Neurotransmission and viscoelasticity in the ovine fetal bladder after in utero bladder outflow obstruction

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Thiruchelvam, N., C. Wu, A. David, A. S. Woolf, P. M. Cuckow, and C. H. Fry. Neurotransmission and viscoelasticity in the ovine fetal bladder after in utero bladder outflow obstruction. Am J Physiol Regul Integr Comp Physiol 284: R1296–R1305, 2003. First published January 23, 2003; 10.1152/ajpregu.00688.2002.—Fetal bladder outflow obstruction, predominantly caused by posterior urethral valves, results in significant urinary tract pathology; these lesions are the commonest cause of end-stage renal failure in children, and up to 50% continue to suffer from persistent postnatal bladder dysfunction. To investigate the physiological development of the fetal bladder and the response to urinary flow impairment, we performed partial urethral obstruction and complete urachal ligation in the midgestation fetal sheep for 30 days. By electrical and pharmacological stimulation of bladder strips, we found that muscarinic, purinergic, and nitric mechanisms exist in the developing fetal bladder at this gestation. After bladder outflow obstruction, the fetal bladder became hypocontractile, producing less force after nerve-mediated and muscarinic stimulation with suggested denervation, and also exhibited greater atropine resistance. Furthermore, fetal bladder urethelium exerted a negative inotropic effect, partly nitric oxide mediated, that was not present after obstruction. Increased compliance, reduced elasticity, and viscoelasticity were observed in the obstructed fetal bladder, but the proportion of work performed by the elastic component (a physical parameter of extracellular matrix) remained the same. In addition to denervation, hypotonicity may result from a reduction in the elastic modulus that may prevent any extramural components from sustaining force produced by detrusor smooth muscle.

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HUMAN FETAL bladder outflow obstruction (BOO) is an almost exclusively male disease, usually caused by posterior urethral valves (PUV). The condition affects 1/5,000 male births (47) and is the commonest cause of end-stage renal failure in children (29, 47). In addition to renal function impairment, up to 70% of boys with PUV suffer significant bladder dysfunction (10, 11) with the commonest urodynamic findings of bladder instability, poor compliance, and poor bladder emptying (22).

The autonomic control of the normal and obstructed adult bladder has been well investigated, but little is known of the neural mechanisms regulating the developing fetal bladder and the fetal bladder exposed to BOO. Aside from histological studies of human fetal bladder characteristics (17, 24, 48), investigators have primarily focused on experimental animal models to study the normally developing and obstructed fetal bladder. For these purposes, the ovine fetus has proved to be a useful model, and an early study (25) showed that bladder contractions were evident at 120 days gestation (sheep gestation is 145 days) and, by intravesical instillation, found that cholinergic and β-adrenergic mechanisms were present. In addition, contractile and relaxant responses to various agonists existed at 95 days gestation and were not altered after short-term BOO (28). Functional assessment (34) found that the ovine fetal bladder had decreased compliance after BOO as measured by delayed stress relaxation during rapid-fill cystometry. Karim et al. (23) reported that accompanying the increased growth of the fetal ovine bladder with BOO, there was a significant increase in total detrusor choline acetyltransferase activity. Cendon et al. (8) also reported increased growth of the fetal bladder subjected to BOO. Our own preliminary study (33) found that the severely obstructed fetal bladder became hypocontractile, denervated, and functionally compliant and flaccid during incremental cystometry. The aims of this current study were to characterize the neural mechanisms in the developing bladder and to determine the causes of the hypotonicity in the obstructed fetal bladder. We found that muscarinic, purinergic, and nitric mechanisms exist in the developing fetal bladder and, to some extent, are perturbed in the obstructed fetal bladder. Furthermore, after in utero BOO, the viscoelastic properties of the obstructed bladder are altered in such a way as to account in part for the reduced contractile state.

MATERIALS AND METHODS

Experimental strategy. In utero BOO was produced in fetal sheep as previously described (33). In brief, male fetuses at 75 days gestation, from time-mated Romney Marsh ewes, Royal Veterinary College, Potters Bar, UK, were placed either in a sham-operated group (n = 5) or an obstructed group.
(n = 5). The obstructed group underwent partial BOO by placement of an omega-shaped silver ring around the urethra and complete ligation of the urachus. The sham-operated group had urethral and urachal exposure only. In addition, five female fetuses underwent the sham operation procedure to investigate possible sex differences in fetal bladder function. After weekly ultrasound examination (to confirm fetal viability and the extent of any urinary tract dilatation), pregnant ewes were killed 30 days after the initial procedure (105 days gestation). At autopsy of the obstructed group, urine leak was evident at the urethral meatus, indicating that the BOO was unlikely to be complete in utero. Fetuses were weighed and fetal bladders collected. In addition, femur length and occipito-snout length were measured as markers of fetal size. Bladders from all male fetuses (sham-operated and obstructed groups) underwent ex vivo filling cystometry, and bladder strips from all fetuses (male sham operated and obstructed and female sham operated) were used for contractility studies, biomechanical stretch studies, and histology. Furthermore, samples from fetal bladders from all groups were also used in a separate study investigating cellular turnover. This study was approved by an ethics committee. Animals (Scientific Procedures Act of 1986).

**Ex vivo filling cystometry.** To measure pressure-volume relationships, fetal bladders underwent ex vivo filling cystometry at postmortem examination with the urinary tract in situ. Experiments were performed as previously described (33). In brief, after noting the initial bladder volume and with all outflow tracts ligated or clipped, bladders were intermittently handfilled with Ca2+-free HEPES-buffered Tyrode solution (for composition see below) at increments of 1 ml in sham-operated bladders and 5 ml in obstructed bladders, and intravesical pressures were recorded continuously. In both groups, the range of filling was from an empty bladder to one where significant changes in intravesical pressure were measurable. Steady-state volume-pressure relationships were used to calculate compliance (ml/cmH2O). Bladder wall stress was also calculated (33) to compare results between the experimental and control groups, despite differences in the in situ bladder volumes.

**Solutions.** Ca2+-free HEPES-buffered Tyrode solution contained (in mM) 105 NaCl, 19.5 HEPES, 3.6 MgCl2·6H2O, 3.6 Na2HPO4·2H2O, 21.5 NaHCO3, 5.5 glucose, 4.5 Na pyruvate, pH 7.1, with 1 M NaOH. Tyrode solution was used for in vitro experiments, containing (in mM) 118 NaCl, 24 NaHCO3, 4.0 KCl, 1.0 MgCl2·6H2O, 0.4 Na2HPO4·2H2O, 1.8 CaCl2, 6.1 glucose, 5.0 Na pyruvate, gassed with 95% O2-5% CO2 (pH 7.4, 37°C). Tetrodotoxin (TTX, 1 µM), atropine (1 µM), αβ-methylene-ATP (10 µM), adenosine (1 mM), and carbachol (1–30 µM) were added to Tyrode solution from 1 or 10 mM aqueous stock solutions. 1H-[1,2,4]oxadiazolo[4,3-]quinoline-1-one (ODQ, 1 µM) was added from a 1 mM stock in chloroform. All chemicals were from Sigma (Poole, Dorset, UK).

**In vitro contractility studies.** After postmortem, a portion of the midbladder (with no bladder trigone) was placed in Ca2+-free HEPES-buffered Tyrode solution. Detrusor smooth muscle function was assessed using bladder strips (diameter <1 mm) after removal of the urethra and adventitia by microdissection (denuded bladder strips). Strips were superfused with Tyrode solution and electrically stimulated with 3-s tetanic trains (1–60 Hz; 0.1-ms pulses; every 90 s) in the presence or absence of pharmacological agents. To determine any effect of the urethra, whole wall bladder strips (diameter <1 mm, mucosal strips) were also studied under electrical field stimulation (EFS), in the presence or absence of ODQ. At the end of the experiments, strips were weighed, and contractile force was expressed as millinewtons per milligram wet tissue weight. Mucosal strips were corrected to muscle thickness wet weight as derived from histological studies (see Histology). Force-frequency relations and dose-response relations were fitted to the empirical equations or absence of ODQ. At the end of the experiments, strips were weighed, and contractile force was expressed as millinewtons per milligram wet tissue weight. Mucosal strips were corrected to muscle thickness wet weight as derived from histological studies (see Histology). Force-frequency relations and dose-response relations were fitted to the empirical equations (2)

\[
T = \frac{T_{\text{max}} \cdot f^n}{K_{\text{ECS}^50} + f^n}
\]

where \(T\) is the tension, \(T_{\text{max}}\) is the estimated maximum tension (or relaxation) at high frequencies or high concentrations, \(f\) is the frequency of stimulation, \(S\) is the agonist concentration, \(K_{\text{ECS}^50}\) and \(EC_{50}\) are the frequency and concentration, respectively, required to achieve \(T_{\text{max}}/2\), and \(n\) is a constant. In results, \(K_{\text{ECS}^50}\) and \(EC_{50}\) are expressed as their logarithmic transforms \(pK_{\text{ECS}^50}\) and \(pEC_{50}\) as these have been shown to be normally distributed parameters (2).

**Biomechanical stretch studies.** The viscoelastic properties of detrusor from the fetal bladder wall were measured using strips (length 4–5 mm, <1 mm diameter) cut from the midbladder and superfused with Tyrode solution. The mucosa was removed as this influences overall viscoelastic parameters (37). Muscle strips were tied between an isometric force transducer and a fixed arm whose position in the horizontal plane could be adjusted by a voltage-operated solenoid (30B Lever Arm System, Cambridge Technology, Watertown, MA). Changes of muscle strain (up to 1.6 mm in 0.3-mm increments) were generated by imposing square-voltage waves (50-s duration) on the solenoid. The resultant changes to muscle stress (tension), normalized to unit cross-section area (mN/mm), were recorded. From the slope of a plot between stress vs. strain, the elastic modulus could be calculated. Note, the inverse of this relationship is a measure of distensibility, and in the physiological context, when volume is plotted as a function of intravesical pressure, this slope is defined as the compliance of the system. Ramp steps (frequency <0.01 Hz) were also imposed to determine any steady-state hysteresis in the stress-strain relationships. Control experiments used a metal bar or a rubber band in place of the muscle strip. With a metal bar the experimental system exhibited a steady-state settling time of <50 µs to an instantaneous length change, i.e., several orders of magnitude faster than changes to muscle stress (see RESULTS). A linear stress-strain relation was recorded with a rubber band, demonstrating no intrinsic hysteresis in the experimental system. The viscoelastic changes of stress, \(T(t)\), were fitted to

\[
T = T' \left( \exp \left( -t/\tau_1 \right) \right) + T_1 \quad \text{for period of stretch (2a)}
\]

\[
T = T' \left[ 1 - \exp \left( -t/\tau_2 \right) \right] + T_2 \quad \text{for period of relaxation (2b)}
\]

where \(T'\) and \(T''\) are the magnitudes of the viscoelastic components of the total change of stress with time constants \(\tau_1\) and \(\tau_2\), and \(T_1\) and \(T_2\) are the steady-state elastic components.

The work in deforming the viscous component, \(W_v\), was calculated from the integral of the time-dependent component of the tension change during the period of stretch, \(t\)

\[
W_v = T' t - T \tau' \left( \exp \left( -t/\tau' \right) \right)
\]

The work to deform the elastic component, \(W_e\), was calculated from

\[
W_e = T_1 t
\]
Histology. At postmortem, a bladder sample was fixed in 10% paraformaldehyde (BDH, Poole, UK); all bladders were empty when samples were dissected. Fixed samples were dehydrated in alcohol, wax-embedded, and sectioned at 4-μm thickness. Sections were dewaxed, rehydrated, and stained with Masson’s trichrome. With the use of a computer program (KS 300, Zeiss, Oberkochen, Germany) that enabled bladder wall measurements from computer-captured images of stained bladder sections, the relative proportion of detrusor thickness to whole wall thickness was calculated for bladders from sham-operated and obstructed groups (n = 5 both groups). From this proportion, the muscle weight was calculated for mucosal strips so that tension could be corrected to muscle weight to allow for direct comparison with denuded strips.

Statistical methods. Results are expressed as means ± SD unless otherwise stated; independent sample Student’s t-tests were used to examine differences in mean values between sham-operated and obstructed groups and between male and female sham-operated groups. To determine whether percent changes to datasets were different from 100% (effect of mucosa on force generation), a Mann-Whitney U-test was used. The null hypothesis was rejected if P < 0.05.

RESULTS

Gross changes, filling cystometry, and bladder wall measurements. Table 1 shows the fetal bladder and body weights and bladder-to-body weight ratios (BBR). Both body and bladder weights were significantly increased in the obstructed vs. sham-operated group. However, the increase in bladder weight was greater than that of body weight as demonstrated by the increase of BBR. Although fetal weight was significantly increased in the obstructed group, the femur length and occipito-snout length were the same in the sham-operated and obstructed groups. This suggests that there was no overall size difference, but there may have been fluid accumulation in the obstructed group. There were no differences between male and female sham-operated fetuses in body or bladder weights. Ultrasonography confirmed dilatation of the obstructed urinary tract (data not shown).

Table 1 also shows data from the filling cystometry; the obstructed bladders had greater intravesical volumes at postmortem. On instillation of fluid, pressure rose instantaneously and then partially relaxed to a new steady-state level that was used to calculate compliance (change in volume/change in pressure). Bladder compliance was significantly greater, and calculated wall stress, at a particular intravesical volume, was significantly lower in the obstructed vs. sham-operated group.

Finally, Table 1 shows that from histological measurements, the obstructed fetal bladder wall was significantly thinner than their sham-operated counterparts. However, there was a similar proportion of detrusor comprising the bladder wall when corrected for this difference in wall thickness. The detrusor component as a proportion of the whole wall was used to calculate the relative muscle weight of mucosal bladder strips (below).

Contractility studies. The lengths (5.8 ± 1.0 mm sham-operated males, 5.2 ± 0.6 mm sham-operated females, 6.5 ± 0.6 mm obstructed males) and weights (4.5 ± 1.9 mg, 4.4 ± 1.5 mg, 10.6 ± 4.2 mg, respectively) of the denuded bladder strips used in the contractility studies were not significantly different between groups, but tension was normalized to unit muscle weight; mucosal strips were heavier than denuded strips (12.4 ± 2.7 mg, 14.3 ± 4.0 mg, 11.5 ± 3.0 mg, respectively). In a small number of preparations from sham-operated bladders, spontaneous contractions developed in later stages of experiments when recordings were terminated; all reported experiments were performed when preparations exhibited no spontaneous contractions.

Contractile properties of bladder strips: nerve-mediated contractions and muscarinic responses. Force-frequency relationships were generated by varying the tetanic stimulation frequency between 1 and 60 Hz in normal Tyrode solution and then in the presence of 1 μM TTX. Nerve-mediated tension was taken as the difference between total and TTX-resistant force. Figure 1A shows that the estimated maximum tension at high frequencies, T_max, was significantly reduced in the obstructed vs. sham-operated group (1.12 ± 0.46 vs. 5.21 ± 2.43 mN/mg, n = 5 both groups). The frequency required for half-maximum tension (K_{1/2}) was not diff-

Table 1. Bladder and fetal dimensions, filling cystometry data, and morphometric results in sham-operated male, obstructed male, and sham-operated female groups

<table>
<thead>
<tr>
<th></th>
<th>Sham-Operated Males (n = 5)</th>
<th>Obstructed Males (n = 5)</th>
<th>Sham-Operated Females (n = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bladder weight, g</td>
<td>1.14 ± 0.25</td>
<td>7.28 ± 4.41*</td>
<td>1.09 ± 0.16</td>
</tr>
<tr>
<td>Fetal weight, kg</td>
<td>1.38 ± 0.29</td>
<td>2.07 ± 0.41*</td>
<td>1.49 ± 0.29</td>
</tr>
<tr>
<td>Bladder/fetal weight ratio, g/kg</td>
<td>0.82 ± 0.11</td>
<td>3.39 ± 1.78*</td>
<td>0.74 ± 0.1</td>
</tr>
<tr>
<td>Femur length, cm</td>
<td>5.5 ± 0.3</td>
<td>6.4 ± 0.8</td>
<td>5.2 ± 0.4</td>
</tr>
<tr>
<td>Occipito-snout length, cm</td>
<td>12.7 ± 0.8</td>
<td>14.6 ± 1.6</td>
<td>13.2 ± 0.8</td>
</tr>
<tr>
<td>Initial intravesical volume, ml</td>
<td>5.7 ± 3.9</td>
<td>49.8 ± 32.1*</td>
<td></td>
</tr>
<tr>
<td>Maximum volume instilled, ml</td>
<td>6.4 ± 2.6</td>
<td>52.0 ± 31.7*</td>
<td></td>
</tr>
<tr>
<td>Maximum pressure, cmH2O</td>
<td>10.6 ± 1.8</td>
<td>12.2 ± 5.0</td>
<td></td>
</tr>
<tr>
<td>Compliance ml/cmH2O</td>
<td>0.64 ± 0.25</td>
<td>6.69 ± 5.4*</td>
<td></td>
</tr>
<tr>
<td>Wall stress, kN/m·m·m−2·ml−1</td>
<td>13.5 ± 2.2</td>
<td>2.7 ± 2.7*</td>
<td></td>
</tr>
<tr>
<td>Whole wall width, mm</td>
<td>2.60 ± 0.45</td>
<td>1.77 ± 0.22*</td>
<td></td>
</tr>
<tr>
<td>Detrusor width, mm</td>
<td>1.45 ± 0.27</td>
<td>1.07 ± 0.15*</td>
<td></td>
</tr>
<tr>
<td>Detrusor width, %wall thickness</td>
<td>56.3 ± 6.3</td>
<td>60.8 ± 9.7</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SD. *P < 0.05 vs. sham-operated males.
Different in the two groups (Fig. 1B): pK$_{1/2}$ values 1.28 ± 0.06 vs. 1.24 ± 0.13, respectively (mean K$_{1/2}$s 19.1 and 17.4 Hz, n = 5 both groups). In addition, sham-operated male and female parameters (T$_{max}$: 5.12 ± 1.37 mN/mg and pK$_{1/2}$ 1.04 ± 0.16; n = 5) were not significantly different. The inotropic response to carbachol in electrically unstimulated preparations was also reduced in the obstructed group. The T$_{max}$ derived from dose-response curves (Fig. 1C) was significantly reduced (4.70 ± 1.73 vs. 10.30 ± 2.38 mN/mg, P < 0.05, n = 5 both groups). However, the potency of carbachol was unchanged (Fig. 1D) as estimated EC$_{50}$ values were similar (pEC$_{50}$ values 5.58 ± 0.29 and 5.79 ± 0.41, respectively; mean EC$_{50}$ values 2.63 and 1.62 µM, respectively, n = 5 both groups). There was no evidence of desensitization to carbachol at the highest concentrations. The responses to carbachol were not significantly different in the sham-operated male and female groups (T$_{max}$ 7.77 ± 1.61 mN/mg, pEC$_{50}$ 5.63 ± 0.19).

Although both interventions revealed a reduced contractile response in the obstructed group, the magnitude of force decline was greater for the nerve-mediated responses compared with the carbachol-evoked contractions. The ratio of T$_{max}$ values from the force-frequency relationship and the carbachol dose-response curve was calculated for each preparation. For sham-operated animals, this was 0.68 ± 0.30 (n = 5) and was significantly less in the obstructed group (0.28 ± 0.16; n = 5, P < 0.05) (Fig. 1E). This may be explained by a reduction not just of the force-generating capacity of the detrusor in the obstructed bladder but also by a denervation to the tissue. The ratio of nerve-mediated and carbachol T$_{max}$ values was also similar in the sham-operated male and female (0.50 ± 0.12, n = 5) groups.

**Contractile properties of bladder strips: atropine-resistant contractions and the effect of adenosine.** The role of the purinergic system in the developing sham-operated and obstructed fetal bladder was examined. Atropine-resistant contractions were recorded in the presence of 1 µM atropine. Three of five strips from obstructed bladders, none from sham-operated male, and one from sham-operated female bladders revealed any atropine resistance. Figure 2A shows an example of force-frequency curves in the presence and absence of 1 µM atropine. The atropine-resistant response (51 ± 27%, n = 4 at 8 Hz) peaked at low frequencies.

As a breakdown product of ATP, the effect of adenosine was determined. This was examined at 8-Hz EFS as this approximates to K$_{1/2}$ and represents a fre-
quency where changes to nerve-mediated tension were easily measured. Figure 2B shows that adenosine (1 mM) reduced significantly tension produced by 8-Hz EFS in both the sham-operated male group (0.66 ± 0.24 to 0.30 ± 0.22 mN/mg, n = 5; P < 0.05) and the obstructed group (0.24 ± 0.12 to 0.08 ± 0.07 mN/mg, n = 5; P < 0.05). The percent reduction of force by adenosine was similar in the sham-operated male (60 ± 25%) and obstructed groups (58 ± 30%). However, the reduction of force was significantly greater in the sham-operated female group (1.85 ± 1.67 to 0.10 ± 0.04 mN/mg, n = 5; 94 ± 8% reduction) vs. the sham-operated male group.

To investigate the site of action of adenosine, its effect on the carbachol contracture in electrically unstimulated preparations was measured (Fig. 2C). Adenosine at 1 mM had no significant effect on the magnitude of the carbachol (1 μM) contracture (107 ± 21, 88 ± 10, and 102 ± 16% of control with adenosine for sham-operated male, sham-operated female, and obstructed male groups, respectively). This implies that adenosine has no direct effect on muscle contractility but acts on earlier steps in the generation of contraction.

Contractile properties of bladder strips: the nitrergic system. ODQ was used to examine the possible role of a nitrergic component to fetal bladder neurotransmission, as this agent has been shown to reduce the relaxant effect of cGMP that is generated by release of nitric oxide (18). Preparations were electrically stimulated when the muscle was contracted by 1 μM carbachol to record any relaxation that might be evoked by release of nitric oxide. Figure 3A shows transient relaxations to EFS in a muscle contracted with carbachol; at higher frequencies the responses became biphasic. TTX (1 μM) abolished these responses, confirming that responses were nerve mediated (data not shown).

The effect of ODQ was variable in different preparations in any group, so that no significance in the magnitude of the effect was seen in muscles from sham-operated male and obstructed bladders (Fig. 3B, 34 ± 43 and 77 ± 28%, respectively, n = 5 both groups). A similar effect of ODQ was seen in three preparations from sham-operated females (data not shown). Chloroform (0.1% vol/vol), used as a solvent for ODQ, had no effect on the relaxant responses (data not shown).

To investigate any effect of the urothelium and lamina propria on muscle contractility and to examine the possible involvement of nitric oxide, force-frequency relations were generated with mucosal strips in the absence and presence of ODQ. Figure 3C shows that the presence of mucosa had a significant (P < 0.05, Mann-Whitney U test) dampening effect on the muscle contractility in the sham-operated male group (T_{max} full thickness strip 69 ± 18% of denuded strip force) and the sham-operated female group (T_{max} EFS full thickness strip 65 ± 17% of denuded strip force). With mucosal strips, ODQ significantly elevated tension in the sham-operated male and female groups (116 ± 7 and 115 ± 11%, respectively, n = 5 both groups). In the obstructed group, the presence of mu-
cosa had no significant effect on $T_{\text{max}}$ EFS ($165 \pm 127\%$, $n = 5$) although the variability of the data was relatively large. In addition, ODQ did not affect force ($121 \pm 20\%$ control).

**Stress-strain relationships of isolated preparations.**

Figure 4 shows the change of stress (tension) when a muscle strip from a sham-operated male or obstructed male bladder was subjected to a step change of strain (length). The preparation demonstrated a rapid increase of stress upon stretch followed by a partial time-dependent relaxation despite the maintenance of strain. On cessation of the step response, muscle stress overshot before recovering to the initial resting level.

Table 2 shows the elastic and viscoelastic properties of denuded muscle strips from the three groups. The steady-state elasticity was significantly lower in the obstructed group vs. sham-operated male group. In addition, steady-state elastic work and viscoelastic work were also significantly lower ($P < 0.005$) in the obstructed vs. sham-operated male group. However, the proportion of total work carried out by the elastic element (and hence the viscoelastic element) as well as the time constant of stress relaxation ($\tau$) was not different in the two groups. There was no difference in any parameter for the sham-operated male and female groups, except a prolongation of the stress-relaxation time constant in the sham-operated female group. Similar conclusions were obtained if the stress response on a return to the control strain from the stretched state (off response) was analyzed, indicating that the magnitude of the strain changes did not exceed the plastic limit of the preparations.

Steady-state stress-strain relationships were generated by imposing ramp stretches to 120% of resting length with a total cycle length of 100 s (50 s stretch, 50 s release). The rate of change of length was approximately five times slower than the viscoelastic time constant so that such ramp-generated stress-strain relationships could be considered to reflect only the elastic properties of the tissue. Figure 5, A and B, shows such relationships for strips from sham-operated male and obstructed bladders, respectively. Both relationships showed hysteresis, i.e., the stretch and relaxation plots were not identical. The area within the loop is work lost during the ramp experiment (presumably as heat) and was less in strips from obstructed vs. sham-operated bladders ($183 \pm 50 \text{ vs. } 305 \pm 17 \text{ mN}\cdot\text{mg}^{-1}\cdot\text{mm}^{-1}$, $n = 3$ and 5, respectively, Fig. 5C).

**DISCUSSION**

The fetal sheep is a useful model to study developmental bladder function. In addition to the advantages such as their large size, maternal and fetal tolerance to in utero surgery, and recognized handling and breeding procedures, numerous investigators have measured bladder function during normal fetal development (25) and after in utero BOO (28, 33, 34). Furthermore, there have been a number of studies examining the effect of surgery, such as their large size, maternal and fetal tolerance to in utero surgery, and recognized handling and breeding procedures, numerous investigators have measured bladder function during normal fetal development (25) and after in utero BOO (28, 33, 34). Furthermore, there have been a number of studies examining the effect of surgery.
changes to the upper urinary tracts in obstructed fetal sheep (1, 14, 50). However, there has been little work on changes to muscle function in the obstructed fetal bladder. We have previously described how the fetal bladder, after partial in utero BOO, becomes denervated, flaccid, and hypocontractile and also accommodated an increase in volume without any change of storage pressure (33). In the current study, we have extended these observations to investigate in more detail the causes of the hypocontractile function and the nature of motor transmission to the fetal bladder. Implications of our findings are explained at the end of the discussion.

Cholinergic neurotransmission. Consistent with our previous study (33), BOO produced a hypocontractile response to EFS and muscarinic stimulation (with carbachol), and this diminished response was greater with EFS, supporting the possibility of denervation. However, the stimulation frequency producing a half-maximal nerve-mediated contraction, $K_{1/2}$, and the half-maximal carbachol concentration, $EC_{50}$, were similar in strips from obstructed and sham-operated bladders. This shows that changes to the excitability of muscle strips to nerve stimulation or detrusor muscarinic sensitivity could not explain the hypocontractility. The lack of denervation supersensitivity to muscarinic agonists is in contrast to observations made in the obstructed adult pig bladder (36) but is in keeping with studies using guinea pig, rabbit, rat, sheep, or human tissue (15, 19). Furthermore, to our knowledge, there have been no conclusive demonstrations of denervation supersensitivity in the obstructed fetal bladder, although it has been implied in a fetal rabbit preparation (35).

Purinergic neurotransmission. In this study, atropine-resistant contractions were primarily found in the obstructed group, in keeping with reports of similar responses in detrusor from overactive human bladders (idiopathic or secondary to obstruction) (2). The sham-operated bladders had little atropine resistance in contrast to many other animal bladders but similar to normal human detrusor (7, 9, 40, 51). It has been proposed that atropine-resistant contractions occur in obstructed bladders due to decreased ectoATPase activity, so that neurally released ATP is more likely to activate detrusor smooth muscle (20). Atropine-resistant contractions are evoked more readily at lower stimulation frequencies (see Fig. 2A). It is possible that neural ATP is depleted at higher frequencies and that at greater frequencies, ectoATPase release itself may be enhanced (46). It is important to note that the similarity of the atropine-resistance responses to human detrusor makes the ovine model of direct relevance to understanding the pathophysiology of human fetal bladder obstruction.

Table 2. Steady-state and viscoelastic properties of muscle strips taken from sham-operated and obstructed fetal male bladders and sham-operated female bladders

<table>
<thead>
<tr>
<th></th>
<th>Sham-Operated Males ($n = 16$)</th>
<th>Obstructed Males ($n = 18$)</th>
<th>Sham-Operated Females ($n = 7$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Elasticity, mN/mm$^3$</td>
<td>$143.3 \pm 173.8$</td>
<td>$289.0 \pm 39.1^*$</td>
<td>$183 \pm 196$</td>
</tr>
<tr>
<td>$W_e$, mN$^s$·mg$^{-1}$</td>
<td>$2.942 \pm 757$</td>
<td>$881 \pm 181^*$</td>
<td>$4.168 \pm 491$</td>
</tr>
<tr>
<td>$W_v$, mN$^s$·mg$^{-1}$</td>
<td>$243 \pm 295$</td>
<td>$40 \pm 16^*$</td>
<td>$243 \pm 26$</td>
</tr>
<tr>
<td>$W_e/W_v \times 100 %$</td>
<td>$90.0 \pm 12.0$</td>
<td>$95.5 \pm 2.0$</td>
<td>$96.0 \pm 5.0$</td>
</tr>
<tr>
<td>$\tau$, s</td>
<td>$11.1 \pm 5.0$</td>
<td>$12.0 \pm 3.8$</td>
<td>$21.4 \pm 2.51^*$</td>
</tr>
</tbody>
</table>

Values are means ± SD. $W_e$, elastic work; $W_v$, viscoelastic work; $\tau$, time constant of stress relaxation. *$P < 0.05$ vs. sham-operated males.

Fig. 5. Stress-strain relationships during a ramp change of strain. Hysteresis loops for steady-state stress relationships of preparations from sham-operated (A) and obstructed (B) bladders. Arrows in A illustrate direction of hysteresis during stretch (top arrow) and relaxation (bottom arrow). C: work lost during increase and decrease of strain (area within hysteresis loops). *$P < 0.05$, SM vs. OM. Inset: hysteresis loop in a rubber band strip.
The breakdown product of purinergic neurotransmission, adenosine, reduced significantly the magnitude of the nerve-mediated contraction in both sham-operated and obstructed groups. However, no effect was observed on contractions evoked by direct muscarinic receptor activation. This suggests that in the ovine fetal bladder adenosine acts on presynaptic, possibly P1, receptors as has been previously described in other species (32, 40, 41). There was no difference in the sensitivity of this response in the sham-operated and obstructed groups, although detrusor from female sham-operated animals was significantly more sensitive.

Nitricergic neurotransmission. Evidence is now accumulating of a role for nitric oxide-mediated relaxation in the lower urinary tract (3, 5, 31, 43). Nitric oxide produces a relaxant response in the urethra and bladder neck, suggesting a role in bladder outlet relaxation during micturition. Evidence supporting the relaxant role of nitric oxide in detrusor smooth muscle remains less convincing despite evidence of nitricergic innervation within fetal detrusor muscle (12). EFS of maximally precontracted detrusor strips produced either a relaxation or a biphasic relaxation-contraction response; the latter occurred at higher frequencies. Stimulation of precontracted obstructed bladder detrusor strips produced a smaller absolute relaxation, in keeping with the decreased force produced by other activators. TTX abolished the electrically stimulated relaxant forces, confirming their neural origin; similar observations have also been made in fetal sheep (28) and cow models (26).

The guanylate cyclase inhibitor ODQ attenuated the relaxations, consistent with the hypothesis that relaxation was nitric oxide mediated. However, responses were abolished incompletely by ODQ, suggesting either that it may not be totally effective or that other neurotransmitters may be involved (16). This study showed that in utero obstruction did not alter the percent attenuation of the relaxations by ODQ in the sham-operated and obstructed groups and is consistent with findings in the obstructed fetal sheep bladder (28) and obstructed rat bladder (38) but in contrast to mice models of bladder dysfunction (6, 27). Interestingly, fetal bladder studies of the nitricergic system did not find relaxant forces in the adult bladder of the same species (26, 28) so that these relaxant forces may be necessary for the protection of renal maturation during development.

Effect of the mucosa and nitricergic neurotransmission. The nitricergic system may be associated with the bladder urothelium (4). Force-frequency relations for mucosal bladder strips (corrected for wet weight of muscle) examined any effect of the urothelium, and these were repeated in the presence of ODQ. Urothelium significantly reduced the force developed by detrusor muscle strips that was partially reversed by ODQ, suggesting nitric oxide mediation. The partial effect of ODQ again may suggest other mediators are involved (13, 21) or that the dampening effect of the mucosa may also be due to a direct mechanical effect. However, the presence of the mucosa in the obstructed fetal bladder strips did not decrease the force of contraction, and ODQ had no effect. Thus any mediator released from the urothelium may not be released after in utero obstruction, consistent with our histological description of an attenuated urothelium and lamina propria after obstruction (33).

Biomechanical studies. These experiments were performed to measure the viscoelastic properties of the developing and obstructed fetal bladder. The purpose was twofold: to complement the findings from filling cystometry that showed the obstructed fetal bladder was more compliant and exhibited less wall stress than the sham-operated counterpart, and to examine the hypothesis that the hypocontractile state of the obstructed bladder may in part result from a change to the passive viscoelastic properties of the bladder wall.

Linear steady-state stress-strain relationships were generated from which an elastic modulus was calculated. In strips from obstructed bladders, elasticity was significantly smaller than in the sham-operated counterparts, showing that the tissue was more flaccid and corroborated qualitatively the cystometry findings. A reduction of elastic modulus in a tissue strip would generate the appearance of a hypocontractile preparation as tension generated by the muscular elements would generate less tension in the whole preparation. Thus reduced contractile force in an isolated muscle strip or reduced wall stress in an intact bladder on activation by an agonist may not reflect any derangement of muscle function but may be simply due to the relative inability of the extramuscular components to sustain that force. This is generally overlooked when attempting to explain the causes of contractile failure and can explain the relative ineffectiveness of positive inotropic agents to reverse the problem on occasion. This explanation is not the only cause for the hypocontractility of the obstructed fetal bladder as nerve-mediated force was reduced more than carbachol-mediated contraction and suggests partial denervation also contributed to the smaller nerve-evoked response. However, these two factors may be sufficient to explain the reduced contractility of the obstructed bladder without the necessity of evoking significant muscle failure. This corroborates other findings (49) that show little difference in the ability of isolated cells from normal and obstructed bladders to generate agonist-mediated intracellular Ca\textsuperscript{2+} transients.

However, an increase of the viscous component of the overall stress-strain relationship may also attenuate transient contractile responses, due to damping of the generated tension. For this reason, we quantified the viscous work as a proportion of total work during a step change of strain and found that this was unchanged in the obstructed bladders. This observation, coupled with the fact that the time constant of viscoelastic relaxation was also unaltered, suggests that the physical properties of the extracellular matrix are unchanged in the obstructed bladder (45). Differences in muscle fiber arrangement and rates of preceding stretch can influence viscoelastic properties (37), and
we took strips from the same area of the bladder and maintained constant rates and magnitudes of stretch in our experiments to minimize these problems.

Clockwise and symmetrical steady-state hysteresis loops were recorded in muscle strips from sham-operated and obstructed groups; this is a phenomenon characteristic of inert substances such as rubber and not organic material such as the lung. This suggests that elastic elements will absorb energy when stretched, which is released in a nonelastic way when relaxed. Of interest in these experiments is that the elastic modulus (the tangent to the hysteresis loop) is not a constant but depends on the magnitude of stretch. For this reason, constant magnitudes of stretch were used to calculate the elastic modulus above.

**Sex differences.** There was no significant difference between the fetal weights and bladder weights in the sham-operated male and sham-operated female fetuses 30 days postoperation and only a small number of functional differences, e.g., the significantly greater effect of adenosine on reducing the nerve-mediated contraction. The lack of sex differences may be surprising as the male and female fetal bladders are exposed to different sex hormones during in utero development (42). Nonetheless, the lack of sex differences may be useful in planning future fetal bladder experiments.

**Human disease.** Although infants with posterior urethral valves typically initially suffer thick-walled, hypercontractile bladders (that progress to a large hypotensive bladder) (22), to date, the contractile properties and pathophysiological progression of bladder dysfunction in human fetal BOO remain poorly understood. Furthermore, dilated bladders have been described in human fetal bladders that suffer in utero bladder obstruction by posterior urethral valves (30, 39) and by the possible bladder obstruction associated with the prune belly syndrome (44). Our experimental model of in utero BOO results in a large dilated hypotensive bladder. This may represent a number of possibilities. First, the model may represent severe obstruction, resulting in a severe bladder phenotype. Alternatively, the hypotensive bladder may represent one end of a spectrum of changes that the fetal bladder undergoes in response to in utero obstruction. We speculate that in response to obstruction, the fetal bladder initially produces a compensatory response followed by a compensation of bladder function; the latter is observed in our current experimental model. Finally, the hypotcontractility was observed in bladder strips taken from an empty or decompressed bladder, which may differ from in vivo whole organ physiology. These issues remain to be addressed by documenting the pressure changes within the fetal bladder and the bladder transformation at different time points.

In conclusion, we have demonstrated that obstruction of the fetal male bladder yields one that is enlarged, hypotcontractile, and compliant. The increased compliance and in part the hypotcontractile state may be explained by a reduction of tissue elasticity. Functional denervation may also contribute to the hypotcontractile state. The urothelium exerts a negative inotropic influence on the detrusor, mediated in part by nitric oxide, that is absent in the obstructed bladder. However, there is little direct evidence that neurotransmitters from the cholinergic, purinergic, or nitricergic systems have significantly different effects on detrusor from sham-operated control or obstructed bladders. The latter point requires, however, direct study by using isolated detrusor myocytes that are free of extracellular influences.

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