Involvement of JAK-STAT signaling/function after cyclophosphamide-induced bladder inflammation in female rats

Bopaiah P. Cheppudira,1 Beatrice M. Girard,2 Susan E. Malley,1 Abbey Dattilio,1 Kristin C. Schutz,2 Victor May,2 and Margaret A. Vizzard1,2

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Cheppudira BP, Girard BM, Malley SE, Dattilio A, Schutz KC, May V, Vizzard MA. Involvement of JAK-STAT signaling/function after cyclophosphamide-induced bladder inflammation in female rats. Am J Physiol Renal Physiol 297: F1038–F1044, 2009. First published July 22, 2009; doi:10.1152/ajprenal.00110.2009.—Cytokines are upregulated in a variety of inflammatory conditions and cytokine/receptor interactions can activate JAK-STAT signaling. Previous studies demonstrated upregulation of numerous cytokines in the urinary bladder following cyclophosphamide (CYP)-induced cystitis. The role of JAK-STAT signaling in urinary bladder inflammation and referred somatic hypersensitivity has not been addressed. The contribution of JAK-STAT signaling pathways in CYP-induced bladder hyperreflexia and referred somatic hypersensitivity was determined in CYP-treated rats using conscious cystometry with intravesical instillation of cyclophosphamide. Phosphorylation status of STAT-3 was demonstrated upregulation of phosphorylated extracellular signal-regulated kinases (ERK) in urinary bladder and lumbosacral spinal cord with CYP-induced cystitis. Mitogen-activated protein kinase kinase (MEK) inhibitors reduce voiding frequency in CYP-treated rats (10, 11).

The involvement of the JAK-STAT pathway in micturnition reflexes and CYP-induced cystitis has not been addressed. The purpose of this study was to determine and quantify 1) phosphorylated (p) STAT3 expression in urinary bladder and modulation with CYP-induced cystitis, 2) urinary bladder function using conscious cystometry with intravesical instillation of AG490, an inhibitor of JAK2, one of the major STAT3 activators, and 3) CYP-induced hind paw mechanical allodynia after administration of AG490.

MATERIALS AND METHODS

Animals

Adult female Wistar rats (200–225 g; Charles River, St. Constant, Canada) were used for this study. Rats were housed two per cage and maintained in standard laboratory conditions with free access to food and water. The University of Vermont Institutional Animal Care and Use Committee approved all animal use procedures (protocols 06-014, 08-085).

Induction of CYP-Induced Cystitis

Rats were anesthetized under isoflurane (2%) and acute cystitis was induced with a single injection of CYP (150 mg/kg ip) and rodents were used in studies at various time points (4 h, 48 h) after treatment (8, 34, 35). Chronic CYP cystitis was induced by administration of CYP (75 mg/kg ip) once every 3 days for 10 days (8, 34, 35). Control rodents received either saline injection or no treatment. Rats were euthanized using isoflurane (5%) and a thoracotomy.
Western Blotting for pSTAT3 Expression in Whole Urinary Bladder

Whole urinary bladders (control, 4 h, 48 h and chronic; n = 5 each) were homogenized separately in tissue protein extraction agent with protease inhibitors (T-PER; Roche, Indianapolis, IN), and aliquots were removed for protein assay as previously described (35). Samples (23 µg) were suspended in sample buffer for fractionation on gels and subjected to SDS-PAGE. Proteins were transferred to nitrocellulose membranes, and efficiency of transfer was evaluated. Membranes were blocked overnight in a solution of 5% milk, 3% bovine serum albumin in Tris-buffered saline with 0.1% Tween. For immunodetection, the following antibodies were used overnight at 4°C: rabbit anti-phospho-STAT3 Tyr705 (1:2,000; Cell Signaling Technology, Danvers, MA) and mouse anti-STAT3 (1:2,000; Cell Signaling Technology). Washed membranes were incubated in species-specific secondary antibodies for 2 h at room temperature for enhanced chemiluminescence detection (Pierce, Rockford, IL). Blots were exposed to Biomax film (Kodak, Rochester, NY) and developed. The intensity of signal predominantly through the JAK-STAT cascades. To study the effects of AG490 in CYP-induced hind paw mechanical sensitivity, four groups (n = 6) of rats were used. Two groups received either 5 or 15 mg/kg ip of AG490 dissolved in 1 ml of sterile saline with 10% DMSO. A vehicle control group and a no treatment group were also included. One hour after AG490 or vehicle administration, all groups except the no treatment group received CYP (150 mg/kg ip). Four hours later, rats from all groups were tested for mechanical pain sensitivity using von Frey filament testing. Previous studies including those from this laboratory demonstrated increased peripheral mechanical sensitivity induced by CYP treatment at this time point with a return to baseline sensitivity 48 h after CYP treatment (48).

Behavioral testing was performed during the light cycle as previously described (48). Mechanical foot withdrawal threshold was determined using a series of von Frey filaments that produce forces ranging from 0.23 to 59.0 g. Rats were placed unrestrained in a plastic cage with a metal mesh floor and permitted to acclimate to this environment (30 min) before testing. The filaments were pressed perpendicularly to the midplantar surface and held approximately for 2 s. Fibers are applied in an increasing and decreasing diameter around the response threshold, with a maximum of nine applications per paw (the up-down method) (6). The minimum interval between stimulations was 5 min. A stimulus-induced response was considered positive when the paw was sharply withdrawn, paw licking occurred, or the animal flinched upon removal of the filament. If the rat did not respond within 2 s, then the filament was removed. This procedure does not produce damage to the hind paw. All somatic testing was performed in a blinded manner with respect to treatment. The groups were decoded after data analysis.

Materials

All standard chemicals were obtained from Sigma or Fisher and were either analytical or laboratory grade.

Statistics

All values are means ± SE. Data were compared using ANOVA. Percentage data from image analysis were arcsin transformed to meet the requirements of this statistical test. Animals, processed and analyzed on the same day, were tested as a block in the ANOVA. When F ratios exceeded the critical value (P ≤ 0.05), the Newman-Keuls post hoc test was used to compare experimental means.

RESULTS

pSTAT3 Expression in Urinary Bladder with CYP-Induced Cystitis

Cytokines, including members of the neuropoietic family, signal predominantly through the JAK-STAT cascades. To

Exclusion Criteria

Rats were removed from study when adverse events occurred that included: ≥20% reduction in body weight postsurgery, a significant postoperative event, lethargy, pain, or distress not relieved by our IACUC-approved regimen of postoperative analgesics or hematuria in control rodents. In the present study, no rats were excluded from the study or from analysis due to any of these exclusion criteria. In addition, behavioral movements such as grooming, standing, walking, and defecation rendered bladder pressure recordings during these events unusable (47). Experiments were conducted at similar times of the day to avoid the possibility that circadian variations were responsible for changes in bladder capacity measurements (15). Rats were euthanized at the conclusion of study as previously described.

Mechanical Sensitivity Testing

To study the effects of AG490 in CYP-induced hind paw mechanical sensitivity, four groups (n = 6) of rats were used. Two groups received either 5 or 15 mg/kg ip of AG490 dissolved in 1 ml of sterile saline with 10% DMSO. A vehicle control group and a no treatment group were also included. One hour after AG490 or vehicle administration, all groups except the no treatment group received CYP (150 mg/kg ip). Four hours later, rats from all groups were tested for mechanical pain sensitivity using von Frey filament testing. Previous studies including those from this laboratory demonstrated increased peripheral mechanical sensitivity induced by CYP treatment at this time point with a return to baseline sensitivity 48 h after CYP treatment (48).

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RESULTS

pSTAT3 Expression in Urinary Bladder with CYP-Induced Cystitis

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tyrosine kinase inhibitor. In control rats, intravesical infusion of AG490 (5 mg/kg) combined with continuous fill cystometry did not affect bladder pressures and had no effect on number of NVCs per micturition cycle or the duration of the intermicturition interval (Table 1) compared with control rats treated with vehicle.

**CYP treatment.** As previously demonstrated (12, 19, 45, 46) and confirmed here, CYP treatment (4 h, 48 h) significantly \((P \leq 0.001)\) decreased the interval between micturition events (i.e., intermicturition interval; Table 1; Fig. 2A). Intravesical infusion of AG490 (5 mg/kg) in 4-h CYP-treated rats significantly \((P \leq 0.001)\) increased the intermicturition interval but produced no effects on the number of NVCs or bladder pressures compared with CYP-treated (4 h) rats treated with vehicle. Similarly, intravesical infusion of AG490 (5 mg/kg) in 48-h CYP-treated rats significantly \((P \leq 0.001)\) increased the intermicturition interval but produced no effects on NVCs or bladder pressures (Table 1; Fig. 2B) compared with CYP-treated (48 h) rats treated with vehicle.

**Mechanical Allodynia**

Previous studies in mice and rats demonstrate increased peripheral mechanical sensitivity after CYP-induced cystitis (23, 48). To assess whether increased cytokine/cytokine signaling mediates some of the sensory changes associated with cystitis, hind paw mechanical sensitivity to von Frey filaments in CYP-treated and control (no inflammation) rats was tested in the presence of AG490 (5, 15 mg/kg ip). After CYP treatment (4 h) and vehicle treatment, the paw withdraw threshold was significantly reduced compared with control (no inflammation; \(P \leq 0.05\); Fig. 3). The reduction in paw withdraw threshold was consistent with previous studies examining hind paw sensitivity after CYP-induced cystitis (23, 48). Treatment with both concentrations of AG490 (5, 15 mg/kg) produced a similar and significant \((P \leq 0.05)\) increase in paw withdraw threshold (Fig. 3) compared with CYP-treated rats with vehicle.

**DISCUSSION**

The present studies demonstrate several novel findings with respect to the involvement of JAK-STAT signaling in CYP-induced bladder hyperreflexia and somatic hypersensitivity. Cytokine receptors signal predominantly through JAK-STAT pathways and we determined whether CYP-induced cystitis of varying duration was associated with enhanced JAK-STAT signaling. We showed that STAT3 phosphorylation/activation was increased after CYP-induced cystitis (4 h, 48 h, and

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**Table 1. Summary of cystometric parameters and numbers of observations**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>N Value</th>
<th>Fill Pressure, cmH2O</th>
<th>Threshold Pressure, cmH2O</th>
<th>Micturition Pressure, cmH2O</th>
<th>NVC/Cycle</th>
<th>Intermicturition Interval, s</th>
</tr>
</thead>
<tbody>
<tr>
<td>No CYP + vehicle</td>
<td>6</td>
<td>12.8±0.5</td>
<td>17.8±1.0</td>
<td>76.8±6.4</td>
<td>0.5±0.2</td>
<td>306.2±16.4</td>
</tr>
<tr>
<td>No CYP + AG490 (5 mg/kg)</td>
<td>6</td>
<td>13.6±0.4</td>
<td>16.8±1.0</td>
<td>79.8±4.0</td>
<td>0.4±0.3</td>
<td>314.8±19.3</td>
</tr>
<tr>
<td>4-h CYP + vehicle</td>
<td>6</td>
<td>13.3±1.0</td>
<td>18.7±1.0</td>
<td>76.1±7.5</td>
<td>0.7±0.1</td>
<td>92.5±17.6*</td>
</tr>
<tr>
<td>4-h CYP + AG490 (5 mg/kg)</td>
<td>6</td>
<td>12.7±0.5</td>
<td>17.3±1.4</td>
<td>69.5±2.2</td>
<td>0.1±0.2</td>
<td>183.8±29.7†</td>
</tr>
<tr>
<td>48-h CYP + vehicle</td>
<td>6</td>
<td>12.8±0.9</td>
<td>17.1±1.6</td>
<td>74.5±10.8</td>
<td>0.5±0.3</td>
<td>155.9±20.4*</td>
</tr>
<tr>
<td>48-h CYP + AG490 (5 mg/kg)</td>
<td>6</td>
<td>12.7±1.1</td>
<td>16.0±0.5</td>
<td>72.8±11.5</td>
<td>0.1±0.2</td>
<td>235.3±25.4‡</td>
</tr>
</tbody>
</table>

Values are means ± SE. Summary of cystometric parameters and numbers of observations in control and cyclophosphamide (CYP)-treated (4 and 48 h) with and without the JAK2 inhibitor, AG490 (5 mg/kg; intravesical instillation) in conscious, unrestrained rats with continuous saline instillation is shown. \(*P \leq 0.01\) compared with control. \(†P \leq 0.01\) compared with 4-h CYP + vehicle. \(‡P \leq 0.01\) compared with 48-h CYP + vehicle. NVC, nonvoiding contractions.
Intravesical instillation of AG490 in CYP-treated rats, voiding frequency was significantly reduced. The intravesical instillation of AG490 in CYP-treated rats, voiding frequency was reduced. With NVCs were not significantly affected by AG490 treatment (see Table 1). Arrows point to some NVCs induced by CYP treatment (see Table 1).

The function recordings in CYP-treated [4 h (A) and 48 h (B)] rats treated with vehicle or the JAK2 inhibitor, AG490 (5 mg/kg; intravesical instillation). Intravesical administration of AG490 reduced voiding frequency after CYP-induced cystitis. A: continuous cystometrogram recordings in a 4-h CYP-treated + vehicle (top trace) and same rat treated with AG490 (bottom trace). Arrows point to some nonvoiding bladder contractions (NVCs). In CYP-treated rats further treated with intravesical instillation of AG490, voiding frequency was reduced. B: continuous cystometrogram recordings in a 48-h CYP-treated + vehicle (top trace) and same rat treated with AG490 (bottom trace). Arrows point to some NVCs induced by CYP treatment (see Table 1). NVCs were not significantly affected by AG490 treatment (see Table 1). With intravesical instillation of AG490 in CYP-treated rats, voiding frequency was reduced. The x-axis represents time (s) and the y-axis represents intravesical pressure (cmH₂O).

Fig. 2. Bladder function recordings in CYP-treated [4 h (A) and 48 h (B)] rats treated with vehicle or the JAK2 inhibitor, AG490 (5 mg/kg; intravesical instillation). Intravesical administration of AG490 reduced voiding frequency after CYP-induced cystitis. A: continuous cystometrogram recordings in a 4-h CYP-treated + vehicle (top trace) and same rat treated with AG490 (bottom trace). Arrows point to some nonvoiding bladder contractions (NVCs). In CYP-treated rats further treated with intravesical instillation of AG490, voiding frequency was reduced. B: continuous cystometrogram recordings in a 48-h CYP-treated + vehicle (top trace) and same rat treated with AG490 (bottom trace). Arrows point to some NVCs induced by CYP treatment (see Table 1). NVCs were not significantly affected by AG490 treatment (see Table 1). With intravesical instillation of AG490 in CYP-treated rats, voiding frequency was reduced. The x-axis represents time (s) and the y-axis represents intravesical pressure (cmH₂O).

chronic). Functionally, blockade of JAK2 with AG490 significantly reduced bladder hyperreflexia and hind paw sensitivity in CYP-treated rats. In aggregate, these studies demonstrate potential roles for JAK-STAT signaling pathways in contributing to bladder hyperreflexia and referred pain of CYP-induced bladder inflammation. These studies extend the list of signal transduction pathways that may represent novel targets for pharmacological intervention in bladder inflammation and referred somatic sensitivity.

IC/PBS is a chronic inflammatory bladder disease syndrome characterized by urinary frequency, urgency, suprapubic and pelvic pain (17, 44). Although the etiology and pathogenesis of IC are unknown, numerous theories including infection, autoimmune disorder, toxic urinary agents, deficiency in bladder wall lining, and neurogenic causes have been proposed (17, 25, 32, 44, 46). We hypothesized that pain associated with IC/PBS involves an alteration of visceral sensation/bladder sensory physiology. Altered visceral sensations from the urinary bladder (i.e., pain at low or moderate bladder filling) that accompany IC/PBS (17, 25, 32, 44, 46) may be mediated by many factors including changes in the properties of peripheral bladder afferent pathways such that bladder afferent neurons respond in an exaggerated manner to normally innocuous stimuli (allodynia). These changes may be mediated, in part, by inflammatory changes in the urinary bladder. Among potential mediators of inflammation, neurotrophins (e.g., nerve growth factor) have been implicated in the peripheral sensitization of nociceptors (13, 16, 38). Proinflammatory cytokines also cause sensitization of polymodal C-fibers (16) and facilitate A-beta input to the spinal cord (2, 53). Several studies from our laboratory demonstrated increased cytokine expression including IL-6 and LIF in the urinary bladder after CYP-induced cystitis (7, 40). Furthermore, a recent study also demonstrated upregulation of IL-6 transcript and protein expression in the submucosal layer of bladder after CYP treatment in mice (43).

Possible mechanisms underlying the neural plasticity and bladder hyperreflexia following chronic CYP-induced cystitis (49, 50, 52) may involve alterations in neurotrophic factors in the urinary bladder (51). In addition, neuroimmune activation, including the production of cytokines, occurs after injury to the central or peripheral nervous system and cytokines are also likely to play a role in the development of pain, exacerbate pathology, or may contribute to repair strategies (1, 9, 24). Individuals with IC/PBS report a predominance of suprapubic pain as well as urethral, genital, and nongenitourinary pain. In addition, hypersensitivity to somatic stimuli has been observed in subjects with IC/PBS (20, 42). A number of reports demonstrated referred somatic hypersensitivity in animal models of urinary bladder inflammation including CYP (23, 48). In this study, we demonstrated a reduction in hind paw sensitivity in rats treated with CYP (4 h) and a reduction in CYP-induced urinary bladder hyperreflexia when rats were also treated with a JAK2 inhibitor, AG490. Tyrosine kinase inhibitors, including AG490, are a class of drugs used to block the activity of tyrosine kinases and the signaling pathways they activate to inflammatory changes in the urinary bladder. Among potential factors including changes in the properties of peripheral bladder afferent pathways such that bladder afferent neurons respond in an exaggerated manner to normally innocuous stimuli (allodynia). These changes may be mediated, in part, by inflammatory changes in the urinary bladder. Among potential mediators of inflammation, neurotrophins (e.g., nerve growth factor) have been implicated in the peripheral sensitization of nociceptors (13, 16, 38). Proinflammatory cytokines also cause sensitization of polymodal C-fibers (16) and facilitate A-beta input to the spinal cord (2, 53). Several studies from our laboratory demonstrated increased cytokine expression including IL-6 and LIF in the urinary bladder after CYP-induced cystitis (7, 40). Furthermore, a recent study also demonstrated upregulation of IL-6 transcript and protein expression in the submucosal layer of bladder after CYP treatment in mice (43).

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reduces cytokine production, inflammatory cell infiltration, blocks STAT3 activation and nitric oxide (NO) production, and improves survival rate in nonseptic shock and peripheral nerve injury models (12). In addition, blockade of the STAT3 pathway with AG490 attenuated mechanical allodynia and thermal hyperalgesia after peripheral nerve injury (14). In the present study, the conditions of our bladder function experiments (e.g., intravesical route of AG490, duration of exposure, and dilution of AG490 with urine production) make confirming the effects of AG490 on JAK2 signaling and pSTAT3 expression extremely challenging. We also only evaluated the acute effects of AG490 on hind paw sensitivity as previous studies demonstrated a return to baseline somatic sensitivity 48 h after CYP treatment (48) so we cannot make any conclusions about the involvement of JAK-STAT signaling in chronically maintained somatic sensitivity induced by CYP-induced cystitis. Although in previous studies in mice we determined pelvic region sensitivity after CYP-induced cystitis (48), this was not possible in the present studies in rats because the length of the von Frey filaments was not adequate to stimulate the pelvic region of the rat. Our present data suggest a role for the activation of the JAK-STAT3 pathway in referred somatic sensitivity of visceral origin of acute duration and bladder hyperreflexia with CYP-induced cystitis at acute (4 h) and intermediate (48 h) time points.

In the present study, intravesical instillation of a JAK2 inhibitor, AG490, reduced voiding frequency (i.e., increased the intercontraction interval) in CYP-treated (4 h, 48 h) rats but was without effect on bladder pressures or NVCs. Intravesical instillation of AG490 also had no effect on bladder function in control rats. We based the dose (5 mg/kg) used in the current study on our somatic testing data, which used a dose consistent with published reports (12, 14); however, this dose may not be optimal as there is no literature precedent for intravesical instillation of AG490. No effects on bladder function were observed in control rats treated with this AG490 dose; the effects were only observed on bladder function in rats treated with CYP and only on intercontraction interval, not on bladder pressure or NVCs. The absence of broad effects on all cystometric parameters and effects only in inflamed urinary bladder suggests specificity in AG490 action at the dose used in this study. The effects of AG490 were similar to those observed in our previous studies with intravesical instillation of U0126, a potent and selective MEK inhibitor (10). U0126 decreased the voiding frequency and exhibited no effects on NVCs. The lack of effect of AG490 on NVCs may be related to the concentration of AG490 tested, and/or lack of penetration to the detrusor smooth muscle with intravesical instillation. It is possible that the observed AG490 effects on bladder function in CYP-treated rats may reflect actions at the level of the urothelium and suburothelial nerve plexus. Previous studies also demonstrated that intrathecal administration of the ERK inhibitor, PD98059, also decreased voiding frequency in rats treated with CYP (200 mg/kg; 4 h) (11). The action of AG490 could be attributed to either preventing a posttranslational change mediated by the JAK-STAT signal transduction pathway or to a reduction in transcription of target genes. We did not evaluate the bladder effects of AG490 in rats treated chronically with CYP because the increased phosphorylation status of STAT3 was maximal with 48-h CYP treatment and no differences in phosphorylation status of STAT3 were observed between 4 h and chronic CYP treatment. We evaluated and demonstrated improvement in bladder function with AG490 at an acute (4 h) and intermediate (48 h) time point of bladder inflammation induced by CYP; however, whether AG490 exhibits similar effects on bladder function after chronic CYP treatment is not currently known so the relevance of these data to the chronic syndrome of PBS/IC is not known. Furthermore, it is not currently known how any of the time points evaluated (4 h, 48 h, and chronic) with CYP-induced cystitis relate to IC/PBS, thus one must always be cautious in interpretation and potential significance of these rodent data to the human IC/PBS syndrome.

STAT3 plays a role in the transcription control of many genes including glial fibrillary acidic protein, gp130, suppressor of cytokine signaling 3 and cyclooxygenase-2 (COX-2) (14) and STAT3 expression is altered with PACAP addition to PC12 cells (29). Previous studies demonstrated involvement of COX-2, prostaglandins, and PACAP in bladder hyperreflexia after CYP treatment (5, 27, 33). The rapid nature of the effect of AG490 in reducing acute pain hypersensitivity and bladder hyperreflexia may be attributed to a posttranslational change downstream of the activation of STAT as has been previously suggested for the rapid effects demonstrated with MEK inhibitors (31). It would be of interest in future studies to determine effects of combined JAK and MEK blockade on bladder function after CYP-induced cystitis. Furthermore, it is not known what is/are the upstream chemical mediator(s) that are activated by CYP-induced cystitis to stimulate JAK-STAT signaling. Future studies involving LIF or IL-6 null mice and subsequent responses to CYP-induced cystitis can begin to address this interesting question.

Conclusions

In summary, these studies suggest the involvement of the JAK-STAT signaling pathway in CYP-induced cystitis. Significant upregulation of pSTAT3 expression was demonstrated in the urinary bladder with CYP-induced cystitis (4 h, 48 h, and chronic). Blockade of JAK2 with AG490 significantly reduced bladder hyperreflexia and referred somatic pain induced by CYP treatment. Blockade of the JAK-STAT signaling pathway may be a novel target to improve bladder function and referred somatic sensitivity after urinary bladder inflammation.

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REFERENCES


