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Alterations in the formation of cyclic nucleotides and prostaglandins in the lower urinary tract of the diabetic rabbit

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Abstract Dysfunction of the urinary bladder is a recognised complication of diabetes mellitus (DM) which has been attributed, in part, to a direct effect on bladder smooth muscle tissue. The objective of this study was to investigate the effect of alloxan-induced DM on endogenous modulators of smooth muscle tone such as cyclic AMP (cAMP), cyclic GMP (cGMP) and prostaglandins. Male New Zealand white rabbits were rendered diabetic (hyperosmolar, non-ketotic) with an i.v. injection of alloxan. After 6 months, the urinary bladders and urethrae were excised, cut into segments, incubated with stimulators and the formation of prostaglandins (PG), cAMP and cGMP measured using radioimmunoassays. PGE_2 and PGI_2 formation was impaired in response to arachidonic acid stimulation, whereas it was increased in response to acetylcholine in DM detrusor, bladder neck and urethra compared to controls. Cyclic AMP and cGMP formation in response to forskolin and sodium nitroprusside, respectively, was significantly reduced in the DM tissues of the lower urinary tract compared to the control. Alterations in the formation of prostaglandins, cAMP and cGMP by the smooth muscle of DM lower urinary tract suggests that these biochemical mediators may have a pathophysiological role in the urinary bladder dysfunction associated with DM.

Key words Diabetic cystopathy · Rabbit · Cyclic AMP · Cyclic GMP · Prostacyclin

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Introduction

Bladder dysfunction has been recognised as a complication of diabetes mellitus (DM) [4, 6, 7]. Based on urodynamic findings, the prevalence of DM-associated bladder dysfunction ranges from 40–100% [13, 25]. Its features include the presence of a large bladder capacity, decreased flow rate, impaired detrusor contractility and increased residual volume [13, 25]. These complications have been attributed in part to peripheral autonomic neuropathy [5, 8]. Functional alterations in autonomic neurotransmission have been reported in DM for noradrenergic, cholinergic, purinergic and non adrenergic, non-cholinergic (NANC) nerves in the bladder and urethra using experimental animal models [14, 16, 20]. In addition, bladder dysfunction has also been attributed to a direct effect of DM on endogenous mediators that modulate smooth muscle function [12].

Adenosine 3′,5′-cyclic monophosphate (cAMP) and guanosine 3′,5′-cyclic monophosphate (cGMP) are recognised as second messengers mediating detrusor and urethral smooth muscle relaxation respectively [2, 19]. Intracellular levels of cAMP are increased by prostaglandins (PGs) or forskolin, which activates adenylyl cyclase. PGs also elicit their effects via activation or modulation of cAMP [1]. Whilst PGs do not play a major role in controlling contraction of the urinary tract smooth

muscle, they may be involved in the enhancement of micturition elicited by cholinergic/adrenergic neurotransmission [1]. For example, PGE_2 elicits detrusor smooth muscle contractions, whereas it relaxes the urethral smooth muscle: both actions are necessary for bladder emptying [9, 24]. Cyclic GMP formation is regulated by nitric oxide (NO) via activation of guanylyl cyclase. In addition, putative interactions between the arachidonic acid and the NO pathways have emerged recently [22].

Alterations in the production of prostacyclins (PGI_2) in the DM rat bladder [12], as well as changes in the formation of cyclic nucleotides in the DM rabbit cavernosa [23] have been documented. We have also shown recently that despite an increase in the NO synthase binding sites in the bladder outlet of diabetic rabbit, cGMP-dependent NANC nerve mediated smooth muscle relaxation was impaired [20].

The most widely used animal model for the study of the effect of DM on bladder function is the rat in which DM is induced by intravenous injection of streptozotocin. However, in this rat model there is marked hypertrophy of the bladder, which at 2 months after induction of DM, is three to five times greater in diabetic animals compared to age-matched controls [15]. In contrast, there is marked growth retardation in these diabetic rats. This results in a two to three-fold decrease in total body weight when compared with control [18]. Since starvation markedly alters smooth muscle biochemistry and function, the validity of the rat model for the investigation of DM bladder dysfunction is questionable. In the rabbit model, however, there is only a modest decrease in body weight and minimal hypertrophy of the urinary bladder (unpublished results).

The effect of DM on the formation of cyclic nucleotides and PGs in the lower urinary tract has not been investigated before. The objective of this study was to investigate the formation of PG, cAMP, cGMP in the alloxan-induced diabetic rabbit.

Materials and methods

Induction of diabetes

Age-matched 3 kg male New Zealand white rabbits ($n = 12$) were used, six of which were injected intravenously with alloxan (via the lateral ear vein) with a single dose of 65 mg/kg. The diabetic rabbits were fed ad libitum with SDS standard rabbit plain chow (SDS, Whitham, UK) and allowed free access to water. Blood was sampled at monthly intervals for serum urea and electrolytes, cholesterol, triglycerides and glucose. Urine was monitored over the duration of diabetes for glucose, ketone bodies and proteins with Multistix (Ames Division, Miles Laboratories Ltd., Stoke Poges, Buckinghamshire, UK).

Preparation of bladder tissue

Following cervical dislocation, bladders and urethrae were excised from the diabetic rabbits at 6 months ($n = 6$) together with the age-matched controls ($n = 6$). The tissues were immediately placed in Dulbecco's Minimum Essential Medium (DMEM) pregassed with $95\%O_2/5\%CO_2$. The bladder was divided into detrusor and bladder neck. The segments were cut longitudinally into two equal lengths and then transversely to give segments of approximately 2 mm^2. These segments of tissue from animals in each study group were pooled and incubated in DMEM at 37°C with regular changes of medium to allow the tissues to recover from preparative handling.

Drugs and solutions

Acetylcholine chloride, antisera against 6-oxo-prostaglandin $F_{1\alpha}$a, arachidonic acid (sodium salt), Dulbecco's minimum essential medium, forskolin, gelatin, isobutylmethylxanthine, Norit activated charcoal, phorbol ester dibutyrate, 6-oxo-prostaglandin $F_{1\alpha}$a and sodium nitroprusside were supplied by Sigma Chemical Co (Poole, Dorset, UK).

The following radiochemicals and kits were obtained from Amersham Radiochemicals (Amersham International, Aylesbury, Bucks, UK): [^{3}H]-prostaglandin E_2, [^{3}H]-6-oxo-prostaglandin $F_{1\alpha}$a and ^{125}I-labelled cAMP and ^{125}I-labelled cGMP radioimmunoassay kits.

PGI_2 and PGE_2 formation

Following preincubation of detrusor, bladder neck and urethral discs for four hours, with frequent changes of medium, one disc, in duplicate for each drug dose was placed in DMEM containing the following drugs which are known to stimulate PGE_2 and PGI_2 synthesis in the rabbit vascular tissue: acetylcholine (receptor agonist) and arachidonate (substrate). Tissues were then incubated for one hour at 37°C. Supernatants were then removed and 6-oxo-$PGF_{1\alpha}$a concentrations (the stable spontaneous hydrolysate of PGI_2) and PGE_2 measured by radioimmunoassay [11]. Briefly, aliquots were diluted with Tris HCl-(1%) gelatin buffer, pH 7.4. To these and 6-oxo-$PGF_{1\alpha}$a or PGE_2 standards (0–10 ng) was added 200 µml diluted 6-oxo-$PGF_{1\alpha}$a or PGE_2 antisera containing 1 µm Ci [^{3}H] 6-oxo-$PGF_{1\alpha}$a or [^{3}H]-PGE_2. Tubes were incubated overnight at 4°C. Activated charcoal (1% w/v) in Tris HCl-gelatin buffer was added to each tube, centrifuged and incubated on melting ice for 15 min. Tubes were then centrifuged at 2500 rpm for 10 min. Supernatants were decanted into vials and scintillation fluid added and counted in a gamma-particle counter (LKB; Copenhagen, Sweden). Standard curves were compiled and unknown values calculated.

Assessment of cyclic nucleotide formation

Following pre-incubation, detrusor, bladder neck and urethral discs were placed in DMEM in polypropylene tubes containing 250 µM isobutylmethylxanthine (a phosphodiesterase inhibitor) and various concentrations of cyclic nucleotide formation stimulators: forskolin (cAMP) and sodium nitroprusside (cGMP). Tubes were incubated for a further 20 min at 37°C. Reactions were stopped by the addition of 1 M perchloric acid and the tissues sonicated (3×30 s; Soniprep, MSE, Bucks, UK), which extracts the cyclic nucleotides. Following centrifugation at 1000 *g* for 15 min, supernatants were taken and neutralised with 1 M K_3PO_4. Aliquots were then taken and acetylated with triethylamine/acetic anhydride (1/2, v/v) and diluted with phosphate buffer, pH 7.4. To these and cAMP and cGMP standards (0–256 fmoles) was added 200 µml diluted cAMP or cGMP antisera containing ^{125}I-labelled cAMP or ^{125}I-labelled cGMP. Tubes were incubated overnight at 4°C. Antisera against rabbit globulins in phosphate buffer was added to each tube and incubated on melting ice for 15 min. Tubes were then centrifuged at 2500 rpm for 10 min. Supernatants were decanted into vials and scintillation fluid added and counted in a gamma particle counter (LKB). Standard curves were compiled and unknown values calculated.

Data analysis

Comparisons of weights, plasma glucose and plasma lipids between the 6 month diabetic groups and the age-matched controls were performed using the Mann-Whitney *U* test (paired values).

For the cAMP, cGMP, PGE_2 and 6-oxo-$PGF_{1\alpha}$a, measurement data were expressed as the mean (SEM) value per milligram of tissue per minute (wet weight) from six samples. Data were analysed using ANOVA for multiple comparisons. Paired comparisons between two groups were performed using paired Student's *t*-test where ANOVA indicated significance for the multiple comparison. Statistical significance was accepted when $P < 0.05$.

Results

Animal weights, serum glucose and cholesterol concentrations

The starting weights in both the control and diabetic rabbit groups were similar (Table 1). At the end of 6 months, the weights of the diabetic rabbits were not significantly different from the non-diabetic group, although there was a smaller weight gain in the diabetic animals (Table 1). Serum glucose concentrations (non-fasting) were significantly ($P < 0.0015$) elevated in the diabetic group when compared to the control group. Serum cholesterol concentrations (non-fasting) were not significantly different between the control and diabetic rabbits (Table 1).

Serum triglycerides were not significantly different between control and diabetic groups (results not shown).

Table 1 The comparison of body weight, serum glucose and cholesterol concentrations before and after 6 months of diabetes

Median (range)	Baseline	6 months
Body weight (kg)		
Control	3.0 (2.7–3.5)	4.1 (3.5–4.6)
Diabetic	3.1 (2.8–3.6)	3.6 (3.0–3.9)
Glucose (mmol/l)		
Control	7.5 (6.8–8.4)	6.4 (6.1–7.5)
Diabetic	7.6 (6.7–8.6)	33.0 (18.3–43.2)
Cholesterol (mmol/l)		
Control	0.7 (0.5–1.4)	1.2 (0.6–2.3)
Diabetic	0.7 (0.6–0.9)	0.9 (0.5–1.3)

Formation of PGI_2, PGE_2, cyclic AMP and cyclic GMP

Both PGI_2 and PGE_2 formation were inhibited in response to arachidonic acid in the detrusor, bladder neck and urethra of the diabetic rabbit compared to age-matched controls, the degree of inhibition being similar for both PGs in all areas (Fig. 1). In contrast, in response to acetylcholine, the formation of both PGI_2 and PGE_2 was significantly enhanced in the detrusor, bladder neck and urethra of the diabetic rabbit compared to age-matched controls, the degree of increase being similar for both PGs in all areas (Fig. 2).

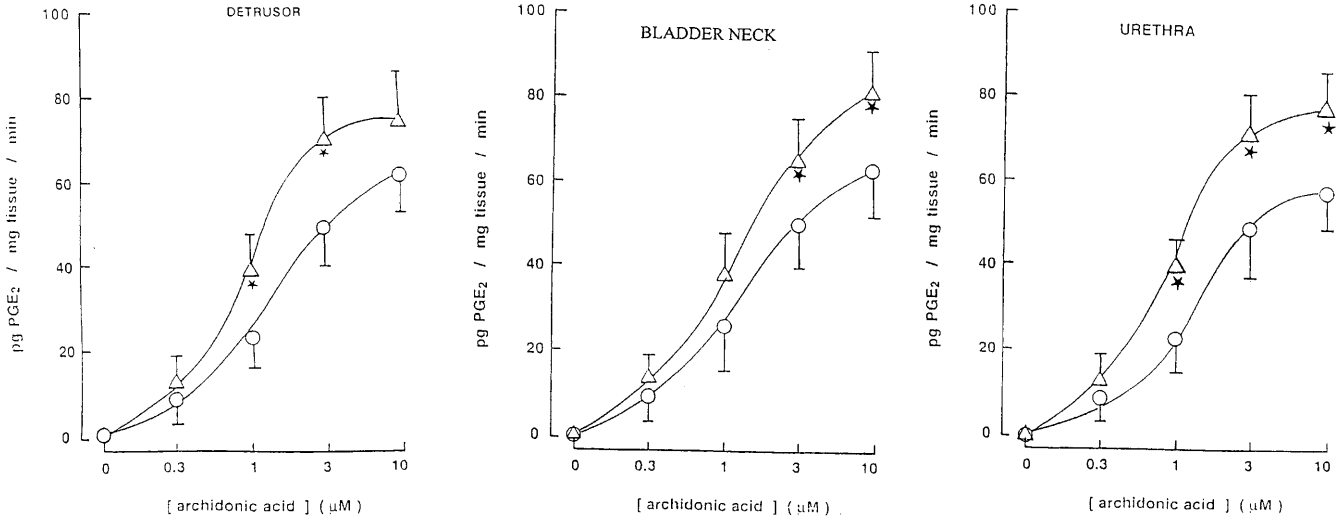
In response to forskolin, cAMP formation was significantly reduced in the detrusor, bladder neck and urethra of the diabetic rabbit compared to age-matched controls. The degree of inhibition was similar in all areas (Fig. 3). In response to sodium nitroprusside, cGMP formation was also significantly reduced in the detrusor, bladder neck and urethra of the diabetic rabbit compared to age-matched controls. A similar degree of inhibition was seen in all areas (Fig. 4).

Discussion

The results of the present study demonstrate significant differential changes in the formation of PGI_2, PGE_2, cGMP and cAMP, in the urinary tract of the diabetic rabbit, which may be of relevance to the pathogenesis of cystopathy associated with DM.

Both PGI_2 and PGE_2 formation were inhibited in response to arachidonic acid in the detrusor, bladder neck and urethra of the diabetic rabbit compared to age-matched controls, the degree of inhibition being similar

Fig. 1 Arachidonic acid-stimulated prostaglandin E_2 formation by the detrusor, bladder neck and urethra from diabetic (O) and control (Δ) rabbits 6 months after the induction of diabetes. Each point equals mean ± SEM, $n = 6$. *$P < 0.05$



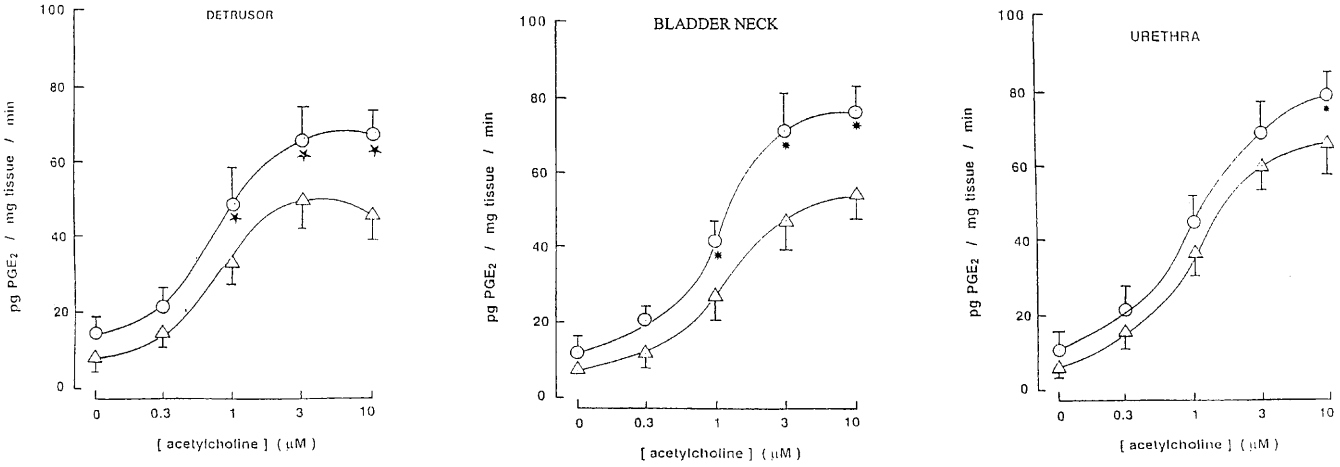
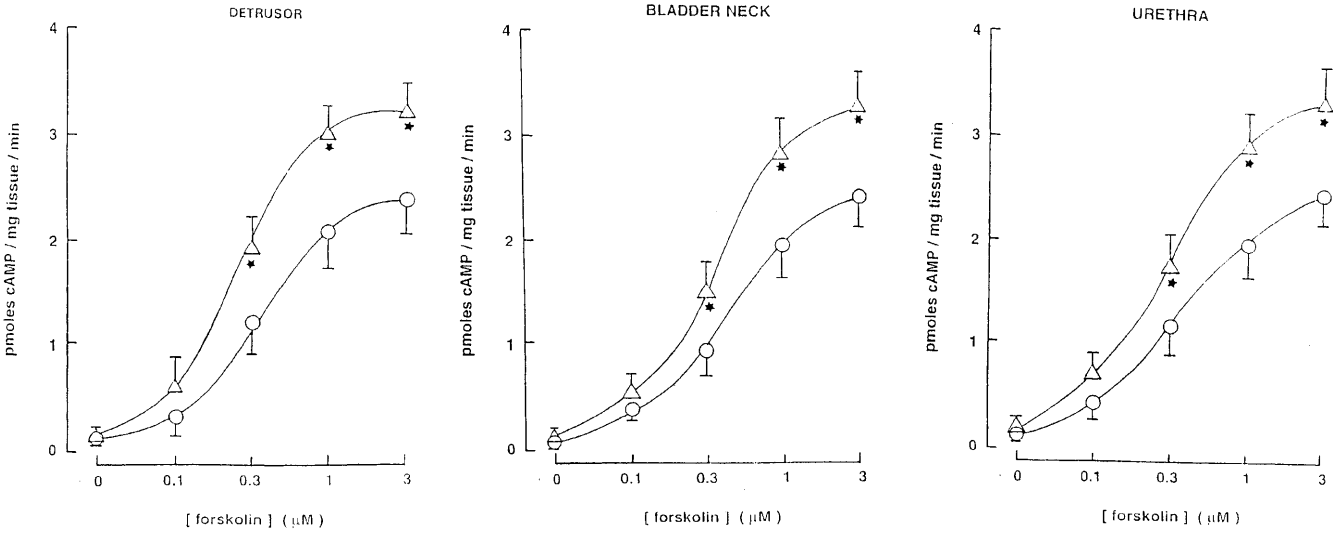


Fig. 2 Acetylcholine-stimulated prostaglandin E_2 formation by the detrusor, bladder neck and urethra from diabetic (O) and control (△) rabbits 6 months after the induction of diabetes. Each point equals mean ± SEM, $n = 6$. $^*P < 0.05$

for both PGs. Since arachidonic acid is the substrate for cyclooxygenase these data suggest that there is a reduction in cyclooxygenase activity rather than of individual PG synthase enzymes. These data confirm the findings of previous studies in a diabetic rat model in which there was a decrease in cyclooxygenase activity in the urinary bladder [12].

With regard to urinary tract smooth muscle tone, it is generally accepted that PGs do not have a primary role, but rather a secondary or facilitatory one [1]. Nonetheless, both PGI_2 and PGE_2, the dominant PGs in the lower urinary tract of the rabbit, promote detrusor contractility and urethral smooth muscle relaxation [9, 24]. A reduction in the formation of these PGs may lead to a reduced contractility of detrusor muscle and impaired urethral smooth muscle relaxation, leading to impaired detrusor contractility with poor flow rate. In contrast to arachidonic acid, the formation of both PGI_2 and PGE_2 was significantly enhanced in response to acetylcholine in the detrusor, bladder neck and urethra of the diabetic rabbit. In the detrusor muscle, acetylcholine elicits contraction and the concomitant release of PGs through an increase in cytosolic calcium [3]. The increase in calcium triggers excitation-contraction coupling, as well as the activation of phospholipase A_2 which releases arachidonic acid from endogenous phospholipid stores [10]. It has been suggested that this concomitant release of PGs may modulate the contraction-relaxation cycles involved in micturition. Smooth muscle contractile responses to agonists such as acetylcholine and phenylephrine are enhanced in the diabetic rabbit detrusor and urethra, respectively, (unpublished results). Thus, although basal cyclooxygenase is reduced, the increased responsiveness to contractile agonists may actually result in an increase in local concentrations of PGs. The distension of the urinary bladder itself promotes PG formation and release, the greater the distension the greater the release. Thus, it has been proposed that as the bladder fills with urine the

Fig. 3 Forskolin-stimulated cyclic AMP formation by the detrusor, bladder neck and urethra from diabetic (O) and control (△) rabbits at 6 months after the induction of diabetes. Each point equals mean ± SEM, $n = 6$. $^*P < 0.05$



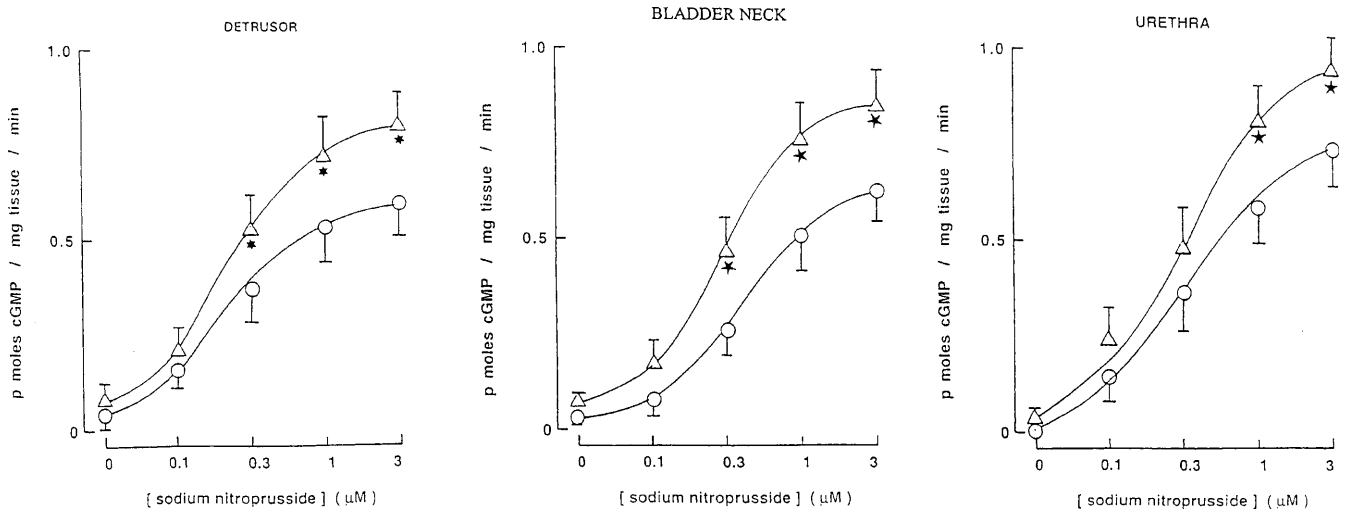


Fig. 4 Sodium nitroprusside-stimulated cyclic GMP formation by the detrusor, bladder neck and urethra from diabetic (O) and control (△) rabbits at 6 months after the induction of diabetes. Each point equals mean ± SEM, $n = 6$. $^*P < 0.05$

increased release of PGs may serve to augment detrusor contraction during micturition at maximal distension. The increase in the formation of PGs in response to cholinergic stimulation, as a result of up-regulation of muscarinic receptors in DM bladder, may be a compensatory response to the failing detrusor, as it gradually distends in DM.

It is often overlooked that PGs have several other roles in the urinary tract other than the control of contraction-relaxation cycles. Firstly, PGI_2 and PGE_2 are produced in large quantities by the urothelium [12]. Since PGs promote the secretion of mucus via activation of adenylyl cyclase in the gastrointestinal tract [21] it was suggested that these PGs play a similar role in maintaining the integrity of the bladder mucosa. A corollary to this is that impairment of urothelial PG formation may lead to increased tissue damage by the urine. An analogy is the gastric mucosa which if compromised by inhibition of endogenous PGI_2 and PGE_2 production leads to an increased risk of ulceration [21]. Little is known of the integrity of bladder or urethral mucosal function in DM but a possible link between diminished cytoprotection and contractile dysfunction warrants consideration. Secondly, in other tissues, PGs play a role in mediating smooth muscle proliferation and in tissue remodelling, in particular in vascular smooth muscle tissue. Diabetes is associated with polyuria and increased micturition rate and as such the bladder adapts by cellular hypertrophy and remodelling [17]. In this context, dividing smooth muscle cells increase PGs production. It is not known what role PGI_2 and PGE_2 play in this adaptive process in bladder/urethral smooth muscle cells. Investigation of this possibility again is warranted.

Since the action of PGs is mediated through the activation of adenylyl cyclase, cAMP formation was also studied. In response to forskolin, cAMP formation was significantly reduced in the detrusor, bladder neck and urethra of the diabetic rabbit compared to age-matched controls. These data indicate a reduced adenylyl cyclase activity since forskolin activates this enzyme directly [19]. Impaired cAMP formation in the lower urinary tract may alter detrusor compliance and impair bladder outlet relaxation leading to inadequate bladder emptying. This is further supported by our functional data (unpublished) which also demonstrated impaired forskolin-stimulated relaxation in the diabetic rabbit detrusor and urethra.

In response to sodium nitroprusside, cGMP formation was also significantly reduced in the detrusor, bladder neck and urethra of the diabetic rabbit compared to age-matched controls. Since nitroprusside activates guanylyl cyclase directly, our study indicates a generalised reduction in the activity of this enzyme. This suggests that urethral relaxation during micturition may be impaired in the diabetic rabbit. In fact, we have recently demonstrated that the NO-mediated relaxation of the bladder neck and urethral smooth muscle is significantly impaired in the DM rabbit [20]. The functional relevance of reduced cGMP formation by the detrusor in DM is not clear. However, impairment of the NO-cGMP pathway might be of relevance to diabetic detrusor smooth muscle hyperplasia, since NO influences cell viability and proliferation.

In conclusion, the present study demonstrates a reduction in the formation cAMP and cGMP and enhanced production of PGI_2 and PGE_2 in response to cholinergic stimulation. These changes may result in altered contractility and/or relaxation of the rabbit diabetic urinary tract smooth muscle. These findings may be useful in devising pharmacological strategies for the treatment of urinary tract smooth muscle dysfunction in diabetic patients.

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