Pudendal nerve injury reduces urethral outlet resistance in diabetic rats

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Pan HQ, Lin DL, Strauch C, Butler RS, Monnier VM, Daneshgari F, Damaser MS. Pudendal nerve injury reduces urethral outlet resistance in diabetic rats. Am J Physiol Renal Physiol 299: F1443–F1450, 2010. First published September 29, 2010; doi:10.1152/ajprenal.00341.2009.—Diabetics have voiding and continence dysfunction to which elevated levels of advanced glycation end products (AGE) may contribute. In addition, pudendal nerve injury is correlated with voiding dysfunction and stress incontinence in rats. The aim of this study was to investigate whether pudendal nerve crush (PNC) in diabetic rats alters urinary function. Female virgin Sprague-Dawley rats (144) were divided equally into diabetic, diuretic, and control groups. Half of the animals in each group were subjected to PNC, and the other half to sham PNC. Diabetes was induced 8 wk before PNC or sham PNC by streptozotocin injection (35 mg/kg). Animals underwent conscious cystometry and leak point pressure (LPP) testing 4 or 13 days after PNC or sham PNC. Tissues of half the animals were tested for levels of AGES. Qualitative histological assessment was performed in the remaining animals. Diabetic rats 4 days after PNC voided significantly greater volume in a shorter time and with significantly less pressure than after sham PNC, suggesting that diabetic rats have a functional outlet obstruction that is relieved by PNC. LPP was significantly reduced 4 days after PNC in diabetic and diuretic animals and returned to normal 13 days after PNC. Diabetic rats with PNC demonstrated increased muscle fiber disruption and atrophy of the external urethral sphincter. AGES were significantly elevated in diabetic rats. PNC relieves a functional outlet obstruction in diabetic rats. AGES are elevated in diabetic rats and could play a role in urinary dysfunction and recovery from PNC.

WOMEN WITH DIABETES have a higher prevalence of lower urinary tract complications than women without diabetes and men with diabetes (2, 3, 42). Diabetes affects women at or before their childbearing years and increases their risk of stress urinary incontinence and overactive bladder, contributing to the high prevalence of incontinence (17). The pudendal nerve courses through Alcock’s canal and is particularly vulnerable to stretch and crush injury during childbirth, leading to denervation and atrophy of the external urethral sphincter (EUS) and reduced urethral closure, resulting in symptoms of incontinence (14, 49). However, the relationship between diabetes, birth trauma, and incontinence is poorly understood.

Hyperglycemia is considered the principal cause of diabetic complications. The formation of sugar-derived advanced glycation end products (AGEs) has been shown to be important for mediating almost all diabetic complications (16, 18). AGEs accumulate in the peripheral nerves of diabetic patients and the use of anti-AGE agents improves nerve conduction velocities and neuronal blood flow (50). Therefore, AGES may play a role in the etiology of incontinence in diabetic women.

Animal models have demonstrated denervation, hypoxia, and atrophy of the EUS after simulated childbirth injuries (9, 10). These injuries reduce urethral closure and leak point pressure (LPP), resulting in symptoms of stress incontinence (20) which recover with time and correlate with clinical data (14, 49). Pudendal nerve crush (PNC) has been used to simulate the injury to the pudendal nerve during vaginal delivery, resulting in reduced urethral closure (10, 26) and voiding dysfunction (39, 40), both of which are reversible following injury (26). Diabetic rats have increased bladder outlet resistance (52), demonstrating that diabetes impacts the urethra in this animal model. The aim of the present study was to determine whether PNC in diabetic animals results in dysfunction of continence or voiding, and to determine whether altered responses in diabetic animals are associated with increased accumulation of AGES.

MATERIALS AND METHODS

This study was approved by the Institutional Animal Care and Use Committee of Cleveland Clinic, Cleveland, OH. To decrease possible variations in anatomy and function due to prior deliveries (26), 144 age-matched female virgin Sprague-Dawley (180–200 g) rats were divided into diabetic, diuretic, and untreated control groups with 48 animals in each group. Each group was further subdivided into bilateral PNC and sham PNC. All rats underwent urodynamic testing 4 or 13 days after injury, respectively, including conscious cystometry (CMG) and LPP testing. The tissues were harvested after LPP testing for histopathology and assessment of accumulation of AGES.

Induction of diabetes. Rats in the diabetic group were made hyperglycemic by a single intraperitoneal (ip) injection of 35 mg/kg body wt of streptozotocin (STZ) in 0.1 M sodium citrate buffer (pH 5.4) after a 24-h fast. The induction of hyperglycemia was confirmed by blood glucose greater than 300 mg/dl (Prestige LX tm, Home Diagnostics, Fort Lauderdale, FL) on the 3rd day after the STZ injection and on the final day of the experiment. The animals had diabetes for 8 wk before PNC or sham PNC. They were usually not seriously ill with this level of diabetes. As in our previous work (27), mortality was below 5%.

Induction of diuresis in the absence of diabetes. To control for the effect of diuresis, we added 5% sucrose to the drinking water of an age-matched group of animals for the 8-wk duration of the study. This group of animals allows us to identify the extent to which diuresis alters bladder function in diabetic bladder dysfunction.

PNC. Bilateral PNC was performed as previously reported (10, 26). Rats were anesthetized with a mixture of ip ketamine (100 mg/kg) and xylazine (10 mg/kg). A midline posterior longitudinal skin incision was made and the ischiorectal fossa was opened bilaterally with retractors. The pudendal nerve was identified and all branches were crushed twice bilaterally with a Castroviejo needle holder for 30 s. Sham PNC rats underwent a dorsal skin incision only. The animals
were given buprenorphine (0.3 mg/kg) subcutaneously for pain control.

Catheter implantation. Two days before LPP testing, all rats underwent suprapubic bladder catheter implantation as previously described (10, 26). The rats were anesthetized as described above and a circular purse-string suture was placed on the bladder wall. A small incision was made in the bladder wall and the catheter (PE-50 tubing with a flared tip) was implanted in the bladder dome. The catheter was then tunneled subcutaneously to the back of neck, where it exited the skin. The catheter was capped and buprenorphine was administrated for pain control as above.

CMG. CMG was performed via a suprapubic bladder catheter 4 and 13 days after injury with the animal in a modified metabolic cage as described previously (38). The bladder catheter was connected to both a syringe pump (KD Scientific 200) and a pressure transducer (Statham P23SL). All bladder pressures were referenced to air pressure at the level of the bladder. A force transducer (model FT10, Grass Instruments, West Warwick, RI) with a beaker on top was calibrated to measure volume and was placed under the cage to record voided volume. Each bladder was filled with saline via the catheter at 5 ml/h while bladder pressure and voided volume were continuously recorded. A voiding contraction was defined as a bladder pressure increase that resulted in urine loss as detected by the force transducer under the cage. Three fills and voids were recorded for each rat. Pressure and volume data were amplified (model P122, Grass Instruments), recorded on a chart recorder, and digitized (10 samples/s) for computer data collection. Mean voided volume, mean duration of void (the time from the beginning of the bladder contraction to the return to baseline pressure), and mean increase in bladder pressure for voiding (baseline bladder pressure subtracted from peak voiding pressure) were calculated for each animal.

LPP. Immediately after cystometry, the animals were anesthetized with urethane (1.2 g/kg body wt ip) and placed supine at the zero pressure level for LPP testing as previously described (10, 22, 23, 26). The bladder catheter was connected via a stopcock to a syringe flow pump (model 200, KD Scientific, New Hope, PA) and a pressure transducer (model P300, Grass Instruments). Pressure signals were amplified (model P122, Grass Instruments), recorded on a chart recorder, and digitized for computer data collection (10 samples/s). The bladder was palpated to empty and filled with saline at 5 ml/h. After 0.3 ml had been infused (approximately half the capacity of a 200-g rat), gentle pressure was applied to the rat’s abdomen to simulate a Credé maneuver, while bladder pressure was recorded. Pressure was slowly increased until saline leaked through the urethra. At the first indication of leakage at the urethral meatus, the applied pressure was rapidly removed. LPP was calculated by subtracting bladder baseline pressure from peak bladder pressure at the moment of leakage in the absence of a bladder contraction, as done previously by ourselves and others (23, 37, 38). LPP provides a consistent measure of urethral resistance, is not sensitive to volume in the bladder, and consistently parallels peak pressure at leakage after injury (1, 4, 8, 9, 44). In general, this external application of gentle pressure does not generate a voiding reflex (5, 9). If a voiding reflex occurred, the result of that test was disregarded and the bladder was emptied, refilled, and the test was repeated. The study was repeated three times in each rat and a mean LPP value was calculated for each rat.

Assessment of AGEs. The bladder, urethra, vagina, and pudendal nerve (6 rats/group of 4 days after injury) were dissected separately and assessed for occurrence of several AGEs, including fructoselysine (furosine), Nε-carboxymethyllysine (CML), Nε-carboxyethyllysine (CEL), and 2-aminoacidic acid using gas chromatography-mass spectrometry (GC-MS; HP-5971, Agilent, Santa Clara, CA) as previously described (43). Animal tissues (30 g) were homogenized using a glass hand-held homogenizer with 1 ml ice-cold Chelex-100 treated (5% Chelex-100, overnight) extraction buffer (PBS containing 0.5 mM EDTA, pH 7.4) plus protease inhibitors (1 μg/ml leupeptin and 1 μg/ml aprotinin). Tissues were then homogenized by hand and the crude homogenate was centrifuged at 15,000 g for 20 min at 40°C. The pellet was washed twice with cold Chelex-treated PBS and with cold water to remove the PBS and delipidated in a chloroform:methanol (2:1) solution overnight at 40°C. The chloroform:methanol was aspirated and the samples were hydrolyzed in 3 ml deionized 6 N HCl for 16 h. For analysis, the HCl was evaporated and a ninhydrin assay was used to determine amino acid content. Tissue samples were aliquoted in three leucine equivalents and spiked with internal standards. Finally, the tissue samples were cleaned using Supelco LC-18 columns, dried, and derivatized for GC-MS analysis as previously described (43).

Histology. Immediately after LPP testing, the remaining anesthetized animals (5–6 rats/group) were euthanized. The urethra at the level of the EUS was dissected en bloc. The urinary bladder was removed at the level of the bladder neck/proximal urethra caudally for morphological study. The bladder was gently dried with gauze and then weighed. All tissues were immersed and fixed in 10% formalin for 24 h. Each piece of tissue was then embedded in paraffin, sectioned transversely (5 μm), and stained with Masson’s trichrome for qualitative histological analysis.

Statistical analysis. A two-way ANOVA was used to compare response means of CMG and LPP across all groups (SAS, v. 9.1). Pairwise mean comparisons were made using the t-test with a Bonferroni correction. A two-way ANOVA was used to compare response means of AGE results across all groups, with a Tukey-Kramer post hoc test for multiple comparisons. In all cases, P < 0.05 was taken to indicate a significant difference between groups. Histological data were analyzed qualitatively.

RESULTS

CMG and LPP. Real-time cystometric pressure recordings were similar to those presented previously (52). Four days after either PNC or sham PNC, diabetic rats had significantly increased voided volume compared with control and diabetic rats (Fig. 1A), characteristic of diabetes (12, 21). Diabetic rats 13 days after PNC had significantly increased voided volume compared with both control and diabetic rats with PNC, whereas rats with sham PNC had significantly increased voided volume compared only with control rats (Fig. 1B). The volume voided by control, diabetic, or diabetic rats either 4 or 13 days after PNC was not significantly different from that of rats with sham PNC (Fig. 1, A and B).

Duration of void was not significantly different in diabetic rats compared with control and diabetic rats either 4 or 13 days after sham PNC or PNC (Fig. 1, C and D). Diabetic rats 4 days after PNC demonstrated significantly decreased duration of void compared with diabetic rats with sham PNC (Fig. 1C), suggesting that PNC reduced a functional outlet obstruction. Duration of void in diabetic rats 13 days after PNC was not significantly different compared with diabetic rats with sham PNC (Fig. 1D).

Four days after sham PNC in diabetic rats, the increase in pressure to void was significantly increased compared with control and diabetic rats with sham PNC (Fig. 1E). Four days after PNC in diabetic rats, the increase in pressure to void was significantly decreased compared with diabetic rats with sham PNC, confirming that PNC reduced a functional outlet obstruction. In contrast, 13 days after PNC, diabetic rats did not have significantly different increased pressure to void compared with diabetic rats with sham PNC (Fig. 1F). Increased pressure to void in diabetic rats 13 days after sham PNC was not significantly different compared with both control and di-

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uretic groups with sham PNC, but increased pressure to void in diabetic rats 13 days after PNC was significantly increased compared with both control and diuretic rats with PNC (Fig. 1F).

LPP was significantly decreased 4 days after PNC in the diuretic and diabetic groups compared with sham PNC (Fig. 2A). These differences were not significant 13 days after PNC or sham PNC, suggesting recovery of urethral resistance to leakage. Peak bladder pressure recorded during the LPP study paralleled the LPP results as observed previously (1, 4, 8, 9, 44). There were no differences between the control, diuretic, and diabetic groups in LPP.

*Bladder weight and histology.* The bladder weight of both diuretic and diabetic rats 4 days after PNC or sham PNC was significantly increased compared with both control and diuretic rats, respectively (Fig. 3A). Thirteen days after sham PNC, the bladder weight of both diabetic and diuretic rats remained significantly increased compared with that of the control rats. The bladder weight of diabetic rats 13 days after sham PNC was also significantly greater than that of diuretic rats with sham PNC. In contrast, the bladder weight of diabetic rats 13
days after PNC was significantly increased compared with only the control rats (Fig. 3B). There were no significant differences in bladder weight between PNC and sham PNC in all groups. In all diabetic and diuretic groups, the bladder cavity was markedly increased compared with the control group (Fig. 4). The bladder of diabetic animals had fewer and thinner muscle layers, as well as more collagen fibers compared with both the diuretic and control rats 4 and 13 days after injury (Fig. 4).

In control animals with sham PNC, the EUS is compact and generally circumferential (Fig. 5A). In contrast, in diabetic rats with sham PNC, the EUS fibers demonstrated tenuous-appearing muscle fibers (Fig. 5, B and C). Histology in diuretic rats with sham PNC was similar to the comparable control group (data not shown). Focal disruption and pathology of the EUS in control animals 4 days after PNC were increased compared with rats with sham PNC in all groups (Fig. 5D). Moreover, there was increased disruption of the EUS in diabetic animals with PNC, with obvious thinning and atrophy of the EUS compared with any other group (Fig. 5E). Thirteen days after injury, mild focal disruption of the EUS was evident in rats with PNC compared with control animals. There was less extensive damage and disruption in the EUS but more collagen infiltration around the EUS in diabetic rats 13 days after PNC compared with the diabetic animals 4 days after PNC (Fig. 5F).

**AGEs assessment.** Fructose-lysine (furosine) and CML were significantly increased in all tissues tested in diabetic rats compared with control and diuretic rats (Fig. 6). There were no significant differences between PNC and sham PNC animals in any group. There were no significant differences between the groups in CEL or 2-aminoadipic acid in any tissue (data not shown).

**DISCUSSION**

Diabetes affects multiple organ systems, including the lower urinary tract, and can cause bladder dysfunction resulting in incontinence (21). Recent research suggests that diabetes also plays a role in severe or recurrent stress incontinence in women (13, 17). Current theories on the pathophysiology of lower urinary tract complications of diabetes include microvascular, neuropathic, and myopathic dysfunction of pelvic components due to hyperglycemia, but mechanistic details remain to be further determined (17). It is likely that diabetes, due to preexisting alterations in their lower urinary tract or pelvic tissues, such as autonomic neuropathy or myopathy, may sustain greater injury and do not recover as well from birth trauma as nondiabetic women (13, 41).

Formation of AGEs in diabetes is also a major contributor to hyperglycemia-induced diabetic neuropathy (30). Diabetes impairs nerve regeneration after a nerve injury (25), in part due to a process initiated by elevated AGE levels (48). When structural and functional proteins of nerves are glycated, impaired nerve function and characteristic pathologic alterations result, including microangiopathy of the nerve, possibly from direct tissue damage to the nerve or by endothelial injury or vascular dysfunction (46). AGEs have been implicated in neuronal degeneration when increased for prolonged periods (50). Thus, impaired pudendal nerve injury may contribute to development of incontinence in diabetic women via an AGE-mediated mechanism.

STZ-induced diabetic rats are widely utilized and are considered to be the closest drug-induced diabetic model to insulin-dependent type 1 diabetes in humans (45, 47). STZ causes...
diabetes by destroying pancreatic β cells, resulting in insulin deficiency. This model has been used extensively for studies of the effects of diabetes on the lower urinary tract and on tissue regeneration after injury (45). In our study, a single ip injection of 35 mg/kg body wt of STZ produced hyperglycemia with blood glucose exceeding 300 mg/dl on the 3rd day after injection and lasting 8 wk until the final day of the experiment. Animals with diabetes are usually not seriously ill after injection of this dosage of STZ and mortality is below 5%, consistent with our previous work (27).

Increased bladder weight and bladder dysfunction are common complications of diabetes (12) and our findings of increased bladder weight in diabetic rats are consistent with reports from other investigators (15, 30). We demonstrated that the bladder weight of diuretic rats with sham PNC increased compared with control rats and the bladder weight of diabetic rats increased further and both were significantly greater than the bladder weight of both diuretic and control rats. In contrast, Tammela et al. (47) reported that bladder weight increased faster in the diabetic group, but by 30 days there were no significant differences in bladder weights between the diabetic and the sucrose groups. In another study, although bladder weight of diabetic and diuretic rats markedly increased compared with the control group, there were no significant differences in bladder weights between the diabetic and the diuretic animals (31). Although the differences in bladder weight between diuretic and diabetic rats in our study were statistically significant, they were not dramatic. Therefore, the differences between our results and those of previous studies could likely be explained by differences in variability between animals and/or differences in statistical tests.

Pudendal nerve injury can affect both voiding and continence in both diabetic and normal rats (39, 40, 52, 53). In our study, diabetic animals 4 days after sham PNC had significantly increased voided volume and voiding pressure without significant alteration of duration of voids, suggesting that the bladder of diabetic rats adapts to increased urine output and increased outlet resistance by producing greater pressures, as has been previously reported (52). Duration of voids and voiding pressure were significantly decreased in diabetic rats after PNC compared with diabetic rats with sham PNC, suggesting that PNC relieves the outlet obstruction caused by diabetes in rats. This enables diabetic rats with PNC to void the same volume of urine in a shorter time and at lower pressures. The significant decrease in LPP in diabetic rats after PNC supports the idea that the urethra is denervated after PNC,
relieving the functional outlet obstruction. These symptoms have been observed in diabetic patients as well (28).

Some anesthetics, including barbiturates, ketamine, and halothane, suppress the micturition reflex in rats (51). Urethane, in contrast, maintains the micturition reflex, but it has a depressant effect on the afferent input to micturition (32). Maggi et al. (34, 35) summarized the rationale for using urethane anesthesia for urodynamic studies, including that it produces only a slight depression of bladder muscle contractility. Urethane has been utilized extensively for investigation of voiding in anesthetized rats (33, 34, 36, 54), including diabetic rats (29, 52). Previous reports demonstrated that compared with ketamine/xylazine anesthesia, urethane anesthesia better reproduces conscious urodynamics of voiding and continence (4, 34, 36).

LPP measures the urethral response to leakage in the absence of a bladder contraction and during continence, rather than during voiding (22, 23). Therefore, utilizing urethane anesthesia to immobilize the rats for LPP testing is reasonable since the measurements are taken when the voiding reflex is not present. Because of the effects of urethane on the voiding reflex, we studied cystometry with the animals awake. The cystometry results support the observations of denervation and decreased outlet resistance, particularly among the diabetic rats, made from the LPP study.

Prior work demonstrated that since LPP parallels peak bladder pressure during leakage (1, 4, 8, 9, 44), either suffices to show dysfunction and recovery compared with a sham group. Previous studies demonstrated that LPP is significantly decreased 4 and 10 days after PNC in control animals and that LPP returns to the control level 2 to 3 wk after PNC (10). In contrast, in our current study, LPP in control rats was decreased 4 days after PNC but was not significantly different from sham PNC animals. This may be due to the statistics used to compare the six experimental groups or to variability in this experiment. Thirteen days after PNC, this trend had normalized as in our previous work (10, 38).

Increased duration of diabetes increases the risk of lower urinary tract symptoms (6, 24). LPP in our current study was not significantly different between the control, diuretic, and diabetic groups, indicating that diabetic pathology of 8- to 10-wk duration in rats might not be of long enough duration to significantly affect EUS resistance and closure function. However, LPP was significantly decreased in diabetic rats 4 days after PNC, indicating a short-term decrease in urethral resistance and urethral function during continence. LPP was not significantly different in diabetic animals 13 days after PNC or sham PNC, indicating that function had returned to normal levels. These results suggest that functional recovery and neuroregeneration are significant ~2-3 wk after PNC, even in diabetic animals, although anatomical recovery appears incomplete, as we previously observed in control animals (10, 27).

Our results that LPP returned to normal 13 days after PNC contrast with our previous work using vaginal distension to simulate childbirth injury, in which diabetic rats demonstrated increased severity of stress incontinence symptoms and slowed recovery (27). Vaginal distension creates hypoxia as well as crush injuries to the lower urinary tract (9, 11) and is a different type of injury from the direct nerve crush used in this study. Comparison of these studies suggests that muscle injuries repair more slowly in diabetic animals than nerve injuries.

Histological results demonstrated muscle damage with fewer and thinner muscle fibers in both the bladder and urethra after PNC, as well as connective tissue infiltration of the EUS in all diabetic animals. Focal disruption and pathology of the EUS 4 days after PNC were increased compared with rats with sham PNC in all groups, indicative of denervation to the EUS. These findings are in agreement with other studies investigating EUS denervation (19, 39). There was increased fiber disruption with thinning and atrophy of the EUS in diabetic animals after PNC, indicating that diabetic animals were more vulnerable to injury, as has been observed previously both clinically and in animal models (21, 24, 27). Thirteen days after PNC in diabetic rats, all tissues showed signs of recovery and repair, as well as functional recovery of LPP. However, anatomic recovery was not complete at this time.

AGE formation is markedly accelerated in diabetes because of the increased availability of glucose (16). Increased levels of AGEs and their receptors affect tissue structure and function by altering the signaling pathways responsible for a variety of cell functions, including muscle contractility (18). They also trigger synthesis of intracellular matrix components, leading to endocytosis, degradation, cellular activation, and prooxidant, proinflammatory events (50). The resultant expansion of extracellular matrix may be responsible for the increased bladder mass and weight observed in the diabetic rats in our study as well as in other studies (30).

We chose to assess AGEs in the bladder, urethra, vagina, and pudendal nerve in this study because AGE assays of blood have not yet been shown to be directly related to tissue AGE content, although blood is more accessible for repeat measurements of AGEs than tissues (7). Our results demonstrate that furosine and CML were significantly increased in all tissues tested in diabetic rats compared with control and diuretic rats. PNC, however, did not affect AGE levels. Further experiments are needed to determine whether animals with longer-duration diabetes will have greater AGE content and whether PNC affects AGE levels in those animals.

Conclusions. PNC relieves a functional outlet obstruction in diabetic rats, enabling them to void increased volumes of urine in a shorter time and with less voiding pressure. PNC also leads to decreased urethral resistance to leakage, as demonstrated by reduced LPP 4 days after injury which returned to normal levels 13 days after PNC. Diabetic rats with PNC demonstrate increased disruption and atrophy of the EUS, suggestive of impaired neuroregeneration. Increased connective tissue content of tissues and elevated levels of certain AGEs in diabetic animals may contribute to the dysfunction.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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