The NLRP3 Inflammasome Mediates Inflammation Produced by Bladder Outlet Obstruction

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Purpose: While bladder outlet obstruction is well established to elicit an inflammatory reaction in the bladder that leads to overactive bladder and fibrosis, little is known about the mechanism by which this is initiated. NLRs (NOD-like receptors) and the structures that they form (inflammasomes) have been identified as sensors of cellular damage, including pressure induced damage, and triggers of inflammation. Recently we identified these structures in the urothelium. In this study we assessed the role of the NLRP3 (NACHT, LRR and PYD domains-containing protein 3) inflammasome in bladder dysfunction resulting from bladder outlet obstruction.

Materials and Methods: Bladder outlet obstruction was created in female rats by inserting a 1 mm outer diameter transurethral catheter, tying a silk ligature around the urethra and removing the catheter. Untreated and sham operated rats served as controls. Rats with bladder outlet obstruction were given vehicle (10% ethanol) or 10 mg/kg glyburide (a NLRP3 inhibitor) orally daily for 12 days. Inflammasome activity, bladder hypertrophy, inflammation and bladder function (urodynamics) were assessed.

Results: Bladder outlet obstruction increased urothelial inflammasome activity, bladder hypertrophy and inflammation, and decreased voided volume. Glyburide blocked inflammasome activation, reduced hypertrophy and prevented inflammation. The decrease in voided volume was also attenuated by glyburide mechanismally as an increase in detrusor contraction duration and voiding period.

Conclusion: Results suggest the importance of the NLRP3 inflammasome in the induction of inflammation and bladder dysfunction secondary to bladder outlet obstruction. Arresting these processes with NLRP3 inhibitors may prove useful to treat the symptoms that they produce.

Key Words: urinary bladder neck obstruction; inflammasomes; inflammation; cystitis; immunity, innate

BLADDER outlet obstruction results from numerous conditions (eg stones, organ prolapse or posterior urethral valves), although the most prevalent cause is benign prostatic hyperplasia. Pharmacotherapy such as α-blockers and 5α-reductase inhibitors treats moderate symptoms but...
does not completely eliminate high intravesical pressure. Persistent pressure provokes detrusor hypertrophy, which may be transmitted to the upper tracts, leading to renal failure. Recent studies demonstrate that elevated pressure produces a chronic inflammatory state in the bladder, leading to irritative symptoms such as frequency, urgency and urge incontinence. These symptoms are often more devastating to quality of life than the original obstructive symptoms. Chronic bladder inflammation eventually leads to fibrosis and decompensation with poor success rates using current therapies. Thus treating underlying inflammation could prevent or delay the progression of bladder dysfunction in BOO.

Sterile inflammation in BOO is likely triggered through the innate immune system by patterns in molecules released from pressure stressed or dying cells. In other tissues these DAMPs are recognized by NLRs, which form multimeric structures called inflammasomes that promote the maturation and release of the pro-inflammatory cytokines IL-1β and IL-18. We recently reported the presence of NLRs in the urothelium and their ability to respond to DAMPs such as adenosine triphosphate and monosodium urate, which are common components of urine. We hypothesized that during BOO NLRP3, which is the best studied NLR and the one known to respond to DAMPs, is activated in the urothelium by high pressure, triggering inflammation, irritative symptoms and eventually fibrosis and decompensation.

To begin to address this hypothesis in the current series we examined NLRP3 activation during BOO and assessed the ability of the NLRP3 inhibitor Gly, also known as glyburide, to block inflammation and voiding dysfunction at an early stage of BOO (day 12) in rats. The results strongly support a causative role for NLRP3 in this disorder.

MATERIALS AND METHODS

Animals, Surgery and Pharmacological Treatments
Female Sprague Dawley® rats at about age 49 days weighing approximately 200 gm were used in all studies. Female rats are the standard for BOO studies based on ease of surgery, lack of tortuosity and short length of the urethra as well as complications arising from ducts associated with the prostate gland and seminal vesicles.

Protocols were approved by the Medical University of South Carolina institutional animal care and use committee. In preparation for all surgeries rats were anesthetized intraperitoneally with ketamine hydrochloride (90 mg/kg) and xylazine (10 mg/kg).

In BOO and sham operated rats a catheter (polyethylene 50 tubing with an outer diameter of 1 mm) was inserted transurethrally. The abdominal cavity was opened and a 5-zero silk suture was passed around the urethra and tied securely in BOO and loosely in sham operated rats. The catheter was then removed.

For urodynamics a suprapubic catheter (polyethylene-50 tubing with a flared end) was implanted at the time of BOO except in 3 rats as described. A 3 to 4 mm piece of silicon tubing placed under the flare served as a spacer between the bladder wall and the catheter opening, preventing urothelial growth over the orifice and occlusion. Before this technical improvement clogging catheters necessitated separate BOO and catheter placement with the latter done 24 hours prior to urodynamics (ie on day 11). In the current study 3 rats, including 2 in the BOO plus vehicle group and 1 in the BOO plus glyburide group, were subjected to multiple surgeries and data were collected. Thereafter we used a spacer that allowed for a single surgery. On final analysis all urodynamic parameters of the 3 rats that underwent multiple surgeries were well within 2 SD of those of the other rats in their respective groups and thus they were included in statistical analysis. Upon placement the catheter was secured with a 6-zero Prolene® purse-string suture, tunneled to the back of the neck and secured to interscapular tissue with 5-zero silk. The abdominal wall and skin were closed separately with 5-zero polyglycolic acid and the catheters were fitted to a Quick Connect™ Harness.

With the first dose given during postoperative recovery BOO animals were given daily glyburide (10 mg/kg in 1 ml) orally or vehicle (40% ethanol). This dose, which is common in the literature, is considerably less than the 500 mg/kg per day used in the original assessment of the ability of glyburide to inhibit NLRP3. Glyburide was prepared by suspending 5 mg/ml in 100% ethanol at 56°C with occasional vortexing until dissolved. It was diluted 2:3 with phosphate buffered saline and immediately administered.

Caspase-1 Assay
As previously described the bladder wall was scraped. Urothelial cells were pelleted and resuspended in 25 μl 10 mM MgCl2 and 0.25% Igepal CA-630, and added to 25 μl 40 mM HEPES (pH 7.4), 20 mM NaCl, 2 mM EDTA (ethylenediaminetetraacetic acid) and 20% glycerol. Extract (20 μl) was combined with 50 μl assay buffer (25 mM HEPES, 5% sucrose and 0.05% CHAPS, pH 7.5), 10 μl 100 mM dithiothreitol and 20 μl 1 mM AFC in black-walled 96-well plates. Fluorescence (excitation 400 and emission 505 nm) was measured every 30 seconds for 15 minutes and the slope was determined. Protein concentrations were assessed and a standard curve of fluorescence vs free AFC was used to calculate specific activity.

Bladder Hypertrophy and Evan Blue Extravasation
At sacrifice the bladders used for caspase-1 and Evan blue extravasation studies were weighed and the results of each experimental group were combined. Rats were injected intravenously with 25 mg/kg Evan blue. One hour later the bladders were weighed and placed in formamide (1 ml overnight at 56°C). Absorbance (620 nm) was measured and results were calculated from a standard curve.
Histology and Immunocytochemistry

Bladders were fixed overnight in 10% neutral buffered formalin at 4°C and paraffin embedded. Sections (5 μm) were stained with hematoxylin and eosin or subjected to immunocytochemistry using standard techniques (antigen retrieval-citrate, pH 6.0, anti-NLRP3, No. SC-66846 and isotype control, No. SC-2027, Santa Cruz Biotechnology®), ImmPRESS™ HRP Anti-Rabbit IgG (No. MP-7401) and the DAB Substrate Kit (No. SK-4100, Vector® Laboratories).

Urodynamics

On postoperative day 12 the rat harnesses were removed. The rats were placed in a restrainer above an analytical balance in a Small Animal Cystometry Lab Station (Catamount Research and Development, St. Albans, Vermont). Sterile saline was infused at 80 μl per minute. Intravesicular pressure (measured with an inline transducer) and voided volume were measured for 60 to 120 minutes and Med-CMG™ software was used. One micturition cycle was defined as the time needed for intravesicular pressure to return to baseline after a previous void until it returned to baseline following the next void. During each cycle voiding pressure was recorded as peak intravesical pressure, voided volume was recorded as the amount of urine voided and ICI was recorded as the time between successive peaks in voiding pressure. Contraction duration was considered the time during which intravesical pressure exceeded the threshold during a void. The duration of the void was considered the time from when the reading changed on the scale to the time that it stabilized. The flow rate was determined as voided volume divided by voiding time. For each rat and each parameter the average of the recorded micturition cycles was used for statistical analysis.

Statistical Analysis

All assays and parameters were assessed by a 1-way ANOVA followed by Tukey post-hoc analysis using InStat®.

![Graphs](image)

**Figure 1.** A, 4, 5, 5 and 3 rats underwent no surgery (Con), sham surgery (Sham) or BOO and were given vehicle (Veh) or Gly, respectively. Gly blocked BOO induced caspase-1 activity in urothelium as analyzed on day 12. Bars indicate mean ± SEM. Asterisk indicates ANOVA and Tukey test p <0.05. B, 11, 16, 27 and 17 rats underwent no surgery, sham surgery or BOO and were given vehicle or Gly, respectively. BOO induced bladder hypertrophy was reduced by Gly. Bladders from animals used for caspase-1 activity and Evans blue extravasation studies were weighed at sacrifice and those in given group were combined. Triple asterisks indicate ANOVA and Tukey test p <0.00. C, 4, 4, 6 and 6 rats underwent no surgery, sham surgery or BOO and were given vehicle or Gly, respectively. Gly prevented BOO induced Evans blue extravasation. Asterisk indicates ANOVA and Tukey test p <0.05 vs control. All other differences were not significant.
RESULTS

BOO Activated NLRP3 in Urothelium
Rats that underwent sham surgery did not show significant changes in urothelial caspase-1 compared to controls (fig. 1, A). In contrast in BOO rats treated with vehicle the activity of this enzyme was increased almost threefold. The increase was prevented by daily administration of glyburide.

NLRP3 Mediation

**BOO Induced Bladder Hypertrophy.** Bladder weight was greatly increased in BOO plus vehicle rats compared to control or sham operated rats (fig. 1, B). This increase was reduced approximately 50% by glyburide.

**BOO Induced Bladder Inflammation.** Bladders from sham operated rats showed a slight but nonsignificant increase in Evan blue dye extravasation compared to controls (fig. 1, C). In contrast BOO plus vehicle rats showed a large increase. Glyburide decreased dye extravasation to levels not significantly different from those in control or sham operated rats.

Glyburide

**Prevented Most Histological Changes Associated with BOO but did Not Affect NLRP3 Tissue Distribution.** Control urothelium was predominantly 3 to 4 cell layers thick with underlying back-to-back smooth muscle fascicles and no significant inflammation (fig. 2). However obstructed bladder urothelium ranged from 3 to 10 cell layers thick. Obstructed bladders also showed marked acute inflammation mostly involving the urothelium and subepithelial connective tissue. Inflammatory cells infiltrated between smooth muscle fascicles, where there was associated collagen deposition, thickening the bladder wall. Outside the smooth muscle layer was a mild to moderate chronic inflammatory infiltrate. Glyburide treated rats demonstrated decreased urothelial thickness and inflammation with a scant to mild infiltrate of acute and chronic inflammatory cells involving the urothelium and subepithelial connective tissue. There was a mild chronic inflammatory infiltrate in the connective tissue surrounding the smooth muscle layer. Sham operated specimens appeared histologically similar to control specimens.

Immunocytochemistry for NLRP3 revealed expression localized to the urothelium with little or no expression in other tissues (fig. 3). Neither BOO nor glyburide appeared to change the tissue distribution.

**Increased Voided Volume by Increasing Duration of Voiding Contraction and Void.** To assess the role of NLRP3 in bladder dysfunction associated with BOO urodynamics was performed. Figure 4, A shows a representative tracing of intravesicular pressure. Figure 4, B shows the tracing derived from the scale during a representative void in the various groups. Figure 5, A and B compares voiding pressure and flow rate among the 4 groups. Voiding pressure in sham operated rats did not significantly differ from that in controls. However BOO rats treated with vehicle showed a highly significant increase in voiding pressure consistent with physical obstruction. Glyburide decreased voiding pressure slightly but significantly relative to that of vehicle treated animals. Importantly voiding pressure remained increased above that in sham operated and control rats, indicating that these animals were obstructed. Further evidence of obstruction was demonstrated by flow rate measurements (fig. 5, B). The flow rate was drastically reduced in vehicle and glyburide treated BOO rats compared to control and sham
operated rats with no difference between the 2 groups.

The parameters most affected by glyburide were voided volume and ICI, which are highly related. Vehicle treated BOO rats showed a significant, approximately 50% decrease in voided volume, an effect that was completely reversed in glyburide treated animals (fig. 5, C). A virtually identical pattern was seen for ICI (fig. 5, D).

Vehicle treated rats demonstrated increased contraction time, which was necessary to push urine past the obstruction (fig. 5, E). This was also evident in the duration of the void (fig. 5, F). However, glyburide significantly increased contraction and voiding times.

DISCUSSION

BOO restricts urethral flow, leading to high voiding pressure as the detrusor contracts with greater force to overcome obstruction. Although there is a consensus that heightened pressure triggers inflammation, little is understood of the underlying mechanism. We suggest a central role for NLRP3 in stimulating inflammation by demonstrating that inhibition of NLRP3 alleviates at least 1 major index of bladder dysfunction.

Previous studies implicated an inflammatory response early in the progression of BOO. Kanno et al reported that IL-1β is particularly relevant in this phase. The functional component of the NLRP3 inflammasome is caspase-1, which cleaves pro-IL-1β to its active form. Our results revealed a fourfold increase in caspase-1 activity 12 days after the creation of BOO while caspase-1 activity in sham operated animals was not significantly elevated. In BOO rats that received the NLRP3 antagonist glyburide caspase-1 levels were similar to those in the sham operated and control groups, demonstrating that this major pathway to inflammation was effectively blocked.

BOO also initiates greater than fivefold increases in bladder weight. This parameter was inhibited only 50% by glyburide, although Evan blue extravasation was completely inhibited. While to our knowledge the reason for this remaining increase in bladder weight is unknown, it may be due to detrusor hypertrophy in response to increased
Figure 5. Average voiding pressure (A), urine flow rate (B), average voided volume (C), ICI (D), and duration of detrusor contraction (E) and void (F) recorded during urodynamics in 8, 6, 9 and 5 rats with no surgery (Con), sham surgery (Sham) or BOO and vehicle (Veh) or Gly, respectively. Single asterisk indicates $p < 0.05$. Triple asterisks indicate $p < 0.00$. All other differences were not significant.
load, fibrosis or nonNLRP3 mediated inflammation independent of extravasation. Similar to the changes in Evan blue extravasation the histological changes were mostly reversed with glyburide. Immunocytochemistry indicated primarily urothelial localization as previously shown with no effect of BOO or glyburide.

Functional bladder analysis on cystometry revealed classic parameters that clinically define BOO, including elevated voiding pressure and a decreased flow rate. Moreover voided volume was decreased in obstructed animals given vehicle, a result consistent with most of the literature. There are at least 2 possible reasons for this observation. 1) The time point assessed may be beyond the compensatory phase when the bladder is capable of generating sufficient pressure to overcome increased resistance and maintain normal voiding efficiency. Instead we may be observing the decompensated phase. If this were the case, serial measurements with time would likely show a peak in voiding pressures followed by a decrease. Constant voided volumes would also be followed by a decrease. Since we did not perform serial measurements to document the temporal appearance of each phase, it is not possible to completely rule out this possibility. However stated we are working within a time frame that would be considered early in rat BOO, making it unlikely that these bladders had decompensated.

2) The more likely explanation for the decreased voided volumes is that the procedure that we used to create obstruction produced a severe enough increase in resistance that the bladder could not generate sufficient force to overcome it even in the early phase. We did not measure post-void residual urine volume in this study as doing so creates unacceptable amounts of artifacts when performing these measurements in nonanesthetized animals. However the observed increase in voiding frequency coupled with the low volumes suggests that we witnessed a decrease in efficiency. In this model of partial obstruction the contractile pressure was sufficient to generate urinary flow but it was not sustained long enough to maintain a normal voided volume.

Blockade of NLRP3 did not affect the elevated voiding pressure and flow rate, which was expected since the severity of obstruction remained unchanged. However voided volume and urinary frequency in glyburide treated animals were close to control values. It is well known that inflamed muscle tissue fatigues more rapidly than unaffected muscle tissue. Therefore, preventing NLRP3 activation likely inhibits the inflammatory response and subsequently preserves the functional capability of the detrusor. Results suggest that NLRP3 inhibitors could serve clinically to prevent bladder deterioration induced by inflammation in the long term and ameliorate lower urinary tract symptoms in earlier phases.

This study provides strong evidence that BOO activates the NLRP3 inflammasome, thus initiating an inflammatory response that contributes to voiding dysfunction. However the mechanism by which NLRP3 is activated remains unclear but it is likely to be multifactorial. One of the most prevalent means by which NLRP3 is activated is oxidative stress. Furthermore the increased load on the detrusor muscle requires a higher rate of metabolism, providing another source of reactive oxygen species. Beyond the contribution of oxidative stress urothelial cells subjected to increased pressure are known to produce adenosine triphosphate, which is a well established DAMP. In other fields of study such as orthopedics and periodontics pressure is known to cause necrosis and/or apoptosis. Necrosis is well established to release DAMPs while apoptosis, classically considered noninflammatory, can also be pro-inflammatory under certain circumstances.

After inflammasomes are activated mature IL-1β is released by a death process called pyroptosis, which is similar to necrosis and releases intracellular DAMPs, thus propagating the resulting inflammation.

While each of these possibilities begs further investigation to not only elucidate the true mechanism but also explore potential targets for intervention, it is apparent that NLRP3 activation has a role in the urinary dysfunction associated with BOO.

CONCLUSIONS

Results suggest the importance of the NLRP3 inflammasome in the induction of inflammation and bladder dysfunction secondary to BOO. Excitingly inhibiting NLRP3 with the FDA (Food and Drug Administration) approved drug glyburide ameliorates inflammation and improves voiding dysfunction even in the continued presence of obstruction. These observations suggest that glyburide or a next generation NLRP3 antagonist may be useful in combating the long-term degeneration of bladder function associated with obstructive uropathies.
REFERENCES


