Estradiol increases urethral tone through the local inhibition of neuronal nitric oxide synthase expression

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The intricate relationship between urine storage and micturition involves a reciprocal balance in the muscle tone of bladder and urethra, which are under spinal and supraspinal controls. Autonomic regulation of the lower urinary tract physiology is driven by all three components of the autonomic nervous system. Nitric oxide (NO), the key neurotransmitter of the nonadrenergic, noncholinergic nerves of the peripheral nervous system, is produced by the neuronal isoform of NO synthase (nNOS). In the lower urinary tract, nNOS is mainly expressed in nerves located in the muscular and inner lamina propria layers of the urethral wall and only sparsely present in the detrusor muscle (5, 14). In line with the ubiquitous relaxing effect of NO (20), variations in local NO production are suspected to play a physiological role in urethral sphincter relaxation during micturition (18).

Because estrogens are known to modulate the expression of nNOS in several target organs, such as the hypothalamus (33) and genital tract (30), we explored the effects of supraphysiological levels of E2 on lower urinary tract function and morphology and urethral nNOS expression, as well as the consequences of acute inhibition of nNOS activity by 7-nitroindazole.

MATERIALS AND METHODS

Animal experiments. Female C57/Bl6 mice (Charles Rivers, Les Oncins, France) were housed in stainless steel cages in a temperature-controlled facility on a 12:12-h light-dark cycle and fed normal laboratory mouse chow diet. All experiments were conducted in conformity with Guide for the Care and Use of Laboratory Animals (Institute for Laboratory Animal Research, National Research Council, Washington, DC: National Academy Press, 1996). All animal studies were approved by the local Animal Care and Use Committee.

For all surgical procedures, mice were anesthetized by intraperitoneal injection of a mixture of 150 mg/kg ketamine (PanPharma, Luitré-Fougères, France) and xylazine (Bayer, Leverkusen, Germany). Body temperature was maintained at 37°C by means of a rectal probe connected to a homeothermic blanket.

Hormonal manipulations. In mice, E2 levels vary threefold with the estrus cycle within a range of 0.1 (proestrus) to 0.3 (estrus) nM, with intermediate values (0.2 nM) observed in noncycling animals (26). After ovarectomy, attempts to restore with exogen depots the physiology of endogenous E2 have shown some limitations. For instance, Modder et al. (19) reported that physiological E2 serum levels were reached with 5 and 10 μg·kg⁻¹·day⁻¹ pellets but that higher dosages (20–40 μg·kg⁻¹·day⁻¹) and supraphysiological serum levels were...
needed to restore the weight of such a select target organ as the uterus in laboratory animals. We therefore opted for classical sham-operated animals as physiological controls to study the influence of E2 on the lower urinary tract.

Mice were sham operated or ovariectomized at 4 wk of age. Two weeks later, the ovariectomized animals were subcutaneously implanted with either a placebo or a 60-day time release E2 pellet (0.1 mg E2; releasing 80 μg·kg⁻¹·day⁻¹; Innovative Research of America, Sarasota, FL). This dose was previously reported to induce plasma levels of 0.3 nM (80 pg/ml), a concentration encountered during pregnancy and ~10 times higher than that found during the estrus cycle (10). Six weeks later, the mice received either an intraperitoneal injection of 7-nitroindazole (Acros Organics, Morris Plains, NJ), a potent selective competitive inhibitor of nNOS with no significant effect on endothelial NO synthase (eNOS) in mice (21), sonicated in peanut oil at a dose of 50 mg/kg or the vehicle alone.

Micturition behavior was then recorded in the animals (6 per group), and 2 days later peak point pressures were measured. A separate set of animals (6 per group) was followed after hormonal manipulation [ovariectomy, sham surgery, ovariectomy plus E2 pel- lets every 2 mo (hereafter reported as E2-treated mice)] until 4 mo of age when they were euthanized for histology and Western blot analysis. In all conditions, the hormonal status was verified by the combination of uterus weights and serum E2 levels.

**Plasma E2.** In all animals, serum samples were taken before death at 12 wk (micturition behavior) or 16 wk (histology) of age. E2 was first extracted under diethyl ether. Plasma E2 was measured with a commercially available double-antibody RIA immulite-kit (Coat-A-Count Estradiol-6; Diagnostic Products, Los Angeles, CA). The interassay and intra-assay coefficients of variation for this kit are reported to be 4.1–15.3% and 3.5–7.6%, respectively. Assay sensitivity was 7.4 pg/ml, and the cross-reactivity with other estrogenic compounds was negligible.

**Micturition behavior.** Micturition behavior was assessed as described by Burnett et al. (5). Briefly, animals were housed individually in hanging stainless cages for 14 h before the experiment and provided with food and water ad libitum. Preweighed absorbent cage paper was placed underneath each cage and weighed at 5-min intervals for 8 h. The sample output was calculated as the sum of the volume urinated and the residual volume per 8 h.

**Leak point pressure study.** The bladder dome was exposed under operative microscopy through a lower midline abdominal incision. A 22-gauge angiocatheter was inserted and fixed to the bladder wall with cyanoacrylate glue. The bladder was then distended with room temperature saline at a filling rate of 20 μl/min. The intravesical pressures were recorded with a TA400 pressure transducer (Gould Electronics, Ballainvilliers, France), and the leak point pressure was defined as the pressure recorded when the first drop was observed at the meatus.

**Histological analysis and histomorphometry.** Mice were euthanized at 16 wk of age for histology analysis. The animals were put under general anesthesia as previously described and injected intraperitoneally with 12.5 mg/kg atropine sulfate (Laboratoires Renaudin, Itxassou, France) to prevent premortem bladder contractions. The bladder was catheterized, emptied, and distended to 50 μl of volume as described for the leak point pressure study. Animals were euthanized by intraperitoneal injection of 250 mg/kg lidocaine (AstraZeneca, Rueil-Malmaison, France).

The upper and lower urinary tracts were removed en bloc, and the kidneys, bladder, and uterus were weighed separately. Bladder dome, midureter, and midurethra specimens were fixed in Dubosc-Brazil-Bouin mixture and routinely processed for Masson trichrome stain.

Digital microscopic pictures of 4-μm-thick slides (ureter = ×400, bladder = ×160, urethra = ×100) were analyzed with the freeware National Institutes of Health (NIH) Image software (http://rsb.info.nih.gov/nih-image). For the ureter and urethra specimens, the areas of the epithelial layer, submucosa, and muscularis layer were measured, as well as the overall area of the section. For the bladder specimens, the mean thickness of the muscularis layer of a bladder section was assessed as the ratio of the muscularis surface to the urothelium segment length.

**Protein extraction and Western blot.** Urethral specimens were removed en bloc, rinsed with ice-cold saline buffer, frozen by immersion in liquid nitrogen, and stored at ~80°C until used for the experiment (within 3 wk).

The frozen tissues were thawed and then homogenized in ~10 vol buffer containing 10% glycerol, 20 mM Tris, 140 mM NaCl, 10 mM sodium pyrophosphate, 10 mM fluoride, 2 mM sodium orthovanadate, 3 mM EDTA, 10 μg/ml trypsin inhibitor, 10 μg/ml leupeptin, and 2 μg/ml aprotinin with a Polytron homogenizer for four periods of 1 min with intermittent cooling pauses of 4 min. The homogenate was centrifuged at 40,000 g for 45 min, and the pellet was discarded. The supernatant was saved.

Aliquots of 60 μl of supernatant were diluted 1:2 in electrophoresis sample buffer (1.8 M Tris–HCl, pH 6.8, 10% glycerol, 10% SDS, 0.01% bromophenol blue, and 5% β-mercaptoethanol) to yield 60 μg of protein per lane. The samples were reduced by boiling for 5 min. Mouse cerebellum cortex was used as a positive control. SDS-PAGE was carried out on a 10% polyacrylamide gel at 125 V for 30 min.

After electrophoretic transfer to a polyvinylidene difluoride membrane at 10–15 V for 30 min, the blots were blocked overnight with dry milk diluted in TBS-Tween 20 at 4°C. The blots were then incubated with primary polyclonal antibodies (nNOS N31030; Transduction Laboratories, Lexington, KY) diluted in TBS-Tween 20-dry milk (nNOS 1:1000) for 1 h at room temperature. Blots were then rinsed in TBS-Tween 20 for 1–15 min followed by 3–5 min and incubated with secondary anti-rabbit IgG-horseradish peroxidase (SC2030; Santa Cruz Biotechnology) diluted in TBS-Tween 20 (1:10 000) for 1 h at room temperature. Blots were then rinsed as above and subjected to the enhanced chemiluminescence system. Autoradiographic film was applied to the blot until satisfactory exposure was obtained. After scanning, films were analyzed by NIH Image software to quantitate nNOS expression.

**Statistical analysis.** Data were analyzed by two-way ANOVA, followed by Student’s t-test when appropriate. Data are presented as means ± SD. \( P < 0.05 \) was considered significant.

### RESULTS

**Uterus and body weight.** Male body weights were similar in all groups (E2-treated mice = 23.3 ± 1.0 g, ovariectomized mice = 22.9 ± 0.9 g, sham-operated mice = 23.1 ± 1.1 g, respectively; not significant).

Compared with sham-operated mice (102 ± 12 mg), mean uterus weights were similar in E2-treated mice (117 ± 22 mg; not significant) and drastically decreased in ovariectomized mice (23 ± 3 mg, \( P = 0.002 \)).

At the time of death (16 wk of age), E2 serum levels were undetectable in ovariectomized animals, whereas a fourfold increase was observed in E2-treated animals compared with

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Body Weight, g</th>
<th>Bladder Weight, mg</th>
<th>Uterus Weight, mg</th>
<th>E2 Serum Level, pg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham-operated</td>
<td>6</td>
<td>23.1 ± 1.1</td>
<td>21.0 ± 1.8</td>
<td>102 ± 12</td>
<td>19 ± 3</td>
</tr>
<tr>
<td>Ovariectomized</td>
<td>6</td>
<td>22.9 ± 0.9</td>
<td>22.1 ± 2.2</td>
<td>23 ± 3</td>
<td>ND</td>
</tr>
<tr>
<td>Supraestrus</td>
<td>6</td>
<td>23.3 ± 3.0</td>
<td>31.2 ± 7.8</td>
<td>117 ± 22</td>
<td>80 ± 9</td>
</tr>
</tbody>
</table>

Values are means ± SD; \( n = \) no. of animals. E2, 17β-estradiol; ND, not detectable.
sham operated animals (80 ± 9 vs. 19 ± 3 pg/ml, \( P < 0.001 \); Table 1). Similar results were observed at 12 wk of age (data not shown).

**Micturition behavior.** E2-treated mice exhibited a lower frequency of micturition than ovariectomized and sham-operated animals (1.2 ± 0.8 vs. 4.0 ± 1.7 and 3.6 ± 1.5 micturition/8 h, respectively; \( P = 0.008 \)). At the end of the experiment, the residual urine volume was determined by bladder catheterization. E2-treated mice exhibited a 30-fold increase in residual volume compared with ovariectomized or sham-operated animals, which were almost devoid of residual (0.32 ± 0.11 ml vs. 0.01 ± 0.01 ml and 0.01 ± 0.01 ml, respectively; \( P = 0.008 \)).

The role of nNOS-derived NO was evaluated by subjecting the animals to pharmacological inhibition by 7-nitroindazole. Acute inhibition of nNOS resulted in a significant decrease in the frequency of micturition in ovariectomized and sham-operated animals, which was paralleled by a significant increase in residual volume (Table 2), giving evidence of the tonic inhibitory effect of nNOS in micturition. In contrast, 7-nitroindazole did not significantly influence the micturition pattern of E2 animals (Table 2). nNOS inhibition significantly reduced urine output in ovariectomized animals (190 vs. 347 µl; \( P = 0.016 \)) but not in sham-operated (352 vs. 389 µl; \( P = 0.45 \)) and in E2-treated animals (261 vs. 474 µl; \( P = 0.11 \)).

**Leak point pressure.** E2-treated mice exhibited a twofold increase in leak point pressure, compared with ovariectomized and sham-operated animals (8.8 ± 2.3 vs. 4.3 ± 0.7 and 4.9 ± 0.9 cmH2O, respectively; \( P < 0.01 \); Table 2). In addition, acute nNOS inhibition significantly increased urethral resistances in sham-operated (7.60 ± 1.55 vs. 4.90 ± 0.89 cmH2O; \( P = 0.03 \)) and ovariectomized animals (9.90 ± 5.13 vs. 4.25 ± 0.66 cmH2O; \( P = 0.03 \)) but not in E2-treated animals (10.67 ± 6.12 vs. 8.75 ± 2.29 cmH2O, not significant, \( P = 0.70 \)), highlighting both the relaxing role of nNOS in sham-operated and ovariectomized animals and its impairment by supraestrus levels of E2 (Table 2).

**Histology and histomorphometry.** Figures 1 and 2 show histology and histomorphometry. As expected in mice, urethral

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### Table 2. Micturition behavior in vigil animals and leak point pressure in anesthetized animals according to hormonal status and nNOS inhibition by 7-nitroindazole intraperitoneal injection

<table>
<thead>
<tr>
<th></th>
<th>Ovariectomized</th>
<th>Sham-operated</th>
<th>E2 Treated</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Without 7NI</td>
<td>7NI</td>
<td>( P )</td>
</tr>
<tr>
<td>Number of micturitions/8 h</td>
<td>4.0±1.7</td>
<td>1.2±0.8</td>
<td>0.0079</td>
</tr>
<tr>
<td>Volume urinated/8 h, µl</td>
<td>332±62</td>
<td>86±69</td>
<td>0.0079</td>
</tr>
<tr>
<td>Residual volume, µl</td>
<td>15±13</td>
<td>104±76</td>
<td>0.016</td>
</tr>
<tr>
<td>Urine output/8 h, µl</td>
<td>347±7</td>
<td>190±12</td>
<td>0.016</td>
</tr>
<tr>
<td>Leak point pressure, cmH2O</td>
<td>4.3±0.7</td>
<td>9.9±5.13</td>
<td>0.03</td>
</tr>
</tbody>
</table>

Values are means ± SD. Urine output was defined as the sum of the volume urinated and the residual volume. Within each condition (ovariectomized, sham-operated, and E2 treated), \( P \) values (ANOVA) refer to the effect of nNOS inhibition by 7-nitroindazole (7NI).
sections were devoid of striated muscle. E2-induced increase in urethral resistances was associated with thicker muscularis layers in ureter and bladder and by a parallel increase in bladder weight (31.2 ± 7.8 vs. 21.0 ± 1.8 vs. 22.1 ± 2.2 mg in E2-treated mice vs. sham-operated and ovariectomized mice, respectively; \( P < 0.01 \)). No differences were observed in various urethral layers, whatever the hormonal status.

**nNOS urethral expression.** E2 strongly affected nNOS urethral expression, as supraestrus E2 resulted in a significant 62% decrease, whereas ovariectomy led to a 69% increase in expression, compared with sham-operated controls (Fig. 3).

**DISCUSSION**

In the present work, we confirmed the influence of E2 on urethral tone and asserted whether it was mediated through local nNOS activity and expression, as reported in other target organs (summarized in Fig. 4). Contrary to sham-operated and ovariectomized animals in which acute nNOS inhibition increased urethral resistances, nNOS inhibition had no effect on the chronically elevated resistances observed in E2-treated animals.

As shown by increased residual volume and fewer micturitions in E2-treated animals, E2 status profoundly influenced the micturition pattern, which raises the concern that supraestrus levels of E2 could lead to overflow incontinence by urinary retention.

However, the NIH consensus definition of overflow incontinence is that of frequent micturitions of low volumes (http://kidney.niddk.nih.gov/kudiseases/pubs/uiwomen/index.htm). We observed in supraestrus animals a reduction in frequency associated with an increase in volume, contrary to this definition.

One limitation of the present study is that it did not address any direct effect on bladder contractility. Although nNOS was shown to be the major NOS isoform in the lower urinary tract (5), its gene invalidation did not affect bladder strip contractility or relaxation after chemical and electrical stimulations (28), suggesting that the reported effects on urodynamics and micturition were secondary to alterations of urethral resistances (5, 18, 28). We therefore focused on the dynamic control of urethral resistances by E2 through the nNOS pathway.

Because urethral resistances may reflect any combination of dynamic obstructions resulting from smooth muscle tonus and static tissue resistances, we investigated their respective contributions depending on E2 status and nNOS activity by measuring the bladder pressure at urine leakage, similar to the leak point pressure in humans, and searched for structural urethral alterations. In contrast to results in sham-operated and ovari-
ectomized controls, E2-treated mice exhibited a twofold increase in urethral resistances that was not further modified by nNOS inhibition. In addition, the structure of the bladder, as assessed by image analysis and weight, was significantly altered. Of note, no differences between groups were observed in the urethral layers, suggesting that the E2-induced urethral resistances were not related to structural changes of the outlet.

In ovariectomized and sham-operated control animals, acute inhibition of nNOS increased the bladder pressure at urine leakage, confirming in the female urinary tract the relaxing effect of NO on urethral tone (28). However, such inhibition had no influences on the increased urethral resistances observed in E2-treated mice.

We therefore assessed nNOS expression and for the first time in urethra highlighted the significant decrease of nNOS expression in E2-treated ovariectomized animals (P < 0.01) and a 69% increase in ovariectomized animals (P < 0.05). *P < 0.05; **P < 0.01; ***P < 0.001.

Ten years ago, Takahashi et al. (29) first reported that short-term high-dose estrogen treatment reduced NOS activity and inhibited the nitricergic nerve stimulation-induced relaxation of rabbit urethral smooth muscle. Al-Hijji and Batra (3) showed that such E2-reduced NOS activity was observed in all segments of the lower urinary tract. Both experiments studied the influence of short-term stimulation (2 and 1 wk, respectively) with high doses of E2 (5 and 1 mg·kg⁻¹·wk⁻¹, respectively) on NOS activity and did not assess selective implication of specific NOS subtypes. To our knowledge, the present report is the first evidence of long-term hormonal modulation of nNOS expression in the female lower urinary tract by su-

Fig. 3. Neuronal nitric oxide synthase (nNOS) expression in female mice urethra according to hormonal status. A: representative Western blot. Standard refers to 0.5, 1, and 1.5 μg of cerebellum protein extract (positive control). B: mean and SD of 6 separate experiments, showing results compared with sham-operated animals (S). There was a significant 62% decrease in nNOS expression in E2-treated ovariectomized animals (P < 0.01) and a 69% increase in ovariectomized animals (P < 0.05). *P < 0.05; **P < 0.01; ***P < 0.001.

Fig. 4. Representation of interactions between estrogens (E2) and nitric oxide production and functional effects on lower urinary tract. A: results from 1) Warembourg et al. (33), 2) Traish et al. (30), 3) Al-Hijji and Batra (3), 4) Takahashi et al. (29), 5) Burnett et al. (5), and 6) Sutherland et al. (28). eNOS, endothelial nitric oxide synthase. B: present experiments. 7-NI, 7-nitroindazole.
EFFECT OF E2 ON URETHRAL nNOS EXPRESSION AND FUNCTION

The present study also confirmed the profound effect of high doses of E2 on kidney function and nNOS renal expression. Indeed, in E2-treated animals, urine output (as represented by the sum of the volume urinated and the residual volume) increased, reminiscent of the report by Carlberg et al. (9), which showed that high-dose supplementation with estrogens increased urine volume by decreasing osmolality. In addition, this was exquisitely influenced by nNOS inhibition, confirming the observation of Alexander et al. (2) in the pregnant rat, which showed that renal hemodynamic changes were related to renal expression of inducible NOS and nNOS isoforms.

With regard to ovariectomized animals, urine output was sharply reduced by nNOS inhibition (347 vs. 190 µl; P = 0.016), whereas no significant effects were observed in sham-operated animals, suggesting that the role of nNOS is more prominent after castration. Recently, Yamaleeye et al. (34) observed in the cortex and medulla of hypertensive rats that ovariectomy enhanced nNOS mRNA but decreased eNOS mRNA, suggesting that increased renal nNOS expression constituted a compensatory mechanism to castration-induced reduction in renal eNOS. This report is consistent with the present observation of a more profound effect of nNOS acute inhibition in ovariectomized animals than in sham-operated animals.

Therefore, one important finding is that, to be of any clinical relevance in terms of urethral tone, E2 supplementation would have to reach selectively supraestrus levels in the urethra. Such a limitation could be addressed by transvaginal administration, which was shown in a Cochrane review of 16 trials encompassing 2,129 women to have a positive effect on vaginal dryness and atrophy, without side effects (27). Another way would be to resort to selective estrogen receptor modulators (SERMs), selected to reproduce some but not all the effects of E2. The identification of numerous coactivators and corepressors that modulate receptor function and the generation of two estrogen receptor subtypes attest to the potential complexity through which SERMs produce diverse tissue-specific responses (17). Contrary to their effects on bone metabolism, SERMs do not appear to have a class effect on the lower urinary tract (1), and the present animal models could prove of value in the preclinical development of “uroSERM,” designed to take advantage of the modulation of urethral tone by estrogens.

Perspectives and Significance

We show here that long-term treatment with high doses of E2 increases the urethral tone and reduces NOS expression in the urethra. This mechanism potentially accounts for the physiological increase in urethral tone during pregnancy. It suggests a beneficial effect of local delivery of estrogens or uroSERM in postmenopausal urinary incontinence.

In conclusion, we here show that supraestrus E2 decreases urethral nNOS expression, resulting in outlet obstruction. No static urethral modifications were observed, in line with a dynamic effect on the urethral smooth muscle tone.

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