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Inhibition of diabetic bladder smooth muscle cell proliferation by endothelin receptor antagonists

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Abstract Urinary bladder hypertrophy and hyperplasia are well recognised in diabetic cystopathy. The urinary bladder is known to synthesise endothelin-1 (ET-1), a potent vasoconstrictor peptide with mitogenic properties. Using diabetic New Zealand White (NZW) rabbits, we investigated the potential role of ET receptor subtypes (ET_A and ET_B) on the proliferation of bladder smooth muscle cells (SMC). Diabetes mellitus was induced in adult male NZW rabbits. After 6 months, control ($n = 6$) and diabetic ($n = 6$) bladders were removed and SMC from the dome and bladder neck were grown using standard explant methodology. At passage two, the cells were made quiescent and then further incubated in foetal calf serum (FCS), control age-matched rabbit serum (CRS) or diabetic rabbit serum (DRS) in the presence or absence of ET_A-antagonist (BQ123) or ET_B-antagonist (BQ788). SMC proliferation was then measured with 5-bromo-2′deoxy-uracil 24 h later and by cell counting (using a haemocytometer) at 48 h. Neither BQ123 nor BQ788 influenced detrusor or bladder neck SMC proliferation in FCS or CRS. However, in the presence of DRS, BQ123 and BQ788 significantly inhibited diabetic detrusor and bladder neck SMC proliferation at 30 and 100 nmol/l ($P < 0.03$ and $P < 0.01$, respectively). Cell counts were also significantly reduced from the diabetic detrusor and bladder neck ($P < 0.01$ and $P < 0.03$ with BQ123 and BQ788, respectively). These results suggest that ET may play a pathophysiological role in the bladder SMC hyperplasia associated with diabetes mellitus.

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Introduction

Alterations in the function of the diabetic bladder have been attributed to peripheral autonomic neuropathy and to changes in the structure of the detrusor as a result of hypertrophy and/or hyperplasia [5, 1, 13, 17].

Recent studies have identified an extensive distribution and synthesis of endothelin-1 (ET-1), a potent vasoconstrictor peptide with mitogenic properties, in the human and rabbit urinary bladder [7, 28, 33]. ET-1 is synthesised by vascular and nonvascular smooth muscle cells (SMC) and by fibroblasts within the urinary bladder [28, 34]. The presence of ET-1 in the urinary bladder in almost all cell types suggests that this peptide plays a role in bladder wall modelling, the control of bladder smooth muscle tone and the regulation of local blood flow. To date, two major ET receptors have been identified and cloned: ET_A and ET_B [2, 21]. The activity of ET-1 is thought to be mediated via both autocrine [28] and paracrine [12] mechanisms. ET-1 elicits concentration-dependent contractions in smooth muscle strips from human and rabbit urinary bladders indicating the presence of functional ET receptors in both these species [7, 18, 19, 28, 33]. Furthermore, we have recently demonstrated alterations in the function and distribution of ET receptor subtypes in the diabetic rabbit bladder [23].

Using alloxan-induced diabetic New Zealand White (NZW) rabbits, we investigated the potential role of ET-1 and its receptor subtypes on the proliferation of bladder SMC.

Materials and methods

Induction of diabetes mellitus

Age matched 3 kg male NZW rabbits ($n = 12$) were selected, six of which were injected intravenously (via the lateral ear vein) with

alloxan (Sigma Chemical Co., Poole, UK) at a single dose of 65 mg/kg body weight, to induce nonketonuric, hyperglycaemic DM. All animals were fed ad libitum with SDS standard plain diet (SDS, Witham, UK) and allowed free access to water.

Blood sampling

Blood was sampled at monthly intervals, via the middle ear vein, for serum glucose, urea and electrolytes.

Proliferation assay with detrusor and bladder neck smooth muscle cells

At 6 months, serum was obtained from control and diabetic rabbits for tissue culture experiments. Following cervical dislocation, control and diabetic urinary bladders were excised and weighed. The bladders were then divided into detrusor ($n = 6$) and bladder neck ($n = 6$) at the level of ureteric orifice and then used for cell culture studies. SMC from the detrusor and bladder neck were obtained as previously described [30]. Detrusor and bladder neck smooth muscle segments from both control and diabetic rabbits were dissected from the urothelium. SMC were then grown using standard explant methods. The segments were then placed in Dulbecco's modified Eagles medium (DMEM; Sigma Chemical, Poole, UK) supplemented with 10% heat inactivated foetal calf serum (FCS) (Gibco, Paisley, UK), 29.2 mg/ml L-glutamine, 10,000 units/ml penicillin G, 10,000 mg/ml streptomycin sulphate and left at 37 °C in a 5% carbon dioxide (CO_2) humidified incubator. The cells were grown to confluency and then passaged with 0.05% trypsin – 0.02% ethylenediamine tetra-acetic acid (Gibco, Paisley, UK) and sub-cultured at a ratio 1:3. Confluent SMC at second passage were sub-cultured into 96 well microtitre tissue culture plates (Falcon, Becton Dickinson, Oxford, UK). The cells were then made quiescent by changing the medium containing 0.4% FCS ($n = 6$), control rabbit serum (CRS) ($n = 6$) or DRS ($n = 6$) and left for further 96 h in a 5% CO_2 humidified incubator. Subsequently, the selective ET_A antagonist BQ123 [29] or the ET_B antagonist BQ788 [9] (10, 30 or 100 nM) or vehicle were dissolved in serum containing 2.5% of the appropriate serum (FCS, CRS or DRS, respectively) and added to the culture. SMC proliferation was then measured 24 h later with 5-bromo-2′deoxy-uracil (BrdU), a thymidine analogue [9]. This substance is taken up by cells actively synthesizing DNA. Hence, BrdU gives an accurate indication of cell proliferation. The BrdU measurement was carried out as previously described [30]. In brief, 10 mol/l of BrdU was added to each of the wells for 24 h in the presence of either ET_A or ET_B antagonists. The supernatant was then discarded and the cells fixed with ethanol. The fixative was then removed and the cells washed with phosphate buffered saline (PBS) and then treated with a nuclease solution and washed three times. A peroxidase-labelled antibody to BrdU containing Fab (fragment antigen binding) was then added and incubated at 37 °C for 30 min. The antibody conjugate was removed and a peroxidase substrate was then added. Cells were incubated until a blue colour developed (2–10 min). Sulphuric acid was then added and absorbance measured using an enzyme-linked immunosorbant assay plate reader (E960; Metertech, Watford, UK) at 450 nm (reference wavelength 690 nm).

Cell counts

For cell counts, the supernatant was discarded at 48 h, and the cells washed with PBS free of calcium and magnesium. Cells were trypsinised (as described above), stained with crystal violet and counted in a Neuber haemocytometer. Data are expressed as cells per well.

Statistical analysis

All the results are presented as mean ± SEM. Statistical analysis was carried out using Student's (unpaired and paired) t test.

Results

Animal weights and serum glucose and cholesterol concentrations

The starting weights in both the control ($n = 6$) and DM rabbits ($n = 6$) 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. There was no significant difference in the serum urea and electrolytes levels (results not shown).

Bladder weights

There was a significant increase ($P < 0.03$) in the bladder weights of the 6-month diabetic rabbits compared to age-matched controls (Table 1).

BrdU incorporation

Incorporation of BrdU by both control and diabetic detrusor and bladder neck SMC was significantly increased in the presence of DRS compared to FCS and CRS (Table 2). The incorporation of BrdU was significantly greater in the diabetic SMC compared to in the controls (Table 2).

BQ123 and BQ788 at concentrations of 30 and 100 nmol/l significantly inhibited BrdU incorporation in the presence of DRS by the diabetic detrusor ($P < 0.03$)

Table 1 Body weight, bladder weight, serum glucose and cholesterol concentrations before and after 6 months of diabetes. The results are expressed as mean ± SEM. The P values are given in the text

	Baseline	+6 Months
Body weight (kg)		
Control	3.0 ± 0.2	4.1 ± 0.5
Diabetic	3.1 ± 0.3	3.6 ± 0.4
Bladder weight (g)		
Control	2.5 ± 0.04	
Diabetic	4.8 ± 0.03	
Glucose (mmol/l)		
Control	7.5 ± 0.2	6.4 ± 0.3
Diabetic	7.6 ± 0.3	33.0 ± 0.4
Cholesterol (mmol/l)		
Control	0.7 ± 0.02	1.2 ± 0.02
Diabetic	0.7 ± 0.03	0.9 ± 0.04

(Fig. 1) and bladder neck ($P < 0.03$) (Fig. 2) SMC. Whereas, BQ123 and BQ788 had no effect on BrdU incorporation in the control detrusor (Fig. 1) and bladder neck (Fig.2) SMC in the presence of DRS. BQ123 and BQ788 also had no effect on BrdU incorporation by the diabetic or control detrusor and bladder neck SMC in the presence of FCS or CRS (data not shown).

Table 2 Effect of control rabbit sera (CRS), fetal calf sera (FCS) and diabetic rabbit sera (DRS) on the level of BrdU incorporation by the control (CT) and diabetic (DM) detrusor and bladder neck SMC. Data is expressed as mean ± SEM

	Detrusor		Bladder neck	
	CT	DM	CT	DM
DRS	1.2 ± 0.1	2.8 ± 0.09[a]	0.68 ± 0.06	1.2 ± 0.08[b]
FCS	0.52 ± 0.05[c]	0.46 ± 0.04[c]	0.32 ± 0.02[c]	0.42 ± 0.05[c]
CRS	0.48 ± 0.03[d]	0.35 ± 0.04[d]	0.28 ± 0.03[d]	0.32 ± 0.03[d]

[a] CT vs DM detrusor in the presence of DRS $P < 0.001$
[b] CT vs DM bladder neck in the presence of DRS $P < 0.01$
[c] FCS vs DRS $P < 0.01$
[d] CRS vs DRS $P < 0.01$

Cell counts

Diabetic detrusor and bladder neck SMC counts were significantly greater than control in the presence of DRS (Table 3). In the presence of BQ123 and BQ788 (30 and 100 nmol/l) diabetic detrusor and bladder neck SMC counts were significantly reduced following 48 h incubation in the presence of DRS (Table 4). These ET receptor antagonists had no influence on either control or diabetic detrusor and bladder neck SMC counts in the presence of FCS or CRS. Furthermore, these antagonists had no effect on the control detrusor and bladder neck SMC counts in the presence of DRS (data not shown).

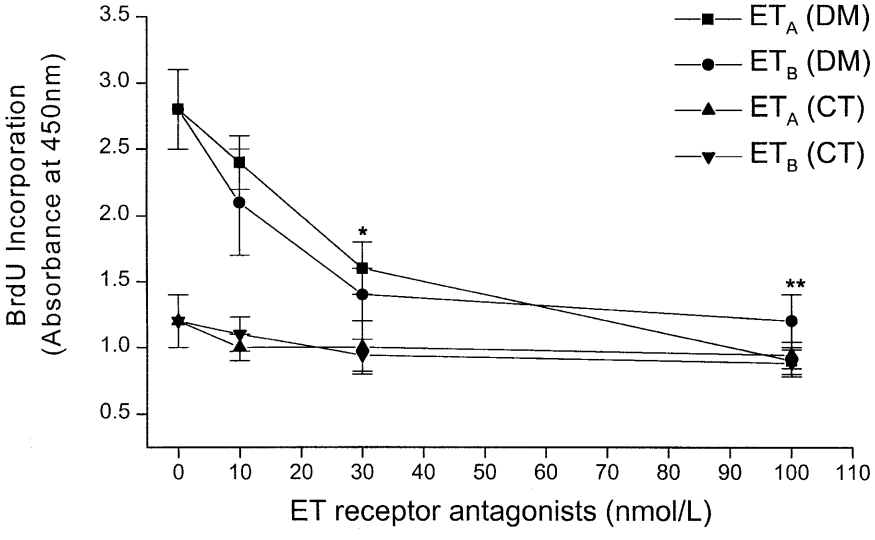

Fig. 1 Control (CT) and diabetic (DM) detrusor SMC: Effect of ET_A (BQ123) and ET_B (BQ788) antagonists on the level of BrdU incorporation by the SMC in the presence of DRS. The results are expressed as mean ± SEM (Absorbance at 450 nm). $*P < 0.03$, $**P < 0.01$

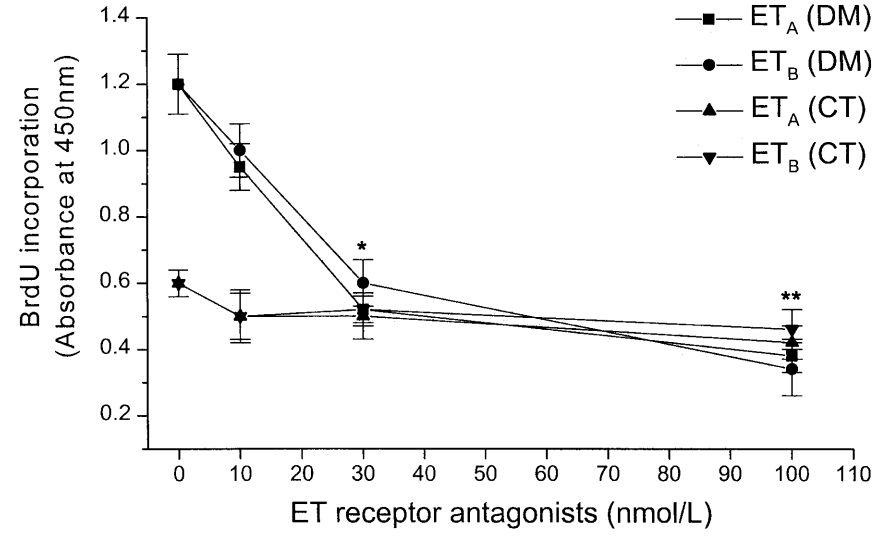

Fig. 2 Effect of ET_A (BQ123) and ET_B (BQ788) antagonists on the level of BrdU incorporation by the SMC in the presence of DRS. The results are expressed as mean ± SEM. (Absorbance at 450 nm). $*P < 0.03$, $**P < 0.01$ (*CT* control, *DM* diabetic, *SMC* bladder neck)

Table 3 Effect of control rabbit sera (CRS), fetal calf sera (FCS) and diabetic rabbit sera (DRS) on SMC counts ($\times 10^{-6}$ cell/ml) in control (CT) and diabetic (DM) detrusor and bladder neck. Data are expressed as mean ± SEM

	Detrusor		Bladder neck	
	CT	DM	CT	DM
DRS	1.1 ± 0.04	2.6 ± 0.05[a]	0.6 ± 0.03	1.9 ± 0.02[b]
FCS	0.5 ± 0.01[c]	0.3 ± 0.02	0.3 ± 0.02[c]	0.4 ± 0.03[c]
CRS	0.4 ± 0.02[d]	0.4 ± 0.03	0.2 ± 0.03[d]	0.3 ± 0.02[d]

[a] CT vs DM detrusor in the presence of DRS $P < 0.001$
[b] CT vs DM bladder neck in the presence of DRS $P < 0.01$
[c] FCS vs DRS $P < 0.03$
[d] CRS vs DRS $P < 0.03$

Discussion

This study has demonstrated, using BrdU and cell count techniques, that at concentrations of 30 and 100 nmol/l, ET_A and ET_B antagonists were able to inhibit diabetic detrusor and bladder neck SMC proliferation in the presence of DRS. This inhibition is unlikely to be due to a non-specific effect of these antagonists since at similar concentrations there was no effect on SMC proliferation in the presence of CRS or FCS. Furthermore, the inhibition of SMC proliferation in other cell culture models has also been reported at similar concentrations of ET antagonists [10, 11, 31].

Interestingly, plasma ET-1 levels are increased in patients with diabetes mellitus [32]. A similar increase in ET-1 levels may also be present in DRS. Unfortunately, it was not possible to measure plasma ET-1 levels in our experiments because the kits used for this assay are based on antibodies that are conjugated against the rabbit.

Since ET_A and ET_B receptors are constitutively expressed in the rabbit bladder SMC [23, 33], it is not surprising that there was a significant increase in proliferation of control detrusor and bladder neck SMC in the presence of DRS. However, this response was significantly greater in the diabetic bladder than in control. This diminished response in control SMC may account for the lack of significant inhibition of proliferation by ET receptor antagonists. Alternatively, the significant inhibition of diabetic SMC proliferation by ET receptor antagonists suggests that the local synthesis of ET-1 by the diabetic urinary bladder was increased. This suggestion would be compatible with the recently demonstrated significant increase in ET-1 binding sites in the diabetic detrusor and bladder neck [23]. Thus, in diabetes an elevated local and/or systemic production of ET-1 may be involved in bladder SMC proliferation. It has also been shown that the proliferation of vascular SMC in response to ET-1 correlates with ET-receptor density [14]. Both ET_A and ET_B receptors have been implicated in this ET-mediated proliferative response. For example, ET_B receptors participate in the development of intimal hyperplasia after endothelial injury [4], while human airway smooth muscle cell proliferation is ET_A receptor-dependent [27]. Thus, the inhibition of bladder SMC proliferation by both ET_A and ET_B antagonists in this study implies that both ET receptor subtypes also play a role in detrusor hyperplasia associated with diabetes mellitus.

One of the most striking features of diabetic cystopathy is the increase in bladder mass [17]. This change has been attributed to connective tissue deposition, tissue oedema and smooth muscle hypertrophy and/or hyperplasia. Several studies have demonstrated that significant proliferative activity is actually involved in this process [8, 22]. Although, it is not known what triggers the proliferative process, it has been postulated that the initial signal for DNA synthesis might be bladder distension associated with diabetic autonomic neuropathy [8]. It is also possible from our results that ET may have a role in the diabetic bladder SMC proliferation. As such, it will be interesting to investigate the effect of urinary diversion (e.g. by cutaneous vesicostomy of the diabetic bladder) on ET receptor density and ET-mediated SMC proliferation, since this procedure produces a decrease in bladder mass, bladder capacity and compliance [6].

Bladder outflow obstruction is another pathological state where an increase in bladder weight occurs [22]. Interestingly, our group has recently demonstrated an increase in the density of ET receptors in a rabbit model with partial bladder outflow obstruction [15]. This increase in ET receptor density appeared to correlate with the duration of obstruction and the increase in bladder weight, thus further strengthening the proposed association between ET-1 and bladder hyperplasia. Furthermore, ET receptor antagonists inhibited detrusor SMC proliferation in this model of partial bladder outflow obstruction [16]. It would, therefore, be of interest to investigate if ET-1 also has a role in bladder hypertro-

Table 4 Effect of ET_A (BQ123) and ET_B (BQ788) antagonists on SMC counts ($\times 10^{-6}$ cell/ml) in diabetic detrusor and bladder neck in the presence of diabetic rabbit sera (DRS). Data is expressed as mean ± SEM

	DRS	DRS + BQ123 (10 nMol)	DRS + BQ123 (30 nMol)	DRS + BQ123 (100 nMol)	DRS + BQ788 (10 nMol)	DRS + BQ788 (30 nMol)	DRS + BQ788 (100 nMol)
Detrusor	2.6 ± 0.02	1.8 ± 0.03	0.7 ± 0.02[a]	0.5 ± 0.03[a]	1.3 ± 0.03	0.8 ± 0.02[b]	0.6 ± 0.02[b]
Bladder neck	1.9 ± 0.04	0.9 ± 0.02	0.5 ± 0.03[c]	0.3 ± 0.03[c]	0.7 ± 0.05	0.4 ± 0.01[d]	0.3 ± 0.01[d]

[a] DRS vs DRS + BQ123 (30 and 100 nMol) $P < 0.01$, [b] DRS vs DRS + BQ788 (30 and 100 nMol) $P < 0.01$. Bladder neck: [c] DRS vs DRS + BQ123 (30 and 100 nMol) $P < 0.03$, [d] DRS vs DRS + BQ788 (30 and 100 nMol) $P < 0.03$

phy following polyuria associated with diabetes insipidus [20].

The functional significance of detrusor SMC proliferation in patients with diabetes and/or bladder outflow obstruction is not clear. However, it may be involved in the development of altered detrusor pressures [26] as part of either a compensatory and/or a pathophysiological response to the underlying disease process. The resulting hyperplasia may enable the bladder to adapt to the polyuria associated with DM. Further work is needed to confirm this.

Lower urinary tract dysfunction is likely to be present in the diabetic rabbit hypertrophic bladder since significant urodynamic alterations have been identified in this model [3]. Furthermore, our in vitro functional studies indicate that bladder neck and urethral smooth muscle responses to ET-1 and nitric oxide are impaired in the diabetic rabbit [24]. In addition, alterations in cAMP and cGMP formation by the diabetic bladder have been described [25].

Conclusion

ET-1 may play a role in modeling the detrusor structure in response to the pathophysiological effects of diabetes mellitus on the urinary bladder. The effect of ET receptor antagonists on diabetic detrusor hyperplasia requires further investigation.

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