Up-Regulation of Phosphorylated CREB but Not c-Jun in Bladder Afferent Neurons in Dorsal Root Ganglia after Cystitis

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ABSTRACT

We examined the changes of two transcription factors, CREB and c-Jun, in dorsal root ganglia (DRG) after acute (8 or 48 hours) or chronic (10 days) cyclophosphamide (CYP)-induced cystitis. Results showed an increase in the number of p-CREB-immunoreactive (-IR) cells in the L1 and L2 DRG (5–7-fold; \( P < 0.05 \)) as well as L6 and S1 DRG (2–4-fold; \( P < 0.05 \)) after acute and chronic cystitis. The number of p-CREB-IR cells in the L4–L5 DRG was not altered with cystitis. The number of c-Jun-IR cells increased in the L1–L2 DRG (L1: 10-fold; L2: 8-fold; \( P < 0.05 \)) only with chronic cystitis, although it increased in the L6–S1 DRG with CYP-induced cystitis of acute (2–3-fold; \( P < 0.05 \)) and chronic (6–10-fold; \( P < 0.05 \)) duration. After CYP treatment, the percentage of bladder afferent cells expressing p-CREB immunoreactivity (3–7-fold; \( P < 0.05 \)) increased in L1, L2, L6, and S1 DRG. The increase occurred 8 hours post-CYP injection and was maintained with chronic cystitis. There were few c-Jun-IR cells in the bladder afferent population. These results demonstrate that CYP induces p-CREB and c-Jun expression in DRG in a time-dependent manner. However, c-Jun expression is not associated with bladder afferent neurons. Resiniferatoxin reduced CYP-induced up-regulation of p-CREB in DRG, suggesting that cystitis can reveal an altered CREB phosphorylation that may be mediated by capsaicin-sensitive bladder afferents. Colocalization of p-CREB and Trk receptor(s) showed that a subpopulation of p-CREB-IR cells expressed p-Trk with cystitis. These results suggest that up-regulation of p-CREB may be mediated by a neurotrophin/Trk signaling pathway. J. Comp. Neurol. 469:262–274, 2004.

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Indexing terms: cystitis; lower urinary tract; neuroplasticity; p-CREB; c-Jun; Trk

Several studies involving a chemically (cyclophosphamide; CYP)-induced bladder inflammation (Cox, 1979; Maggi et al., 1992; Lanteri-Minet et al., 1995; Vizzard, 2000a–c) model have demonstrated alterations in neurochemical (Vizzard and Boyle, 1999; Vizzard, 2000a–c), electrophysiological (Jennings and Vizzard, 1999; Yoshimura and de Groat, 1999) and organizational (Vizzard, 2000c) properties of bladder afferent neurons in dorsal root ganglia (DRG) and in central reflex micturition pathways. These changes suggest considerable reorganization of reflex connections in the spinal cord and marked changes in the properties of micturition reflexes with CYP treatment. Altered visceral sensation from the urinary bladder with bladder inflammation may be mediated by at least two factors: 1) changes in the properties of the urinary bladder and 2) changes in the properties of peripheral bladder afferent pathways such that bladder afferent neurons respond in an exaggerated manner to normally innocuous stimuli.
Recent findings suggested that neurotrophins may contribute to neuroplasticity of lower urinary tract pathways with cystitis because of increases in the level of neurotrophins and high-affinity receptors (Trks) in the bladder as well as in bladder afferent neurons with bladder dysfunction, including cystitis (Steers et al., 1991, 1996; Chen et al., 1995; Lowe et al., 1997; Oddiah et al., 1998; Okragly et al., 1999; Vizzard, 2000a; Qiao, 2002a–c; Zvara et al., 2002). Elevated levels of neurotrophins have also been detected in the urine of women with interstitial cystitis (IC; Lowe et al., 1997). Increased expression of neurotrophins in the inflamed bladder could influence biological function through 1) activation of signal transduction cascades mediated by receptor tyrosine kinase (Trk) phosphorylation at nerve terminals in the target tissue or 2) retrograde transport (molecules or signals) to innervating neurons (MacInnis and Campenot, 2002; Miller and Kaplan, 2002). In the neuronal cell body, the activated neurotrophin/Trk complex (Mu et al., 1993; McMahon et al., 1994) could facilitate a signal cascade(s) to induce neurochemical changes. In vitro studies demonstrate that the activation of downstream intracellular signaling molecules, including Ras/Raf/MAPK, PI-3K/Akt, and PLC-γ (Ji and Woolf, 2001; Ji et al., 2002), as well as transcription factors (cAMP-responsive element binding protein (CREB), c-Jun, Elk-1, etc. (Hawley et al., 1992; Gold et al., 1993; Riccio et al., 1999; Avelino et al., 2002)) is an important step in the neurotrophin signaling cascades. These signals finally induce gene transcription, leading to gene expression and long-term changes in cells. These long-term changes include 1) mediating neurotransmitter phenotype, 2) influencing dendrite size and synaptic reorganization, 3) increasing synaptic efficacy and promoting cell survival or differentiation, and 4) controlling innervation density and function in a target organ (Ji and Woolf, 2001; Liu et al., 2001).

Several studies have demonstrated the expression of phosphorylated CREB and c-Jun in the spinal cord and DRG in response to peripheral stimulation. Chronic hind paw inflammation (Ji and Rupp, 1997; Messersmith et al., 1998) dramatically induced phosphorylation of CREB and c-Jun in nociceptive-specific neurons in the lumbar rat spinal cord. Peripheral nerve injury (Jenkins et al., 1993; De Leon, et al., 1995; Gillardon et al., 1996; Broude et al., 1997) increased c-Jun expression in the DRG neurons. Recent experiments also suggested a significant increase in the number of Jun-positive colon DRG neurons in inflammatory bowel disease (Birder et al., 2003). In addition, electrical stimulation at C-fiber intensity (Ji et al., 2000) as well as renal artery occlusion (Lishnak and Vizzard, 2001) evoked CREB phosphorylation in the spinal cord and DRG.

In the present study, by using antibodies that specifically recognize the phosphorylated form of CREB (p-CREB) or c-Jun, we examined the distribution of p-CREB and c-Jun in lumbosacral DRG cells as a function of the duration (acute: 8 and 48 hours; chronic: 10 day) of CYP-induced bladder inflammation. We also determined whether cystitis-induced p-CREB or c-Jun is associated with 1) bladder afferent cells in the DRG, by retrograde labeling of bladder afferent neurons with a conventional tracing dye, fast blue (FB), and 2) neurotrophin signal transduction by colabeling DRG neurons with phosphorylated Trk (p-Trk) antibody (Qiao and Vizzard, 2002a,b). The contribution of C-fiber afferents to p-CREB expression with cystitis was also assessed by pretreatment of rats with resiniferatoxin (RTX; Craft et al., 1995; Szallasi et al., 1999).

### MATERIALS AND METHODS

#### Experimental animals and reagents

Adult female Wistar rats (120–150 g) were purchased from Charles River Canada. For each set of experiments, six control and six experimental animals were used. To minimize the number of rats used in this study, ipsilateral and contralateral DRG were harvested, and sections were equally and sequentially divided into several groups in order to satisfy all studies. Chemicals used in this experiment were purchased from Sigma (St. Louis, MO). Primary antibodies against c-Jun and p-Trk were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). p-CREB antibody was purchased from New England Biolabs (Beverly, MA). Cy3- or Cy2-conjugated secondary antibodies were purchased from Jackson Immunoresearch (West Grove, PA).

#### CYP-induced cystitis

Acute and chronic CYP-induced cystitis rat models were examined in these studies as previously described (Qiao and Vizzard, 2002b). For chronic CYP-induced cystitis, rats received drug injection (75 mg/kg, i.p.) every third day for 10 days. For acute CYP-induced cystitis, rats received a single injection (150 mg/kg, i.p.) and survived for 8 or 48 hours after injection. Control rats received volume-matched injections of saline (0.9%; i.p.). All injections were performed with animals under isoflurane (2%) anesthesia. All experimental protocols involving animal use were approved by the University of Vermont Institutional Animal Care and Use Committee (IACUC 03-030). Animal care was under the supervision of the University of Vermont 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.

#### Retrograde labeling of bladder afferent cells

One week before euthanasia, FB (4%, weight/volume; Polyul, Gross Umstadt, Germany) was injected into the bladder wall for retrograde labeling of bladder afferent neurons in the L1, L2, and L6–S1 DRG. As described previously (Vizzard, 2000c; Qiao and Vizzard, 2002b), a total volume of 40 μl divided into six to eight injections was injected into the dorsal surface of the bladder wall, with particular care taken to avoid injections into the bladder lumen, major blood vessels, or overlying fascial layers. At each injection site, the needle was kept in place for several seconds after injection, and the site was washed with saline to minimize contamination of adjacent organs with FB.

#### RTX treatment

It has been well documented that systemic RTX, an ultrapotent analog of capsaicin, produces long-lasting desensitization of unmyelinated nociceptive C-fiber afferents (Szallasi et al., 1989, 1999; Dray et al., 1990; Craft et al., 1995). To assess the contribution of C-fiber afferents in CYP-induced cystitis and subsequent changes in tran-
perfusion, and tissue harvesting

After control or CYP treatment, animals were deeply anesthetized with isoflurane (3–4%) and then perfused with oxygenated Krebs buffer (pH 7.4; 95% O₂, 5% CO₂), followed by 4% paraformaldehyde. After perfusion, the spinal cord and DRG were quickly removed and postfixed for 6 hours. Tissue was then rinsed in phosphate-buffered saline (PBS; 0.1 M NaCl in phosphate buffer, pH 7.4) and placed in ascending concentrations of sucrose (20%) in 0.1 M PBS for cryoprotection. Spinal cord segments were identified based on at least two criteria: 1) The T13 DRG exists after the last rib, and 2) the L6 vertebra is the last moveable vertebra, followed by the fused sacral vertebrae. Another less precise criterion is the observation that the L6 DRG are the smallest ganglia following the largest, L5 DRG. DRG sections from the (L1, L2, L4–S1) spinal cord segments were sectioned parasagittally at a thickness of 20 μm on a freezing microtome. Some DRG (L1, L2, L6, S1) were specifically chosen for analysis based on the previously determined segmental representation of urinary bladder circuitry (Donovan et al., 1983; Keast and de Groat, 1992; de Groat et al., 1994; Nadelhaft and Vera, 1995). Bladder afferents are not distributed within the L4–L5 DRG (Donovan et al., 1983; Keast and de Groat, 1992), which contain only somatic afferents, nor are neurons involved in urinary bladder function observed in the L4–L5 spinal segments (de Groat et al., 1994; Nadelhaft and Vera, 1995). Thus, the L4–L5 DRG serves as internal controls for these studies. Tissues from control animals were handled in a manner identical to that described above.

Immunohistochemistry

cell and a Cy2-conjugated species-specific secondary antibody was used for detection. According to the manufacturer, the antibody sc-7996 reacts with phosphorylated TrkA and phosphorylated TrkB and shows partial cross-reactivity with phosphorylated TrkC. To determine whether the order of processing affected the immunostaining, we reversed the order of antibody presentation such that DRG sections were first processed for p-Trk immunostaining, followed by p-CREB immunostaining. No difference in DRG immunostaining was detected between the different presentation orders of antibodies, so all results were pooled. Control sections incubated in the absence of primary or secondary antibody were also processed and evaluated for specificity or background staining levels.

Assessment of positively stained DRG cells

Staining observed in experimental tissue was compared with that observed in experiment-matched negative controls. For both p-CREB and c-Jun immunoreactivity, DRG cells exhibiting intense nuclear staining were considered positively stained. Cells with intense nuclear staining may or may not have exhibited cytoplasmic staining that was greater than the background level observed in experiment-matched negative controls. DRG cells exhibiting cytoplasmic staining but no nuclear staining were not considered as positive in this experimental protocol. Positively counted cells were not further divided into categories of different staining intensities.

Data analysis

Sections were examined under an Olympus fluorescence photomicroscope (model BX51; Optical Analysis Corp., Nashua, NH) with a multiband filter set for simultaneous visualization of Cy3 and Cy2 fluorophores. Cy2 staining was viewed by using a filter with an excitation range of 447–501 nm and an emission range of 510–540 nm. Cy3 staining for p-Trk immunoreactivity was observed. The antibody sc-7996 reacts with phosphorylated TrkC. To determine whether the order of processing affected the immunostaining, we reversed the order of antibody presentation such that DRG sections were first processed for p-Trk immunostaining, followed by p-CREB immunostaining. No difference in DRG immunostaining was detected between the different presentation orders of antibodies, so all results were pooled. Control sections incubated in the absence of primary or secondary antibody were also processed and evaluated for specificity or background staining levels.

In the absence of primary antibody, no positive immunostaining for p-Trk or c-Jun was observed.

Double staining of p-CREB and p-Trk

Sections were processed as described above for p-CREB immunoreactivity. Subsequently, these same sections were processed for p-Trk immunoreactivity detection. A pan-p-Trk antibody (sc-7996) was used to recognize the p-Trk-IR
between control and experimental means. Animals, processed and analyzed on the same day, were tested as a block in the analysis of variance. Thus, day was treated as a blocking effect in the model. Two variables were being tested in the analysis: 1) experimental manipulation vs. control situation and 2) effect of day [i.e., tissues from groups (experimental and control) of animals were processed on different days]. When F ratios exceeded the critical value (P ≤ 0.05), Dunnett’s test was used for comparisons between control and experimental means.

**Figure preparation**

Digital images were obtained with a CCD camera (MagnaFire SP; Optronics Optical Analysis Corp.) and LG-3 frame grabber attached to an Olympus microscope. Exposure times were held constant when acquiring images from control and experimental rats processed and analyzed on the same day. Images were imported into Adobe Photoshop 7.0 (Adobe Systems, San Jose, CA), in which groups of images were assembled and labeled. Composite figures were printed on a Hewlett Packard Color LaserJet 4550 HDN.

**RESULTS**

**Time course of p-CREB immunoreactivity in DRG with CYP-induced cystitis**

p-CREB immunoreactivity was examined from L1, L2, and L4–S1 DRG after CYP treatment for 8 hours, 48 hours, or 10 days. In control animals, p-CREB-IR cell profiles were observed in all DRG levels examined (L1, L2, L4–S1; Figs. 1A,E, 2). Significantly (P ≤ 0.05) greater numbers of p-CREB-IR cell profiles were observed in the L4 and L5 DRG compared with the L1, L2, L6, or S1 DRG (Fig. 2). In all DRG examined, p-CREB immunoreactivity was observed in nuclear profiles or nuclear profiles with cytoplasmic staining (Fig. 1). p-CREB immunoreactivity was primarily restricted to relatively small-diameter cells in all DRG examined. After CYP injection, rats were killed at specific time points (8 or 48 hours after a single CYP injection or 10 days after multiple CYP injections). p-CREB immunoreactivity significantly (P ≤ 0.05) increased in all DRG levels (rostralumbal L1 and L2: 5–7-fold; caudal lumbar L6 and S1: 2–4-fold), except for L4 and L5 DRG following acute (8 or 48 hours; Figs. 1B,F,C,G, 2) or chronic (Figs. 1D,H, 2) cystitis.

**Time course of c-Jun immunoreactivity in DRG with CYP-induced cystitis**

In control animals, c-Jun-IR cell profiles were observed in all DRG examined (L1, L2, L4–S1), and the immunostaining was observed in nuclear profiles (Fig. 3Aa,e; arrowheads). In control animals, significantly (P ≤ 0.05) greater proportions of c-Jun-IR cell profiles were observed in the L4 and L5 DRG compared with the L1, L2, and S1 DRG (Fig. 3B). After acute CYP treatment (8 and 48 hours), c-Jun immunoreactivity was primarily restricted to relatively small-diameter cells in all DRG examined (Fig. 3Ab,f,cg, arrowheads). In chronic cystitis, c-Jun immunoreactivity was observed in both small (20 μm)– and medium (30–40 μm)-diameter cells (Fig. 3Ad,b). After acute (8 and 48 hours) CYP treatment, c-Jun immunoreactivity was increased in the L6 and S1 DRG (2–3-fold; P ≤ 0.05) but not in the L1 and L2 DRG (Fig. 3B). With chronic CYP treatment, the number of c-Jun-IR cells increased in the L1–L2 DRG (L1: 10-fold; L2: 8-fold; P ≤ 0.05) and in the L6 DRG (6-fold) and S1 DRG (10-fold; P ≤ 0.05) compared with control (Fig. 3B). No changes were observed in c-Jun expression in the L4 and L5 DRG with CYP treatment of any duration.

**p-CREB and c-Jun immunoreactivity in bladder afferent neurons**

To evaluate the distribution of p-CREB and c-Jun immunoreactivity in bladder afferent neurons before or after CYP treatment, FB was injected into the wall of the urinary bladder for retrograde labeling of bladder afferent neurons in DRG (Figs. 4B, 5B). FB-labeled bladder afferent cells in the L1, L2, L6, and S1 DRG. No FB-labeled cells were observed in the L4 or L5 DRG. The presence of FB did not affect the number of p-CREB- or c-Jun-IR cell profiles in DRG either in control or in experimental animals (data not shown). In control rats, 4–8% of bladder afferent (FB-labeled) cells expressed p-CREB immunoreactivity (Fig. 4D). It should be noted that not all bladder afferent cells (FB-labeled) expressed p-CREB immunoreactivity (Fig. 4, green arrow). In addition, not all p-CREB-IR cells in the DRG were FB-labeled bladder afferent cells (Fig. 4, white arrows). In control rats, under 5% of bladder afferent cells expressed c-Jun immunoreactivity (data not shown).

**Increased p-CREB but not c-Jun immunoreactivity in bladder afferent cells with CYP-induced cystitis**

Changes in p-CREB and c-Jun immunoreactivity were evaluated from FB-labeled bladder afferent cells after CYP treatment. After CYP treatment, the percentage of bladder afferent cells expressing p-CREB immunoreactivity was significantly increased in all levels of DRG determined: L1 (3-fold for 8 hours; 2-fold for 48 hours and 10 days; P ≤ 0.05), L2 (3–4-fold; P ≤ 0.05), L6 (6–7-fold for acute and 2.5-fold for chronic cystitis; P ≤ 0.05), and S1 (2–3-fold; P ≤ 0.05) compared with DRG from control animals (Fig. 4D). The largest change in p-CREB immunoreactivity in bladder afferents was observed in L6 DRG with acute CYP treatment (8 and 48 hours). In contrast, no significant changes in c-Jun immunoreactivity (Fig. 5C, yellow arrows, red staining) were observed in FB-labeled bladder afferent cells (Fig. 5C, white arrows, blue cells) in any level of DRG, L1, L2, L6, or S1, after CYP treatment of any duration.

**Colocalization of p-CREB and p-Trk immunoreactivity in DRG**

Previous studies have demonstrated increased expression and phosphorylation of tyrosine kinase receptor (TrkA, TrkB) in lumbar DRG after CYP-induced cystitis (Qiao and Vizzard, 2002b). Phosphorylation of CREB has been reported to be an important step in Trk-mediated neurotrophin signal transduction (Hawley et al., 1992; Riccio et al., 1999). To determine whether p-CREB is expressed in Trk-IR neurons in DRG, double staining of p-CREB and p-Trk was performed in L1, L2, L6, and S1 DRG before or after CYP treatment. It was noted (see Fig. 6) that a subpopulation of p-CREB-IR cells expressed p-Trk (yellow arrows) with acute and/or chronic cystitis. However, not all p-Trk-IR cells expressed p-CREB (Fig. 6,
Fig. 1. Expression of phosphorylated CREB (serine 133) in L1 and L6 DRG in control and CYP-treated rats. Immunostaining of p-CREB in L1 (A–D) and L6 (E–H) DRG in control (A,E) and CYP-treated rats was performed 8 hours (B,F) or 48 hours (C,G) after acute CYP treatment or 10 days (D,H) after chronic CYP treatment. In all experimental groups, p-CREB immunoreactivity was observed in relatively small-diameter cell profiles with nuclear staining or nuclear plus cytoplasmic staining in the DRG. CYP-induced cystitis upregulated p-CREB immunoreactivity at all time points with CYP treatment in L1, L2, L6, and S1 DRG (see Fig. 2 for time course of up-regulation). Arrowheads indicate several examples of positively stained cell profiles. Scale bar = 80 μm.
control, CYP for 48 hours, RTX pretreatment without CYP, and RTX pretreatment with CYP for 48 hours. RTX vehicle with or without CYP for 48 hours was also studied to serve as a control. Immunofluorescence labeling showed reduced vanilloid receptor 1 immunoreactivity in DRG cells of RTX-treated rats in this research protocol (data not shown). Consistently, 48 hours after CYP treatment, the number of p-CREB-IR cells in the L1, L2, L6, and S1 DRG (Fig. 7) was markedly increased. One week of RTX treatment did not influence the expression of p-CREB in the DRG in control (no CYP treatment) rats. However, RTX suppressed the up-regulation of p-CREB immunoreactivity in DRG induced by CYP treatment compared with the rats treated with RTX vehicle and CYP. RTX vehicle showed no regulation of p-CREB immunoreactivity in DRG (Fig. 7).

**DISCUSSION**

The aim of this work was to determine whether CYP-induced cystitis involved changes in the transcription factor p-CREB and the immediate early gene c-Jun in lumbar sacral DRG. Results demonstrated that there were significant increases in the expression of p-CREB and c-Jun in the DRG following bladder irritation. However, CYP induces the phosphorylation of CREB and expression of c-Jun in lumbar sacral DRG in a time-dependent manner. The number of p-CREB-IR cells significantly increased in the rostral lumbar L1 and L2 DRG (5–7-fold; $P \leq 0.05$) as well as caudal lumbar sacral L6 and S1 DRG (2–4-fold; $P \leq 0.05$) after acute and chronic cystitis compared with control rats. The number of c-Jun-IR cells increased in the L1–L2 DRG (L1: 10-fold; L2: 8-fold; $P \leq 0.05$) only with chronic cystitis. In contrast, c-Jun-IR cells increased in the L6–S1 DRG (P $\leq 0.05$) with CYP-induced cystitis of acute (2–3-fold) or chronic (6–10-fold) duration compared with control rats. As a result of the differential expression of p-CREB immunoreactivity and c-Jun immunoreactivity in DRG following CYP treatment, the association of p-CREB or c-Jun with bladder afferent neurons was also studied. We have shown that p-CREB but not c-Jun expression increased in bladder afferent neurons after cystitis. We also have shown that a population of p-CREB-IR DRG cells exhibited p-Trk immunoreactivity. p-CREB up-regulation induced by cystitis was prevented by RTX pretreatment, suggesting a role for C-fiber bladder afferents in this up-regulation.

CYP-induced cystitis is a well-established animal model and is widely used in many laboratories in studying bladder inflammation and interstitial cystitis (IC; Watson and Notley, 1973; Cox, 1979; Stillwell and Benson, 1988; Lecci et al., 1994; Lanteri-Minet et al., 1995; Wood et al., 2001). Intraperitoneal injection of CYP induces bladder irritation through the toxic metabolite of CYP, acrolein (Cox, 1979). Urodynamic studies demonstrate that CYP injection resulted in bladder overactivity as early as 4 hours after treatment (Hu et al., 2003). In addition to the alteration in the level of neurotrophic factors in the inflamed bladder (Vizzard, 2000a), a recent study demonstrated changes in the levels of cytokine transcripts and proteins (Malley and Vizzard, 2002). Both neurotrophins and cytokines may elicit the changes in p-CREB and c-Jun expression after CYP treatment. This possibility has been suggested by studies including lipopolysaccharide-induced spleen inflammation (Zhou et al., 2003), sciatic nerve injury (Gold...
Fig. 3. Expression of c-Jun immunoreactivity in lumbosacral DRG from control and CYP-treated rats. Immunostaining (A) of c-Jun in L1 and L6 DRG from control (a,e) or CYP-treated rats was performed at the designated times, 8 hours (b,f), 48 hours (c,g), or 10 days (d,h) of CYP treatment. In control and acute CYP treatment (8 and 48 hours), c-Jun immunoreactivity was primarily restricted to relatively small-diameter cell profiles (a–c,e–g) with nuclear staining. After chronic cystitis, c-Jun immunoreactivity was observed in both small- and medium-diameter cell profiles (d,h) with nuclear staining or nuclear plus cytoplasmic staining. CYP-induced cystitis up-regulated c-Jun immunoreactivity in the L1 DRG after chronic (10 days) but not acute (8 or 48 hours) duration. Expression of c-Jun in the L6 DRG was up-regulated in both acute and chronic CYP injection. Arrowheads indicate several examples of positively stained cell profiles. B: Histogram showing the number of cells in DRG (L1, L2, L4–S1) expressing c-Jun immunoreactivity in control and CYP-treated rats. In control rats, the number of c-Jun-IR cells in the L4 and L5 DRG was significantly greater than in the L1 or L2 DRG (*P ≤ 0.05). Significant increases in the number of DRG cells expressing c-Jun immunoreactivity were observed in the L1, L2, L6, and S1 DRG 10 days after CYP treatment. L6 and S1 DRG also exhibited increased c-Jun immunoreactivity after acute CYP treatment (8 and 48 hours). No changes in c-Jun immunoreactivity were observed in the L4–L5 DRG following cystitis (*P ≤ 0.05). Scale bar = 40 μm.
et al., 1993), and sympathetic neuron survival (Riccio et al., 1999).

Our previous studies have demonstrated increased expression and phosphorylation of tyrosine kinase receptors (TrkA, TrkB) in lumbosacral DRG after CYP-induced cystitis (Qiao and Vizzard, 2002b). The increased expression of Trks in DRG may result from changes in the properties or functions of the urinary bladder (Lowe et al., 1997; Okragly et al., 1999; Vizzard, 2000a–c; Qiao and Vizzare, 2002b; Saban et al., 2002; Hu et al., 2003). In patients with IC, neurotrophins, including nerve growth factor (NGF), neurotrophin-3 (NT-3), and glial-derived neurotrophic factor (GDNF), have been detected in the urine (Okragly et al., 1999). Increased expression of NGF is also present in bladder biopsies from women with IC (Lowe et al., 1997). In addition, acute intravesical administration of NGF induces bladder overactivity in rats (Chuang et al., 2001). Inflammation-induced changes in neurotrophins and/or neural activity arising in the bladder (Vizzare, 2000a) may sensitize afferent nerves, induce bladder overactivity, mediate changes in Trk expression and phosphorylation, and further induce the activation of Trk-mediated signal transduction pathway in DRG.

Trk and Trk-mediated signal transduction have been predicted to be key components in cell survival during neuro-
trophic factor deprivation or cell survival after chemical or physical injury (Miller and Kaplan, 2002). Signaling mediated by Trks involves ligand-induced Trk autophosphorylation (Schlessinger and Ulrich, 1992; Guiton et al., 1994), and subsequent activation of intracellular signaling molecules. TrkA uses phosphatidylinositol-3 kinase (PI-3K) and TrkB uses PI-3K and mitogen-activated protein kinase (MAPK) signaling pathways to stimulate cell survival (Atwal et al., 2000; Liu et al., 2001). Another critical step in the Trk-mediated signaling pathway is the involvement of tran-

Fig. 5. Lack of c-Jun Immunoreactivity in bladder afferent neurons (FB labeled). Bladder afferent cells in DRG were retrogradely labeled by injecting FB (B, white arrows) into urinary bladder smooth muscle 1 week prior to euthanasia. In A, c-Jun-IR cells showed nuclear staining (yellow arrows); however, the same cells were not labeled by FB (B, yellow arrows); likewise, FB-labeled (B, white arrows) cells did not exhibit c-Jun immunoreactivity in the cell nuclei (A, white arrows). In C, bladder afferent cells were pseudocolored blue and c-Jun cells were pseudocolored red. C represents a color merge from images of c-Jun staining and FB labels of the same DRG section (L6, 48 hours) that was viewed with optical filters with different excitation wavelengths to visualize FB-labeled bladder afferents (blue cells) or c-Jun-IR cells (red nuclear staining). Note that c-Jun immunoreactivity (yellow arrows) is not associated with bladder afferent neurons in DRG (FB-labeled neurons, white arrows). Arrows point to several examples of indicated cells. Scale bars = 80 μm.
scription factors (Hawley et al., 1992; Gold et al., 1993; Riccio et al., 1999; Avelino et al., 2002). In the present study, we examined the changes of two downstream transcription factors, CREB and c-Jun, in DRG after CYP-induced cystitis. CREB is activated via phosphorylation at serine 133 (Sassone-Corsi, 1995; Ji and Rupp, 1997; Ji and Woolf, 2001) and binding to the cAMP-responsive element on target genes (Sassone-Corsi, 1995). c-Jun and c-Fos dimerize to form activating protein (AP)-1 complex (Wisdom, 1999). Therefore, antibodies specific to phosphorylated CREB (serine 133) or c-Jun were used for this study. Here, p-CREB immunoreactivity showed sustained up-regulation in the L1, L2, L6, and S1 DRG after acute and chronic cystitis. In the L1 and L2 DRG, p-CREB immunoreactivity was up-regulated following chronic cystitis (8–10-fold). No changes were detected in the L1 and L2 DRG after acute CYP treatment. In the L6 and S1 DRG, a modest increase of c-Jun immunoreactivity (2–3-fold) was noted with acute cystitis, and a marked increase (6–10-fold) in the number of c-Jun-IR cells was detected in the L6 and S1 DRG after chronic cystitis compared with control. Our retrograde tracing studies also indicated the increased p-CREB immunoreactivity in bladder afferent neurons; however, c-Jun expression is not associated with bladder afferent cells in the DRG, suggesting that cystitis-induced p-CREB and c-Jun up-regulation may occur via different mechanisms.

Table 1. Proportion of Cells with p-Trk + p-CREB Immunoreactivity in the p-Trk-IR or p-CREB-IR Population in DRG

<table>
<thead>
<tr>
<th>Segment (DRG)</th>
<th>Control (%)</th>
<th>CYP-48 hours (%)</th>
<th>CYP-10 days (%)</th>
</tr>
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<tbody>
<tr>
<td>p-Trk + p-CREB</td>
<td>16.8 ± 8.1</td>
<td>35.0 ± 8.2*</td>
<td>30.9 ± 5.2*</td>
</tr>
<tr>
<td>L1</td>
<td>17.5 ± 3.4</td>
<td>43.7 ± 9.4*</td>
<td>30.4 ± 9.3*</td>
</tr>
<tr>
<td>L2</td>
<td>18.2 ± 3.5</td>
<td>27.2 ± 5.6</td>
<td>43.9 ± 15.4*</td>
</tr>
<tr>
<td>L6</td>
<td>16.8 ± 3.3</td>
<td>26.0 ± 5.9*</td>
<td>33.9 ± 11.2*</td>
</tr>
<tr>
<td>S1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p-Trk + p-CREB</td>
<td>25.6 ± 5.9</td>
<td>48.1 ± 6.7*</td>
<td>56.8 ± 9.8*</td>
</tr>
<tr>
<td>L1</td>
<td>30.4 ± 3.7</td>
<td>49.7 ± 3.3*</td>
<td>42.1 ± 13.5*</td>
</tr>
<tr>
<td>L2</td>
<td>30.1 ± 3.8</td>
<td>53.3 ± 7.6*</td>
<td>29.8 ± 8.0</td>
</tr>
<tr>
<td>L6</td>
<td>24.5 ± 6.8</td>
<td>34.3 ± 4.6*</td>
<td>25.7 ± 5.7</td>
</tr>
<tr>
<td>S1</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*P ≤ 0.05.

Fig. 6. Colocalization of phosphorylated CREB with phosphorylated Trk immunoreactivity in DRG following CYP treatment. p-CREB immunostaining (A, blue and yellow arrows) was pseudocolored red with nuclear staining. p-Trk-IR cells (B, white and yellow arrows) were pseudocolored green with cytoplasmic staining. C represents color merges of A and B. Note that neurons expressing both p-CREB and p-Trk exhibit a red nucleus surrounded by green cytoplasm (C, yellow arrows). Color merges of images were viewed with optical filters with different excitation wavelengths to visualize p-Trk-IR or p-CREB-IR cells. Scale bar = 40 μm.

Fig. 7. Histogram showing the effects of resiniferatoxin (RTX) pretreatment on the expression of p-CREB immunoreactivity in DRG before and after CYP injection. Pretreatment with RTX (300 μg/kg) has no effect on p-CREB immunoreactivity in control rats; however, it significantly suppressed the increased p-CREB immunoreactivity in the L1, L2, L6, and S1 DRG in CYP-treated rats (*P ≤ 0.05).
The regulation of p-CREB and c-Jun by neurotrophins or cytokines in DRG during pathological conditions demonstrated diverse patterns (Hawley et al., 1992; Gold et al., 1993; Riccio et al., 1999; Finkbeiner, 2000; Avelino et al., 2002; Ventura et al., 2003). It has also been reported that neurotrophins exhibit diverse effects on C-jun expression. For example, the temporary deprivation of bladder-derived neurotrophic factors by intravesical RTX application markedly increased the number of c-Jun-IR cells in bladder sensory neurons in L6 DRG (Avelino et al., 2002). In addition, continuous intrathecal infusion of NGF for 1 week reduced the injury-induced increase in c-Jun immunoreactivity in DRG (Gold et al., 1993). Lipopolysaccharide-elicited proinflammatory cytokine up-regulation markedly induces c-Jun expression but increases p-CREB to a lesser extent (Zhou et al., 2003). In taking these findings together with the present findings, a diverse response of CREB and c-Jun to lower urinary tract inflammation is also suggested.

The influence of target organ-to-neuron interactions has been extensively reported and may also contribute to up-regulation of the two transcription factors examined in the present study (Steers et al., 1991; Eide et al., 1993; Tuttle et al., 1994). For example, the hypertrophied bladder after bladder outlet obstruction exhibits markedly increased levels of NGF, and autoimmunization against NGF reduces the MPG neuronal hypertrophy (Steers et al., 1991, 1996; Dupont et al., 1995). This suggests that neurotrophin(s) released in the hypertrophied bladder is partially responsible for the change in neuronal morphology. Increased access to bladder neurotrophins and/or increased retrograde transport may increase expression of Trks (Qiao and Vizzard, 2002b) and/or Trk-mediated p-CREB expression in bladder afferent neurons. Recent studies also showed that in vitro NGF application to pelvic ganglion cultures increased p-CREB immunoreactivity (Furves-Tyson and Keast, 2003). Thus, neurotrophic factor expression in the urinary bladder after cystitis may contribute to p-CREB expression in DRG. The percentage of bladder afferent cells in DRG expressing p-CREB immunoreactivity markedly increased to 30% from basal level (4–8%) after CYP treatment. However, the expression of c-Jun in bladder afferent cells remained unchanged with CYP treatment, suggesting that the increase in c-Jun immunoreactivity after chronic CYP may not result from bladder afferent sensitization. Urthral afferents and/or caudal urethral afferents may result in c-Jun expression, in that inflammation/irritation of these tissues may occur from CYP treatment. Alternatively, cross-sensitization of DRG cells by a paracrine mechanism has been demonstrated (Amir and Devor, 1996; Pezzone et al., 2003). Thus, there is precedent for secondary activation of a DRG population from a primary locus.

An involvement of unmyelinated C-fiber bladder afferents in the up-regulation of p-CREB after cystitis was suggested by systemic administration of RTX prior to CYP treatment. RTX, an ultrapotent analog of capsaicin and C-fiber desensitizer, has been widely used to study the action of nociceptive C-fiber afferents (Szallasi et al., 1989, 1999; Dray et al., 1980; Craft et al., 1995). Systemic RTX produces long-lasting desensitization of unmyelinated nociceptive C-fiber afferents (Szallasi et al., 1989, 1999). In these RTX-pre-treated animals, CYP-induced up-regulation in the number of p-CREB-IR cells in L1, L2, L6, and S1 DRG was significantly reduced. This suggested that cystitis-induced CREB phosphorylation in lumbosacral DRG was, at least partially, mediated by capsaicin-sensitive (presumptive C-fibers) bladder afferents. Recently, the use of capsaicin and RTX therapy for overactive bladder treatment has been extensively reviewed (Chancellor and de Groat, 1999; Kim and Chancellor, 2000). In addition, RTX has been shown to be a potent in vitro inhibitor of the nuclear transcription factor-κB (NF-κB) pathway (Singh et al., 1996), a major pathway in inducing inflammatory and growth-regulatory genes. Therefore, we cannot rule out the possibility that the reduction of p-CREB with RTX treatment in vivo may be secondary to the inhibition of inflammatory genes.

We have previously demonstrated p-TrkA- and p-TrkB immunoreactivity in lumbosacral DRG after cystitis. Sustained expression in both Trk- and p-Trk may contribute to the increased expression of p-CREB in DRG after cystitis. The CREB family of transcription factors is involved in NGF-induced survival of sympathetic neurons (Hu et al., 2000; Manji et al., 2003) and PC12 cells (Bedogni et al., 2003). However, the relationship between Trk activation and CREB phosphorylation in sensory neurons in a visceral model of inflammation is unknown. We examined, based on the evidence that both p-Trk immunoreactivity and p-CREB immunoreactivity are up-regulated in DRG after cystitis, the colocalization of p-CREB and p-Trk in the DRG from L1, L2, L6, and S1 segments. The results showed that 30–40% of p-Trk-IR cells expressed p-CREB with acute and chronic durations of CYP treatment compared with control (17%). The percentage (57%) of p-CREB-IR cells expressing p-Trk immunoreactivity also increased in rats with cystitis compared with control. These data suggested that not all p-CREB immunoreactivity was associated with p-Trk and that not all p-Trk-IR cells expressed p-CREB in DRG following CYP treatment. Trk-mediated signaling can involve multiple intracellular signaling cascades, including Ras/Raf/MEK, PI-3KAkt, and PLC-γ/PKC, and involves several transcription factors, such as CREB, c-Fos/Jun, and Elk. With these results taken together, CREB phosphorylation may play a role but may not be the only transcription factor that is involved in lower urinary tract plasticity with cystitis. To date, no studies have examined transcription factor activation in lumbosacral DRG after CYP-induced cystitis or any other model of cystitis, and little is known regarding the neurotrophin-mediated signal transduction in a model of visceral inflammation.

In summary, the present study has examined changes in the immunoreactivity of two transcription factors, p-CREB and c-Jun, in lumbosacral DRG cells after acute (8 or 48 hours) or chronic (10 days) CYP-induced cystitis. These data indicate that CYP treatment differentially affects p-CREB and c-Jun expression in bladder afferent cells in the DRG. Further studies on p-CREB expression demonstrated that CREB phosphorylation (serine 133) was triggered by C-fiber bladder afferent activation and related, at least partially, to the increased expression and phosphorylation of Trk in DRG after cystitis. Thus, these data add to a growing body of literature implying involvement of unmyelinated bladder afferents in bladder overactivity after cystitis. Specifically, these studies have demonstrated that increases in the expression of p-CREB immunoreactivity in bladder afferent cells in the DRG after cystitis are mediated by C-fiber bladder afferents.
LITERATURE CITED


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