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Elevated hydrostatic pressure stimulates ATP release which mediates activation of the NLRP3 inflammasome via P2X₄ in rat urothelial cells

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Abstract

Partial bladder outlet obstruction (pBOO) is a prevalent urological condition commonly accompanied by increased intravesical pressure, inflammation and fibrosis. Studies have demonstrated that pBOO results in increased NLRP3 inflammasome and caspase-1 activation and that ATP is released from urothelial cells in response to elevated pressure. In the present study, we investigated the role of elevated pressure in triggering caspase-1 activation via purinergic receptors activation in urothelial cells. Rat urothelial cell line, MYP3 cells, were subjected to hydrostatic pressures of 15 cmH₂O for 60 minutes, or 40 cmH₂O for 1 minute to simulate elevated storage and voiding pressure conditions, respectively. ATP concentration in the supernatant media and intracellular caspase-1 activity in cell lysates were measured. Pressure experiments were repeated in the presence of antagonists for purinergic receptors to determine the mechanism for pressure-induced caspase-1 activation. Exposure of MYP3 cells to both pressure conditions resulted in an increase in extracellular ATP levels and intracellular caspase-1 activity. Treatment with P2X₇ antagonist led to a decrease in pressure-induced ATP release by MYP3 cells, while P2X₄ antagonist had no effect but both antagonists inhibited pressure induced caspase-1 activation. Moreover, when MYP3 cells were treated with extracellular ATP (500 μM), P2X₄ antagonist inhibited ATP-induced caspase-1 activation, but not P2X₇ antagonist. We concluded that pressure-induced extracellular ATP in urothelial cells is amplified by P2X₇ receptor activation and ATP-induced-ATP release. The amplified ATP signal then activates P2X₄ receptors, which mediate activation of the caspase-1 inflammatory response.

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Keywords

hydrostatic pressure; NLRP3; inflammasome; inflammation; purinergic receptors; P2X4; P2X7; pressure mechanobiology

Introduction

Partial bladder outlet obstruction (pBOO) is a prevalent urological condition that is expected to affect approximately 1.1 billion people worldwide by 2018 [1]. Clinically, pBOO is defined in patients who demonstrated low urinary flow rates while concurrently demonstrating elevated voiding pressures. It occurs most commonly due to prostate enlargement in aging males or organ prolapse in females, but can also occur as a result of anatomic birth defects in the lower urinary tract or from functional obstruction in patients with neurological pathology. Partial obstruction of the bladder neck or urethra is commonly accompanied by inflammation, fibrosis, and reduced bladder compliance [2] and patients often experience lower urinary tract symptoms (LUTS) such as hesitancy, weak stream, and incomplete bladder emptying. The standard pharmacotherapy with α_1 -adrenergic (AR) antagonists has proven effective against LUTS by relaxing the prostate and/or bladder neck in which AR receptors are present [3]; however, AR antagonists do not abolish the underlying etiology generating the obstruction [4]. In many instances, persistent storage symptoms such as urinary frequency, urgency, and urge incontinence stemming from inflammation and reduced compliance of the bladder tissue remain and can even show progression. If the inflammation-driven damage to the bladder reaches a state where fibrosis and nerve damage occur, symptoms can persist even after removal of the obstruction via surgical intervention [5–8]. A number of studies using animal models have demonstrated that pBOO results in upregulation of pro-inflammatory cytokines such as IL-1 β and increased bladder weight [2, 9–11]. Based on these findings, we speculate that a link exists between pBOO-associated mechanical insults, such as elevated intravesical pressure, and inflammation of the bladder tissue; however, no study to date has ever examined a link between elevated pressure and activation of the inflammasome.

A urodynamic study conducted on 38 unobstructed and 96 pBOO human patients, categorized by endoscopic evaluation, determined the average detrusor pressure at bladder capacity to be 3–16 cmH₂O in unobstructed patients versus 3–25 cmH₂O in pBOO patients [12]. In the same study, the average detrusor pressure at maximum flow rate ($P_{\text{det}}Q_{\text{max}}$) during micturition was observed to be 17–48 cmH₂O for unobstructed patients versus 35–88 cmH₂O in pBOO patients [12]. A similar study conducted on surgically induced pBOO rat models determined that intraluminal pressure (IP) ranges between 7–12 cmH₂O in unobstructed rats and 7–16 cmH₂O in pBOO rats during storage periods, while obstruction of the urethra resulted in increasing voiding pressure from ~60 cmH₂O in unobstructed rats to ~90 cmH₂O in pBOO rats [13, 14]. Previous investigation into the role of IP in bladder physiology and pathophysiology demonstrated that ATP is released from rat urothelium *in vivo* upon distension of the bladder under IP of 10–30 cmH₂O [15, 16]. Moreover, studies have shown that ATP is released from primary mouse and rat urothelial cells *in vitro* upon the application of pressure (up to 20 cmH₂O) [17], distention (~127%) [18], or both (30

cmH₂O pressure to the bladder mucosa in an Ussing Chamber) [19]. Together, these results suggest that various mechanical insults present in the obstructed bladder may trigger biochemical events that lead to ATP release from the urothelium.

Upon its release from cells, ATP regulates numerous biochemical processes including inflammation and fibrosis [20, 21]. Riteau and colleagues established a correlation between elevated levels of extracellular ATP and fibrosis in a murine model of pulmonary fibrosis induced by airway-administered bleomycin (BLM) [20]. Treatment with ATP derivative ATP- γ S enhanced pulmonary tissue inflammation, while removal of extracellular ATP via administration of an ATPase (apyrase) reduced the upregulation of the inflammasome-dependent pro-inflammatory cytokine interleukin(IL)-1 β and decreased metalloprotease (TIMP)-1 production, both indicators of tissue inflammation [20]. Similarly, blood and liver tissue from lipopolysaccharide (LPS; a common stimulus for pathogen-associated inflammation)-treated mice displayed an upregulation in IL-1 β as well as tissue necrosis factor (TNF) and IL-10, while treatment with apyrase abolished the LPS-induced proinflammatory response [21]. These results indicate that extracellular release of ATP in response to various stimuli leads to activation of the inflammasome and fibrosis of the tissue through pathways involving pro-inflammatory cytokines such as IL-1 β .

Recently, Hughes et al. demonstrated that the NLRP3 inflammasome is an important mediator in regulating pBOO-associated inflammation and hypertrophy in rat bladder tissue [10]. After surgically inducing pBOO in rats, administration of an NLRP3 inhibitor, glyburide, blocked inflammasome activation and prevented inflammation of the bladder tissue [10]. The NLRP3 inflammasome can be activated by pathogen-associated molecular patterns (PAMPs) such as LPS and flagellin [22], or damage-associated molecular patterns (DAMPs) such as ATP [23]. Upon activation, the NLRP3 inflammasome functions by converting pro-caspase-1 to active caspase-1 which then processes pro-inflammatory cytokines such as IL-1 β and IL-18 [24]. Based on these findings to date, we hypothesized that elevated pressure of pBOO triggers extracellular ATP release, which activates caspase-1 via purinergic receptor activation leading to subsequent inflammation in urothelial cells. In the present study, we examined the mechanisms of pressure-induced purinergic signaling and NLRP3-mediated caspase-1 activation in a rat urothelial cell line *in vitro*.

Materials and Methods

Animals and Surgery

All animal protocols were approved by the Institutional Animal Care and Use Committee at Duke University Medical Center and performed in accordance with the guidelines in the NIH Guide for the Care and Use of Laboratory Animals. Sprague Dawley female rats ($\approx$ 200 g, $\approx$ 45–50 days old) (Harlan, Prattville AL) were used as previously described [10, 25, 26]. Animals were housed in the vivarium and maintained for 12 days until sacrificed for immunocytochemistry, as described below.

Urothelial Cell Culture

MYP3 cells (an immortalized non-tumorigenic rat urothelial cell line) were originally developed by Dr. Ryoichi Oyasu (Northwestern University, Chicago, IL) [27] and generously provided to us by Samuel M. Cohen through the lab of Lora L. Arnold, both at the University of Nebraska Medical Center, Omaha, NE [28, 29]. We have previously shown that they possess NLRP3 which is capable of activation by exogenous ATP [30]. These cells were cultured in F-12K media (HyClone Laboratories, Logan, UT) supplemented with 10% low-endotoxin dialyzed fetal bovine serum (HyClone Laboratories, Logan, UT), 10 μ M non-essential amino acids (HyClone Laboratories, Logan, UT), 1.0 μ g/ml hydrocortisone (Sigma-Aldrich, St. Louis, MO), 10 μ g/ml insulin (Gibco Laboratories, Gaithersburg, Maryland), 5 μ g/ml transferrin (Gibco Laboratories, Gaithersburg, MD), and 6.7 ng/ml selenium (Gibco Laboratories, Gaithersburg, MD).

Hydrostatic Pressure Experiments

A custom apparatus [31] was used to expose MYP3 cells to sustained hydrostatic pressure over designated time intervals. Briefly, the system consisted of a computer that ran a custom Lab View program (National Instruments Corporation, Austin, TX) with a pump and pressure transducer to monitor and regulate the pressurized environment within a closed chamber via opening and closing solenoid valves to allow for 95% air 5% CO₂ gas mixture to move into or out of the chamber as needed. The pressure chamber and control samples were housed within a cell culture incubator to maintain standard cell culture conditions of 37°C, 95% air and 5% CO₂. Previous research from our lab confirmed that the pH, pO₂, and pCO₂ of the culture media were not affected by the application of hydrostatic pressure at levels similar to those used in this study [31].

Cells were plated at 1.2×10^6 cells/well in sterile 6 well cell culture plates and incubated for 48 hours until the cells reached 90% confluence. The complete F-12K media was then replaced with serum free F-12K media and left to incubate an additional 18 hours to allow for the cells to become quiescent. 60 minutes prior to pressure exposure, the culture media was replaced with serum free F-12K media containing 100 μ M ATPase inhibitor ARL67156 (Tocris Bioscience, Bristol, UK) in order to prevent degradation of extracellular ATP before measurements could be taken. Media comprised of serum free media and ARL67156 was replaced immediately prior to exposure to hydrostatic pressure in order to minimize the effects of basal extracellular ATP levels on the results after exposure to pressure.

The cells were then subjected to hydrostatic pressure of either 15 cmH₂O for 60 minutes to simulate elevated storage pressure conditions, or 40 cmH₂O for 1 minute to simulate elevated voiding pressure. Determination of the voiding pressure used in the present study was calculated as the differential pressure between storage pressure and voiding pressure in pBOO rat models, which was determined to be ~40 – 60 cmH₂O based on previous studies [13, 14]. Cells prepared in a similar manner but maintained under atmospheric pressure served as a baseline control and cells exposed to a hypotonic condition, prepared by adjusting Hank's Balanced Salt Solution (Thermo Fisher Scientific, Waltham, MA) to 240 mOsm by diluting with dH₂O, for 60 minutes served as a positive control. Following

exposure, the media was collected for ATP quantification and the cells were lysed to determine the intracellular caspase-1 activity.

Inhibition Experiments

Pressure experiments were repeated in the presence or absence of antagonists for purinergic receptors, vesicular exocytosis, and ion channels before exposure to hydrostatic pressure (Table 1). Cells were subjected to drug pretreatment for 60 minutes, then fresh media comprised of serum free media, ARL67156, and purinergic antagonists was added immediately prior to exposure to hydrostatic pressure.

ATP Dose Response Determination

Cells were plated under complete F-12K media at 50,000 cells/well in sterile black walled 96-well plates and cultured 24 hours until 90% confluent. The media were replaced with serum free F-12K media and incubated overnight to allow the cells to become quiescent. Cells were incubated with ATP disodium salt (Sigma-Aldrich, St. Louis, MO) in a range of concentrations (500, 250, 50, 25, 5, 2.5, and 0 μ M) under standard culture conditions for 60 minutes. The cells were lysed and the intracellular caspase-1 levels were quantified as described below.

ATP quantification

ATP concentration in the supernatant media was measured using a commercially available luciferin-luciferase assay kit (Life Technologies, Carlsbad, CA) and following the manufacturer's instructions. Briefly, 90 μ L of ATP standard reaction solution (SRS) containing 8.9 mL dH₂O, 0.5 mL 20x reaction buffer, 0.1 mL of 0.1 M DTT, 0.5 mL of 10 mM D-luciferin, and 2.5 μ L of firefly luciferase 5 mg/mL stock, was added to each well along with 10 μ L of supernatant. The luminescence values of the media were measured using a Tecan Genios microplate reader (Tecan US, Inc., Durham, NC). The raw luminescence values were adjusted by subtracting out the background luminescence, determined by measuring 100 μ L of SRS containing no supernatant. The data in each individual experiment were normalized to the value of the no-pressure, no-drug control group for that experiment and reported as the fold-change in ATP concentration.

Caspase-1 Activity Determination

Intracellular caspase-1 activity was measured as previously described [10, 13, 30]. Briefly, following removal of the culture media, cells were lysed in 250 μ L of lysis buffer (10 mM MgCl₂ and 0.25% Igepal CA-630) for 5 minutes, then 250 μ L of storage buffer (40 mM HEPES (pH 7.4), 20 mM NaCl, 2 mM EDTA and 20% glycerol) was added. The cell lysates were frozen at -80°C until used in the assay. For each sample, 20 μ L of cell lysate was combined with 50 μ L assay buffer containing 25 mM HEPES, 5% sucrose and 0.05% CHAPS (pH 7.5), 10 μ L 100 mM dithiothreitol and 20 μ L 1 mM Z-YVAD-AFC in a black walled 96-well plate. Fluorescence (excitation 400 and emission 505 nm) was measured using Tecan Genios microplate reader. Raw fluorescence values were adjusted by subtracting the background fluorescence then normalized to the value of the no-pressure, no-

drug control group from that experiment and recorded as the fold-change in caspase-1 activity.

Immunohistochemistry

For analysis of tissue sections, animals were sacrificed one at a time by overdose of isoflurane and the bladders immediately removed and placed in cold PBS on ice for 5–10 minutes. They were then fixed in 10% neutral buffered formalin overnight. They were then stored in 70% ethanol before being embedded in paraffin and sectioned (5 μ m). Transverse cross-sections from the lower one-third of the bladder were used for staining. Sections were deparaffinized, rehydrated with a series of graded alcohols and subjected to citrate-based antigen retrieval (14 min in a high temperature pressure cooker submerged in Enzo Cat#ADI-950-270-0500 antigen retrieval reagent).

For analysis of MYP3 cells the cells were plated at 100,000 cells per well in 0.5 ml cell culture media in 8 well BD Falcon CultureSlides (Cat # 354118; BD Biosciences, Franklin Lakes, NJ). Following an overnight incubation to allow the cells to adhere they were fixed with 10% neutral buffered formalin for 10 min at room temperature and rinsed in 70% ethanol followed by PBS.

Both tissues sections and culture slides were subsequently blocked for 30 min with normal goat serum (5%) followed by incubation with primary (overnight at 4°C) and secondary (1 h at room temperature) antibodies using standard techniques. The primary antibodies used were rabbit anti-P2X₄ (Lifespan Biosciences, Seattle, WA; catalog# LS-C501672/105989; 1:100 dilution) and rabbit anti-P2X₇ (Thermo Fisher Scientific, Waltham, MA; catalog# PA5-28020; 1:100 dilution). The secondary antibody used was a goat anti-rabbit IgG conjugated to Alexa Flour 488 (SouthernBiotech, Birmingham, AL; catalog# 4050-30; 1:500 dilution). The resulting stained slides were visualized using a Zeiss Axio Imager 2 microscope (20x: Carl Zeiss AG, Oberkochen, Germany) running Zen software (Zeiss).

Statistical Analysis

Each experiment was performed in triplicate, with a minimum of eight repetitions (n=8) and inhibition experiments each had six repetitions (n = 6). The mean values of the data from the repetitions were calculated using SAS software (SAS Institute, Cary, NC) and compared using single-factor analysis of variance (ANOVA). When a statistically significant difference was displayed, a *post hoc* pairwise analysis was conducted using the Tukey-test. P-values less than 0.05 were considered statistically significant.

Results

pBOO causes elevated voiding pressure coupled with low urinary outflow upon voiding, eventually leading to decreased tissue compliance and decompensation resulting in elevated storage pressure during bladder filling [38]. In the present study, MYP3 cells were exposed to hydrostatic pressure that simulated elevated storage (15 cmH₂O for 60 minutes) or voiding (40 cmH₂O for 1 minute) pressures and, under both conditions, responded with a significant release of ATP into the extracellular environment (Figure 1A). Treatment with a known stimulator of urothelial ATP release, 240 mOsm hypotonic solution, for 60 minutes

also resulted in significant ($p < 0.05$) release of ATP from MYP3 cells, producing extracellular ATP levels in response to osmotic cell swelling similar to those seen in pressure treated groups (Figure 1A). Concomitant with ATP release, exposure of MYP3 cells to both time/pressure combinations resulted in a significant increase in intracellular caspase-1 activity (Figure 1B).

To determine if a direct connection exists between ATP released into the extracellular environment and caspase-1 activation, MYP3 cells were exposed to increasing concentrations of ATP resulting in a dose dependent increase in intracellular caspase-1 activity (Figure 2A). To determine the response to the application of hydrostatic pressure, pressure experiments were repeated with a cocktail of inhibitors for vesicular exocytosis of ATP, Brefeldin A, and its release via ion transporters, Probenecid, in conjunction with an extracellular ectoATPase, Apyrase. Extracellular ATPase inhibitor, ARL67156, was omitted from the cocktail to prevent inhibition of Apyrase. Treatment of MYP3 cells with this cocktail resulted in significant ($p < 0.05$) reduction in extracellular ATP concentration in both the control and cells exposed to 40 cmH₂O for 1 minute (Figure 2B). Moreover, intracellular caspase-1 activity did not increase in the cocktail-treated cells exposed to pressure (Figure 2C).

Examination of various purine receptors involved in NLRP3 activation indicated that P2X₇ and P2X₄ receptors are involved in inflammasome activation in various tissue types [4, 5, 13, 20, 39–43]. Immunostaining of rat bladder tissue as well as MYP3 cells indicated that both P2X₇ and P2X₄ receptors are expressed both intracellularly and on the surface of the rat bladder urothelium and cultured MYP3 cells (Figure 3). These results are consistent with previous reports of intracellular and surface expression of P2X₇ [33] and other purinergic receptors [44]. To assess a possible role for P2X₇ and P2X₄ receptors in hydrostatic pressure-induced caspase-1 activation, cells were pre-treated with non-specific, P2X₇, or P2X₄ purinergic antagonists for 60 minutes before exposure to hydrostatic pressure (40 cmH₂O for 1 min). Cells treated with various antagonists and maintained under atmospheric pressure (0 cmH₂O for 60 min) displayed similar extracellular ATP concentration or intracellular caspase-1 activity compared to the non-treated control (Figure 4). Treatment of MYP3 cells with a non-specific purinergic antagonist, PPADs, or with a purinergic receptor antagonist specific for P2X₇ [45], A-438079, attenuated the pressure-induced ATP release and activation of caspase-1 (Figure 4). In contrast, treatment of MYP3 cells with a P2X₄ specific antagonist, 5-BDBD, did not alter the ATP release, but intracellular caspase-1 levels were reduced compared to the non-treated control (Figure 4). These results indicate that P2X₇ receptors may mediate ATP-dependent ATP release, while P2X₄ receptors may mediate ATP-induced NLRP3 activation downstream of P2X₇ activation.

To further examine the different roles of different purinergic receptors, MYP3 cells were treated with extracellular ATP (500 μ M ATP disodium salt solution) in the presence and absence of purinergic antagonists to demonstrate that activation of caspase-1 is blocked by P2X₄ specific antagonists, but not P2X₇ (Figure 5). Treatment of MYP3 cells with the P2X₇ antagonist had no effect on the caspase-1 levels compared to the no-drug treated group. The addition of the non-specific purinergic inhibitor resulted in a significant change in caspase-1 activity compared to the no-drug treated control at a concentration of 500 μ M ATP, while the

addition of a P2X₄ purinergic inhibitor resulted in a significant change in caspase-1 activity compared to the no-drug treated group at a 500 μ M ATP concentration (Figure 5).

Discussion

Although inflammation, hypertrophy, and eventual decompensation, fibrosis, and denervation of bladder tissue have been associated with pBOO [2], its mechanism has yet to be completely elucidated. In the present study, we hypothesized that elevated pressure triggers extracellular ATP release, which activates purinergic receptors mediating caspase-1 activation in urothelial cells. Using a custom experimental setup, a monolayer culture of MYP3 cells were exposed to sustained hydrostatic pressure at 15 cmH₂O for 60 minutes or 40 cmH₂O for 1 minute to represent the urothelium under elevated storage and voiding pressures, respectively. Following exposure to pressure, ATP in the media and intracellular caspase-1 activation were both significantly increased (Figure 1). In addition, exposure of MYP3 cells to hypotonic buffer (240 mOsm) for 60 minutes also triggered ATP release and caspase-1 activation (Figure 1). The result of ATP release in response to hypotonic cells swelling is in agreement with a number of previous studies [19, 46–48]; however, the present study demonstrated for the first time that hypotonic cell swelling also results in caspase-1 activation (Figure 1B) and provided additional evidence that elevated extracellular ATP concentration can lead to the inflammatory response. These results indicate that hypotonic cell swelling may be a good model for studying the effects of cell membrane stretch, but under normal physiological conditions or in obstructed bladders the cells are not subjected to hypotonic conditions. Within the bladder lumen, urothelial cells are constantly exposed to hydrostatic pressure for containment of fluid, and the intraluminal pressure rises 3- to 6- fold [12] during voiding due to muscle contraction. We previously demonstrated that after exposure to 10 cmH₂O for 5 and 10 minutes and 15 cmH₂O for 5 minutes primary urothelial cells exhibited a significant increase in extracellular ATP concentration [17]. In addition to urothelial cells, other cell types including retinal ganglion [49] and lung epithelium [50] have been shown to release ATP in response to elevated hydrostatic pressure. These reports support the concept that pressure is a stimulus that can induce ATP release thru various mechanisms including pannexin and/or connexin hemichannels [49–51], vesicular exocytosis [51], and ATP-binding cassette (ABC) family proteins [51]. The results of the present study suggest that short-term exposure of urothelial cells to elevated pressure can also induce inflammatory responses. As high voiding pressure is an early indicator of pBOO, and commonly used for diagnoses (when coupled with a decrease in maximum flow rate [38]), it may be responsible for initiating the inflammatory response in the urothelium and therefore a precursory event to sustained elevated storage pressure. For this reason, a condition that simulated elevated voiding pressure (40 cmH₂O 1 minute) was used for the remaining pressure experiments conducted in the present study. Still, our finding that long-term exposure of urothelial cells to a lower-pressure stimulus results in ATP release and increased caspase-1 activity is important as it potentially further implicates storage pressure in pBOO disease progression, and provides a working theory for the ever-present basal level of inflammasome activity found in the urothelium [13].

The results of the present study indicate that urothelial cells are sensitive to hydrostatic pressure and respond by releasing ATP, an established DAMP, and this ATP is responsible

for activating caspase-1. When MYP3 cells were exposed to increasing concentrations of ATP, a dose dependent increase in caspase-1 activity was observed (Figure 2A), as previously demonstrated [30]. Moreover, pharmacological inhibition of ATP release and enzyme digestion of ATP in MYP cell cultures diminished both extracellular ATP levels (Figure 2B), and pressure-induced activation of caspase-1 (Figure 2C). The maximum increase in caspase-1 activity to the ATP dose-response (Figure 2A) is lower than that induced by pressure stimulation (Figure 2C). We speculate that this may be due to the use of purinergic agonist ATP-disodium salt to stimulate caspase-1 activity. It has been reported that ATP-disodium salt generates a slightly acidic environment when dissolved in cell culture media, which results in decreased intracellular Ca^{2+} and increased intracellular K^{+} levels compared to treatment with a neutral pH ATP solution [52]. As purinergic receptor activation results in Ca^{2+} influx coupled with K^{+} efflux [53], it is likely that the slight pH shift of the media has an effect of purinergic receptors resulting in lower caspase-1 activity. Since purinergic receptors, especially P2X_7 [20, 39, 54–59] and P2X_4 [40, 54], have been implicated in inflammasome activation, we focused on examining the role of these channels in ATP release and caspase-1 activation in response to hydrostatic pressure. The results of the present study provided evidence that treatment of MYP3 with the non-specific purinergic inhibitor PPADs attenuated the pressure-induced increase in extracellular ATP and intracellular caspase-1 activity (Figure 4), indicating the role of purinergic receptors in both of these events. Similarly, inhibition of the P2X_7 receptor with a specific inhibitor A-438079 resulted in a reduction in pressure-induced ATP release and caspase-1 activation (Figure 4). While not significant due to the current method of detection, there also appears to be a small increase in extracellular ATP in cells exposed to pressure in the presence of A-438079 when compared to cells treated with P2X_7 receptor antagonist and maintained under atmospheric pressure (+0 cmH_2O). In contrast, inhibition of P2X_4 with 5-BDBD did not affect extracellular ATP levels in response to pressure, but did result in a decrease in caspase-1 activation (Figure 4). These results suggest that P2X_7 receptors mediate ATP-induced ATP release in response to pressure and P2X_4 receptors mediate the activation of caspase-1 downstream of ATP release.

The different roles of P2X_7 and P2X_4 in the propagation of signals vital to the inflammatory response in the bladder were supported further by the results of the present study. MYP3 cells treated with a P2X_4 inhibitor prior to exposure to a 500 μM ATP solution reduced caspase-1 activity (Figure 5). However, inhibition of P2X_7 did not affect ATP-induced caspase-1 activity (Figure 5). Many studies have concluded that P2X_4 receptors interact with other purinergic receptors, including P2X_2 , 4, 6, & 7, to make heterodimers or heterotrimers upon activation [60–62]. The interconnected nature of purinergic receptors is important to consider as various purinergic receptors may be involved in regulating ATP-induced caspase-1 activity. While it is often considered that activation of NLRP3 inflammasome *in vitro* requires priming step with lipopolysaccharide (LPS) [63–65], it is not the case for all cell types [66]. The results of the present study and a previous report by Hughes and colleagues both demonstrated that urothelial cells appear to have no requirement for priming with LPS prior to activation of the inflammasome [67]. These findings shed some light on the mechanisms by which different purinergic receptors present in the bladder urothelium and other organ systems are involved in inflammatory responses. A number of previous

studies have argued that P2X₇ receptor is the lone activator of the NLRP3 inflammasome [43, 68–70]. While our results are in agreement with the theory that P2X₇ receptors play an important role in NLRP3 activation, as is evident by the lack of caspase-1 activity upon treatment with a P2X₇ antagonist (Figure 4B), our study also implicates P2X₄ receptors in pressure induced NLRP3 activation. Currently it is believed that small amounts of ATP are released from the urothelium in response to hydrostatic pressure via similar mechanisms demonstrated in bovine retina exposed to hydrostatic pressure [49] and in the bladder urothelium during the filling stage [42]. The results of the present study suggest that released extracellular ATP then binds to P2X₇ receptors, which initiates further release of ATP and amplification of the extracellular ATP signal. The amplified ATP signal then acts on P2X₄ receptors, which mediate activation of the caspase-1 inflammatory response (Figure 6). Our proposed concept that P2X₄ receptors mediate NLRP3 activation downstream of P2X₇ may provide an explanation for a previous report that active NLRP3 was present in P2X₇ receptor knockout mice [68]. Moreover, as the NLRP3 inflammasome plays an important role in inflammatory responses from a wide variety of tissues including the lung [71], cerebral cortex [72], and myocardium [73], this mechanically triggered, purinergic-dependent inflammatory paradigm may be applicable beyond bladder disease alone.

Conclusions

The present study demonstrated for the first time that elevated hydrostatic pressure can activate the caspase-1 response in urothelial cells, which is controlled by a purinergic dependent mechanosensory pathway. Based on these results it can be speculated that elevated voiding pressure resulting from pBOO triggers inflammation of the urothelium, which has been shown in other studies to result in reduced bladder compliance, bladder dysfunction and eventually decompensation, fibrosis, and denervation. Chronic exposure to elevated storage pressure may then exacerbate the conditions by furthering inflammation in the bladder. Our results also indicate inhibition of P2X₇ and P2X₄ receptors in the urothelium as potential targeted treatment options for reducing pressure-induced inflammation. However, further study is still required to determine the exact mechanisms of hydrostatic pressure-induced caspase-1 activation and the effects of P2X₇ and P2X₄ inhibition on normal bladder function.

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Fig 1. Exposure to hydrostatic pressure increases extracellular ATP concentration and caspase-1 activity

MYP3 cells were exposed to 15 cmH₂O for 60 minutes and 40 cmH₂O for 1 minute to simulate elevated storage and voiding pressure respectively. After 60 minutes total incubation time the supernatant media was removed and the ATP concentration was determined (A). Cells were lysed and the lysates were measured for caspase-1 activity as described in the material and methods section (B). Cells exposed to atmospheric pressure (0 cmH₂O for 60 min) served as a negative control, while cells exposed to a 240 mOsm hypotonic solution served as a positive control. Fold change was determined by normalizing the data by the 0 cmH₂O control. Results are the mean $\pm$ SEM. Brackets above the graph indicate group comparisons and their level of significance. All groups were compared using one-way ANOVA followed by a post-hoc Tukey-test. * $P < 0.05$ and ** $P < 0.01$, $n = 14$ except for 15 cmH₂O 60 minute group where $n = 8$.

Fig 2. Inhibition of ATP release and the addition of an ectoATPase removes extracellular ATP and prevents pressure induced caspase-1 activation

MYP3 cells were exposed to varying concentrations of ATP disodium salt and left to incubate for 60 minutes. After exposure, cells were lysed and lysates were measured for caspase-1 activity (A). MYP3 cells were divided into two groups: 1) not treated with inhibitors of ATP release and ectoATPase, and 2) pretreated with 1 unit/mg Apyrase, 100 μM Probenecid, and 10 μg/mL Brefeldin A for 60 minutes prior to being exposed to 40 cmH₂O for 1 minute in the presence of the aforementioned antagonists. After exposure to pressure the supernatant media was removed and the ATP concentration was determined (B). Cells were lysed and the lysates were measured for caspase-1 activity (C). Cells exposed to atmospheric pressure (0 cmH₂O for 60 min) served as a control. Fold change was determined by normalizing the data by the 0 cmH₂O + no drug control. Results are the mean ± SEM. Brackets above the graph indicate group comparisons and their significance. All

groups were compared using a one-way ANOVA followed by a post-hoc Tukey-test.
*P<0.05, n=4.

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Fig 3. Immunofluorescence staining of rat bladder sections and MYP3 cells for P2X₇ and P2X₄ purinergic receptors

Tissue sections of the rat bladder from healthy rats along with MYP3 cells were stained with antibodies to P2X₇ or P2X₄ purinergic receptors as described in the materials and methods sections or with normal rabbit serum substituting for the primary antibodies (isotype controls). Brackets demarcate the urothelial layer while arrows point to the smooth muscle. All images were taken at 20x magnification.

Fig 4. Inhibition of purinergic receptors reduces pressure induced extracellular ATP concentration and caspase-1 activation

MYP3 cells were pretreated with 10 μ M A-438079, 20 μ M 5-BDBD, 25 μ M PPADs or no drug for 60 minutes before being exposed to 40 cmH₂O for 1 minute in the presence of the aforementioned antagonists. After exposure to pressure the supernatant media was removed and the ATP concentration was determined (A). Cells were lysed and the lysates were measured for caspase-1 activity (B). Cells exposed to atmospheric pressure (0 cmH₂O for 60 min) served as a control. Fold change was determined by normalizing the data by the 0 cmH₂O + no drug control. Results are the mean $\pm$ SEM. Brackets above the graph indicate group comparisons and their significance. All groups were compared using a one-way ANOVA followed by a post-hoc Tukey-test. * $P < 0.05$ and ** $P < 0.01$, $n = 8$.

Fig 5. ATP-induced caspase-1 response

MYP3 cells were pretreated with 10 μ M A-438079, 20 μ M 5-BDBD, or no drug for 60 minutes before being exposed to a 500 μ M ATP disodium salt solution and left to incubate for 60 minutes. Cells were then lysed and the lysates were measured for caspase-1 activity. Fold change was determined by normalizing the data by the 0 μ M ATP + no drug control. Results are the mean $\pm$ SEM. Data is displayed on a log base 10 scale. * $P < 0.05$, $n = 6$.

Fig 6. Mechanisms of pressure-induced ATP release and NLRP3 inflammasome activation

Upon exposure to hydrostatic pressure, small amounts of ATP are released from the urothelium via Pannexin-1 hemi-channels and vesicular exocytosis. Released extracellular ATP then binds to P2X₇ receptors, which initiates amplification of the extracellular ATP signal via ATP induced ATP release. The amplified ATP signal then acts on P2X₄ receptors which mediate activation of the caspase-1 inflammatory response.

Table 1

Drug	Concentration	Action
5-BDBD (Tocris Bioscience, Bristol, UK)	20 μ M	P2X ₄ antagonist [32]
A-438079 (R&D Systems, Minneapolis, MN, USA)	10 μ M	P2X ₇ antagonist [33]
Pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid (PPADS) (Tocris Bioscience, Bristol, UK)	25 μ M	Non-specific purinergic antagonist [34]
Apyrase (Sigma-Aldrich, St. Louis, Missouri, USA)	1 U/mL	ATPase [35]
Brefeldin A (Sigma-Aldrich, St. Louis, Missouri, USA)	10 μ g/mL	Vesicular exocytosis inhibitor [36]
Probenecid (Sigma-Aldrich, St. Louis, Missouri, USA)	100 μ M	Ion channel antagonist [37]