Research Article

Effect of pro-inflammatory cytokines on urothelial cell adenosine triphosphate release and breakdown

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Abstract

Objectives: Urinary symptoms of urgency, frequency, and pain are thought to be the result of inflammation in several bladder pathologies although the cause of these symptoms remains uncertain. Extracellular adenosine triphosphate (ATP) released from the bladder urothelium during normal bladder stretch is believed to bind to purinergic receptors on afferent nerves to signal bladder sensation. This study examined pro-inflammatory cytokines in the urine of women with detrusor overactivity (DO) with or without urinary tract infection (UTI) compared to controls and then determined the effect of pro-inflammatory cytokines on ATP signaling (release and breakdown) from the urothelium. **Methods:** The urinary concentrations of interferon-gamma (IFN-γ), tumor necrosis factor-alpha (TNF-α), and interleukin-1 beta (IL-1β) were determined in women with DO with or without UTI compared to female controls. The effect of pro-inflammatory cytokines (IFN-γ, TNF-α, and IL-1β) on control and hypotonic-induced ATP release using human UROtsa urothelial cells was examined, as was the effect of these cytokines on nucleotide (ATP, adenosine diphosphate and adenosine monophosphate) breakdown. **Results:** Urinary concentrations of IFN-γ, TNF-α, and IL-1β were increased in women with DO and UTI. Pre-treatment of urothelial cells with individual cytokines stimulated a decrease rather than an increase in ATP release whereas pre-treatment with a cocktail of all three cytokines stimulated a small but significant increase in hypotonic-induced ATP release. Pre-treatment of urothelial cells with cytokines significantly enhanced nucleotide breakdown. **Conclusion:** Using a simple cell culture model we have demonstrated that the response of the urothelium to pro-inflammatory cytokines is complex, affecting both release and breakdown of ATP.

Keywords: Urothelium, Extracellular adenosine triphosphate, Inflammation, Cytokine, Ectonucleotides

1. INTRODUCTION

Inflammation is involved in the etiology of several bladder pathologies, including detrusor overactivity (DO) and urinary tract infections (UTIs). Inflammatory cell infiltration has been demonstrated in patients with DO [1,2] and, pyuria, the presence of white blood cells in the urine, is a characteristic finding in patients with UTI [3] and is an indicator of active infection [4,5]. Inflammation of the urinary bladder is commonly associated with symptoms, including urinary urgency, frequency, and pain in bladder filling [6]. While the underlying cause of these symptoms remains uncertain, the sensitization of bladder afferent nerves has been demonstrated in UTI [7], with inflammatory cytokines inducing hyperactivity of afferent nerves [8], and is proposed as a pathological mechanism for urgency [9].

One of the main signaling molecules known to activate sub-urothelial afferent nerves is extracellular adenosine triphosphate (ATP) [10]. Much research over the last two decades has demonstrated the importance of extracellular ATP, released from the urothelial cell layer, during normal bladder stretch [11-13]. ATP has been shown to bind to purinergic receptors located on the sub-urothelial afferent nerves [14] and myofibroblasts [15] to signal bladder filling. Increased ATP release was demonstrated in tissue models of inflammatory bladder pathologies [16-18].

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The effect of ATP and other extracellular nucleotides (adenosine diphosphate [ADP], UTP, and UDP) is terminated by cell surface enzymes called ectonucleotidases [19]. For instance, CD39, an ectoATPDase important in inflammation, converts ATP to ADP, and ADP to adenosine monophosphate (AMP), with the release of an inorganic phosphate at each step in the pathway [20]. Little is known about the expression of ectonucleotidases in the bladder but mRNA of ectonucleotidases (CD39) has been found in cancerous urothelial cell lines [21].

Laboratory studies using human urinary tract epithelial cells demonstrated that a cocktail of pro-inflammatory cytokines (including interleukin-1 alpha, tumor necrosis factor-alpha [TNF-α], and interferon-gamma [IFN-γ]) were able to increase purinergic receptor expression in the urothelium [22], indicating an interaction between inflammation and the purinergic system. These cytokines have an important role in coordinating immune responses. Inflammatory cytokines can be produced by both immune cells, such as macrophages, and non-immune cells, such as urothelial cells [23].

Urinary levels of pro-inflammatory cytokines are increased under bladder conditions such as interstitial cystitis [24], overactive bladder [25,26], and UTI [27-30]. High amounts of interleukin-1 beta (IL-1β) in urine are associated with bacterial cystitis [28,31,32]. In addition, IL-1β mRNA and protein expressions were increased following acute cyclophosphamide-induced inflammation in a rat model [33]. Elevated urinary concentrations of IFN-γ [25,29,30] and TNFα [28] have been reported in patients with UTI. There are variable reports of changes in IFN-γ in other inflammatory bladder conditions with either no change [34] or significantly increased IFN-γ mRNA expression [35] reported in biopsies from patients with bladder pain syndrome/ interstitial cystitis. TNFα was elevated in response to lipopolysaccharide treatment *in vitro* [36]. It has been suggested that, in chronic inflammatory conditions, prolonged elevation of TNFα may predispose individuals to bladder cancer [37].

The aim of this study was to determine the concentration of three key pro-inflammatory cytokines, *i.e.*, IFN-γ, TNF-α, and IL-1β, in urine collected from control women and women with DO, with or without UTI. The second aim of this study was to examine the effect of these pro-inflammatory cytokines on urothelial cell ATP release and breakdown. As inflammation is associated with symptoms of urinary urgency and frequency, it was hypothesized that cytokine treatment would enhance urothelial cell ATP release.

2. METHODS

2.1. Participant selection and recruitment

Patients were recruited from a single urogynecology clinic over a 12-month period with approval obtained from the Ethics Review Committee (HREC18/G/219 South East Sydney Local Health District). Sixty-five post-menopausal women over 50 years of age with urodynamically proven idiopathic refractory DO were recruited. Refractory DO was defined as persistent urinary urgency and urge incontinence symptoms despite lifestyle modification, bladder training, and two anticholinergics for more than 1 year in conjunction with urodynamic findings of DO. Age-matched controls were also enrolled (n = 30). These included women with pure stress incontinence or pelvic organ prolapse, without urge incontinence symptoms. Women were excluded from this study if they were diagnosed with neurological diseases (i.e. Parkinson's disease/Multiple sclerosis), neurogenic DO, voiding dysfunction (defined as post-void residual >100 mL), interstitial cystitis/bladder pain syndrome, or any malignancy.

2.2. Sample collection and urinary cytokine measurement

Midstream urine samples were collected with careful labial toilets and refrigerated at 4°C immediately. Half of each sample was sent to the hospital microbiology department for routine culture. UTI was determined by the presence of a single bacterial species (>10^5 colony forming units/L), usually with pyuria >10 white blood cells/high power field [3]. All control patients were negative for UTI on the day of urine collection. The remaining half of the urine sample was centrifuged (160 g for 10 min) to remove exfoliated urothelial cells before being stored at −80°C.

Urinary cytokine concentrations were measured using a magnetic bead-based immunoassay kit (Bio-Plex Pro Human Th17 Cytokine Panel, Bio-Rad Laboratories Pty. Ltd, NSW Australia). Measurements were recorded on a Luminex-MAGPIX® multiplex reader using Luminex xPONENT software. While this kit provided data on the concentration of 16 individual cytokines, results for IFN-γ, TNF-α, and IL-1β are presented here. These three cytokines were selected for inclusion as they are core cytokines associated with the initiation of the innate immune response. They have been shown to impact urothelial purinergic expression [22] and are expected to be increased in patients with UTI [28].

2.3. Culture of urothelial cells

UROtsa cells (kindly provided by Dr. Catherine McDermott, Bond University, Gold Coast, Australia) were grown in low glucose Dulbecco's Modified Eagle Medium (DMEM) supplemented with 1% glucose, 5% fetal calf serum, 100 units/mL penicillin and 100 μg/mL streptomycin. Cells were incubated at 37°C (with 5% CO_2 and 95% air) with the culture medium changed every 2–3 days. Cells were passaged when confluent (approximately 4–7 days) using 0.5% trypsin, incubated for 5 min, and subsequently plated into T-75 flasks for continuous passage

or 24-well plates used for experiments. The UROtsa cell line is an immortalized cell line originally isolated from a primary culture of normal human urothelia. The cells display morphology consistent with the intermediate layers of the *in situ* urothelium [38].

2.4. ATP release studies

Confluent cells, in 24-well plates, were grown in serum-free DMEM overnight before an ATP release experiment. Cells were pre-treated with cytokines (IFN-γ and TNF-α at 25 ng/mL, and IL-1β at 5 ng/mL; Sigma Chemical Co., St Louis, MO, USA) for 10 min, 1 h, or 24 h. In some experiments, cells were pre-treated for 1 h with a cytokine cocktail, containing IFN-γ and TNF-α at 25 ng/mL and IL-1β at 5 ng/mL. Cytokine concentrations were chosen to match those described previously [22]. After pre-treatment, cells were washed three times with phosphate-free buffer (120 mM NaCl, 20 mM HEPES, 5 mM KCl, 10 mM glucose, 2 mM $CaCl_2$, 1 mM $MgSO_4$, pH 7.4) to remove culture medium which interferes with ATP measurements. ATP release was measured over 10 min in cells incubated in control (phosphate-free buffer) or hypotonic media (a 1:2 dilution of phosphate-free buffer with water) at 37°C. Ten minutes was chosen as the time point for ATP measurement as this has been used routinely for ATP determinations [39]. The concentration of ATP was quantified using a luciferin-luciferase assay (Sigma Chemical Co) as previously described [40].

2.5. Nucleotide breakdown studies

Confluent cells, cultured in 24-well plates, were washed three times with phosphate-free buffer and pre-treated for 1 h with the chosen cytokines (IL-1β, IFN-γ, and TNF-α at 25 ng/mL). Following cytokine pre-treatment, cells were incubated with ATP, ADP, or AMP (at 100 μM) (Sigma Chemical Co.) in phosphate-free buffer for 30 min at 37°C.

Phosphate liberated from nucleotides was determined as previously described [46]. Briefly, equal volumes of cell supernatant and color reagent (containing 1% ammonium molybdite, 0.3 mM H_2SO_4, and 4% $FeSO_4$) were mixed, and the absorbance at 750 nm was measured. Phosphate concentration in the cell supernatant was determined relative to a standard curve (KH_2PO_4, 10–150 nM) and results were expressed as the percentage of phosphate liberation compared to untreated (control) cells on the same plate [40].

2.6. Statistical analysis

Initial observations indicated that the concentrations of urinary cytokines were non-normal and positively skewed. Therefore, the log of the cytokine concentrations was used in all statistical comparisons. Comparisons between groups (control and DO or control and DO with UTI) were undertaken with a two-tailed unpaired Student's *t*-test. For the cell culture studies, experimental design and number of replicates were determined from previously published work [39-41]. All experiments were conducted in triplicate with the mean ATP concentration or phosphate liberation in the three wells calculated. At least four replicate plates were examined for each treatment ($n = 4$).

The hypotonic stimulus was used as a positive control for ATP release. After the initial series of experiments ($n = 20$), normality was confirmed using a Shapiro-Wilk normality test ($P = 0.59$ for control and $P = 0.42$ for hypotonic). The effect of cytokines on control or hypotonic ATP release was analyzed by a two-tailed paired Student's *t*-test compared to untreated UROtsa cells on the same 24-well plate.

3. RESULTS

3.1. Enhancement of cytokine release in women with bladder inflammation

Cytokines were detected in urine taken from controls ($n = 30$) and in women with DO, either with ($n = 20$) or without UTI ($n = 45$). The concentration of IFN-γ was significantly increased in DO women with UTI (Figure 1A, $P = 0.015$, Control 0.14 ± 0.07 pg; DO + UTI 0.44 ± 0.13 pg) and with DO without UTI (Figure 1D, $P = 0.030$, 0.85 ± 0.29 pg). Similarly, the concentration of TNF-α was significantly elevated in DO women with UTI (Figure 1B, $P = 0.029$, Control 0.34 ± 0.06 pg; DO = UTI 2.33 ± 1.54 pg) and with DO (Figure 1E, $P = 0.020$, 0.64 ± 0.13 pg). The concentration of IL-1β was significantly higher in women with UTI (Figure 1C, $P = 0.007$, Control 0.06 ± 0.01 pg; DO + UTI 0.64 ± 0.27 pg) but not in women with DO (Figure 1F, $P = 0.75$, 0.13 ± 0.07 pg).

3.2. Effect of cytokines on urothelial cell ATP release

Treatment with neither IFN-γ, TNF-α nor IL-1β stimulated the expected increase in urothelial cell ATP release (Table 1). In contrast, pre-treatment of urothelial cells with IFN-γ (25 ng/mL) for 10 min significantly decreased ATP release in both control (Figure 2A, $P = 0.0027$) and hypotonic media (Figure 2D, $P = 0.0104$). A 10-min pre-treatment with either TNF-α or IL-1β did not reduce ATP release in either control or hypotonic media (Figure 2). Similarly, pre-treatment of urothelial cells with cytokines for other time frames (1 h and 24 h) was unable to effect significant changes in ATP release induced by hypotonic media. A 1-h pre-treatment with IFN-γ significantly reduced ATP release in control cells (Table 1) and a 24-h pre-treatment with TNF-α significantly lowered control ATP release (Table 1).

3.3. Effect of a cytokine cocktail on urothelial cell ATP release

To determine if cytokines interact to elicit an increase in ATP release, urothelial cells were pre-treated with a cytokine cocktail [22]. In the control conditions, pre-treatment of urothelial

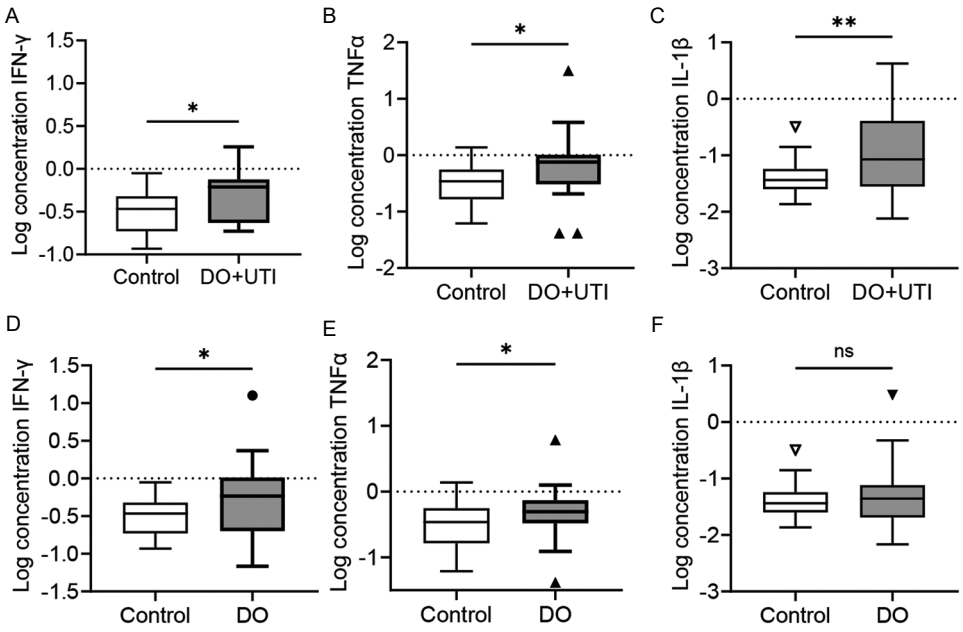

Figure 1. Enhanced release of cytokines into urine in women with detrusor overactivity (DO) with urinary tract infection (UTI) (n = 20, A-C) or DO (without UTI, n = 45, D-F) compared to control women (n = 30). IFN-γ (A and D) and TNF-α (B and E) were significantly increased in both patient groups. IL-1β was significantly increased in women with DO and UTI (C) but not in women with DO without UTI (F). Notes: *P < 0.05. Data represents mean cytokine concentration with standard error of the mean. IFN-γ: Interferon-gamma; IL-1β: Interleukin-1 beta; TNF-α: Tumor necrosis factor-alpha.

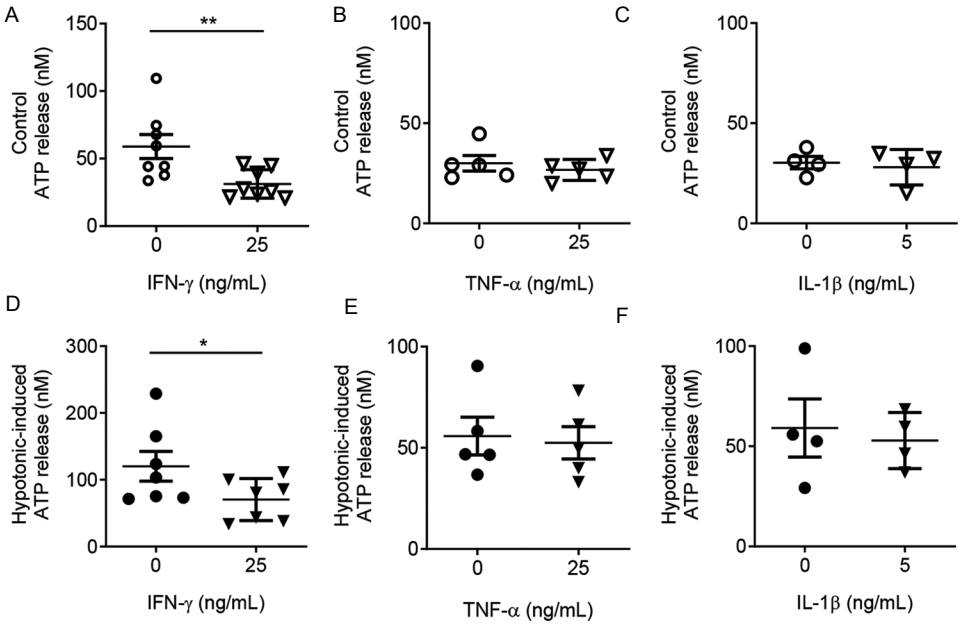

Figure 2. Adenosine triphosphate (ATP) release from untreated urothelial cells (○,●) and urothelial cells pre-treated with IFN-γ (A and D), TNF-α (B and E) or IL-1β (C and F) and for 10 min (▽,▼). ATP release was stimulated by control (A-C, open symbols) or hypotonic (D-F, filled symbols) media. ATP release significantly decreased in both control and hypotonic in cells pre-treated with IFN-γ (A and D). Notes: *P < 0.05, **P < 0.01 compared to untreated (0 ng/mL) cells (n = 4–8). Data represents mean ATP release with standard error of the mean. IFN-γ: Interferon-gamma; IL-1β: Interleukin-1 beta; TNF-α : Tumor necrosis factor-alpha.

cells with the cytokine cocktail had no effect on ATP release (Figure 3A). However, the cytokine cocktail was able to stimulate a slight but significant increase in hypotonic-induced ATP release (Figure 3B, P = 0.022, untreated mean 100 ± 5.031 nM, cytokine cocktail pre-treated mean 111 ± 3.5 nM, n = 4).

3.4. Effect of cytokines on urothelial cell nucleotide breakdown

Pre-treatment of urothelial cells with cytokines enhanced nucleotide breakdown (Figure 4). A significant increase in the

Table 1. Effect of cytokine pre-treatment, for three different time frames, on urothelial cell ATP release

Pre-treatment duration	Cytokine treatment					
	IFN-γ		TNF-α		IL-1β	
	0 ng/mL	25 ng/mL	0 ng/mL	25 ng/mL	0 ng/mL	5 ng/mL
10 min						
Control	58.9±8.9	31.3±3.7 (*P*=0.003)	30.0±3.9	26.7±2.3	30.3±3.1	28.0±4.4
Hypotonic	120.1±22.2	70.4±11.9 (*P*=0.01)	55.8±9.3	52.5±8.0	59.1±14.5	52.9±7.0
1 h						
Control	68.2±8.3	49.8±3.3 (*P*=0.05)	20.8±2.4	27.3±11.7	20.9±0.8	22.3±2.0
Hypotonic	160±27.7	163.6±24.6	45.2±10.7	66.8±29.4	48.5±4.377	44.0±4.6
24 h						
Control	33.68±6.8	29.0±1.8	66.7±9.1	53.3±9.2 (*P*=0.04)	27.8±4.9	24.7±3.8
Hypotonic	68.06±9.6	73.6±4.0	115.3±21.0	90.1±13.1	52.3±6.4	52.0±4.0

ATP: Adenosine triphosphate; IFN-γ: Interferon-gamma; TNF-α: Tumor necrosis factor-alpha; IL-1β: Interleukin-1 beta.

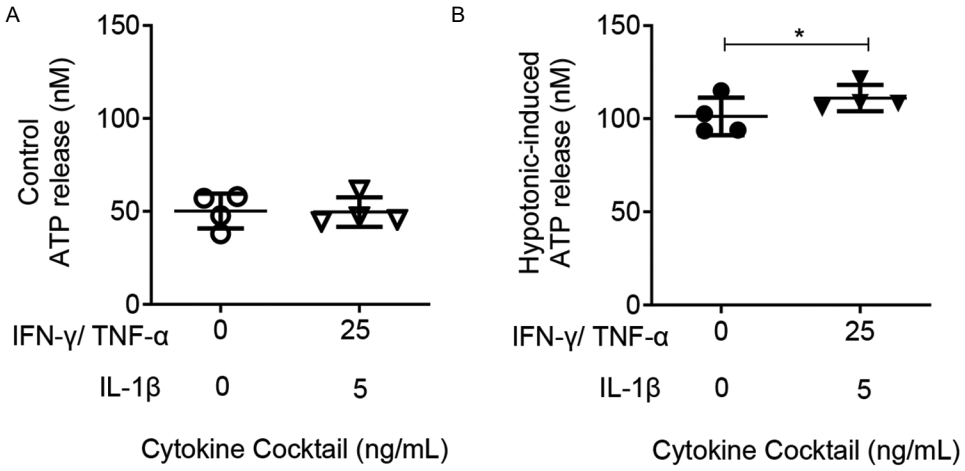

Figure 3. Adenosine triphosphate (ATP) release from untreated urothelial cells (○,●) and urothelial cells pre-treated with a cytokine cocktail (IFN-γ, TNF-α and IL-1β) for 24 h (▽,▼). ATP release was stimulated by control (A, open symbols) or hypotonic (B, filled symbols) media. The cytokine cocktail stimulated a slight but significantly increase in hypotonic-induced ATP release compared to no cytokines ($P = 0.02$, $n = 4$). Data represents mean ATP release with standard error of the mean. IFN-γ: Interferon-gamma; IL-1β: Interleukin-1 beta; TNF-α : Tumor necrosis factor-alpha.

capacity of urothelial cells to breakdown ATP was observed following pre-treatment with all three of the cytokines, i.e., IFN-γ, TNF-α, and IL-1β) (Figure 4A, $P < 0.05$). IL-1β pre-treatment also enhanced the breakdown of ADP (Figure 4B, $P = 0.0083$). None of the cytokines exerted any effect on AMP breakdown (Figure 4C).

4. DISCUSSION

Inflammation within the bladder is being increasingly recognized as an etiological factor of bladder pathology not only for UTI but also for refractory DO. Approximately, one-third of the refractory DO patients recruited for this study were found to have a current UTI. This was comparable to a previous finding that 25–40% of patients with urge incontinence had underlying infection [42-45]. This study demonstrated the inflammatory cytokines IFN-γ and TNF-α were elevated in DO patients with and without UTI, and IL-1β was increased in DO patients with UTI. This may reflect the well-described priming of cells by bacterial products to induce IL-1β synthesis before its release [46].

The three cytokines examined in this study are all involved in the activity of the innate immune system. IFN-γ is a critical coordinator of the innate and adaptive immune responses. IFN-γ modulates an array of immune responses, including activation of macrophages, promoting cytokine release from these cells [47]. However, interestingly, in terms of UTI pathogenesis, IFN-γ has been demonstrated to reduce macrophage phagocytosis of *Escherichia coli* [48], the most common causative organism of UTI. TNF-α is a major regulator of inflammation. In UTI, release of TNF-α

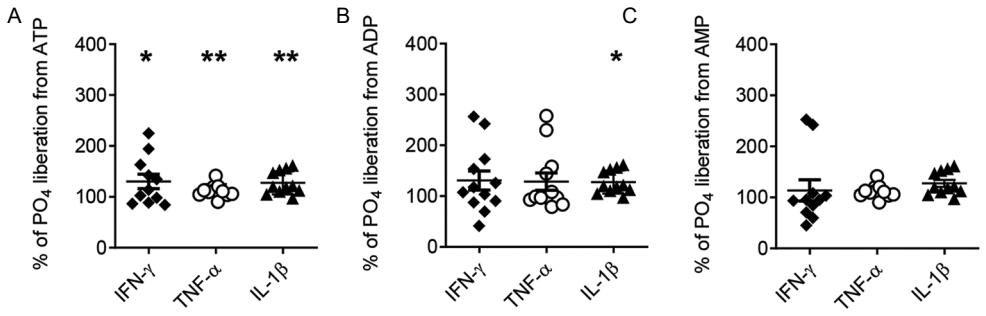

Figure 4. The effects of cytokine pre-treatment on (A) adenosine triphosphate, (B) adenosine diphosphate, and (C) adenosine monophosphate breakdown by urothelial cells. Data represents percentage of phosphate (PO_4) concentration compared to untreated urothelial cells (100%). Notes: $*P < 0.05$ or $**P < 0.01$ compared to untreated (0 ng/mL) cells ($n = 11$). Data represents mean PO_4 percentage with standard error of the mean.

leads to rapid recruitment of innate immune cells, including neutrophils, into the bladder, and promotes clearance of uropathogenic bacteria and exfoliation of infected bladder urothelial cells [49]. IL-1β is one of the cytokines produced by activated macrophages. IL-1β, one of the most potent pro-inflammatory cytokines, is elevated in UTI and is considered a marker of bacterial cystitis [32].

Urgency is thought to be mediated by activation of afferent sensory nerves following ATP release from the urothelium and it was hypothesized that pro-inflammatory cytokines stimulate ATP release [7,9]. At first glance, the current study showed ATP release was decreased in response to cytokine pre-treatment, and ATP breakdown was enhanced, which appeared to be in conflict with the known role of ATP in triggering urgency. However, this is not the only report of reduced ATP in the presence of an inflammatory stimulus. We have previously demonstrated a reduction in intravesical ATP in patients with DO and acute UTI [45]. This is in contrast to the reports of increased ATP release in human tissues obtained from patients with diseases such as interstitial cystitis/bladder pain syndrome, which are characterized by chronic inflammation. ATP release was also reportedly increased substantially in urothelial tissue strips collected from patients with painful bladder syndrome [16], while enhanced urothelial cell ATP release has been demonstrated in isolated cells derived from patients with interstitial cystitis [18]. This could possibly indicate that the acute response to inflammation, as seen in UTI, involves a decrease in ATP from the urothelium, or enhanced ATP breakdown while chronic inflammation, implicating multiple inflammatory cytokines and cells, could stimulate ATP release.

In this study, urothelial cells were treated with a single cytokine which was found to decrease urothelial cell ATP release and to enhance the breakdown of nucleotides, i.e., ATP and ADP, which would be expected to limit the effect of ATP as a stimulus for afferent nerve activity. However, in an *in vivo* inflammatory response, it is unlikely that a single cytokine would be released in isolation. The current study demonstrated that a cytokine cocktail, containing IFN-γ, TNF-α, and IL-1β, was able to induce a small but significant increase in urothelial cell ATP release, perhaps mirroring the results of previous studies that have shown an increase in ATP in other models of bladder inflammation [16-18].

It is also important to note that, in the current study, most changes in ATP release following pre-treatment with cytokines occurred in resting (control) and not the stretch-induced (hypotonic) condition. These results suggest that cytokines have a tendency to affect basal levels of ATP released from the urothelium and are possibly involved in mechanisms other than stretch-induced ATP release that initiates voiding. This is similar to findings that pyuria (presence of white blood cells in the urine) was associated with enhanced basal, but not stretch-evoked release of ATP in bladder biopsy specimens from patients with overactive bladder [50].

Another discrepancy between the current and past results is that the current study used an *in vitro* model of cell culture for the inflammatory stimuli. The aforementioned studies by Kumar *et al*. [16] and Smith *et al*. [17] involved more complex preparations in that they were undertaken with human bladder strips [16] or whole rat bladders [17]. In these models of chronic inflammation, the urothelial cell layer within the bladder wall would be exposed to inflammatory cells such as mast cells [51], as well as any inflammatory mediators and cytokines induced as part of the inflammatory response. Therefore, it is possible that augmented ATP release, as shown in these studies, requires the presence of other inflammatory cells that are absent from the single-cell model used. It is also possible that the increase in ATP release with inflammation described in the literature may not be coming directly from the urothelium but from other tissues within the bladder wall, such as sub-urothelial myofibroblasts or detrusor muscle cells, both of which can release ATP [51].

5. CONCLUSION

In conclusion, this study showed urinary concentrations of IFN-γ, TNF-α, and IL-1β were increased in women with DO

with or without UTI. Furthermore, this study demonstrated that pre-treatment of urothelial cells with a single cytokine caused a decrease rather than an increase in urothelial cell ATP release as well as an increase in the breakdown of ATP and ADP. However, treatment of urothelial cells with a cytokine cocktail resulted in a small but significant increase in ATP release from the urothelium, suggesting that the interaction between the urothelium and inflammatory stimuli is complex and multifactorial.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Conceptualization: Ronald Sluyter, Kylie J Mansfield
Investigation: Belinda Kaleska, Zhuoran Chen
Methodology: All authors
Writing – original draft: Belinda Kaleska, Kylie J Mansfield
Writing – review & editing: All authors

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Ethics approval for the collection of specimens from patients was obtained from the Ethics Review Board of the South East Sydney Local Health District (approval no.: HREC/G/219).

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA

Data used in this work are available from the corresponding author upon reasonable request.

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