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Urodynamic Effects of Estradiol (E_2) in Ovariectomized (ovx) Rats

D. Seidlová-Wuttke, A. Schultens, H. Jarry, and W. Wuttke

Department of Clinical and Experimental Endocrinology, University of Goettingen, Germany

Whether estrogens have a beneficial effect in the urinary bladder to prevent or to delay occurrence of urinary bladder incontinence is an open question. Good animal models are missing. Therefore, in ovariectomized (ovx) rats we studied the effects of estradiol (E_2) administered with food for 3 mo on urodynamic properties of the urinary bladder and the urethra. A biluminal catheter with one outlet in the bladder and another in the urethra in juxtaposition to the external sphincter was inserted in isoflurane anaesthetized animals. Within 2 × 30 s (1 min apart) 0.5 mL Ringer's solution was infused into the bladder and the inner vesicular and urethral pressure were recorded. In comparison to ovx estradiol-treated rats, ovx animals had significantly ($p < 0.05$) lower vesicular and urethral pressure. In the sham-treated ovx animals vesicular and urethral pressures were unstable and appeared uncoordinated, whereas estrogens increased vesicular and urethral pressure in a coordinated way, such that in these animals leaked volume was significantly lower than in the sham-treated ovx animals. By means of quantitative RT-PCR we demonstrate that the upper and lower part of the bladder and the urethra express estrogen receptor of the alpha- and beta-subtype (ERα and β) and nerve growth factor, which is associated with painful sensations in inflamed urinary bladders. E_2 downregulated both ERs in the bladder but not in the urethra, while NGF gene expression was downregulated in the urethra but unaffected by E_2 in the bladder. It is concluded that estrogen deprivation causes uncoordinated function of the detrusor and sphincter muscles and that this effect can be prevented by estradiol.

Key Words: Urinary bladder; estradiol; ERα; ERβ; NGF.

Introduction

In women menopause is often associated with changes in the function of the urinary bladder, which may lead to two types of incontinence: stress incontinence is mostly due to anatomical changes, whereas urge incontinence in its pure form has mostly functional, often nervous reasons *(1–3)*. Estrogen receptors have been demonstrated in urethral and urinary bladder tissue *(4,5)*. It is therefore likely that the functional changes of the urinary bladder observed after menopause may be due at least in part to a lack of estrogens. Attempts to study the effect of estrogens in the urinary bladder have been made and surprisingly contradictory results have been published *(6–8)*.

Studies of estrogen effects in the urethra and the urinary bladder of animal origin are scarce. Morphological changes in response to ovariectomy and E_2 replacement have been reported *(9–12)*. For functional studies most authors used either isolated urinary bladder tissue strips or intact bladders of ovariectomized (ovx) rats and rabbits *(12–17)*, both kept under in vitro conditions, and these few experiments indicated that E_2 affects the function of the voiding mechanisms *(14–16)*. It was shown that detrusor contractility in rodents *(16)* and its innervation are sensitive to estrogen deficiency *(16–18)*. Estrogen withdrawal causes an overreactivity of urinary bladder tissue, which can be "smoothened" by estrogens, and it appears that β2- or β3-adrenergic receptor subtypes may be involved in this process. This effect may involve sympathetic and parasympathetic preganglionic neurons innervating the bladder, which express the estrogen receptor of the α- and β-subtype *(19,20)*. Owing to the presence of ERβ in the urothelium and ERα in the fibroblasts and detrusor muscle cells, a direct action of estrogens on these cell types is also likely *(4,5)* and, indeed, histological changes in ovx estrogen-replaced animals have been demonstrated when compared to ovx controls *(9–12)*. However, the delicate homeostasis between sphincter and detrusor functions cannot be studied.

Of the family of neurotropic factors, nerve growth factor (NGF) appears to play an important role in smooth muscle function in many organs including the bladder *(21–23)*. NGF mRNA and protein expression is subject to estrogenic modulation in the bladder *(24–29)*. Whether only the bladder or urethral tissue also express NGF has not been published so far.

Good models to study the urodynamic properties of the urinary bladder–urethral unit, which would allow establishing the physiological importance of E_2 in the urinary blad-

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Author to whom all correspondence and reprint requests should be addressed: Prof. Dr. W. Wuttke, Department of Clinical and Experimental Endocrinology, University of Goettingen, Medical School, Robert-Koch-Str. 40, D-37075 Goettingen, Germany. E-mail: ufkendo@med.uni-goettingen.de

der, are missing. Recently, we developed the catheterized ovx rat as a reasonable model to study effects of E_2 on bladder function similar to an in vitro model described earlier *(13)*. Therefore, for the present experiment we utilized the ovx rat model to study the in vivo effects of E_2 replacement therapy on urinary bladder and urethra functions by measuring the intravesicular and urethral pressures in animals fitted with a biluminal catheter of which one opening is located in the urinary bladder, the other in the sphincter area of the upper urethra/urinary neck area following a 3 mo of ovx with or without E_2 treatment. In addition, we studied the gene expression of the ERα and ERβ in urethra and urinary bladder as determined by RT-PCR.

Results

Figure 1 details the intravesicular and intraurethral pressures in two individual rats under and following the 2 × 0.5 mL infusion periods (30 s each, 60 s apart). While the urinary bladder and the urethra of ovx rats kept for 3 mo under E_2-containing food built up a much higher intravesicular and intraurethral pressure, the response of the urinary bladder and the urethra of an ovx control rat was much lower. In addition, it appears that both the bladder and urethra contracted in an uncoordinated fashion in the ovx non-E_2-substituted animal. Owing to these uncoordinated contractions the urinary bladder pressure occasionally exceeded the sphincter pressure, which resulted in urine discharge.

Figure 2 shows the mean vesicular and urethral pressures in both animal groups. During the first infusion period filling the bladder with 0.5 mL fluid neither urethral nor bladder pressure changed significantly in both groups of rats. However, the uncoordinated contractions seen in the individual ovx rats shown in Fig. 1 reflect also in ups and downs of the mean urethral and bladder pressures. The uncoordinated contractions in ovx sham-treated rats (shown in Fig. 1) reflect in the larger fluctuations also of the mean pressure values particularly during the first 60 s of the two infusion periods. In comparison to the controls, the mean pressures built up in the urinary bladder and in the sphincter were much higher in the estrogen-primed animals and remained at these high values during the remaining 180 s in which the bladders remained filled. Even though urethral and vesicular pressures were higher in the E_2-treated animals, the leaked volume was significantly higher in the control animals.

Uterine and urinary bladder weights and serum E_2 levels are detailed in Table 1. Although uterine weights were largely stimulated in the E_2-treated animals, no such effect was seen in the urinary bladders. Serum E_2 levels clearly indicated the presence of E_2 in the serum. Gene expression of ERα, ERβ, and NGF in the upper and lower half of the urinary bladder are shown in Figs. 3 and 4. E_2 inhibited both ERα and ERβ gene expression and, with the exception of ERβ in the upper part of the bladder, these effects were statistically significant. Gene expression of NGF was not significantly affected by the E_2 treatment in either part of the urinary bladder. Although ERα gene expression remained unchanged in the urethra, expression of the ERβ and NGF genes were significantly reduced by the E_2 treatment (Fig. 5).

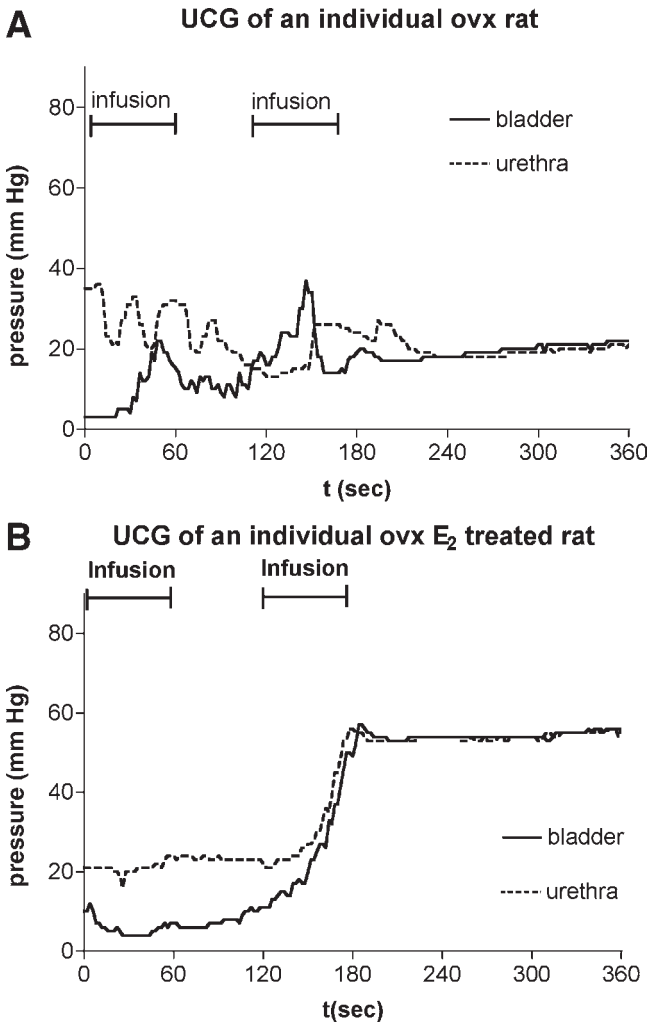

Fig. 1. The inner vesicular and urethral pressures of one individual ovx (**A**) and another ovx E_2-treated animal (**B**) are shown. During the first 60 s of instillation of 0.5 mL NaCl, the urinary bladder and urethra of the ovx rat reacted with uncoordinated contractions, such that the pressure in both system fluctuated widely. The fluctuations continued during the following 60-s period, during which no fluid was infused. During the second instillation period, bladder pressure was higher than urethral pressure, and this resulted in uncontrolled leaking of the animal. During the following 3 min, in which the urinary bladder remained instilled with the total volume of 1 mL, a relatively stable low pressure developed in the urethra and bladder. In contrast, the urinary bladder and urethral pressures of the ovx E_2-treated rat remained low during the first instillation period and during the 60 s thereafter and increased steadily over the total time of instillation of the second 0.5 mL physiological saline such that the pressures in the bladder and the urethra remained more than twice as high during the follow-up period than in the ovx nonestrogen-treated animals.

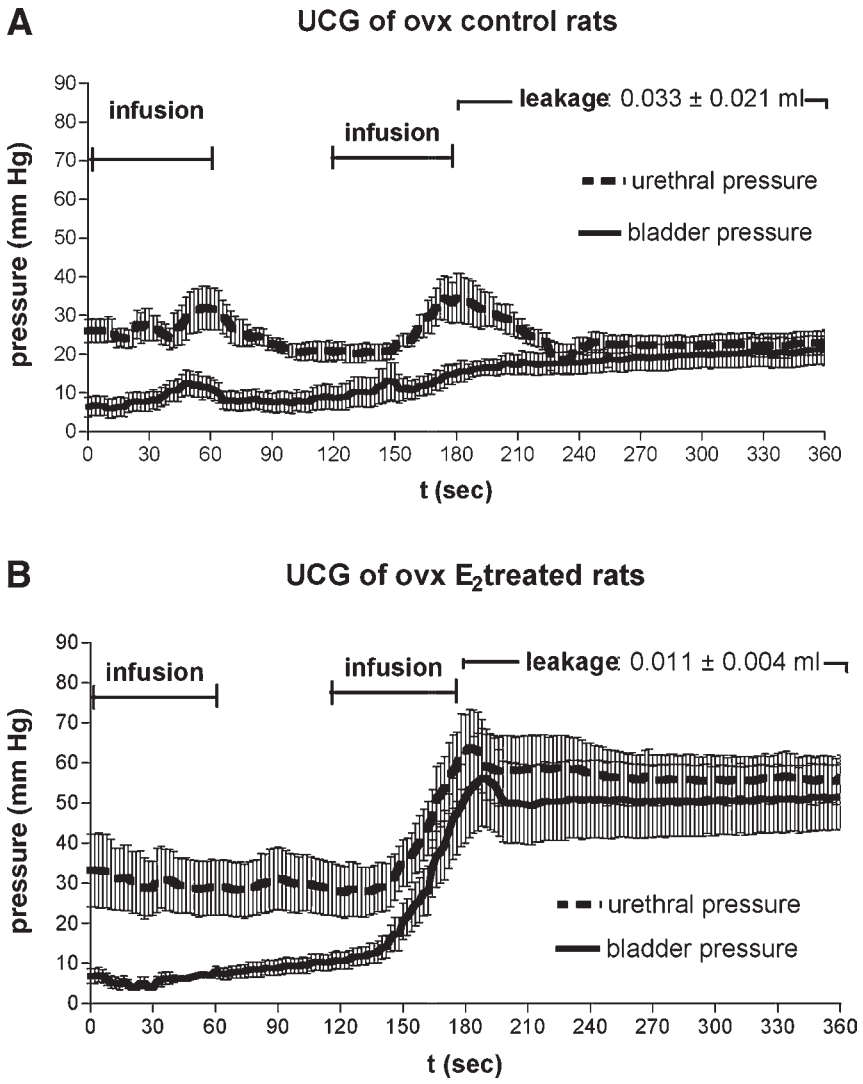

Fig. 2. Mean urinary bladder and urethral pressures in ovx animals kept on soy-free diet for 3 mo (**A**). The relatively unstable pressure situation in both the bladder and urethral systems shown reflects in slightly increased mean pressures. During the second infusion period, the urinary bladder and urethra of the ovx rats reacted again with only a slight increase in pressure, such that the mean pressures were significantly lower than those observed in the ovx rats kept on E_2-supplemented diet (**B**). In the E_2-treated animals the first instillation period did not cause any significant reaction of the urinary bladder or the urethra, while a marked increase in pressure was noted during the second instillation period, resulting in significantly higher mean bladder and urethral pressures during and following this second infusion period in comparison to the controls. The leakage volume in the estrogen-supplemented animals was significantly lower than the leakage volume in the animals kept under estrogen-free conditions.

Discussion

In these experiments E_2 was applied orally with pelleted food. The measurable E_2 concentrations in the blood and the large uteri of the E_2-treated animals clearly indicate that E_2 was absorbed and passed the liver resulting in slightly supraphysiological levels. Importantly, these E_2 concentrations did not result in changed weights of the urinary bladders indicating that no gross morphological changes were induced.

Table 1
Serum E_2 Concentrations,
Weights of Uteri and Urinary Bladders (Means ± SEM)

Treatment (3 months with food)	Serum E_2 (pg/mL)	Uterine weights (mg)	Urinary bladder weights (mg)
Control	7.1 ± 0.34	119.2 ± 11.1	108.8 ± 14.7
E_2-benzoat	156.7 ± 15.6*	665.6 ± 32.2*	104.8 ± 8.6

*$p < 0.05$ vs controls.

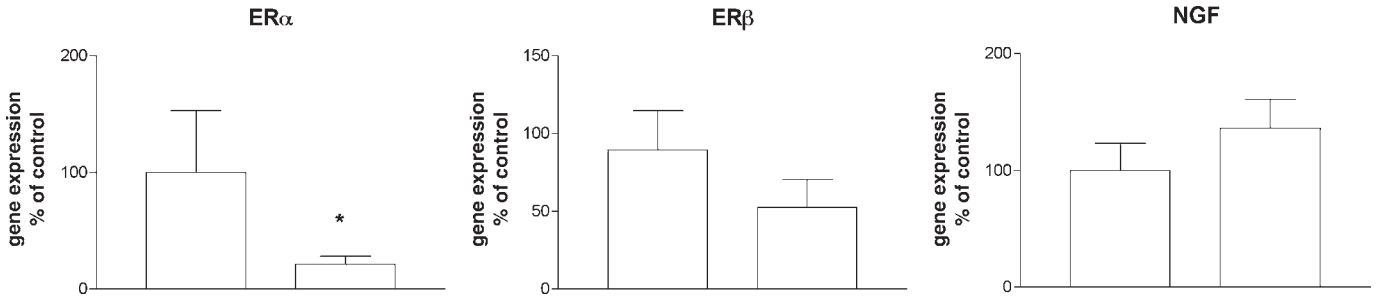

Fig. 3. Effects of E_2 (3 mo per food in ovx rats) on gene expression of ERα, ERβ, and NGF in the upper part of urinary bladder. The E_2 treatment inhibited ERα gene expression significantly. Means + SEM are shown, $*p < 0.05$.

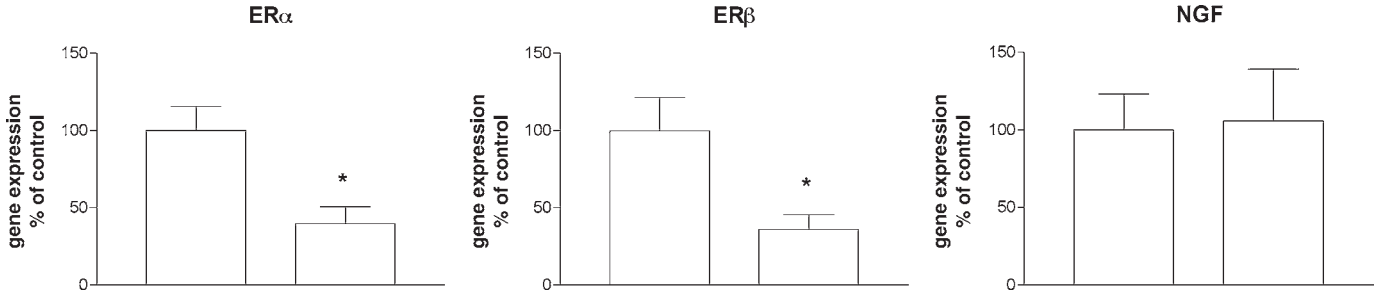

Fig. 4. Effects of E_2 (3 mo per food in ovx rats) on gene expression of ERα, ERβ, and NGF in the lower part of urinary bladder. The E_2 treatment suppressed both ERα and ERβ gene expression significantly. Means + SEM are shown, $*p < 0.05$.

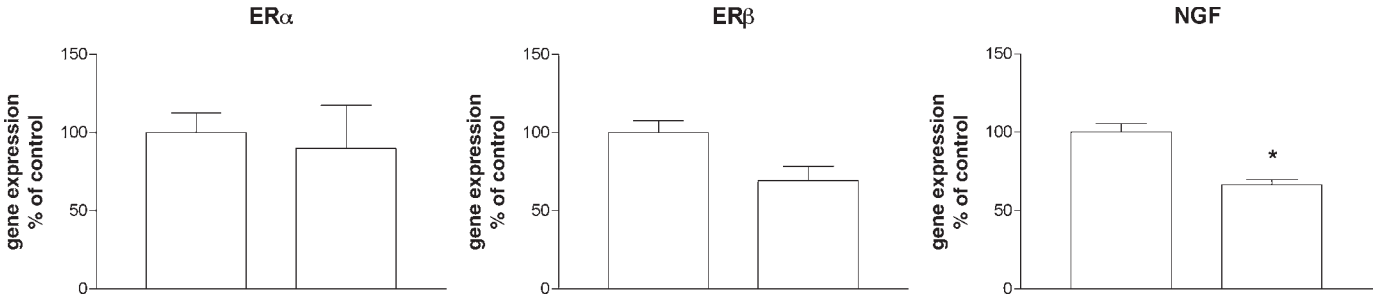

Fig. 5. Effects of E_2 (3 mo with food in ovx rats) on gene expression of ERα, ERβ, and NGF in urethra. The E_2 treatment had no effect on ERα and ERβ but suppressed NGF gene expression significantly. Means + SEM are shown, $*p < 0.05$.

For the demonstration of estrogenic effects in the urinary bladder of ovx rats, we introduced a new experimental model. We catheterized the urinary bladder with a biluminal catheter with an outlet located within the urinary bladder and another in the sphincter area of the urethra. This allowed inflation of the urinary bladder with NaCl and simultaneous recording of the intravesicular and intraurethral pressure. In response to exogenous filling with initially 0.5 mL of saline, the network of the detrusor muscular system of the urinary bladder of ovx rats as well as the sphincter of the urethra reacted with increased contractions in a relatively uncoordinated way. In individual ovx animals these uncoordinated contractions of the urinary bladder and the urethra resulted occasionally in higher intravesicular pressure than that in the urethra, which prompted uncontrolled leaking. Such detrusor instability or spontaneous contractions were also reported to occur in strips of urinary bladders of ovx rabbits *(15)* and of postmenopausal women *(27)*. These spontaneous contractions of rabbit detrusor muscle strips were readily inhibited by E_2 *(15)*. In the present experiments, the erratic contractions of the detrusor muscles were also not observed in the chronically ovx animals, which were substituted with E_2 over the entire investigation period of 3 mo, indicating that E_2 has a similar detrusor stabilizing effect in ovx rats as in the detrusor strips of ovx rabbits *(15)*. Similar rhythmic contractions as those of the detrusor muscles were observed in the sphincter muscle of the urethra, again only in ovx and not in ovx E_2-treated rats. Sphincter and detrusor contractions occurred asynchronously during both filing periods. This asynchrony often resulted in higher intravesicular than urethral pressure and thus in leaking. Hence, the leaked volume was significantly higher in the

ovx than in the ovx E_2-treated animals. The mean pressures measured in both structures were identical at the beginning of and during the first infusion period. The second infusion period resulted in significantly increased bladder and urethral pressures in both animal groups; however, in the estrogen-substituted animals, this increase was much sharper and reached a significantly higher level. The urethral pressure in the E_2-treated animals was constantly maintained at the high level even though infusion was stopped and the bladder remained filled. In the estrogen-free kept animals, the urethral pressure decreased significantly after the second filling period while the intravesicular pressure continued to increase slightly but steadily such that both pressures equalized each other quite often, which resulted in significantly higher leaking volume in comparison to the estrogen-treated animals.

The uncoordinated contractions of detrusor muscles as a result of estrogen deficiency have been reported previously in a variety of experimental models. Thus, Palea and Angel *(13)* reported increased sensitivity of urinary bladder and urethra tissue kept under in vitro conditions to the contractile effects of carbachol and norepinephrine in comparison to the estrogen-pretreated tissue samples. Pinna et al. *(30)* gave experimental evidence that abnormalities of the urethra and bladder function caused by ovx of diabetic rats could be restored by estrogen treatment. In these studies the authors used rat urethra and detrusor strips of which the mechanical contractions to norepinephrine or K^+ were determined. Impaired detrusor contractility in response to ovariectomy of long-term ovariectomized rats was also studied utilizing a muscle strip prepared by Zhu et al. *(9)*. These authors conclude that not only the rat detrusor but also its innervation are sensitive to prolonged ovarian hormone deficiency. Yono et al. *(17)* reported increased relaxant effect of E_2 on rabbit detrusor muscle strips. On the other hand, some authors also observed detrusor contractant effects of E_2 *(16)*, which, however, was never reported in in vivo experiments. Spontaneous, uncoordinated, and inappropriate bladder contractions are the basis for urge incontinence *(1, 3,13)*, and therapy of urge incontinence is largely based on treatment with neurally active substances *(1,3)*. Indeed, sympathetic neurons innervating the urinary bladder were shown to express the ER genes *(18,19)* and most of the above-described effects were attributed to impaired sympathetic or parasympathetic mechanisms. A rapid stabilizing effect of E_2 on spontaneous rhythmic contractions of the rabbit bladder was reported by Shenfeld et al. *(15)* in response to K^+ depolarization indicating rapid direct effects of E_2 in the bladder. Direct actions of E_2 in urinary bladder and urethral tissue are therefore also likely. This prompted us to determine by RT-PCR the presence of ERα and ERβ mRNA in bladder and urethral tissue. In the male bladder and urethra *(31)* the urothelium expresses the ERβ gene, whereas connective tissue cells, i.e., the collagen-producing fibroblasts, express primarily the ERα. Nerve growth factor (NGF) belongs to the family of neurophilins *(25,28)* and is not only expressed in neurons innervating the urinary bladder and the urethra but also in other cells of these organs *(25)*. In spontaneously hypertensive rats, hyperactive voiding was associated with increased neurophilin including NGF gene and protein expression *(28,29)*, and this increase may be related to increased smooth muscle proliferation *(21)*. There is some evidence that estrogens may modulate NGF concentrations in the bladder: ovx or treatment of intact mice with the estrogen antagonist 182780 reduced NGF gene expression and the ovx-induced decrease could be counteracted by E_2 replacement *(24,25)*. These experiments were done under acute (few to 24 h) conditions. In the present experiments we did not observe significant effects of a chronic E_2 substitution of ovx rats on NGF gene expression. In tendency, a slight but statistically insignificant increase in NGF mRNA was observed in the upper but not the lower part of the bladder. However, we did observe a significant reduction of NGF gene expression under the chronic E_2 treatment in the urethra, which may indicate an anti-inflammatory effect of E_2 in this organ. Such effect has been clinically reported as a result of hormone replacement therapy in postmenopausal women *(32,33)*. Taken collectively the NGF data appear to indicate that this peptide may not be involved in the urinary bladder dysfunction induced by ovx.

In the present experiments we demonstrate that both ERα and ERβ are regulated by E_2, and gene expression of both ERs was downregulated by the chronic E_2 replacement. Downregulation of ERβ by E_2 has been observed in other organs *(34)*. Estrogenic regulation of the ERα gene, however, appears to be much more seldom. Whether the changed gene expression of the ERs reflect also in relevant protein expression and may therefore be one of the reasons for the observed tonus stimulating effects of E_2 remains to be determined. Recently, we observed a stimulation of urothelium proliferation and an increase in collagenous tissue in the bladder of chronically E_2-treated rats (Schultens et al., unpublished).

In summary, we describe urinary bladder and urethra pressure stabilizing effects of a 3-mo E_2 substitution of ovx rats: the irregular and uncoordinated muscle activity in both the bladder and the urethra seen in ovx rats did not occur in the E_2-treated rats. The in vivo response of the urinary bladder and urethral contractile elements were significantly higher in E_2-substituted ovx rats. Both the coordinated and the significantly higher intravesicular pressures resulted in less leaking volume in the E_2-substituted animals. Hence, we feel that the ovariectomized rat fitted with a biluminal urinary bladder catheter is a suitable model to study the effects of sex hormones and possibly of other substances on the coordinated function of the urinary bladder and the sphincter urethra. ERα and ERβ are estrogens downregulated in the bladder, while in the urethra only ERβ mRNA

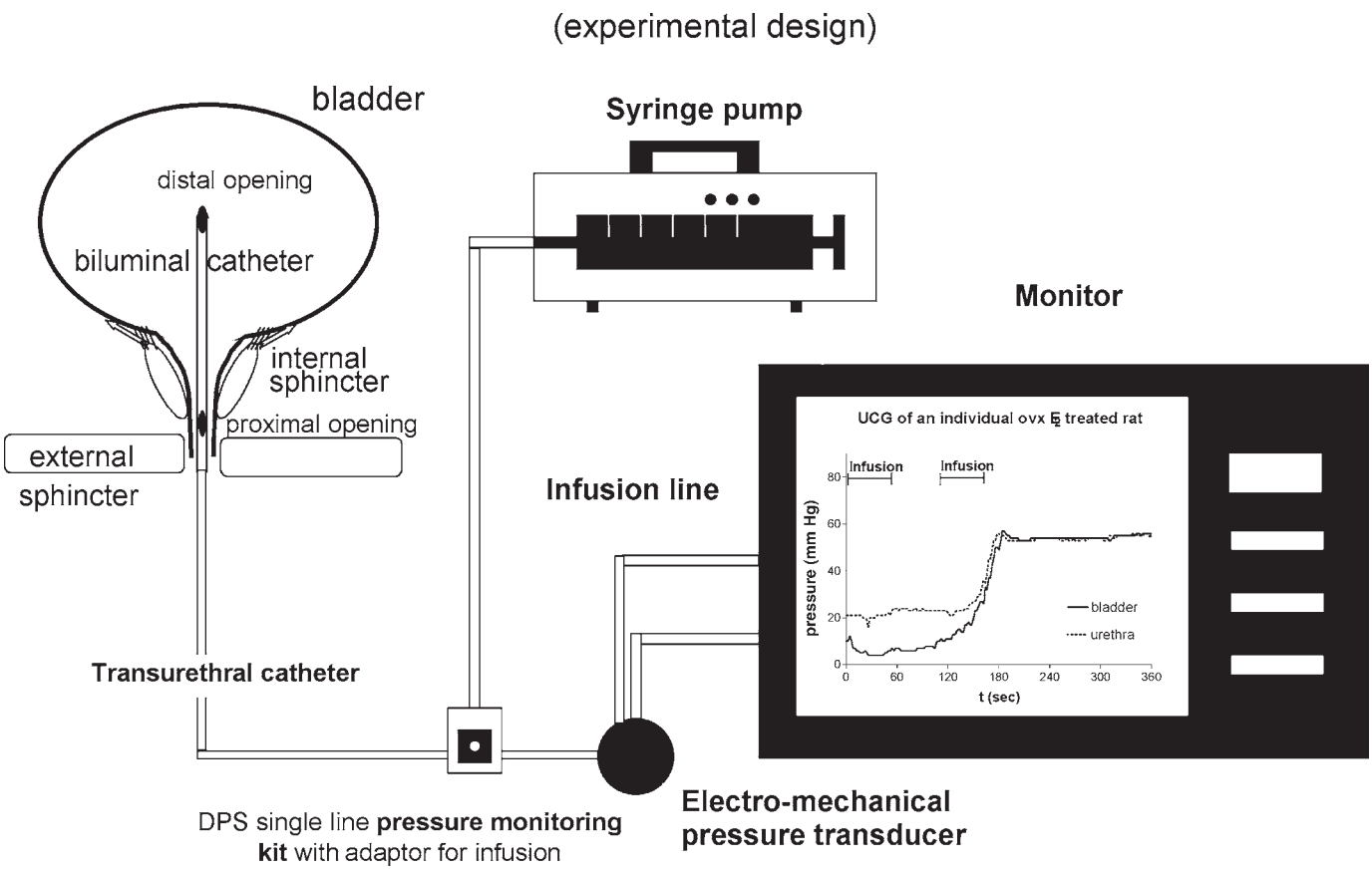

Fig. 6. Schematic drawing of experimental set-up. The biluminal catheter was inserted such that one outlet was located within the bladder, the other in the urethra at the level of the internal sphincter. The inner catheter was used to infuse the Ringer's solution. This part as well as the outer catheter were connected to pressure-measuring devices. The intravesicular and urethral pressures were continuously recorded at 1 s intervals, of which the means were calculated and statistically evaluated.

levels are reduced under E_2 treatment. NGF, a neurophilin involved in inflammation induced pain transmission in the urinary bladder and in the urethra is downregulated in the urethra possibly indicating a reduction of inflammatory painful sensation.

Material and Methods

Thirty female Sprague Dawley rats weighing approx 280–300 g were purchased from Winkelmann (Borchen, Germany). Permission to perform these experiments was obtained from the Landesregierung Braunschweig (Permission No. Az 509.42502/01-13.00). After an acclimatization period of 2 wk, they were ovariectomized under isoflurane anaesthesia. From there on, 15 rats were kept under soy-free pelleted food in which proteins were replaced by potato proteins. The other 15 rats were kept under the same food but the pellets were supplemented with estradiol 17β such that the mean daily food intake of 14 g per animal substituted each animal with 0.3 mg of the steroid. This food was also given immediately after ovx. Animals were kept on this food for a period of 3 mo. They were then again isoflurane anaesthetized and the urinary bladder was fitted with a biluminal catheter (Fig. 6). The opening of the inner catheter was located within the urinary bladder, the opening of the outer catheter was located in the urethra in juxtaposition to the sphincter muscle. The inner catheter was connected to a syringe and a pump, which allowed infusion of 0.9 NaCl into the urinary bladder. In addition, both catheters were connected to pressure-measuring devices. The urinary bladder was then filled with 0.5 mL saline over a period of 60 s. After a course of 60 s, another 0.5 mL of saline was infused into the bladder. The inner vesicular as well as the urethral pressure were continuously recorded during and for 180 s after these two filling periods. In preliminary experiments it was noted that drops of liquid appeared at the orificium of the urethra. Therefore, a piece of blotting paper surrounding the catheter at the level of the orificium of the urethra served to suck up any leaked fluid. The piece of blotting paper was weighed prior to and after experimentation, which allowed calculation of the leaked volume. Animals were then sacrificed and uteri, urinary bladder, and urethra (which included the sphincter of the urethra) were removed. The uterus and urinary bladder of

Table 2
PCR Primers, Probes, and References for Real-Time PCR

Gene	Primer sequence forward and reverse	Taqman probe	Prod size	Reference/ ACC #
ERα	5'-AAGCTGGCCTGACTCTGCAG-3' 5'-GCAGGTCATAGAGAGGCACGA-3'	5'FAM-CGTCTGGCCCAGCTCCTCCTCATC-TAMRA3'	144 bp	*(36)*/ X61098
ERβ	5'-GAGGAGATACCACTCTTCGCAATC-3' 5'-GGAGTATCTCTGTGTGAAGGCCAT-3'	5'FAM- CAGGGCATCTGTCACCGCGTTCAG-TAMRA3'	159 bp	*(37)*/ U57439
NGF	5'-CAC CTC TTC GGA CAC TCT GGA TT-3' 5'- AGC GCT TGC TCC TGT GAG TC-3'	5'FAM-TCC AGG CCC ATG GTA CAA TCT CCT TCA-TAMRA3'	80 bp	*(38)*/ V00836

each animal was weighed and the bladder was cut in upper and lower halves and immediately frozen in liquid nitrogen for determination of ERα, ERβ, and NGF gene expression.

Taqman Quantitative PCR for ERα, ERβ, and NGF

The deep frozen tissue specimens were pulverized and the RNA was isolated using RNeasy columns (Quiagen, Hilden, Germany). For ERα, ERβ, and NGF one-step Taqman RT-PCRs were performed as previously described *(34)*. In short: Deep-frozen urinary bladders were homogenized and 50 mg of the tissue powder was suspended in 600 µL RT-lysis buffer. After loading on QIA shredder columns (Quiagen) eluates were loaded onto extraction columns. Total RNA was eluted with diethylpyrocarbonate-treated water. Concentration of RNA solution was adjusted to 50 ng RNA per µL. This solution was stored at –70°C. The RT reaction was carried out with 500 ng total RNA. The real-time PCR reaction was based on the 5' nuclease assay *(35)*, which was run on the ABI prism 77OO sequence detection system (TaqMan, PE Applied Biosystems, Foster City, CA, USA). Each PCR run included six duplicate cDNA samples of defined concentrations to generate a standard curve and a no-template control. Primers and appropriate references are given in Table 2 *(36–38)*.

Statistical Treatment of Data

For evaluation of quantitative PCR data, the RT values of the control animals were set to 100% and the values for the E_2-treated animals were calculated accordingly. Data were statistically evaluated by Student's *t*-test or by Mann–Whitney *U* test. A significant value of $p < 0.05$ was considered to be significant.

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