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Regulation of IGF-1 but not TGF- β 1 by NGF in the smooth muscle of the inflamed urinary bladder

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Abstract

Intraperitoneal injection of cyclophosphamide (CYP) causes haemorrhagic cystitis with excess growth of muscular layer leading to bladder hypertrophy; this could be attributable to changes in the expression profiles of growth factors in the inflamed urinary bladder. The growth factors characterized in the current study include nerve growth factor (NGF), insulin-like growth factor (IGF)-1, and transforming growth factor (TGF)- β 1. We found that following CYP injection for 8h and 48h, the mRNA levels of all three factors were increased in the inflamed bladder when compared to control. The level of NGF mRNA was mainly increased in the urothelium layer while the levels of IGF-1 mRNA and TGF- β 1 mRNA were increased in the smooth muscle layer. The level of NGF high affinity receptor TrkA mRNA was also increased in both the urothelium and the smooth muscle layers during bladder inflammation. When we blocked NGF action with NGF neutralizing antibody *in vivo*, we found that the up-regulation of IGF-1 in the inflamed bladder was reversed while the up-regulation of TGF- β 1 was not affected by NGF neutralization. The effect of NGF on regulating IGF-1 expression was further confirmed in bladder smooth muscle culture showing that exogenous NGF increased the mRNA level of IGF-1 after 30 min to 1h stimulation. These results suggest that bladder inflammation induced region-specific changes in the expression profiles of NGF, IGF-1 and TGF- β 1. The up-regulation of NGF in the urothelium may have a role in affecting bladder smooth muscle cell physiology by regulating IGF-1 expression.

Keywords

NGF; IGF-1; TGF- β 1; urinary bladder; inflammation

1. INTRODUCTION

Cyclophosphamide (CYP) is used to treat various types of cancer and some autoimmune disorders. CYP treatment frequently results in haemorrhagic inflammation and enlargement

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QLZ designed and conducted most of the experiments, and wrote part of the manuscript. LYQ designed the project, conducted some of the experiments, and wrote and finalized the manuscript.

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of the urinary bladder in patients and animals [1–2], which is largely due to the urotoxicity effects of CYP metabolite, acrolein [3]. During bladder inflammation, numerous inflammatory factors such as cytokines, chemokines and growth factors are generated by the inflamed urinary bladder [4–5]. Among these factors, growth factors may play significant roles in regulating gene expression, cell survival and growth, and contribute to the increased thickness of the detrusor muscle wall during bladder inflammation. Our previous study showed that blockade of nerve growth factor (NGF) action attenuated CYP-induced urinary bladder enlargement [2].

NGF was first identified as a signaling protein promoting neuronal survival [6]. In the central and peripheral nervous systems, NGF is critical for the axonal growth and maintenance of neurons without undergoing apoptosis. Recent studies also provide strong evidence demonstrating that NGF is expressed in inflammatory conditions [7–11]. The increased NGF levels were observed in bronchoalveolar lavage fluid and serum from patients with asthma [7] and in the urinary bladder and the urine from patients with bladder inflammation [9–11]. In a CYP-induced bladder inflammation model, rats treated with CYP acutely (single dose for 4h) or chronically (multiple dose for 10 days) [12] showed increased amount of NGF mRNA or protein in the inflamed urinary bladder. Cellular responses mediated by NGF require ligand-binding and subsequent activation of specific receptor tyrosine kinase TrkA that is embedded in the plasma membrane [13–14]. Although previous studies showed that cystitis increased the protein levels of TrkA and p75 neurotrophin receptor (p75NTR) in the urinary bladder [15–16], it is not known if the bladder smooth muscle expresses TrkA thus is able to respond to NGF and leads to the morphological and cellular changes in the muscular layer of the inflamed bladder.

Bladder inflammation-induced detrusor muscle growth may also involve changes in the growth factors such as insulin-like growth factor (IGF)-1 and transforming growth factor (TGF)- β 1. Both factors are reported to play critical roles in regulating cellular proliferation, survival and growth [17–20]. IGF-1 stimulates proliferation of intestinal smooth muscle cells and regulates muscle cell development and survival [17], and is involved in mechanical forces-induced bladder smooth muscle cell growth and remodeling [21]. IGF-1 and its binding proteins secreted into the urine have also been used as inflammatory markers to differentiate cystitis and normal bladder of patients [22]. The role of TGF- β in regulating cellular proliferation and survival has also been observed in chronic myeloid leukaemia [19]. During bladder inflammation, the bladder tissue and urine exhibited significantly higher levels of TGF- β 1 protein [23].

In the present study, we aim to characterize the expression profiles of NGF in the inflamed bladder, and compared them with the expression profiles of IGF-1 and TGF- β 1 post CYP treatment in rats. We characterized the expression level of NGF, IGF-1 and TGF- β 1 in the separated urothelium layer and the detrusor smooth muscle layer. The results that NGF up-regulated in the urothelium regulated IGF-1 but not TGF- β 1 in the detrusor muscle suggest distinct roles of these three growth factors during inflammation.

2. MATERIALS AND METHODS

2.1. Experimental animals and reagents

Adult male rats (150–200 g) from Harlan Sprague Dawley, Inc. (Indianapolis, IN) were used. All experimental protocols involving animal use were approved by the Institutional Animal Care and Use Committee at the Virginia Commonwealth University. Animal care was 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 as well as to reduce the number

of animals used. CYP and other chemicals used in this experiment were purchased from Sigma-Aldrich (St. Louis, MO). Real-time PCR reagents were purchased from Applied Biosystems (Foster City, CA).

2.2. Cyclophosphamide-induced Cystitis

CYP cystitis was induced in rats by the technique previously described [2]. Briefly, cystitis was induced in rats by injecting CYP intraperitoneally at a single dose of 150 mg/kg for 8h or 48h. Control rats received volume-matched injections of saline. All injections were performed under isoflurane (2 %) anesthesia.

2.3. Whole mount dissection of the urinary bladder

The urinary bladder from control animals and animals treated with CYP was freshly dissected out and cut open parasagittally from the bladder neck to the top of the bladder dome. The opened urinary bladder was pinned flatly urothelium (inside) up to a sylgard-coated dish containing Krebs buffer bubbled with 95% O₂/5% CO₂. While pinned, the urothelium layer was separated from the underlying muscular layer of the urinary bladder with fine tip forceps and iris scissors. The urothelium and the muscular layer were then collected separately and homogenized for analysis. The expression level of smooth muscle α -actin was examined in both urothelium and detrusor muscle preparation in order to determine the purity of the dissection of the tissue layers.

2.4. Drug treatment of bladder smooth muscle culture

The urinary bladder smooth muscle layer was dissected out via a whole mount preparation and minced for acute culture. NGF (100 ng/mL) was added to the cultured bladder smooth muscle explants for 30 min, 1h and 3h.

2.5. Drug treatment of animals

Immediately following CYP or saline injection, animals also received intraperitoneal injections of either NGF neutralizing antibody (30 μ g/kg) or control IgG (30 μ g/kg). Each independent experiment includes four animals: saline +IgG, CYP+IgG, NGF antibody, CYP +NGF antibody.

2.6. RNA extraction and quantitative real-time PCR

Total RNA was extracted using a RNA extraction kit RNAqueous (Ambion, TX). RNA concentration was determined spectrophotometrically. cDNA was synthesized using Cloned AMV First-Strand Synthesis Kit (Invitrogen) with random hexamers. Following reverse transcription, quantitative real-time PCR (qPCR) was performed for NGF, TrkA, IGF-1, and TGF- β 1. β -actin, GAPDH, and ribosomal protein large P2 (RPLP2) were used for validation of the best internal control under inflammatory condition. qPCR was done with Taqman probes (see Table 1) mixed with PCR Master-Mix for 40 cycles (95 °C for 15 sec, 60 °C for 1 min) on a 7300 real-time PCR system (Applied Biosystems). The level of target mRNA was normalized against the expression of one of these internal controls in the same sample that was calculated with Δ Ct method (Δ Ct=Ct target gene Ct internal control in the same sample). The expression level of target mRNA in control group from each independent experiment was considered as 1, and the relative expression level of target mRNA in experimental groups was adjusted as a ratio to its control in each independent experiment and expressed as fold changes ($2^{-\Delta\Delta$ Ct fold) ($\Delta\Delta$ Ct = Δ Ct experimental group – Δ Ct control group).

2.7. Statistical analysis

All data were presented as mean $\pm$ SEM. Comparison between control and experimental groups was made by using one-way ANOVA followed by Dunnett's test, or using student's *t*-test. Differences between means at a level of *p* 0.05 were considered to be significant.

3. RESULTS

3.1. Validation of internal controls

The expression level of some commonly used internal controls may also be regulated by inflammation. To find a proper internal control for the current study, we compared the relative mRNA expression levels of β -actin, GAPDH and RPLP2 in the whole bladder before and post CYP treatment (Figure 1A–C). Results showed that β -actin and RPLP2 had similar expression levels in the control and the inflamed urinary bladder, while GAPDH had a higher level in the inflamed urinary bladder 8h post CYP treatment when these three molecules were cross-used as internal controls for one and another (Figure 1A–C). Further experiments on characterizing the relative mRNA levels of these three internal controls in the urothelium and muscle layers showed that post CYP treatment for 8h, the relative levels of GAPDH were also higher in the smooth muscle layer of the inflamed urinary bladder (Figure 1D–F). In the urothelium layer, the relative levels of all three internal controls were similar when these three molecules were cross-used as internal controls for one and another (Figure 1D–F). Considering that β -actin expression level (Figure 1A, D) was relatively more consistent than GAPDH (Figure 1B, E) during bladder inflammation when using RPLP2 as an internal control, we decided to use β -actin as the internal control when only one was chosen.

3.2. Up-regulation of NGF, IGF-1 and TGF- β 1 in the inflamed urinary bladder

The whole urinary bladders from control and CYP-treated animals were freshly dissected out and subjected to RNA extraction and real-time PCR analysis. The results showed that the level of NGF mRNA was increased in the inflamed urinary bladder at both 8h and 48h post CYP injection when compared to control (Figure 2A and B). The levels of IGF-1 (Figure 2C and D) and TGF- β 1 (Figure 2E and F) mRNA were also increased in the inflamed urinary bladder at both 8h and 48h post CYP injection when compared to control. There were greater levels for NGF, IGF-1, and TGF- β 1 mRNA at 48h when compared to 8h post CYP injection, suggesting an increment in the expression of these factors during the progression of bladder inflammation. The changes in NGF, IGF-1 and TGF- β 1 mRNA levels were not significantly affected when either β -actin or GAPDH was used as the internal control.

3.3. Expression of NGF, IGF-1 and TGF- β 1 in the urothelium and detrusor muscle

After separation of the urothelium layer from the detrusor smooth muscle layer of both control and the inflamed urinary bladder, we characterized the relative levels of NGF, IGF-1 and TGF- β 1 in these two types of tissues. We chose 8h post CYP injection in the current study. Results showed that NGF mRNA was largely increased in the urothelium (Figure 3A). In contrast, the mRNA levels of both IGF-1 (Figure 3B) and TGF- β 1 (Figure 3C) were increased in the detrusor smooth muscle layer during bladder inflammation.

3.4. Expression of TrkA in the urothelium and detrusor smooth muscle

Examination of the urothelium and detrusor smooth muscle before and during bladder inflammation showed that the mRNA level of TrkA, a high affinity receptor for NGF, was up-regulated in both tissues (Figure 4). The magnitude of increases in the relative level of

TrkA mRNA in the muscle layer was significantly higher than that in the urothelium layer during bladder inflammation when compared to their respective controls (Figure 4).

3.5. Effects of NGF on IGF-1 and TGF- β 1 expression in the urinary bladder

Up-regulation of TrkA in the detrusor smooth muscle during bladder inflammation may increase the responsiveness of the muscle cells to NGF. Thus, we examined if NGF had a role in regulating IGF-1 and TGF- β 1 expression in the detrusor muscle. To do so, we injected NGF antibody or control IgG to animals with or without bladder inflammation and found that treatment with control IgG had no effects on CYP-induced up-regulation of IGF-1 or TGF- β 1 mRNA levels in the inflamed urinary bladder (Figure 5). However, treatment with anti-NGF attenuated CYP-induced IGF-1 expression in the urinary bladder, but had no effect on the increased level of TGF- β 1 mRNA (Figure 5). To further examine the role of NGF in IGF-1 expression in the detrusor smooth muscle, we treated the cultured detrusor muscle with NGF and found that NGF treatment increased IGF-1 mRNA level at 30 min to 1h following treatment (Figure 6).

4. DISCUSSION

The present study demonstrated that bladder inflammation induced significant increases in the expression levels of NGF, IGF-1 and TGF- β 1 in the inflamed urinary bladder 8h to 48h post CYP injection. There is a positive correlation between the expression levels of these three growth factors and the severity of the bladder inflammation. The expression levels of all three growth factors were higher at 48h than that at 8h post induction of bladder inflammation. However, the expression profiles of these three growth factors in the inflamed urinary bladder were distinct showing that NGF expression level was increased in the urothelium layer while IGF-1 and TGF- β 1 expression levels were increased in the smooth muscle layers. The smooth muscle layers also demonstrated an increased level of NGF high affinity receptor TrkA, which could increase the responsiveness of the detrusor muscle cells to NGF during bladder inflammation, and regulate gene expression in these cells. The present study showed that the up-regulation of IGF-1 in the detrusor smooth muscle layer during bladder inflammation was attenuated by NGF neutralizing antibody, suggesting a paracrine role of NGF in regulating IGF-1 expression in the detrusor muscle. Although TGF- β 1 was also increased in the inflamed bladder, NGF failed to regulate TGF- β 1 expression during bladder inflammation. It is noteworthy that exogenous NGF acutely increased IGF-1 production. Thus, the progressive increases in IGF-1 transcripts in the inflamed urinary bladder may require a sustained increase in NGF during bladder inflammation. This notion is supported by our results that the levels of NGF and IGF-1 mRNA were increased in parallel in the inflamed urinary bladder following CYP treatment.

The urinary bladder is made of four layers. The innermost urothelium layer, which is made of epithelium cells, acts as a permeability barrier protecting underlying tissues against noxious urine components; the next suburothelium space contains nerves, blood vessels and connective tissues; the muscular layer is called the detrusor muscle which controls the distension and contraction of the urinary bladder, and is surrounded by the outer layer, a serous membrane. Histological examination of the urinary bladder demonstrated that CYP treatment induced significant ulcerative damage of the urothelium and increased the thickness of the detrusor wall. The increases in the thickness of the bladder wall may be attributable to several factors including increases in the production of extracellular matrix [24–26] and increases in the muscular growth [25, 27]. Our recent study showed that injection of NGF neutralizing antibody significantly reduced the increased bladder weight during bladder inflammation [2], suggesting a positive role of NGF in bladder enlargement. The current study demonstrated that NGF was increased in the urothelium layer during

bladder inflammation. Thus, NGF neutralizing antibody could bind to NGF that was secreted out of the urothelium cells, and attenuate NGF action within the urinary bladder.

To further characterize the action of NGF within the bladder, we characterized its receptor TrkA. We found that TrkA expression was increased in both the urothelium layer and the smooth muscle layer during bladder inflammation, suggesting that cells within these two layers were able to respond to NGF. IGF-1 and TGF- β 1, two growth factors that play critical roles in smooth muscle survival and growth, were also increased in the smooth muscle layer of the inflamed bladder. Thus, we asked questions if NGF had a role in regulating IGF-1 and TGF- β 1 expression in the detrusor smooth muscle. Our results showed that NGF was able to regulate IGF-1 but not TGF- β 1 level in the detrusor muscle. The transcription of *IGF1* involves association with transcription factors the signal transducer and activator of transcription (Stat)5 [28–29], and activating protein (AP)1 [30]; both of them could be activated by NGF [31–35].

The mechanism underlying NGF up-regulation in the urothelium during bladder inflammation is unknown. The urothelium is the first layer that gets contact with the toxic components in the urine. After intraperitoneal injection, CYP is metabolized to acrolein [3] and excreted into the urinary bladder. The concentrated chemical in the urinary bladder can cause haemorrhagic cystitis or even bladder cancer. It is not clear if NGF up-regulation is directly in response to the chemical irritation to the urothelium, or a secondary response to factors that are initiated prior to NGF production in the inflamed bladder. Studies with instillation of urine from patients with interstitial cystitis to the experimental animal models showed an increased level of NGF in the bladder of these animals, suggesting that factors secreted into the patient's urine might elicit NGF up-regulation [36]. Recent studies with a colitic animal model showed that NGF was markedly increased in the epithelial cells [37]. In patients with persistent allergic rhinitis, it was also shown that NGF was expressed in the nasal mucosa [38–39]. The current findings further support the notion that NGF was up-regulated in the mucosa coat of the urinary bladder in response to inflammation. The driving force that induces the NGF expression is unknown, pro-inflammatory mediators may have a role in NGF up-regulation [40]. It has been suggested that the activated mast cells could be a source to release NGF [41]. However, a recent study showed that a NGF precursor, the pro-NGF, but not the mature NGF was detected in a mucosal mast cell line [37]. It is noteworthy that the NGF antibody we used (Santa Cruz) does not distinguish the pro-form and the mature form of NGF, and it could block the activity of both. The primers used for detecting NGF also could detect both because of their shared mature NGF sequence. Thus, the findings from this study do not exclude that the urothelium produced pro-NGF as well.

Previous studies have suggested an association of NGF, IGF-1 and TGF- β 1 with bladder inflammation [9–12, 22–23]. The current study revealed distinct expression patterns of these three growth factors during bladder inflammation, which was that NGF was mainly up-regulated in the urothelium while IGF-1 and TGF- β 1 were up-regulated in the detrusor smooth muscle layer. We also found that NGF could act on TrkA that was expressed in the detrusor smooth muscle and regulate IGF-1 expression. These results suggest a paracrine role of NGF in regulating the cytological changes of the urinary bladder.

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Abbreviations

CYP	cyclophosphamide
NGF	nerve growth factor
IGF	insulin-like growth factor
TGF	transforming growth factor
RPLP	ribosomal protein large P
Stat	signal transducer and activator of transcription
AP	activating protein

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Highlights

1. CYP-induced bladder inflammation increased the level of nerve growth factor (NGF) in the urothelium.
2. CYP-induced bladder inflammation increased the levels of insulin-like growth factor (IGF)-1, and transforming growth factor (TGF)- β 1 in the detrusor muscle.
3. NGF regulates IGF-1 but not TGF- β 1 in the detrusor muscle.

Figure 1. Expression of β -actin, GAPDH and RPLP2 in the urinary bladder before and during bladder inflammation

After CYP treatment for 8 or 48h, the whole urinary bladder was homogenized and real-time PCR was performed to detect the relative levels of β -actin, GAPDH and RPLP2 in the urinary bladder from control animals and animals with bladder inflammation (A–C). Histograms showed that the levels of β -actin were not affected by inflammation when RPLP2 was used as internal control (A); however, the level of GAPDH was increased by inflammation when RPLP2 was used as internal control (B). In other words, β -actin was decreased by inflammation when GAPDH was used as internal control (C). After CYP treatment for 8h, the levels of β -actin were not affected by inflammation in both the urothelium (compare CYP urothelium [CYP U] to control urothelium [Contr U]) and muscle layers (compare CYP muscle [CYP M] to control muscle [Contr M]) when RPLP2 was used as internal control (D); the level of GAPDH did not change in the urothelium but was increased in the muscle layer when RPLP2 (E) or β -actin (F) was used as internal control. *, $p < 0.05$ versus control. #, $p < 0.05$ versus control. $n=5$ animals for each time points.

Figure 2. Up-regulation of NGF, IGF-1 and TGF-β1 in the inflamed urinary bladder

The mRNA levels of NGF (A and B), IGF-1 (C and D) and TGF-β1 (E and F) were increased in a time-dependent manner after CYP treatment. All three factors were up-regulated in the urinary bladder 8h post CYP injection and continuously increased 48h post CYP injection. To confirm the changes, we used both β-actin and GAPDH as internal controls. *, p < 0.05 versus control. #, p < 0.05 versus CYP 8h. n=5 animals for each time points.

Figure 3. Expression of NGF, IGF-1 and TGF-β1 in the urothelium and the detrusor muscle before and during bladder inflammation

The increased expression of NGF in the urinary bladder 8h post CYP injection was mainly produced by the urothelium (A); the increased expression of IGF-1 (B) and TGF-β1 (C) in the urinary bladder 8h post CYP injection was mainly produced by the detrusor muscle. The comparison was made between urothelium from control animals (Contr U) versus urothelium from the inflamed animals (CYP U), and detrusor muscle from control animals (Contr M) versus detrusor muscle from the inflamed animals (CYP M). No comparison was made between control urothelium versus control muscle, or CYP urothelium versus CYP muscle. *, $p < 0.05$ versus corresponding controls. $n = 4$ animals for each experimental group.

Figure 4. Expression of TrkA in the urothelium and the detrusor muscle before and during bladder inflammation

The TrkA mRNA levels were increased in both the urothelium and the detrusor muscle layers. The comparison was made between urothelium from control animals (Contr U) versus urothelium from the inflamed animals (CYP U), and detrusor muscle from control animals (Contr M) versus detrusor muscle from the inflamed animals (CYP M). *, $p < 0.05$ versus corresponding controls. The magnitude of increases in the relative level of TrkA mRNA in the smooth muscle layer (ΔM) was higher than that in the urothelium layer (ΔU), #, $p < 0.05$. $n = 4$ animals for each experimental group.

Figure 5. Effects of NGF antibody on the expression levels of IGF-1 and TGF-β1 in the urinary bladder before and during bladder inflammation

In the control IgG-treated animals, cystitis increased the expression levels of IGF-1 (A) and TGF-β1 (B) in the inflamed urinary bladder. Administration of NGF neutralizing antibody had no effects on the basal level of IGF-1 and TGF-β1 in the non-inflamed bladder. NGF neutralizing antibody significantly attenuated IGF-1 but not TGF-β1 up-regulation in the inflamed bladder. *, $p < 0.05$ versus IgG; #, $p < 0.05$ versus CYP+IgG. $n = 6$ animals for each experimental group.

Figure 6. Up-regulation of IGF-1 by NGF in the urinary bladder smooth muscle

In cultured detrusor smooth muscle, the level of IGF-1 mRNA was significantly increased by NGF treatment (100 ng/mL) for 30 min and also kept significantly high till 1 h. The results were from 3 independent experiments and presented as mean $\pm$ SE. *, $p < 0.05$ vs 0 treatment.

Table 1

Catalogue numbers of the Taqman probes used in the current study.

NGF:	Rn01533872
TrkA:	Rn00572130
IGF-1:	Rn00710306
TGF- β 1:	Rn00572010
β -actin:	Rn4352931E
GAPDH:	Rn01462662
RPLP2:	Rn01479927