2017

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Honors College Thesis/CALS Distinguished Undergraduate Research Project Thesis

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Abstract

Interstitial cystitis is a serious chronic condition that causes bladder pain and increased voiding frequency in millions of adults in the US, most of them women. A possible biomarker that may be linked to bladder inflammation is the CXCL chemokine family, specifically CXCL9, CXCL10, and CXCL11. The goal of this project is to investigate the expression and regulation of these CXCL chemokines during acute and chronic inflammation of the urinary bladder. Wild-type C57BL/6J mice were injected with cyclophosphamide (CYP) to induce bladder inflammation. RT-PCR and ELISAs were used to determine mRNA and protein expression of CXCL9, CXCL10, and CXCL11 chemokines. During CYP-induced cystitis, the detrusor muscle exhibited more CXCL mRNA regulation in both males and females compared to the urothelium. CYP-induced cystitis significantly ($p \leq 0.05$) upregulated CXCL10, while CXCL9 and CXCL11 were significantly ($p \leq 0.05$) downregulated. CXCL chemokines were also more regulated during acute and intermediate inflammation versus chronic inflammation. Females had significantly ($p \leq 0.05$) decreased CXCL9 and CXCL11 protein levels after chronic inflammation. There were no statistical differences between CXCL chemokine protein levels in males. Future research such as immunohistochemistry to focus on tissue distribution of chemokines and use of chemokine receptor antagonist should be performed to further explore the functional role of these chemokines in male and female urinary bladders.

Introduction

Interstitial cystitis (IC), also known as Painful Bladder Syndrome (PBS), is a chronic condition that causes bladder pain, discomfort, and urinary urgency in as many as 7.9 million US adult women (1). The prevalence of IC is very disproportionate; many studies on the topic have discovered a 5:1 ratio of women to men with IC. While IC is not necessarily life-threatening, the chronic pain it causes can lead to a severely decreased quality of life equivalent to rheumatoid arthritis, another debilitating chronic condition (6). The cause of IC is uncertain since there are many different factors that could work in tandem to cause disease (9). However, it has been discovered that many IC patients have some form of defect in the urothelium, the tissue that lines the interior of the bladder (Figure 1), and it is theorized that chemicals in the urine can leak through the urothelium to cause pain and inflammation (8).

The resulting inflammation has been attributed to a variety of signaling molecules, including the CXCL chemokine family. Chemokines are a group of small cytokines that are characterized by their ability to induce chemotaxis in cells. The two groups of chemokines are homeostatic and inflammatory, though some chemokines can be both types. Homeostatic chemokines migrate leukocytes such as lymphocytes to locations in the body by using concentration gradients (12). As their name suggests, they are responsible for maintaining a homeostatic concentration of leukocytes. Inflammatory chemokines are activated during infection or other conditions where inflammation is necessitated and result in the migration of immune cells to their destination. An example of an inflammatory chemokine is the CXCL chemokine family.

CXCL chemokines are named for their C-X-C amino acid motif, as their N-terminus contains two cysteine residues separated by another amino acid designated as "X." The CXCL chemokine family plays several roles throughout the human body, but CXCL9, CXCL10, CXCL11, and their receptor CXCR3 have been found to play a significant role in inflammation including in the bladder (13). Their pro-inflammatory properties play a role in the mediation of angiostasis (4) but can also impair bladder function, leading to conditions such as IC (7). Some studies have been conducted on CXCL9, CXCL10, and CXCL11 and their general response to inflammation (11) but not on the specifics of location of activity and expression. The goal of this project is to determine the expression and regulation of these CXCL chemokines in cystitis in male and female mice, the mRNA and protein levels in the urothelium and detrusor muscle, and the effect of varying duration of bladder inflammation on CXCL regulation in urinary bladder inflammation. Learning more about the expression and regulation of CXCL chemokines in the bladder with cystitis may eventually be translated into a future treatment for human cystitis or biomarker for the disease.

Materials and Methods

The mice used in this study were wild-type (WT) C57BL/6J mice and were bred at the Larner College of Medicine at The University of Vermont. All experimental protocols involving animal use were approved by the UVM Institutional Animal Care and Use Committee (IACUC #08-085 and #14-060). Animal care was under the supervision of the UVM Office of Animal Care Management with the Association for Assessment and Accreditation of Laboratory Care and National Institutes of Health guidelines. All efforts were made to minimize the potential for animal pain, stress, or distress.

Both male and female adult (3-4 months) mice (n = 6-7) were subjected to cyclophosphamide (CYP) injections administered intraperitoneally to stimulate urinary bladder inflammation. Cyclophosphamide is metabolized by the body into acrolein, which builds up in the urinary bladder and irritates the urothelium to cause inflammation. The groups were differentiated based on whether the inflammation was acute or chronic and on the gender of the mice. The acute groups consisted of mice treated with one CYP treatment (200 mg/mL per kg mouse), whereas the chronic group received a CYP treatment (75 mg/mL per kg mouse) every three days for a total of three treatments. The control group received no CYP treatments. Previous studies have demonstrated no differences in inflammatory mediators when rodents receive saline injections compared to no treatment. The mice were deeply anesthetized with isoflurane (5%) and then euthanized via thoracotomy. Bladders were harvested from the acute inflammation group either 4 hours (acute inflammation) or 48 hours (intermediate inflammation) after CYP treatment. Bladders were harvested from the chronic inflammation group 10 days after CYP treatment. The L6 and S1 sections of the spinal cord and dorsal root ganglia were also harvested for future analysis of CXCL transcript levels. The urinary bladder and lumbosacral (L6-S1) dorsal root ganglia were quickly dissected under RNase-free conditions. For mRNA analysis, the bladder was cut open along the midline and pinned to a sylgard-coated dish and the urothelium and suburothelium were removed from the detrusor muscle with the aid of fine forceps and a dissecting microscope. In this study, the use of the term urothelium refers to the urothelium and suburothelial layers. For protein analysis, the bladders were kept whole rather than being separated into

urothelium and detrusor components due to time constraints. All tissues were snap-frozen on dry ice prior to processing.

Quantitative RT-PCR was used to determine CXCL9, CXCL10, and CXCL11 mRNA transcript levels in the urothelium and in the detrusor muscle. Total RNA was extracted using STAT-60 total RNA/mRNA isolation (Tel-Test 'B', Friendswood, TX, USA). One microgram of RNA per sample was used to synthesize complementary DNA using a mix of random hexamer and oligo dT primers with M-MLV reverse transcriptase (Promega Corp., Madison, WI, USA) in a 25-uL final reaction volume. The quantitative PCR standards for all transcripts were prepared with the amplified cDNA products ligated directly into pCR2.1 TOPO vector using the TOPO TA cloning kit (Invitrogen, Carlsbad, CA, USA). The nucleotide sequences of the inserts were verified by automated fluorescent dideoxy dye terminator sequencing (Vermont Cancer Center DNA Analysis Facility). To estimate the relative expression of the receptor transcripts, 10-fold serial dilutions of stock plasmids were prepared as quantitative standards. The range of standard concentrations was determined empirically. Complementary DNA templates, diluted 10-fold to minimize the inhibitory effects of the reverse transcription reaction components, were assayed using HotStart-IT SYBR Green qPCR Master Mix (USB, Cleveland, OH, USA) and 300 nM of each primer in a final 25-μL reaction volume. Real-time quantitative PCR was performed on an Applied Biosystems 7500 Fast real-time PCR system (Applied Biosystems, Foster City, CA, USA) using the following standard conditions: (1) serial heating at 94 °C for 2 minutes and (2) amplification over 45 cycles at 94 °C for 15 seconds and 60°C for 30 seconds. The amplified product from these amplification parameters was subjected to SYBR Green I melting analysis by

ramping the temperature of the reaction samples from 60 to 95 °C. A single DNA melting profile was observed under these dissociation assay conditions demonstrating the amplification of a single unique product free of primer dimers or other anomalous products. Oligonucleotide primer sequences were: CXCL9: upper primer (5'-GTGGAGTTCGAGGAACCCTAG-3'); lower primer (5'-ATTGGGGCTTGGGGCAAAC-3'); CXCL10: upper primer (5'-GTGGGACTCAAGGGATCCCTC-3'); lower primer (5'-CAGGATAGGCTCGCAGGGATG-3'); CXCL11: upper primer (5'-GCTCAAGGCTTCCTTATGTTCAAAC-3'); lower primer (5'-CTTTGTCGCAGCCGTTACTCG-3')

For data analyses, a standard curve was constructed by amplification of serially diluted plasmids containing the target sequence. Data were analyzed at the termination of each assay using sequence detection software (Sequence Detection Software, version 1.3.1; Applied Biosystems, Norwalk, CT, USA). In standard assays, default baseline settings were selected. The increase in SYBR Green I fluorescence intensity (ΔRn) was plotted as a function of cycle number, and the threshold cycle was determined by the software as the amplification cycle at which the ΔRn first intersects the established baseline. All data are expressed as the relative quantity of the gene of interest normalized to the relative quantity of the housekeeping gene ribosomal protein L32.

ELISAs were used to determine CXCL9, CXCL10, and CXCL11 content in the urinary bladder of mice ($n = 6–7$) with and without CYP treatment. Whole urinary bladders were homogenized separately in tissue protein extraction agent (T-PER; Roche, Indianapolis, IN), a commercially available, mild zwitterionic dialyzable detergent in

25 mM bicine, 150 mM sodium chloride (pH 7.6) containing a protease inhibitor mix (Sigma-Aldrich, St. Louis, MO; 16 μg/ml benzamidine, 2 μg/ml leupeptin, 50 μg/ml lima bean trypsin inhibitor, and 2 μg/ml pepstatin A), and aliquots were removed for protein assay. The supernatants were used for quantification. Total protein was determined with the Coomassie Plus (Bradford) Protein Assay Kit (Fisher Scientific, Pittsburgh, PA). According to the manufacturer (R&D Systems, Minneapolis, MN), the CXCL9, CXCL10, and CXCL11 DuoSets do not show cross-reactivity or interference with similar recombinant human and mouse proteins at concentrations up to 50 ng/ml. The standards provided with these systems generated linear standard curves ($R^2 = 0.985–0.998$, $p \leq 0.001$). Absorbance values of standards and samples were corrected by subtraction of the background value (absorbance due to nonspecific binding). No samples fell below the detection limits of the assays, and samples were not diluted before assay. Curve fitting of standards and evaluation of CXCL9, CXCL10, and CXCL11 content of samples were performed with a least-squares fit.

All values were expressed as means ± S.E.M. Comparisons among experimental groups were made using one-way analysis of variance (ANOVA). Animals, processed and analyzed on the same day, were tested as a block in the ANOVA. Outliers determined by using a Q test were removed from analysis. When *F* ratios exceeded the critical value ($p \leq 0.05$), Tukey's multiple comparison test was used to compare experimental means among groups.

Results

Male mice exhibited no significant changes in CXCL9 or CXCL10 mRNA levels in the urothelium when experimental groups were compared to the control (Figure 2).

However, there was a significant ($p = 0.0035$ for acute, $p = 0.0013$ for intermediate, $p < 0.0001$) downregulation of CXCL11 in the male urothelium in all CYP treatment groups. Female mice exhibited significant upregulation in CXCL10 in the urothelium for the acute (4 hour) and intermediate (48 hour) groups ($p = 0.0003$ for acute, $p = 0.0266$ for intermediate) and CXCL11 in the urothelium for the acute group ($p = 0.0009$) (Figure 3). CXCL9 was significantly downregulated in male detrusor muscle for all inflammatory groups ($p < 0.0001$ for acute, $p = 0.0002$ for intermediate, $p < 0.0001$ for chronic) but was not significantly regulated in females. CXCL10 was significantly upregulated in male detrusor muscle in the acute and intermediate groups ($p = 0.0099$ for acute, $p = 0.0472$ for intermediate) and significantly upregulated in female detrusor muscle in the acute and chronic groups ($p = 0.0006$ for acute, $p = 0.0259$ for chronic). CXCL11 was significantly downregulated in male detrusor muscle in the acute and chronic groups ($p = 0.0032$ for acute, $p = 0.0032$ for chronic) and in the female detrusor intermediate group ($p = 0.0092$ for intermediate).

Urinary bladders of female mice exhibited significantly decreased protein levels of CXCL9 and CXCL11 in the chronic inflammation group when compared to the control ($p = 0.0242$ for CXCL9 chronic, $p = 0.0498$ for CXCL11 chronic) (Figure 4). No significant differences were found in the protein levels of CXCL9, CXCL10, and CXCL11 in male mice with CYP-induced cystitis.

Discussion

This study produced several interesting findings. First, CXCL10 mRNA was found to be upregulated during urinary bladder inflammation, whereas CXCL9 and CXCL10 mRNA were almost always downregulated instead. The detrusor muscle was

also found to be more susceptible to CXCL mRNA regulation than the urothelium with CYP-induced cystitis. Acute and intermediate bladder inflammation led to more CXCL mRNA regulation than chronic inflammation. Similar to the downregulation of CXCL9 and CXCL11 mRNA, CXCL9 and CXCL11 protein levels were also decreased during chronic inflammation in females. No other changes in CXCL protein levels were demonstrated in either males or females with CYP-induced cystitis.

The upregulation of CXCL11 mRNA in the female urothelium was opposite to that of the downregulation of CXCL11 mRNA in the male urothelium. In addition, CXCL10 mRNA was significantly upregulated in all groups when compared to the control. However, CXCL9 and CXCL11 mRNA were almost always downregulated except for CXCL11 in the female urothelium. This suggests that the inflammatory pathway in males and females may be regulated differently with regards to CXCL mRNA and that CXCL10 may be mechanistically different from CXCL9 and CXCL11 in the pro-inflammatory pathway in the bladder. CXCL9, CXCL10, and CXCL11 all share common transcription factors such as NF-κB, STAT1, and IFN regulatory factor (IRF)-1 (2, 13), though they each have specific factors as well. However, it is unknown how CXCL9, CXCL10, and CXCL11 are specifically regulated in the bladder with regards to transcription. Future research should be conducted to study the pathway in the bladder.

With CYP-induced cystitis, the detrusor muscle was more susceptible to regulation of CXCL mRNA than the urothelium in both males and females, indicating significant regulation of CXCL mRNA does occur in the detrusor muscle. The urothelium was expected to exhibit regulation of CXCL chemokines because CYP causes inflammation in the urothelium characterized by urothelial erosion and infiltration of

inflammatory cells. However, inflammatory cell infiltrates are observed throughout the detrusor muscle as well suggesting that it may be more vulnerable to CYP and subsequent inflammation.

Acute and intermediate inflammation led to more regulation of CXCL mRNA compared to chronic inflammation. This may mean CXCL mRNA is more regulated during the early stages of inflammation. Inflammation is a complex and multi-stage process so certain mRNA may be activated and deactivated earlier than other transcripts, and CXCL may be part of the early response rather than later.

Most CXCL protein levels were not significantly different when compared to the control, which conflicts with the mRNA expression levels. However, CXCL9 and CXCL11 protein levels were significantly decreased in the female chronic inflammation group. Again, this means CXCL9 and CXCL11 could play a role as early response proteins and then be downregulated as inflammation progresses. No other CYP treatment in males or females exhibited significant differences.

Based on the mRNA expression levels, one might expect some statistical significance to be present in the ELISA results. Although whole bladders were used and no bladders separated into urothelium and detrusor, the detrusor muscle accounts for most of the bladder mass, so CXCL levels should be skewed towards the level of activity in the detrusor muscle. The lack of variance may indicate that the CXCL mRNA transcripts are unstable or post-translationally processed in some way that leads to decreased protein expression. CXCL chemokines might also not remain in the bladder due to limited storage during inflammation. Chemokine half-lives have been found to be around 2 hours in blood plasma (3), but it is unknown how stable they are in urinary

bladder tissue. Further research should be conducted to determine exactly how CXCL mRNA or protein is processed during inflammation.

Interestingly, when comparing the male and female ELISA results, it appears that CXCL chemokine levels are higher in females compared to males, especially CXCL10. However, the male and female data cannot be statistically compared to each other because samples were not run on the same ELISA plates. This is another potential experiment to perform in the future.

As mentioned previously, the ELISAs were performed using whole bladders rather than the urothelium and detrusor muscle separately due to low protein yield and the difficulty in analyzing low protein content in the urothelium and detrusor muscle. Regardless, in the future, ELISAs should be repeated with split bladders to accurately compare to the mRNA expression results.

Immunohistochemistry and cystometry with chemokine receptor antagonist treatment were planned after RT-PCR and ELISAs. Unfortunately, there was not enough time to pursue those morphological and functional studies. These procedures should be performed in the future to further explore the role of these CXCLs in the bladder function during urinary bladder inflammation. For instance, since more regulation is present in the detrusor muscle, immunohistochemistry should help to determine the level of expression of each CXCL chemokine in detrusor muscle compared to urothelial cells. Cystometry would also help to determine if administering a chemokine receptor antagonist to block the CXCL receptor, CXCR3, would improve voiding outcomes (e.g., reduce voiding frequency) in mice with CYP-induced cystitis. Both

immunohistochemistry and chemokine receptor antagonist treatment will give us a better picture of the expression and possible function of CXCL chemokines in the bladder.

Additional studies should be conducted to better understand the inflammation pathway in the bladder activated by CYP treatment. This means learning more about the protein-protein interactions of CXCL9, CXCL10, CXCL10, and CXCR3, the activation/deactivation of specific proteins, the downstream signaling molecules, and the aforementioned processing of CXCL transcript and proteins.

A limitation in this study may be the male CXCL ELISA results. There were no statistical differences between the control and experimental groups for CXCL9, CXCL10, and CXCL11. The results yielded only a slight, non-significant regression as inflammation progressed. Although the bladders appeared to be inflamed based on past gross appearances during bladder extractions, there is still the possibility of some procedural error or defective cyclophosphamide. The ELISAs should be repeated for the males to replicate the results.

One possible alternative is the usage of a different normalizer other than or in addition to ribosomal protein L32. We were only able to test two normalizers, 18S and L32. Both genes yielded slight regulation despite being housekeeping genes, so testing other normalizers or using a combination of housekeeping genes could result in better correction of mRNA variance and more accurate statistical significance.

The results of this experiment were unexpected, as most regulation of the CXCL chemokines during inflammation occurred in the detrusor muscle rather than the urothelium. It was also interesting that only CXCL10 was upregulated, and CXCL9 and CXCL11 were downregulated. Further procedures and research are necessitated to delve

further into the role of these CXCL chemokines in urinary bladder inflammation, such as using split bladders for ELISAs, immunohistochemistry to confirm location and activity of CXCL chemokines in bladder cells, and antagonist treatment to determine potential benefits of CXCR3 blockade.

Figures

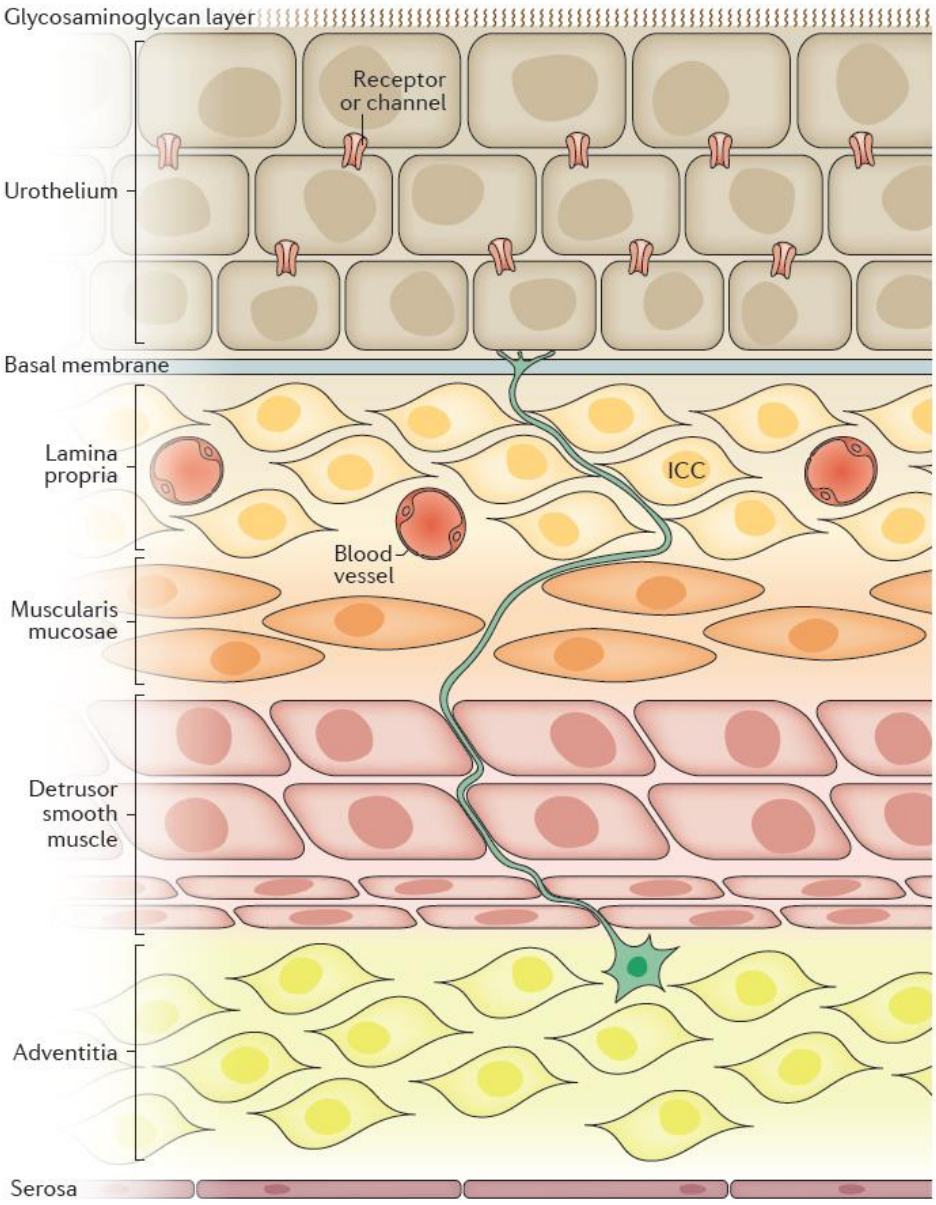

Figure 1. Anatomical components of the urinary bladder wall (5). Many receptors (including CXCR3, the receptor for CXCL chemokines) and ion channels (transient receptor potential channels) are expressed by the anatomical components of the urinary bladder wall including the urothelium, bladder sensory nerves, interstitial cells of Cajal (ICC), and detrusor smooth muscle. The tissues of interest in this study are the urothelium and detrusor muscle.

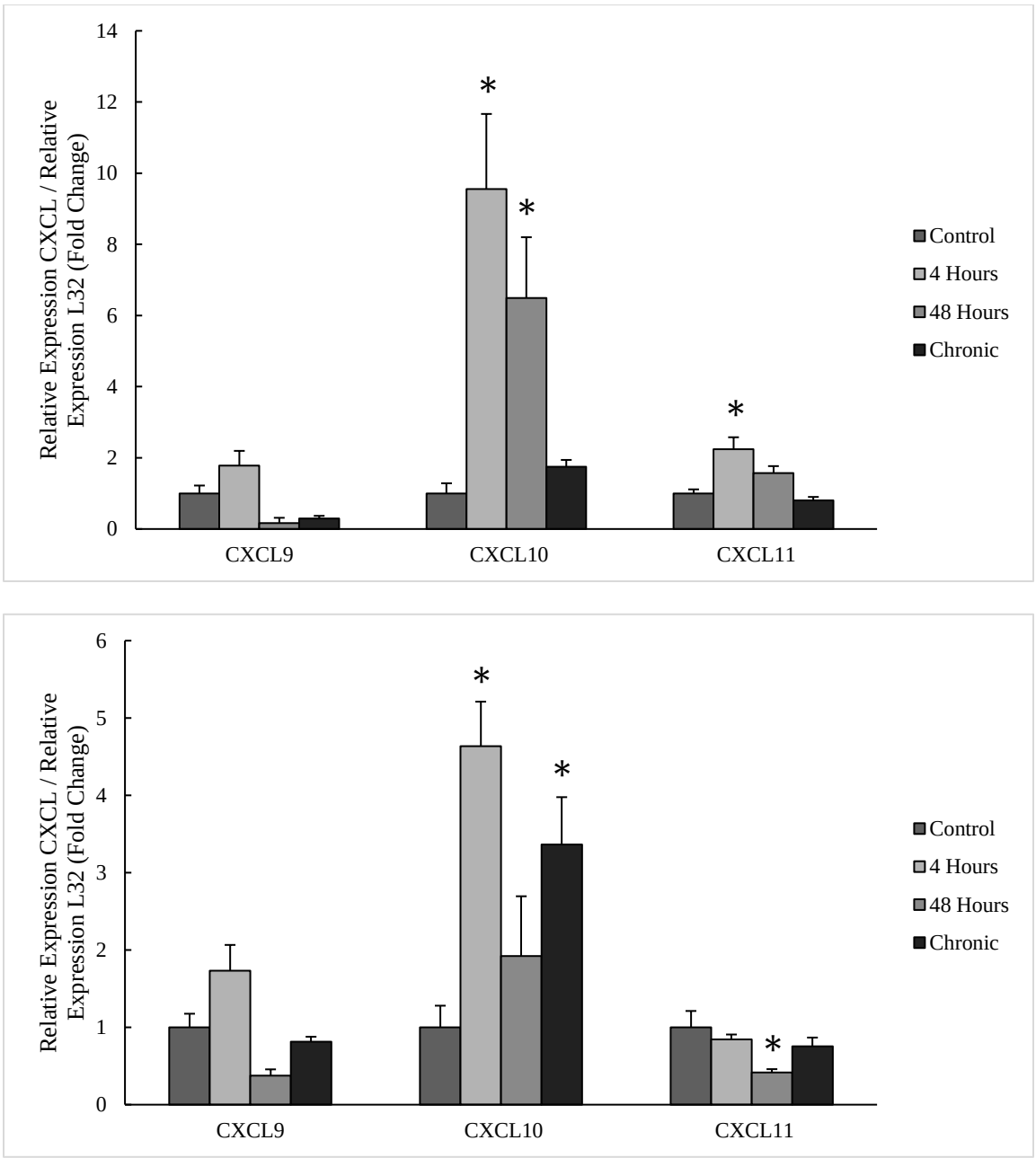

Figure 2. Mean CXCL mRNA levels (fold change) of female mouse urothelium (top) and detrusor muscle (bottom) with CYP treatment (4 hours, 48 hours, chronic). Groups that are statistically significant when compared to the control are marked with an asterisk (Tukey's multiple comparison test, $p \leq 0.05$). n = 6 for all groups.

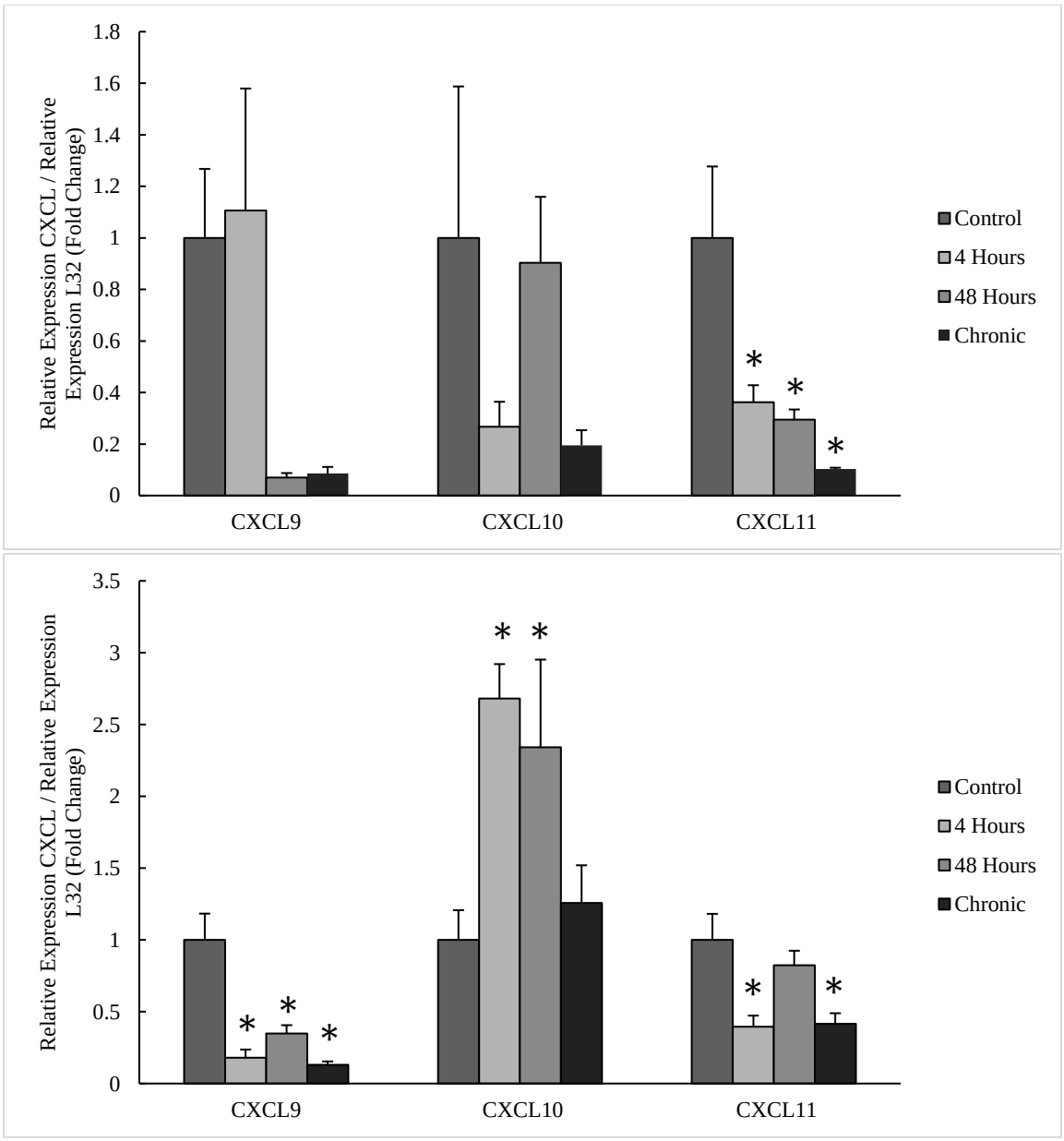

Figure 3. Mean CXCL mRNA levels (fold change) of male mouse urothelium (top) and detrusor muscle (bottom) with CYP treatment (4 hours, 48 hours, chronic). Groups that are statistically significant when compared to the control are marked with an asterisk (Tukey's multiple comparison test, $p \leq 0.05$). n = 6 for control, 4 hour, and 48 hour CYP treatment groups. n = 7 for chronic CYP treatment group.

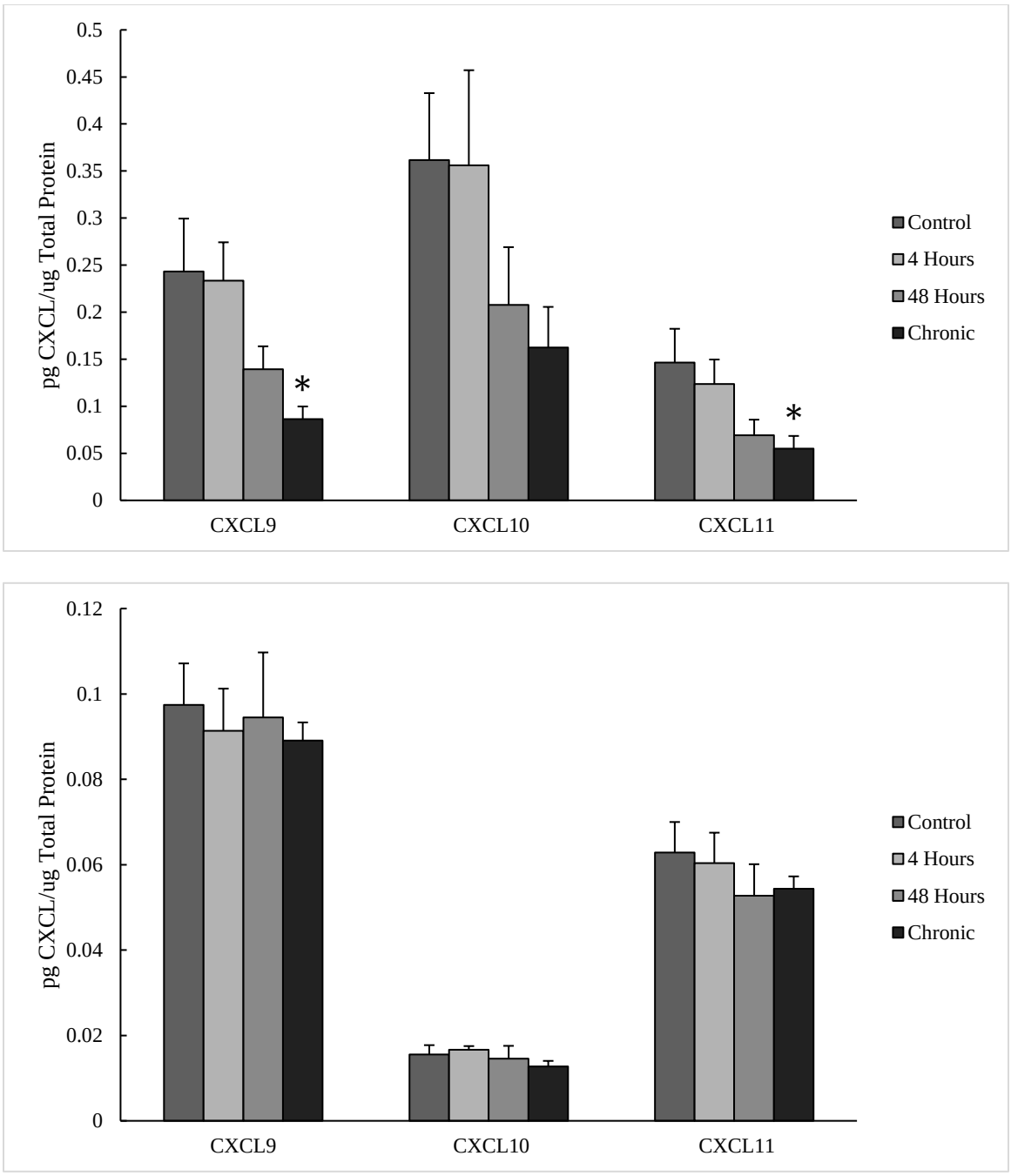

Figure 4. Mean CXCL protein level per total protein of female (top) and male (bottom) mouse bladders with CYP treatment (4 hours, 48 hours, chronic). Groups that are statistically significant when compared to the control are marked with an asterisk (Tukey's multiple comparison test, $p \leq 0.05$). n = 6 for all groups.

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