Immunopathology and Infectious Disease

Role of Nicotinic and Estrogen Signaling during Experimental Acute and Chronic Bladder Inflammation

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Inflammation is a physiological process that characterizes many bladder diseases. We hypothesized that nicotinic and estrogen signaling could down-regulate bladder inflammation. Cyclophosphamide was used to induce acute and chronic bladder inflammation. Changes in bladder inflammation were measured histologically and by inflammatory gene expression. Antagonizing nicotinic signaling with mecamylamine further aggravated acute and chronic inflammatory changes resulting from cyclophosphamide treatment. Estrogen and nicotinic signaling independently attenuated acute bladder inflammation by decreasing neutrophil recruitment and down-regulating elevated lipocalin-2 and cathepsin D expression. However, the combined signaling by the estrogen and nicotinic pathways, as measured by macrophage infiltration and up-regulation of interleukin-6 expression in the bladder, synergistically reduced chronic bladder inflammation. The elevated expression of p65 nuclear localization in bladders treated with cyclophosphamide or cyclophosphamide with mecamylamine suggested nuclear factor- κ B activation in the chronic inflammatory process. The complementary treatment of 17 β -estradiol and the nicotinic agonist anabasine resulted in the translocation of p65 to the cytoplasm, again greater than either alone. Activation of nuclear factor- κ B can result in macrophage activation and/or elevation in epithelial proliferation. These data suggest that 17 β -estradiol and anabasine reduce chronic bladder inflammation through reduction of nuclear translocation of p65 to suppress cytokine expression. (*Am J Pathol* 2008, 172:59–67; DOI: 10.2353/ajpath.2008.070529)

significant burden on health care expenditures and utilization in the United States resulting in annual costs estimated at \$750 million US dollars.¹ Although the pathophysiology of these diseases is currently unknown, they are thought to be linked to an occult inflammatory process that mediates the clinical symptoms of disease. Inflammation is a physiological process that characterizes many bladder diseases such as interstitial cystitis that disproportionately affects women. The etiology of interstitial cystitis is not known; however, afferent innervation on the bladder is thought to be involved. The nicotinic acetylcholine receptor (nAChR), associated with afferent neuronal signaling, is a gated ion channel responsible for signaling between cells at the neuron-neuron and nerve-muscle synapse. Interestingly, the activation of the nicotinic pathway is reported to down-regulate the production of inflammatory cytokines in mouse pancreatitis,² acute lung injury,³ and experimental sepsis.⁴ Systemic administration of 17 β -estradiol has been shown to differentially regulate the nicotinic receptor signaling.⁵ The concentration of nAChR's cell surface expression is elevated after estrogen treatment.⁵ Yet 17 β -estradiol and other steroids such as progesterone are reported to inhibit nAChR calcium signaling in human neuronal and muscle cell lines.⁶ Thus it is difficult to predict a combinatorial role of nicotinic and estrogen signaling in regulation of inflammation in the bladder.

In this study we used cyclophosphamide to induce bladder inflammation in female mice. Cyclophosphamide is an alkylating agent with immunosuppressive and antineoplastic activities. Cyclophosphamide is used to treat several solid tumors, notably breast cancer and B-cell malignancies as well as autoimmune diseases such as lupus, rheumatoid arthritis, and Wegener's granulomatosis.^{7,8} The toxicity of cyclophosphamide by a hepatic metabolite, acrolein, accumulates in the bladder to cause inflammation.^{8–10} Based on the hypothesis that nicotinic and estrogen signaling can independently suppress bladder inflammation, the objective of this study was to

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Inflammatory diseases of the urinary bladder (ie, interstitial cystitis, painful bladder syndrome, and others) have a

determine the possibility of cooperation of estrogen and the nicotinic signaling on anti-inflammatory pathways to regulate host responses during cyclophosphamide-induced bladder inflammation in mice. Here, we show that the combined estrogen and nicotinic signaling resulted in decreased acute and chronic cyclophosphamide-induced bladder inflammation. The clinical implications of this study impact bladder inflammatory diseases as well as bladder inflammation resulting from side effects of cyclophosphamide treatment.

Materials and Methods

Animals and Experimental Groups

Female C57/B6 mice, 7 to 12 weeks old, were housed and maintained in a pathogen-free environment at the Vanderbilt University Medical Center animal facility under the Institutional Animal Care and Use Committee regulations. Animals received food and water *ad libitum* with a 12-hour light cycle. In this study, mice were ovariectomized at day 0. Two weeks after ovariectomy, mice were treated with daily subcutaneous injections of 17β -estradiol ($1\ \mu\text{g}$ as a suspension in vegetable oil) throughout the study (see Figure 1). Acute bladder inflammation was induced by one intraperitoneal injection of cyclophosphamide ($200\ \text{mg/kg}$). Chronic bladder inflammation was induced with two doses of intraperitoneal injections of cyclophosphamide ($150\ \text{mg/kg}$). The first dose was given at day 1, the second dose was given at day 4, and bladders were collected at day 5. In both models, inflammation was induced after 1 week of daily doses of 17β -estradiol, and bladder inflammation was preceded by one pretreatment with mecamylamine (nicotinic receptor antagonist, $1\ \text{mg/kg}$) or anabasine (nicotinic receptor agonist, $4\ \text{mg/kg}$). Control mice for mecamylamine, anabasine, and cyclophosphamide received saline, and 17β -estradiol control mice received vegetable oil. Each experimental group consisted of four mice. Tissues were isolated 18 hours after the last cyclophosphamide injection.

Tissue Collection and Histological Examination

Bladders were removed and split longitudinally. One section of the bladder was fixed in 4% paraformaldehyde, and the second section was used for RNA isolation. To assess inflammatory changes in the bladder, bladder tissue was embedded in paraffin, and $5\text{-}\mu\text{m}$ sections were cut and stained with hematoxylin and eosin (H&E). Slides were examined using an Olympus BX41TF microscope (Olympus Corporation, Tokyo, Japan) and photographed using an Olympus DP70 microscope digital camera (Olympus Corporation). Histological examination of the bladder was used to determine the severity of inflammation. Bladder inflammation was assessed by a six-point scale based on the presence and degree of inflammatory infiltrate (lymphocytes, neutrophils) in the lamina propria and muscularis propria, the presence and extent of edema and hemorrhage, and the presence of surface epithelial changes, including erosion and ulcer-

ation. Grade 0 was assigned to those specimens that appeared morphologically unremarkable with no or very minimal inflammation or epithelial changes. Grade 1 represented a minimal inflammatory infiltrate composed of occasional neutrophils or lymphocytes within the lamina propria, in the absence of inflammation in the muscularis propria or significant edema, hemorrhage, or urothelial changes. Grade 2 was assigned to specimens with a minimal to mild inflammatory infiltrate within the lamina propria with scattered neutrophils or lymphocytes accompanied by mild edema or hemorrhage, but in the absence of inflammation in the muscularis propria or significant urothelial changes. Grade 3 represented the presence of a mild or mild to moderate inflammatory infiltrate in the lamina propria and focal extension of the inflammation into the muscularis propria. Grade 4 represented moderate inflammation with scattered to frequent neutrophils and lymphocytes in both the lamina propria and muscularis propria. Grade 5 was assigned to specimens with severe inflammation in the lamina propria and muscularis propria in association with other significant findings such as urothelial ulceration, severe edema, hemorrhage, and fibrin deposition. This grading scheme was determined blindly in each specimen by a pathologist.

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

RNA was isolated from bladder tissue using the RNeasy Mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The resultant RNA was spectrophotometrically qualified and quantified at 260 nm and 280 nm on a Bio-Rad SmartSpec 3000 spectrophotometer (Bio-Rad Laboratories, Inc., Hercules, CA). RNA ($1\ \mu\text{g}$) was reversed-transcribed using the iScript cDNA synthesis kit (Bio-Rad) and following the manufacturer's instructions. Thermal cycling was performed using an iCycler (Bio-Rad). The real-time PCR was performed by using SYBR supermix (Bio-Rad) in a total volume of $25\ \mu\text{l}$, at 95°C for 15 seconds and 60°C for 1 minute. The target amplicon was detected between 20 to 28 cycles for all primers. PCR efficiency was examined by serially diluting the template cDNA and the melting curve data were collected to check the PCR specificity. An internal loading control of GAPDH expression was used for all genes of interest. Results were quantified using the relative standard curve method. No PCR product was detected in control samples in which reverse transcriptase was omitted. The primers used were as follows: glyceraldehyde phosphodehydrogenase (GAPDH) antisense, 5'-AGTGGGAGTTGCTGTTGAAGTC-3', and sense, 5'-CGTGCCGCCTGGAGAAAC-3'; lipocalin-2 (Lcn2) antisense, 5'-CGTCCTTGAGGCCCAGAGA-3', and sense, 5'-CCCCTGAAGGAACGTT-3'; interleukin 6 (IL-6) antisense, 5'-GCATAACGCACTAGGTTTGCCGAG-3', and sense, 5'-AGCTGGAGTCACAGAAGGAGTGGC-3'; and cathepsin D antisense, 5'-GACAGCTCCCCGTGGTAGT-A-3', and sense, 5'-GGCATGGGCTACCCTTTTAT-3'.

Figure 1. Acute and chronic models used to induce inflammation in mice. In this study, ovariectomized mice were treated with daily injections of 17 β -estradiol (E₂) 2 weeks after surgery until the end of the study. Acute bladder inflammation was induced by one injection of cyclophosphamide (Cyp). Chronic bladder inflammation was induced with two doses of cyclophosphamide. In both models, inflammation was induced after 1 week of daily doses of 17 β -estradiol, and bladder inflammation was preceded by one pretreatment with mecamlamine (Mec) or anabasine (Ana). Tissues were isolated 18 hours after the last cyclophosphamide injection.

Immunohistochemistry

For immunohistochemical analysis, bladder sections were dewaxed in two changes of xylene and rehydrated in a series of descending concentrations of alcohol. Bladder antigens were retrieved by incubation in 10 mmol/L citrate buffer (pH 6.0) for nuclear factor (NF)- κ B p65, F4/80, and Ki-67. The primary antibodies against NF- κ B p65 (1:100 dilution; Santa Cruz Biotechnology, Santa Cruz, CA), F4/80 (1:50 dilution; Serotec, Raleigh, NC), and Ki-67 (1:1000 dilution; Upstate USA Inc., Charlottesville, VA) were incubated overnight in a humid chamber at 4°C. All immunohistochemistry was detected by DAKO Envision+ system peroxidase kit (DAKO, Carpinteria, CA) according to the manufacturer's instructions.

Statistical Analysis

Statistical analysis was performed using one-way analysis of variance test, and $P < 0.05$ was considered statistically significant. Tukey's multiple comparison test was used to compare means. Graphs were generated using GraphPad Prism v.4 (GraphPad Software, San Diego, CA).

Results

Acute Bladder Inflammation Is Attenuated by Nicotinic and Estrogen Signaling

Nicotinic and estrogen signaling independently mediate inflammatory responses. To determine whether the nicotinic and estrogen pathway had a role in acute bladder inflammation, we used a cyclophosphamide model to induce inflammation in ovariectomized C57/BL6 female mice (Figure 1). In this study the mice were ovariectomized at the beginning of the study to have similar levels of estrogen among all treatments. Control mice (ovariectomized mice treated with oil and saline) had bladders showing normal histology with normal epithelium and lamina propria (Figure 2, A and B). Treating female mice with cyclophosphamide induced histological inflama-

Figure 2. Representative photomicrographs showing the histological changes of the acute bladder inflammation. Mecamlamine enhanced the severity of bladder inflammation whereas estrogen reduced the severity of inflammation in the bladder. **A–H:** H&E sections showing normal bladder histology with normal epithelium and lamina propria in control ovariectomized mice (**A** and **B**); bladder inflammation with a predominance of PMN infiltrates induced with cyclophosphamide (**C** and **D**); increased severity of bladder inflammation showing loss of epithelium, ulceration, PMN infiltrates, edema, and infiltration of inflammatory cells into the muscle when mice were treated with cyclophosphamide and mecamlamine (**E** and **F**); bladder inflammation in which histological inflammatory levels were restored to levels lower than cyclophosphamide alone with treatment of 17 β -estradiol in addition to cyclophosphamide and mecamlamine (**G** and **H**). **I:** Acute effects of cyclophosphamide evaluated with a six-point histological score of bladder inflammation based on the infiltration of polymorphonuclear neutrophils, lymphocytes, edema, and loss of epithelium. Results shown are the mean $\pm$ SE ($n = 4$ in each group). * $P < 0.05$ when compared with control group and with estrogen plus cyclophosphamide plus mecamlamine group. **Circles** in **D**, **F**, and **H** represent presence of neutrophils. Scale bars: 20 μ m (**A**, **C**, **E**, **G**); 5 μ m (**B**, **D**, **F**, **H**). Original magnifications: $\times 100$ (**A**, **C**, **E**, **G**); $\times 400$ (**B**, **D**, **F**, **H**).

tory changes in the bladder after 18 hours (Figure 2, C and D). As expected, a predominance of polymorphonuclear infiltrates (PMNs) was observed in the bladder during acute inflammation. The combination of cyclophosphamide and mecamlamine resulted in histologically increased inflammation when compared with mice that were treated with cyclophosphamide alone. Treatment with mecamlamine and cyclophosphamide consistently resulted in loss of epithelium, presence of PMNs, edema, and infiltration of PMNs into the muscle (Figure 2, E and F). Incidentally, at the extreme, one of four mice treated with mecamlamine and cyclophosphamide resulted in ulceration of the bladder. However, the additional treatment with 17 β -estradiol restored histological inflammatory levels below that of treatment with cyclophosphamide alone and significantly reduced inflammation when compared with mice treated with the combination of mecamlamine and cyclophosphamide ($P < 0.05$, Figure 2, G and H). We used a superphysiological dose of 17 β -estradiol (1 μ g/day) to observe maximal changes on bladder inflammation.¹¹ The extent of neutrophil infiltration in the lamina propria and detrusor muscle correlated with the histological scores given for the individual treatments (Figure 2, B, D, F, and H). The median and mean of the data provided similar results (data are shown as mean $\pm$ SE). All of the treatment permutations were summarized based on a six-point histological score of bladder inflammation, in which anabasine, mecamlamine, or 17 β -estradiol treatments alone did not result in histological changes statistically different from control (Figure 2I). Similarly, treatments with 17 β -estradiol in combination with mecamlamine and 17 β -estradiol in combination with anabasine did not produce levels of inflammation that were statistically different from control mice. Compared to cyclophosphamide treatment alone, treatment combinations of cyclophosphamide with anabasine or with both anabasine and 17 β -estradiol did not induce any histological changes. In contrast, acute histological changes in bladder inflammation, resulting from cyclophosphamide treatment, could be further aggravated by antagonizing nicotinic signaling and attenuated by estrogen signaling.

The molecular basis for the histological changes observed was determined by the assessment of lipocalin-2, cathepsin D, and IL-6 mRNA expression. Lipocalin-2 has been described as a marker and potential positive modulator of acute inflammation.¹² Cathepsin D is an ubiquitous enzyme that is present in neutrophils.¹³ IL-6 is associated with chronic inflammatory processes and is involved in the switch from neutrophils to macrophage chemoattraction.¹⁴ The differences of lipocalin-2 mRNA levels of mice treated with mecamlamine were not statistically significant compared to control (Figure 3A). However, when mice were treated with cyclophosphamide, the expression of lipocalin-2 increased ninefold when compared with control. Antagonizing the nicotinic pathway with mecamlamine in addition to cyclophosphamide caused an 11-fold increase in lipocalin-2 expression over mice treated with cyclophosphamide alone ($P < 0.001$). However, when mice, treated with mecamlamine and cyclophosphamide, were also

Figure 3. Quantitative RT-PCR analysis of inflammation markers. Mice were treated with estrogen, mecamlamine (nicotinic receptor antagonist), cyclophosphamide, and/or anabasine (nicotinic receptor agonist) to look at the effect of estrogen and nicotinic signaling during acute bladder inflammation. Mecamlamine increased the expression of lipocalin-2, cathepsin D, and IL-6 whereas treatment with estrogen and/or anabasine down-regulates their expression. mRNA levels of lipocalin-2, * $P < 0.001$ when compared with all treatments (A); mRNA levels of cathepsin D, * $P < 0.001$ when compared with all treatments (B); mRNA levels of IL-6, * $P < 0.001$ when compared with control and mecamlamine treatments (C).

treated with 17 β -estradiol, the expression of lipocalin-2 decreased 40-fold ($P < 0.001$). In addition, combining cyclophosphamide treatment with anabasine, 17 β -estradiol, or both 17 β -estradiol and anabasine resulted in a 2.5-fold decrease in lipocalin-2 expression when compared with mice treated with cyclophosphamide alone. However, combinatorial treatments of 17 β -estradiol with anabasine or mecamlamine were not statistically different from control mice.

Cathepsin D expression in control mice did not differ significantly from individual treatments with mecamlamine or cyclophosphamide (Figure 3B). However,

antagonizing the nicotinic pathway caused a fivefold increase in cathepsin D expression compared to mice treated with cyclophosphamide alone ($P < 0.001$). The elevated cathepsin D expression by the combined treatment with mecamylamine and cyclophosphamide was reduced 12-fold when 17 β -estradiol was added ($P < 0.001$). Also, treating mice with cyclophosphamide in combination to the nicotinic agonist anabasine caused a 2.5-fold reduction in cathepsin D expression when compared with mice treated with cyclophosphamide alone. Combinatorial treatments with 17 β -estradiol, with cyclophosphamide, mecamylamine, anabasine, or cyclophosphamide and anabasine were not statistically different from control mice.

IL-6 expression among treatments followed a similar trend as observed with lipocalin-2 and cathepsin D. IL-6 mRNA expression was significantly elevated in bladders of mice given cyclophosphamide alone or in combination with mecamylamine compared to control ($P < 0.001$, Figure 3C). Both anabasine and 17 β -estradiol reduced the elevated levels of IL-6 resulting from cyclophosphamide and mecamylamine approximately twofold. The combination of anabasine and 17 β -estradiol reduced the cyclophosphamide-mediated IL-6 expression nearly fourfold.

Chronic Bladder Inflammation Is Attenuated by Nicotinic and Estrogen Signaling

To determine whether the nicotinic and estrogen signaling pathways had a role in chronic bladder inflammation, we used the cyclophosphamide-induced inflammation model for 5 days.¹⁵ Histologically a pattern of inflammation similar to the acute inflammation model was identified. Control mice showed normal bladder histology (Figure 4, A and B). Chronic cyclophosphamide treatment in mice resulted in a significant increase in recruitment of inflammatory cells (Figure 4, C and D). However, when the nicotinic pathway was antagonized bladder inflammation was dramatically enhanced (Figure 4, E and F). Bladder sections of mice treated with mecamylamine and cyclophosphamide resulted in denudation of the epithelium, presence of more inflammatory cells, hemorrhage, and inflammatory cells in the muscle. However, additional treatment with 17 β -estradiol significantly decreased ($P < 0.05$) inflammation when compared with mice treated with the combination of mecamylamine and cyclophosphamide (Figure 4, G and H). Histologically, additional treatment with anabasine or 17 β -estradiol had little effect on cyclophosphamide-induced chronic inflammation, but when anabasine and 17 β -estradiol were used in combination, it reduced inflammation 2.2-fold when compared with mice treated with cyclophosphamide (Figure 4I).

Next, the nature of the chronic inflammatory cells was identified through F4/80 staining for macrophage localization. No F4/80-positive cells were detected in the acute model of bladder inflammation (data not shown) as in the control bladders (Figure 5A). However, macrophage infiltration induced by cyclophosphamide (Figure 5B) was further enhanced by the additional antagonism

Figure 4. Representative photomicrographs showing the histological changes of the chronic bladder inflammation. Mecamylamine enhanced the severity of bladder inflammation whereas estrogen reduced the severity of inflammation in the bladder. **A–H:** H&E sections showing normal bladder histology with normal epithelium and lamina propria in control ovariectomized mice (**A** and **B**); bladder inflammation with a predominance of inflammatory cells induced with cyclophosphamide (**C** and **D**); increased severity of bladder inflammation showing loss of epithelium, edema, infiltration of inflammatory cells into the muscle when mice were treated with cyclophosphamide and mecamylamine (**E** and **F**); bladder inflammation in which histological inflammatory levels were restored to levels lower than cyclophosphamide alone with treatment of 17 β -estradiol in addition to cyclophosphamide and mecamylamine (**G** and **H**). **I:** Chronic effects of cyclophosphamide evaluated with a six-point histological score of bladder inflammation based on the infiltration of inflammatory cells, edema, and loss of epithelium. Results shown are the mean $\pm$ SE ($n = 4$ in each group). * $P < 0.05$ when compared with estrogen plus cyclophosphamide plus mecamylamine group. Scale bars: 20 μ m (**A**, **C**, **E**, **G**); 5 μ m (**B**, **D**, **F**, **H**). Original magnifications: $\times 100$ (**A**, **C**, **E**, **G**); $\times 400$ (**B**, **D**, **F**, **H**).

Figure 5. Expression of macrophages in bladders treated with saline, cyclophosphamide, mecamylamine, anabasine, and/or 17β -estradiol. Immunohistochemistry analysis using an F4/80 antibody showed the presence of macrophages during chronic bladder inflammation. **A:** Control bladders were negative for the presence of macrophages. **B:** Cross sections of mouse bladders treated with cyclophosphamide showed infiltration of macrophages. **C:** However, when the nicotinic pathway was antagonized, the expression of macrophages was higher when compared with cyclophosphamide alone. **D:** When mice treated with cyclophosphamide and mecamylamine were also treated with 17β -estradiol, the expression of macrophages was reduced. **E–G:** Treatments with 17β -estradiol and cyclophosphamide (**E**), anabasine and cyclophosphamide (**F**), and the combination of 17β -estradiol, anabasine, and cyclophosphamide (**G**) decreased macrophage recruitment resulting from cyclophosphamide treatment. Scale bar = 20 μ m. Original magnifications, $\times 100$.

of the nicotinic pathway with mecamylamine (Figure 5C). However, 17β -estradiol was able to reduce macrophage induced by the combination of cyclophosphamide and mecamylamine treatment (Figure 5D). 17β -Estradiol (Figure 5E), anabasine (Figure 5F), and the combination of 17β -estradiol and anabasine (Figure 5G) decreased macrophage recruitment resulting from cyclophosphamide treatment. Chronic histological changes in bladder inflammation, resulting from cyclophosphamide treatment, could be attenuated by estrogen signaling or estrogen combined with anabasine.

As for the acute model of inflammation, the molecular basis of the observed histological changes was assessed by measuring the expression of lipocalin-2, cathepsin D, and IL-6 mRNA. Lipocalin-2 and cathepsin D expression was not statistically different among treat-

Figure 6. Quantitative RT-PCR analysis of inflammation markers. Mice were treated with 17β -estradiol, mecamylamine (nicotinic receptor antagonist), cyclophosphamide, and/or anabasine (nicotinic receptor agonist) to examine the effect of estrogen and nicotinic signaling during chronic bladder inflammation. mRNA levels of lipocalin-2 (**A**) and cathepsin D (**B**) were not different among treatments. **C:** However, mecamylamine increased the expression of IL-6 whereas treatment with 17β -estradiol and/or anabasine down-regulates their expression. Asterisks indicate statistical significance of IL-6 mRNA expression when mecamylamine treatments were compared to control ($P < 0.001$).

ments and did not change because of chronic inflammatory stimulus (Figure 6, A and B). In contrast, chronic bladder inflammation induced with cyclophosphamide significantly increased IL-6 mRNA in the urinary bladder by 23-fold when compared with control mice ($P < 0.01$,

Figure 7. Immunohistochemistry analysis using Ki-67 antibody to determine changes in proliferation. Treatment with cyclophosphamide and/or mecamylamine induced changes in epithelial proliferation. **A:** Cross sections from control bladders showed normal urothelial proliferation. However, when inflammation was induced with either cyclophosphamide (**B**) or cyclophosphamide and mecamylamine (**C**), the proliferation in the urothelium was enhanced. **D:** The additional treatment with 17 β -estradiol restored proliferation to normal levels. Scale bar = 5 μ m. Original magnifications, $\times 400$.

Figure 6C). When the nicotinic pathway was antagonized, IL-6 expression was further elevated by threefold when compared with mice treated with cyclophosphamide alone ($P < 0.01$). The elevated expression of IL-6 by treatment of mecamylamine or cyclophosphamide was down-regulated by 17 β -estradiol or anabasine. In a combinatorial treatment of mecamylamine and cyclophosphamide, the addition of 17 β -estradiol decreased IL-6 mRNA expression fourfold ($P < 0.001$). Elevated expression of IL-6 induced by cyclophosphamide was reduced 2.5-fold by anabasine or 17 β -estradiol and anabasine together, compared with mice treated with cyclophosphamide alone.

One of the most consistent histological abnormalities in cystitis is elevation in urothelial proliferation.¹⁶ An alteration in epithelial proliferation, by Ki-67 immunohistochemistry, was found in the chronic inflammation model. Control bladders showed a relatively low level of urothelial proliferation (Figure 7A). However, when inflammation was induced with either cyclophosphamide (Figure 7B) or cyclophosphamide and mecamylamine (Figure 7C) the proliferation in the urothelium was enhanced. The additional treatment with estrogen decreased urothelial proliferation (Figure 7D).

NF- κ B is a transcription factor that has a role in mediating immune and inflammatory processes.¹⁷ More specifically it has been shown that IL-6 is up-regulated by NF- κ B.¹⁸ Thus, expression and distribution of p65 (Rel A) in bladders treated with saline, cyclophosphamide, mecamylamine, anabasine, and/or 17 β -estradiol was examined by immunohistochemistry to determine NF- κ B activation. Bladders of control mice had diffuse p65 cellular distribution that was predominantly in the cytoplasm (Figure 8A). Not surprisingly, we observed active nuclear p65 expression in bladders with chronic cyclophosphamide-induced inflammation, by cyclophosphamide or cyclophosphamide with mecamylamine treatments (Figure

Figure 8. Expression and distribution of p65 in bladders treated with saline, cyclophosphamide, mecamylamine, anabasine, and/or 17 β -estradiol to determine NF- κ B activation. Immunohistochemistry analysis of p65 suggested activation of NF- κ B during chronic bladder inflammation. **A:** The expression of p65 in normal bladder was low, but when present its distribution was predominantly in the cytoplasm. However, the expression of p65 in bladders treated with cyclophosphamide (**B**) or cyclophosphamide with mecamylamine (**C**) was higher, and the distribution of p65 in the bladders that showed more inflammation was located predominantly in the nucleus, suggesting NF- κ B activation. Treatments of 17 β -estradiol with mecamylamine and cyclophosphamide (**D**), 17 β -estradiol and cyclophosphamide (**E**), and anabasine with cyclophosphamide (**F**) changed the distribution of p65 becoming more intense in the cytoplasm and less in the nucleus, suggesting less NF- κ B activation. **G:** The combined treatment of 17 β -estradiol, anabasine, with cyclophosphamide resulted in cytoplasmic expression of p65. Scale bars: 5 μ m (**A–G**); 2.5 μ m (**insets**). Original magnifications $\times 400$ (**A–G**); $\times 800$ (**insets**).

8, B and C). The additional treatment with estrogen (Figure 8, D and E) or anabasine (Figure 8F) resulted in the translocation of p65 to the cytoplasm, suggesting decreased NF- κ B activity. In addition, there was an observed synergy between estrogen and anabasine (Figure 8G) for the translocation of p65 to the cytoplasm, suggesting decreased NF- κ B activity.

In the chronic model we found that treatment with a nicotinic antagonist increased inflammation, as observed histologically, as well as the infiltration of macrophages. Inhibition of nicotinic signaling also increased the expression of IL-6 and caused changes in proliferation and translocation of NF- κ B into the nucleus. The cooperation between 17 β -estradiol and anabasine caused translocation of p65 to the cytoplasm and concomitant decrease in

IL-6 expression to contribute to the observed histological resolution of bladder inflammation.

Discussion

The nicotinic and estrogen signaling pathways have been implicated as important anti-inflammatory pathways. The results from this study show for the first time that estrogen signaling and the nicotinic pathway are involved in the down-regulation of bladder inflammation during experimental acute and chronic bladder inflammation in mice based on histological examination and inflammatory gene expression patterns. Antagonizing the nicotinic pathway by the administration of mecamylamine enhanced the severity of acute and chronic bladder inflammation only in the presence of co-administration of cyclophosphamide. However, additional treatment with 17 β -estradiol reduced inflammation at both histological and molecular levels. Although the nicotinic agonist resulted in little histological change in acute bladder inflammation, mRNA analysis demonstrated a reduction in inflammatory marker expression. In the chronic bladder inflammation model, the cooperation of estrogen and the nicotinic agonist anabasine was very important in reducing inflammation at the histological level.

One inflammatory marker that was down-regulated in the acute model by both estrogen and anabasine either alone or in combination was lipocalin-2. It is involved in inflammation and detoxification processes caused by immune system activation in mammals. The expression of lipocalin-2 was not different among treatments in the chronic model, and it may be because it has been used as a marker of neutrophil inflammation in conditions such as ulcerative colitis and proctitis.¹⁹ Histological analysis of bladder sections demonstrated the presence of neutrophils in the acute model but very few or none in the chronic model. Lipocalin-2 expression in ovariectomized mice treated with 17 β -estradiol was slightly increased when compared with control mice as reported previously in the uterus.²⁰ Another marker for acute inflammation, cathepsin D, is important in the recruitment of neutrophils. Consistent with the lipocalin-2 results, cathepsin D was altered in the acute model but not in the chronic model. IL-6 is a cytokine produced by leukocytes, endothelial cells, and many other cell types. IL-6 changes were detected both in the acute and chronic model. Generally the mRNA expression of the respective inflammatory markers was elevated when the nicotinic pathway was antagonized and down-regulated when 17 β -estradiol and/or anabasine were given. Strikingly, the combined treatment of anabasine and 17 β -estradiol reduced cyclophosphamide-induced chronic inflammation histologically, when anabasine and 17 β -estradiol alone had little effect on chronic bladder inflammation. The reduction of inflammation observed histologically after administration of 17 β -estradiol or anabasine may in part be mediated by inactivation of the inflammatory markers measured.

The clinical relevance of a bladder inflammation model in mice is primarily at the level of chronic inflammation. As

such, the phenotype of the chronic inflammation model followed the same histological pattern as the acute but with expected differences in the inflammatory infiltrates. The presence of macrophages was confirmed by immunohistochemical F4/80 staining. Treatment with estrogen and/or anabasine reduced significantly the expression of macrophages caused by inflammation. Additionally, epithelial cell proliferation has been significantly correlated with inflammatory processes of the bladder,¹⁶ gastric inflammation,²¹ and other conditions associated with tissue injury and wounding.²² Such chronic proliferative conditions may lead to neoplastic transformation of the respective tissues. During chronic bladder inflammation, urothelial proliferation paralleled the level of inflammation. Changes in proliferation have been related with NF- κ B activation.²³ NF- κ B is a family of ubiquitously expressed transcription factors that are widely believed to trigger both the onset and the resolution of inflammation.²⁴ This transcription factor has an important role in the control of genes involved in inflammation, cell proliferation, and apoptosis.²⁵ NF- κ B has been shown to mediate lipopolysaccharide-induced inflammation in the bladder.²⁶ Previous studies have shown that IL-6 expression in response to bacillus Calmette-Guerin requires nuclear factor NF- κ B-mediated signal transduction.²⁷ Physiologically high concentrations of 17 β -estradiol modulate NF- κ B signaling in human T cells.²⁸ In this study we found that NF- κ B is activated during chronic bladder inflammation induced by cyclophosphamide or furthered by cyclophosphamide and mecamylamine together. However, the additional treatment with estrogen and/or anabasine resulted in the translocation of p65 to the cytoplasm, suggesting decreased NF- κ B activity. Previous studies showed that nicotine inhibited the transcriptional activity of NF- κ B.²⁹ The distribution of NF- κ B in bladder sections was both in the epithelium and in the stroma. NF- κ B distribution in the epithelium was cytoplasmic, and its distribution in the stroma was nuclear when inflammation was induced using cyclophosphamide alone or in combination with mecamylamine. We found that NF- κ B p65 was not present in either the cytoplasm or nucleus during acute bladder inflammation. In our model, the activation of NF- κ B may be a result of either or both the activation of macrophages and because of the changes in epithelial proliferation. The data suggest that a mechanism by which estrogen or anabasine may reduce inflammation might be through reduction of nuclear translocation of p65 to suppress cytokine expression.

In summary, results from this study suggested that a nicotinic antagonist promotes the expression of lipocalin-2, cathepsin D, and IL-6 and infiltration of neutrophils in the acute model and it increases the expression of IL-6 and infiltration of macrophages in the chronic model. However, when a nicotinic agonist or 17 β -estradiol was administered it reduced the expression of the same markers. Also, the cooperation of estrogen and anabasine was important in reducing the expression of inflammatory markers. Results suggested that estrogen and the nicotinic anti-inflammatory pathways regulate host responses during cyclophosphamide-induced chronic bladder inflammation in mice through NF- κ B sig-

naling. A better understanding of the molecular mechanisms of the apparent cooperation of the estrogen and nicotinic anti-inflammatory pathways in the bladder could be used in the control of bladder inflammation such as interstitial cystitis. Further, the data support methods of reducing the toxicity caused by cyclophosphamide when it is used as a chemopreventive agent or to treat several autoimmune diseases and cancer.

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