199, 1:1000). Secondary antibodies were purchased from Jackson Immuno Research Labs (West

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Grove, PA): Cy3 AffiniPure Goat Anti-Rabbit IgG (H+L) (111-165-144, 1:500) and Cy3 AffiniPure Donkey Anti-Goat IgG (H+L) (705-166-147, 1:500). Primary antibodies were diluted in 0.1 M phosphate buffered saline (PBS) containing either 1% goat or donkey serum.

Real-Time Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)

Female Wistar rats (n=5-7 per condition) were anaesthetized with 2% isoflurane and urinary bladders were harvested under RNase-free conditions following a thoracotomy. Components of the urinary bladder (urothelium and detrusor smooth muscle) were separated as previously described (9, 32). For transcript analyses, the term urothelium refers to both the urothelial cell layers (basal, intermediate and apical) and accompanying suburothelial structures. Total RNA was extracted from urothelium and detrusor smooth muscle using STAT-60 total RNA/mRNA isolation reagent (Tel-Test ‘B’, Friendswood, TX) and complementary DNA (cDNA) was synthesized using random hexamer and oligo dT primers with M-MLV reverse transcriptase (Promega Corp, Madison, WI) (20). cDNA templates were assayed using HotStart-IT SYBR Green qPCR Master Mix (USB Corporation, Cleveland, Ohio) containing 5 mM MgCl2, 0.4 mM dATP, dGTP, dCTP and dTTP, HotStart-IT Taq DNA polymerase and 300 nM of each primer (Table 1) in a final 25 µL reaction volume (3, 20, 32). qRT-PCR was performed on an Applied Biosystems 7500 Fast real-time PCR system (Foster City, CA) using previously defined conditions (3, 20, 32). Amplicons were subjected to a SYBR Green I melting curve analysis by ramping the reaction temperature from 60°C to 95°C. A single hyperchromic effect was observed under these dissociation conditions.
demonstrating amplification of a specific product free of primer-dimers or other contaminants.

*Analysis:* Data were analyzed at the termination of each assay using the Sequence Detection Software (version 1.3.1; Applied Biosystems, Norwalk, CT). A standard curve was constructed by amplification of serially diluted plasmids containing the target sequence. The increase in SYBR Green I fluorescence intensity (ΔRn) was plotted as a function of cycle number and the threshold cycle (CT) was the amplification cycle at which ΔRn intersects baseline (3, 20, 32). qRT-PCR data are expressed as relative quantity of the gene of interest normalized to the relative quantity of the ribosomal reference gene, L32 (19).

*Enzyme-Linked Immunosorbent Assays (ELISAs)*

Female Wistar rats (*n*=8 per condition) underwent the aforementioned CYP treatment and tissue harvest. Individual whole urinary bladders were weighed and solubilized for immunoassays in tissue protein extraction (Pierce Biotechnology, Woburn, MA) solution, a mild zwitterionic dialyzable detergent, supplemented with a complete protease inhibitor cocktail (Roche, Indianapolis, IN) (36). Tissue was homogenized using a Kinematica Polytron homogenizer (Fischer Scientific, Pittsburgh, PA) and centrifuged (3,000 rpm at 10°C for 10 min). Supernatant was removed and protein was quantified according to manufacturer instructions using a Coomassie Plus Protein Assay Kit (Pierce Biotechnology).

Ninety-six (96)-well microtiter plates (R&D Systems, Minneapolis, MN) were coated overnight with a capture antibody specific to the analyte (DuoSet Development
Systems, R&D Systems). Plates were washed (1X PBS with 0.05% Tween20), blocked (1X PBS with 5% Tween20) and then standards and samples were added in duplicate. Standards provided by the manufacturer consisted of a seven point standard curve ($r^2=$ 0.989-0.997) in Reagent Diluent (1% BSA in PBS) beginning at 2,000 pg/mL. Following a 2 h incubation, standards and samples were vigorously washed three times (3x) and a biotinylated detection antibody specific to the analyte was added and allowed to incubate for 2 h. Plates were washed (3x) and a detection reagent (streptavidin-horseradish peroxidase) was added and incubated for 20 min. Plates were washed (3x) and a substrate solution (hydrogen peroxide and tetramethylbenzidine) was added. Following 20 min incubation, reactions were terminated with 2N sulfuric acid and the optical density was read at 450 nm and 570 nm.

Analysis: Absorbance values were corrected for optical imperfections by subtracting readings at 570 nm (49). Samples were diluted to bring absorbance values onto the linear portion of the standard curve. Samples did not fall below the minimum detection limits of the assay. Curve fitting of standards and sample were performed using least-squares fit regression analysis (49).

Immunohistochemistry

Female Wistar rats (n=4 per condition) underwent the aforementioned CYP treatment and tissue harvest. Urinary bladder tissue was immediately allowed to incubate in 4% paraformaldehyde for 24 h at 4°C and was then transferred to 30% sucrose in 0.1 M PBS overnight at 4°C. Tissue was embedded in optimal cutting temperature compound (Tissue-Tek, Batavia, IL), sectioned at 20 μm and mounted on 0.5% gel-
coated slides (5, 9, 11). Sections were incubated overnight in polyclonal primary antibody (rabbit anti-TGF-β1, rabbit anti-TβR-1, rabbit anti-TβR-2 and goat anti-TβR-3), washed (3 x 10 min) and then incubated in Cyanine 3 (Cy3) AffiniPure conjugated secondary antibody for 2 h. Following washing (3 x 10 min), slides were mounted with an anti-fading media (Citifluor, Fischer Scientific) and coverslipped.

**Visualization and semi-quantitative analysis:** Immunoreactivity was captured using an Olympus fluorescent photomicroscope with a charge-coupled device (CCD) camera (MagnaFire SP, Optical Analysis, Nashua, NH) and LG-3 frame grabber (Scion, Frederick, MD). Each experimental series was processed on the same day with exposure time, brightness and contrast held constant throughout image acquisition (5, 9). To visualize Cy3, the filter was set with an excitation range of 560-569 nm and an emission range of 610-655 nm (5, 9). Images (6 per animal) were acquired, saved as 24-bit RGB tagged image file format and imported into MetaMorph Image Analysis Software (v4.5r4, Microscope Imaging Center, Downingtown, PA) (5, 9).

The focus of our analyses was on the urothelium and detrusor smooth muscle whereas other parts of the lower urinary tract including the suburothelial plexus and lumbosacral dorsal root ganglia (DRG) are the focus of a separate study. A free hand drawing tool was used to trace and measure total pixels area in the urothelium (basal, intermediate, apical) and excluded any suburothelial structures (11). For the detrusor smooth muscle, six rectangles of fixed dimensions (125 x 125 pixels) were placed without overlap according to random x and y coordinates (9, 32). As previously described (9, 32), a threshold encompassing an intensity range of 100-250 grayscale
values was applied to the region of interest in the least immunoreactive condition first and was maintained throughout the series. The threshold was adjusted for each experimental series with negative controls as a guide for setting background fluorescence (9, 32).

Assessment of immunoreactivity: Immunohistochemistry and evaluation of TGF-β1 and TβR (1, 2 and 3) immunoreactivity in urothelium or detrusor smooth muscle were performed on control and experimental tissues simultaneously to reduce the incidence of staining variation that can occur between tissues processed on different days (5, 9, 11). Staining in experimental tissue was compared with that in experiment-matched negative controls. Negative controls following the same experimental procedures were processed in the absence of a polyclonal primary antibody to assess specificity and background staining. In the absence of primary antibody, positive immunoreactivity was not observed (data not shown). Immunoreactivity was considered positive only when intensity measurements of the target exceeded the established threshold. Percent immunoreactivity above threshold as a function of total area selected was calculated and reported. For the detrusor smooth muscle, percent target expression above threshold was averaged across the six regions.

Cystometry

Intravesical Catheter Implant: A dorsal (below scruff of the neck) incision and lower midline abdominal incision was performed under 2% isoflurane using aseptic techniques. The end of a polyethylene (PE-50; Clay Adams, Parsippany, NJ) tube was flared, tunneled and inserted into the bladder dome. The proximal end was secured to the
dome with a 6-0 nylon purse-string suture whereas the distal end was sealed, coiled and stored in a dorsal subcutaneous pouch. Adult female Wistar rats (n=4-5 per condition) recovered for 72 h with post-operative analgesia (Buprenorphine, 0.05 mg/kg, s.c.) being maintained for 48 h. No animals met our exclusion criteria (3, 5).

Conscious, freely moving cystometry with an open outlet: The dorsal subcutaneous polyethylene tubing was externalized under 2% isoflurane and the animal was placed unrestrained into a Small Animal Cystometry System (Med Associates, St. Albans, VT) recording cage over a scale and pan used to quantify voided volume. The animals were each given a 10 min acclimation period prior to the continuous intravesical infusion of 0.9% sodium chloride (NaCl) injection, USP (Baxter, Deerfield, IL) at a rate of 10 mL/h. To avoid micturition variation resulting from circadian rhythms, functional tests were conducted at similar times of the day (15). Each animal was run for at least six reproducible micturition cycles (pre-drug baseline) after an initial stabilization period (30 min). Intravesical pressure (non-voiding, filling, threshold and peak micturition pressures), intercontraction interval and infused and voided volume were recorded for each micturition cycle. Bladder capacity was quantified as the total infused 0.9% NaCl at the time micturition commenced (3, 5). Non-voiding bladder contractions were defined as rhythmic intravesical pressure increases 0.69 kilopascals (kPa) above baseline without the release of fluid from the urethra and are not reported due to their infrequency in the current study (3, 5).

To determine the role of TGF-β on urinary bladder function in control (no inflammation) or CYP-treated rats, we intravesically instilled 1 mL of a TβR-1 small
molecule inhibitor, SB505124 (5 µM; R&D Systems), under 2% isoflurane for 30 min immediately following the baseline measurements. SB505124 (5 µM) was chosen due to its in vitro potency and selectivity for TGF-β activated activin receptor-like kinases (ALKs) (12). Intravesical instillation of 1 mL 0.01% dimethyl sulfoxide (DMSO) in 0.9% NaCl under 2% isoflurane was used for the vehicle control conditions. Rats remained anesthetized during instillation to suppress the micturition reflex and prevent expulsion of SB505124 (5 µM) or 0.01% DMSO (2, 3, 5). At the conclusion of drug instillation, urinary bladder function tests were repeated and ran for at least 6 reproducible micturition cycles (post-drug). The animals were euthanized following functional testing as described above.

Materials

SB505124 (R&D Systems) was reconstituted in DMSO (99.5%) and stored at -20°C. Prior to its use, stock solutions were diluted to a working concentration (5 µM) with 0.9% NaCl injection, USP (Baxter).

Statistical Analyses

All values represent mean ± SEM. Data were compared with one-way or repeated measures analysis of variance (ANOVA) where appropriate. When F(test statistic) exceeded the critical value (p ≤ 0.05), the Bonferroni or Newman-Keuls multiple comparisons test was used to compare group means.
Results

TGF-β (1, 2 and 3) and TβR (1, 2 and 3) transcript expression in urothelium and detrusor smooth muscle following CYP-induced cystitis.

The regulation of TGF-β (1, 2 and 3) and TβR (1, 2 and 3) transcripts in urothelium and detrusor smooth muscle was examined by qRT-PCR analyses (Figure 1, A-F).

TGF-β1

TGF-β1 transcript expression significantly increased relative to control in the urothelium (p ≤ 0.001) and detrusor smooth muscle (p ≤ 0.01) following intermediate (48 h) CYP-induced cystitis (Figure 1A). Significant differences were not observed in urothelium or detrusor smooth muscle following acute (4 h) or chronic CYP-induced cystitis (Figure 1A).

TGF-β2

Basal TGF-β2 transcript expression significantly (p ≤ 0.05) differed between urothelium and detrusor smooth muscle under control conditions (Figure 1B). TGF-β2 transcript expression in the urothelium significantly increased relative to control following intermediate (48 h; p ≤ 0.01) or chronic (p ≤ 0.01) CYP-induced cystitis; whereas, no significant differences were observed following acute (4 h) CYP-induced cystitis (Figure 1B). Acute (4 h), intermediate (48 h) or chronic CYP-induced cystitis did not significantly regulate TGF-β2 transcript expression in the detrusor smooth muscle (Figure 1B).

TGF-β3
TGF-β3 transcript expression significantly increased in the urothelium following intermediate (48 h; p ≤ 0.001) or chronic (p ≤ 0.05) CYP-induced cystitis relative to control; whereas, no significant differences were observed following acute (4 h) CYP-induced cystitis (Figure 1C). Acute (4 h), intermediate (48 h) or chronic CYP-induced cystitis did not significantly regulate TGF-β3 transcript expression in the detrusor smooth muscle (Figure 1C).

\textit{TβR-1}

TβR-1 transcript expression significantly increased relative to control in the urothelium following intermediate (48 h; p ≤ 0.01) or chronic (p ≤ 0.001) CYP-induced cystitis (Figure 1D). In the detrusor smooth muscle, significant increases in TβR-1 transcript expression were only observed following chronic (p ≤ 0.05) CYP-induced cystitis relative to control (Figure 1D). Significant differences in detrusor smooth muscle TβR-1 transcript expression were not observed following acute (4 h) or intermediate (48 h) CYP-induced cystitis (Figure 1D).

\textit{TβR-2}

Intermediate (48 h) CYP-induced cystitis significantly increased TβR-2 transcript expression in both the urothelium (p ≤ 0.05) and detrusor smooth muscle (p ≤ 0.05); whereas, significant transcript increase following chronic CYP-induced cystitis was only seen in the urothelium (p ≤ 0.001) (Figure 1E). Acute (4 h) CYP-induced cystitis did not significantly regulate urothelial or detrusor smooth muscle TβR-2 transcript expression (Figure 1E).

\textit{TβR-3}
TβR-3 transcript expression significantly increased relative to control in the urothelium following intermediate (48 h; p ≤ 0.001) and chronic (p ≤ 0.05) CYP-induced cystitis (Figure 1F). Significant transcript increases were similarly seen in the detrusor smooth muscle following intermediate (48 h; p ≤ 0.05) and chronic (p ≤ 0.05) CYP-induced cystitis (Figure 1F). Acute (4 h) CYP-induced cystitis did not significantly regulate TβR-3 transcript expression in the urothelium or detrusor smooth muscle (Figure 1F).

*TGF-β (1, 2 and 3) protein expression in whole urinary bladder following CYP-induced cystitis.*

The regulation of TGF-β (1, 2 and 3) urinary bladder protein was examined by ELISAs (Figure 2, A-C). The selection of analyte was limited by commercial availability. Intermediate (48 h) CYP-induced cystitis significantly increased urinary bladder TGF-β1 (p ≤ 0.001) and TGF-β3 (p ≤ 0.05) protein expression relative to control (Figure 2, A and C). Significant differences relative to control were not observed in TGF-β1 and TGF-β3 urinary bladder protein expression following acute (4 h) or chronic CYP-induced cystitis (Figure 2, A and C). TGF-β2 urinary bladder protein expression did not significantly differ relative to control across acute (4 h), intermediate (48 h) or chronic CYP treatments (Figure 2B).

*TGF-β1 and TβR (1, 2 and 3) immunoreactivity (IR) in urothelium and detrusor smooth muscle following CYP-induced cystitis.*

Based on the transcriptional and translational expression profiles observed, we focused immunostaining on TGF-β1 and TβR variants whose mRNA and protein
exhibited tissue and temporal regulation by CYP treatment (Figure 1A, 1D, 1E, 1F and Figure 2A). Regional differences in IR were not observed in the dome, body or neck of the rat urinary bladder.

**TGF-β1**

Low-intensity, basal TGF-β1-IR was present in all urothelial layers (basal, intermediate and apical) and detrusor smooth muscle of control rat urinary bladders (Figure 3, A and E). Acute (4 h) CYP-induced cystitis revealed no observable differences in urothelial or detrusor smooth muscle TGF-β1-IR relative to control (Figure 3, B and F). Intermediate (48 h) or chronic CYP-induced cystitis, however, resulted in a high-intensity TGF-β1-IR in both the urothelium and detrusor smooth muscle (Figure 3, C-D and G-H). Semi-quantitative analyses revealed a significant suprathreshold increase relative to control in the urothelium following intermediate (48 h; p ≤ 0.001) or chronic (p ≤ 0.001) CYP-induced cystitis (Figure 3I). Similarly, semi-quantitative analyses in the detrusor smooth muscle revealed a significant suprathreshold increase relative to control following intermediate (48 h; p ≤ 0.01) or chronic (p ≤ 0.01) CYP-induced cystitis (Figure 3J).

**TβR-1**

Low-intensity, basal TβR-1-IR was present in all urothelial layers (basal, intermediate and apical) and detrusor smooth muscle of control rat urinary bladders (Figure 4, A and E). Acute (4 h) CYP-induced cystitis resulted in moderate urothelial IR relative to control, whereas intermediate (48 h) or chronic CYP-induced cystitis resulted in a high-intensity urothelial TβR-1-IR (Figure 4, B-D). Semi-quantitative analyses
revealed a significant suprathreshold increase relative to control in the urothelium following intermediate (48 h; p ≤ 0.001) or chronic (p ≤ 0.01) CYP-induced cystitis (Figure 4I). There were no observable differences in detrusor smooth muscle TβR-1-IR following acute (4 h) CYP-induced cystitis, while intermediate (48 h) or chronic CYP-induced cystitis resulted in moderate IR relative to control (Figure 4, F-H). Semi-quantitative analyses revealed an emerging trend of robust suprathreshold IR following intermediate (48 h) or chronic CYP-induced cystitis (Figure 4J).

TβR-2

Low-intensity, basal TβR-2-IR was present in all urothelial layers (basal, intermediate and apical) and detrusor smooth muscle of control rat urinary bladders (Figure 5, A and E). Acute (4 h) CYP-induced cystitis resulted in moderate urothelial and detrusor smooth muscle IR relative to control, while intermediate (48 h) or chronic CYP-induced cystitis resulted in a high-intensity TβR-2-IR (Figure 5, B-D and F-H). Semi-quantitative analyses revealed a significant suprathreshold increase relative to control in the urothelium following intermediate (48 h; p ≤ 0.001) or chronic (p ≤ 0.01) CYP-induced cystitis (Figure 5I). Similarly, semi-quantitative analyses in the detrusor smooth muscle revealed a significant suprathreshold increase relative to control following intermediate (48 h; p ≤ 0.001) or chronic (p ≤ 0.01) CYP-induced cystitis (Figure 5J).

TβR-3

Low-intensity, basal TβR-3-IR was present in the detrusor smooth muscle of control rat urinary bladders (Figure 6A). Acute (4 h) CYP-induced cystitis resulted in moderate IR relative to control, while intermediate (48 h) or chronic CYP-induced
cystitis resulted in a high-intensity detrusor smooth muscle TßR-3-IR (Figure 6, B-D). Semi-quantitative analyses revealed a significant suprathreshold increase relative to control in the detrusor smooth muscle following intermediate (48 h; p ≤ 0.01) or chronic (p ≤ 0.01) CYP-induced cystitis (Figure 6E). TßR-3-IR was not present in the urothelium following any control or CYP treatment paradigm (data not shown).

**Urinary bladder function following TßR-1 inhibition in control (no inflammation) or intermediate (48 h) CYP-induced cystitis rats.**

Urinary bladder function was determined using open outlet, continuous cystometry in conscious, freely moving control or intermediate (48 h) CYP-induced cystitis rats (Table 2). Intermediate (48 h) CYP-induced cystitis was chosen for analysis due to a robust increase in urinary bladder TGF-β ligand and receptor transcript and protein expression.

**Control (no inflammation)**

Intravesical instillation of SB505124 (5 µM) significantly (p ≤ 0.05) decreased voiding frequency and increased bladder capacity (1.3-fold), void volume (1.4-fold) and intercontraction intervals (1.3-fold) relative to pre-drug baseline measurements (Table 2). Intravesical pressure (filling, threshold, peak) was not significantly affected following SB505124 (5 µM) instillation (Table 2). Residual volume before or after SB505124 (5 µM) instillation was minor (≤ 35 µL) and the effects of treatment persisted throughout the entirety of the experiment (1.5-2 h).

**Intermediate (48 h) CYP-induced cystitis**
As previously demonstrated (3, 5, 29), intermediate (48 h) CYP-induced cystitis increased voiding frequency and intravesical pressure (filling, threshold, peak) and decreased bladder capacity, void volume and intercontraction intervals relative to control (no inflammation) rats (Table 2). Intravesical instillation of SB505124 (5 µM) significantly (p ≤ 0.001) decreased voiding frequency and increased bladder capacity (2.5-fold), void volume (2.6-fold) and intercontraction intervals (2.5-fold) relative to pre-drug baseline measurements (Table 2, Figure 7). SB505124 (5 µM) did not significantly affect intravesical pressure (filling, threshold, peak) in CYP-treated (48 h) rats (Table 2, Figure 7). Residual volume before or after SB505124 (5 µM) instillation was minor (≤ 20 µL) and the effects of treatment persisted throughout the entirety of the experiment (1.5-2 h).
Discussion

The present study characterized the expression of urinary bladder TGF-β (1, 2 and 3) and TβR (1, 2 and 3) following CYP-induced cystitis of varying durations and established a role for TGF-β signaling in micturition reflex function. We confirmed the presence of a delayed TGF-β proinflammatory phenotype following acute urinary bladder inflammation and extended previous findings (45, 55) by demonstrating the functional expression and chronicity of urinary bladder TGF-β. These results provide evidence for a novel molecular component of the urinary bladder inflammatory response that, when inhibited, reduces voiding frequency with urinary bladder dysfunction.

The expression of TGF-β ligands in the urinary bladder proinflammatory cytokine milieu has been previously suggested to reflect the inflammatory state of the urinary bladder (55). Zhang and Qiao (2012) have observed a step-wise TGF-β1 transcript upregulation following acute (8 h) and intermediate (48 h) CYP-induced cystitis, while Tyagi et al. (2009) observed significant upregulation of TGF-β1 urinary bladder protein following acute (24 h) CYP-induced cystitis. Similarly, the present study demonstrates an upregulation of TGF-β ligand and receptor expression following intermediate (48 h) or chronic CYP-induced cystitis, but not acute (4 h) CYP-induced cystitis. The temporal regulation of TGF-β transcript and protein expression may aid in monitoring the urinary bladder inflammatory response and suggests an involvement in the resolution of urinary bladder inflammation to initiate tissue remodeling (45).

In the remedial stages of inflammation, the urinary bladder undergoes alterations characterized by a deposition of extracellular matrix constituents and proliferation of
activated fibroblasts (13). Although the precise contribution of TGF-β to pathological tissue remodeling is unknown, TGF-β is capable of promoting deposition of profibrotic extracellular matrix proteins and preventing matrix degradation by inhibiting expression of metalloproteinases and plasminogen-activators (8). While neither the present nor past studies address the role of TGF-β in urinary bladder remodeling, our results suggest the profibrotic profile may be partly mediated by a delayed, long lasting TGF-β upregulation.

Chronic urinary bladder inflammation is characterized by increased mononuclear cell infiltration, mucosal ulceration and the systemic release of proinflammatory cytokines and chemokines from distinct tissue compartments (40, 42). The present study not only demonstrated the temporal regulation of TGF-β but also the differential distribution of TGF-β ligands and its cognate receptors. The regulation of TGF-β1 and TGF-β3 transcript expression in the urothelium appeared to contribute most to the global translational profiles observed in our conditions. However, significant upregulation of TGF-β2 transcript in the urothelium was not sufficient to increase urinary bladder protein at any CYP-treatment duration. These differences may reflect the elevated basal TGF-β2 transcript expression in the detrusor smooth muscle relative to the urothelium. TβR (1, 2 and 3) transcript expression in the urothelium and detrusor smooth muscle was, in general, significantly increased following intermediate (48 h) or chronic CYP-induced cystitis. These profiles were maintained in TβR-1 and TβR-2 antigen immunoreactivity in the urothelium, as well as TβR (1, 2 and 3) immunoreactivity in the detrusor smooth muscle. The absence of urothelial TβR-3 immunoreactivity in control or CYP-treated animals may reflect transcript expression and regulation in the
suburothelium, transcript instability (27), decreased translation efficiency and/or increased receptor internalization (17).

While the clinical presentation of IC/BPS is diverse, there remain histopathologic and immunologic features that may contribute to urinary bladder dysfunction. Cystoscopic and bladder biopsy examinations indicate IC/BPS patients may present with a constellation of signs and symptoms including: an exaggerated inflammatory response, intrafascicular fibrosis and detrusor mastocytosis (37, 39, 46). Our findings combined with the established role of TGF-β in cellular proliferation, differentiation and migration, suggests TGF-β ligand overexpression and subsequent intracellular signaling may contribute, in part, to the pathophysiology of our experimental cystitis model of IC/BPS. To determine if aberrant TGF-β signaling contributes to urinary bladder dysfunction, we intravesically instilled a competitive ATP small molecule inhibitor, SB505124 (5 µM). Due to the structural similarities between TGF-β activated ALKs, it is possible for SB505124 to have off target effects on activin A receptor, type IB (ACVR1B, ALK4). While further studies are necessary to define the specific role(s) of ACVR1B and TβR-1 on urinary bladder dysfunction, SB505124 demonstrates 2.5-fold less potency in vitro on ACVR1B SMAD phosphorylation suggesting it may have a greater influence on TGF-β signaling in vivo (12, 51).

Instillation of SB505124 (5 µM) decreased voiding frequency in both control (no inflammation) and intermediate (48 h) CYP-induced cystitis rats, however, differences in fold change suggest TGF-β signaling has a more prominent role in micturition reflex function following urinary bladder inflammation. These results not
only remain consistent with the transcriptional and translational overexpression profiles observed following intermediate (48 h) CYP-induced cystitis but also demonstrate a novel, albeit small, role for TGF-β in normal micturition reflex function.

Cystometrogram recordings in control (no inflammation) rats following SB505124 (5 µM) instillation revealed a role for basal urothelial TGF-β signaling in micturition reflex function due to the barrier properties provided by intact junction complexes and specialized apical proteins (4). In addition, the absence of intravesical pressure changes suggests little to no effect on the urethral outlet and efferent limb of the micturition reflex. Similar parameters (void frequency, infused and voided volume, intercontraction intervals) were altered following intermediate (48 h) CYP-induced cystitis and SB505124 (5 µM) suggesting a role for aberrant TGF-β signaling in the afferent limb of the micturition reflex. Specific tissues or cell types in the urinary bladder that contribute to the functional effects are less clear due to increases in urinary bladder permeability after CYP metabolism (16) and global TβR-1 overexpression. Future studies can address suburothelial-specific overexpression of TβR-1 and/or determine the contributions of urothelial and detrusor smooth muscle TGF-β signaling to resolve our observed phenotypes.

The combination of systemic proinflammatory cytokine release, mononuclear cell infiltration and the profibrotic resolution of inflammation may contribute to peripheral and central sensitization that may facilitate viscerosomatic hypersensitivity and bladder hyperreflexia observed in IC/BPS (42). Given the biological contributions TGF-β may have on urinary bladder dysfunction (45, 55) and its recently established role
in peripheral sensitization (56), the present study characterized TGF-β transcript and protein expression at the level of the urinary bladder and provided evidence for a functional role of TGF-β signaling in the afferent limb of the micturition reflex. These studies demonstrate that targeting one component of the urinary bladder inflammatory response may be an effective strategy to reduce voiding frequency in an experimental cystitis model of IC/BPS.
Acknowledgements

The authors gratefully acknowledge the technical expertise and support provided by Susan Malley, Abbey Peterson and the Vermont Cancer Center DNA Analysis Facility.

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Disclosures

No conflict of interests are declared by the authors.
References


**Table 1: Primer sequences**

rTGF-β, Rat Transforming Growth Factor-Beta; rTβR, Rat Transforming Growth Factor-Receptor.

<table>
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<th>Sequence</th>
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<tr>
<td>rTGF-β1</td>
<td>5’-AGGTCCTCCTCCTAAAGTCAAT-3’</td>
</tr>
<tr>
<td>rTGF-β2</td>
<td>5’-TTTAGGAATGTGCAGGATAA-3’</td>
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Table 2: Cystometrogram recordings for control (no inflammation) or intermediate (48 h) CYP-induced cystitis rats with SB505124 (5 µM) or 0.01% DMSO instillation

Intravesical instillation of SB505124 (5 µM) significantly decreased voiding frequency and increased bladder capacity (total infused 0.9% NaCl at the time micturition commenced), void volume and intercontraction intervals relative to pre-SB505124 baseline measurements in both control (no inflammation) and intermediate (48 h) CYP-induced cystitis rats. n=4-5 per condition; values are mean ± SEM; *p ≤ 0.05, ***p ≤ 0.001 versus pre-SB505124 baseline.

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<th>Infused Volume (mL)</th>
<th>Void Volume (mL)</th>
<th>Intercontraction Interval (s)</th>
<th>Filling Pressure (kPa)</th>
<th>Threshold Pressure (kPa)</th>
<th>Peak Micturition Pressure (kPa)</th>
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<td>1.3 ± 0.1</td>
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<td>1.5 ± 0.2</td>
<td>4.4 ± 0.5</td>
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<td>1.3 ± 0.2</td>
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<td>1.7 ± 0.2</td>
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<td>1.8 ± 0.3*</td>
<td>641 ± 101*</td>
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<td>1.5 ± 0.2</td>
<td>4.3 ± 0.8</td>
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<td>0.2 ± 3 x 10^{-2}</td>
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<td>1.9 ± 0.3</td>
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<td>0.2 ± 3 x 10^{-2}</td>
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<td>2.1 ± 0.3</td>
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<tr>
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<td>0.2 ± 4 x 10^{-2}</td>
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<td>1.9 ± 0.3</td>
<td>1.9 ± 0.3</td>
<td>8.3 ± 1.6</td>
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<tr>
<td>Post-SB505124</td>
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<td>217 ± 35***</td>
<td>1.8 ± 0.4</td>
<td>2.0 ± 0.3</td>
<td>5.3 ± 0.6</td>
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Figure 1: Time- and tissue-dependent regulation of TGF-β (1, 2 and 3) and TβR (1, 2 and 3) transcript expression following cyclophosphamide (CYP) treatment

TGF-β (1, 2 and 3) and TβR (1, 2 and 3) transcript expression was not significantly regulated following acute (4 h) induced cystitis. Intermediate (48 h) CYP-induced cystitis significantly increased TGF-β1 (A; urothelium/detrusor smooth muscle), TGF-β2 (B; urothelium), TGF-β3 (C; urothelium), TβR-1 (D; urothelium), TβR-2 (E; urothelium/detrusor smooth muscle) and TβR-3 (F; urothelium/detrusor smooth muscle) transcript expression relative to control. Chronic CYP-induced cystitis significantly increased TGF-β2 (B; urothelium), TGF-β3 (C; urothelium), TβR-1 (D; urothelium/detrusor smooth muscle), TβR-2 (E; urothelium) and TβR-3 (F; urothelium/detrusor smooth muscle) transcript expression relative to control. n=5-7 per condition; values are mean ± SEM; *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001 versus control.
Figure 2: Time-dependent regulation of TGF-β (1, 2 and 3) protein expression in whole rat urinary bladder following CYP treatment

(A) TGF-β1 urinary bladder protein expression was significantly increased following intermediate (48 h) CYP-induced cystitis. (B) TGF-β2 urinary bladder protein expression did not significantly differ across any condition relative to control. (C) TGF-β3 urinary bladder protein expression was significantly increased following intermediate (48 h) CYP-induced cystitis. n=8 per condition; values are mean ± SEM; *p ≤ 0.05, ***p ≤ 0.001 versus control.
Figure 3: Time- and tissue-dependent regulation of TGF-β1 immunoreactivity (IR) following CYP treatment

(A-D) Representative images of TGF-β1-IR in urothelium following control or CYP treatment paradigms. (E-H) Representative images of TGF-β1-IR in detrusor smooth muscle following control or CYP treatment paradigms. (I) Semi-quantitative analyses in urothelium revealed a significant suprathreshold increase following intermediate (48 h) or chronic CYP-induced cystitis. (J) Semi-quantitative analyses in detrusor smooth muscle revealed a significant suprathreshold increase following intermediate (48 h) or chronic CYP-induced cystitis. L, lumen; U, urothelium; SU, suburothelium; sm, detrusor smooth muscle.

Figure 3: Time- and tissue-dependent regulation of TGF-β1 immunoreactivity (IR) following CYP treatment.
Figure 4: Time- and tissue-dependent regulation of TβR-1 immunoreactivity (IR) following CYP treatment

(A-D) Representative images of TβR-1-IR in urothelium following control or CYP treatment paradigms. (E-H) Representative images of TβR-1-IR in detrusor smooth muscle following control or CYP treatment paradigms. (I) Semi-quantitative analyses in urothelium revealed a significant suprathreshold increase following intermediate (48 h) or chronic CYP-induced cystitis. (J) Semi-quantitative analyses in detrusor smooth muscle revealed a robust suprathreshold trend following intermediate (48 h) or chronic CYP-induced cystitis. L, lumen; U, urothelium; SU, suburothelium; sm, detrusor smooth muscle. Calibration bar represents 25 µm. n=4 per condition; values are mean ± SEM; **p ≤ 0.01, ***p ≤ 0.001 versus control.
Figure 5: Time- and tissue-dependent regulation of TβR-2 immunoreactivity (IR) following CYP treatment

(A-D) Representative images of TβR-2-IR in urothelium following control or CYP treatment paradigms. (E-H) Representative images of TβR-2-IR in detrusor smooth muscle following control or CYP treatment paradigms. (I) Semi-quantitative analyses in urothelium revealed a significant suprathreshold increase following intermediate (48 h) or chronic CYP-induced cystitis. (J) Semi-quantitative analyses in detrusor smooth muscle revealed a significant suprathreshold increase following intermediate (48 h) or chronic CYP-induced cystitis. L, lumen; U, urothelium; SU, suburothelium; sm, detrusor smooth muscle. Calibration bar represents 25 µm. n=4 per condition; values are mean ± SEM; **p ≤ 0.01, ***p ≤ 0.001 versus control.
Figure 6: Time- and tissue-dependent regulation of TβR-3 immunoreactivity (IR) following CYP treatment

(A-D) Representative images of TβR-3-IR in detrusor smooth muscle following control or CYP treatment paradigms. (E) Semi-quantitative analyses in detrusor smooth muscle revealed a significant suprathreshold increase following intermediate (48 h) or chronic CYP-induced cystitis. L, lumen; U, urothelium; SU, suburothelium; sm, detrusor smooth muscle. Calibration bar represents 25 µm. n=4 per condition; values are mean ± SEM; **p ≤ 0.01 versus control.
Figure 7: Cystometrogram traces of TβR-1 inhibition following intermediate (48 h) CYP-induced cystitis

(A) Representative pre-SB505124 baseline traces. (B) Representative post-SB505124 (5 µM) traces. Intravesical instillation of SB505124 (5 µM; B) significantly (p ≤ 0.001) decreased voiding frequency and increased bladder capacity (total infused 0.9% NaCl at the time micturition commenced; 2.5-fold), void volume (2.6-fold) and intercontraction intervals (2.5-fold) relative to pre-SB505124 baseline measurements (A). Representative traces (A, B) were from the same rat.
CHAPTER 3: PURINERGIC SIGNALING UNDERLIES TRANSFORMING GROWTH FACTOR-BETA MEDIATED BLADDER AFFERENT NERVE HYPEREXCITABILITY

Eric J. Gonzalez, Thomas J. Heppner, Mark T. Nelson, and Margaret A. Vizzard

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Key Points

- The sensory components of the urinary bladder are responsible for the transduction of bladder filling and are often impaired with neurological injury or disease.

- Elevated extracellular ATP contributes, in part, to bladder afferent nerve hyperexcitability during neurogenic bladder inflammation or irritation.

- TGF-β1 stimulates ATP release from the urothelium through vesicular exocytosis mechanisms with minimal contribution from pannexin-1 channels to increase bladder afferent nerve discharge.

- Bladder afferent nerve hyperexcitability and urothelial ATP release with CYP-induced cystitis is decreased with TGF-β inhibition.

- These results establish a causal link between an inflammatory mediator, TGF-β, and intrinsic signaling mechanisms of the urothelium that may contribute to the altered sensory processing of bladder filling.
Abstract

The afferent limb of the micturition reflex is often compromised following bladder injury, disease and inflammatory conditions. We have previously demonstrated that transforming growth factor-beta (TGF-β) signaling contributes to increased voiding frequency and decreased bladder capacity with cystitis. Despite the functional presence of TGF-β in bladder inflammation, the precise mechanisms of TGF-β mediating bladder dysfunction are not yet known. Thus, the present studies investigated the sensory components of the urinary bladder that may underlie the pathophysiology of aberrant TGF-β activation. We utilized bladder-pelvic nerve preparations to characterize bladder afferent nerve discharge and the mechanisms of urothelial ATP release with distention. Our findings indicate that bladder afferent nerve discharge is sensitive to elevated extracellular ATP during pathological conditions of neurogenic bladder inflammation or irritation. We determined that TGF-β1 increased bladder afferent nerve excitability by stimulating ATP release from the urothelium via vesicular exocytosis mechanisms with minimal contribution from pannexin-1 channels. Furthermore, blocking aberrant TGF-β signaling in cyclophosphamide-induced cystitis with TβR-1 inhibition decreased afferent nerve hyperexcitability with a concomitant decrease in urothelial ATP release. Taken together, these results establish a role for purinergic signaling mechanisms in TGF-β mediated bladder afferent nerve activation that may ultimately facilitate increased voiding frequency. The synergy between intrinsic urinary bladder signaling mechanisms and an inflammatory mediator provides novel insight into bladder dysfunction and supports new avenues for therapeutic intervention.
Abbreviations. BFA, Brefeldin A; CYP, cyclophosphamide; Det, detrusor smooth muscle; ICC, interstitial cells of Cajal; P2X/P2Y, purinergic receptor; Panx1, pannexin 1; PPADS, pyridoxal phosphate-6-azophenyl-2',4'-disulfonic acid; PSS, physiological saline solution; TGF-β, transforming growth factor-beta; TβR, transforming growth factor-β receptor.

Keywords: urinary bladder, TGF-β, ATP, inflammation
Introduction

The transitional epithelial cells that line the bladder lumen are now recognized to act as a passive barrier to urinary metabolites (Min et al., 2003) and contribute to the sensory transduction of bladder filling (Birder, 2005). These cells, termed the urothelium, respond to mechanical (Olsen et al., 2011) or chemical (Everaerts et al., 2010) stimuli by releasing urinary proteins (Deng et al., 2001) or neuroactive signaling molecules (Ferguson et al., 1997; Birder et al., 2002; Hanna-Mitchell et al., 2007; Nile & Gillespie, 2012) from their apical and basolateral surfaces (Lewis & Lewis, 2006). The release of these mediators presumably permits the urothelium to communicate with nerve endings (Morrison, 1999; Andersson, 2002) and other cell types such as interstitial cells of Cajal (ICC) (Sui et al., 2008; Drumm et al., 2014) or smooth muscle cells (Hashitani et al., 2004) that are proximal to the mucosa. Given the role that the urothelium has in bladder sensation, any disruption to these signaling mechanisms, such as what may occur with inflammation (de Groat & Yoshimura, 2009), may contribute to the development of lower urinary tract symptoms (Gonzalez et al., 2014b).

Purinergic signaling has garnered much of the focus in bladder sensory processing ever since the urothelium was discovered to release ATP (Ferguson et al., 1997). The mechanisms of ATP release from the urothelium have since been demonstrated to include vesicular exocytosis (Wang et al., 2005; Sui et al., 2014; McLatchie & Fry, 2015), ion channels (Wang et al., 2005; Sui et al., 2014; Beckel et al., 2015; McLatchie & Fry, 2015) and transporters (Wang et al., 2005). Extracellular ATP and other associated nucleotides may then bind to purinergic receptors located on various
cell types (e.g. urothelial (Tempest et al., 2004; Chopra et al., 2008), smooth muscle (Heppner et al., 2005) and ICC (Wu et al., 2004)) or nerve terminals (Namasivayam et al., 1999; Andersson, 2002) to activate autocrine or paracrine pathways and facilitate bladder sensory signaling (Birder & Andersson, 2013). Purinergic signaling in these cell types has been demonstrated to increase sensory neuron excitability (Chen et al., 1995) and to modulate smooth muscle tone (Sui et al., 2014). Furthermore, aberrant purinergic signaling in the urothelium has been suggested to contribute to bladder dysfunction in injury or disease since ATP release is increased in many functional disorders of the urinary bladder (Sun et al., 2001; Ruggieri, 2006; Sun & Chai, 2006; Silva-Ramos et al., 2013).

In addition to alterations in the intrinsic properties of the urothelium, functional disorders of the bladder that activate an inflammatory response may also involve an upregulation of proinflammatory mediators (Gonzalez et al., 2014a). Previous studies from our laboratory have established the roles of cytokines (Malley & Vizzard, 2002) and chemokines (Arms et al., 2010; Arms et al., 2013) in the maintenance and development of lower urinary tract symptoms. In particular, we have demonstrated that transforming growth factor-beta (TGF-β) overexpression contributes to the afferent limb of the micturition reflex with cyclophosphamide (CYP)-induced cystitis (Gonzalez et al., 2013). In addition to its role(s) in cystitis, increased urinary TGF-β has also been observed in diabetic nephropathy (Chen et al., 2003) and other nephropathic conditions (Goumenos et al., 2002; De Muro et al., 2004) with bladder dysfunction (Golbidi & Laher, 2010). Despite its functional presence in the bladder inflammatory milieu, the precise
mechanisms of TGF-β mediating voiding dysfunction are not yet known. Therefore, we hypothesized that TGF-β signaling contributes to afferent nerve excitability through purinergic mechanisms that may ultimately facilitate increased voiding frequency. A comprehensive understanding of the downstream signaling effectors that interact in the afferent limb of the micturition reflex with bladder dysfunction will allow for novel therapeutic approaches to improve the quality of life of these patients.
Materials and Methods

Ethical Approval

Experimental procedures were approved by the University of Vermont Institutional Animal Care and Use Committee (protocol 08-055) and experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (8th edn).

Animals

Male C57Bl/6 mice (3-6 months old) purchased from Jackson Laboratories (Bar Harbor, ME) were housed with littermates and maintained in standard laboratory conditions with food and water available ad libitum.

Induction of CYP-induced cystitis

Male C57Bl/6J mice (n=4) received a 150 mg/kg I.P. injection of CYP (Sigma-Aldrich, St. Louis, MO). Mice were harvested 48 h following CYP treatment for bladder-pelvic nerve electrophysiology or ATP release experiments.

Bladder-pelvic nerve electrophysiology

The bladder-pelvic nerve model was developed and described in detail by Namasivayam et al. (1998). Briefly, male C57Bl/6J mice (n=3-4) were euthanized by an injection of pentobarbital sodium (I.P.) followed by decapitation. The abdominal cavity was opened by a midline incision and the urinary bladder and surrounding tissues (urethra, ureters, major pelvic ganglia, postganglionic nerves and pelvic nerves) were excised and transferred to ice-cold dissection solution. The ureters were isolated, dissected and ligated with nylon suture adjacent to the bladder wall. The pelvic nerves
were isolated and the ends were cleaned of connective tissue to facilitate recording. The urinary bladder, urethra, ureters and pelvic nerves were then transferred to a recording chamber on a vibraplane platform and superfused with biological atmosphere gas (20% O2, 5% CO2, 75% N2) physiological saline solution (PSS) circulating at 37 °C. One arm of a triple lumen cannula was inserted through the urethra into the bladder and ligated. The remaining arms were attached to a remote-controlled syringe pump and pressure transducer to monitor intravesical pressure or used to empty the bladder. One of the pelvic nerves was attached to a suction electrode to record distention-evoked multifiber afferent nerve activity. With this recording arrangement, we did not identify the relative contribution(s) of myelinated Aδ fibers and the normally quiescent unmyelinated C fibers. Vehicle or drugs were administered at a rate of 30 µl/min intravesically up to 25 mmHg for the filling phase and then manually emptied. There was a 10 min rest period between the emptying phase and the start of the next filling phase. For the co-administration studies, bladders were first pretreated with the various inhibitors followed by the inhibitors plus TGF-β1. Administration of drugs began once bladder afferent nerve activity to the vehicle plateaued for two consecutive filling phases (usually after 5-6 filling cycles). Bladder afferent nerve activity was collected and amplified with a Neurolog head stage (NL104, Digitimer), filtered (band pass 200-4000 Hz) using a Digitimer NL125/NL126 filter and digitized with a Power 1401 analog to digital interface (Cambridge Electronic Design, Cambridge, UK). The acquisition rates for nerve activity and bladder pressure were 25,000 Hz and 100 Hz, respectively. Data were analyzed
offline via Spike 2 software (version 5.11, Cambridge Electronic Design, Cambridge, UK).

**Analysis**: Action potentials were determined by using twice the root mean square amplitude of the recording signal for detection threshold. Action potential events were quantified in 5 mmHg increments to determine activity throughout multiple pressures in the filling phase. The mean frequency (imp/sec) graphed for each condition (vehicle or drug) per animal was the average of two consecutive filling phases. A two-way repeated measures ANOVA followed by Sidak’s multiple comparisons test was then performed to compare these frequency group means. Fold change group means were compared with Student’s unpaired t test.

**ATP release**

Male C57Bl/6J mice (n=4-8) were anesthetized with 2% isoflurane and euthanized by decapitation. The abdominal cavity was opened by a midline incision and the urinary bladder, urethra and ureters were excised and transferred to ice-cold dissection solution. The ureters were isolated, dissected and ligated with nylon suture adjacent to the bladder wall. The urinary bladder, urethra and ureters were then transferred to a recording chamber with oxygenated (95% O2-5% CO2) PSS circulating at 37 °C and cannulated through the urethra with a 22 gauge blunt needle. The cannula was attached to a remote-controlled syringe pump and a pressure transducer to monitor intravesical pressure. Vehicle or drugs were administered at a rate of 30 µl/min intravesically up to 25 mmHg for the filling phase and then manually emptied via a three-way stop cock. There was a 10 min rest between the emptying phase and the start of the
next filling phase. After 1-1.5 h of vehicle or drug administration, the instillate was collected from two separate but consecutive emptying cycles and immediately flash frozen until ATP analysis. Upon collection, CYP instillate samples also received adenylyl-imidodiphosphate (AMP-PNP, 200 µM) to limit ATP hydrolysis that may be present from a compromised barrier. ATP quantification was determined with the ATP bioluminescent assay kit (Sigma-Aldrich, St. Louis, MO) following their procedural instructions with the exception of halving the recommended concentration of reagents and samples. ATP measurements were taken with the BioTek H4 Plate Reader (Winooski, VT) within the UVM Advanced Genome Technologies Core.

**Analysis:** The bioluminescence emitted by the sample was plotted against the calibration curve to determine the final concentration (pmol/ml) of ATP. Calibration curves were not affected by the drugs used in the current study (data not shown). Statistical analysis was performed on the ATP concentration (pmol/ml) of the instillate before or after drug administration as Student’s paired t test for all group means.

**Materials**

The dissection solution consisted of (in mM) 55 NaCl, 6 KCl, 80 MSG, 10 HEPES, 2 MgCl2, 10 glucose, and adjusted to pH 7.3. The PSS consisted of (in mM) 119 NaCl, 4.7 KCl, 24 NaHCO3, 1.2 KH2PO4, 1.2 MgCl2, 2 CaCl2, 7 glucose, and adjusted to pH 7.4. Recombinant mouse transforming growth factor-beta 1 (TGF-β1, R&D Systems) was reconstituted to 50 µg/ml in 4 mM HCl and 0.1% BSA and stored at -20 °C. Bladder tissues respond to a range of TGF-β1 concentrations (Hiti et al., 1990; Duan et al., 2015) and 10 ng/ml was selected for as the working concentration as it does not
induce EMT related genes in the urothelium (Islam et al., 2014). SB505124 (R&D Systems) was reconstituted to 100 mM in DMSO (99.5%) and stored at -20 °C. SB505124 is a potent inhibitor of TGF-β type I receptor kinase activity with an IC50 of 47 ± 5 nM (DaCosta Byfield et al., 2004). Before its use, stock solutions were diluted to a working concentration (5 μM) with PSS. Concentrations up to 5 μM maintain inhibition specificity of downstream targets of TβR-1 and other closely related receptors and have been demonstrated to decrease voiding frequency with cystitis (DaCosta Byfield et al., 2004; Gonzalez et al., 2013). Brefeldin A (BFA, R&D Systems) was reconstituted to 50 mM in DMSO (99.5%) and stored at -20 °C. Stock solutions were diluted to a working concentration (10 μM) with PSS to remain consistent with previous literature demonstrating decreased ATP release from the urothelium (Sui et al., 2014; McLatchie & Fry, 2015). 10Panx (R&D Systems) was reconstituted to 1 mg/ml in PSS and stored at -20 °C. The IC50 of 10Panx for the inhibition of panx1 currents in overexpressed panx1 HEK cells is 52 ± 12 μM (Pelegrin & Surprenant, 2006). We utilized 10Panx at a working concentration of 50 μM because intravesical 10Panx near the IC50 concentration was functional in the urinary bladder to decrease voiding frequency (Timoteo et al., 2014). Pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid tetrasodium salt (PPADS, R&D Systems) was reconstituted to 100 mM in distilled water and stored protected from light at -20 °C to prevent photodecomposition. A working concentration of 300 μM was selected for PPADS because Vlaskovska et al. (2001) reported decreased distention-evoked afferent nerve discharge in control tissues that was not observed at lower concentrations (Yu & de Groat, 2008).
Statistics

All values represent means ± SEM. Outliers were identified using the extreme studentized deviate test on GraphPad Prism (v. 6.07, La Jolla, CA) and excluded from further analysis. Data were then compared with two-way repeated measures ANOVA and two-tailed Student’s unpaired or paired t test where appropriate. When F-test statistic exceeded the critical value at $\alpha = 0.05$, the Sidak’s multiple comparisons test was used to compare group means.
Results

*TGF-β1 increased bladder afferent nerve discharge and ATP release from the urothelium.*

The continuous intravesical instillation of recombinant TGF-β1 (10 ng/ml) had a significant (p = 0.0447) treatment effect on mean bladder afferent nerve frequency (imp/sec) (Figure 1A, Figure 1B). Nerve firing increased (50.5 ± 9.9 to 71.6 ± 13.3 imp/sec, n=4) by 41.8% from 0-25 mmHg with TGF-β1 instillation. More specifically, nerve discharge significantly increased from 5-10 (p = 0.0337), 10-15 (p = 0.0003), 15-20 (p ≤ 0.0001) and 20-25 (p ≤ 0.0001) mmHg (Figure 1B). Distention-evoked ATP release from the urothelium at 25 mmHg was also significantly (p = 0.0066) increased with the intravesical instillation of TGF-β1 (Figure 1C).

The concurrent intravesical instillation of TGF-β1 and SB505124 (5 µM), a TGF-β type I receptor inhibitor, attenuated (p = 0.4396) afferent nerve hyperexcitability (76.8 ± 17.7 to 61.8 ± 12.9 imp/sec, n=4) from 0-25 mmHg (Figure 2C, Figure 2D). SB505124 co-administration also attenuated (p = 0.3245) TGF-β1 mediated distention-evoked urothelial ATP release at 25 mmHg (Figure 2E). The intravesical administration of SB505124 (5 µM) alone did not have a significant (p = 0.6506) treatment effect on mean nerve firing (76.8 ± 17.7 to 63.9 ± 13.6 imp/sec, n=4) from 0-25 mmHg (Figure 2A, Figure 2B). Furthermore, SB505124 did not have a significant (p = 0.0782) effect on distention-evoked urothelial ATP release at 25 mmHg (4.8 ± 1.7 to 7.2 ± 2.5 pmol/ml, n=4).
**TGF-β1 stimulated ATP release from the urothelium through vesicular exocytosis mechanisms to mediate bladder afferent nerve hyperexcitability.**

**Vesicular ATP release.** The continuous intravesical instillation of BFA (10 µM) did not have a significant (p = 0.8960) treatment effect on mean bladder afferent nerve firing (68.7 ± 12.6 to 58.7 ± 15.1 imp/sec, n=3) from 0-25 mmHg (Figure 3A, Figure 3B). Similarly, the intravesical co-administration of recombinant TGF-β1 (10 ng/ml) and BFA (10 µM) did not have a significant (p = 0.5646) treatment effect on mean nerve firing (68.7 ± 12.6 to 63.4 ± 21.4 imp/sec, n=3) from 0-25 mmHg (Figure 3C, Figure 3D). The intravesical co-administration of BFA also attenuated (p = 0.2155) TGF-β1 mediated distention-evoked urothelial ATP release at 25 mmHg (Figure 3E).

**Pannexin-1 channel ATP release.** The continuous intravesical instillation of 10Panx (50 µM) did not have a significant (p = 0.1340) treatment effect on mean bladder afferent nerve firing (41.9 ± 1.8 to 42.8 ± 3.9 imp/sec, n=4) from 0-25 mmHg (Figure 4A, Figure 4B). The intravesical co-administration of recombinant TGF-β1 (10 ng/ml) and 10Panx (50 µM), however, had a significant (p = 0.0213) treatment effect on mean nerve frequency with nerve firing increased (41.9 ± 1.8 to 50.1 ± 3.4 imp/sec, n=4) by 19.4% from 0-25 mmHg (Figure 4C, Figure 4D). More specifically, nerve discharge significantly increased from 10-15 (p = 0.0014), 15-20 (p ≤ 0.0001) and 20-25 (p = 0.0008) mmHg (Figure 4D). Relative to TGF-β1 alone (1.67 fold, Figure 1B), TGF-β1 and 10Panx co-administration (1.18 fold, Figure 4D) significantly (p = 0.0397) decreased the magnitude of change only from 20-25 mmHg. Distention-evoked urothelial ATP
release at 25 mmHg was also significantly (p = 0.0465) increased with the intravesical co-administration of TGF-β1 and 10Panx (Figure 4E).

Purinoceptor signaling. The continuous intravesical instillation of PPADS (300 µM) did not have a significant (p = 0.2339) treatment effect on mean bladder afferent nerve firing (75.9 ± 25.1 to 64.0 ± 16.9 imp/sec, n=4) from 0-25 mmHg (Figure 5A, Figure 5B). The intravesical co-administration of recombinant TGF-β1 (10 ng/ml) and PPADS (300 µM) attenuated (p = 0.2941) afferent nerve hyperexcitability (75.9 ± 25.1 to 59.2 ± 11.9 imp/sec, n=4) from 0-25 mmHg (Figure 5C, Figure 5D). The intravesical co-administration of TGF-β1 and PPADS, however, significantly (p = 0.0403) increased distention-evoked urothelial ATP release at 25 mmHg (Figure 5E). Bladder afferent nerve hyperexcitability and urothelial ATP release with CYP-induced cystitis is decreased with TGF-β inhibition.

CYP-induced cystitis had a significant (p ≤ 0.0001) treatment effect on mean bladder afferent nerve frequency (imp/sec) and nerve firing increased (62.2 ± 7.2 to 179.8 ± 20.9 imp/sec, n=4-19) by 188.9% from 0-25 mmHg relative to control (Figure 6A, Figure 6B). Nerve discharge significantly increased from 5-10 (p ≤ 0.0001), 10-15 (p ≤ 0.0001), 15-20 (p ≤ 0.0001) and 20-25 (p ≤ 0.0001) mmHg (Figure 6B). The continuous intravesical instillation of SB505124 (5 µM) significantly (p = 0.0028) decreased mean nerve frequency with CYP-induced cystitis (Figure 6C, Figure 6D). Mean nerve firing decreased (179.8 ± 20.9 to 103.6 ± 6.0 imp/sec, n=4) by 40.6% from 0-25 mmHg with a significant decrease in discharge from 5-10 (p = 0.0041), 10-15 (p = 0.006) and 15-20 (p = 0.0197) mmHg following SB505124 instillation (Figure 6D). TβR-1 inhibition with
SB505124 also significantly (p = 0.0190) decreased distention-evoked urothelial ATP release at 25 mmHg following CYP-induced cystitis (Figure 6E). The vehicle wash out of SB505124 increased nerve firing (103.6 ± 6.0 to 172.8 ± 21.9 imp/sec, n=4) and urothelial ATP release (6.3 ± 2.9 to 17 ± 3.7 pmol/ml, n=4) back to baseline.
Discussion

The present study revealed that purinergic signaling mechanisms underlie TGF-β mediated bladder afferent nerve excitability in both control and CYP-treated tissues. We determined that TGF-β1 contributes to bladder afferent nerve hyperexcitability through increased ATP release from the urothelium by vesicular exocytosis mechanisms. We also demonstrated that blocking aberrant TGF-β signaling in cystitis with TβR-1 inhibition decreased bladder afferent nerve discharge and urothelial ATP release. These studies delineate the sensory components of the urinary bladder that may underlie the interface between the upregulation of inflammatory mediators and bladder dysfunction.

ATP release. The urothelium participates in the sensory processing of bladder filling by releasing neuroactive factors, such as ATP (Wang et al., 2005; Birder & Andersson, 2013). The altered release of ATP in many functional disorders of the urinary bladder (Sun et al., 2001; Sun & Chai, 2006; Silva-Ramos et al., 2013) suggest purinergic signaling may contribute to the pathophysiology of bladder dysfunction (Ruggieri, 2006). We determined that a previously identified inflammatory mediator of cystitis, TGF-β1 (Gonzalez et al., 2013), was able to stimulate an increase in distention-evoked release of ATP from the urothelium (Figure 1D). The release of ATP was specific to ligand/receptor activation because the co-administration of TGF-β1 with a TβR-1 inhibitor, SB505124 (DaCosta Byfield et al., 2004), attenuated an elevation in extracellular ATP (Figure 2F). Not surprisingly, TGF-β1 continued to stimulate distention-evoked ATP release from the urothelium when P2 receptors were inhibited downstream of the release pathways with the co-administration of PPADS (Figure 5F). Aberrant TGF-β activation that was
previously observed with CYP-induced cystitis (Gonzalez et al., 2013) also contributed to altered ATP release. In our studies with cystitis, the inhibition of aberrant TGF-β signaling significantly reduced urothelial ATP release that recovered immediately with vehicle washout (Figure 6F). These results suggest that TGF-β signaling may directly contribute to the altered sensory processing of bladder filling by increasing ATP release from the urothelium.

Urothelial cells release ATP through various mechanisms that include vesicular exocytosis (Wang et al., 2005; Sui et al., 2014; McLatchie & Fry, 2015), connexin/pannexin channels (Wang et al., 2005; Sui et al., 2014; Beckel et al., 2015; McLatchie & Fry, 2015) and nucleoside transporters (Wang et al., 2005). Although each mechanism may play a part in release, our studies focused on the pharmacological manipulation of vesicular exocytosis and pannexin-1 channels due to the disputed role of transporters in uroepithelial cells (Knight et al., 2002) and because TGF-β1 downregulates connexin hemichannels in the bladder (Neuhaus et al., 2009) suggesting an alternative mechanism for increased ATP release. We intravesically instilled the general secretory inhibitor, BFA, for vesicular release inhibition. BFA has previously been shown to decrease mechanical- and stretch-evoked ATP release from the urothelium (Wang et al., 2005; Sui et al., 2014; McLatchie & Fry, 2015). We also intravesically instilled the inhibitory peptide, 10Panx, to block pannexin-1 channels. These channels have recently been implicated in distention-evoked ATP release from the urothelium with pharmacological or genetic manipulation (Negoro et al., 2014; Timoteo et al., 2014; Beckel et al., 2015).
Our studies with BFA or 10Panx co-administration with TGF-β1 demonstrated that the majority of urothelial ATP release stimulated by TGF-β1 occurred through vesicular exocytosis (Figure 3F, Figure 4F). While these results suggest a vesicular secretory mechanism, the effects of BFA may also generalize to inhibit the transport of cell surface proteins, like hemichannels, to attenuate release (Wang et al., 2005). Further studies are needed to separate the effects of vesicle release and hemichannel inhibition, however, given that TGF-β1 downregulates connexin hemichannels and pannexin-1 channels had a minimal role in TGF-β1 stimulated ATP release, it is likely that the effects we observed are due to vesicular secretory release. Future studies in our lab will aim to resolve the intracellular signaling cascade that may promote ATP release through these mechanisms.

**Bladder afferent nerve excitability.** Purinergic signaling is recognized to directly influence the afferent limb of the micturition reflex and bladder function. The intravesical instillation of ATP enhanced spinal bladder neuron excitability (Munoz et al., 2011) and increased bladder activity (Pandita & Andersson, 2002). Non-voiding bladder contractions and voiding frequency, on the other hand, were decreased when ATP release was attenuated with the inhibition of vesicular (Smith et al., 2005) or pannexin-1 channel (Timoteo et al., 2014; Beckel et al., 2015) release mechanisms, respectively. Our studies determined that the decrease in distention-evoked ATP release from the urothelium through vesicular exocytosis or pannexin-1 channels did not significantly contribute to mechanosensitive afferent nerve discharge. This suggests that the decrease in bladder
function previously observed occurred through an as yet unknown mechanism that is independent of afferent nerve excitability.

Extracellular ATP may contribute to bladder sensory processing through the activation of purinergic receptors on various cell types within the bladder wall including afferent nerve terminals. The activation of P2 receptors with intravesical alpha,beta-methylene ATP or ATP increased bladder afferent nerve discharge (Yu & de Groat, 2008) that was mediated mainly through capsaicin-insensitive C fibers (Aizawa et al., 2011). Following intravesical P2 receptor inhibition, bladder activity decreased (Beckel et al., 2015) and afferent nerve discharge decreased (Namasivayam et al., 1999; Vlaskovska et al., 2001) or remained the same (Yu & de Groat, 2008). Our current study determined that intravesical PPADS decreased nerve frequency by 15.7% but did not have a significant effect on afferent nerve discharge. Taken together, these conflicting studies indicate that purinergic receptors may only be activated on C fibers during pathological conditions of elevated extracellular ATP to contribute to the sensation of bladder filling.

The aforementioned contributions of purinergic signaling to bladder sensory transduction and function suggests that an insult, like inflammation, that alters ATP release may also affect nerve excitability. Given the functional overexpression of TGF-β in CYP-induced cystitis (Tyagi et al., 2009; Zhang & Qiao, 2012; Gonzalez et al., 2013) and its ability to stimulate ATP release in our current studies, we sought to determine if TGF-β1 also contributes to bladder afferent nerve hyperexcitability. The intravesical instillation of TGF-β1 increased nerve excitability by 41.8% and its significant effects on
nerve discharge were observed nearly throughout the entirety of the filling phase (Figure 1D). Nerve hyperexcitability was dependent on receptor activation and subsequent release of ATP because the intravesical co-administration of TGF-β1 and a TβR-1 inhibitor, SB505124, attenuated ATP release and bladder afferent nerve discharge (Figure 2F).

In addition to the necessity of TGF-β receptor activation, we also demonstrated that TGF-β1 stimulates ATP release through vesicular exocytosis to increase afferent nerve firing. Decreasing the release of ATP from the urothelium with BFA co-administration attenuated TGF-β1 mediated afferent nerve hyperexcitability (Figure 3F). The co-administration of TGF-β1 and 10Panx, however, significantly increased distention-evoked ATP release and afferent nerve discharge suggesting minimal contribution from pannexin-1 channels in this response (Figure 4F). The elevated extracellular ATP stimulated by TGF-β1 is likely activating nerve terminals within the bladder wall to increase excitability. We demonstrated that the intravesical co-administration of PPADS attenuated TGF-β1 mediated bladder afferent nerve hyperexcitability suggesting a role for these purinergic receptors in suburothelial afferent nerve firing (Figure 5F). Intravesical PPADS may also be working at the level of the urothelium to inhibit further basolateral release of ATP or other mediators that enhance excitability (Figure 5F) (Sun & Chai, 2006). Future studies are still needed to determine the basolateral release of ATP in this model because the concentrations of ATP that we quantified in the lumen likely underrepresents basolateral release onto sensory fibers due to volume differences in the interstitium, tissue-associated
ectonucleotidases/exonucleotidases (Wang et al., 2005) and cell-type specific ATP release (Cheng et al., 2011; McLatchie & Fry, 2015). Currently, the direct activation of purinergic receptors within the wall of an intact urinary bladder remains difficult to investigate without disrupting normal bladder physiology.

TGF-β is increased in the urine (Tyagi et al., 2009), urothelium (Tyagi et al., 2009; Gonzalez et al., 2013) and detrusor smooth muscle (Zhang & Qiao, 2012; Gonzalez et al., 2013) following CYP-induced cystitis. CYP-induced cystitis is characterized by voiding dysfunction and TGF-β is considered to contribute, in part, to the development of these lower urinary tract symptoms because TβR-1 inhibition decreased voiding frequency and increased bladder capacity (Gonzalez et al., 2013). We confirmed that bladder afferent nerve hyperexcitability is increased in CYP-induced cystitis (Yu & de Groat, 2008) and demonstrated a functional role for aberrant TGF-β signaling in this response (Figure 6F). Our current studies determined that TβR-1 inhibition may improve voiding dysfunction by decreasing ATP release from the urothelium and decreasing nerve firing by 40.6%. These effects were specific to aberrant TGF-β activation because vehicle washout increased ATP release and recovered nerve hyperexcitability to baseline. The decreased release of ATP with TβR-1 inhibition may normalize purinergic signaling and work comparably to intravesical P2X inhibition that has been previously reported to decrease afferent nerve excitability in CYP-induced cystitis (Yu & de Groat, 2008). It should also be noted that while TβR-1 inhibition significantly decreased distention-evoked ATP release and afferent nerve excitability, nerve frequency did not reach control
activity suggesting that other neuroactive mediators may be influencing
mechanosensitive nerve discharge with cystitis.

Conclusion. In summary, our studies highlight the role of purinergic signaling
in TGF-β mediated bladder afferent nerve hyperexcitability. The apparent synergy
between a component of the bladder inflammatory milieu and intrinsic signaling
mechanisms discussed here will contribute to the understanding of the pathophysiology
of bladder injury or disease. The distinct interactions of multiple signal transducers
underscore the challenges for single target therapies and support the development of
combinatory therapeutics for bladder dysfunction.
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Disclosures

The authors declare no competing interests.
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Figure 1: TGF-β1 increased distention-evoked bladder afferent nerve discharge and ATP release

(A) Representative traces of vehicle (gray) and recombinant TGF-β1 (10 ng/ml, black) instillation from the same preparation. (B) TGF-β1 significantly increased mean bladder afferent nerve frequency (imp/sec) by 41.8% relative to control with significant nerve discharge increase from 5-10, 10-15, 15-20 and 20-25 mmHg. (C) TGF-β1 significantly increased distention-evoked ATP release at 25 mmHg. (D) Proposed mechanisms of purinergic signaling with TβR activation. n=4-8; values are mean ± SEM; *p ≤ 0.05, ***p ≤ 0.001, ****p ≤ 0.0001 by two-way repeated measures ANOVA followed by Sidak’s multiple comparisons test. #p ≤ 0.01 by Student’s paired t test.
Figure 2: Distention-evoked bladder afferent nerve discharge and ATP release are unchanged with the co-administration of TGF-β1 and SB505124

(A) Representative traces of vehicle (gray) and SB505124 (5 μM, black) instillation from the same preparation. (B) SB505124 did not have a significant treatment effect on mean bladder afferent nerve frequency (imp/sec) relative to control. (C) Representative traces of vehicle (gray) and recombinant TGF-β1 (10 ng/ml, black) and SB505124 (5 μM, black) co-instillation from the same preparation. Concurrent instillation of TGF-β1 and SB505124 attenuated distention-evoked bladder afferent nerve hyperexcitability (D) and ATP release (E) mediated by TGF-β1. (F) Proposed mechanisms of purinergic signaling with TβR inhibition. n=4-7; values are mean ± SEM.
Figure 3: Distention-evoked bladder afferent nerve discharge and ATP release are unchanged with the co-administration of TGF-β1 and BFA

(A) Representative traces of vehicle (gray) and BFA (10 μM, black) instillation from the same preparation. (B) BFA did not have a significant treatment effect on mean bladder afferent nerve frequency (imp/sec) relative to control. (C) Representative traces of vehicle (gray) and recombinant TGF-β1 (10 ng/ml, black) and BFA (10 μM, black) co-instillation from the same preparation. Concurrent instillation of TGF-β1 and BFA attenuated distention-evoked bladder afferent nerve hyperexcitability (D) and ATP release (E) mediated by TGF-β1. (F) Proposed mechanisms of purinergic signaling with TβR activation and BFA. n=3-8; values are mean ± SEM.
Figure 4: TGF-β1 and 10Panx co-administration increased distention-evoked bladder afferent nerve discharge and ATP release

(A) Representative traces of vehicle (gray) and 10Panx (50 μM, black) instillation from the same preparation. (B) 10Panx did not have a significant treatment effect on mean bladder afferent nerve frequency (imp/sec) relative to control. (C) Representative traces of vehicle (gray) and recombinant TGF-β1 (10 ng/ml, black) and 10Panx (50 μM, black) co-instillation from the same preparation. (D) Concurrent instillation of TGF-β1 and 10Panx significantly increased mean bladder afferent nerve frequency (imp/sec) by 19.4% relative to control with significant nerve discharge increase from 10-15, 15-20 and 20-25 mmHg. (E) TGF-β1 significantly increased distention-evoked ATP release at 25 mmHg when co-administered with 10Panx. (F) Proposed mechanisms of purinergic signaling with TβR activation and 10Panx. n=4-7; values are mean ± SEM. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001 by two-way repeated measures ANOVA followed by Sidak’s multiple comparisons test. #p ≤ 0.05 by Student’s paired t test.
Figure 5: Distention-evoked bladder afferent nerve discharge is unchanged but ATP release is increased with the co-administration of TGF-β1 and PPADS

(A) Representative traces of vehicle (gray) and PPADS (300 μM, black) instillation from the same preparation. (B) PPADS did not have a significant treatment effect on mean bladder afferent nerve frequency (imp/sec) relative to control. (C) Representative traces of vehicle (gray) and recombinant TGF-β1 (10 ng/ml, black) and PPADS (300 μM, black) co-instillation from the same preparation. (D) Concurrent instillation of TGF-β1 and PPADS attenuated distention-evoked bladder afferent nerve hyperexcitability mediated by TGF-β1. (E) TGF-β1 significantly increased distention-evoked ATP release at 25 mmHg when co-administered with PPADS. (F) Proposed mechanisms of purinergic signaling with TβR activation and PPADS. n=4-5; values are mean ± SEM. #p ≤ 0.05 by Student’s paired t test.
Figure 6: SB505124 decreased distention-evoked bladder afferent nerve hyperexcitability and ATP release with CYP-induced cystitis

(A) Representative traces of CYP-induced cystitis (gray) and control (black) instillation. (B) CYP-induced cystitis significantly increased mean bladder afferent nerve frequency (imp/sec) by 188.9% relative to control with significant nerve discharge increase from 5-10, 10-15, 15-20 and 20-25 mmHg. (C) Representative traces of CYP-induced cystitis (gray) and SB505124 (5 μM, black) instillation from the same CYP preparation. (D) SB505124 significantly decreased mean bladder afferent nerve frequency (imp/sec) by 40.6% relative to CYP with significant nerve discharge decrease from 5-10, 10-15 and 15-20 mmHg. (E) SB505124 significantly decreased distention-evoked ATP release with CYP-induced cystitis. (F) Proposed mechanisms of purinergic signaling with TβR inhibition and CYP-induced cystitis. n=4-19; values are mean ± SEM; *p ≤ 0.05, **p ≤ 0.01, ****p ≤ 0.0001 by two-way repeated measures ANOVA followed by Sidak’s multiple comparisons test. #p ≤ 0.05 by Student’s paired t test.
CHAPTER 4: CONCLUSIONS AND FUTURE DIRECTIONS

4.1. Summary

BPS/IC is a chronic pain syndrome characterized by lower urinary tract symptoms and pain or discomfort thought to be bladder related. BPS/IC impacts the quality of life of the 3.3-7.9 million women (>18 years old) in the United States who exhibit symptoms and burdens the economy with significant financial ramifications (Berry et al., 2011). Currently, the etiology of altered visceral sensation that accompanies BPS/IC is not known. We propose that neurochemical alterations in the urinary bladder contribute to peripheral afferent activation and sensitization to underlie bladder dysfunction (Figure 1). The studies in this dissertation identified and described one potential inflammatory mediator, TGF-β, that may be used as a diagnostic marker and/or target in the lower urinary tract to improve function with urinary bladder inflammation. The following sections will discuss its clinical implications and the potential to expand this work.

4.2. Summary and Future Directions: ATP Release

The sensory processing of bladder filling is proposed to involve the release of ATP from the urothelium and the activation of P2X3 and P2X2/3 receptors on afferent nerve terminals (Kaan et al., 2010; Rong, Spyer, & Burnstock, 2002). Any perturbation that increases ATP release may therefore alter nerve excitability and underlie the development of micturition reflex dysfunction. We first demonstrated that aberrant TGF-β signaling contributed, in part, to altered ATP release following CYP-induced cystitis (Figure 1). We next determined that TGF-β1 can directly stimulate the release of ATP
from the urothelium and that the effect was specific to receptor activation because the co-
administration with a TβR-1 inhibitor attenuated ATP release (Figure 1). We also
determined that vesicular exocytosis mechanisms contributed more than pannexin-1
cannels to ATP release mediated by TGF-β1 (Figure 1).

Our current studies were limited to measuring the luminal content of ATP due to
methodological limitations. To confirm that TGF-β1 also stimulates basolateral ATP
release that may then directly activate bladder afferent nerve terminals, we would use
urothelial sheets in an Ussing chamber. The Ussing chamber would allow us to separate
the mucosal and serosal compartments of the urothelium to measure ATP release into
each chamber. Similar to other studies, we would expect that the luminal application of
ATP would increase serosal ATP release (Sun & Chai, 2006). We would also expect the
luminal application of TGF-β1 to increase serosal ATP release. While the addition of
these experiments would strengthen our overall conclusions, the functional changes we
observed in bladder-pelvic nerve preparations suggest we are altering the basolateral
release of neuroactive transmitters.

A mechanism that is not yet known in our model is the intracellular signaling
cascade that TGF-β activates to stimulate ATP release. ATP release can be regulated, in
part, by increases in intracellular calcium (McLatchie & Fry, 2015) and PKA second
messenger cascades (Wang et al., 2005). Previous studies have reported that TGF-β can
induce a sustained increase in intracellular calcium through type III IP3 receptors
(McGowan et al., 2002). TGF-β has also been demonstrated to activate PKA, a response
that was independent to an increase in cAMP (Zhang et al., 2004). We can examine these
mechanisms through various experiments that include chelating and reducing intracellular calcium with BAPTA-AM, inhibiting calcium release from IP3-dependent stores in the ER with 2-aminoethoxydiphenylborate (APB) (Ma, Venkatachalam, Parys, & Gill, 2002) and blocking PKA with cell permeable inhibitors H89 or PKI (5-24). These experiments would give us further insight into the intracellular mechanisms that underlie TGF-β mediated ATP release and may provide another target to improve voiding dysfunction.

Figure 1: Schematic of TGF-β and purinergic signaling within the sensory components of the urinary bladder

TβR activation evokes ATP release from urothelial cells through vesicular exocytosis mechanisms. These extracellular nucleotides may activate P2X/P2Y receptors on underlying cells, including afferent nerve terminals, to increase excitability. The resultant afferent nerve hyperexcitability may alter the sensory processing of bladder filling and contribute to increased voiding frequency. Abbreviations: ATP, adenosine triphosphate; BFA, brefeldin A; Ca++, calcium; Det, detrusor; G, g-protein coupled receptor; ICC, interstitial cells of Cajal; Na+, sodium; P2, purinergic receptor; Panx, pannexin; PPADS, pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid; TβR, transforming growth factor-beta receptor.
4.3. Summary and Future Directions: Afferent Limb of the Micturition Reflex

Our studies demonstrated a role for TGF-β signaling in the afferent limb of the micturition reflex because intravesical TGF-β1 instillation increased bladder afferent nerve frequency and intravesical TβR-1 inhibition with SB505124 decreased bladder afferent nerve excitability and decreased voiding frequency following CYP-induced cystitis. TGF-β may alter nerve excitability indirectly by enhancing the release of neuroactive molecules from the urothelium or directly by activating receptors on nerve terminals. Our studies suggest that the intravesical treatment instillation was likely having an indirect effect on nerves through the urothelium and purinergic signaling (Figure 1). In addition to TGF-β1 stimulating ATP release, TGF-β1 affected nerve excitability in control tissues with an intact barrier suggesting a role for urothelial sensory processing (Figure 1). These experiments, however, cannot discount the possible direct effect on nerve terminals that TGF-β1 may be having in CYP-induced cystitis.

Future studies can use whole-cell patch-clamp recording techniques to examine the direct role of TGF-β1 on primary afferent cell bodies (dorsal root ganglia) innervating the urinary bladder. Neurons projecting from the urinary bladder would first be labeled with fast blue and then selected for intracellular recordings. We would expect TGF-β1 to contribute to peripheral sensitization by increasing spontaneous and evoked action potentials as previously described (Zhu et al., 2012). Several mechanisms have been proposed to underlie this TGF-β mediated excitability. The phosphorylation of TRPV1 by TGF-β mediated upregulation of Cdk5 has previously been shown to induce hyperexcitability in sensory neurons (Utreras et al., 2012). Similarly, TGF-β has been...
previously shown to modulate KCNA4 transcript expression in sensory neurons resulting in a decrease of IA currents and hyperexcitability (Zhu et al., 2012). Future experiments may look at these mechanisms within urinary bladder sensory neurons to determine if they have a role in CYP-induced cystitis.

4.4. Future Directions: Detrusor Smooth Muscle

TGF-β can affect smooth muscle cell contractility by altering cytoskeletal (Hu et al., 2006) and contractile protein expression (Grainger, Metcalfe, Grace, & Mosedale, 1998). TGF-β1 has also been demonstrated to increase contractility of primary human bladder smooth muscle cells through the effector JunB (Ramachandran et al., 2013). Since TGF-β1 is increased in the detrusor smooth muscle with intermediate (48 h) CYP-induced cystitis, we would expect to see similar functional changes in contractility. We would determine the contribution of aberrant TGF-β signaling to detrusor contractility with CYP-induced cystitis using detrusor smooth muscle strips suspended from force transducers. We would first confirm previous studies that showed an increase in spontaneous contractions with CYP-induced cystitis (Okinami et al., 2014). We would next superfuse the TβR-1 inhibitor, SB505124, through the bath solution and expect to observe an inhibition of TGF-β1-induced contractility.

The isolation of smooth muscle strips may not completely reflect intravesical pressure changes in open outlet cystometry because of not having an intact micturition reflex. As a result, these studies must be interpreted cautiously and in the context of the in vivo cystometrogram measurements. Our cystometry data demonstrated no changes in intravesical pressure (filling, threshold and peak) following CYP-induced and the
intravesical instillation of SB505124. This suggests that the detrusor smooth muscle was not involved in the decreased voiding frequency and increased bladder capacity, void volume and longer intercontraction intervals we observed. An alternative explanation is that while TβR-1 inhibition may alter contractility, SB505124 is simply not reaching the detrusor smooth muscle with intravesical instillation. Future studies can circumvent this problem and determine its involvement by administering SB505124 intravenously so that it can more readily access the smooth muscle.

4.5. Future Directions: Somatic Hypersensitivity

BPS/IC is characterized by pain perceived to emanate from the urinary bladder. In modeling this syndrome, CYP-induced cystitis has been demonstrated to increase hindpaw and pelvic somatic sensitivity (Arms, Girard, Malley, & Vizzard, 2013). Given the role of TGF-β1 in central and peripheral sensitization (Lantero, Tramullas, Diaz, & Hurle, 2012), we would expect to see a contribution of TGF-β1 to somatic hypersensitivity. Von Frey monofilaments can be used to determine referred pelvic and hindpaw sensitivity in CYP-treated animals with TβR-1 inhibition. The animals would be placed in a plexiglass chamber with a wire mesh floor and then tested for tactile allodynia and secondary hyperalgesia using these calibrated filaments to the abdomen and hindpaw. We would expect the intravesical instillation of SB505124 to decrease tactile allodynia and secondary hyperalgesia as evidenced by decreased withdrawal frequencies relative to baseline measurements in CYP-treated animals. The addition of these experiments would determine the role(s) of TGF-β1 in the development of somatic pain symptoms with cystitis.
4.6. Clinical Implications

The neural circuitry underlying the micturition reflex is complex and is often compromised with neural injury, disease or inflammatory conditions. Our studies utilized the preclinical CYP-induced cystitis model to characterize the neurochemical plasticity in the sensory components of the urinary bladder. We demonstrated elevated levels of TGF-β ligands and their cognate receptors following cystitis and determined their functional expression in the afferent limb of the micturition reflex. Given the diversity of bladder syndromes, it remains to be determined if certain inflammatory mediators and downstream signaling pathways are unique to specific conditions. Nevertheless, the inhibition of cytokine/receptor signaling may represent a therapeutic avenue for inflammation-associated bladder dysfunction.

The presence of inflammatory molecules, like TGF-β, in the urine or urothelium may be a useful biomarker for monitoring inflammation or tracking responses to treatment in bladder syndromes. At this moment, the diagnostic criteria of BPS/IC are dependent on cystoscopy and symptom-based lower urinary tract dysfunction (Kuo, 2014). Potential markers, such as elevated urinary proteins (Erickson et al., 2002) and serum cytokines (Jiang, Peng, Liu, & Kuo, 2013), have been suggested to permit an earlier treatment intervention to control or slow disease progression. Currently, however, these markers are likely not specific enough to differentially diagnose bladder syndromes (BPS/IC, OAB, etc.) (Kuo, 2014). While TGF-β may not be a diagnostic marker for BPS/IC, it does appear to offer an alternative method to track the progression of inflammation and tissue repair due to its temporal regulation in CYP-induced cystitis.
Future longitudinal studies must determine if inflammatory mediators, like TGF-β, follow this time course in clinical populations. The continued characterization of neurochemical alterations in bladder syndromes may improve patient outcome by offering more selective treatment options that are dependent on the profile of markers expressed.
References for Conclusions and Future Directions


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APPENDIX A: THE EFFECTS OF TEMPO ON CYCLOPHOSPHAMIDE (CYP)-INDUCED OXIDATIVE STRESS IN RAT MICTURITION REFLEXES

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Abstract

Bladder pain syndrome (BPS)/interstitial cystitis (IC) is a chronic bladder disorder characterized by pressure, discomfort, and pain perceived to be related to the urinary bladder, with at least one urinary symptom. We hypothesized that cyclophosphamide (CYP)-induced cystitis results in oxidative stress and contributes to urinary bladder dysfunction. We determined: (1) the expression of oxidative stress markers (3-nitrotyrosine (3-NT), reactive oxygen species (ROS)/reactive nitrogen species (RNS), inflammatory modulators, neuropeptides (calcitonin gene-related peptide (CGRP); substance P (Sub P), and adenosine triphosphate (ATP) that contribute to the inflammatory process in the urinary tract and (2) the functional role of oxidative stress in urinary bladder dysfunction with an antioxidant, Tempol (1 mM in drinking water) combined with conscious cystometry. In CYP-treated (4 hr of 48 hr; 150 mg/kg, i.p.) rats, ROS/RNS and 3-NT significantly (p ≤ 0.01) increased in urinary bladder. CYP-treatment increased ATP, Sub P and CGRP expression in the urinary bladder and cystometric fluid. In CYP-treated rats, Tempol significantly (p ≤ 0.01) increased bladder capacity, and reduced voiding frequency compared to CYP-treated rats without Tempol. Tempol significantly (p ≤ 0.01) reduced ATP expression, 3-NT and ROS/RNS expression in the urinary tract of CYP-treated rats. These studies demonstrate that reducing oxidative stress in CYP-induced cystitis improves urinary bladder function and reduces markers of oxidative stress and inflammation.

Keywords: reactive oxygen species, reactive nitrogen species, 3-nitrotyrosine, ATP, neuropeptides, cystometry
Introduction

Bladder pain syndrome (BPS)/interstitial cystitis (IC) is a chronic syndrome characterized by pressure, discomfort, and pain thought to arise from the urinary bladder, with at least one urinary symptom [1, 2]. While the underlying etiology of BPS/IC is not known, the majority of biopsies from BPS/IC patients reveal inflammation [3]. Mediators of inflammation, including cytokines, chemokines, growth factors and neuropeptides, have been shown to contribute to urinary bladder dysfunction and somatic sensitivity in animal models of cystitis and in the clinical syndrome of BPS/IC [3, 4]. In addition, reactive oxygen species (ROS) and reactive nitrogen species (RNS) generated by inflammation result in oxidative stress and may contribute to urinary bladder dysfunction [5, 6]. The role(s) oxidants may have in inducing inflammation has been extensively studied in diverse experimental models [7]. Although it is widely accepted that ROS/RNS are fundamentally involved, antioxidant therapy as a valid means of arresting inflammation remains largely unresolved especially in the context of urinary bladder inflammation.

Accompanying oxidative stress and inflammatory mediator upregulation, the urothelium may also respond to cystitis by increasing the secretion of neuroactive factors, such as ATP, that signal to the underlying nerve plexus [8]. Altered release of these neuromodulatory compounds has been suggested to contribute to increased sensory transduction and result in urinary bladder dysfunction [4, 8]. In BPS/IC, for example, distention-evoked release of ATP was increased in tissue biopsies that may have resulted in the elevated urinary ATP observed in patients with BPS/IC [9]. Similarly, numerous
animal models of cystitis have demonstrated increased distention-evoked release of ATP from the urothelium [10, 11]. Further, purinergic receptor activation has been associated with increased cellular production and release of multiple inflammatory mediators, including superoxide anion, nitric oxide and other ROS [12]. Purinergic receptor activation induces ROS generation in numerous cell types resulting in a variety of downstream effects including, transcription factor activation [13], pro-inflammatory cytokine release [14, 15] and cell death [16].

Using a rat model of urinary bladder inflammation induced by cyclophosphamide (CYP) [3, 4, 17, 18], we determined: (1) the expression of oxidative stress markers (3-nitrotyrosine (3-NT), ROS/RNS) and other modulators of inflammation, the neuropeptides calcitonin gene-related peptide (CGRP) and substance P (Sub P) in the urinary tract; (2) the contribution of ROS/RNS to ATP expression in the urinary bladder and urine with an antioxidant and superoxide dismutase mimetic, Tempol (1 mM in drinking water); and (3) the role of ROS/RNS in urinary bladder function with Tempol combined with open outlet, conscious and continuous intravesical infusion [19]. Although the etiology of BPS/IC is unknown, previous studies [3, 4, 17, 18] have demonstrated that the rat CYP model of urinary bladder inflammation is a reliable and reproducible model with face validity (e.g., increased voiding frequency and referred somatic sensitivity) to BPS/IC. Previous studies have demonstrated roles for neuropeptides, including CGRP and Sub P, in CYP-induced bladder dysfunction [39, 57] and the current studies have continued to focus on these two neuropeptides, abundantly expressed in bladder sensory pathways.
Materials and Methods

Animals

Adult female, Wistar rats (200-225 g; Charles River, St. Constant, Canada) were used for this study. Rats were housed two per cage and maintained in standard laboratory conditions with free access to food and water. The University of Vermont Institutional Animal Care and Use Committee approved all animal use procedures (protocol 08-085). Animal experimentation was carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize the potential for animal pain, stress or distress.

Induction of cyclophosphamide (CYP)-induced cystitis

Rats were anesthetized under isoflurane (2%) and acute cystitis was induced with a single injection of CYP (150 mg/kg, i.p.) and used in studies at various time points (4 hours (hr) or 48 hr) after treatment [20-22]. Control rats received volume-matched injections of saline (0.9%; i.p.) or no treatment and no difference among the control groups was observed. All injections were performed under isoflurane (2%) anesthesia. The CYP model of bladder inflammation produces an increase in voiding frequency with small micturition volumes and is associated with inflammatory cell infiltrates in the urinary bladder including mast cells, macrophages and neutrophils [23, 24]. Rats were euthanized using isoflurane (5%) and a thoracotomy.

For cystometry in conscious rats, an unrestrained animal was placed in a Plexiglas cage with a wire bottom. Before the start of the recording, the bladder was emptied and the catheter was connected via a T-tube to a pressure transducer (Grass...
Model PT300, West Warwick, RI) and microinjection pump (Harvard Apparatus 22, South Natick, MA). A Small Animal Cystometry Lab Station (MED Associates, St. Albans, VT) was used for urodynamic measurements [19, 28]. Saline solution was infused at room temperature into the bladder at a rate of 10 ml/h to elicit repetitive bladder contractions. At least six reproducible micturition cycles were recorded after the initial stabilization period of 25–30 min [19, 28]. The following cystometric parameters were recorded in each animal: baseline pressure (BP; pressure at the beginning of the bladder filling), threshold pressure (TP; bladder pressure immediately prior to micturition), peak micturition pressure (MP), intercontraction interval (ICI; time between micturition events), bladder capacity (BC), void volume (VV), presence and amplitude of non-voiding bladder contractions (NVCs) [19, 28]. NVCs were defined as rhythmic intravesical pressure increases 7 cm H2O above baseline, during the filling phase, without the release of fluid from the urethra. Bladder capacity (BC) was measured as the volume of saline infused into the bladder at the time when micturition commenced [35]. In these rats, residual volume was less than 10 μl; therefore, VV and BC were similar. After the initial stabilization period, cystometric fluid expelled during micturition events was collected and frozen on dry ice and stored at -80 °C for future use in the assays described above. At the conclusion of the experiment, the rat was euthanized (5% isoflurane plus thoracotomy), the urinary bladder was harvested and used in the assays described above.

Split bladder preparation and assessment of potential contamination of bladder layers

The urothelium + suburothelium was dissected from the detrusor smooth muscle using fine forceps under a dissecting microscope as previously described [20, 21]. To
confirm the specificity of our split bladder preparations, urothelium + suburothelium and detrusor samples were examined for the presence of α-smooth muscle actin (1:1000; Abcam, Cambridge, MA) and uroplakin II (1:25; American Research Products, Belmont, MA) by western blotting or reverse transcription PCR [20, 25]. In urothelium + suburothelium layers, only uroplakin II was present (data not shown). Conversely, in detrusor samples, only α-smooth muscle actin was present (data not shown). All subsequent measurements of urinary bladder and cystometric fluid samples and conscious cystometry in rat groups were performed in a blind manner.

Substance P (Sub P), calcitonin gene-related peptide (CGRP), 3-nitrotyrosine (3-NT) by enzyme-linked immunosorbent assays (ELISAs)

Tissue processing and ELISAs were performed as described previously [18, 26]. Briefly, rats from control (n = 6 each) and all experimental groups (n = 6 each) were deeply anesthetized (4% isoflurane), and a thoracotomy was performed. Individual rat bladders were dissected, weighed, and placed in Tissue Protein Extraction Reagent (1 g tissue/20 ml; Pierce Biotechnology, Woburn, MA) with Complete protease inhibitor cocktail tablets (Roche Applied Science, Mannheim, Germany), and stored at −80 °C. On the day of assay, individual bladders were disrupted with a Polytron homogenizer until homogeneous and centrifuged (10,000 rpm for 10 min) [18, 26], and the supernatant was used for total protein estimation and CGRP (Phoenix Pharmaceuticals, Inc. Burlingame, CA), Sub P (Phoenix Pharmaceuticals) and 3-NT (Millipore Corporation, Bellerica, MA) quantification. Total protein was determined by the Coomassie Plus Protein Assay Reagent Kit (Pierce) [18, 26] and CGRP, Sub P and 3-NT were quantified using standard
96-well ELISA plates (Phoenix Pharmaceuticals; Millipore Corporation) according to the manufacturer's recommendations. For determination of Sub P and CGRP content in voided cystometric fluid, void volumes were collected following each micturition event in control and CYP-treated groups with and without Tempol (only vehicle) administration during conscious cystometry (see below). For Sub P, CGRP and 3-NT measurements in voided cystometric fluid, 6–8 individual voids/animal were collected and immediately frozen on dry ice. Data from multiple voids were averaged, and the mean value was used for each animal.

**ELISAs for CRGP, Sub P, 3-NT expression in urinary bladder and voided cystometric fluid**

The microtiter plates (Phoenix Pharmaceuticals; Millipore Corporation) were coated with mouse anti-rat CGRP, anti-rat Sub P or anti-rabbit-NT antibody. Sample and standard solutions were run in duplicate. Horseradish peroxidase (HRP)-streptavidin or HRP-conjugated goat anti-rabbit IgG and LumiGLO was used to detect the antibody complex. Tetramethylbenzidine or LumiGLO was the substrate, and the enzyme activity or luminescence was measured. The CRGP standard provided with this protocol generated a standard curve from 0 to 100 ng/ml (R2 = 0.998, P ≤ 0.0001) for bladder samples [26]. The Sub P standard provided with this protocol generated a standard curve from 0 to 25 ng/ml (R2 = 0.998, P ≤ 0.0001) for bladder samples [26]. The nitrated-BSA standard provided with this protocol generated a standard curve from 1.5 to 100 µg/ml (R2 = 0.997, P ≤ 0.0001) for bladder samples. The absorbance values of standards and samples were corrected by subtraction of the background value (absorbance due to
nonspecific binding) [18, 26]. No samples were diluted and all samples had absorbance values that fell onto the linear portion of the standard curve. Curve fitting of standards and evaluation of protein content of samples were performed using a least-squares fit.

**Measurement of cellular oxidative stress**

The levels of cellular oxidative stress were determined by a spectrofluorimetric method, using the dichlorofluorescein DiOxyQ (DCFH-DiOxyQ) assay (OxiSelect In Vitro ROS/RNS Assay Kit (Green Fluorescence; Cell Biolabs, Inc., San Diego, CA, USA). The urinary bladder was removed from control (n = 6 each) and all experimental groups (n = 6 each) and rapidly homogenized in 50 mM Tris–Cl, pH 7.4. The homogenate was centrifuged at 2400g for 15 min at 4 °C and a low-speed supernatant fraction was used for assays. To determine levels of cellular oxidative stress, the supernatant from the urinary bladder homogenate was diluted (1:10) in 50 mM Tris–HCl (pH 7.4) and incubated with 10 μl of 2′,7′-DCHF-DA (1 mM), at 37 °C for 30 min. The DCHF-DA is enzymatically hydrolyzed by intracellular esterases to form nonfluorescent DCFH, which is then rapidly oxidized to form highly fluorescent 2′,7′-dichlorofluorescein (DCF) in the presence of cellular oxidative stress species. DCF fluorescence intensity is proportional to the amount of cellular oxidative stress species that is formed. The DCF fluorescence intensity emission was recorded at 520 nm (with 480 nm excitation) 30 min after the addition of DCHF-DA to the sample. The cellular oxidative species levels were expressed as fluorescence arbitrary units (FAU).

**Collection and measurement of urinary ATP**
Sample collections. For determination of ATP in urinary bladder, urinary bladder harvest and subsequent processing was performed as described above for ELISAs. For determination of ATP content in voided cystometric fluid, void volumes were collected following each micturition event in animal groups (control, CYP-treated groups, with and without Tempol) during conscious cystometry. For ATP measurements in voided cystometric fluid, 6–8 individual voids/animal were collected and immediately frozen on dry ice and stored at -80 °C until use. Undiluted cystometric fluid samples were defrosted till 25°C, centrifuged at 3000g at room temperature for 20 seconds to remove cellular debris and the supernatant was separated. Data from multiple voids were averaged, and the mean value was used for each animal.

ATP determination. A mixture of luciferin-luciferase was added according to the manufacturer instructions using the ATP Bioluminescence Assay Kit HS II (Adenosine Triphosphate (ATP) Bioluminescent Assay Kit, Sigma-Aldrich, St. Louis, MO, USA) as previously described [27]. ATP detection was evaluated using a multi-mode microplate reader (Synergy HT, BioTek Instruments Inc., Vermont, USA) controlled with Gen5™ Data Analysis Software (BioTek). Sample bioluminescence was compared to that of standard amounts of ATP used in the same concentration range; standard ATP samples were prepared daily. All samples were run in duplicate. Cystometric void data were obtained from at least three voids/animal from control (n = 6 each) and all experimental groups (n = 6 each) and urinary bladder data were obtained from control (n = 6 each) and all experimental groups (n = 6 each). Vehicle for Tempol did not affect the ATP determinations. The ATP in cystometric fluid or ATP content in urinary bladder was
calculated relative to the standard curve, and expressed as nmol per total protein or nmol per ml of urine.

**Conscious Cystometry and effects of Tempol, a superoxide dismutase (SOD) mimetic**

Rats were anesthetized with isoflurane (3-4%), a lower midline abdominal incision was made, and polyethylene tubing (PE-50, Clay Adams, Parsippany, New Jersey) was inserted into the bladder dome and secured with a nylon purse-string sutures (6-zero) [19, 28]. The end of the PE tubing was heat flared, but the catheter did not extend into the bladder body or neck and it was not associated with inflammation or altered cystometric function [19, 28]. The distal end of the tubing was sealed, tunneled subcutaneously and externalized at the back of the neck out of reach of the animal [19, 28]. Abdominal and neck incisions were closed with nylon sutures (4-zero).

Postoperative analgesics were given and animals were maintained for 72 to 96 hours after survival surgery to ensure recovery.

Tempol (4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl), a superoxide dismutase (SOD) mimetic, is a stable nitroxyl antioxidant. Previous studies have suggested that Tempol is protective in disorders involving ROS [29-32]. The effects of Tempol on urinary bladder function in CYP-treated (4 hr and 48 hr; n = 6 each) rats and control rats (n = 6 each) were assessed using conscious, open outlet, cystometry with continuous instillation of intravesical saline [19, 28]. Tempol was prepared daily with distilled water purified with a Millipore Milli-Q system and administered in the drinking water (1 mM) that was provided to rats ad libitum; untreated rats received water ad libitum. Covered bottles were used to minimize degradation by light. The addition of
Tempol to the drinking water did not affect water consumption (data not shown). For CYP-treated rat groups (4 hr, 48 hr), Tempol was provided for approximately two weeks (13-15 days) while the intravesical tube was implanted on day 10, CYP or vehicle was injected on day 13 and rats were used in experiments on day 13-15. Due to the daily administration and route of delivery (oral) of the Tempol, these experiments were performed in different groups of control and CYP-treated rats treated with vehicle or Tempol. The concentration (1 mM) of Tempol and duration of treatment used in these studies was based upon previous studies [33, 34].

Exclusion Criteria

Rats were removed from the study when adverse events occurred that included 20% reduction in body weight post-surgery, a significant postoperative event, lethargy, pain, or distress not relieved by our IACUC-approved regimen of postoperative analgesics or hematuria in control rodents [19, 28]. In the present study, no rats were excluded from the study. In addition, behavioral movements such as grooming, standing, walking, and defecation rendered bladder pressure recordings during these events unusable.

Statistical Analyses

All values represent mean ± SEM. Cystometry data were compared using repeated measures ANOVA, where each animal served as its own control. Animals, processed and analyzed on the same day, were tested as a block in the ANOVA. When F-ratios exceeded the critical value (p ≤ 0.05), the Newman-Keuls or Dunnett's post hoc tests were used to compare group means. Data obtained from the ATP assays violated the
assumptions of the ANOVA. Thus, these data were analyzed using a non-parametric analysis, the Mann-Whitney Rank Sum test. When F ratios exceeded the critical value (p ≤ 0.05), the Newman-Keul's post-hoc test was used to compare the experimental means. P ≤ 0.05 (two-tailed) values were considered statistically significant.
Results

ROS/RNS Expression in Urinary Bladder with CYP-induced Cystitis and the Effects of Tempol

We determined the levels of reactive oxygen species (ROS) and reactive nitrogen species (RNS) in the urinary bladder following CYP treatment (4 hr, 48 hr) and in the presence of the antioxidant, Tempol. 4 hr and 48 hr CYP-induced cystitis significantly (p ≤ 0.01) increased the total free radical presence in the detrusor and urothelium that was significantly reduced (p ≤ 0.01) by Tempol (1 mM) delivered in the drinking water (Figure 1A). With both 4 hr and 48 hr CYP-induced cystitis, the increase in total free radical presence was significantly (p ≤ 0.01) greater in the detrusor compared to the urothelium (Figure 1A). Basal expression of total free radical presence in the detrusor was significantly (p ≤ 0.01) greater in the detrusor compared to the urothelium (Figure 1A).

3-Nitrotyrosine (3-NT) Expression in Urinary Bladder with CYP-induced Cystitis and the Effects of Tempol

3-NT, a product of tyrosine nitration mediated by RNS such as peroxynitrite anion and nitrogen dioxide, is considered a marker of NO-dependent, RNS-induced nitrative stress [36]. 4 hr and 48 hr CYP-induced cystitis significantly (p ≤ 0.01) increased 3-NT expression in the urinary bladder that was significantly reduced (p ≤ 0.01) by Tempol (1 mM) delivered in the drinking water (Figure 1B). 3-NT expression in the urinary bladder was significantly (p ≤ 0.01) greater following 4 hr CYP-induced cystitis compared to 48 hr CYP-induced cystitis (Figure 1B).
Substance P (Sub P) and Calcitonin Gene-Related Peptide (CGRP) Expression in Urinary Bladder and Cystometric Fluid with CYP-induced Cystitis and the Effects of Tempol

The neuropeptides, Sub P and CGRP, are known modulators of inflammation and may contribute to the pathogenesis of many diseases including migraine, asthma, and urinary bladder inflammation [4, 37-39]. 4 hr and 48 hr CYP-induced cystitis significantly (p ≤ 0.01) increased Sub P and CGRP expression in the urinary bladder (Figure 2A; Figure 3A) and cystometric fluid (Figure 2B; Figure 3B) that was significantly reduced (p ≤ 0.01) by Tempol (1 mM) delivered in the drinking water (Figure 2A, 2B; Figure 3A, 3B). Sub P expression in the urinary bladder was similar following 4 hr and 48 hr CYP-induced cystitis in the urinary bladder and cystometric fluid (Figure 2A, 2B). In contrast, 48 hr CYP-induced cystitis resulted in significantly (p ≤ 0.01) greater CGRP expression in the urinary bladder and cystometric fluid compared to the 4 hr time point (Figure 3A, 3B).

Adenosine Triphosphate (ATP) Expression in Urinary Bladder and Cystometric Fluid with CYP-induced Cystitis and the Effects of Tempol

Numerous studies have described a role(s) for ATP in urinary bladder dysfunction, pain and altered ATP release mechanisms in animal models and clinical studies of BPS/IC [8]. 4 hr and 48 hr CYP-induced cystitis significantly (p ≤ 0.01) increased ATP expression in the urinary bladder and cystometric fluid that was significantly reduced (p ≤ 0.01) by Tempol (1 mM) delivered in the drinking water (Figure 4A, 4B). ATP expression in the urinary bladder was similar following 4 hr and
48 hr CYP-induced cystitis (Figure 4A). In contrast, 4 hr CYP-induced cystitis resulted in significantly ($p \leq 0.01$) greater ATP expression in the cystometric fluid compared to the 48 hr time point (Figure 4B).

**Effect of an Antioxidant, Tempol, on Bladder Function**

Conscious, open outlet cystometry with continuous intravesical infusion of saline was performed in separate groups (n = 6 each) of control and CYP-treated (48 h) rats with or without Tempol (vehicle only) in the drinking water to determine bladder function (Figure 5; Figure 6; Figure 7; Figure 8).

**Control (no CYP treatment).**

Tempol (1 mM) treatment had no effects on ICI, BC, or VV in control rats (no CYP treatment) compared with control rats (no CYP treatment) treated with vehicle (Figure 5). There were no changes in BP, TP, or peak MP with Tempol treatment compared to control rats (no CYP treatment) treated with vehicle (Figure 6). Residual volume in control rats with or without Tempol (vehicle) treatment was minimal ($\leq 10 \mu l$).

**CYP treatment.**

As previously demonstrated [20-22], and confirmed here, CYP treatment (4 hr and 48 hr) increased void frequency and decreased BC, ICI, and VV compared with control rats (no CYP treatment) (Figure 5; Figure 6; Figure 7; Figure 8). Additionally, 48 hr CYP-induced cystitis significantly ($p \leq 0.01$) increased BP and TP (Figure 6). Tempol in the drinking water (1 mM) of CYP-treated rats (4 hr and 48 hr) significantly ($p \leq 0.01$) increased the ICI (i.e., decreased voiding frequency) (Figure 5A; 2.0-2.9-fold), increased BC (Figure 5C; 2.8-3.1-fold), and increased VV (Figure 5B; 2.9-5.4-fold) compared to
rats treated with CYP (4 hr and 48 hr) receiving vehicle (Figure 7; Figure 8). Tempol treatment of CYP-treated rats (4 hr and 48 hr) increased BC to 70% of control rats (no CYP treatment) (Figure 5C; Figure 7; Figure 8). Effects of Tempol on bladder function in CYP-treated rats persisted for at least 2 hr. Residual volume in CYP (4 hr and 48 hr)-treated rats with or without Tempol treatment was minimal and similar to that observed in control (no CYP treatment) (≤ 10 μl). CYP-treated rats (4 hr and 48 hr) treated with or without Tempol (vehicle) exhibited no differences in BP, TP or peak MP (Figure 6A-C). Tempol also significantly (p ≤ 0.01) reduced the number (2.4 ± 0.4/micturition cycle vs. 0.6 ± 0.2/micturition cycle) and amplitude (11.2 ± 1.3 cm H20 vs. 7.5 ± 0.5 cm H20) of NVCs (increases in bladder pressure during the filling phase without the release of fluid) in 4 hr CYP-treated rats (Figure 7). The effects of Tempol on NVCs in the 48 hr CYP-treated group were not determined due to the dramatically increased voiding frequency that made the presence of NVCs difficult to determine.
Discussion

The present studies demonstrate several novel findings with respect to the induction and reduction of oxidative stress with the antioxidant, Tempol, following cyclophosphamide (CYP)-induced cystitis. CYP-induced cystitis (4 hr and 48 hr) increased ROS/RNS and 3-NT expression in the urinary bladder. In addition, CYP-induced cystitis increased expression of neuropeptides, CGRP and Sub P, in the urinary bladder as well as cystometric fluid collected during conscious cystometry. CYP-induced cystitis also increased ATP in the urinary bladder and cystometric fluid. Providing rats with the antioxidant, Tempol, in the drinking water prior to and during the induction of CYP-induced cystitis significantly reduced the expression of ROS/RNS, CGRP, Sub P and ATP in urinary bladder and cystometric fluid. Further, Tempol decreased voiding frequency, increased the intercontraction interval and bladder capacity without effects on urinary bladder pressures (baseline, threshold, peak) in rats with CYP-induced cystitis. Tempol did not alter bladder function in control (no CYP treatment) rats. These studies demonstrate that CYP-induced cystitis is associated with oxidative stress in the urinary tract and that use of the antioxidant, Tempol, reduces oxidative stress and improves urinary bladder function.

Molecular oxygen reduction results in the production of several reactive intermediates that must be actively scavenged [40]. Under conditions of inflammation, there may be insufficient scavenging of reactive intermediates that can lead to oxidative stress and damage to cellular structure and function. Recent studies suggest the generation of these reactive intermediates contribute to the pathogenesis of urinary
bladder dysfunction with CYP administration [41-43]. The contribution of oxidative stress in bladder inflammation following CYP is further supported by the attenuation of tissue damage following the administration of agents with antioxidant properties, like taurine [41], flavonoid [42], beta-carotene and others [43]. Our studies using the CYP model of urinary bladder inflammation at two time points (4 hr and 48 hr) confirm the expression of reactive intermediates including ROS/RNS and 3-NT in the urinary bladder. Expression of ROS/RNS following CYP-induced cystitis was significantly greater in the detrusor smooth muscle compared to the urothelium but both tissues exhibited increased ROS/RNS compared to control (no CYP treatment) urinary bladder. 3-NT expression has been previously demonstrated in lower urinary tract tissues following injury or inflammation. Partial outlet obstruction in rabbit is associated with increased NT in mucosa [44] that is correlated with progressive decrease in contractility of detrusor smooth muscle [45]. Previous studies demonstrated iNOS and NT immunoreactivity in the urothelium and inflammatory infiltrates in the lamina propria of individuals with BPS/IC with a Hunner’s lesion [47]. Consistent with this clinical study, the present study demonstrates increased 3-NT in the urinary bladder following CYP-induced cystitis (4 hr and 48 hr) in a rat model. The amino acid tyrosine is particularly susceptible to nitration and the formation of 3-NT may represent a biomarker for the generation of reactive nitrogen intermediates in vivo [47]. In addition to being a biomarker, 3-NT could also have a detrimental impact on cell function and viability by inhibiting protein phosphorylation by tyrosine kinases and interfering with the signal transduction mechanism [48]. In addition, in vitro nitration of a specific tyrosine residue
inactivates manganese superoxide dismutase [49] that may lead to a greater concentration of ROS/RNS perpetuating tissue damage and altered function.

As a major sensory component of the urinary bladder, the urothelium is able to respond to various extracellular stimuli by releasing neuroactive factors like ATP, acetylcholine and nitric oxide [8]. The sustained release of these factors, such as what may occur with inflammation, may underlie the development of urinary bladder dysfunction and lower urinary tract symptoms. The role of oxidative stress with ATP release in epithelial cells, however, is not well defined. In endothelial cells, oxidative stress has been demonstrated to mediate the direct release of ATP and inhibit the catabolism of extracellular ATP [50, 51]. Further, purinergic receptor activation has been associated with increased cellular production and release of multiple inflammatory mediators, including superoxide anion, nitric oxide and other ROS. Purinergic receptor activation induces ROS generation in numerous cell types resulting in a variety of downstream effects including, transcription factor activation [13], pro-inflammatory cytokine release [14, 15] and cell death [16]. Our studies determined the contribution of ROS/RNS to extracellular ATP expression with CYP-induced cystitis and Tempol. CYP-induced cystitis increased ATP expression in the urinary bladder and cystometric fluid that was significantly reduced by Tempol administration. It is not known from the present studies whether antioxidant treatment directly inhibits the release of ATP through purinergic receptor blockade. Additional studies in CYP-treated rats involving assessment of oxidative stress in the presence of purinergic receptor (P2) blockade as well as the effects of P2 activation on oxidative stress in controls should be considered.
Tempol is a membrane-permeable, redox-cycling agent that scavenges superoxide anions and decreases the formation of hydroxyl radicals [32]. The protective effects of Tempol to tissues with inflammation and oxidative damage have been widely established. For example, Tempol has been shown to decrease NF-kappaB activation with acute inflammation [52], decrease neutrophil infiltration and PARP activation with periodontitis [53], and decrease cytokine release stimulated by an inflammatory soup [54]. In animal studies, purinergic neuromuscular transmission and propulsive motility were significantly restored in the inflamed colon treated with the free radical scavenger, Tempol [55]. Furthermore, intrathecal Tempol administration has been demonstrated to decrease thermal and mechanical hypersensitivity with neuropathic pain [56]. Due to the central and peripheral anti-inflammatory properties of Tempol, our studies determined the role of oxidative stress on bladder function with CYP-induced cystitis and Tempol administration. Pretreatment with Tempol and continued treatment with Tempol during the progression of CYP-induced cystitis significantly improved urinary bladder function. In CYP-treated (4 hr and 48 hr) rats, Tempol in the drinking water increased the intercontraction interval and bladder capacity and reduced urinary frequency. The presence and amplitude of NVCs during the filling phase of the urinary bladder were also reduced in 4 hr CYP-treated rats given Tempol. Given the demonstration of Tempol decreased mechanical hypersensitivity from neuropathic pain [56], future studies should examine the effects of Tempol administration on somatic (i.e., pelvic, hindpaw) sensitivity in CYP-treated rats.
Previous studies from our laboratory have demonstrated roles for neuropeptides, including pituitary adenylate cyclase-activating polypeptide (PACAP), CGRP and Sub P in CYP-induced bladder dysfunction [39, 57]. Increased expression of PACAP, CGRP and Sub P were demonstrated in the urinary bladder, lumbosacral spinal cord and dorsal root ganglia of CYP-treated rats and pharmacological blockade of the PACAP specific PAC1 receptor improved urinary bladder function in CYP-treated rats [39, 57]. The present studies confirm increased expression of Sub P and CGRP in the urinary bladder and cystometric fluid with CYP-induced cystitis (4 hr and 48 hr). Tempol treatment significantly reduced Sub P and CGRP expression in the urinary bladder and cystometric fluid. Previous studies demonstrated that Sub P via neurokinin (NK) receptor facilitates bladder afferent signaling and ROS formation in bladder in association with neurogenic inflammation [58]. Increased Sub P release increased ROS in the bladder via increased mast cell degranulation, intercellular adhesion molecule expression, and leukocyte adhesion. Future studies can determine the involvement of Sub P/NK receptor signaling with these sources of ROS in the inflamed bladder following CYP-induced cystitis. The neuropeptides, PACAP27, PACAP38 and VIP, evoked ATP release from rat urothelial cell cultures and significantly blocked by the PAC1 receptor selective antagonist, M65 [27]. The present studies suggest two possibilities by which CYP-induced cystitis increases ATP expression in the urinary bladder and cystometric fluid: 1) neuropeptide (Sub P and CGRP) evoked release of ATP and 2) ROS evoked ATP release. Current research is consistent with the suggestion that neuropeptide as well as ROS signaling are regulators of bladder physiology at the level of the urinary bladder and possibly, the
urothelium [27]. The present studies demonstrate that use of the antioxidant, Tempol, not only reduces the presence of oxidative stress markers in the urinary tract but also reduces modulators/mediators of inflammation including neuropeptides (CGRP, Sub P) also known to contribute to micturition reflex plasticity and dysfunction with CYP-induced cystitis. In the context of CYP-induced cystitis, Tempol treatment may be more beneficial given its broader impact on oxidative stress markers and other modulators of urinary bladder inflammation while also improving urinary bladder function.

The present studies demonstrate oxidative stress in the urinary tract following CYP-induced cystitis and improvement in urinary bladder function and markers of oxidative stress with antioxidant treatment; however, there are additional issues to be considered. Future studies may include: 1) determining the effects of Tempol after the induction of CYP-induced cystitis, rather than as a pretreatment, on urinary bladder function; 2) determining if Tempol treatment can reduce somatic sensitivity in the CYP model of urinary bladder inflammation with referred, somatic hypersensitivity; 3) determining the effects of Tempol in a more chronic model of CYP-induced urinary bladder inflammation that we have used extensively [23, 24]. The present studies demonstrate that CYP-induced cystitis results in oxidative stress in the urinary tract and that the antioxidant, Tempol, ameliorates CYP-induced bladder dysfunction. These studies suggest that pharmacological interventions directed at oxidative stress mediators/markers may be a promising strategy to address inflammation of the urinary tract and target organ dysfunction.
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Disclosures

The authors declare that there is no conflict of interest in this study.
References


Figure 1: CYP-induced cystitis increases reactive oxygen species (ROS)/reactive nitrogen species (RNS) and 3-nitrotyrosine (3-NT) in the urinary bladder and the antioxidant, Tempol, reduces expression.

A. 4 hr and 48 hr CYP-induced cystitis significantly (p ≤ 0.01) increased ROS/RNS expression in urothelium and detrusor that was significantly (p ≤ 0.01) reduced with Tempol. Basal expression and CYP-induced ROS/RNS expression and was significantly (p ≤ 0.01) greater in detrusor compared to urothelium. Tempol was without effect on ROS/RNS expression in urothelium and detrusor from control (no CYP) rats.

B. 4 hr and 48 hr CYP-induced cystitis significantly (p ≤ 0.01) increased ROS/RNS expression in urinary bladder that was significantly (p ≤ 0.01) reduced with Tempol. Tempol was without effect on 3-NT expression in urinary bladder from control (no CYP) rats. *, p ≤ 0.01. n=6 for control and treatment groups.
Figure 2: CYP-induced cystitis increases substance P (Sub P) in the urinary bladder and cystometric fluid and the antioxidant, Tempol, reduces expression

A. 4 hr and 48 hr CYP-induced cystitis significantly (p ≤ 0.01) increased Sub P expression in urinary bladder that was significantly reduced with Tempol. Tempol was without effect on Sub P expression in urinary bladder from control (no CYP) rats. B. 4 hr and 48 hr CYP-induced cystitis significantly (p ≤ 0.01) increased Sub P expression in cystometric fluid that was significantly (p ≤ 0.01) reduced with Tempol. Tempol was without effect on Sub P expression in cystometric fluid from control (no CYP) rats. *, p ≤ 0.01. n=6 for control and treatment groups.
Figure 3: CYP-induced cystitis increases calcitonin gene-related peptide (CGRP) in the urinary bladder and cystometric fluid and the antioxidant, Tempol, reduces expression.

A. 4 hr and 48 hr CYP-induced cystitis significantly (p ≤ 0.01) increased CGRP expression in urinary bladder that was significantly (p ≤ 0.01) reduced with Tempol. Tempol was without effect on CGRP expression in urinary bladder from control (no CYP) rats. B. 4 hr and 48 hr CYP-induced cystitis significantly (p ≤ 0.01) increased CGRP expression in cystometric fluid that was significantly (p ≤ 0.01) reduced with Tempol. *, p ≤ 0.01. n=6 for control and treatment groups.
Figure 4: CYP-induced cystitis increases adenosine triphosphate (ATP) in the urinary bladder and cystometric fluid and the antioxidant, Tempol, reduces expression

A. 4 hr and 48 hr CYP-induced cystitis significantly (p ≤ 0.01) increased ATP expression in urinary bladder that was significantly (p ≤ 0.01) reduced with Tempol. Tempol was without effect on ATP expression in urinary bladder from control (no CYP) rats. B. 4 hr and 48 hr CYP-induced cystitis significantly (p ≤ 0.01) increased ATP expression in cystometric fluid that was significantly (p ≤ 0.01) reduced with Tempol. Tempol was without effect on ATP expression in cystometric fluid from control (no CYP) rats. *, p ≤ 0.01. n=6 for control and treatment groups.
Figure 5: Summary histograms of the effects of Tempol (1 mM) on intercontraction interval (ICI; s), bladder capacity (BC; µl) and void volume (VV; ml) in CYP-treated (4 hr; 48 hr) rats.

Tempol in the drinking water significantly (p ≤ 0.01) increased ICI (A), VV (B) and BC (C) in CYP-treated (4 hr and 48 hr) rats. Tempol was without effect in control (no CYP treatment) rats. *, p ≤ 0.01. Sample sizes are n = 6 in control and treatment groups.
Figure 6: Tempol in the drinking water was without effect on baseline pressure (A; BP), threshold pressure (B; TP) or peak micturition pressure (C; MP; cm H2O) in control or CYP-treated (4 hr; 48 hr) rats

Sample sizes are n = 6 in control and treatment groups. *, p ≤ 0.01 compared to control + vehicle (between-group difference).
A,B: Bladder function in a CYP-treated (4 hr) rat without Tempol (vehicle only; A1-A3) and in a CYP-treated (4 hr) rat with Tempol (1 mM in the drinking water; B1-B3) during continuous intravesical infusion of saline. Bladder function recordings in A and B are from different rats. Infused volume (IF, µl; A1, B1), bladder pressure (BP, cm H2O; A2, B2), and void volume (VV, ml; A3, B3) with vehicle (A1-A3) and with Tempol treatment (B1-B3) are shown. Arrows (A2, B2) indicate examples non-voiding contractions that were significantly (p ≤ 0.01) reduced in number and amplitude in CYP-treated (4 hr) rats with Tempol.
Figure 8: Representative cystometrogram recordings using continuous intravesical infusion of saline in conscious rats with an open outlet from a CYP-treated (48 hr) rat with vehicle (A1-A3) and a CYP-treated (48 hr) rat with Tempol (1 mM; B1-B3).

A,B: Bladder function in a CYP-treated (48 hr) rat without Tempol (vehicle only; A1-A3) and in a CYP-treated (48 hr) rat with Tempol (1 mM in the drinking water; B1-B3) during continuous intravesical infusion of saline. Bladder function recordings in A and B are from different rats. Infused volume (IF, µl; A1, B1), bladder pressure (BP, cm H2O; A2, B2), and void volume (VV, ml; A3, B3) with vehicle (A1-A3) and with Tempol treatment (B1-B3) are shown.