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Artemin Immunotherapy Is Effective in Preventing and Reversing Cystitis-Induced Bladder Hyperalgesia via TRPA1 Regulation

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Abstract: Injury- or disease-induced artemin (ARTN) signaling can sensitize primary afferents and contribute to persistent pain. We demonstrate that administration of an ARTN neutralizing antibody, anti-artemin (α-ARTN), can block the development of, and reverse already established, bladder hyperalgesia associated with cyclophosphamide-induced cystitis in mice. We further demonstrate that α-ARTN therapy blocks upregulation of TRPA1, an ion channel contributing to persistent bladder pain during cyclophosphamide-induced cystitis, and decreases phospho-ERK1/2 immunoreactivity in regions of the spinal cord receiving bladder afferent input. Thus, α-ARTN is a promising novel therapeutic approach for treatment of bladder hyperalgesia that may be associated with interstitial cystitis/painful bladder syndrome, as well as cystitis associated with antitumor or immunosuppressive cyclophosphamide therapy.

***Perspective:* α-ARTN therapy effectively prevented and reversed ongoing bladder hyperalgesia in an animal model of cystitis, indicating its potential as an efficacious treatment strategy for ongoing bladder pain associated with interstitial cystitis/painful bladder syndrome.**

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Key words: *Bladder, visceral, pain, cystitis, TRPA1, artemin, growth factor.*

Pelvic/suprapubic pain is a cardinal symptom of interstitial cystitis/painful bladder syndrome (IC/PBS), a chronic condition affecting 2.7 to 6.5% of women in the United States.[3] In contrast to bacterial cystitis, IC/PBS characteristically occurs in the absence of ongoing infection but may be accompanied by varying degrees of inflammation.[21] There is no single defining etiology or pathogenesis of IC/PBS; thus, there is no consistently effective pain management strategy to improve quality of life in individuals with the disease.

Animal studies have shown that visceral injury and inflammation are accompanied by increased peripheral growth factor expression, which in turn can drive hyperalgesia.[4,12,24,32,33] Studies of individuals with IC/PBS have reported increases in nerve growth factor (NGF) and glial cell line–derived neurotrophic factor (GDNF) in urethral and bladder tissue.[23,30,31,41] We have previously shown in animal models of skin and colon inflammation that target-derived mRNA for artemin (ARTN), a member of the GDNF family, increases up to 5-fold more than the mRNAs for either NGF or GDNF.[32,35] Moreover, a single injection of ARTN into uninflamed skin increases the duration of painlike behavior in vivo and potentiates nociceptor function in vitro.[35] ARTN sensitizes nociceptive afferents, in part, through upregulation and/or augmented function of the ion channels TRPV1 and TRPA1.[32,35] Recent studies from our laboratories revealed that bladder afferent TRPA1 contributes to persistent hyperalgesia in a mouse model of cyclophosphamide (CYP)–induced cystitis.[16] Because of the previously demonstrated relationship between ARTN and TRPA1 expression, we hypothesized that treatment with an ARTN-neutralizing antibody (α-ARTN) might be effective in blocking bladder hyperalgesia.

To test our hypothesis, we used a mouse model of cystitis shown previously to elicit changes in TRPA1 expression and function[16] and quantified urinary

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bladder expression of growth factor messenger RNAs (mRNAs), bladder primary afferent TRPA1 expression and function, nociceptive behavior, and spinal phospho-ERK1/2 (pERK) immunoreactivity. A single injection of α-ARTN, at either the initiation or the conclusion of CYP treatment, effectively blocked or reversed, respectively, bladder hyperalgesia. α-ARTN prevented CYP-induced upregulation of bladder afferent TRPA1 and reduced the number of activated second-order neurons in the spinal cord that receive bladder primary afferent input. These results suggest that α-ARTN treatment could be effective for people experiencing bladder pain associated with IC/PBS.

Methods

Animals

Experiments were performed on female 8- to 12-week-old C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME) housed in the Division of Laboratory Animal Resources at the University of Pittsburgh Medical Center. Mice received food and water ad libitum. All procedures conformed to National Institutes of Health guidelines and were in accordance with those of the University of Pittsburgh Institutional Animal Care and Use Committee and the Committee for Research and Ethical Issues of the International Association for the Study of Pain.

CYP-Induced Cystitis and α-ARTN Treatment

Cystitis was induced by intraperitoneal injection of CYP (100 mg/kg; Sigma-Aldrich, St. Louis, MO) every other day for a period of 5 days (3 injections total). A control group was administered intraperitoneal sterile saline injections on the same schedule. Some mice additionally received an intraperitoneal injection of α-ARTN (10 mg/kg; R&D Systems, Minneapolis, MN) or immunoglobulin G (IgG, 10 mg/kg; R&D Systems) 30 minutes prior to the first CYP or saline injection on day 1; a final group received α-ARTN 30 minutes following the final CYP injection on day 5 (Fig 1). Mice were randomly assigned to all groups. Endpoint measurements were assessed at 1 or 7 days following the final CYP injection.

Real-Time Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

RNA isolation and real-time RT-PCR were performed as previously described.[12,35] Extracted RNA (5 μg) was treated with DNase (Invitrogen, Carlsbad, CA) to remove genomic DNA; then, 1 μg was reverse transcribed using Superscript II (Invitrogen). SYBR Green PCR amplification was performed using a 7000 real-time thermal cycler (Applied Biosystems, Waltham, MA). Each sample was run in duplicate, and the threshold cycle (Ct) values were recorded as a measure of initial template concentration of NGF, GDNF, and ARTN and were normalized to Ct values for β-actin. A relative change in mRNA expression was calculated as a ratio of the control group mean for each gene using the Pfaffl method.[43] Primer sequences used were NGF (F-TCCAATCCTGTTGAGAGTGG, R-CAGGCTGTGTCTATGC GGAT), GDNF (F-AAGGTCACCAGATAAACAAGCGG, R-T CACAGGAGCCGCTGCAATATC), ARTN (F-CTCAGTCTCCTC AGCCCG, R-TCCACGGTCCTCCAGGTG), β-actin (F-AGAGG GAAATCGTGCGTGAC, R-CAATAGTGATGACCTGGCCGT).

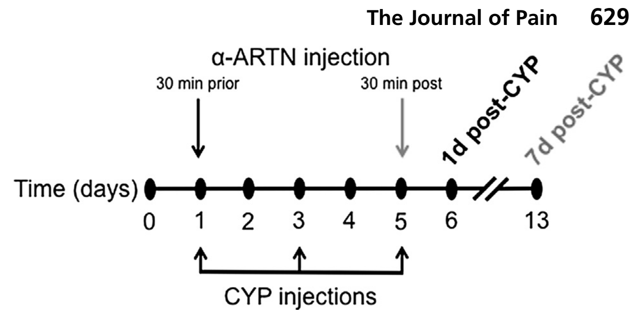

Figure 1. The experimental time line was as follows: cystitis was induced by injection of CYP (100 mg/kg, intraperitoneal) on days 1, 3, and 5; an ARTN neutralizing antibody (α-ARTN; 10 mg/kg, intraperitoneal) was administered to one group of mice 30 minutes prior to CYP on day 1, and to another group of mice 30 minutes following CYP on day 5. Experimental endpoints were collected from these groups on days 6 and 13, respectively.

Retrograde Labeling of Bladder Afferents

Mice were anesthetized using isoflurane, and the urinary bladder was exposed via laparotomy. Three injections (12 μL total volume) of Alexa Fluor–conjugated cholera toxin-β (CTβ) (Life Technologies, Carlsbad, CA) were made into the bladder wall, and abdominal incisions were sutured. Mice were returned to their home cages and allowed to recover for at least 3 days before initiation of CYP injections.

Single-Cell RT-PCR

Mice were deeply anesthetized with isoflurane and transcardially perfused with cold Ca^{2+}/Mg^{2+}-free Hank's balanced salt solution (HBSS; Invitrogen). Bilateral L5-S1 dorsal root ganglia were dissected into cold HBSS and dissociated.[34] Cells were plated in Dulbecco's modified Eagle medium–nutrient mixture F-12 media (Invitrogen) containing 10% fetal bovine serum and antibiotics (penicillin/streptomycin, 50 U/mL). Coverslips were flooded with media 2 hours after plating and used the same day.

Individual $CT\beta^{+}$ neurons were collected with large-bore (50 μm) glass pipettes and expelled into microcentrifuge tubes containing RT mix (Invitrogen). For each preparation of isolated cells on which single-cell PCR was performed, 2 negative controls were included: 1 omitting RT and 1 using a cell-free mix as template. The first-strand cDNA from $CT\beta^{+}$ neurons was used as template in a reaction containing 1 × GoTaq reaction buffer (Promega, Fitchburg, WI), 20 μM external primers, .2 M deoxynucleoside triphosphates, and .2 μL GoTaq DNA polymerase (Promega). Each initial PCR product served as template in a subsequent reaction using a nested (internal) primer pair. Products were electrophoresed on

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