Bladder Outlet Obstruction Mediates Muscarinic Acetylcholine Receptor Expression in the Urothelial Layer

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Abstract

Bladder outlet obstruction (BOO) is an important condition which occurs primarily in older men due to the prevalence of benign prostatic hyperplasia (BPH). BPH costs four billion dollars annually in healthcare expenditures in the US alone. BOO induces inflammation in the bladder leading to bladder epithelial (urothelial) layer reorganization and eventually to fibrosis and irreversible bladder dysfunction. Recently Dr. J. Todd Purves’ lab has discovered a critical role for a family of supramolecular complexes called inflammasomes in the induction of this inflammation, specifically the NLRP3 inflammasome. However, it is not known how these inflammasomes influence the population of cell surface receptors in the urothelium. Muscarinic acetylcholine receptors (mAChRs) in the urothelium have been specifically implicated in inducing irritative bladder symptoms such as increased frequency and voiding trouble. To gain insight into this phenomenon, changes in the expression level of several critical cell surface receptors in the urothelium of a rat model of BOO were measured in the presence and absence of NLRP3 inflammasome inhibition. After quantification of receptor expression levels with flow cytometry and immunofluorescence microscopy, BOO was discovered to have direct effects on mAChR expression, through a physiological pathway demonstrated to be mediated by NLRP3 inflammasome activation. The discovery of this relationship moves the field one step closer to understanding ways in which the irritative symptoms associated with BPH and BOO may be alleviated.
Introduction

Benign prostatic hyperplasia (BPH) presents society with one of medicine’s most prevalent issues, with 40 to 50 percent of men aged 51 to 60, and over 80 percent of men >80, affected [1]. The healthcare costs associated with this condition amount to over $4 billion annually in the US alone [2]. BPH is a noncancerous increase in size of the prostate gland and can lead to a number of issues, most importantly to this research a restriction in the diameter of the urethra (i.e. a bladder outlet obstruction or BOO) causing a resistance of the outflow of urine from the bladder (Fig. 1) [3]. BOO leads to increased bladder pressure (as the smooth muscle must contract with greater force to overcome the obstruction), stretch (due to incomplete emptying), and hypoxia (due to compression of blood vessels during stretch) [4, 5]. Together, these insults can lead to bladder epithelial layer (urothelium) restructuring and chronic inflammation, both contributors to overactive bladder (OAB) symptoms such as increased frequency and urgency of urination [4, 5, 6]. The urothelium is the immediate layer surrounding the bladder lumen where urine is held has been shown to sense bladder conditions, containing several different types of cell-surface receptors able to respond to a variety of different stimuli [7, 8]. The response of the receptors in the urothelium is relayed to afferent nerves, and the reorganization of this layer following BOO may contribute to bladder dysfunction and OAB symptoms [8]. Chronic inflammation during BOO leads to fibrosis (a deposition of connective tissue) which can decrease the capacity of the bladder, and prevent a return to normalcy even if the obstruction is relieved [9, 19]. Fibrosis can eventually cause end-stage bladder dysfunction which may result in overflow incontinence and kidney failure [10, 11].
The inflammation associated with BOO is triggered by activation of the innate immune system. Dying cells and cells under stress due to the increased bladder pressure, stretch, and hypoxia during BOO release molecules with damage-associated molecular patterns (DAMPs) [12, 13]. These DAMPs are recognized by nucleotide-binding oligomerization domain-like receptors (NOD-like receptors, or simply NLRs) and activate multimeric protein structures called inflammasomes [12, 13]. Inflammasomes then trigger inflammation by promoting the maturation and release of the pro-inflammatory cytokines interleukin-1 beta (IL-1β) and interleukin-18 (IL-18) (Fig. 2) [12]. The NLRP3 inflammasome, in particular, plays a critical role in BOO-induced chronic inflammation [12]. What remains enigmatic is if the activation of the NLRP3 inflammasome directly influences the reorganization of the urothelial layer seen in BOO. As previously stated, urothelial cells contain many cell-surface receptors that aid in sensing bladder conditions. Alteration of the expression of these receptors may change perception of bladder conditions and trigger the irritative voiding symptoms associated with OAB. Thus, experimentally, quantitative changes in the expression level of these receptors would reflect the reorganization and altered perception of the urothelial layer. Delineation of a relationship between inflammasome activation and expression of these receptors in the urothelia could lead to further progress on a solution to OAB symptoms that afflict BPH patients.
Muscarinic acetylcholine receptors have been shown to mediate bladder smooth muscle (detrusor) contraction and relaxation, both integral aspects of OAB symptoms [14, 15]. Five subtypes of muscarinic receptors have been identified (M1-M5), while two subtypes (M2 and M3) appear to play critical roles in bladder detrusor contractile responses, a response that may be relayed from the urothelial layer to the detrusor. M2 and M3 expression levels have been shown.

**Figure 2.** Outline of the BOO induced NLRP3 inflammasome activation pathway leading to downstream inflammation, fibrosis, and irritative voiding symptoms. Also shown are the location of inhibition by Glyburide and Anakinra.
to increase during BOO, but the specific source of this increase is not known [16]. Consequently, these two receptors are the most common targets of pharmaceutical intervention in treating the symptoms of OAB, although such medication is largely ineffective and is accompanied by many harsh side effects [17, 18]. As such, new forms of treatment are desired.

Based on the above information, I postulate that changes in the expression levels of M2 and M3 during BOO are mediated by the activation of the NLRP3 inflammasome. To investigate the relationship between NLRP3 inflammasome activity and changes in M2 and M3 expression levels, BOO was surgically introduced in rats and receptor expression levels quantitated in the presence and absence of daily treatments with both Glyburide, an FDA approved diabetes medication and antagonist to NLRP3 inflammasome activation, as well as Anakinra, an IL-1β receptor antagonist currently FDA-approved for treatment of rheumatoid arthritis. Receptor expression levels were quantified by flow cytometry and localization observed using immunofluorescence microscopy.

Materials and Methods

Experimental Groups

The cells used for analysis were scraped from the urothelial layer of four experimental rat groups: a control group with no surgical operation, a BOO group administered a vehicle solution, and two BOO groups that had the disorder reversed by a Glyburide or Anakinra injection. For BOO rats, a catheter (P50 tubing; O.D. 1 mm) was inserted into the urethra, the abdominal cavity was opened, a 5-0 silk suture passed around the urethra and tied securely. The catheter was then removed (Fig. 3). With the first dose given during post-operative recovery, BOO rats were given daily glyburide (10 mg/kg in 1 ml, by mouth.), Anakinra (25mg/kg; diluted 1:3 with PBS), or
vehicle (40% ethanol in PBS, by mouth) for 12 days. Protocols were approved by the Institutional Animal Care and Use Committee at the Medical University of South Carolina.

**Figure 3.** Rat BOO surgery consisting of urethral insertion of a catheter, silk suture passed around the urethra and tied securely, followed by removal of the catheter [12].

*Flow Cytometry*

To obtain cells for flow cytometry, rats were sacrificed, bladders were inverted and filled with PBS, and tied off with a purse string suture. Inverted bladders were digested with 1mg/ml collagenase P solution and incubated for 1 hour at 37 °C [20]. After intense shaking and incubation for an additional 15 minutes, the bladders were scraped for cells and centrifuged. Pellets were fixed in 250μL of Cytofix and incubated for 30 minutes at 4°C. Pellets were washed in wash buffer (Perm/Wash, 1:10 dilution) and incubated with 250μL blocking buffer (1% BSA in wash buffer) for 0.5 hour on ice. 5μL of mAChR M2, sc-9107 or mAChR M3, sc-
primary antibody (1:50 dilution of antibody in wash; Santa Cruz Biotechnology) were added and the cells incubated overnight at 4°C. Cells were then washed and resuspended in 250μL goat anti-rabbit IgG AF-488 secondary antibody (Southern Biotech; 1:500 dilution antibody in wash) for 1 hour on ice. Cells were washed once more and resuspended in 500μL PBS for flow cytometry.

Flow cytometry parameters for data acquisition were adjusted for each sample. Analysis included two graphs, one showing individual cells of a sample plotted by laser forward scatter (cell size) and laser side scatter (cell complexity), and the second graph a histogram that measured cell fluorescence (cell receptor expression). A region of interest was manually drawn around groups of cells based on forward and side scatter so that a majority of cells analyzed for fluorescence were individual cells and not cellular debris and/or cellular clusters (Fig. 4). Mean fluorescence per cell was used to compare sample muscarinic receptor expression.

**Immunofluorescence Microscopy**

Bladders were dissected, cleaned of surrounding fat and placed in 10% neutral buffered formalin overnight at 4°C. Bladders were then transferred to 70% ethanol and stored at 4°C for 1 - 7 days until they were embedded in paraffin blocks using standard techniques [21]. Cross-sections (5μm) from approximately the middle of the bladder were used for staining and were deparaffinized and hydrated through a graded alcohol series before being subjected to antigen retrieval (Vector labs citrate-based antigen unmasking solution for 30 min using the manufacturers recommended protocol; Vector Labs, Burlingame, CA). Following washing in PBS, sections were blocked with normal goat serum for 30 min and incubated with primary antibody (mAChR M2, sc-9107 or mAChR M3, sc-9108 primary antibody; 1:50 dilution of
antibody in wash; Santa Cruz) overnight at 4°C. Slides were washed and secondary antibody added (goat anti-rabbit IgG AF-488; 1:500 dilution; Southern Biotech). Slides were incubated for 30 min, washed, coverslipped, and analyzed by fluorescence microscopy. The sections were imaged using upright microscopy at 10 X (Zeiss Axio Imager 2, Carl Zeiss AG, Oberkochen, Germany).

**Figure 4.** Example flow cytometric analysis for a sample. **Top:** Each dot represents an event passing the flow cytometer laser plotted by forward and side scatter. The R1 region of interest is the manually gated individual urothelial cells based on typical forward and side scatter. **Bottom:** A histogram of the gated cells measuring cell fluorescence.
Results

Flow cytometry data allowed analysis of mean fluorescence for each experimental group, whereby fluorescence was a measure of receptor expression in each cell. M2 expression levels significantly increased in BOO (129.41±12.55%; \( n=9 \)) compared to control (\( n=9; \ p<0.05 \)) and decreases compared to BOO in BOO+Gly (88.52±7.10%; \( n=4 \)) and BOO+Ana (99.82±12.81%; \( n=4 \)) (Fig. 5). Furthermore, M3 expression levels significantly increased in BOO (126.36±6.28%; \( n=12 \)) compared to control (\( n=13; \ p<0.05 \)) and decreases compared to BOO in BOO+Gly (92.69±14.87%; \( n=9 \)) and BOO+Ana (73.84±19.26%; \( n=4 \)) (Fig. 6).

Immunofluorescence microscopy slides show M2 and M3 expression localization to the urothelial layer (Fig. 7 and 8). Although it appears M2 and M3 receptor expression is upregulated in BOO and correspondingly decreased in BOO+Gly sections, this is qualitative and cannot be confirmed or quantified without sophisticated imaging techniques not available to us at the present time. Additionally, due to time and resource constraints, immunostaining for M2 and M3 in BOO+Ana bladders was not completed.

Bladder weights were used as the indicator of inflammation in the current study. Kanno et al. demonstrated that BOO-induced inflammation and bladder weight gain were decreased during BOO using a mouse in which IL-1β had been knocked out [22], suggesting that bladder weight is a fair proxy for inflammation. Bladders increased in weight after induction of BOO (367.62±29.34; \( n=23 \)) compared to control (92.31±4.178; \( n=24 \)) and decreased closer to control levels in both BOO+Gly (149.05±13.40; \( n=15 \)) and BOO+Ana (192.85±17.79; \( n=8 \)) (Fig. 9).
Figure 5. Mean fluorescence as a percentage of control fluorescence for M2 expression. Flow cytometry indicated M2 expression levels significantly increased in BOO (129.41±12.55%; n=9) compared to control (n=9) and decreases compared to BOO in BOO+Gly (88.52±7.10%; n=4) and BOO+Ana (99.82±12.81%; n=4). *p<0.05 significantly different than control M2 expression levels.
**Figure 6.** Mean fluorescence as a percentage of control fluorescence for M3 expression. Flow cytometry indicated M3 expression levels significantly increased in BOO (126.36±6.28%; n=12) compared to control (n=13) and decreases compared to BOO in BOO+Gly (92.69±14.87%; n=9) and BOO+Ana (73.84±19.26%; n=4). *p<0.05 significantly different than control M2 expression levels
**Figure 7.** M2 immunofluorescence microscopy slides (20X). Each image in a column is the same slide viewed from an alternative angle. The bladder lumen is seen in black, with the urothelial layer immediately surrounding it. M2 expression shows localization to the urothelium in control, BOO, and BOO+Gly bladders.
Figure 8. M3 immunofluorescence microscopy slides (20X). Each image in a column is the same slide viewed from an alternative angle. The bladder lumen is seen in black, with the urothelial layer immediately surrounding it. M3 expression shows localization to the urothelium in control, BOO, and BOO+Gly bladders.
**Figure 9.** Mean bladder weights in milligrams. Bladders increased in weight after induction of BOO (367.62±29.34; n=23) compared to control (92.31±4.178; n=24) and decreased closer to control levels in both BOO+Gly (149.05±13.40; n=15) and BOO+Ana (192.85±17.79; n=8).
Discussion

The results of this study give interesting insight into the relationship between muscarinic expression levels in the urothelial layer and the induction of inflammation through BOO. In the analysis of mean expression levels of both M2 and M3, receptor expression increased in BOO (as expected based on previous work [16]) but importantly returned to at or below control expression levels in BOO+Gly and BOO+Ana groups. As inhibitors of NLRP3 inflammasome activation and IL-1β (the major product of NLRP3 activation), respectively, Glyburide and Anakinra specifically implicate the NLRP3 inflammasome in the changes in M2 and M3 expression levels during inflammation caused by BOO. The combined results indicate both M2 and M3 expression levels are mediated by NLRP3 inflammasome activation.

Results for bladder weights confirmed BOO and corresponding inflammation were induced and inhibited by Glyburide and Anakinra. Hypertrophy (enlargement of a tissue or organ due to an increase in the size of its cells) and hyperplasia (enlargement of a tissue or organ due to an increase in the reproduction of its cells) of the urothelium and bladder wall is to be expected during BOO as the bladder muscle works harder to overcome the outflow resistance. Perhaps this explains the increase (albeit insignificant) in weight even in the presence of Glyburide and Anakinra: bladder muscle enlarges independent of inflammation.

Immunofluorescence microscopy confirmed that M2 and M3 receptors localize to the urothelial layer, and although it appears they are upregulated in BOO and decrease with inflammation inhibition by Glyburide and Anakinra, this cannot be verified without further quantitation, as mentioned previously. Localization to the urothelium supports the idea that the urothelium serves as a responsive structure, able to sense bladder conditions, rather than a passive barrier, with both receptors localized primarily in the urothelium compared to other
bladder layers. The “response” of the urothelium to sensing bladder conditions may be to signal to the bladder detrusor muscle to contract or relax, but the exact mechanism of this pathway is not known, nor is the extent of muscarinic receptors’ involvement in this pathway. However, the primary findings of this study, in conjunction with previous research showing M2 and M3 receptor involvement in detrusor contraction, and detrusor dysfunction in OAB, begins to construct a model whereby M2 and M3 expression in BOO are increased due to activation of the NLRP3 inflammasome, possibly contributing to improper detrusor activity [14, 23].

The principal discovery of this study that the NLRP3 inflammasome mediates M2 and M3 expression leads to a possible paradigm explaining M2 and M3 overexpression in BOO. The onset of BOO and consequent inflammation via NLRP3 activation leads to increased M2 and M3 expression levels in the urothelium. OAB symptoms associated with BOO are in part due to bladder detrusor muscle overactivity, a response that may be relayed from the urothelial layer to the detrusor [24]. M2 and M3 activation is known to stimulate the release of ATP in the bladder, and ATP is a quintessential activator of the NLRP3 inflammasome [25, 26]. This raises the possibility of a positive feedback loop whereby OAB symptoms and the corresponding increased M2 and M3 activation releases excessive ATP, activating the NLRP3 inflammasome and further upregulating M2 and M3 expression levels. Additionally, the NLRP3 inflammasome may have basal levels of activation independent of BOO as it is involved in the general inflammatory response in the bladder. Glyburide and Anakinra may block this basal inflammasome activity, and based on the central finding of this study that the NLRP3 inflammasome mediates M2 and M3 expression levels, this would explain the decrease in M2 and M3 expression in BOO+Gly and BOO+Ana below control levels.
The next steps in this research are to quantify changes in the expression levels of M1, M4, and M5 during BOO and to see if they too are mediated by the NLRP3 inflammasome. M1 and M4 receptors have been shown to be involved in detrusor relaxation in the bladder, and these receptors may also be contributing to OAB symptoms [16]. In fact, studies have shown that it may be the ratio of muscarinic receptor subtypes in the urothelium during BOO that contribute to OAB symptoms, rather than the expression levels themselves [27, 28]. Further studies directly comparing receptor subtype expression levels in the urothelial layer during BOO would be helpful in elucidating this relationship. In addition, the present study did not look at muscarinic expression in bladder layers other than that of the urothelium, whereas quantification of muscarinic receptors in the detrusor and interstitial space may be necessary to fully understand the paradigm of muscarinic expression and OAB symptoms. Greater delineation of how urothelial expression of muscarinic receptors brings about bladder dysfunction and OAB symptoms is needed.

The long-range goal of this research and similar studies is seeing if manipulation of these muscarinic receptor expression levels can be used in therapeutic treatment of BPH. While current treatment for BPH and OAB involve antagonizing muscarinic receptors after their expression levels have already shifted because of the inflammation associated with BOO, a more preemptive approach would involve preventing the change in expression levels in the first place, which could aid in better preventing OAB symptoms.

This study works to show how the NLRP3 inflammasome mediates the expression levels of muscarinic receptors in the bladder urothelial layer during BOO. This finding is a step towards elucidating how muscarinic receptors and bladder conditions interact to induce many of the irritative urinary symptoms associated with OAB and on a broader level, BPH.
References


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