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Exaggerated Expression of Inflammatory Mediators in Vasoactive Intestinal Polypeptide Knockout (VIP$^{-/-}$) Mice with Cyclophosphamide (CYP)-Induced Cystitis

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Abstract

VIP is an immunomodulatory neuropeptide distributed in micturition pathways. VIP$^{-/-}$ mice exhibit altered bladder function and neurochemical properties in micturition pathways after cyclophosphamide (CYP)-induced cystitis. Given VIP's role as an anti-inflammatory mediator, we hypothesized that VIP$^{-/-}$ mice would exhibit enhanced inflammatory mediator expression after cystitis. A mouse inflammatory cytokines and receptors RT^2 profiler array was used to determine regulated transcripts in the urinary bladder of WT and VIP$^{-/-}$ mice with or without CYP-induced cystitis (150 mg/kg; i.p.; 48 hour (h). Four binary comparisons were made: wildtype (WT) control versus CYP treatment (48 h); VIP$^{-/-}$ control versus CYP treatment (48 h); WT control versus VIP$^{-/-}$ control and WT with CYP treatment (48 h) versus VIP$^{-/-}$ with CYP treatment (48 h). The genes presented represent (1) greater than 1.5-fold change in either direction and (2) the p value is less than 0.05 for the comparison being made. Several regulated genes were validated using enzyme-linked immunoassays. The data suggest that in VIP$^{-/-}$ mice with bladder inflammation, inflammatory mediators are increased above that observed in WT with CYP. This shift in balance may contribute to increased bladder dysfunction in VIP$^{-/-}$ mice with bladder inflammation and altered neurochemical expression in micturition pathways.

Keywords

Micturition; Cytokines; Chemokines; Inflammation

Introduction

Recent studies with a chemically (cyclophosphamide, CYP)-induced bladder inflammation model have demonstrated alterations in neurochemical (Vizzard, 2000d, 2001), electrophysiological (Yoshimura and de Groat, 1999) and organizational (Vizzard and Boyle, 1999) properties of micturition reflex elements. These changes suggest considerable reorganization of reflex connections in the spinal cord and marked changes in micturition reflexes with CYP-treatment. These changes may be mediated by chemical mediators produced in the bladder, spinal cord or dorsal root ganglia with cystitis (Vizzard, 2000b, 2000d, 2001;

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Malley and Vizzard, 2002; Braas et al., 2006). Possible mechanisms underlying the neural plasticity following chronic CYP-induced cystitis (Vizzard and de Groat, 1996; Jennings and Vizzard, 1999; Yoshimura and de Groat, 1999; Vizzard, 2000d, 2000a, 2001) may involve alterations in neurotrophic factors and/or neural activity arising in the urinary bladder (Vizzard, 2000b). 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 (Cominelli and Pizarro, 1996; Wong et al., 1997; Hill et al., 1999; Anderson and Rao, 2001; Mason et al., 2001; Samad et al., 2001; Winkelstein et al., 2001). The concept that target organs can influence the neurons that innervate them now is widely accepted and readily demonstrated during embryonic or postnatal development (Unsicker et al., 1992; Vantini and Skaper, 1992; Eide et al., 1993; Korsching, 1993; McMahon et al., 1994; Snider and Silos-Santiago, 1996; Lentz et al., 1999).

Neuropeptides are potential mediators or modulators of inflammation and are found in human micturition pathways (Chapple et al., 1992; Lasanen et al., 1992; Morgan et al., 1999). Changes in the expression of neuropeptides have been observed with bladder overactivity (Chapple et al., 1992; Lasanen et al., 1992) and in animal models of bladder inflammation (Vizzard, 2000d, 2001). We have recently demonstrated the contribution of vasoactive intestinal polypeptide (VIP) in afferent pathways to the urinary bladder by using wildtype and $VIP^{-/-}$ mice under control conditions or after induction of bladder inflammation. VIP is a member of the glucagon/secretin superfamily of hormones (Dickinson et al., 1999) and acts through two high affinity receptors, the $VPAC_1$ and $VPAC_2$ receptors (Harmar et al., 1998). VIP may exert excitatory or inhibitory actions in neural pathways controlling micturition and these functions may be altered with neural injury, disease or inflammation. Previous studies have demonstrated increased expression of phosphorylated cAMP response element binding protein (p-CREB) in bladder afferent cells in the lumbosacral dorsal root ganglia, increased NGF content in the urinary bladder and increased bladder hyperreflexia after CYP-induced cystitis compared to littermate, wildtype (WT) mice (Vizzard et al., 2007; Jensen et al., 2008). In addition to roles as neurotransmitter or neuromodulator in autonomic nervous system pathways, VIP exhibits considerable anti-inflammatory properties (Said, 1991; Voice et al., 2002; Szema et al., 2006) (Delgado et al., 2000). It has been shown that VIP inhibits the production of pro-inflammatory compounds (Chorny et al., 2006) whereas VIP upregulates production of anti-inflammatory cytokines (Delgado et al., 1999). We have suggested that differences in bladder hyperreflexia, pCREB expression and NGF expression in urinary bladder after bladder inflammation in $VIP^{-/-}$ mice may be attributed to a positive shift in the balance of proinflammatory mediators in the absence of VIP (Jensen et al., 2008).

Patients with interstitial cystitis (IC) or painful bladder syndrome (PBS), a painful, chronic urinary bladder inflammation syndrome, exhibit urinary frequency, urgency, suprapubic and pelvic pain and pain at low to moderate bladder pressure (Petrone et al., 1995). An involvement of C-fibers has been suggested (Chancellor and Yoshimura, 2004). In this study, we hypothesize that chemokines and cytokines and associated receptors expressed in the urinary bladder may contribute to the neuroplasticity of the lower urinary tract following bladder inflammation and contribute to inflammatory-induced changes including bladder hyperreflexia and changes in sensory processing. Furthermore, we hypothesize the $VIP^{-/-}$ mice will exhibit greater changes in proinflammatory mediators after CYP-induced cystitis. In this study, we: (1) examined a panel of inflammation-associated genes that may be regulated in urinary bladder of WT and $VIP^{-/-}$ with and without CYP-induced cystitis; and (2) selected several genes for validation using enzyme-lined immunoassays.

Materials and Methods

Animals

VIP$^{-/-}$ mice (Dr. James Waschek, University of California, LA, USA) (Colwell et al., 2003) were used in these experiments. The VIP$^{-/-}$ mouse model was prepared using a VIP gene disruption strategy with confirmation of targeted mutation in mice and subsequent backcrossing to the C57BL/6 strain for at least six generations (Colwell et al., 2003). Mice used were bred locally at The University of Vermont and lack of the VIP gene was confirmed by PCR genotyping of tailsnips. Mice were housed (12-hr light/dark cycle) in groups (5) in the UVM animal vivarium with water and food provided ad libitum. VIP$^{-/-}$ and wildtype (WT) controls from the same litter were analyzed in most cases (Jensen et al., 2008). When a WT littermate was unavailable, an age-matched control mouse from another C57BL/6 litter was used. Previous studies (Girard et al., 2006) have demonstrated that the closely related neuropeptide, pituitary adenylate cyclase activating polypeptide (PACAP), does not compensate for VIP in VIP$^{-/-}$ mice. Thus, the VIP and PACAP systems appear distinct. All experimental protocols involving animal use were approved by the University of Vermont Institutional Animal Care and Use Committee (IACUC # 06-014). Animal care was under the supervision of the University of Vermont's Office of Animal Care Management in accordance with the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) and National Institutes of Health guidelines. All efforts were made to minimize the potential for animal pain, stress or distress.

Acute cyclophosphamide (CYP)-induced cystitis

To produce an acute inflammation of the bladder, the cytostatic drug, cyclophosphamide (CYP; Sigma-Aldrich, 40 mg/ml in saline), was injected (150 mg/kg; i.p.) (Qiao and Vizzard, 2002, 2004; Zvarova and Vizzard, 2006). After CYP-treatment, animal health status was observed daily. Control rats received volume-matched injections of saline (0.9%; i.p.) or no treatment and no difference among the control groups was observed. All injections were performed under isoflurane (2%) anesthesia. The CYP model of bladder inflammation produces an increase in voiding frequency with small micturition volumes and is associated with inflammatory cell infiltrates in the urinary bladder including mast cells, macrophages and neutrophils (Vizzard, 2000c; Hu et al., 2003; LaBerge et al., 2006). The animals were euthanized 48 hours (h) after treatment.

Tissue Harvest

After CYP treatment or in control situations (control, 4 h, 48 h; $n = 7 – 9$ for each), animals were deeply anesthetized with isoflurane (3–4%) and then euthanized via thoracotomy. The urinary bladder and was quickly removed under RNase-free conditions.

Quantitative real time polymerase chain reaction (qPCR)

The PCR Array System is a quantitative SYBR® Green-based real-time PCR for analyzing the expression of a focused panel of genes. The Mouse Inflammatory Cytokines and Receptors RT^2 Profiler Array: PAMM-011 (Superarray, Gaithersburg, MD) is a 96 well plate containing RT^2 qPCR Primer Assays for a set of 84 related genes, plus five housekeeping genes, and three controls. Each qPCR assay on the array is uniquely designed for use in SYBR Green real-time PCR analysis. The assay design criteria ensure that each qPCR reaction will generate single, gene-specific amplicons and prevent the co-amplification of non-specific products. The qPCR Assays used in PCR Arrays are optimized to work under standard conditions enabling a large number of genes to be assayed simultaneously.

Whole bladders from C57BL6 and $VIP^{-/-}$ mice with or without CYP treatment (48 h) ($n = 3$ for each group) were dissected under RNAse free conditions. Total RNA was extracted using the STAT-60 total RNA/mRNA isolation reagent (Tel-Test'B', Friendswood, TX, USA) as previously described. Quantity and quality of the total RNA for each preparation was determined using a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). 1 μg of each experimental RNA sample was converted into PCR template with the RT^2 First Strand Kit. The templates were then combined with RT^2 SYBR Green qPCR Master Mix. Equal aliquots of this mixture (25 μl for 96-well plate) are loaded to each well of the same PCR array plate containing the pre-dispensed gene-specific primer sets, and PCR is performed on a 7900HT Sequence Detection System (Applied Biosystems, Norwalk, CT, USA). Sequence Detector 2.2 software (Applied Biosystems, Norwalk, CT, USA) was used to calculate the threshold cycle (Ct) values for all the genes on each PCR Array. Relative gene expressions were calculated by using the ΔΔCt method. The delta-delta method uses the normalized ΔCt value of each sample, calculated using a total of endogenous control genes. Examination of Ct value consistency for the housekeeping genes allowed gene selection and proper normalization method. For these analyses, three control genes that did not exhibit regulation with treatment or between mouse strains were used: hypoxanthine guanine phosphoribosyl transferase 1 (Hprt1); heat shock protein 90kDa alpha (cytosolic), class B member 1 (Hsp90ab1); actin, beta, cytoplasmic (Actβ). An evaluation of the built-in RNA quality controls elements provides the relative levels of genomic DNA contamination and inhibitors of either the reverse transcription or the PCR itself. All plates were exempt of DNA contamination. Four binary comparisons were made: wildtype (WT) control versus CYP treatment (48 h); $VIP^{-/-}$ control versus CYP treatment (48 h); WT control versus $VIP^{-/-}$ control and WT with CYP treatment (48 h) versus $VIP^{-/-}$ with CYP treatment (48 h). Visual representations of the comparisons were prepared as two-dimensional volcano plots with $\log_2$ fold change as the abscissa and the p value as the ordinate. The genes presented in tables and volcano plots represent (1) greater than 1.5-fold change in either direction and (2) the p value is less than 0.05 for the comparison being made.

Preparation of Immunoassay Samples

Several genes were selected for further confirmation of regulated expression by immunoassay. Adult rats were euthanized as above, and the bladder ($n = 4 – 6$ for each treatment; $n = 4 – 6$ for control) was rapidly dissected and weighed. Individual bladders were solubilized in T-PER tissue protein extraction reagent (1 g tissue/20 ml; Pierce, Rockford, IL) with Complete (protease inhibitors cocktail tablets; Roche Diagnostics, Germany). Bladder tissue was disrupted with a Polytron homogenizer and then centrifuged (10,000 rpm for 5 min). The supernatants were used for IL-1α, IL-1β, IL-10, monocyte chemoattractant protein (MCP1/CCL2), or CXCL1 quantification. We used the Coomassie Plus protein assay reagent kit (Pierce) to determine total protein.

Enzyme-linked Immunoassays

Monoclonal antibodies against IL-1α, IL-1β, IL-10, MCP1/CCL2, or CXCL1 were adsorbed to microtiter (R&D Systems, Minneapolis, MN) plates. After addition of the sample or standard solution, the second antibody (polyclonal) was applied. Sample and standard solutions were run in duplicate. The antibody complex was detected with a horseradish peroxidase-labeled immunoglobulin. Enzyme activity was quantified by the change in optical density, using tetramethylbenzidine as substrate. The standards provided with this system generated linear standard curves from 5 to 2,000 pg/ml ($R^2 = 0.983–0.997$, $P \leq 0.001$) for IL-1α, IL-1β, IL-10 or CXCL1 and from 5 to 250 pg/ml ($R^2 = 0.983$, $P \leq 0.001$) for MCP1/CCL2. The absorbance values of standards and samples were corrected by subtraction of the background value (absorbance due to nonspecific binding). Samples were diluted to bring the absorbance values onto the linear portion of the standard curve. No samples fell below the minimum detection

limits of the assay. Curve fitting of standards and evaluation IL-1α, IL-1β, IL-10, MCP1/CCL2, or CXCL1 content of samples was performed using a least squares fit. No significant cross-reactivity or interference is observed to other cytokines or chemokines with any immunoassay kit used in the present study according to the manufacturer.

Myeloperoxidase assay

Inflammation of the urinary bladder was also assessed with an assay for myeloperoxidase (MPO). Polymorphonuclear (PMN) cell infiltration is a characteristic of inflammation and MPO is a naturally occurring enzyme contained in the primary granules of the PMN cells (Vizzard, 2000a). Greater MPO activity in a tissue represents increased PMN cell infiltration in inflamed tissue. Thus an MPO assay was performed on freshly harvested urinary bladder tissue obtained from mice euthanized as described above. The MPO assay was performed as described previously (Vizzard, 2000a). Briefly, MPO was extracted from homogenized bladder tissue by suspending the material in 0.5% hexadecyltrimethylammonium bromide (Sigma-Aldrich) in 50 mM potassium phosphate buffer (pH 6.0) before sonication in an ice bath for 30 s. The specimens were freeze-thawed three times, and the sonication was repeated. Suspensions were then centrifuged (40,000 *g*) for 15 min, and the supernatant was assayed spectrophotometrically. Supernatant (0.1 ml) was mixed with 2.9 ml of potassium phosphate buffer containing 0.167 ml/ml of *o*-dianisidine dihydrochloride (Sigma-Aldrich) and 0.0005% hydrogen peroxide. The change in absorbency was measured at 460 nm. One unit of MPO activity was defined as that degrading one micromole of peroxide per minute at 25°C.

Materials

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

Statistical Analysis

All values are means ± SEM. Comparisons of MPO activity, cytokine or chemokine protein concentration in urinary bladder CYP-induced cystitis were made using analysis of variance. When F ratios exceeded the critical value ($P \leq 0.05$), the Newman-Keuls post hoc test was used to compare the experimental means.

Results

Using the established model of CYP-induced cystitis, we treated wildtype (WT) and VIP$^{-/-}$ mice with CYP (48 hr; 150 mg/kg, i.p.) to compare inflammation-induced responses in the urinary bladder. Separate groups of WT and VIP$^{-/-}$ mice were also used to establish baseline expression of inflammatory mediators in the urinary bladder in the absence of inflammation. The following comparisons were made: wildtype (WT) control versus CYP treatment (48 h); VIP$^{-/-}$ control versus CYP treatment (48 h); WT control versus VIP$^{-/-}$ control and WT with CYP treatment (48 h) versus VIP$^{-/-}$ with CYP treatment (48 h). When the data sets were filtered for fold changes ≥ 1.5 (either up or down) and p-values of ≤ 0.05, 24 target genes were identified for the WT control versus CYP treatment (48 h) comparison (Table 1), 31 genes for the VIP$^{-/-}$ control versus CYP treatment (48 h) comparison (Table 2), 14 genes for the WT control versus VIP$^{-/-}$ control comparison (Table 3) and 6 genes for WT with CYP treatment (48 h) versus VIP$^{-/-}$ with CYP treatment (48 h) (Table 4) comparison. In visual two-dimensional representations of the data in volcano plots, where $\log_2$ fold change for each gene was expressed as a function of the p-value, all of the upregulated genes were noted in the upper right quadrant and all of the downregulated genes were noted in the upper left quadrant (Fig. 1A–D).

Of the genes upregulated in the urinary bladder after CYP-treatment in WT and VIP$^{-/-}$ mice, several were selected for validation using enzyme-linked immunoassays. In addition, a

myeloperoxidase (MPO) assay was used to determine the extent of neutrophil infiltration present in the urinary bladder after CYP-induced cystitis (48 h). As expected, MPO levels significantly increased in the urinary bladder after CYP treatment (48 h) in both WT and $VIP^{-/-}$ mice (Fig. 2A). CYP treatment of a shorter duration (4 h) only increased MPO levels in the urinary bladder of $VIP^{-/-}$ mice (Fig. 2A). MPO expression in the urinary bladder of $VIP^{-/-}$ mice at both 4 or 48 h CYP treatment was significantly ($p \leq 0.001$) greater than that observed in WT mice (Fig. 2A). No differences in MPO expression in the urinary bladder were observed between WT and $VIP^{-/-}$ mice in the absence of inflammation (control) (Fig. 2A). This change in MPO expression with CYP-induced cystitis and the differences observed in MPO levels between WT and $VIP^{-/-}$ mice is consistent with the dramatic upregulation of the chemokines, CXCL5 and CXCL1, known to be involved in neutrophil recruitment in other organs systems following inflammation (Fong et al., 1998;Haskell et al., 2000).

We evaluated IL-1α, IL-1β, IL-10, MCP1/CCL2, or CXCL1 expression in urinary bladder in WT and $VIP^{-/-}$ mice with and without CYP treatment (48 h). The expression patterns for CXCL1 and IL-1β in the urinary bladder of control and CYP-treated mice were similar (Fig. 2B, D). No differences in CXCL1 or IL-1β expression in urinary bladder of control WT or $VIP^{-/-}$ mice were observed (Fig. 2B, D). In contrast, CYP-treatment significantly ($p \leq 0.001$) increased expression of CXCL1 and IL-1β in the urinary bladder of WT and $VIP^{-/-}$ (Fig. 2B, D) mice but expression in $VIP^{-/-}$ mice with CYP treatment was significantly ($p \leq 0.001$) greater (4.2–13-fold increase) than that observed in WT urinary bladder (Fig. 2) (3.6–5-fold increase) (Fig. 2B, D). MCP1/CCL2 expression in the urinary bladder was significantly ($p \leq 0.001$) increased after CYP-treatment (48 h) in both WT and $VIP^{-/-}$ mice; however, no differences in the magnitude of the change were observed between mouse strains (Fig. 2C). No differences in MCP1/CCL2 expression were observed in non-inflamed (control) urinary bladder of WT or $VIP^{-/-}$ mice (Fig. 2C). The changes in urinary bladder protein expression of CXCL1, CCL2 and IL-1β complement the increased urinary bladder transcript expression demonstrated in the superarray with CYP-treatment in WT and $VIP^{-/-}$ mice (Table 1, Table 2).

In contrast to robust changes in urinary bladder protein and transcript expression for CXCL1, CCL2 and IL-1β, no change in protein expression of IL-1α or IL-10 were observed in the urinary bladder with CYP treatment (48 h) in WT or $VIP^{-/-}$ mice. No differences in non-inflamed (control) urinary bladder expression of IL-1α or IL-10 were observed between WT or $VIP^{-/-}$ mice (data not shown). The absence of changes in protein expression in urinary bladder IL-1α or IL-10 in response to CYP treatment (48 h) complement the absence of effect on IL-1α and IL-10 urinary bladder transcript using the superarray. Changes in IL-1α and IL-10 transcripts in urinary bladder with CYP treatment in WT or $VIP^{-/-}$ mice did not meet the selection criteria described.

Discussion

The present studies demonstrate the increased expression of inflammatory gene transcripts and protein in urinary bladder of $VIP^{-/-}$ mice as demonstrated with a superarray and confirmed with enzyme-lined immunoassays. Previous studies have demonstrated that p-CREB expression in lumbosacral DRG was greater in $VIP^{-/-}$ mice under basal conditions and after bladder inflammation induced by cyclophosphamide (CYP) (Jensen et al., 2008). Urinary bladder from $VIP^{-/-}$ mice exhibit greater mass, increased thickness of detrusor smooth muscle and increased neurotrophic factor (e.g., nerve growth factor) content compared to WT mice (Jensen et al., 2008). With respect to urinary bladder function, $VIP^{-/-}$ mice exhibit more pronounced urinary bladder hyperreflexia in response to CYP-induced cystitis compared to WT mice (Vizzard et al., 2007). We have previously suggested that these differences may be due, in part, to differences in expression in urinary bladder inflammatory mediators under non-inflamed and inflamed conditions (Jensen et al., 2008). The present studies demonstrate more

robust upregulation of numerous inflammatory mediator genes in $VIP^{-/-}$ mice treated with CYP compared to WT mice treated with CYP. Thus, these studies demonstrate a positive shift in the balance of proinflammatory mediators in urinary bladder of $VIP^{-/-}$ mice with bladder inflammation.

Interstitial cystitis (IC)/painful bladder syndrome (PBS) is a chronic inflammatory bladder disease syndrome characterized by urinary frequency, urgency, suprapubic and pelvic pain (Petrone et al., 1995; Driscoll and Teichman, 2001). 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 (Petrone et al., 1995; Ho et al., 1997; Johansson et al., 1997; Driscoll and Teichman, 2001; Sant and Hanno, 2001). We have hypothesized that pain associated with IC 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 (Petrone et al., 1995; Ho et al., 1997; Johansson et al., 1997; Driscoll and Teichman, 2001; Sant and Hanno, 2001) 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 (eg., nerve growth factor) (Woolf and Doubell, 1994; Dray, 1995; Baba et al., 1999) as well as pro-inflammatory cytokines have been implicated in this sensitization process. Specifically, cytokines cause sensitization of polymodal C-fibers and facilitate A-beta input to the spinal cord (Woolf and Doubell, 1994; Dray, 1995; Baba et al., 1999). The present studies suggest that cytokines produced in the urinary bladder after CYP-induced cystitis may also contribute to this sensitization process. Sources of cytokines in the CYP-induced cystitis model may include a variety of cell types (resident and/or infiltrating) in the urinary bladder: lymphocytes, macrophages, mast cells, microglial cells, urothelial cells, urinary bladder smooth muscles cells, and fibroblasts (Bartfai and Schultzberg, 1993; Gadient and Otten, 1997; Parkin and Cohen, 2001). Previous studies from this laboratory (Malley and Vizzard, 2002) have suggested that cytokines produced in the urinary bladder after CYP-induced cystitis may also contribute to this sensitization process.

Changes in synthesis and/or release of cytokines by target organs may also have an impact on the changes in lower urinary tract function as well as reorganization of reflex connections in the spinal cord after CYP-induced cystitis. Saban et al. (Saban et al., 2001a; Saban, 2001; Saban et al., 2001b) were the first to demonstrate gene-regulation during inflammatory bladder responses in the mouse (lipopolysaccharide, substance P or antigen-induced inflammation) using cDNA expression array technology. Consistent with the results of the present study using the CYP-induced cystitis model in the mouse, Saban et al. (Saban et al., 2001a; Saban, 2001; Saban et al., 2001b) have also demonstrated urinary bladder upregulation of cytokines. Interestingly, several cytokines (i.e., IL-1β, IL-6, IL-8 among others) have also been reported to be upregulated during active states of inflammatory bowel disease (Sartor, 1991, 1994; Cominelli and Pizarro, 1996; MacDermott, 1996; Debreceni et al., 2001). A significant body of literature exists to support the concept that cytokines (e.g., IL-1β) are key signals that are released in the periphery to signal the central nervous system that infection/inflammation has occurred (Lindholm et al., 1987; Dinarello, 1998; Poole et al., 1998; Poole and Woolf, 1998; Maier and Watkins, 1999). IL-1β has also been implicated in inflammatory hyperalgesia (Ferreira et al., 1988; Poole et al., 1998). Several lines of evidence further suggest that IL-1β may cause hyperalgesia by releasing cyclooxgenase products through stimulation of cyclooxygenase-2 (COX-2) (Geng et al., 1995; Samad et al., 2001) and phospholipase A_2 (Burch et al., 1993) or by inducing arachidonic acid release (Dinarello, 1997), respectively. In the present study, more robust upregulation of IL-1β transcript in urinary bladder was demonstrated in $VIP^{-/-}$ mice with CYP-induced cystitis compared to WT. Similarly, greater

IL-1β protein expression in the urinary bladder was demonstrated in $VIP^{-/-}$ mice treated with CYP compared to WT mice treated with CYP. Thus, inflammatory processes in visceral organs and associated changes in neurochemistry (Vizzard, 1997, 2000d, 2001), and reflex organization (Vizzard, 2000a) may involve cytokines.

The present study also demonstrates upregulation of transcripts and protein for numerous chemokines and associated receptors in urinary bladder after CYP-induced cystitis. Chemokines, chemotactic cytokines, are another class of neuromodulatory agents that may contribute to inflammatory-induced changes (Wieseler-Frank et al., 2004; Marchand et al., 2005). Chemokine expression has been demonstrated at sites of inflammation (Garcia et al., 2000; Raychaudhuri et al., 2001; Yamashita et al., 2003; Ahn et al., 2004; Nagarsekar et al., 2005; Sung et al., 2005) and chemokines are potential mediators of nociceptive facilitation (Johnston et al., 2004; Milligan et al., 2004; Verge et al., 2004; Lindia et al., 2005). Some classes of chemokines are upregulated in the periphery by tumor necrosis factor-alpha (TNF-α), interleukin-1β, and lipopolysaccharide (Bazan et al., 1997; Garcia et al., 2000; Ahn et al., 2004; Sung et al., 2005). Chemokines have numerous roles including adhesion molecules, chemotactic agent to T cells and monocytes (Fong et al., 1998; Haskell et al., 2000), and involvement in the establishment and/or maintenance of inflammatory pain (Marchand et al., 2005), neuropathic pain (Verge et al., 2004), thermal hyperalgesia and mechanical allodynia (Watkins et al., 2001; Watkins and Maier, 2002; Milligan et al., 2004; Verge et al., 2004). In the present study, robust changes in CXCL5 and CXCL1 transcript expression were demonstrated in $VIP^{-/-}$ mice with CYP-induced cystitis. CXCL5 was also robustly expressed in urinary bladder of WT mice with CYP-induced cystitis. The magnitude of change for both CXCL5 and CXCL1 transcripts was greater in the $VIP^{-/-}$ mouse. Both CXCL5 and CXCL1 cause neutrophil infiltration in various organ systems and the demonstration of increased urinary bladder MPO, an indicator of neutrophil presence, is consistent with this potent chemotactic role (Fong et al., 1998; Haskell et al., 2000). Immunoassay for CXCL1 in urinary bladder validated the transcript data obtained with the superarray. MCP1/CCL2 is another chemokine with a role in the recruitment of inflammatory cells into tissue during inflammation (Castellani et al., 2007). MCP1/CCL2 exhibited more robust transcript expression in $VIP^{-/-}$ mice compared to WT with CYP-induced cystitis. However, no difference in the magnitude of MCP1/CCL2 protein expression in the urinary bladder between WT and $VIP^{-/-}$ mice was demonstrated. Inhibition of MCP1 is currently being explored as a possible therapeutic intervention that may provide benefits in different clinical scenarios including cancer, inflammation, and autoimmune diseases (Castellani et al., 2007).

In summary, the present study has demonstrated upregulation of numerous inflammatory genes in the urinary bladder of WT and $VIP^{-/-}$ mice after CYP-induced cystitis (48 h). Several inflammatory genes were downregulated in the urinary bladder of both mouse strains with CYP-induced cystitis but these downregulated genes were fewer in number compared to upregulated genes. Furthermore, the magnitude of upregulation for inflammatory genes in urinary bladder common to both WT and $VIP^{-/-}$ mice was more robust in the $VIP^{-/-}$ mouse. This difference in magnitude of change may be due to the absence of the anti-inflammatory influence of VIP to the context of bladder inflammation. We selected several transcripts for validation in immunoassays and the protein expression generally complemented the transcript expression. Future studies will address the contribution of robustly regulated inflammatory genes and protein to urinary bladder hyperreflexia and altered sensory processing in the CYP-induced cystitis model of bladder inflammation (Vizzard et al., 2007).

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References

Ahn SY, Cho CH, Park KG, et al. Tumor necrosis factor-alpha induces fractalkine expression preferentially in arterial endothelial cells and mithramycin A suppresses TNF-alpha-induced fractalkine expression. Am. J. Pathol 2004;164:1663–1672. [PubMed: 15111313]

Anderson LC, Rao RD. Interleukin-6 and nerve growth factor levels in peripheral nerve and brainstem after trigeminal nerve injury in the rat. Arch. Oral Biol 2001;46:633–640. [PubMed: 11369318]

Baba H, Doubell TP, Woolf CJ. Peripheral inflammation facilitates A beta fiber-mediated synaptic input to the substantia gelatinosa of the adult rat spinal cord. J. Neurosci 1999;19:859–867. [PubMed: 9880605]

Bartfai T, Schultzberg M. Cytokines in neuronal cell types. Neurochem. Int 1993;22:435–444. [PubMed: 8485449]

Bazan JF, Bacon KB, Hardiman G, et al. A new class of membrane-bound chemokine with a CX3C motif. Nature 1997;385:640–644. [PubMed: 9024663]

Braas KM, May V, Zvara P, et al. Role for pituitary adenylate cyclase activating polypeptide in cystitis-induced plasticity of micturition reflexes. Am. J. Physiol. Regul. Integr. Comp. Physiol 2006;290:R951–R962. [PubMed: 16322346]

Burch RM, Connor JR, Axelrod J. Interleukin-1 amplifies receptor-mediated activation of phospholipase A2 in 3T3 fibroblasts. Proc. Natl. Acad. Sci. (U.S.A.) 1993;85:6306–6309. [PubMed: 2901097]

Castellani ML, Bhattacharya K, Tagen M, et al. Anti-chemokine therapy for inflammatory diseases. Int. J. Immunopathol. Pharmacol 2007;20:447–453. [PubMed: 17880758]

Chancellor MB, Yoshimura N. Treatment of interstitial cystitis. Urology 2004;63:85–92. [PubMed: 15013658]

Chapple CR, Milner P, Moss HE, Burnstock G. Loss of sensory neuropeptides in the obstructed human bladder. Br. J. Urol 1992;70:373–381. [PubMed: 1450844]

Chorny A, Gonzalez-Rey E, Varela N, Robledo G, Delgado M. Signaling mechanisms of vasoactive intestinal peptide in inflammatory conditions. Regul. Pept 2006;137:67–74. [PubMed: 16949684]

Colwell CS, Michel S, Itri J, et al. Disrupted circadian rhythms in VIP-and PHI-deficient mice. Am. J. Physiol. Regul. Integr. Comp. Physiol 2003;285:R939–R949. [PubMed: 12855416]

Cominelli F, Pizarro TT. Interleukin-1 and interleukin-1 receptor antagonist in inflammatory bowel disease. Aliment Pharmacol. Ther 1996;10:49–53. [PubMed: 8899101]

Debreceni A, Okazuchi O, Matsushima Y, et al. mRNA expression of cytokines and chemokines in the normal gastric surface mucous epithelial cell line GSM06 during bacterial infection with Helicobacter felis. J. Physiol. (Paris) 2001;95:461–467. [PubMed: 11595476]

Delgado M, Munoz-Elias EJ, Gomariz RP, Ganea D. Vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide enhance IL-10 production by murine macrophages: in vitro and in vivo studies. J. Immunol 1999;162:1707–1716. [PubMed: 9973433]

Delgado M, Gomariz RP, Martinez C, Abad C, Leceta J. Anti-inflammatory properties of the type 1 and type 2 vasoactive intestinal peptide receptors: role in lethal endotoxic shock. Eur. J. Immunol 2000;30:3236–3246. [PubMed: 11093139]

Dickinson T, Mitchell R, Robberecht P, Fleetwood-Walker SM. The role of VIP/PACAP receptor subtypes in spinal somatosensory processing in rats with an experimental peripheral mononeuropathy. Neuropharmacology 1999;38:167–180. [PubMed: 10193908]

Dinarello, CA. Overview of inflammatory cytokines and their role in pain. In: Watkins, LR.; Maier, SF., editors. Cytokines and Pain. Boston: Birkhauser Verlag; 1998. p. 1-20.

Dinarello CAD. Proinflammatory and anti-inflammatory cytokines as mediators in the pathogenesis of septic shock. Chest 1997;112:321S–329S. [PubMed: 9400897]

Dray A. Inflammatory mediators of pain. Br. J. Anaesth 1995;75:125–131. [PubMed: 7577246]

Driscoll A, Teichman JMH. How do patients with interstitial cystitis present? J. Urol 2001;166:2118–2120. [PubMed: 11696718]

Eide FF, Lowenstein DH, Reichardt LF. Neurotrophins and their receptors--current concepts and implications for neurologic disease. Exp. Neurol 1993;121:200–214. [PubMed: 8339771]

Ferreira SH, Lorenzetti BB, Bristow AF, Poole S. Interleukin-1-beta as a potent hyperalgesic agent antagonized by a tripeptide analogue. Nature 1988;334:698–700. [PubMed: 3137474]

Fong AM, Robinson LA, Steeber DA, et al. Fractalkine and CX3CR1 mediate a novel mechanism of leukocyte capture, firm adhesion, and activation under physiologic flow. J. Exp. Med 1998;188:1413–1419. [PubMed: 9782118]

Gadient RA, Otten UH. Interleukin-6 (IL-6)-A molecule with both beneficial and destructive potentials. Prog. Neurobiol 1997;52:379–390. [PubMed: 9304698]

Garcia GE, Xia Y, Chen S, et al. NF-kappaB-dependent fractalkine induction in rat aortic endothelial cells stimulated by IL-1beta, TNF-alpha, and LPS. J. Leukoc. Biol 2000;67:577–584. [PubMed: 10770292]

Geng Y, Blanco FJ, Corenelisson M, Lotz M. Regulation of cyclooxygenase-2 expression in normal human chondrocytes. J. Immunol 1995;155:796–801. [PubMed: 7608556]

Girard BA, Lelievre V, Braas KM, et al. Noncompensation in peptide/receptor gene expression and distinct behavioral phenotypes in VIP-and PACAP-deficient mice. J. Neurochem 2006;99:499–513. [PubMed: 17029602]

Harmar AJ, Arimura A, Gozes I, et al. International Union of Pharmacology. XVIII. Nomenclature of receptors for vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide. Pharmacol. Rev 1998;50:265–270. [PubMed: 9647867]

Haskell CA, Cleary MD, Charo IF. Unique role of the chemokine domain of fractalkine in cell capture. Kinetics of receptor dissociation correlate with cell adhesion. J. Biol. Chem 2000;275:34183–34189. [PubMed: 10940307]

Hill JK, Gunion-Riner L, Kulhanek D, et al. Temporal modulation of cytokine expression following focal cerebral ischemia in mice. Brain Res 1999;820:45–54. [PubMed: 10023029]

Ho, N.; Koziol, JA.; Parsons, CL. Epidemiology of interstitial cystitis. In: Sant, GR., editor. Interstitial Cystitis. Philadelphia: Lippincott-Raven Publishers; 1997. p. 9-16.

Hu VY, Malley S, Dattilio A, Folsom JB, Zvara P, Vizzard MA. COX-2 and prostanoid expression in micturition pathways after cyclophosphamide-induced cystitis in the rat. Am. J. Physiol. Regul. Integr. Comp 2003;284:R574–R585.

Jennings LJ, Vizzard MA. Cyclophosphamide-induced inflammation of the urinary bladder alters electrical properties of small diameter afferent neurons from dorsal root ganglia. FASEB J 1999;13:A57.

Jensen DG, Studeny S, May V, Waschek J, Vizzard MA. Expression of Phosphorylated cAMP Response Element Binding Protein (p-CREB) in Bladder Afferent Pathways in VIP(−/−) Mice with Cyclophosphamide (CYP)-Induced Cystitis. J. Mol. Neurosci. 2008[epub ahead of print]

Johansson, SL.; Ogawa, K.; Fall, M. The pathology of interstitial cystitis. In: Sant, GR., editor. Interstitial Cystitis. Philadelphia: Lippincott-Raven Publishers; 1997. p. 143-152.

Johnston IN, Milligan ED, Wieseler-Frank J, et al. A role for proinflammatory cytokines and fractalkine in analgesia, tolerance, and subsequent pain facilitation induced by chronic intrathecal morphine. J. Neurosci 2004;24:7353–7365. [PubMed: 15317861]

Korsching S. The neurotrophic factor concept: a reexamination. J. Neurosci 1993;13:2739–2748. [PubMed: 8331370]

LaBerge J, Malley SE, Zvarova K, Vizzard MA. Expression of corticotropin-releasing factor and CRF receptors in micturition pathways after cyclophosphamide-induced cystitis. Am. J. Physiol. Regul. Integr. Comp. Physiol 2006;291:R692–R703. [PubMed: 16614059]

Lasanen LT, Tammela TL, Liesi P, Waris T, Polak JM. The effect of acute distension on vasoactive intestinal polypeptide (VIP), neuropeptide Y (NPY) and substance P (SP) immunoreactive nerves in the female rat urinary bladder. Urol. Res 1992;20:259–263. [PubMed: 1380745]

Lentz SI, Knudson CM, Korsmeyer SJ, Snider WD. Neurotrophins support the development of diverse sensory axon morphologies. J. Neurosci 1999;19:1038–1048. [PubMed: 9920667]

Lindholm D, Heumann R, Meyer M, Thoenen H. Interleukin-1 regulates synthesis of nerve growth factor in non-neuronal cells of the rat sciatic nerve. Nature 1987;330:658–659. [PubMed: 3317065]

Lindia JA, McGowan E, Jochnowitz N, Abbadie C. Induction of CX3CL1 expression in astrocytes and CX3CR1 in microglia in the spinal cord of a rat model of neuropathic pain. J. Pain 2005;6:434–438. [PubMed: 15993821]

MacDermott RP. Alterations of the mucosal immune system in inflammatory bowel disease. J. Gastroenterol 1996;31:907–916. [PubMed: 9027661]

Maier SF, Watkins LR. Bidirectional communication between the brain and the immune system: implications for behaviour. Animal Behaviour 1999;57:741–751.

Malley SE, Vizzard MA. Changes in urinary bladder cytokine mRNA and protein after cyclophosphamide-induced cystitis. Physiol. Genomics 2002;9:5–13. [PubMed: 11948286]

Marchand F, Perretti M, McMahon SB. Role of the immune system in chronic pain. Nat. Rev. Neurosci 2005;6:521–532. [PubMed: 15995723]

Mason JL, Suzuki K, Chaplin DD, Matsushima GK. Interleukin-1 beta promotes repair of the CNS. J. Neurosci 2001;21:7046–7052. [PubMed: 11549714]

McMahon SB, Armanini MP, Ling LH, Phillips HS. Expression and coexpression of Trk receptors in subpopulations of adult primary sensory neurons projecting to identified peripheral targets. Neuron 1994;12:335–340.

Milligan ED, Zapata V, Chacur M, et al. Evidence that exogenous and endogenous fractalkine can induce spinal nociceptive facilitation in rats. Eur. J. Neurosci 2004;20:2294–2302. [PubMed: 15525271]

Morgan CW, Ohara PT, Scott DE. Vasoactive intestinal polypeptide in sacral primary sensory pathways in the cat. J. Comp. Neurol 1999;407:381–394. [PubMed: 10320218]

Nagarsekar A, Hasday JD, Singh IS. CXC chemokines: A new family of heat-shock proteins? Immunol. Invest 2005;34:381–398. [PubMed: 16136787]

Parkin J, Cohen B. An overview of the immune system. The Lancet 2001;357:1777–1789.

Petrone RL, Agha AH, Roy JB, Hurst RE. Urodynamic findings in patients with interstitial cystitis. J. Urol 1995;153290A

Poole, S.; Woolf, CJ. Cytokine-nerve growth factor interactions in inflammatory hyperalgesia. In: Watkins, LR.; Maier, SF., editors. Cytokines and Pain. Boston: Birkhauser Verlag; 1998. p. 59-88.

Poole, S.; de Queiroz Cunha, F.; Henriques Ferreira, S. Hyperalgesia from subcutaneous cytokines. In: Watkins, LR.; Maier, SF., editors. Cytokines and Pain. Boston: Birkhauser Verlag; 1998. p. 59-88.

Qiao LY, Vizzard MA. Cystitis-induced upregulation of tyrosine kinase (TrkA, TrkB) receptor expression and phosphorylation in rat micturition pathways. J. Comp. Neurol 2002;454:200–211. [PubMed: 12412144]

Qiao LY, Vizzard MA. Up-regulation of phosphorylated CREB but not c-Jun in bladder afferent neurons in dorsal root ganglia after cystitis. J. Comp. Neurol 2004;469:262–274. [PubMed: 14694538]

Raychaudhuri S, Jiang W-Y, Farber E. Cellular localization of fractalkine at sites of inflammation: antigen-presenting cells in psoriasis express high levels of fractalkine. Br. J. Dermatol 2001;144(6): 1105–1113. [PubMed: 11422028]

Saban MR, Hellmich H, Nguyen NB, Winston J, Hammond TG, Saban R. Time course of LPS-induced gene expression in a mouse model of genitourinary inflammation. Physiol. Genomics 2001a;5:147–160. [PubMed: 11285368]

Saban R. Gene-regulation during bladder neurogenic inflammation. Urology 2001;57:103. [PubMed: 11378061]

Saban R, Saban MR, Nguyen NB, Hammmond TG, Wershil BK. Mast cell regulation of inflammation and gene expression during antigen-induced bladder inflammation in mice. Physiol. Genomics 2001b;10:35–43.

Said SI. Vasoactive intestinal polypeptide (VIP) in asthma. Ann. N. Y. Acad. Sci 1991;629:305–318. [PubMed: 1683200]

Samad TA, Moore KA, Sapirstein A, et al. Interleukin-1 beta-mediated induction of Cox-2 in the CNS contributes to inflammatory pain hypersensitivity. Nature 2001;410:471–475. [PubMed: 11260714]

Sant G, Hanno PM. Interstitial cystitis: current issues and controversies in diagnosis. Urology 2001;57:82. [PubMed: 11378054]

Sartor RB. Pathogenetic and clinical relevance of cytokines in inflammatory bowel disease. Immunol Res 1991;10:465–471. [PubMed: 1955773]

Sartor RB. Cytokines in intestinal inflammation: pathophysiologic and clinical considerations. Gastroenterology 1994;106:533–542. [PubMed: 8299918]

Snider WD, Silos-Santiago I. Dorsal root ganglion neurons require functional neurotrophin receptors for survival during development. Philos. Trans. R. Soc. Lond. Biol. Sci 1996;351:395–403. [PubMed: 8730777]

Sung MJ, Kim W, Ahn SY, et al. Protective Effect of {alpha}-Lipoic Acid in Lipopolysaccharide-Induced Endothelial Fractalkine Expression. Circ. Res 2005;97(9):880–890. [PubMed: 16166554]

Szema AM, Hamidi SA, Lyubsky S, et al. Mice lacking the VIP gene show airway hyperresponsiveness and airway inflammation, partially reversible by VIP. Am. J. Physiol. Lung Cell Mol. Physiol 2006;291:L880–L886. [PubMed: 16782752]

Unsicker K, Reichert-Preibsch H, Wewetzer K. Stimulation of neuron survival by basic FGF and CNTF is a direct effect and not mediated by non-neuronal cells: evidence from single cell cultures. Dev. Brain Res 1992;65:285–288. [PubMed: 1572071]

Vantini G, Skaper SD. Neurotrophic factors: from physiology to pharmacology. Pharmacol. Res 1992;26:1–15. [PubMed: 1513747]

Verge GM, Milligan ED, Maier SF, Watkins LR, Naeve GS, Foster AC. Fractalkine (CX3CL1) and fractalkine receptor (CX3CR1) distribution in spinal cord and dorsal root ganglia under basal and neuropathic pain conditions. Eur. J. Neurosci 2004;20:1150–1160. [PubMed: 15341587]

Vizzard MA. Increased expression of neuronal nitric oxide synthase in bladder afferent and spinal neurons following spinal cord injury. Dev. Neurosci 1997;19:232–246. [PubMed: 9208207]

Vizzard MA. Alterations in spinal Fos protein expression induced by bladder stimulation followng cystitis. Am. J. Physiol. Reg. Integr. Comp. Physiol 2000a;278:R1027–R1039.

Vizzard MA. Changes in urinary bladder neurotrophic factor mRNA and NGF protein following urinary bladder dysfunction. Exp. Neurol 2000b;161:273–284. [PubMed: 10683293]

Vizzard MA. Alterations in spinal cord Fos protein expression induced by bladder stimulation following cystitis. Am. J. Physiol. Regul. Integr. Comp. Physiol 2000c;278:R1027–R1039. [PubMed: 10749792]

Vizzard MA. Up-regulation of pituitary adenylate cyclase-activating polypeptide in urinary bladder pathways after chronic cystitis. J. Comp. Neurol 2000d;420:335–348. [PubMed: 10754506]

Vizzard MA. Alterations in neuropeptide expression in lumbosacral bladder pathways following chronic cystitis. J. Chem. Neuroanat 2001;21:125–138. [PubMed: 11312054]

Vizzard MA, de Groat WC. Increased expression of neuronal nitric oxide synthase (NOS) in bladder afferent pathways following chronic bladder irritation. J. Comp. Neurol 1996;370:191–202. [PubMed: 8808730]

Vizzard MA, Boyle MM. Increased expression of growth-associated protein (GAP-43) in lower urinary tract pathways following cyclophosphamide (CYP)-induced cystitis. Brain Res 1999;844:174–187. [PubMed: 10536274]

Vizzard MA, Braas KM, Studeny S, et al. Vasoactive intestinal polypeptide knockout (VIP−/−) mice exhibit altered bladder function and somatic sensitivity with cyclophosphamide (CYP)-induced cystitis. J. Mol. Neurosci 2007;33:311.

Voice JK, Dorsam G, Chan RC, Grinninger C, Kong Y, Goetzl EJ. Immunoeffector and immunoregulatory activities of vasoactive intestinal peptide. Regul. Pept 2002;109:199–208. [PubMed: 12409234]

Watkins LR, Maier SF. Beyond neurons: Evidence that immune and glial cells contribute to pathological pain states. Physiol. Rev 2002;82:981–1011. [PubMed: 12270950]

Watkins LR, Milligan ED, Maier SF. Glial activation: a driving force for pathological pain. Trends Neurosci 2001;24:450–455. [PubMed: 11476884]

Wieseler-Frank J, Maier SF, Watkins LR. Glial activation and pathological pain. Neurochem Int 2004;45:389–395. [PubMed: 15145553]

Winkelstein BA, Rutkowski MD, Sweitzer SM, Pahl JL, DeLeo JA. Nerve injury proximal or distal to the DRG induces similar spinal glial activation and selective cytokine expression but differential behavioral responses to pharmacologic treatment. J. Comp. Neurol 2001;439:127–139. [PubMed: 11596043]

Wong M-L, Rettori V, McCann SM, Licinio J. Interleukin (IL) 1-beta, IL-1 receptor antagonist, IL-10 and IL-13 gene expression in the central nervous system and anterior pituitary during systemic

inflammation: pathophysiological implications. Proc. Natl. Acad. Sci. (U.S.A.) 1997;94:227–232. [PubMed: 8990190]

Woolf CJ, Doubell TP. The pathophysiology of chronic pain-increased sensitivity to low threshold A-beta fiber inputs. Curr. Opin. Neurobiol 1994;4:525–534. [PubMed: 7812141]

Yamashita K, Imaizumi T, Hatakeyama M, et al. Effect of hypoxia on the expression of fractalkine in human endothelial cells. Tohoku J. Exp. Med 2003;200:187–194. [PubMed: 14580149]

Yoshimura N, de Groat WC. Increased excitability of afferent neurons innervating rat urinary bladder following chronic bladder inflammation. J. Neurosci 1999;19:4644–4653. [PubMed: 10341262]

Zvarova K, Vizzard MA. Changes in galanin immunoreactivity in rat micturition reflex pathways after cyclophosphamide-induced cystitis. Cell Tissue Res 2006;324:213–224. [PubMed: 16491427]

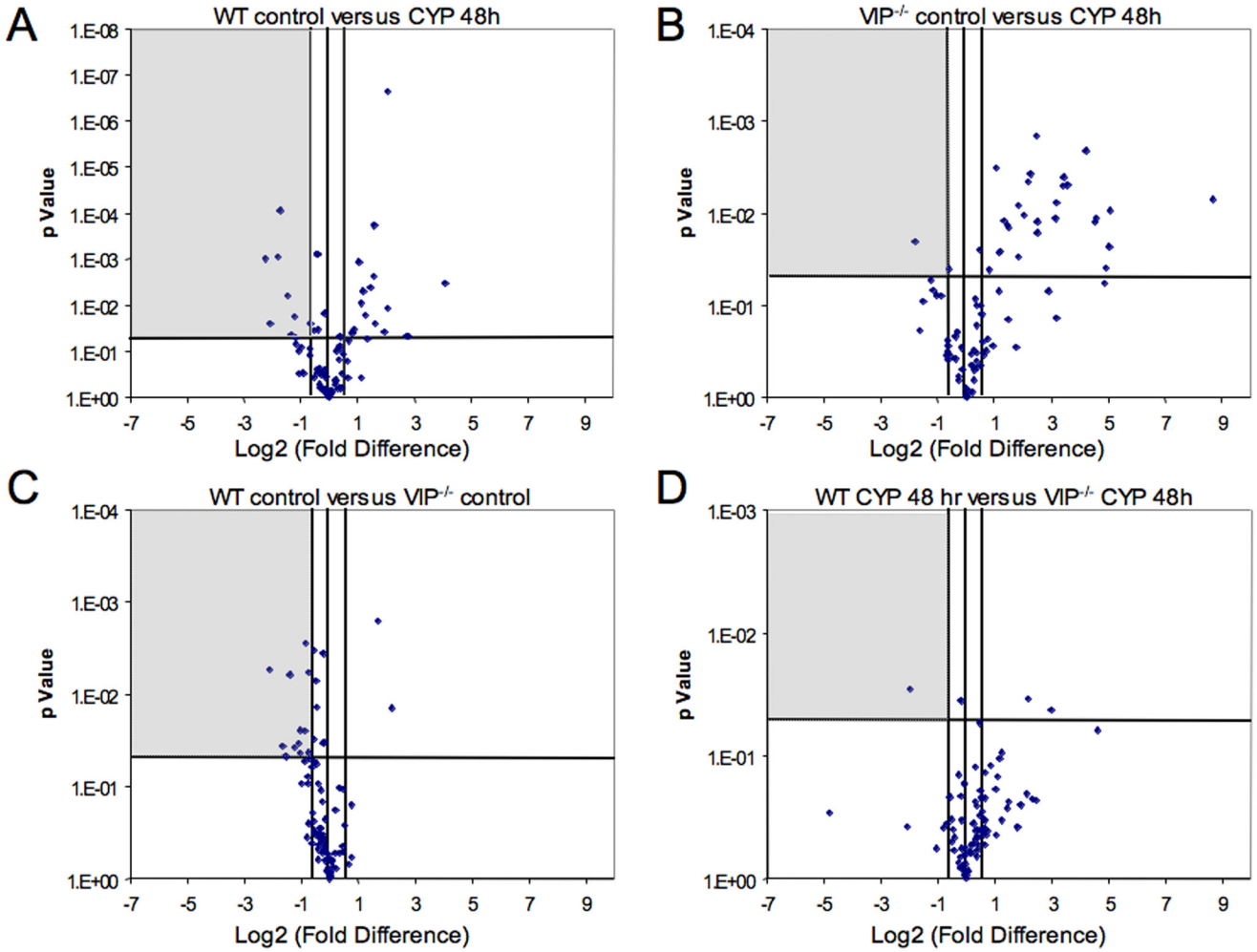

Figure 1.

Volcano plots demonstrate differential regulation of mouse inflammatory cytokines and receptors gene expression in urinary bladder. Criteria boundaries are shown for each plot. Transcripts upregulated are designated by diamonds located in the upper right quadrants; transcripts downregulated are designated by diamonds in upper left quadrants (**A–D**). **A**: Profile of wildtype whole bladder gene regulation after 48 hour (h) cyclophosphamide (CYP) treatment. **B**: Profile of $VIP^{-/-}$ whole bladder gene regulation after 48h CYP treatment. **C**: Baseline comparison between wildtype and $VIP^{-/-}$ whole bladders. **D**: Comparative effect of 48h CYP treatment on urinary bladder transcript expression between wildtype and $VIP^{-/-}$ whole bladders. $n = 3$ for each group.

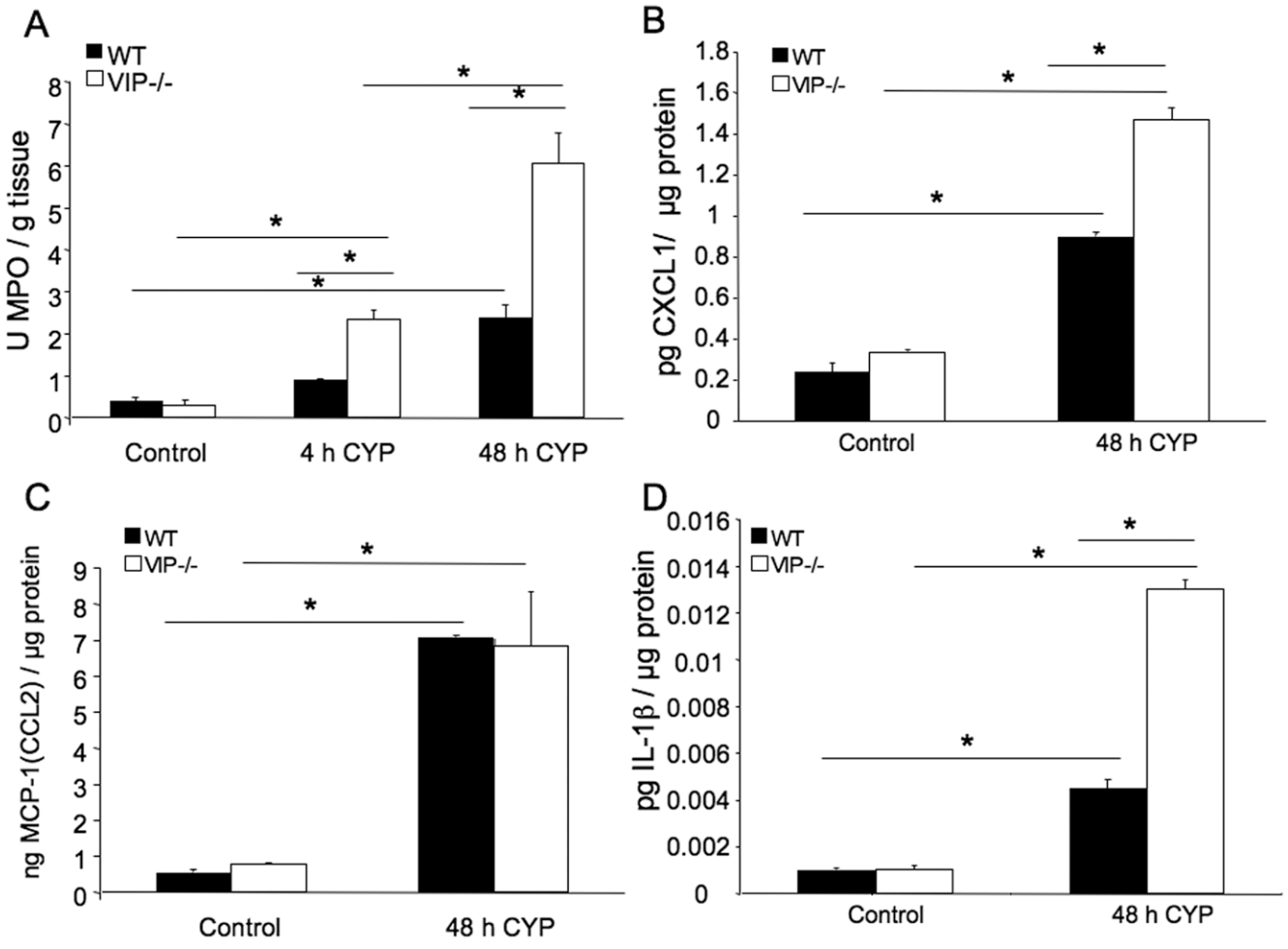

Figure 2.

Immunoassay validation of inflammatory mediator protein expression in urinary bladder of wildtype (WT) and VIP$^{-/-}$ mice after 48 hour (h) cyclophosphamide (CYP) treatment. **A**. Myeloperoxidase (MPO) expression in urinary bladder of WT and VIP$^{-/-}$ mice with and without CYP-induced cystitis. **B**. CXCL1 expression in urinary bladder of WT and VIP$^{-/-}$ mice with and without CYP-induced cystitis. **C**. MCP1/CCL2 expression in urinary bladder of WT and VIP$^{-/-}$ mice with and without CYP-induced cystitis. **D**. IL-1β expression in urinary bladder of WT and VIP$^{-/-}$ mice with and without CYP-induced cystitis. *, $p \leq 0.001$. $n = 4 – 6$ for each group.

Table 1

Effect of 48 hour (h) cyclophosphamide (CYP) treatment on whole urinary bladder from wildtype mice. Regulated genes with fold changes ≥ 1.5 (either up or down) and p-values of ≤ 0.05 are presented expressed in urinary bladder after CYP treatment (150 mg/kg; i.p.; 48 h) in wildtype mice are presented. $n = 3$ for each group.

Symbol	Accession	Description	p value	fold change
		Chemokine ligands		
Cxcl5	NM_009141	Chemokine (C-X-C motif) ligand 5	0.0033	16.9100
Ccl8	NM_021443	Chemokine (C-C motif) ligand 8	0.0000	4.1153
Ccl2	NM_011333	Chemokine (C-C motif) ligand 2	0.0380	3.8279
Ccl9	NM_011338	Chemokine (C-C motif) ligand 9	0.0002	2.9963
Ccl7	NM_013654	Chemokine (C-C motif) ligand 7	0.0250	3.0740
Ccl6	NM_009139	Chemokine (C-C motif) ligand 6	0.0023	2.8996
Ccl12	NM_011331	Chemokine (C-C motif) ligand 12	0.0040	2.6934
Cxcl4	NM_019932	Chemokine (C-X-C motif) ligand 4	0.0051	2.2833
Ccl3	NM_011337	Chemokine (C-C motif) ligand 3	0.0339	1.8379
Ccl5	NM_013653	Chemokine (C-C motif) ligand 5	0.0255	−1.5855
Ccl22	NM_009137	Chemokine (C-C motif) ligand 22	0.0172	−2.3518
Ccl17	NM_011332	Chemokine (C-C motif) ligand 17	0.0254	−4.2494
Cxcl13	NM_018866	Chemokine (C-X-C motif) ligand 13	0.0010	−4.7788
		Chemokine receptors		
Ccr1	NM_009912	Chemokine (C-C motif) receptor 1	0.0164	2.4280
Ccr5	NM_009917	Chemokine (C-C motif) receptor 5	0.0091	2.1778
Ccr4	NM_009916	Chemokine (C-C motif) receptor 4	0.0391	1.7624
		Interleukins		
Il1b	NM_008361	Interleukin 1 beta	0.0544	2.5620
		Interleukin receptors		
Il8rb	NM_009909	Interleukin 8 receptor, beta	0.0119	4.1965
Il2rb	NM_008368	Interleukin 2 receptor, beta chain	0.0063	−2.7474
		others		
Spp1	NM_009263	Secreted phosphoprotein 1	0.0458	6.9931
Itgam	NM_008401	Integrin alpha M	0.0011	2.0759
Ltb	NM_008518	Lymphotoxin B	0.0510	−2.2706
Xcr1	NM_011798	Chemokine (C motif) receptor 1	0.0001	−3.2867
Ifng	NM_008337	Interferon gamma	0.0009	−3.4887

Table 2

Effect of 48 hour (h) cyclophosphamide (CYP) treatment on whole urinary bladder in VIP−/− mice. Regulated genes with fold changes ≥ 1.5 (either up or down) and p-values of ≤ 0.05 expressed in urinary bladder after CYP treatment (150 mg/kg; i.p.; 48 h) in VIP−/− mice are presented. *n* = 3 for each group.

Symbol	Accession	Description	P value	fold change
		Chemokine ligands		
Cxcl5	NM_009141	Chemokine (C-X-C motif) ligand 5	0.0072	413.8539
Cxcl1	NM_008176	Chemokine (C-X-C motif) ligand 1	0.0232	33.1444
Ccl7	NM_013654	Chemokine (C-C motif) ligand 7	0.0114	24.1522
Ccl2	NM_011333	Chemokine (C-C motif) ligand 2	0.0127	23.4502
Ccl8	NM_021443	Chemokine (C-C motif) ligand 8	0.0021	18.9456
Ccl12	NM_011331	Chemokine (C-C motif) ligand 12	0.0050	11.9712
Ccl6	NM_009139	Chemokine (C-C motif) ligand 6	0.0051	10.5684
Cxcl15	NM_011339	Chemokine (C-X-C motif) ligand 15	0.0124	5.7238
Ccl9	NM_011338	Chemokine (C-C motif) ligand 9	0.0162	5.6637
Cxcl10	NM_021274	Chemokine (C-X-C motif) ligand 10	0.0106	4.1592
Cxcl4	NM_019932	Chemokine (C-X-C motif) ligand 4	0.0082	3.5455
Ccl1	NM_011329	Chemokine (C-C motif) ligand 1	0.0402	−1.5035
Ccl17	NM_011332	Chemokine (C-C motif) ligand 17	0.0204	−3.5047
		Chemokine receptors		
Ccr1	NM_009912	Chemokine (C-C motif) receptor 1	0.0041	10.8650
Ccr5	NM_009917	Chemokine (C-C motif) receptor 5	0.0076	9.2075
Ccr3	NM_009914	Chemokine (C-C motif) receptor 3	0.0014	5.5398
Ccr2	NM_009915	Chemokine (C-C motif) receptor 2	0.0038	4.7891
		Interleukins		
Il1b	NM_008361	Interleukin 1 beta	0.0397	30.3111
Il10ra	NM_008348	Interleukin 10 receptor, alpha	0.0414	1.7533
Il13	NM_008355	Interleukin 13	0.0540	−2.3133
		Interleukins receptors		
Il8rb	NM_009909	Interleukin 8 receptor, beta	0.0574	29.0341
Il1r2	NM_010555	Interleukin 1 receptor, type II	0.0116	8.9317
Il2rg	NM_013563	Interleukin 2 receptor, gamma chain	0.0120	2.5213
		Integrin		
Itgam	NM_008401	Integrin alpha M	0.0046	4.5982
Itgb2	NM_008404	Integrin beta 2	0.0264	2.3103
		TNF and receptors		
Tnf	NM_013693	Tumor necrosis factor	0.0297	3.5450
Tnfrsf1b	NM_011610	Tumor necrosis factor receptor superfamily, member 1b	0.0143	2.8444
Tnfrsf1a	NM_011609	Tumor necrosis factor receptor superfamily, member 1a	0.0268	2.2318
		others?		
Spp1	NM_009263	Secreted phosphoprotein 1	0.0093	34.2836

Symbol	Accession	Description	P value	fold change
Tgfb1	NM_011577	Transforming growth factor, beta 1	0.0138	2.7552
Casp1	NM_009807	Caspase 1	0.0033	2.0622

Table 3

Baseline comparison of whole bladder gene expression between wildtype and VIP−/− mice. Only genes with fold changes ≥ 1.5 (either up or down) and p-values of ≤ 0.05 are presented. $n = 3$ for each group.

Symbol	Accession	Description	p value	fold change
		Chemokine ligands		
Ccl7	NM_013654	Chemokine (C-C motif) ligand 7	0.0377	−2.3387
Ccl22	NM_009137	Chemokine (C-C motif) ligand 22	0.0470	−2.8898
Ccl8	NM_021443	Chemokine (C-C motif) ligand 8	0.0371	−3.1834
Cxcl1	NM_008176	Chemokine (C-X-C motif) ligand 1	0.0053	−4.3074
		Chemokine receptors		
Ccr4	NM_009916	Chemokine (C-C motif) receptor 4	0.0016	3.2747
Xcr1	NM_011798	Chemokine (C motif) receptor 1	0.0059	−1.6405
Ccr3	NM_009914	Chemokine (C-C motif) receptor 3	0.0428	−1.6705
Ccr2	NM_009915	Chemokine (C-C motif) receptor 2	0.0442	−2.0518
Ccr5	NM_009917	Chemokine (C-C motif) receptor 5	0.0247	−2.0658
		Interleukins		
Il1b	NM_008361	Interleukin 1 beta	0.0061	−2.6180
		Interleukin receptors		
Il10ra	NM_008348	Interleukin 10 receptor, alpha	0.0494	−1.6000
Il2rg	NM_013563	Interleukin 2 receptor, gamma chain	0.0250	−1.8303
		others		
Ltb	NM_008518	Lymphotoxin B	0.0028	−1.7911
Tnf	NM_013693	Tumor necrosis factor	0.0339	−2.0972

Table 4

Comparative effect of 48 hour (h) cyclophosphamide (CYP) treatment on whole bladder between wildtype and $VIP^{-/-}$ mice. Regulated genes with fold changes ≥ 1.5 (either up or down) and p-values of ≤ 0.05 expressed in the urinary bladder of $VIP^{-/-}$ mice relative to wildtype mice after 48 h CYP treatment. $n = 3$ for each group.

Symbol	Accession	Description	p value	fold change
		Chemokine ligands		
Cxcl5	NM_009141	Chemokine (C-X-C motif) ligand 5	0.0190	8.5400
Cxcl15	NM_011339	Chemokine (C-X-C motif) ligand 15	0.0424	7.8647
		Interleukins		
Il13	NM_008355	Interleukin 13	0.0286	−3.8917
		Interleukin receptors		
Il1r2	NM_010555	Interleukin 1 receptor, type II	0.0343	4.4162
		others		
Tollip	NM_023764	Toll interacting protein	0.0355	−1.1449
Hsp90ab1	NM_008302	Heat shock protein 90kDa alpha (cytosolic), class B member 1	0.0012	−1.2250