Effect of inflammatory mediators on ATP release of human urothelial RT4 cells

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Abstract
Inflammation is an important contributor to the aetiology of a number of bladder dysfunctions including interstitial cystitis, painful bladder syndrome, and overactive bladder. The aim of this study was to examine the effects of inflammatory mediators on urothelial ATP release. Human urothelial RT4 cells were exposed to normal buffer or varying concentrations of inflammatory mediators (bradykinin, histamine, and serotonin) in the presence or absence of hypotonic stretch stimuli (1:2 dilution of Krebs-Henseleit buffer). Others have demonstrated that bradykinin increased stretch-induced ATP release; however, we observed no change in control or stretch-induced ATP release with bradykinin. Pretreatment of RT4 cells with histamine or serotonin decreased stretch-induced ATP release (P = 0.037, P = 0.040, resp.). Previous studies have demonstrated increased ATP release in response to inflammation utilising whole bladder preparations in contrast to our simple model of cultured urothelial cells. The current study suggests that it is unlikely that there is a direct interaction between the release of inflammatory mediators and increased ATP release, but rather more complex interactions occurring in response to inflammation that lead to increased bladder sensation.

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Research Article

Effect of Inflammatory Mediators on ATP Release of Human Urothelial RT4 Cells

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Inflammation is an important contributor to the aetiology of a number of bladder dysfunctions including interstitial cystitis, painful bladder syndrome, and overactive bladder. The aim of this study was to examine the effects of inflammatory mediators on urothelial ATP release. Human urothelial RT4 cells were exposed to normal buffer or varying concentrations of inflammatory mediators (bradykinin, histamine, and serotonin) in the presence or absence of hypotonic stretch stimuli (1:2 dilution of Krebs-Henseleit buffer). Others have demonstrated that bradykinin increased stretch-induced ATP release; however, we observed no change in control or stretch-induced ATP release with bradykinin. Pretreatment of RT4 cells with histamine or serotonin decreased stretch-induced ATP release \((\overline{P} = 0.037, \overline{P} = 0.040, \text{resp})\). Previous studies have demonstrated increased ATP release in response to inflammation utilising whole bladder preparations in contrast to our simple model of cultured urothelial cells. The current study suggests that it is unlikely that there is a direct interaction between the release of inflammatory mediators and increased ATP release, but rather more complex interactions occurring in response to inflammation that lead to increased bladder sensation.

1. Introduction

Inflammation is an important contributor to the aetiology of a number of bladder dysfunctions. Pyuria (the presence of white blood cells in the urine) has been associated with lower urinary tract symptoms [1] and biopsy specimens from patients with interstitial cystitis (IC)/painful bladder syndrome (PBS) are commonly characterised by the presence of inflammatory cells in the lamina propria, especially infiltration with mast cells [2–7]. Recently, histological evidence for chronic inflammatory infiltrate has been demonstrated in patients with refractory overactive bladder (OAB) [8] together with pyuria in these patients [8, 9]. Proinflammatory cytokines are increased in the urine from patients with OAB [10–12].

These conditions (IC/PBS/OAB) are all characterised by urinary urgency, together with frequency and nocturia [13]. It is believed that bladder sensation is associated with the interaction of ATP with purinergic receptors located on suburothelial afferent nerves [14, 15] and myofibroblasts [15]. ATP is released from the urothelium in response to stretch of the urothelium triggered by bladder filling [16]. An increase in stretch-induced ATP release has been demonstrated in tissue strips and biopsies from patients with IC/PBS [17–19] and OAB [20]. In addition, the concentration of ATP in intravesical fluid has been shown to correlate with urinary urgency as indicated by the volume at first desire to void in patients with OAB [21, 22].

Similar to the changes in bladder histological structure described with PBS/IC, chemical-induced cystitis causes histological changes in the bladder wall including infiltration of inflammatory cells (e.g., mast cells and macrophages) into the submucosa [4–7], together with increased bladder weight and oedema. In addition, chemical cystitis is associated with activation of previously silent C-fibre afferents and sensitisation of mechanosensitive Aδ-fibres within the submucosa [23–26].

The effect of inflammation causing an increase in urothelial ATP release has been examined in animal models of inflammation, including feline interstitial cystitis [27],
cyclophosphamide cystitis [28], and ketamine cystitis [2]. However, the agents responsible for the increased ATP release are unknown. The aim of this study was to examine the effects of bradykinin, histamine, and serotonin, mediators commonly associated with bladder inflammation, on urothelial cell ATP release.

2. Materials and Methods

2.1. Cell Culture. Human urothelial RT4 cells (obtained from the ECACC) were grown at 37°C with 5% CO₂ in McCoy’s 5A culture medium supplemented with 10% foetal bovine serum, 100 units/mL of penicillin, 100 µg/mL of streptomycin, and 0.25 µg/mL of fungizone. At confluence, cells were passaged and then replated onto T75 flasks for continuous passage or onto 24 well plates for use in ATP release when confluent (approximately 3 to 5 days after passage).

2.2. ATP Release. ATP release was determined as previously described [29]. Urothelial cells were washed (three times) with carbogenated Krebs-Henseleit solution (containing mM: NaCl 118, KCl 4.7, NaHCO₃ 25, KH₂PO₄ 1.2, MgSO₄ 1.2, CaCl₂ 2.5, and D-glucose 11.7). The basal level of ATP release was then determined by 10-minute incubation in 500 µL Krebs-Henseleit solution. Cells were then exposed to normal Krebs-Henseleit (control) or the indicated concentration of mediator (bradykinin, histamine, and serotonin) in the presence or absence of hypotonic Krebs-Henseleit (1:2 dilution of Krebs-Henseleit in distilled water). Hypotonic Krebs-Henseleit was used as a stress stimulus to examine the effect of the mediators on stretch-induced ATP release. Cells were treated for 10 min before the supernatant (200 µL) was collected and used for ATP determinations.

ATP concentration in the supernatant was measured using the bioluminescence assay. Equal volumes of the cellular supernatant or ATP standard solutions (10⁻⁶ to 10⁻¹⁰ M) were mixed with the bioluminescence assay mix and the luminescence generated was measured immediately using a plate reader (BMG Labtech Polarstar). The ATP concentration in the cell supernatant was calculated relative to the standard curve. Treatments were carried out in triplicate and the mean ATP concentration (in nM) per treatment was determined.

2.3. Statistics. As results are nonnormally distributed and as such are expressed as median with interquartile range (IQR); two different treatments were compared using a Wilcoxon matched-pairs t-test. Dose response relationships were examined using a sigmoidal dose response curve. All statistics were performed using Graphpad Prims (version 6) (San Diego, CA).

2.4. Materials. All cell culture reagents were purchased from Invitrogen (Mount Waverley, Australia). Bioluminescence ATP assay kit and mediators (bradykinin, histamine, and serotonin) were from Sigma-Aldrich (Sydney, Australia). All other reagents were of high analytical grade.

3. Results and Discussion

3.1. Effect of Bradykinin on Urothelial Cell ATP Release. Bradykinin is a peptide neurotransmitter released from sensory afferent nerves. Bradykinin release is closely associated with inflammatory responses. Distension of the bladder has been shown to stimulate release of bradykinin in patients with IC indicating a potential role for this peptide in the pathophysiology of this disorder [30]. Bradykinin exerts its physiological actions via activation of B1 and B2 receptors. Bradykinin B2 receptors are believed to be important in inflammatory pain with animal models showing that block of this receptor reduces inflammatory hyperalgesia [31, 32]. In contrast, bradykinin B1 receptors are expressed at low levels under normal circumstances with their expression upregulated following tissue damage or inflammation [33]. This includes upregulation of bradykinin B1 receptors following cystitis induced by intravesical injection of the detergent Triton X100 [34]. Expression of bradykinin B1 receptors is increased in biopsies obtained from patients with interstitial cystitis [35] and, in cyclophosphamide-induced cystitis, B1 receptor mediated bladder responses are significantly increased [36, 37]. In addition, cyclophosphamide cystitis induced a bladder hyperactivity that was dependent on bradykinin B2 receptor activation and was inhibited by the P2 receptor antagonist PPADS [38]. This indicates a role for both bradykinin and ATP in cyclophosphamide-induced cystitis [38].

Other studies have observed that activation of bradykinin B2 receptors by bradykinin increases stretch-induced ATP release in UROtsa urothelial cells [39] and in primary cultures of rat urothelial cells [38]. However, in the current study, using urothelial RT4 cells, we saw no change in either control or stretch-induced ATP release in the presence of bradykinin (Figure 1). Hypotonic Krebs which was used as a positive control was seen to induce an approximate threefold increase in ATP release (baseline ATP release 34.06 (23.8–99.1) nM; stretch-induced ATP release 146.7 (72.4–217.6) nM, P = 0.002). It is possible that the differences in these findings relate to the individual cell lines used and that the bradykinin receptors usually present on urothelial cells are not functional on RT4 urothelial cells.

3.2. Effect of Mast Cell Mediators on Urothelial Cell ATP Release. Infiltration of inflammatory cells such as mast cells and macrophages into the bladder submucosa has been demonstrated in cyclophosphamide-induced cystitis [3] and ketamine-induced cystitis [2]. In addition, it is well known that there is an increased density of mast cells in the bladder wall in patients with IC [4–7]. Mast cells lie in close proximity to both urothelial cells and afferent fibres in the submucosa of the urinary bladder, and degranulation of these cells has been shown to release a wide range of neurotransmitters and cytokines [38].

Mast cells are granulated immune system cells which make up the major sensory arm of the innate immune system [40]. Mast cells respond to allergens as well as non-immunologic stimuli such as bacteria, chemicals, kinins, and neuropeptides [41] to release mediators such as histamine and
Inflammation of the bladder wall including defects in urothelial junction proteins, increased suburothelial inflammation, and increased urothelial cell apoptosis [2]. Bladder inflammation is associated with disruption of the glycosaminoglycan layer that covers the urothelium [48–50]. Disruption of this layer may allow urine contents to evoke irritation and inflammation within the bladder wall. There is also increased density of submucosal afferent nerves in the inflamed bladder [43, 51, 52]. Urinary nerve growth factor (NGF) levels are increased in patients with IC [53] and OAB [54]. In addition, cyclophosphamide-induced cystitis is associated with increased NGF mRNA levels in the bladder [55], increases the density of afferent nerves, and increases expression of P2X\textsubscript{2} and P2X\textsubscript{3} receptors in afferent nerves [56]. It is likely that the increase in NGF expression in patients with bladder dysfunction is associated with the increased density of suburothelial nerves in these patients. NGF is also thought to alter expression of neurotransmitters, modulate ion channels, and increase excitability of afferent nerves [57]. In rats, exogenously applied intravesical NGF can induce bladder nociceptive responses and bladder overactivity [58]. In addition to NGF, inflammatory mediators themselves may sensitize the afferent nerves to the effects of ATP [56, 59], which could increase the bladder sensations in response to bladder filling in the inflamed bladder.

4. Conclusions

It is well accepted that bladder inflammation is associated with increased signalling from the bladder and increased sensation. Studies using whole bladder models of inflammation have shown that this is due to increased release of ATP from the urothelium. However, based on the results of the current study, it appears unlikely that there is a simple

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**Figure 1:** Effect of bradykinin (1 μM, \(n = 10\)) on baseline level of ATP release and release induced by hypotonic media. Symbols are representative of individual data points for the four groups. Data are shown as median with interquartile range.
interaction between the release of the inflammatory mediators (bradykinin, histamine, and serotonin) and increased ATP release leading to increased bladder sensation. It is more likely that there are numerous factors involved in the interaction between bladder inflammation and increased sensation. These factors include alterations to receptor expression on the urothelium and afferent nerves, increased density of afferent nerves, and hyperresponsiveness of the afferent nerves, altering the neuronal component of afferent signalling.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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