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Nerve-mediated bladder contraction is impaired by cytokines: involvement of inducible nitric oxide synthase

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Abstract

We investigated the possible involvement of inducible nitric oxide synthase (iNOS) in the effect of cytokines on neuromuscular function in isolated rat bladder strips. Bladder strips were incubated in cell culture medium for 24 h with or without tumour necrosis factor-α (TNF-α) + interleukin-1β. Mechanical activity in response to electrical field stimulation and carbachol was recorded in organ baths. Both the electrical field stimulation- and carbachol-induced contractions were reduced by the incubation. The electrical field stimulation-induced contraction was significantly further impaired after prolonged exposure to TNF-α + interleukin-1β. This impairment was restored by dexamethasone, the iNOS inhibitor aminoguanidine and partially by brain-derived neurotrophic factor (BDNF). In contrast, carbachol-induced contractions were not affected by cytokines. iNOS protein expression was detected in cytokine-incubated bladder strips by immunohistochemistry and Western blot analysis. The results demonstrated that TNF-α + interleukin-1β impaired nerve-mediated bladder contractions. Aminoguanidine, and to some extent BDNF, exerted neuroprotective effects.

Keywords: Aminoguanidine; Brain-derived neurotrophic factor (BDNF); Nitric oxide (NO)

1. Introduction

Various bladder infections and diseases such as urinary tract infection, interstitial cystitis and bladder cancer are associated with elevated levels of cytokines (Davidoff et al., 1997; Hang et al., 1998; Martins et al., 1994). Secretion of cytokines contributes to immune activation, but non-immune cells in the bladder, like smooth muscle cells and nerves, are also likely to be exposed to cytokines. Exposure of aortic smooth muscle preparations to inflammatory mediators is known to impair the contractility (McKenna, 1990; Robert et al., 1992). Induction of nitric oxide (NO) has been demonstrated to play a role in cytokine-mediated dysfunction in vascular smooth muscle (French et al., 1991; Lu and Fiscus, 1999). NO is produced by three different isoforms of the enzyme NO synthase (NOS). Neuronal NOS (nNOS) and endothelial NOS (eNOS) are constitutively expressed, while inducible NOS (iNOS) is transcriptionally induced by proinflammatory stimuli such as cytokines and microbial agents (Xie and Nathan, 1994).

iNOS has been observed in vivo in the bladder upon experimental urinary tract infections (Olsson et al., 1998; Poljakovic et al., 2001) and following bladder outflow obstruction (Johansson et al., 2002a; Lemack et al., 1999). Furthermore, isolated bladder smooth muscle cells have been found to express iNOS upon direct exposure to cytokines (Johansson et al., 2002b; Xu et al., 2001). iNOS induction in the urinary bladder by Bacillus Calmette-Guerin treatment of bladder cancer (Jansson et al., 1998) and due to cyclophosphamide-induced cystitis (Xu et al., 2001) have been considered to be secondary to an increase in cytokines.

Induction of iNOS in inflammatory conditions is associated with production of high local levels of NO. NO derived from iNOS is primarily thought to exert an anti-microbial and immunoregulatory effect. However, exaggerated production of NO may be cytotoxic to the host and cause tissue damage. NO forms cytotoxic peroxynitrite upon reaction with superoxide which may cause lipid peroxidation (Hogg and Kalyanaraman, 1999) and nitrotyrosine formation (Ischiropoulos, 1998). Cytoskeletal and contractile fila-

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ments, as well as neurofilaments, are known to be susceptible to nitration (Beckman and Koppenol, 1996). In the bladder, NO release may cause damage of both smooth muscle cells and neurons and thus contribute to urinary tract dysfunctions.

The aim of the present study was to investigate a possible involvement of iNOS in the effect of cytokines on neuromuscular function in isolated rat bladder strips. A method involving prolonged exposure of bladder strips to cytokines was employed and characterized.

2. Materials and methods

2.1. Incubation of bladder strips

Female Sprague–Dawley rats (200 g) were sacrificed by CO_2 asphyxia and the bladder was carefully removed. Bladder strips, taken in the circular direction, were prepared from middle third of the detrusor. The strips were transferred to 12-well plates with 2 ml cell Dulbecco's modified Eagle's medium with 10% fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin and 0.1 mg/ml streptomycin (all from Sigma). The bladder strips were incubated with the inflammatory cytokines tumour necrosis factor-α (TNF-α; 50 ng/ml) + interleukin-1β (2 ng/ml), alone and in combination with the iNOS inhibitor aminoguanidine (0.5 mM), the general inflammatory inhibitor dexamethasone (0.1 μM) and brain-derived neurotrophic factor (BDNF; 100 ng/ml) for 24 h. The cytokine combination used in this study has previously been found to stimulate iNOS induction in the rat bladder (Johansson et al., 2002b).

2.2. Recording of mechanical activity

The strips were transferred to 5-ml tissue baths containing Krebs solution (in mM; NaCl 119, KCl 4.6, $CaCl_2$ 1.5, MgCl 1.2, $NaHCO_3$ 15, NaH_2PO_4 1.2, glucose 5.5) bubbled with carbogen gas. After mounting, the strips were stretched to a passive tension of 4 mN and allowed to equilibrate for 45–60 min before further experiments were performed. The experiments were started by exposing the preparations to a K^+ (124 mM) Krebs solution until two reproducible contractions had been obtained. The contractile responses were expressed in percent of these KCl-induced contractions. Transmural stimulation of nerves was performed using a Grass stimulator delivering single square wave pulses (duration 0.5 ms). Stimulation was accomplished by means of two platinum electrodes placed on either side of the preparations. The voltage was maximal and determined individually for each strip. The train duration was 5 s and the polarity was shifted after each pulse by means of a polarity-changing unit. The stimulation interval was 2 min. Frequency–response relations (2–60 Hz) to electrical field stimulation were obtained and concentration–response curves were constructed in response to carbachol (0.01–100 μM). Some experiments were performed in the presence of tetrodotoxin (1 μM). The acute effect of aminoguanidine (0.5 mM) during electrical field stimulation-induced contraction at 20 Hz was investigated in cytokine-treated and incubated control bladder strips.

2.3. Immunohistochemistry

Bladder strips were incubated for 24 h with TNF-α + interleukin-1β as described above and fixed in cold 4% formaldehyde in phosphate-buffered saline (PBS) for 4 h. The tissue was rinsed in PBS containing 15% sucrose for 2–3 days. Both fixation and rinsing were performed at 4 °C, after which the specimens were frozen in isopenthane at −40 °C and stored at −70 °C until sectioning. Tissue sections were cut at a thickness of 10 μm and pre-incubated with PBS containing 0.2% Triton X-100 in 0.1% bovine serum albumin for 2 h at room temperature. Incubation with rabbit polyclonal antibody raised against iNOS (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA) or rabbit anti-nitrotyrosine polyclonal antibody (1:10,000; Chemicon International, CA, USA) was performed overnight. For the visualization of the immunoreactive products, the sections were rinsed in PBS and then incubated for 90 min with fluorescein isothiocyanate-conjugated donkey anti-rabbit immunoglobulin G (IgG) (1:80; Jackson Immunoresearch Laboratories, West Grove, PA) diluted in PBS. All incubations with primary and secondary antisera were performed at room temperature in moisture chambers. The sections were finally rinsed and mounted in PBS/glycerol with *p*-phenylenediamine to prevent fluorescence fading. In control experiments, no immunoreactivity was detected in sections incubated with only the secondary antibody. All micrographs of the immunolabelled sections were obtained using a digital camera system (Olympus BX60F-3 microscope and Olympus Digital camera DP-50; Olympus Optical, Tokyo, Japan), and the pictures were captured using appropriate filter settings for fluorescein isothiocyanate. Adobe® Photoshop™ was used for image handling.

2.4. Western blot analysis

Bladder strips were incubated for 24 h with TNF-α + interleukin-1β as described above and were analyzed by Western blot for detection of iNOS protein expression. The bladder strips were homogenized with an electric homogeniser in ice-cold homogenization buffer containing Tris–HCl (0.067 M), glycerol (11%), sodium dodecyl sulfate (SDS; 2.2%), protease inhibitor cocktail (Complete, mini EDTA-free, Roche Diagnostics, Mannheim, Germany). The homogenate was centrifuged at 20,000 × *g* for 30 min at 4 °C. The protein content was determined by BioRad DC protein assay (Bio-Rad Laboratories, Hercules, CA, USA) with bovine serum albumin as standard (Pierce, Rockford, IL, USA). 5% β-Mercaptoethanol (Sigma) and bromphenol-blue were added and the samples were boiled

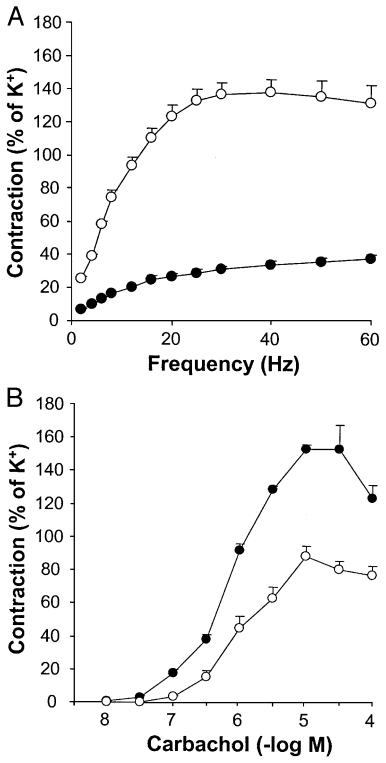

Fig. 1. Contractile responses of freshly isolated bladder strips and bladder strips incubated in cell culture medium for 24 h. (A) Electrical field stimulation- and (B) carbachol-induced contractions of freshly isolated strips (○) and strips incubated for 24 h in cell culture medium (●). Contractions were expressed as percent of the K^+-induced contraction. Data are given as mean ± S.E.M. ($n = 4–6$).

for 5 min. Samples containing 50–100 μg protein were separated by 7.5% SDS-polyacrylamide gel electrophoresis (Bio-Rad Laboratories) and blotted onto a polyvinylidine difluoride membrane (Bio-Rad Laboratories). The membrane was blocked with 5% non-fat milk for 4 h in room temperature and incubated with a rabbit polyclonal antibody raised against iNOS (1:1000; Santa Cruz Biotechnology) or rabbit anti-nitrotyrosine polyclonal antibody (1:10,000; Chemicon International) overnight at 4 °C. Subsequently, the membrane was incubated with a secondary donkey anti-rabbit IgG antibody conjugated with horseradish peroxidase (1:20,000; Santa Cruz Biotechnology) for 1 h in room temperature. The bound antibodies were visualized using enhanced chemiluminescence reagents (ECL) and exposure to X-ray film (both from Amersham Pharmacia Biotech, Buckinghamshire, UK).

2.5. Analysis of data

Data are given as mean ± standard error of the mean (S.E.M.). Analysis of variance (ANOVA) followed by Bonferroni–Dunn post hoc test performed the statistical analysis of data. Statistical significance was accepted when $P < 0.05$.

3. Results

3.1. Contractility of incubated bladder strips compared with freshly isolated strips

The contractile response was decreased in bladders strips incubated in cell culture medium for 24 h compared to freshly isolated strips. The contractile response to K^+ (124 mM) amounted to 27 ± 2.6 mN ($n = 7$) in freshly isolated strips and to 6.1 ± 0.8 mN ($n = 8$) in incubated strips. The electrical field stimulation-induced response (40 Hz) was markedly attenuated in incubated strips (2.3 ± 0.5 mN; $n = 8$) compared to freshly isolated bladder strips (41 ± 4.2 mN; $n = 7$). The response to carbachol (10 μM) was also affected by incubation (incubated strips, 9.6 ± 0.8 mN; $n = 8$, freshly isolated strips 24 ± 2.4 mN; $n = 7$).

The contractile response to electrical field stimulation, expressed as percent of the K^+-induced contraction, was markedly reduced in incubated bladder strips (Fig. 1A). In contrast, the carbachol-induced contraction increased in incubated bladder strips when expressed as percent of the K^+-induced contraction (Fig. 1B).

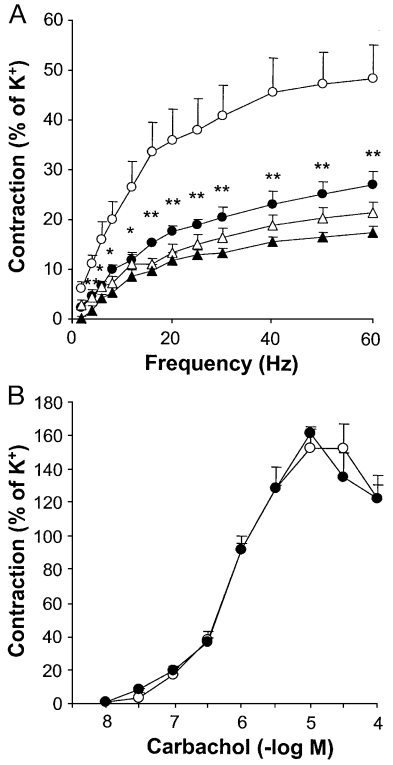

Fig. 2. The effect of stimulation with TNF-α + interleukin-1β for 24 h on bladder contractility. (A) Electrical field stimulation- and (B) carbachol-induced contractions in control (○) and cytokine-stimulated (●) strips. Electrical field stimulation-induced contractions in the presence of tetrodotoxin (1 μM) in control (△) and cytokine-stimulated (▲) strips are shown in panel A. Contractions were expressed as percent of the K^+-induced contraction. Data are given as mean ± S.E.M. ($n = 3–8$). $^{**}P < 0.01$, $^{*}P < 0.05$ control vs. cytokine-stimulated strips.

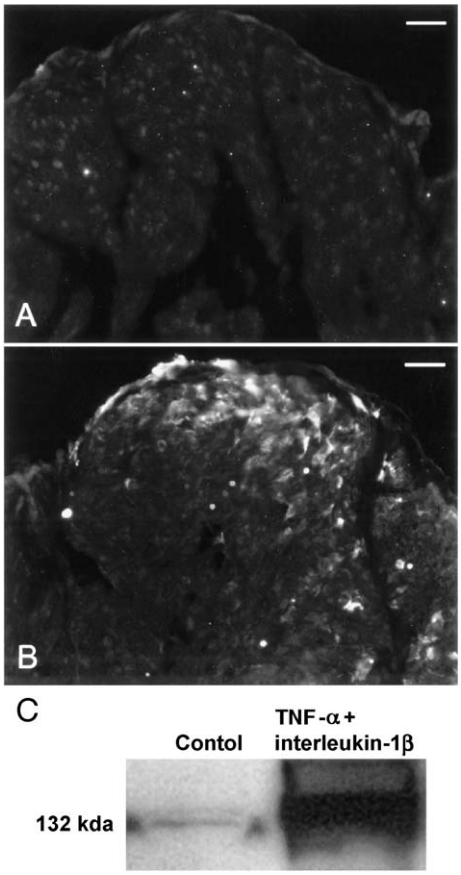

Fig. 3. iNOS protein expression in bladder muscle strips. (A) Unstimulated bladder strips showed no iNOS immunoreactivity. (B) iNOS immunoreactivity in cytokine-stimulated bladder strips at the serosal side of the smooth muscle layer and in interstitial cells. Scale bar 60 μm. (C) Western blot analysis of iNOS protein in homogenate of bladder strips incubated with TNF-α + interleukin-1β and in control strips.

3.2. The effect of TNF-α+interleukin-1β on bladder contractility

The contractile response to K^+ was not altered by prolonged incubation with the cytokines TNF-α + interleukin-1β (5.7 ± 1.1 mN; $n = 8$) compared to incubated control strips (6.1 ± 0.8 mN; $n = 7$). The response to electrical field stimulation was significantly decreased in bladder strips incubated with TNF-α + interleukin-1β for 24 h compared to incubated control strips (Fig. 2A). Electrical field stimulation-evoked contractions in incubated strips had a non-neurogenic component as revealed by tetrodotoxin experiments. Approximately 55% of the electrical field stimulation-evoked contraction was nerve-mediated in control strips, whereas 35% of the contraction in cytokine-treated strips was nerve-mediated (Fig. 2A). The immediate effect of TNF-α + interleukin-1β on electrical field stimulation-induced contraction was also investigated. Freshly isolated bladder strips were incubated with the cytokines TNF-α + interleukin-1β for 30 min. Electrical field stimulation-induced contractions were not impaired after immediate cytokine stimulation (data not shown). The contractile response evoked by carbachol was not affected by TNF-α + interleukin-1β exposure (Fig. 2B).

3.3. iNOS protein expression in cytokine-stimulated bladder strips

iNOS positive cells were demonstrated by immunohistochemistry in bladder strips stimulated with TNF-α + interleukin-1β for 24 h. The iNOS positive cells were located mainly at the serosal side and within the smooth muscle layer (Fig. 3B). No iNOS immunoreactivity was found in nerve fibers. iNOS protein expression in TNF-α + interleukin-1β stimulated bladder strips was confirmed by Western blot analysis (Fig. 3C). A faint iNOS protein band was also detected in unstimulated control strips. Nitrotyrosine was not detected with Western blot analysis or immunohistochemistry in cytokine-stimulated bladder strips (data not shown).

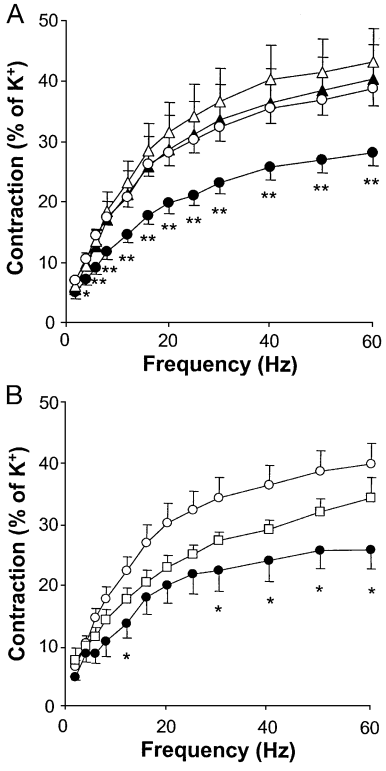

Fig. 4. (A) Effect of inhibitors on the cytokine-mediated impairment of electrical field stimulation induced bladder contractility. The contractile response was measured in unstimulated strips (○), in strips stimulated with TNF-α + interleukin-1β alone (●) and in combination with dexamethasone (△) and aminoguanidine (▲); $n = 6–14$. (B) Effect of BDNF on the cytokine-mediated decrease in electrical field stimulation-induced contractility. Electrical field stimulation in unstimulated strips (○), in strips incubated with TNF-α + interleukin-1β alone (●) and in the presence of BDNF (□); $n = 6$. Data are expressed as mean ± S.E.M. $^{**}P < 0.01$, $^{*}P < 0.05$ unstimulated vs. cytokine-stimulated strips.

3.4. Restoration of electrical field stimulation-evoked contraction by aminoguanidine, dexamethasone and BDNF

Addition of the iNOS inhibitor aminoguanidine (0.5 mM) during the incubation completely restored the cytokine-mediated decrease in contraction (Fig. 4A). Direct addition of aminoguanidine during electrical field stimulation-induced contractions (20 Hz) had no effect on contractility in cytokine-treated (106 ± 2%; $n = 4$) or incubated control strips (103 ± 2%; $n = 4$). Dexamethasone (0.1 μM) also completely restored the cytokine-induced decrease in contraction (Fig. 4A). BDNF is known to exert a restorative effect on cholinergic neurons. Incubation with BDNF (100 ng/ml) slightly, although not significantly, improved the electrical field stimulation-induced contractions in cytokine-stimulated bladder strips (Fig. 4B).

4. Discussion

The effect of cytokine exposure on urinary bladder function has not been established. In this study, we found that cytokine exposure for 24 h impaired electrical field stimulation-evoked bladder contractions by a prejunctional effect. The cytokine-mediated impairment of the nerve-induced contraction may involve iNOS, since the iNOS inhibitor aminoguanidine completely restored the response.

To be able to expose bladder strips to cytokines for a prolonged time, a ‘‘culture’’ method was employed. Compared to fresh bladder strips, strips incubated in cell culture medium for 24 h showed an overall reduced contractile response to K^+. The decreased contractile response to K^+ after incubation is consistent with observations made in aorta strips (Wylam et al., 2001). The electrical field stimulation-evoked response was more suppressed after incubation than the response evoked by carbachol when expressed in absolute values (mN). This suggests that the bladder nerves were more affected by the incubation conditions than the smooth muscle. However, the contractile response to carbachol, relative the K^+ response, increased in incubated compared to fresh bladder strips. Denervation is known to cause supersensitivity to the transmitter released from neurons (Westfall, 1981). Such a mechanism may contribute to the increased carbachol response, since several studies have shown that denervation of the urinary bladder can lead to supersensitivity to muscarinic receptor stimulation (Ekstrom and Malmberg, 1984; Persson et al., 1998).

The cytokines TNF-α + interleukin-1β reduced electrical field stimulation-mediated contractions in bladder strips, but the contractile response to carbachol was unaltered. This indicates that bladder nerves but not the postjunctional muscarinic receptor response was affected by cytokines. Thus, the decrease in electrical field stimulation-evoked contractility caused by cytokines may be explained by a prejunctional effect. Moreover, in the presence of cytokines, the tetrodotoxin-sensitive part of the contraction was nearly abolished which further demonstrates that cytokines have pronounced effects on bladder nerves.

TNF-α + interleukin-1β had no immediate effects on bladder muscle contractility, but a reduced nerve-mediated contraction was consistently observed after incubation for 24 h. This suggests that the effect of cytokines in incubated strips is caused by induction of secondary mediators. Dexamethasone exerts anti-inflammatory actions by inhibiting inflammatory transcription factors and by inducing anti-inflammatory proteins (Saklatvala, 2002). The addition of dexamethasone during the incubation was found to completely restore the cytokine-mediated reduction of electrical field stimulation-induced contractions, which indicates involvement of inflammatory mediators. The iNOS inhibitor aminoguanidine caused a complete restoration of the effect. iNOS protein expression was confirmed by Western blot analysis and immunohistochemistry in cytokine-stimulated bladder strips. These data suggest that the impaired nerve-evoked contraction after cytokine exposure involves induction of iNOS. A weak positive iNOS protein band was found in unstimulated incubated bladder strips. This may be due to the possible tissue damage during handling or oxygen deprivation. Both hypoxia and shear stress are known to induce iNOS (Miyajima et al., 2000; Saito and Miyagawa, 1999).

NO was found to inhibit acetylcholine release in the rabbit urinary bladder (Miyamoto et al., 2001). NO produced after cytokine exposure may thus influence bladder nerves by inhibiting acetylcholine release. In the present study, direct addition of aminoguanidine had no acute effect on electrical field stimulation-induced contractility in cytokine-treated bladder strips indicating that aminoguanidine did not restore electrical field stimulation by affecting neurotransmitter release. Aminoguanidine, at least in our experiments, may rather exert a protective effect by inhibiting the production of neurotoxic NO from iNOS. Indeed, NO is suggested to be involved in several neurodegenerative diseases by various mechanisms (Dawson and Dawson, 1998). NO can mediate its effect by depolarizing the plasma- and mitochondrial membranes of neurons (Mongin et al., 1998) that may lead to decreased excitability. High levels of NO derived from iNOS may form cytotoxic peroxynitrite (Beckman and Koppenol, 1996; Ischiropoulos, 1998). Motoneuron degeneration is suggested to involve peroxynitrite formation (Estevez et al., 1998). We investigated the expression of nitrotyrosine that is commonly used as a marker for peroxynitrite formation. Nitrotyrosine expression was, however, not detected in our tissue.

An increase in BDNF and other nerve growth factors following bladder dysfunction due to spinal chord injury, cyclophosphamide-induced cystitis and bladder outflow obstruction, has been observed (Vizzard, 2000; Zvara et al., 2002), indicating that BDNF plays a role in the pathophysiology of bladder dysfunction. BDNF is known to mediate survival of cholinergic neurons in culture (Nonomura and Hatanaka, 1992) and to exert a neuroprotective

effect via the regulation and inhibition of NOS expression in neurons (Novikov et al., 1995; Sharma et al., 1998). BDNF was found to suppress increased NOS activity in degenerating motoneurons and was thought to be important in halting NO-mediated neurodegeneration (Tsuzaka et al., 2001). In our study, BDNF slightly improved the cytokine-mediated impairment of electrical field stimulation-evoked contraction of incubated bladder strips. The observed protective effect of BDNF demonstrated in our study may be due to inhibition of cytokine-evoked iNOS expression and/or NO production.

Bladder dysfunctions may involve cytokine-mediated induction of iNOS and other mediators. We have demonstrated that cytokines impair nerve-induced contraction of bladder muscle strips and that an iNOS inhibitor, and to some part BDNF, exert neuroprotective effects.

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