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Role of Urothelial Nerve Growth Factor in Human Bladder Function

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Abstract

Aims—To test whether nerve growth factor (NGF) concentration in human bladder urothelium/suburothelium is related to detrusor overactivity (DO), bladder sensation, detrusor contractility, or other aspects of lower urinary tract function.

Materials and Methods—Concentration of NGF was measured (using ELISA) in superficial bladder biopsies from 27 women (mean age 52 years, range 22–82) after comprehensive videourodynamics and bladder diary. Approximately half (12/27) showed clear DO and half did not.

Results—There was no evidence for increased NGF concentration in subjects with DO (association negative by Mann–Whitney test, $P = 0.23$). NGF was not significantly associated with two measures of detrusor contractility (Spearman's $r = -0.29$, $P = 0.17$; $r = -0.20$, $P = 0.33$); nor with four measures inversely related to sensation: volume at strong desire to void and maximum capacity on cystometry ($r = -0.13$, $P = 0.53$; $r = -0.23$, $P = 0.28$), and maximum voided volume and mean daytime voided volume on bladder diary ($r = -0.29$, $P = 0.16$; $r = -0.16$, $P = 0.44$). It was significantly associated with 24-hr urine output on bladder diary (Spearman's $r = -0.55$, $P = 0.004$).

Conclusions—Elevated NGF levels in human urothelium/suburothelium are not strongly associated with DO, detrusor contractility or increased bladder sensation. NGF levels are lower in subjects with higher 24-hr urine output. This observation is consistent with a role for NGF in an active process (trafficking) involved in bladder filling.

Keywords

bladder sensation; detrusor contractility; detrusor overactivity; nerve growth factor; urine output

INTRODUCTION

Nerve growth factor (NGF) is involved in many active processes. Its role in bladder behavior is unknown, but it may contribute to the abnormalities that are characteristic of pathological conditions such as spinal cord injury, urethral obstruction, and chronic bladder inflammation. In spontaneously hypertensive rats, for example, elevated NGF levels in bladder muscle are associated with hyperactive voiding¹ and bladder overactivity.²

Increased NGF expression has been linked to alterations in afferent excitability^{3–5} and to mechanical stretch.⁶ In spinal cord injured patients, treatment of the bladder with botulinum toxin not only reduces neurogenic detrusor overactivity (DO), but also leads to a reduction in NGF levels.⁷ Bladder NGF levels are elevated in men with bladder outlet obstruction⁸ and they diminish following treatment with an alpha-blocker which reduces obstruction and DO.⁹ Elevated NGF levels in human urothelium are associated with increased bladder sensation.¹⁰ Finally, long-standing diabetes leads to reduced bladder contractility and sensation in humans, and to reduced NGF levels in rats.¹¹

Thus, there is evidence that NGF is involved in bladder dysfunction, especially abnormal afferent signaling and bladder sensation, and perhaps DO or underactivity.¹² Because urothelial tissue has neuronal-like properties, and afferent nerve endings are located in the urothelium and suburothelial layer,¹³ the concentration of NGF in this location may be important for bladder function.

This is a secondary study of biopsy material gathered in the course of a study (of detrusor ultrastructure), which determined the number and type of subjects and the assessments they underwent. We have measured the concentration of NGF in superficial bladder biopsies obtained from women who had undergone comprehensive testing of lower urinary tract function including careful classification of DO status (see below). Postulating that urothelial or suburothelial NGF concentration might be related to DO, bladder sensation, or detrusor contractility, we identified the relation of urodynamic variables in these three domains to NGF concentration. We expected that the results would illuminate the role of NGF and the urothelium in bladder function and dysfunction.

MATERIALS AND METHODS

Subjects

Subjects were female volunteers over 20 years of age, either with or without symptoms suggestive of DO. Males were excluded because of potential difficulty in obtaining biopsies and the possible confounding effect of urethral obstruction. Potential subjects were excluded if there was a history of vitamin B12 deficiency; multiple sclerosis, Alzheimer's or other dementing disease, diabetes mellitus, alcohol abuse, bladder carcinoma, pelvic irradiation, anaphylactic reaction to local anesthetic, abdominoperineal resection, disc disease, detrusor-sphincter dyssynergia; current urinary tract infection, pregnancy, current anticholinergic medication or acetylsalicylic acid that could not be stopped for the study, or current coumadin. Suprapontine lesion was not an exclusion criterion because a few subjects with such a lesion were required for the main study. However, the majority of subjects had no overt neurological abnormality and no subject had had a stroke.

Subjects were volunteers recruited by advertizing and gave written informed consent. Study procedures were approved by the University of Pittsburgh institutional review board.

Clinical Assessment

Baseline evaluation included medical history, physical examination, bladder diary, and comprehensive urodynamics. Dipstick testing and urinalysis were performed to rule out infection.

Voiding Diaries

Following instruction, subjects completed a baseline voiding diary for 3 days, recording voiding times and volumes and leakage episodes. They estimated the amount of leakage as small (drops, about 5 ml), moderate (wets outer clothes, about 15 ml), or large (soaks

through outer clothes, runs to floor, about 60 ml). We included these estimated amounts in the urine output totals.

Urodynamics

Videourodynamic evaluation (Laborie Aquarius, OEC C-arm and Sonesta X-ray table) included uroflowmetry and post-void residual (PVR) urine; fluoroscopically-monitored provocative medium-fill (30 ml/min) cystometry, initially in the supine position, using a 7 Fr twin-lumen urethral catheter, a rectal balloon catheter, and room-temperature Cystografin; seated pressure-flow study with the same catheters; and isovolumetric testing using a 3-channel balloon catheter to occlude the urethra.¹⁴ No bladder volume limit was imposed other than patient discomfort. Maneuvers aimed at provoking DO included: coughing, suprapubic tapping, running water, hand-washing, catheter manipulation, change of posture from supine to standing, Valsalva, heel jouncing, and 2-min wait with full bladder after sitting on a commode. Methods, definitions, and units conform to the standards recommended by the International Continence Society (ICS), except where specifically noted.

After urodynamics, subjects were classified either as “cases,” with DO on urodynamics and one or more of the following symptoms both on history and voiding diary: urgency (precipitant urge to void); increased frequency (≥ 9 voids/day not accounted for by increased fluid output); nighttime frequency (≥ 2 voids/night not accounted for by fluid output); or as “controls” without DO and without these symptoms. Subjects who could not be classified in this way (e.g., with DO but without the above symptoms), or whose classification was doubtful, did not participate in this study. Thus, the final study population consisted of two clearly distinct subgroups: “cases” with DO, confirmed by corresponding symptoms; and “controls” without DO, confirmed by absence of such symptoms.

Biopsies

Biopsies (diameter 1–1.5 mm) were taken 15–20 mm superior to the left and right ureteric orifices, under 2% lidocaine anesthesia of urethra and biopsy sites, using a rigid cystoscope (Storz 19 Fr) and cold cup forceps under direct vision. At each site three successively deeper biopsies were taken. The two deeper biopsies were used for the main study. The superficial biopsies used for this study consisted mainly of urothelium and suburothelium, although a small amount of detrusor muscle may sometimes have been present. Biopsies were immediately immersed in HBSS (5 mM KCl, 0.3 mM KH_2PO_4 , 138 mM NaCl, 4 mM NaHCO_3 , 0.3 mM Na_2HPO_4 , 5.6 mM Glucose, and 10 mM HEPES, pH 7.4; Sigma, St. Louis, MO) containing complete protease inhibitor cocktail tablets (Roche, Penzburg, Germany) added at 1 tablet/10 ml. Following biopsy, the sites were cauterized and subjects were observed until the urine became clear. Biopsies from left and right were combined to maximize the amount of material from each subject.

Tissue Preparation

Biopsies were homogenized in 100 μl of HBSS with protease inhibitor using a tissue tearor (Biospec Products, Bartlesville, Oklahoma) and frozen at -80°C immediately after preparation for later analysis in batches. For analysis, the homogenate was centrifuged at 13,000 rpm for 15 min. The supernatant is referred to as the cytosol lysate. The membrane protein fraction was prepared by suspending the membrane pellets in lysis buffer containing 0.3 M NaCl, 50 mM Tris-HCl (pH 7.6), and 0.5% Triton X-100 and the same concentration of protease inhibitors as above. The suspensions were incubated on ice, sonicated and centrifuged at 13,000 rpm for 15 min at 4°C . A whole cell lysate was prepared by combining both the cytosol and membrane protein fractions. The protein concentrations were determined using the BCA protein assay (Pierce).

NGF Elisa

Lysates were diluted with 4 volumes of D-PBS (0.02% KCl, 0.8% NaCl, 0.02% KH₂PO₄, 0.115% Na₂HPO₄, 0.0133% CaCl₂ · 2H₂O, and 0.01% MgCL₂ · 6H₂O, pH 7.35, Sigma). The samples were then acidified with 1 N HCl to pH 2.0–3.0 for 15 min at room temperature and neutralized by 1 N NaOH to pH 7.5–8.0. The samples were assayed in duplicate by ELISA (Promega, Madison, Wisconsin). Plates were read at 450 nm using a SpectraFluor Plus (Tecan, Maennedorf, Switzerland) and the absorbance of the blank value (the mean of the assay wells without sample) was subtracted from each sample's absorbance. Tissue NGF values were normalized against the protein concentrations of each sample and expressed as picograms per microgram protein. To investigate possible NGF changes during storage, some samples were assayed a second time after further storage.¹⁵

Statistics

Spearman's rank correlation coefficient was used to examine bivariate associations between normalized NGF concentration and variables in the three domains specified a priori, such as bladder sensation and detrusor contractility. For the dichotomous variable DO we used the Mann–Whitney test. We also examined associations with age, presence of suprapontine lesion, and voiding diary variables. We used stepwise multiple linear regression ($P < 0.05$ for insertion; $P > 0.10$ for removal) to examine the relative importance of potential predictors of NGF concentration revealed by bivariate associations and to estimate the effect of variable storage time.

Significance Levels and Multiple Comparisons

Bivariate associations were taken as significant if two-tailed $P < 0.05$. The number of multiple comparisons was limited by selecting, a priori, representative variables from only three domains.

Statistical Power

With two-tailed $P < 0.05$, 26 subjects would be required to provide 80% power to detect a (Pearson) correlation coefficient of 0.5 between NGF concentration and other variables. Assuming a standard deviation of 0.9 pg/μg for NGF concentration, there is 80% power to detect a difference of 1.0 pg/μg between two groups.

Domains and Variables Examined

Detrusor overactivity was classified by “cases” versus “controls” (see Table I). For bladder sensation, we used the standard urodynamic variables bladder volume at strong desire to void and maximum cystometric capacity.¹⁶ To better represent daily life, we added voiding diary variables, including mean and maximum voided volumes, and mean daytime voiding frequency (commonly taken as a proxy for sensation). For detrusor contractility we used maximum isovolumetric detrusor pressure, both as measured during a mechanical or continuous-occlusion stop test,¹⁴ and as estimated from the contractility index $PIP_1 (= Q_{\max} + p_{\det, Q_{\max}})$ in a voiding study;¹⁷ we also used the minimum PVR urine after free uroflow.

RESULTS

Biopsies were obtained from 27 women, mean age 52 years, range 22–82 years. One biopsy contained insufficient protein to examine. Twelve women were “cases” with DO and 15 were “controls” with no such activity. Mean ages of “cases” and “controls” were 53 and 50 years, respectively. Seven subjects (three “cases” and four “controls”) had a suprapontine lesion due to brain tumor. Except possibly for these three “cases”, all DO was idiopathic, and even in these three cases there was no obvious clinical or urodynamic evidence of

neurological involvement. None of the subjects was taking specific bladder relaxant medication. Only one subject, with DO, used a medication (amitryptiline 25 mg/day, continued throughout the study) other than estrogen that might have affected bladder or sphincter function.

Median 24-hr urine output was 1,587 ml, with a range from 858 up to 3,680 ml. Such high values are common in the normal population in our region. Estimated 24-hr urine leakage on bladder diary ranged from 0 to 38 ml, with a median value of 0 ml. Thus any uncertainty in this estimate did not greatly affect the calculation of total urine output. Mean values, standard deviations, and ranges of measured variables are shown in Table I. NGF concentrations ranged from 0.28 to 3.41 pg/ μ g of protein, with mean value 1.37. NGF concentration was not significantly different in “cases” (1.16 ± 0.84 pg/ μ g, mean $\pm$ SD) and “controls” (1.54 ± 0.89 pg/ μ g), although it tended to be lower in the “cases” ($P = 0.23$), as shown in Figure 1.

The storage time before assay ranged from 1 to 157 days with a mean of 50 days. In order to check the effect of storage time, repeated evaluations were made on 14 samples after further storage intervals of 13, 22, or 58 days. They suggested that on average NGF concentration decreased in storage at $(3.0 \pm 0.9)\%$ per day. Table II shows the mean and standard error (SE) of the change in NGF concentration after each of these three fixed intervals, both overall and broken down according to presence/absence of DO and of elevated fluid output (cut point at median value, 1,587 ml/day). Although numbers are small, the trend to decreasing NGF concentration with longer storage interval is visible. For all 27 women, however, the normalized NGF concentrations (the initial value if measurement was repeated) showed no monotonic dependence on storage time, consistent with others’ observations showing an initial increase followed by a decrease.¹⁵ Therefore, we did not correct for storage time, but we later performed multiple regressions with storage time as one independent variable. We also checked critical results after stratification into two subgroups with storage times less or greater than the median value of 34 days (corresponding approximately to the expected peak value of concentration).

Table I shows the bivariate associations between NGF concentration and the variables representing DO, bladder sensation, and detrusor contractility. All were negative but none was significant. The association of NGF concentration with DO remained negative in the two subgroups stratified with respect to storage time, although it was significant in neither ($P = 0.35$ and 0.53 , respectively). The only a priori variable with a near-significant association with NGF concentration was 24-hr voiding frequency. Since voiding frequency depends also on urine output as well as sensation, we also examined the association of NGF concentration with 24-hr urine output. It was negative and significant (see Table I and Fig. 2). This association remained negative in each of the two stratified subgroups ($r = -0.59$, $P = 0.04$ and $r = -0.38$, $P = 0.23$ for shorter and longer storage times, respectively).

There was no significant association between NGF concentration and age or the presence of an upper motor neuron lesion ($P = 0.6$ and 0.7 , respectively).

Multiple regression analyses based on the above variables confirmed that 24-hr urine output was the only independent predictor of NGF concentration, accounting for 25% of the variance. The time of storage of the samples had no significant linear relation to NGF concentration ($P > 0.9$ in all analyses).

DISCUSSION

In our subjects, contrary to hypothesis, NGF concentration in superficial layers of the bladder was not significantly associated with DO (Table I). It remains possible that NGF

concentration in the deep detrusor is positively associated with DO.² The study was designed so as not to be confounded by the known association between high NGF concentration in bladder tissue and bladder outlet obstruction⁸ with concomitant DO. Thus, this association was not tested.

NGF concentration was not positively associated with detrusor contraction strength, but showed a trend to negative association (Table I). Thus, there is no evidence that an elevated NGF concentration in the superficial layers of the bladder is related to increased detrusor contractility. Consistent with this finding, NGF concentration showed no significant correlation with PVR urine, a different aspect of contractility.¹⁸ The largest PVR urine was only 75 ml, however, implying that no subject had very poor contractility.¹⁹

NGF concentration showed negative but non-significant associations with four volume variables in the domain of bladder sensation (Table I). Previous results¹⁰ have shown a more conclusive relationship between sensation and NGF, consistent with recent findings suggesting that urothelial cells exhibit specialized sensory and signaling properties that could allow release of mediators (including NGF) to alter excitability of nearby bladder afferents.

If the fifth variable in the domain of bladder sensation, mean voiding frequency, was a good proxy for sensation, voiding should be more frequent when the NGF level is high. In fact, it tends to be less frequent (Table I). Because voiding frequency depends on urine output as well as bladder capacity, we added 24-hr urine output to the list of variables examined. It was negatively and significantly associated with NGF concentration (Table I). Thus, if NGF level is elevated, urine output is lower, and voiding frequency tends to be correspondingly reduced.

This unexpected result suggests that the main determinant of NGF levels in the superficial layers of bladder is the urine output (fluid load) handled by this organ. The higher the fluid load, the lower is the concentration of NGF in superficial bladder samples. Such an association is supported by observations in animals of a relationship between NGF concentration and bladder-wall stretch or fluid load in vitro⁶ and in vivo.¹¹ At first sight there are several plausible explanations: (1) a high fluid load (more urine) simply washes NGF out of the urothelium (non-specific dilution); (2) a large volume of urine in the bladder stretches the wall, allowing NGF to leak out passively into the urine; (3) the actual process of filling the bladder actively reduces the concentration of NGF in the bladder wall.

Possibility (1) would imply that the normal concentration of NGF in urine was high enough to limit the rate of diffusion out of the bladder wall. More dilute urine would then allow increased washout of NGF. It seems unlikely however that the normal NGF concentration in urine is so high. Possibility (2) would imply that subjects who void larger volumes should have smaller NGF concentrations in the bladder wall. In fact, neither mean nor maximum voided volume showed any trend to association with NGF concentration. Stratification by mean voided volume indicated that the association between urine output and NGF level was similar for all voided volumes. This explanation is therefore unlikely. Regarding possibility (3), as urine accumulates in the bladder, the wall is extended and the urothelial surface area increases, requiring an active process (trafficking).²⁰ The observed relation between NGF levels and 24-hr urine output is consistent with NGF involvement in such an active process.

This study is one of the first to measure NGF levels in superficial bladder biopsies from human subjects whose lower urinary tract function has been meticulously characterized. Its exploratory nature required multiple statistical comparisons, the impact of which was minimized by the experimental design. The number of subjects, although large for a group of human biopsies, provides limited statistical power to reject hypotheses concerning the

relation of NGF to other variables. In fact, however, most hypotheses were rejected quite decisively, with correlation coefficients having the opposite sign to that predicted.

It is possible that a small amount of detrusor muscle was included in some of the superficial biopsies used for this study. However, the underlying deeper biopsies (examined for another study) usually included suburothelium as well as muscle, indicating that the superficial biopsies were primarily (sub)urothelial. Another limitation of the study is the variable storage time between preparation of the homogenate and assay, due to batch processing. Table II shows that the effect of increasing storage interval on NGF concentration is quite variable, so reducing the power to detect dependence of NGF concentration on other variables. More serious biases might be introduced if the effect of storage time depended on the presence of DO or of elevated fluid output. In fact, Table II suggests that NGF concentration may decrease more rapidly in samples from control subjects than in those from cases with DO. Such a bias, however, cannot easily account for the unexpectedly low levels in cases, compared with controls. Moreover, scatterplots (not shown) of NGF concentration against storage time, divided according to presence/absence of DO or of elevated fluid output, provide no suggestion of any systematic bias that could account for these results. Thus, the dependence of measured NGF levels on storage time may have increased the variability of the results, but it does not seem to have biased the conclusions drawn from them.

The specificity of the assay used in this study has been called into question when sample results are below the linear portion of the assay's standard curve. To check whether such an effect was important, the statistical calculations were re-run after omitting three samples whose absorbance was at or below the mean plus three times the standard deviation of the assay blank. No correlation coefficient changed sign or significance/non-significance status and none of the conclusions was altered.

CONCLUSIONS

An elevated concentration of NGF in human urothelium and suburothelium seems not to be strongly associated with DO or increased detrusor contractility. It shows at most a weak trend to association with increased bladder sensation. Unexpectedly, the concentration of NGF in the superficial bladder layers is significantly lower in subjects with higher 24-hr urine output. A possible explanation is that NGF is involved in an active process (trafficking) by which urothelial surface area is created as the bladder is filled.

Abbreviations

NGF	nerve growth factor
PIP ₁	projected isovolumetric pressure (modified)

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Fig. 1. Median, interquartile range, and range of NGF levels in 14 “controls” (without detrusor overactivity) and 12 “cases” (with detrusor overactivity).

Fig. 2.
Relation between NGF concentration in (sub)urothelial tissue and fluid load, measured as mean 24-hr fluid output.

TABLE I
Domains and Variables Examined, With Mean Values, Standard Deviations, Ranges, and Bivariate Associations With NGF Concentration

Domain	Variable	Mean	Median	Standard deviation	Range	Correlation with NGF concentration
Detrusor overactivity	NGF concentration (pg/ μ g protein)	1.37	1.23	0.87	0.28–3.41	—
	“case” (1) or “control” (0)	—	—	—	—	See text: Mann–Whitney test ($P = 0.23$)
Sensation	Volume at strong desire to void (ml)	366	342	156	128–725	–0.13 ($P = 0.53$)
	Maximum cystometric capacity (ml)	498	490	162	227–815	–0.23 ($P = 0.28$)
	Maximum voided volume on bladder diary (ml)	532	500	181	240–950	–0.29 ($P = 0.16$)
	Mean daytime voided volume on bladder diary (ml)	249	235	122	121–733	–0.16 ($P = 0.44$)
	Mean voiding frequency on bladder diary (# voids/24 hr)	7.8	7.2	3.3	3.0–19.7	–0.38 ($P = 0.07$)
Detrusor contractility	Isovolumetric detrusor pressure (cm H ₂ O)	48	48	24	9–96	–0.29 ($P = 0.17$)
	PIP ₁ (cm H ₂ O)	52	49	13	29–83	–0.20 ($P = 0.33$)
	Post-void residual urine (ml)	15	6	20	0–75	–0.07 ($P = 0.75$)
Post hoc	24-hr urine output on bladder diary (ml)	1,859	1,587	800	858–3,680	–0.55 ($P = 0.004$)

TABLE II

Change in NGF Concentration After One of Three Fixed Storage Intervals, in 14 Repeated Evaluations, Both Overall and Also Divided According to Presence/Absence of DO and Presence/Absence of Elevated 24-hr Fluid Output

Storage interval (days)	Mean change in NGF concentration (pg/ μ g)				
	Overall (n = 14)	Cases (DO) (n = 8)	Controls (no DO) (n = 6)	High fluid output (>1,587 ml/24 hr) (n = 8)	Low fluid output (<1,587 ml/24 hr) (n = 6)
13 (n = 7)	-0.43 (SE 0.16)	-0.25 (n = 4)	-0.68 (n = 3)	-0.55 (n = 5)	-0.14 (n = 2)
22 (n = 3)	-0.89 (SE 0.61)	-0.28 (n = 1)	-1.19 (n = 2)	-0.27 (n = 2)	-2.11 (n = 1)
58 (n = 4)	-0.93 (SE 0.30)	-0.63 (n = 3)	-1.83 (n = 1)	-0.62 (n = 1)	-1.03 (n = 3)

SE, standard error; DO, detrusor overactivity.