
Acute Stress and Intravesical Corticotropin-Releasing Hormone Induces Mast Cell Dependent Vascular Endothelial Growth Factor Release From Mouse Bladder Explants

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Purpose: Corticotropin-releasing hormone is typically released from the hypothalamus but it has proinflammatory effects outside of the brain, possibly through the activation of mast cells. These cells express corticotropin-releasing hormone receptors with selective secretion of vascular endothelial growth factor, which may be involved in the pathogenesis of painful bladder syndrome/interstitial cystitis. This condition is characterized by bladder inflammation and worsened by stress. We investigated the effect of intravesical corticotropin-releasing hormone and acute restraint stress on vascular endothelial growth factor release from mouse bladder explants and the role of mast cells.

Materials and Methods: The bladder of C57BL/6 mast cell deficient (W/W^v) and normal congenic (+/+) female mice (Jackson Laboratories, Bar Harbor, Maine) at ages 10 to 12 weeks was catheterized using anesthesia. After emptying urine 1) normal saline or corticotropin-releasing hormone was introduced for 45 minutes, urine was collected and the mice were allowed to recover for 4 hours before sacrifice or 2) the mice were stressed by placing them in a restrainer for 4 hours before sacrifice and urine was collected 2 hours after stress. The bladder was removed 4 hours after stress and processed for corticotropin-releasing hormone immunohistochemical staining. In other experiments the bladder was removed, minced into 1 mm² pieces and cultured with or without corticotropin-releasing hormone overnight. Urine and medium were frozen for histamine, interleukin-6, tumor necrosis factor- α and vascular endothelial growth factor assay.

Results: Corticotropin-releasing hormone (100 nM) or acute restraint stress (4 hours) increased histamine release in urine and vascular endothelial growth factor release in medium without increasing interleukin-6 or tumor necrosis factor- α in the bladder explants of C57BL/6 or +/+ but not W/W^v mice. No vascular endothelial growth factor, interleukin-6 or tumor necrosis factor- α was detected in urine before or after stimulation. Corticotropin-releasing hormone immunoreactivity was present in control bladders but it increased dramatically in the bladder of stressed mice.

Conclusions: Intravesical corticotropin-releasing hormone and acute restraint stress induced mast cell dependent vascular endothelial growth factor release from bladder explants. These findings suggest that stress, corticotropin-releasing hormone, mast cells and vascular endothelial growth factor might participate in the pathogenesis of painful bladder syndrome/interstitial cystitis, which is worsened by stress, and provide for new therapeutic targets.

Key Words: bladder; cystitis, interstitial; corticotropin-releasing hormone; mast cells; stress

Stress stimulates the hypothalamic-pituitary-adrenal axis through the release of CRH, leading to the secretion of glucocorticoids that down-regulate immune responses.¹ However, CRH is also secreted outside of the brain, where it exerts proinflammatory effects of stress that are apparently mediated through mast cells.²

Mast cells are normally distributed throughout the body and they are necessary for the development of allergic reactions, during which they secrete vasoactive and nociceptive molecules as well as numerous cytokines.^{3,4} However, increasing evidence indicates that mast cells are also involved

in innate and acquired immunity⁴ as well as in neuroinflammatory conditions affected by stress,³ such as PBS/IC.⁵ PBS/IC is a sterile bladder condition that occurs primarily in women and is characterized by pelvic pain, pressure and discomfort as well as irritative voiding symptoms.⁶ The 2 main theories of PBS/IC pathophysiology are 1) some defect in the protective glycosaminoglycan layer of the bladder mucosa⁷ and 2) an increased number of activated bladder mast cells.⁸ Interestingly about 45% of patients with PBS/IC experience or have a history of allergies.⁹ In particular there are twice as many mast cells in the urothelium and 10 times as many in the detrusor of patients with PBS/IC compared to controls.¹⁰ An increased number of bladder tryptase-positive mast cells was the only pathological finding that correlated with nocturia in bladder biopsies in patients with PBS/IC.¹¹ Bladder mast cells are located close to neurons and are activated by neuropeptides, antidromic nerve stimulation and acute stress.¹² Mast cells secrete tryptase, which causes microvascular leakage,¹³ and VEGF, of which an isoform is particularly vasodilatory.¹⁴ VEGF expression

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was increased in bladder tissue from patients with IC and it was considered to be involved in the development of bladder glomerulations.¹⁵

CRH has been reported to increase mast cell dependent vascular permeability in rodent² and human¹⁶ skin. We recently reported that HMC-1 human leukemic mast cells and human umbilical cord blood derived mast cells express CRH-R and CRH stimulates mast cells to release newly synthesized VEGF without the preformed mediators tryptase and histamine, or the cytokines IL-6, IL-8 or TNF- α .¹⁷ However, to our knowledge a direct effect of CRH on bladder mast cells *in vivo* is still unknown. We investigated the effect of CRH and acute restraint stress on the release of proinflammatory mediators in urine and in bladder explants of normal and mast cell deficient mice.

MATERIALS AND METHODS

Animals and Protocol

Ten to 12-week-old female C57BL/6, mast cell deficient (W/W^v) mice and their +/+ normal counterparts were used.

Experimental Design

Three groups of animals, including C57BL/6 mice, W/W^v mast cell deficient mice and their congenic normal (+/+) controls, were used in experiments. Each group underwent treatment, including 1) intravesical NS (0.9% NaCl) or intravesical NS plus NS in culture as the control, 2) intravesical CRH or intravesical CRH plus CRH in culture, 3) non-stress control and 4) acute restraint stress. This protocol (50-02) was approved by the University Animal Care and Use Committee.

CRH Stimulation

The animals were anesthetized with intraperitoneal injection of a mixture of ketamine (80 mg/kg) and xylazine (10 mg/kg) in NS. With its tip lubricated with glycerin, a translucent 24 gauge 3/4-inch catheter was introduced transurethally into the bladder and advanced until the first drop of urine appeared in the hub (fig. 1, A). Pyrogen-free NS (0.15 ml) was instilled into each bladder to wash and NS together with urine was then removed. NS (0.15 ml) as the control or CRH was instilled into the bladder and allowed to remain for 45 minutes. The syringe was kept on the catheter during this time to prevent leakage. Subsequently, urine and instilled solution were removed and stored at -80C for mediator assays.

After the mice awakened from anesthesia they were allowed to remain in the cages for 4 hours after NS or CRH was first instilled. They were then sacrificed by cervical dislocation. Each bladder was removed, placed in chilled RPMI 1640 medium and minced into 1 mm² pieces in a cold Petri dish on ice. The bladder explants were plated in separate wells in a 24-well cell culture plate in 0.5 ml RPMI 1640 medium containing various concentrations of CRH, 10% fetal bovine serum, 100 U/ml penicillin/streptomycin and a protease inhibitor cocktail (Sigma, St. Louis, Missouri). The bladder explants were incubated overnight at 37C in 5% CO₂. The next day the medium was removed by aspiration and stored at -80C for mediator assays. The remaining tissue was blotted and weighed.

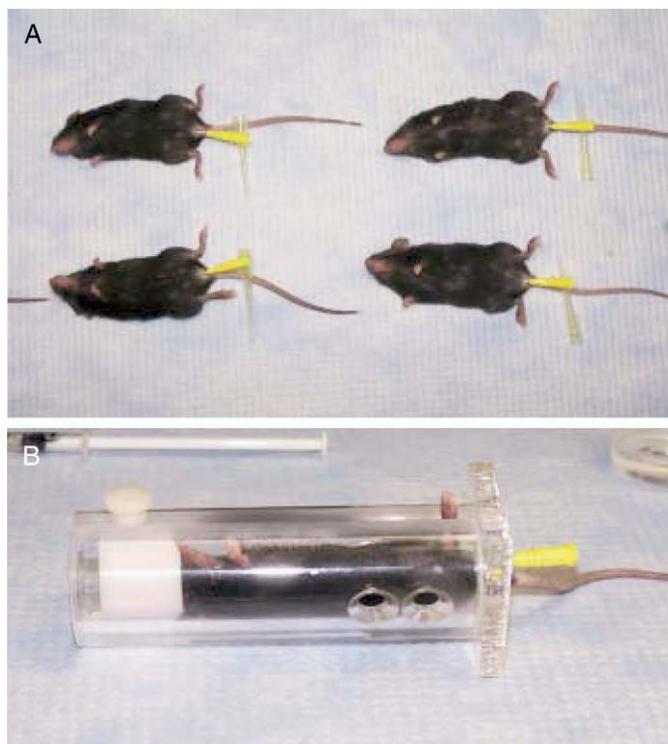


FIG. 1. Female C57BL/6 mice with catheter inserted intravesically. Note anesthetized (A) and conscious (B) mice inside restrainer.

Restraint Stress

The animals were anesthetized and a catheter was introduced transurethally as described for CRH stimulation. The animals were then placed in restraint chambers (Harvard Apparatus, Holliston, Massachusetts) and stressed for 4 hours (fig. 1, B). After the first 2 hours urine and instilled solution were removed and stored at -80C for mediator assays. Nonstressed control animals remained anesthetized throughout the experiment.

After 4 hours of stress the animals were sacrificed by cervical dislocation. Each bladder was removed, placed in chilled RPMI 1640 medium and minced into 1 mm² pieces in a cold Petri dish on ice. Bladder explants were then plated in separate wells in a 24-well cell culture plate in 0.5 ml RPMI 1640 medium containing 10% fetal bovine serum, 100 U/ml penicillin/streptomycin and a protease inhibitor cocktail. The bladder explants were incubated overnight at 37C in 5% CO₂. The next day medium was removed by aspiration and stored at -80C for mediator assays. The remaining tissue was blotted and weighed.

Immunohistochemical Staining for CRH

The bladders were fixed in freezing medium (Triangle Bio-medical Sciences, Durham, North Carolina) immediately after collection. Cryostat sections (8 μ m) were prepared, dried and stored at -80C until staining. These sections were stained for CRH using an ABC kit (Santa Cruz Biotechnology, Santa Cruz, California). The sections were fixed with Carnoy's solution (60% ethanol, 30% chloroform and 10% glacial acetic acid) for 5 minutes. They were incubated with 0.03% hydrogen peroxide for 5 minutes and with 1.5% blocking serum for 1 hour at room temperature. Following this the sections were incubated with CRH goat polyclonal IgG

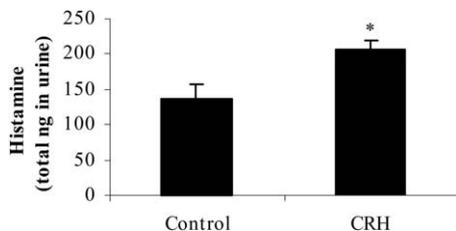


FIG. 2. Effect of CRH on histamine release in urine of C57BL/6 mice. NS or CRH (100 nM) was instilled transurethraly into bladder of C57BL/6 mice and allowed to remain for 45 minutes. Urine was removed for histamine assay. Data represent mean \pm SEM of 4 preparations. Asterisk indicates $p < 0.05$ vs control.

(Santa Cruz Biotechnology) (1:50 dilution) overnight at 4°C and then with biotinylated secondary antibody (donkey anti-goat IgG) for 1 hour at room temperature. The slides were incubated with avidin and biotinylated horseradish peroxidase for 1 hour and with peroxidase substrate solution (diaminobenzidine chromogen) for 10 minutes at room temperature. The slides were washed with tris-buffered saline between incubations. For control staining CRH blocking peptide, normal goat serum (Santa Cruz Biotechnology) and no primary antibody was used.

Mediator Assays

Histamine, VEGF, IL-6 and TNF- α were measured in urine collected after 45 minutes of instillation and in tissue-free medium supernatant collected after overnight incubation of bladder explants using ELISA (R and D Systems, Minneapolis, Minnesota).

Statistical Analysis

The results of mediator release are presented as the mean \pm SEM. They were compared using the paired Student *t* test or by nonparametric analysis using the Mann-Whitney *U* test. Significance was considered at $p < 0.05$.

RESULTS

Effect of Intravesical CRH

C57BL/6 mice were first used to investigate the effect of intravesical CRH because other genetically altered mice considered for future investigations are C57BL/6-derived. Histamine was measured as an index of a pre-stored mast cell mediator that could be secreted soon after stimulation. Total histamine release in the urine of C57BL/6 control mice was 137.2 ± 20.1 ng. This increased to 204.0 ± 13.1 ng in 4

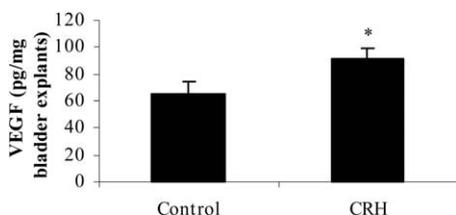


FIG. 3. Effect of CRH on VEGF release in medium from bladder explants of C57BL/6 mice. NS or CRH (100 nM) was instilled transurethraly into bladder of C57BL/6 mice, allowed to remain for 45 minutes and transferred to bladder explants overnight. Medium was collected next day and assayed for VEGF release by ELISA. Data represent mean \pm SEM of 6 preparations. Asterisk indicates $p < 0.05$ vs control.

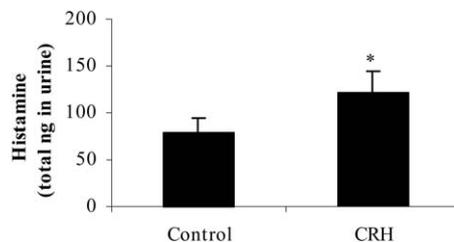


FIG. 4. Effect of CRH on histamine release in urine of congenic, normal (+/+) mice. NS or CRH (100 nM) was instilled transurethraly into bladder of +/+ mice and allowed to remain for 45 minutes. Urine was removed for histamine assay. Data represent mean \pm SEM of 3 preparations. Asterisk indicates $p < 0.05$ vs control.

preparations in response to intravesical CRH (100 nM) ($p = 0.007$, fig. 2).

VEGF release in the medium from bladder explants of C57BL/6 control mice that received only NS intravesically and in culture was 64.7 ± 9.4 pg/mg. This increased to 90.0 ± 8.7 pg/mg in 6 preparations when stimulated with CRH (100 nM) ($p = 0.002$, fig. 3). Administration of CRH intravesically but not in culture or intravesical NS and CRH only in culture did not yield significant results (data not shown).

Mast Cell Requirement of the Effect of Intravesical CRH

Total histamine release in the urine of +/+ mice was 79.8 ± 14.9 ng. This increased to 120.3 ± 23.1 ng in 3 preparations after CRH (100 nM) stimulation ($p = 0.042$, fig. 4). VEGF release in the medium from bladder explants of +/+ mice was 43.0 ± 5.6 pg/mg. This increased to 70.4 ± 6.8 pg/mg in 6 preparations in response to CRH (100 nM) stimulation ($p < 0.001$, fig. 5, A). A dose-response study of CRH (10 nM to

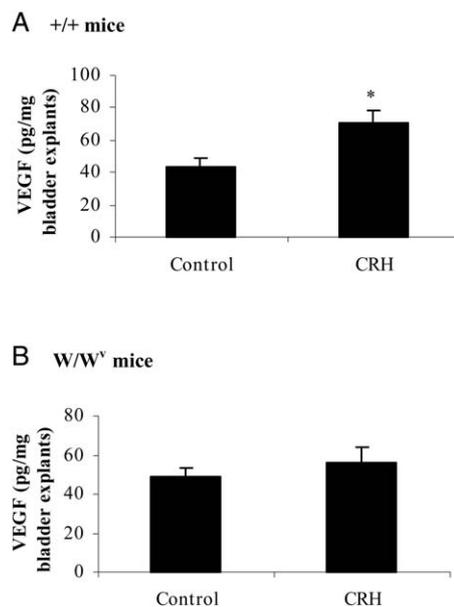


FIG. 5. Effect of CRH on VEGF release in medium from bladder explants of congenic normal (+/+) (A) and W/W^v mast cell deficient (B) mice. NS or CRH (100 nM) was instilled transurethraly into bladder of +/+ or W/W^v mice, allowed to remain for 45 minutes and transferred to bladder explants overnight. Medium was collected next day and assayed for VEGF release by ELISA. Data represent mean \pm SEM of 6 preparations. Asterisk indicates $p < 0.05$ vs control.

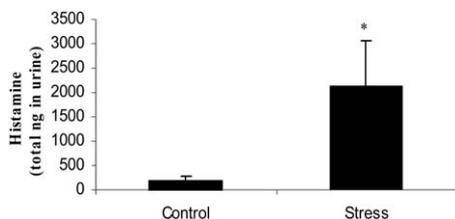


FIG. 6. Effect of acute restraint stress on histamine release in urine of C57BL/6 mice stressed in restraint chamber for 4 hours. Urine was collected for first 2 hours of stress for histamine assay. Data represent mean \pm SEM of 6 preparations. Asterisk indicates $p < 0.05$ vs control.

1 μ M) in 4 preparations showed that the best effect was achieved with 100 nM (results not shown).

Total histamine release in the urine and medium of W/W^v mast cell deficient mice was under the detection level. VEGF release in the medium from bladder explants of W/W^v mice that received only NS was 49.1 ± 4.7 pg/mg. This remained similar at 55.9 ± 8.4 in response to CRH (100 nM) stimulation in 6 preparations ($p = 0.42$, fig. 5, B).

In C57BL/6, W/W^v and +/+ control mice no VEGF, IL-6 or TNF- α was detected in urine before or after CRH stimulation. There was also no change in histamine and IL-6 release in medium and TNF- α was under detection level (data not shown).

Effect of Acute Restraint Stress

Total histamine release in the urine of C57BL/6 control mice was 194.5 ± 85.4 ng. This increased to 2133.0 ± 937.6 ng in 6 preparations in response to stress ($p = 0.015$, fig. 6).

VEGF release in the medium from bladder explants of unstressed C57BL/6 control mice was 50.2 ± 9.2 pg/mg. This increased to 83.9 ± 8.7 pg/mg in 6 preparations in stressed samples ($p = 0.002$, fig. 7).

Mast Cell Requirement of the Effect of Acute Restraint Stress

Total histamine release in the urine of +/+ mice was 95.1 ± 36.6 ng. This increased to 696.4 ± 209.2 ng in 7 preparations in stressed samples ($p = 0.002$, fig. 8). VEGF release in the medium from bladder explants of +/+ mice was 47.2 ± 2.1 pg/mg. This increased to 72.0 ± 2.7 pg/mg in 5 preparations in response to stress ($p < 0.001$, fig. 9, A). VEGF release in the medium from bladder explants of unstressed W/W^v mice was 48.7 ± 4.2 pg/mg. This remained similar at 50.4 ± 4.7 pg/mg in response to stress in 5 preparations ($p = 0.79$, fig. 9, B).

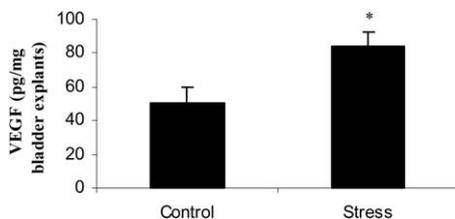


FIG. 7. Effect of acute restraint stress on VEGF release in medium from bladder explants of C57BL/6 mice stressed in restraint chamber for 4 hours before sacrifice. Bladder explants were incubated overnight. Medium was collected next day and assayed for VEGF release by ELISA. Data represent mean \pm SEM of 6 preparations. Asterisk indicates $p < 0.05$ vs control.

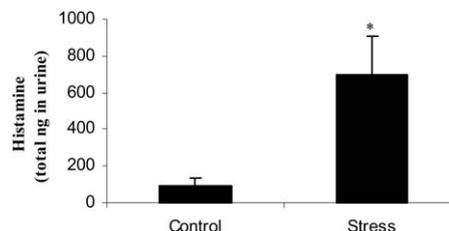


FIG. 8. Effect of acute restraint stress on histamine release in urine of congenic normal (+/+) mice stressed in restraint chamber for 4 hours. Urine was collected for first 2 hours of stress for histamine assay. Data represent mean \pm SEM of 7 preparations. Asterisk indicates $p < 0.05$ vs control.

In C57BL/6 mice, W/W^v and +/+ control mice no VEGF, IL-6 or TNF- α was detected in urine before or after stress. There was also no change in histamine or IL-6 release in medium and TNF- α was under the detection level (data not shown).

Effect of Acute Restraint Stress on Bladder CRH Expression

Immunocytochemistry for CRH showed patchy, faint staining in the bladder of nonstressed control animals (fig. 10, A). However, following acute restraint stress bladder sections stained strongly for CRH (fig. 10, B and C). Negative controls included pre-absorption of immune antibody with a blocking peptide, inclusion of nonimmune goat serum or absent primary antibody, which did not show a positive reaction for CRH (fig. 10, D to F).

DISCUSSION

To our knowledge this is the first report showing that CRH could be released locally in the bladder under stress and

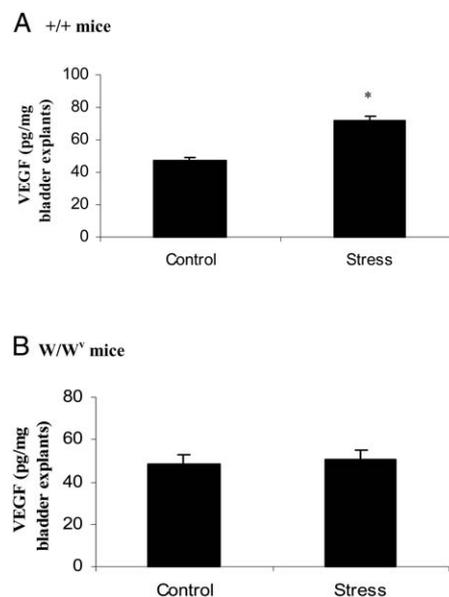


FIG. 9. Effect of acute restraint stress on VEGF release in medium from bladder explants of congenic normal (+/+) (A) and W/W^v mast cell deficient (B) mice stressed in restraint chamber for 4 hours before sacrifice. Bladder explants were incubated overnight. Medium was collected next day and assayed for VEGF release by ELISA. Data represent mean \pm SEM of 5 preparations. Asterisk indicates $p < 0.05$ vs control.

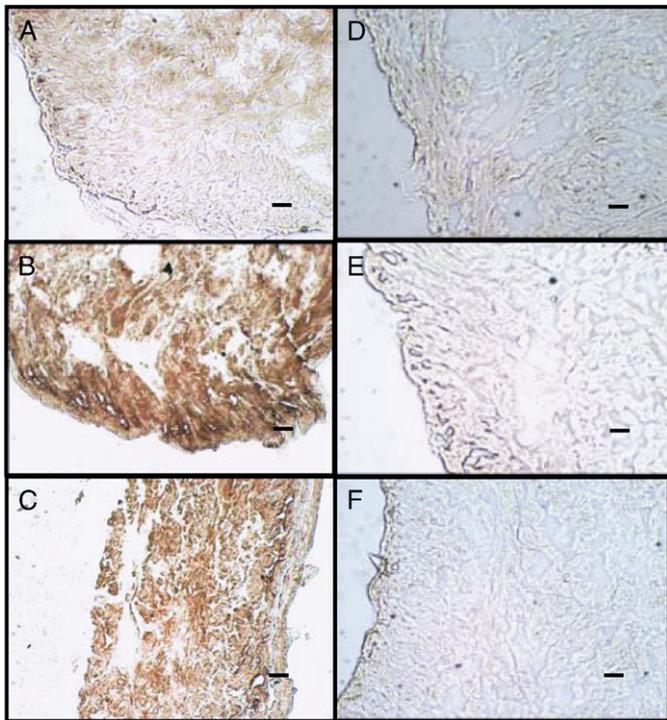


FIG. 10. Bladder sections were stained with CRH goat polyclonal IgG. Brown areas indicate positive reaction for CRH. Control bladder shows weak positive CRH staining (A) compared to bladder from restraint stressed animals (B and C). Bladder tissues incubated with blocking peptide (D), normal goat serum (E) and no primary antibody (F) show no positive reaction for CRH. Scale bars indicate 10 μ m.

induce selective VEGF release from that dependent on mast cells. Histamine was released in the urine of control mice, indicating that there may be immediate and delayed effects in response to CRH and stress or that histamine release requires an intact organism. For instance, *in vivo* stimulation may also require the synergistic action of other neuropeptides, such as substance P¹⁸ or neurotensin.¹⁹ Mast cells have previously been shown to secrete VEGF in response to immunological stimulation¹⁴ and by CRH selectively.¹⁷ VEGF expression is higher in bladder biopsies from patients with PBS/IC and it was suggested that it may contribute to the development of glomerulations in these patients.¹⁵

Neuroimmune interactions involving CRH released from sacral nerves and mast cells in the bladder were recently described as having a key role in the pathogenesis of IC.⁵ Bladder mast cells are increased and activated in IC.⁸ Acute immobilization stress triggered bladder mast cell degranulation¹² and bladder mucosal damage reminiscent of IC.²⁰ IC symptoms are worsened by stress²¹ and they are associated with panic disorder,²² suggesting that there may be local release of CRH in the bladder. CRH is present in the dorsal root ganglia²³ and in pelvic nerve afferent projections in the dorsal part of the sacral spinal cord. Moreover, bladder gene array analysis of mouse experimental cystitis showed that there was up-regulation of CRH-R.²⁴ Acute stress has also been shown to increase the CRH content in the skin,²⁵ while intradermal administration of CRH induces mast cell dependent vascular permeability in rodents² and humans.¹⁶ CRH had been considered a potential mediator in PBS/IC and

anxiety.²⁶ It was recently shown to lower the micturition threshold in the rat, but only when administered intrathecally or intraperitoneally, while it had no direct contractile effect on the detrusor.²⁷ Finally, cerebrospinal fluid levels of CRH were shown to be increased in a feline model of PBS/IC.²⁸

Our findings show that acute restraint stress could increase CRH immunoreactivity in the bladder. CRH released from nerve endings outside of the brain may activate mast cells,² thus, explaining how stress could worsen a number of inflammatory diseases, including PBS/IC.³ We recently reported that human mast cells express CRH-R, of which activation by CRH leads to the selective release of VEGF without degranulation.¹⁷ This effect may initiate the inflammatory process in the bladder and possibly contribute to the clinical and pathological symptoms that characterize many patients with PBS/IC, a disorder associated with bladder inflammation and pain.¹⁵

CONCLUSIONS

Intravesical CRH and acute stress induce VEGF release from bladder explants through a mast cell dependent process. These findings support the premise that CRH and mast cell-derived VEGF are implicated in bladder inflammation and they justify the consideration of CRH-R antagonists²⁹ for PBS/IC treatment.

ACKNOWLEDGMENTS

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Abbreviations and Acronyms

CRH	=	corticotropin-releasing hormone
CRH-R	=	CRH receptor
ELISA	=	enzyme-linked immunosorbent assay
IC	=	interstitial cystitis
IL	=	interleukin
NS	=	normal saline
PBS	=	painful bladder syndrome
TNF- α	=	tumor necrosis factor- α
VEGF	=	vascular endothelial growth factor

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