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Effects of Herpes Simplex Virus Vectors Encoding Poreless TRPV1 or Protein Phosphatase 1α in a Rat Cystitis Model induced by Hydrogen Peroxide

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Abstract

Enhanced afferent excitability is considered to be an important pathophysiological basis of interstitial cystitis/bladder pain syndrome (IC/BPS). In addition, transient receptor potential vanilloid-1 (TRPV1) receptors are known to be involved in afferent sensitization. Animals with hydrogen peroxide (HP)-induced cystitis have been used as a model exhibiting pathologic characteristics of chronic inflammatory condition of the bladder. This study investigated the effect of gene therapy with replication-defective herpes simplex virus (HSV) vectors encoding poreless TRPV1 (PL) or protein phosphatase 1 α (PP1α), a negative regulator of TRPV1, using a HP-induced rat model of cystitis. HSV vectors encoding green fluorescent protein (GFP), PL or PP1α were inoculated into the bladder wall of female rats. After one week, 1% HP or normal saline was administered into the bladder, and the evaluations were performed 2 weeks after viral inoculation. In HP-induced cystitis rats, gene delivery of PL or PP1α decreased pain behavior as well as a reduction in the intercontraction interval. Also, both treatments reduced NGF expression in the bladder mucosa, reduced bladder inflammation characterized by infiltration of inflammatory cells, and increased bladder weight. Taken together, HSV-mediated gene therapy targeting TRPV1 receptors could be effective for the treatment of IC/BPS.

INTRODUCTION

Interstitial cystitis/bladder pain syndrome (IC/BPS) is a chronic inflammatory bladder disorder characterized by pelvic pain with bladder symptoms including urinary frequency

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

and urgency, which substantially reduces patient's quality of life (ref. (1, 2)). Its pathophysiology and etiology is still largely unknown, but there has been increasing evidence that increased afferent excitability is an important pathophysiological basis of IC/BPS (ref. 3–6). Previous studies also reported the significant contribution of transient receptor potential vanilloid-1 (TRPV1) receptors to afferent sensitization (ref. 7, 8).

It has recently been reported that a single intravesical administration of hydrogen peroxide (HP) induces relatively long-lasting bladder inflammation and bladder dysfunction for up to two weeks in mice and rats, and that changes in histology and urothelial permeability were similar to those observed in IC/BPS (ref. 9, 10). We have also recently reported using the HP-induced cystitis rat model that liposome-based intravesical application of nerve growth factor (NGF) antisense reduced enhanced bladder pain sensitivity and bladder overactivity in association with a reduction in NGF expression in the bladder mucosa (ref. 11).

Furthermore, we have previously shown that replication-defective herpes simplex virus (HSV) vectors expressing poreless TRPV1 (PL), a dominant-negative mutant of TRPV1 or protein phosphatase 1α (PP1α), a negative regulator of TRPV1 reduced thermal sensitivity following HSV vector injection into rat footpads (ref. 12). We have also reported that replication-deficient HSV vector-mediated gene delivery of PL significantly improved bladder overactivity and pain behavior induced by TRPV1 activation in rats with chemically induced acute cystitis (ref. 13). However, it is not known whether HSV vectors encoding PP1α are effective to treat bladder dysfunction induced by long-lasting bladder inflammation. Therefore, we extend our previous study to investigate the effect of gene therapy using HSV vectors encoding PL or PP1α (Figure 1) in a rat model of longer-lasting cystitis induced by HP administration.

RESULTS

Cystometry

In cystometric analysis, animals with HP-induced cystitis injected with green fluorescent protein (GFP) control vector (HP-GFP, n = 6) showed significantly ($P < 0.01$) shorter intercontraction intervals (ICI) than normal saline administration in the GFP control vector treatment group (NS-GFP, n = 6) (ICI: 400 ± 28 and 981 ± 70 sec, respectively). HP-induced cystitis in the poreless TRPV1 treatment group (HP-PL, n = 8) and PP1α treatment group (HP-PP1α, n = 8) showed significantly ($P < 0.01$) longer ICIs than the HP-GFP group (ICI:, 632 ± 40, 672 ± 51 and 400 ± 28 sec, respectively, Figure 2). There were no significant differences in other cystometric parameters among the 4 groups.

Nociceptive Behavior Observation

The HP-PL and HP-PP1α vector groups showed a significant ($P < 0.05$) decrease in licking behavior compared to the HP-GFP control vector group only during the first 5-minute period after RTX stimulation (24 ± 2, 24 ± 2 and 30 ±3, respectively), but there was no significant difference in the 5 to 15-minute period after RTX stimulation among the 4 groups (Figure 3).

The number of freezing behavior was significantly ($P < 0.001$) lower in HP-PL (n = 10) and HP-PP1α vector groups (n = 8) compared to the HP-GFP control vector group (4 ± 1, 2 ± 0 and 25 ± 6, respectively, in the entire 15-minute period after RTX stimulation) (Figure 4).

Bladder Weight

Compared with the NS-GFP control vector group (n = 8), the HP-GFP control vector group (n = 8) demonstrated significantly heavier bladder weight (74 ± 2 and 155 ± 7 mg, respectively, $P < 0.001$). On the other hand, the HP-PL vector group (n = 8) and HP-PP1α vector group (n = 9) showed significantly ($P < 0.05$ and $P < 0.01$, respectively) lighter bladder weights than the HP-GFP control vector group (133 ± 5, 128 ± 5 and 155 ± 7 mg, respectively) (Figure 5).

GFP Expression in the Bladder and L6 DRG

To examine the transport of HSV vectors from the bladder to bladder afferent pathways, GFP expression was investigated in rats inoculated with the control vector encoding GFP driven by the strong CMV promoter (Figure 1). A fluorescent microscope was used to identify GFP-positive cells in tissue sections of the bladder wall and L6 DRG that contain bladder afferent neurons (Figure 6).

Hematoxylin and Eosin Staining

Hematoxylin and eosin staining of the bladder showed substantial inflammation characterized by the infiltration of inflammatory cells, which consisted of lymphocytes with some neutrophils and mast cells, damaged urothelium, and submucosal bleeding in the bladder wall in the HP-GFP control vector group (Figure 7B) compared with NS-GFP group (Figure 7A), which were alleviated in the HP-PL and HP-PP1α vector groups (Figure 7B & C).

Gene Expressions in the Bladder

Using RT-PCR, the HP-GFP control vector group showed significantly higher expression of NGF ($P < 0.05$) mRNA in the bladder mucosa than the NS-GFP group, whereas the HP-PL and HP-PP1α vector groups showed a significant decrease in NGF expression ($P < 0.05$) than the HP-GFP control vector group. In contrast, there was no significant difference in the NGF expression in the detrusor among the 4 groups (Figure 8).

DISCUSSION

The results of the current study indicate that (1) HSV vectors are transported to L6 DRG neurons through bladder afferent pathways after the bladder wall inoculation; (2) bladder overactivity caused by hydrogen peroxide is ameliorated using replication-defective HSV vectors expressing either PL or PP1α (HSV-PL or HSV-PP1α) treatment; (3) bladder pain induced by RTX is reduced by HSV-PL or HSV-PP1α treatment; (4) mRNA levels of NGF in the bladder mucosa increased in HP cystitis rats is reduced in HSV-PL or HSV-PP1α treated rats; (5) bladder weight as well as the extent of inflammatory infiltrates, urothelial damage increased in HP cystitis rats is reduced by HSV-PL or HSV-PP1α treatment.

It is known that hydrogen peroxide is a reactive oxygen species, which leads to lipid peroxidation and oxidation of DNA and proteins (ref. 14). Homan et al. reported that intravesical instillation of HP caused urothelial damage characterized by increased permeability of the urothelium, increased bladder weight and infiltration of inflammatory cells in the mouse and rat bladder (ref. 9, 10). Therefore, it is likely that in our study, HP-induced bladder inflammation via urothelial damage was caused by lipid peroxidation and oxidation of DNA and proteins.

Our previous studies showed that HSV vector-mediated gene therapy using various genes like encephalin or soluble TNF-α receptor (TNF-αsR) was effective in reducing bladder overactivity and nociceptive responses in rats (ref. 15, 16). HSV has been shown to be suitable for a gene therapy vector because of its natural biology, especially when treating peripheral diseases with increased afferent sensitivity. First, wild-type HSV shows high affinity for the infection of afferent neurons and wild-type HSV infection can cause disease symptoms in including pain (ref. 17). Second, replication-defective HSV vectors can be readily engineered for neuronal gene transfer that is safe and do not alter the biology of the transduced neuron (ref. 18, 19). Third, the HSV genome is relatively large whereas much of it is not required for the growth and propagation of the virus in the cell cultured system. Multiple or large transgenes can therefore be inserted with relative ease (ref. 18, 19). Fourth, because the HSV genome is not integrated into the host genome, tumorigenic effects of HSV due to insertional mutagenesis into the target cell DNA is not a concern (ref. 20). Lastly, HSV replication-defective vectors can deliver the desired gene product locally at a high level, without causing systemic untoward effects. Therefore, HSV is considered to be ideal in treating disorders in which afferent sensitization greatly contributes to disease etiology such as seen with IC/BPS. In addition, it can also be used to deliver genes locally that, when administered systemically, display potentially severe side effects. In the past, clinical use of TRPV1 antagonists for chronic pain conditions has been hindered because partially of their untoward effects, including drug-induced hyperthermia and impairment of noxious heat sensation (ref. 21). HSV vector-mediated local therapy in the bladder could avoid systemic side effects while at the same time the treatment was dramatically effective in reducing bladder pain and overactivity, as shown in this study. Moreover, the treatment with HSV-PL or HSV-PP1α was similarly effective in reducing NGF mRNA expression in bladder mucosa, bladder weight, and bladder inflammation assessed by histological findings, which were induced in our HP model of cystitis. NGF is known as an activator of immune cells such as mast cells (ref. 22), lymphocytes and monocytes/macrophages (ref. 23), which express NGF and TrkA receptors. NGF is also known to activate TRPV1 through multiple pathways (ref. 12, 17). It has been reported that the local inflammatory state itself activates p38 MAP kinase pathway, thereby increasing NGF expression and TRPV1 activation (ref. 24). Thus, it seems reasonable to assume that HSV-PL or HSV-PP1α treatment can alleviate bladder inflammation and decreased NGF expression in the bladder mucosa through deactivation of TRPV1, and mitigate the vicious cycle of the TRPV1-NGF-local inflammatory process. However, there is the possibility that other pathophysiological mechanisms other that the enhanced TRPV1-NGF system may also contribute to HP-induced bladder overactivity because it was significantly, but not completely, reduced after the TRPV1-targeting gene therapy (Figure 2) whereas the enhanced bladder pain sensitivity

(i.e., freezing behavior) was almost completely suppressed by the therapy in this study (Figure 4).

As previously described, HP-induced cystitis in mice and rats (ref. 9, 10, 25) exhibits several similar aspects to IC/BPS in human such as urinary frequency, bladder pain sensation, and upregulation of NGF, TRPV1, and TRPA1 (ref. 26). Therefore, the HP–induced cystitis model seems suitable for evaluation of the effect of HSV-PL or HSV-PP1α gene therapy. Furthermore, our recent study also demonstrated that the NGF-targeting therapy in the bladder using liposome-mediated antisense-NGF conjugates ameliorated bladder overactivity and enhanced bladder pain sensitivity in HP-induced cystitis rats (ref. 11). Taken together with our current results, it is likely that over-expression of NGF and activation of TRPV1 play a great role in this chronic inflammatory bladder disorder, and that targeting NGF or TRPV1 could be an effective strategy for treating IC/BPS. However, to design the clinically relevant strategy for IC/BPS treatment, further studies are required to examine the effects of HSV vector-mediated gene therapy after the establishment of cystitis.

In conclusion, the results of the present study indicate that HSV vectors-mediated gene delivery of PP1α or poreless TRPV1 significantly reduced bladder overactivity and bladder pain sensitivity enhanced in HP cystitis rats. It seems likely that both treatments reduced bladder inflammation characterized by infiltration of inflammatory cells, as well as increased bladder weight at least in part through amelioration of NGF over-expression in the bladder mucosa. Therefore, HSV-mediated TRPV1-targeting gene therapy could be a novel modality for the treatment of IC/BPS, including IC with Hunner's lesion that is usually associated with bladder inflammatory changes (ref. 27).

MATERIALS AND METHODS

Vectors

The recombinant HSV virus vectors was engineered by deleting the essential immediately early (IE) genes, ICP4 and ICP27, as well as the TAATGARAT elements in the promoters of ICP22 and ICP47 IE genes, in order to reduce their ICP4 and ICP27-dependent expression as early genes from the complementing cell line used to reproduce the vectors (ref 28). The HSV-PL, HSV-PP1α vectors also contains two copies of PL or PP1α gene driven by the strong HCMV promoter in place of the enhanced GFP cassettes at the ICP4 loci of the HSV-GFP vector (Figure 1). In the PL mutant, ^{684}Glu-^{721}Arg was deleted from the wild-type TRPV1 channel (ref. 29). PP1α is a protein identified as a negative regulator of TRPV1 using a selection system of PC12 cell-derived cDNA as previously described (ref. 10). The vectors were purified following the production in the 7b ICP4-ICP27-expressing cell line (ref. 28).

Viral Vector Administration

All animal experiments were performed in accordance with the animal research protocol approved by the University of Pittsburgh Institutional Animal Care and Use Committee (IACUC). Nine-weeks-old Sprague-Dawley female rats were purchased from Envigo (Frederick, MD, USA). Under pentobarbital anesthesia (30mg/kg), a laparotomy at the lower

abdomen was performed, followed by injection of viral suspension (20 μl, total of 1×10^8 plaque forming units) of HSV-GFP, HSV-PL or HSV-PP1α at 4 sites (5μl per site) of the bladder wall using a 30-gauge Hamilton syringe on day 0 as previously described (ref. 13).

Hydrogen peroxide-induced cystitis

A polyethylene (PE) 10 transurethral catheter was inserted into the bladder under isoflurane anesthesia on day 7. After emptying the bladder, either saline or 1% HP solution (0.3 ml each) was administered into the bladder (ref. 11). Rats were placed in a supine position, followed by right and left lateral decubitus position, 10 minutes each, for 30 minutes in total. Then, the bladder was completely emptied. All the assessments were performed on day 14.

Cystometry

Under isoflurane anesthesia, a polyethylene catheter (PE-50, Clay-Adams, Parsippany, NJ, USA) was inserted into the bladder through the bladder dome, and the catheter was secured with a purse suture. Rats were then placed in restraining cages (W 80 mm × L 300 mm × H 150 mm, Yamanaka Chemical Ind., Ltd., Japan). After the recovery from anesthesia, animals were placed in the cages for at least 2 hours for acclimation, and bladder activity was recorded in the awake condition through the bladder catheter connected via a three-way stopcock to a pressure transducer and a pump to infuse saline solution at a rate of 0.04 ml/min. After rhythmic bladder contractions became stable for at least 60 min, the following cystometric parameters were measured; basal pressure (BP), micturition threshold (MT), intercontraction intervals (ICI), peak pressure (PP), voided volume (VV), residual volume (RV), voiding efficiency (VE), and bladder compliance. Chart 5 software (AD Instruments, Milford, MA, USA) was used for data analysis.

Bladder Weight

The bladder was harvested from each rat and weighed before homogenization for RT-PCR analyses.

Evaluation of Nociceptive Behavior

Our previous studies showed that intravesical application of resiniferatoxin (RTX; aTRPV1 agonist) induces 2 different nociceptive behavior; licking behavior evidenced by lower abdominal licking and freezing behavior evidenced by motionless head-turning to the lower abdomen, and that freezing behavior corresponds to pelvic nerve-mediated bladder pain in contrast to licking behavior, which is predominantly caused by urethral pain sensation conveyed through the pudendal nerve (ref. 30, 31). Rats were acclimated in a metabolic cage for at least 2 hours before behavior evaluation. Thereafter, a volume of 0.3mL of 3 μM RTX (Sigma Aldrich) was infused into the bladder via a temporary inserted urethral catheter, and kept for 1 minute, and the animals were placed again to the metabolic cage. Licking and freezing events were then scored for the duration of 15 minutes that were divided into 5-second intervals in a blinded manner. Licking or freezing event was counted as one positive event when it was observed in a 5-second interval. The RTX-mediated induction of nociceptive behavior was needed because HP-induced cystitis rats exhibited hyperalgesia

upon bladder irritation shown by increases in RTX-induced freezing and licking behaviors, without affecting baseline pain behavior, as shown in our recent study (ref. 10).

Hematoxylin and Eosin Staining

After the intracardial perfusion with cold heparinized-saline and 4% paraformaldehyde (JT Baker-Avantor, Central Valley, PA, USA), the bladder and L6 DRG were harvested on day 14. Those tissues underwent overnight post-fixation with 4% paraformaldehyde, followed by incubation with 20% sucrose for cryoprotection for 48 hours. Then, they were frozen after embedding into the OCT compound (Sakura Finetek USA, Inc.). The 10-μm bladder sections were made using a cryostat, followed by staining with hematoxylin and eosin.

Gene Expression in the Bladder

Bladder tissue was collected and dissected into mucosa and detrusor layers on ice using a microscope. Each tissue was homogenized with Trizol (Thermo Fisher Scientific, Pittsburgh, PA, USA). Then total RNA was extracted using RNeasy Mini Kit (QIAGEN, Germantown, MD, USA) according to the manufacturer's manual. Then, it was transcribed to cDNA using a High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). mRNA levels were quantified with an Mx3000P™ Real-Time PCR System using SYBR® Green PCR Master Mix (ABI-Thermo Fisher Scientific). cDNA was amplified for 40 cycles (95°C for 15 minutes, 94°C for 30 seconds, and 55°C for 60 seconds). The oligonucleotide primers used for NGF were 5′-ACCTCTTCGGACACTCTGG-3′ and 5′-CGTGGCTGTGGTCTTATCTC-3′. Specificity of each primer was confirmed by melting curve analysis. mRNA expression was normalized against the expression of GAPDH that was used as the house keeping gene, and compared among 4 groups in terms of fold difference determined by the delta-delta-CT method.

Statistical Analysis

Statistical program R commander (version 3.3.1; the Comprehensive R Archive Network) was used for data analysis. Data were expressed as mean ± standard errors. The statistical significance of differences among groups was determined using nonparametric Mann-Whitney U test due to unequal data distribution. A p-value of less than 0.05 was considered to be statistical significant.

Acknowledgments

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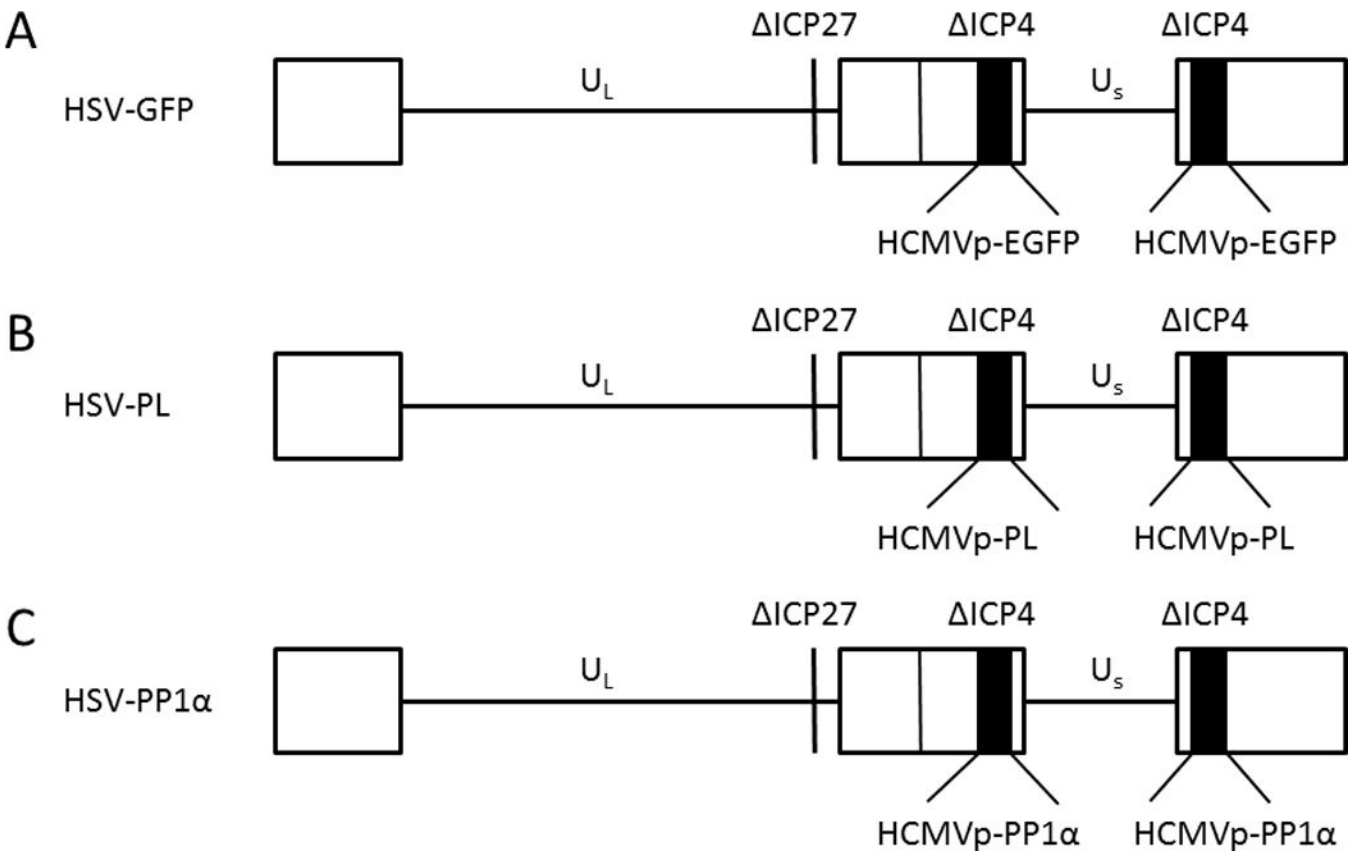

Figure 1.

Replication-deficient herpes simplex virus (HSV) vector constructs. The HSV-GFP (A), HSV-PL (B) and HSV-PP1α (C) vector have a deletion of the essential immediately early (IE) genes, ICP4 and ICP27, as well as IE regulatory elements within the promoters of IE genes, ICP22 and ICP47, making their expression dependent on ICP4 and ICP27, and thus they are expressed as early genes only within the complementing cell line used to propagate the vectors. In the genome of the HSV-GFP, an HCMV IE promoter driving enhanced green fluorescent protein (EGFP) was inserted into both ICP4 loci, while in the poreless TRPV1 or PP1α genome, an HCMV promoter driving each gene was inserted.

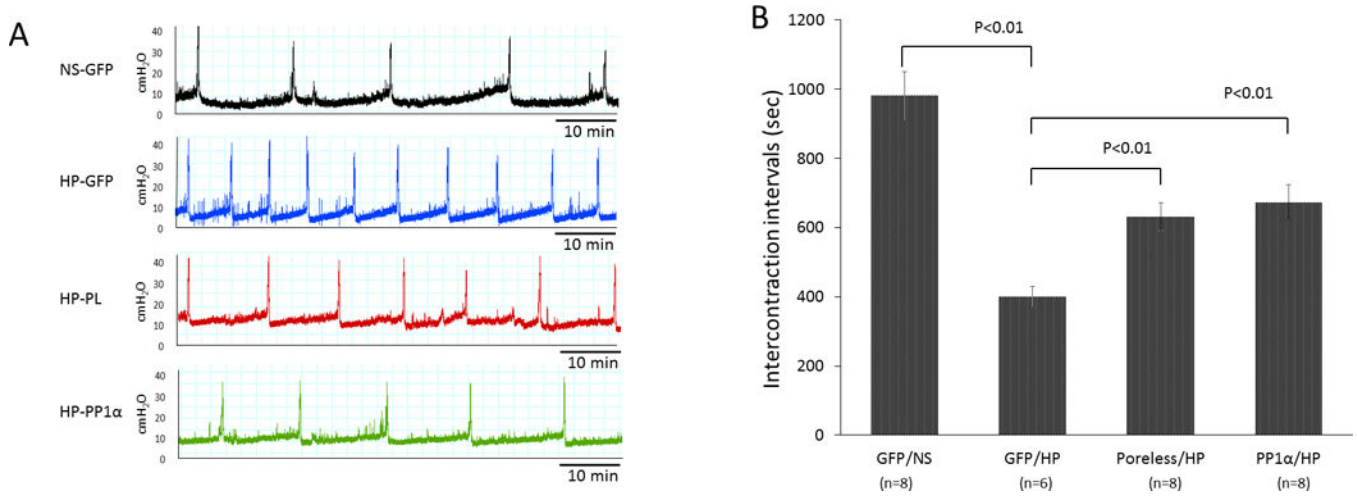

Figure 2.

Cystometry under an awake condition. Representative traces of cystometry in the groups of normal saline administration with GFP treatment (NS-GFP), cystitis with GFP treatment (HP-GFP), cystitis with poreless TRPV1 treatment (HP-PL), and cystitis with PP1α treatment (HP-PP1α) (A) and the comparison of intercontraction intervals (ICIs) (B). ICI was significantly shorter in the HP-GFP group compared with NS-GFP group ($P<0.01$), which was significantly prolonged ($P<0.01$) in both HP-PL and HP-PP1α groups. These results indicate that the HP-PL or HP-PP1α treatment is effective to reduce HP-induced bladder overactivity.

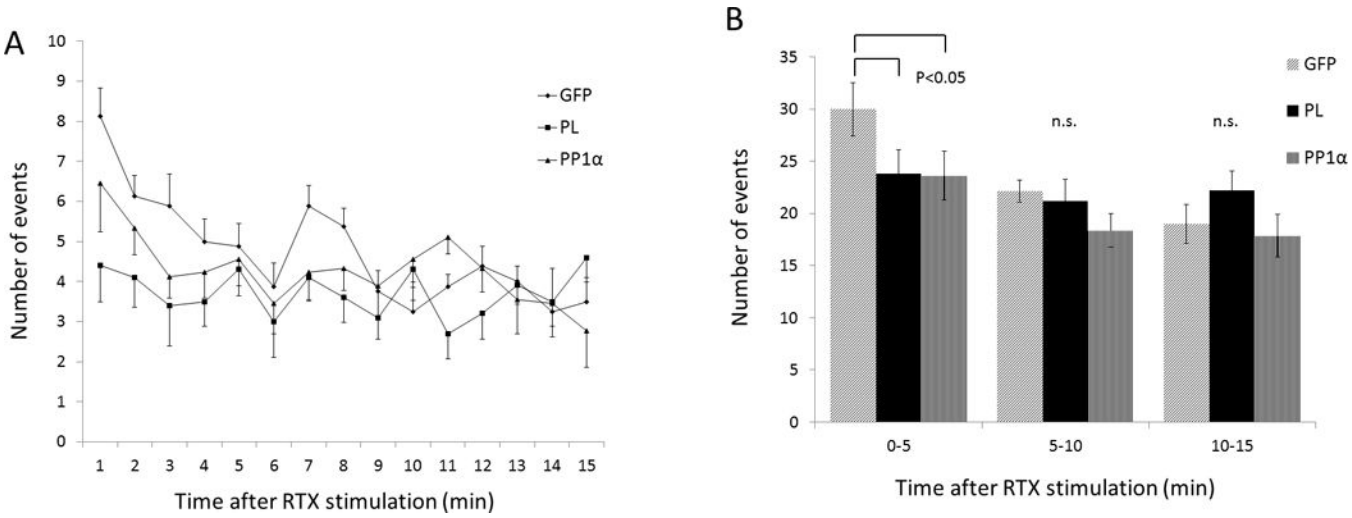

Figure 3.

Resiniferatoxin (RTX)-induced licking behavior. RTX (3μM) was administered into the bladder through a temporary indwelled urethral catheter and kept there for 1 minute. The number of licking events was counted for a 15-minute period with 5-second intervals. Time-course changes in the number of licking behavior events (A). Comparison of licking behavior events in the 5-minute periods (B). HP-PL group and HP-PP1α groups showed a significant decrease ($P<0.05$) in licking behavior compared to the HP-GFP group only during the first 5-minute period, but there was no significant difference among 4 groups in the 5 to 15-minute period after RTX stimulation. These results indicate that the HP-PL or HP-PP1α treatment is effective to ameliorate nociceptive responses that predominantly represent urethral pain sensation induced by intravesical RTX instillation in rats with HP-induced cystitis.

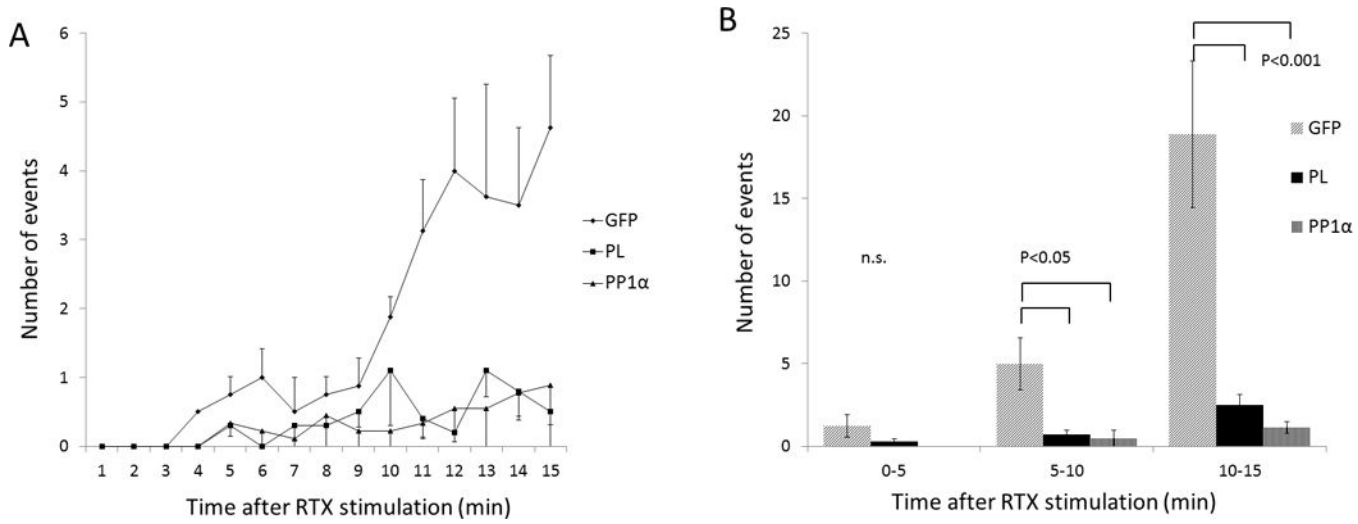

Figure 4.

Resiniferatoxin (RTX)-induced freezing behavior. RTX (3μM) was administered into the bladder through a temporary indwelled urethral catheter and kept there for 1 minute. The number of freezing events was counted for a 15-minute period with 5-second intervals. Time-course changes in the number of freezing behavior events (A). Comparison of freezing behavior events in the 5-minute periods (B). The number of freezing behavior was significantly lower in HP-PL and HP-PP1α groups compared to the HP-GFP group in the 5 to 10 ($P<0.05$) and 10 to 15-minute periods ($P<0.05$). These results indicate that the HP-PL or HP-PP1α treatment is effective to ameliorate bladder-related nociceptive responses induced by intravesical RTX instillation in rats with HP-induced cystitis.

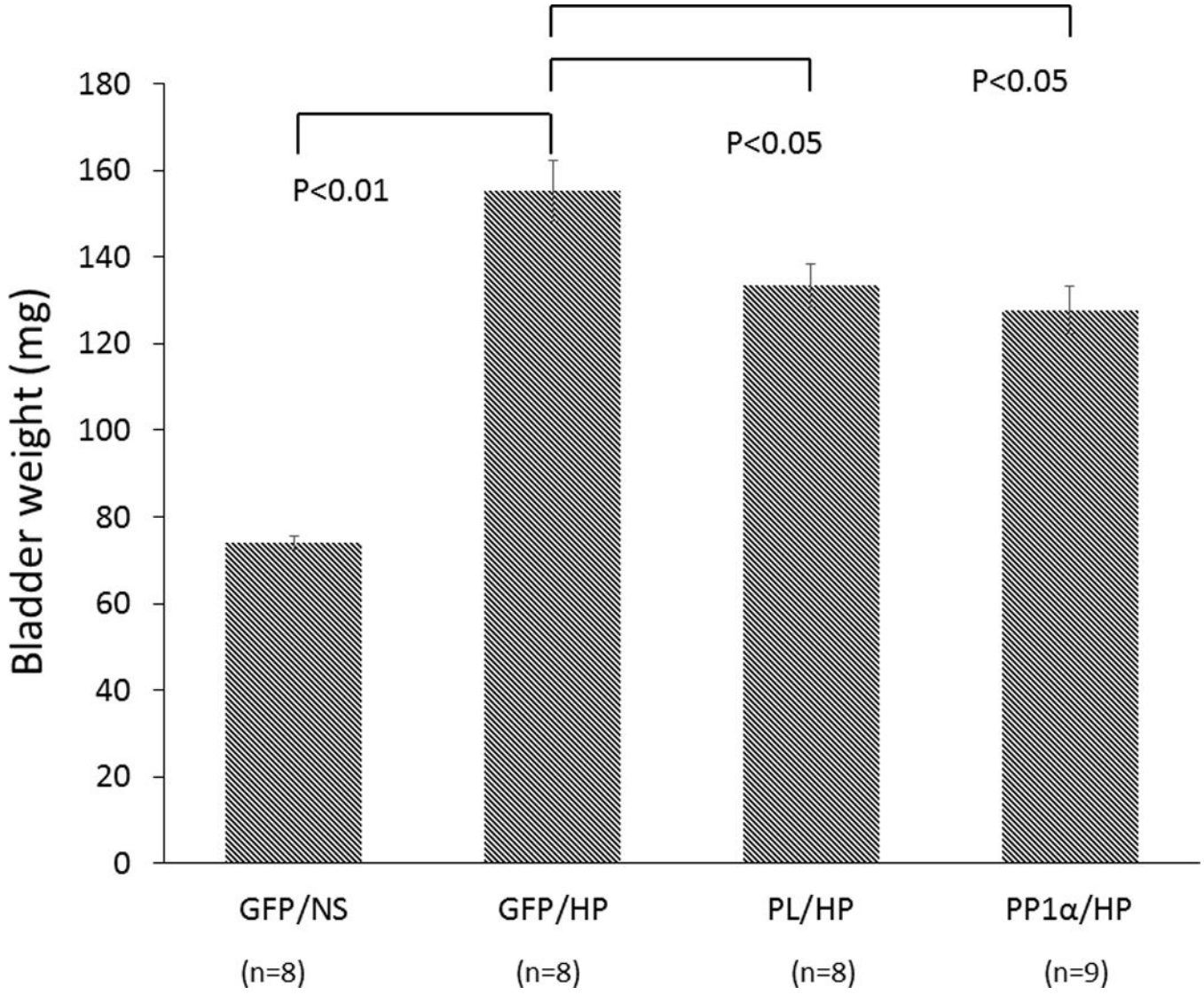

Figure 5.

Bladder weight. Compared with the NS-GFP group, the HP-GFP group had significantly heavier bladder weight ($P<0.01$). On the other hand, the HP-PL and HP-PP1α groups showed significantly lighter bladder weight ($P<0.05$) than the HP-GFP group. These results indicate that the HP-PL or HP-PP1α treatment is effective to reduce the bladder weight increased by HP-induced cystitis.

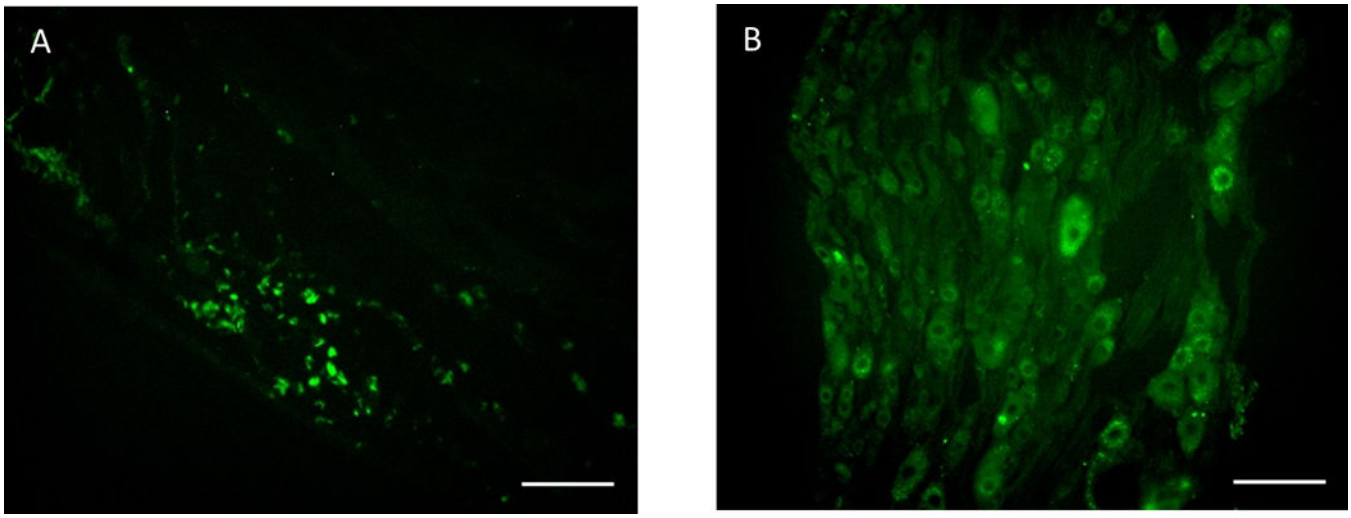

Figure 6.

HSV vector-mediated GFP expression in the bladder (A), L6 dorsal root ganglia (DRG) (B). GFP positive cells were observed in the bladder and L6 DRG, indicating that HSV vectors injected into the bladder wall were transported to DRG neurons through afferent nerves. Scale bars indicate 100 μm

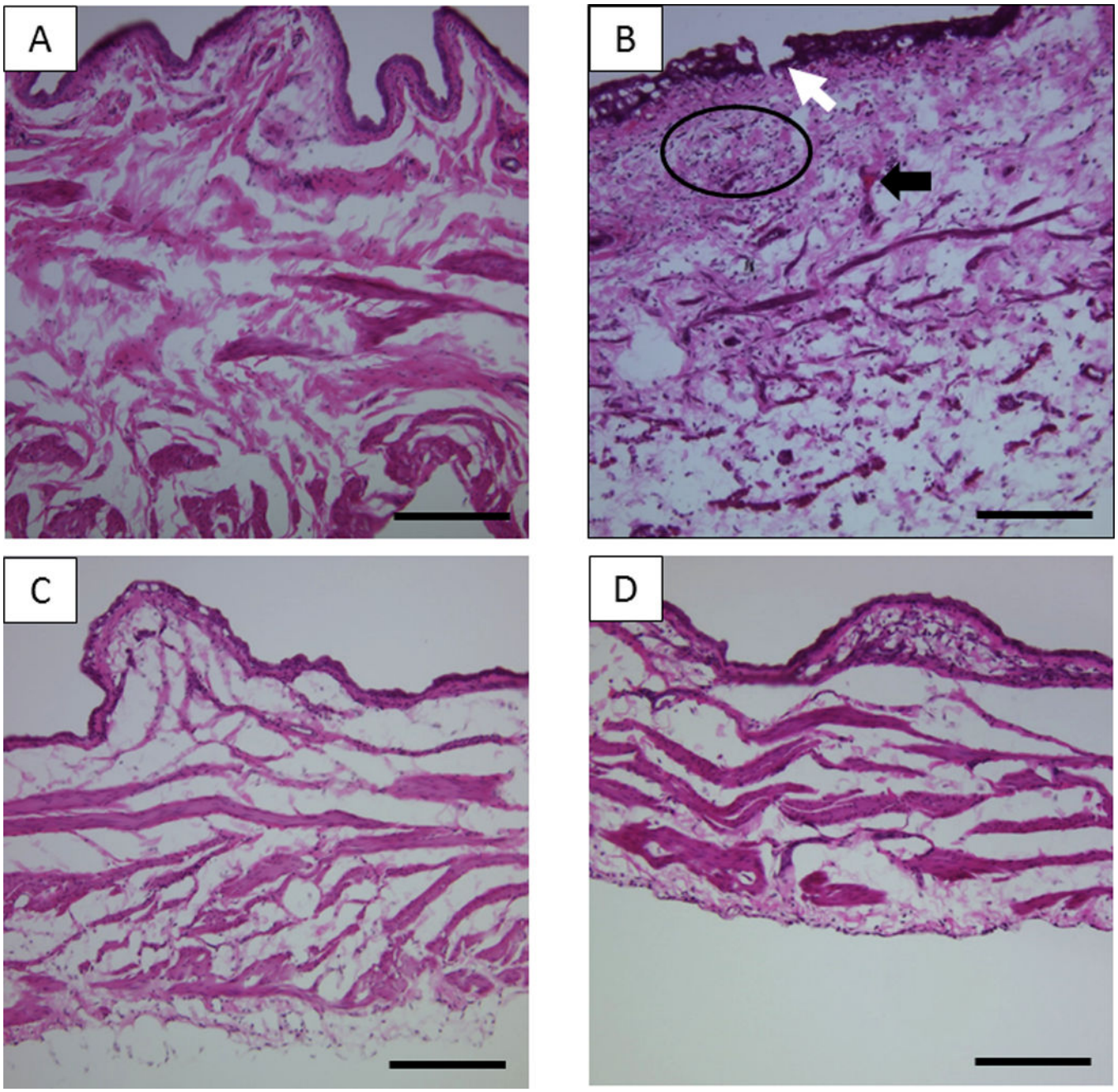

Figure 7.

Hematoxylin-Eosin staining of the bladder. There is substantial inflammation characterized by the infiltration of inflammatory cells (circled area), damaged urothelium (white arrow), and submucosal bleeding (black arrow) in the bladder wall in the HP-GFP (B) group compared with NS-GFP (A) group, which were alleviated in the HP-PL (C) and HP-PP1α (D) groups. These results indicate that the HP-PL or HP-PP1α treatment is effective to reduce bladder inflammatory changes in rats with HP-induced cystitis. Scale bars indicate 200 μm

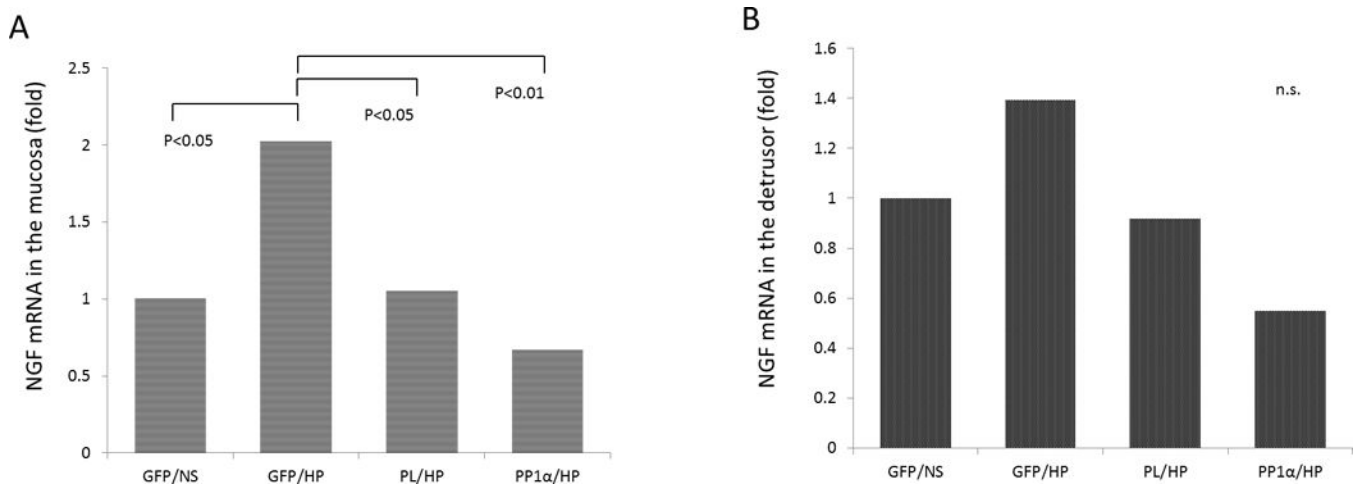

Figure 8.

Levels of NGF mRNA in the bladder mucosa (A), and in the detrusor (B). The data are expressed as relative values of mRNA levels in each of three HP groups vs. NS-GFP group whereas the statistical analysis was performed using the NGF mRNA expression ratio against the house keeping gene (GAPDH) calculated in each rat. In RT-PCR, the HP-GFP group showed significantly higher expression of NGF mRNA in the bladder mucosa ($P<0.05$) than the NS-GFP group, whereas the HP-PL and HP-PP1α groups showed significantly lower expression of NGF ($P<0.05$ and $P<0.01$, respectively) than the HP-GFP group. In the detrusor, there was no significant difference in the expression of NGF among the 4 groups. These results indicate that the HP-PL or HP-PP1α treatment is effective to reduce NGF overexpression in the bladder mucosa of rats with HP-induced cystitis.