INVESTIGATIONS INTO THE ROLE OF ENDOTHELIN, NITRIC OXIDE AND PROSTAGLANDINS IN THE PATHOGENESIS OF DIABETIC CYSTOPATHY.

A thesis presented for the degree of Doctor of Medicine in the Faculty of Medicine of the University of London

Submitted

By

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ABSTRACT

Endothelin-1 (ET-1) and its receptors (ET\textsubscript{A} and ET\textsubscript{B}) have been identified on the urothelium and smooth muscle of the urinary bladder. ET-1 has potent smooth muscle contractile and mitogenic properties. In contrast, the nitric oxide (NO)-cyclic-guanine 3'5' monophosphate (cGMP) pathway mediates bladder outlet smooth muscle relaxation. In addition, the prostaglandin (PG)-cyclic-adenosine 3'5' monophosphate (cAMP) pathway regulates urinary tract smooth muscle tone. The role of these mediators in the pathogenesis of diabetic cystopathy has not been elucidated.

Detrusor, bladder neck and urethral tissue from control and six month alloxan-induced diabetic New Zealand White (NZW) rabbits were obtained. Using organ bath, autoradiographic, histochemical, biochemical and tissue culture techniques, the role of these mediators in the pathogenesis of diabetic cystopathy was investigated.

These studies demonstrate: 1) Impaired bladder neck and urethral smooth muscle responses to ET-1 and NO in diabetes mellitus (DM) despite a significantly increased expression of ET\textsubscript{B} receptors and NO synthase (NOS) binding sites. This upregulation may be a compensatory pathophysiological response to smooth muscle dysfunction and/or to alterations in the bioactivity of ET-1 and NO in DM. 2) Decreased formation of cGMP and cAMP by the diabetic smooth muscle that may be a consequence of a decrease in NO and PG bioactivity, respectively. 3) Increased muscarinic receptor-linked PGE\textsubscript{2} and PGI\textsubscript{2} production by the diabetic detrusor and bladder outlet. This may be a compensatory response to a hypotonic bladder and impaired bladder outlet relaxation in response to NO. 4) Inhibited diabetic smooth muscle cell proliferation by ET\textsubscript{A} and ET\textsubscript{B} receptor antagonists indicating a mitogenic role for ET-1 in detrusor hyperplasia.
The ET, NO and PG pathways may contribute to the pathogenesis of diabetic cystopathy. The experimental model described may be useful for the evaluation of pharmacological interventions in diabetic cystopathy.
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<td>ACh</td>
<td>acetylcholine</td>
</tr>
<tr>
<td>AGE's</td>
<td>advanced glycosylation end products</td>
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<td>ALP</td>
<td>alkaline phosphatase</td>
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<tr>
<td>ALT</td>
<td>alanine transaminase</td>
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<tr>
<td>APES</td>
<td>aminopropyletoxysilane</td>
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<td>AST</td>
<td>aspartate transaminase</td>
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<tr>
<td>BCG</td>
<td>Bacille Calmette-Guerin</td>
</tr>
<tr>
<td>BPH</td>
<td>benign prostatic hyperplasia</td>
</tr>
<tr>
<td>Ca^{2+}</td>
<td>calcium</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine 3'5' monophosphate</td>
</tr>
<tr>
<td>cGMP</td>
<td>cyclic guanosine 3'5' monophosphate</td>
</tr>
<tr>
<td>CGRP</td>
<td>calcitonin gene related peptide</td>
</tr>
<tr>
<td>CRC</td>
<td>concentration response curves</td>
</tr>
<tr>
<td>CRS</td>
<td>control rabbit serum</td>
</tr>
<tr>
<td>CT</td>
<td>control</td>
</tr>
<tr>
<td>DM</td>
<td>diabetes mellitus</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's minimum essential medium</td>
</tr>
<tr>
<td>dpm</td>
<td>disintegration per minute</td>
</tr>
<tr>
<td>DRS</td>
<td>diabetic rabbit serum</td>
</tr>
<tr>
<td>EC_{50}</td>
<td>concentration producing 50% of maximum response</td>
</tr>
<tr>
<td>EFS</td>
<td>electrical field stimulation</td>
</tr>
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<td>eNOS</td>
<td>endothelial nitric oxide synthase</td>
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<tr>
<td>Abbreviation</td>
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<tr>
<td>ET</td>
<td>endothelin</td>
</tr>
<tr>
<td>FCS</td>
<td>fetal calf sera</td>
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<td>FGF</td>
<td>fibroblast growth factor</td>
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<td>GGT</td>
<td>gamma glutamyl transferase</td>
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<tr>
<td>IC₅₀</td>
<td>concentration inhibiting maximum response by 50%</td>
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<tr>
<td>iNOS</td>
<td>inducible nitric oxide synthase</td>
</tr>
<tr>
<td>-ir</td>
<td>immunoreactivity</td>
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<td>K⁺</td>
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<td>L-NOARG</td>
<td>L-N⁰-nitroarginine</td>
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<td>nNOS</td>
<td>neuronal nitric oxide synthase</td>
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<tr>
<td>MCH</td>
<td>mean corpuscular haemoglobin</td>
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<tr>
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<td>mean corpuscular haemoglobin concentration</td>
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<tr>
<td>MCV</td>
<td>mean corpuscular volume</td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>magnesium</td>
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<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>NA</td>
<td>noradrenaline</td>
</tr>
<tr>
<td>Na⁺</td>
<td>sodium</td>
</tr>
<tr>
<td>NADPH-d</td>
<td>nicotinamide adenine dinucleotide phosphate-diaphorase</td>
</tr>
<tr>
<td>NANC</td>
<td>nonadrenergic, noncholinergic</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>NOS</td>
<td>nitric oxide synthase</td>
</tr>
<tr>
<td>NZW</td>
<td>New Zealand White</td>
</tr>
<tr>
<td>O$_2^-$</td>
<td>oxygen</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PE</td>
<td>phenylepherine</td>
</tr>
<tr>
<td>PGE$_2$</td>
<td>prostaglandin E$_2$</td>
</tr>
<tr>
<td>PGI$_2$</td>
<td>prostaglandin I$_2$</td>
</tr>
<tr>
<td>PO$_4^{2-}$</td>
<td>phosphate</td>
</tr>
<tr>
<td>RBC</td>
<td>red blood cells</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of mean</td>
</tr>
<tr>
<td>SMC</td>
<td>Smooth muscle cells</td>
</tr>
<tr>
<td>SNP</td>
<td>sodium nitroprusside</td>
</tr>
<tr>
<td>Tbil</td>
<td>total bilirubin</td>
</tr>
<tr>
<td>TGF</td>
<td>transforming growth factor</td>
</tr>
<tr>
<td>TH</td>
<td>tyrosine hydroxylase</td>
</tr>
<tr>
<td>TP</td>
<td>total protein</td>
</tr>
<tr>
<td>WBC</td>
<td>white blood cells</td>
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ACKNOWLEDGEMENTS

I owe a great debt of gratitude to Mr R J Morgan FRCS, Consultant Urologist, Royal Free Hospital, London, who was instrumental in starting this project. I thank him for his unlimited support and constant encouragement.

To Dr D P Mikhailidis, FRCPath, Reader in the Department of Molecular Pathology and Clinical Biochemistry, Royal Free Hospital, London, my special thanks for providing me with laboratory space. I am also indebted for his practical help. Much more importantly, he gave me his invaluable time, help, criticism and encouragement throughout my research.

Very special thanks to Prof M Winslet, Head of the Academic Department of Surgery, Royal Free Hospital, London for his invaluable support of my research.

I would like to express my gratitude to Dr M R Dashwood PhD, Senior Research Fellow, for providing me with guidance and support for the receptor analysis studies.

My special thanks to Dr C S Thompson PhD, Senior Research Fellow, for his assistance with the diabetic rabbit model and setting up the in vitro organ baths. His help and good humour sustained me through many hours of laboratory-based work.

Thanks and recognition to Dr JY Jeremy for his help with the biochemical analysis and valuable comments

My thanks to Dr KM Naseem and Prof. RK Bruckdorfer in providing me with nitric oxide solutions for the functional studies. I also thank Dr A Naylor, Pfizer Ltd, Discovery Biology for providing the necessary equipment and drugs. A Charles Wolfson Research Grant also supported this thesis.
To Talat my wife, my love and gratitude. She bore the frustration of research with fortitude.

Finally, I dedicate my thesis to my late brother Mazhar Mumtaz, whose irreplaceable loss, during my research period, left us with a large vacuum in our life. I miss him very much.


STATEMENT OF ORIGINALITY

This thesis embodies results that were obtained entirely from work carried out by myself. Using functional, autoradiographic, tissue culture and biochemical techniques I, for the first time, demonstrated that ET-1, NO and PGs may play a role in the pathophysiology of diabetic cystopathy.
HYPOTHESIS

The pathophysiological changes in diabetic cystopathy have predominantly been attributed to autonomic neuropathy and alterations in the structure and function of the detrusor smooth muscle (Kaplan et al 1988). However, the underlying mechanisms involved are not clearly established. Over the last decade, it has become evident that important vasoactive mediators such as ET-1, NO and PGs may also have significant physiological actions in the urinary tract. ET-1 and its receptors (ET\(_A\) and ET\(_B\)) identified in the urothelium and smooth muscle of the urinary bladder possess contractile and mitogenic properties (Saenz de Tejada et al 1992). In contrast, NO, synthesized by NOS, mediates bladder neck and urethral smooth muscle relaxation via cGMP formation (Ehern et al 1994b). The PG-cAMP pathway also regulates urinary tract smooth muscle tone (Jeremy et al 1986). Interactions between these mediators and alterations in their function are now well recognized to play a key role in the pathogenesis of vascular smooth muscle dysfunction associated with diabetes. Since all these mediators have been identified in the lower urinary tract, I utilized a diabetic rabbit model to investigate whether ET, NO and PGs could play a role in the pathogenesis of diabetic cystopathy.
CHAPTER 1

GENERAL INTRODUCTION
1.1. DIABETIC CYSTOPATHY – DEFINITION AND PREVALENCE

Following the first reported description of the diabetic bladder (Jordan et al 1935), several studies have described the clinical manifestations of diabetic cystopathy. These include impaired bladder sensation, prolonged intervals between voiding, weak urinary stream, urinary retention and/or overflow incontinence (Spring et al 1953, Frimodt-møller 1976a, Kaplan et al 1988). With the development of urodynamic techniques and methods of evaluating bladder sensation, the micturition abnormalities associated with DM were characterised (Fagerberg et al 1967, Frimodt-møller et al 1976ab, Kaplan et al 1995). These abnormalities included a large bladder capacity, increased residual urine, detrusor areflexia, low flow rate and detrusor instability (Fagerberg et al 1967, Frimodt-møller et al 1976c, Kaplan et al 1995). Based on these findings, the prevalence of diabetic cystopathy has been reported to vary between 40% (Frimodt-møller et al 1976a) and 90% (Kaplan et al 1995). The reported frequency of diabetic cystopathy among insulin-dependent diabetics being greater than non-insulin-dependent diabetics (Frimodt-møller et al 1976d)
1.2. THE PHYSIOLOGY OF MICTURITION.

The collection and storage of urine by the detrusor at low intravesical pressures followed by its periodic expulsion through the bladder neck and urethra (bladder outlet) are dependent upon complex neural pathways. Under normal circumstances, the detrusor and the bladder outlet exhibit a reciprocal relationship in effecting their functional role. This role is also supported by the inhibitory impulses from the suprapontine centers, which allows the bladder to fill at low intravesical pressures. At the beginning of micturition, the initial event is the reduction of intraurethral pressure as a result of relaxation of the pelvic floor and the paraurethral striated muscles. This is immediately followed by a reflex inhibition of the smooth and striated muscles of the urethra accompanied with a mechanical shortening of the urethra, which opens the bladder neck. Detrusor contraction with a concomitant rise in intravesical pressures is maintained to allow the bladder to empty completely (de Groat et al 1993).

This complex process is dependent on a number of factors, including (a) the state of neuronal innervation and the neurotransmitters involved, (b) the structure of the organ as a whole, (c) the contractile response of the smooth muscle elements and (d) the availability of metabolic energy (in the form of cytosolic adenosine triphosphate and oxidative metabolism).

The activities of the neural pathways to the lower urinary tract are coordinated by the parasympathetic (pelvic), sympathetic (hypogastric) and somatic ( pudendal) nerves, containing both afferent and efferent nerve fibers (de Groat et al 1993, Andersson et al 1993).
The preganglionic parasympathetic efferents originating from the intermediolateral column of 2nd to 4th sacral segments of the spinal cord provide the principal excitatory input to the bladder. These efferent neurons activate postsynaptic nicotinic receptors (ganglionic type N2) by the release of acetylcholine (ACh) (Jaing et al 1986, Lundberg 1996a). Several other transmitters, such as enkephalins, galanin and cholecystokinin (de Groat et al 1986a, Morris et al 1989, Dunn et al 1993) are thought to regulate the nicotinic transmission. For example, Leucine enkephalins inhibit cholinergic transmission in the pelvic ganglia (de Groat 1986b), in contrast to vasoactive intestinal polypeptide (VIP) and substance P, which facilitate ganglionic transmission in the pelvic ganglia (Kawatani et al 1986, 1989). These mediators may be responsible for gating, patterning or adjusting the frequency of neuronal stimulation to the lower urinary tract. This capability could allow the nervous system to adjust the degree of excitatory and inhibitory input to the bladder. Furthermore, it is also evident that atropine (an antimuscarinic agent) only partially antagonises the contraction elicited by parasympathetic nerves in several animal species including humans. Thus, the nature of postganglionic neurotransmission in the mammalian urinary bladder appears to be dual in nature; an atropine sensitive (cholinergic) and atropine-resistant (non-cholinergic) component. The proportion and importance of each in the micturition process is controversial being dependent on species and the functional status of the bladder. (Ambache et al 1970, Burnstock et al 1972, Hoyle et al 1989, Luheshi et al 1990a). For example, normal male human detrusor strips exhibit little if any atropine resistant contractions (Sjogren et al 1982, Sibley et al 1984). In contrast, detrusor strips from male patients with bladder hypertrophy and normal female human bladders, demonstrate an
atropine resistant component of up to 50% (Nergardh et al 1983, Sjorgen et al 1982, Cowan et al 1983). Unlike in humans, a large atropine resistant component is demonstrable in several animal species, including the rat, rabbit, guinea pig and cat. Thus, in the rabbit, it has been estimated that 48-60% of the electrically evoked contractions is non-cholinergic (Levin et al 1994).


The sympathetic preganglionic pathways to the lower urinary tract arise from the intermediolateral cell column 11th thoracic to 2nd lumbar segments of the spinal cord. These link with the postganglionic neurons in the inferior mesenteric, paravertebral and pelvic ganglia. Sympathetic ganglionic transmission is also mediated by ACh acting on N2 nicotinic receptors. Norepinephrine (NE) released by the sympathetic postganglionic terminals constricts bladder neck and urethral smooth muscle via α1-adrenoceptors, whereas, it relaxes the detrusor via β2-adrenoceptor activation (Yoshimura et al 1997).

The somatic cholinergic efferents innervating the external urethral sphincter and pelvic floor emerge from the anterior horn of the 2nd to the 4th sacral spinal cord segments and travel via the pudendal nerve (Roppolo et al 1985). The activation of nicotinic receptors on striated muscle elicits contraction.

Afferent activity, including the feeling of bladder fullness or bladder pain, is conveyed to the spinal cord via the pelvic and hypogastric nerves (Jaing et al 1993, Maggi et al 1993, de Groat 1986b). The afferent fibers initiating micturition are small myelinated (Aδ) and unmyelinated (C) fibers (Mallory et al 1989, de Groat et al 1981).
These afferents convey impulses from tension receptors and nociceptors in the bladder wall. Electrophysiological studies in the cat show that Aδ afferent fibers respond in a graded manner to passive distension as well as active contraction of the bladder. Although, mechanoreceptor afferents from the lower urinary tract consisting of myelinated and unmyelinated axons have also been identified in sympathetic nerves, their exact function in the micturition process remains unclear. These afferent pathways respond to stimulus modalities similar to those triggering afferents in the pelvic nerve. Afferent pathways from the urethra, which induce the sensations of temperature, pain and passage of urine, travel in the pudendal nerve to the lumbosacral spinal cord. These afferents, as well as pudendal nerve afferents from the striated sphincter muscles have a modulatory influence on micturition. Stimulation of somatic afferent nerve fibers reflexly inhibits bladder function (Roppolo et al 1985).

Immunohistochemical studies have shown that a large percentage of bladder afferent neurons in the submucosal and subepithelial layer stain for CGRP, VIP, SP, EK, and CCK (Yoshimura et al 1997). Afferent neurons in the sacral dorsal root ganglia and spinal cord have a similar distribution. Therefore it is not surprising that the use of anticholinergic drugs alone has little effect on sensory disorders of the lower urinary tract.

The neurotransmitters and the mechanisms that regulate the micturition process are not only of research interest but also provide a basis for therapeutic intervention in patients with voiding dysfunction. A decade ago, the study of amine mediators such as NE and ACh dominated pharmacological research in the lower urinary tract. Recently, several nonadrenergic, noncholinergic (NANC) mediators have been recognised to play a
significant role in the physiology of the lower urinary tract (Anderson et al 1993). NO and ET, are increasingly recognised as important cell mediators with a broad range of functions in the lower urinary tract.

1.3. ENDOTHELINS IN THE LOWER URINARY TRACT

1.3.1 Synthesis and localisation of receptors

Following the identification of ET in 1988 as a 21 amino acid peptide, three ET isoforms (ET-1, ET-2 and ET-3) have been identified (Yanagisawa et al 1988, Arai et al 1990). These isoforms are synthesised from a precursor, proET-1 (big ET), by the action of ET converting enzymes whose cDNA have been cloned and functionally expressed (Ikura et al 1994, Schmidt et al 1994). The presence of ET-like immunoreactivity and ET-1 mRNA in the epithelium, smooth muscle and fibroblasts of the urinary bladder provides evidence that ET is synthesised locally (Saenz de Tejada et al 1992). ET-like immunoreactivity and ET converting enzyme-1 mRNA has also been detected in the prostatic epithelium (Langenstroer et al 1993, Walden et al 1998). Furthermore, genes coding for ET-1 biosynthesis and its receptors are expressed in the human prostate indicating that this peptide is being produced in this organ (Prayer-Galetti et al 1997). The regulation of the transcription of ET mRNA, which plays an important role in the production of ET isoforms is modulated by several factors. For example, cultured endothelial cells have been shown to increase message levels after treatment with transforming growth factor-β, insulin and stress. Furthermore, ET-1 increases the release of NO by the endothelium, while NO depresses the production and/or release of ET-1
(Hirata et al 1993, Owada et al 1994, Gellali et al 1997, Goligorsky et al 1994). ET-1 by activating phospholipase A$_2$ as well as phospholipase C in cultured vascular smooth muscle cells (SMC), leads to the release of arachidonic acid from membrane phospholipids. Arachidonic acid can then metabolised to various prostaglandins, thromboxanes and leukotrienes, depending on the metabolic activity of the cells concerned. Thus, ET-1 by stimulating the release of PG and NO may impair its own contractile activity. NO and ET, therefore, appear to form a paracrine/endocrine control cycle with a negative feedback mechanism. It has also been suggested that ET-1 acts as an autocrine and/or a paracrine mediator of bladder and prostatic smooth muscle contraction and proliferation. This proposed mechanism is similar to that observed in vascular tissues where ET-1 is synthesised in the endothelium and smooth muscle and exerts several physiological and pathophysiological effects (Rubanyi et al 1994). ET$_A$ and ET$_B$ receptors have been demonstrated using radioligand binding studies in the epithelium and smooth muscle of the bladder and prostate in humans and animal species (Kondo et al 1993, Kobayshi et al 1994, Traish et al 1995, Le-Burn et al 1996). The density of both ET receptors in the bladder dome is greater than in the bladder base and urethra (Latifpour et al 1995). The predominant receptor subtype in the bladder dome is ET$_A$ whereas both ET$_A$ and ET$_B$ receptors are of approximately equal proportions in the bladder base and urethra (Latifpour et al 1995). Regional variations in the distribution of ET receptor subtypes have also been described in the human prostate (Prayer-Galetti et al 1997). For example, within the peripheral zone, where prostate cancer is initiated, the ET$_A$ and ET$_B$ receptor binding sites are found predominantly in the glandular epithelium and smooth muscle, respectively (Prayer-Galetti et al 1997). In contrast, in the zone
where benign prostatic hyperplasia (BPH) predominates, the $ET_A$ and $ET_B$ receptor subtypes are found only in the smooth muscle (Prayer-Galetti et al. 1997). This suggests that the function of ET-1 may vary depending on the region and the distribution pattern of its receptors in the lower urinary tract.

1.3.2 Effect on endothelin on smooth muscle tone

ET-1 elicits potent and long-lasting receptor-dependent contractions in smooth muscle strips from the urinary tract and prostate in humans and several animal species (Maggi et al. 1989, 1990, Garcia-Pascual et al. 1990, Saenz de Tejada et al. 1992, Langenstroer et al. 1993, 1997, Kobayashi et al. 1994a,b). In addition, ET has also been shown to modulate cholinergic (Saenz de Tejada et al. 1992), adrenergic (Garcia-Pascual et al. 1990) and NANC (Donoso et al. 1994) neurotransmission in the lower urinary tract.

The contractile responses to ET-1 in the rat bladder are predominantly mediated via the $ET_A$ receptor subtype (Donoso et al. 1994). Although, the exact role of $ET_B$ receptors in the bladder is not clear, its activation has been shown to release NO in vascular tissues (Moritoki et al. 1993). Whether a similar response occurs in the urinary tract smooth muscle remains speculative. In vitro studies on human and canine prostatic smooth muscle strips indicate that both receptors mediate contraction (Kobayashi et al. 1994b, Langenstroer et al. 1994). These studies show that the magnitude of ET-1-induced contractions in vitro is at least 80% of the contraction elicited by the stimulation of $\alpha$-adrenergic receptors. In comparison, in vivo experiments indicate that although prostatic urethral pressure is raised following the intravenous administration of ET-1, the magnitude of this change is only 30% of that elicited by alpha-adrenergic receptors.
(Imajo et al 1997). The discrepancy between the in vitro and in vivo studies leads to the hypothesis that the systemic administration of ET-1 may stimulate reverse compensatory mechanisms such as NO release by the prostatic epithelium. This additional effect may result in prostatic smooth muscle relaxation (Takeda et al 1995).

1.3.3 Mechanisms responsible for endothelin-mediated smooth muscle contraction
The calcium (Ca\(^{2+}\)) dependence of ET-1-mediated contractile responses in the urinary tract is tissue and species specific. For example, ET-1-induced contractile responses in the rabbit urinary bladder smooth muscle can be antagonised by dihydropyridine Ca\(^{2+}\) channel blockers, whereas human prostate smooth muscle contractions to ET-1 are independent of these Ca\(^{2+}\) channels. ET-1 elicits its effects by binding to and activating its receptors, which subsequently leads to an increase in intracellular Ca\(^{2+}\) concentration via either transmembrane Ca\(^{2+}\) influx and/or Ca\(^{2+}\) liberation from intracellular storage sites (Maggi et al 1989, Garcia-Pascual et al 1990). Despite the fact that the peak elevations in intracellular Ca\(^{2+}\) are known to be transient, ET-1 is able to elicit long-lasting and sustained contractions. This may be mediated via Ca\(^{2+}\) sensitisation mechanisms, which have been described in diverse smooth muscles (Kitazawa et al 1991).

1.3.4 Effects of endothelin on cell proliferation.
In addition to its contractile properties, ET-1 is also a potent mitogen of prostatic SMC (Nelson et al 1996, Walden et al 1998). The proliferation of SMC in response to ET-1 has been shown to correlate with ET-receptor density (Kanse et al 1995). ET receptors also mediate proliferation in other tissues. For example, ET\(_B\) receptors
participate in the development of intimal hyperplasia after endothelial vascular injury (Azuma et al. 1995). In contrast, in the human airway SMC proliferation is $\text{ET}_A$ receptor-dependent (Panettieri et al. 1996). However, both $\text{ET}_A$ and $\text{ET}_B$ receptors have been shown to exert a mitogenic effect on prostatic SMC (Nelson et al. 1996, Walden et al. 1998).

1.3.5 Pathophysiological role of endothelins

Both glucose and insulin enhance the release of ET-1 from endothelial cells (Hu et al. 1993) and from mesenteric arteries (Takeda et al. 1991). Also, blood (Collier et al. 1992) and urine levels (Morabito et al. 1994) of ET-1 are elevated in DM. The stimulation of ET-1 production and enhancement of its mitogenic action on SMC by glucose and insulin is thought to accelerate the progression of vascular diseases associated with DM (Frank et al. 1993). In addition, alterations in the expression of ET receptors have been shown to have a pathophysiological role in the vascular system (Rubanyi et al. 1994). In this context, recent studies have also implicated alterations in ET bioactivity in the pathophysiology of lower urinary tract dysfunction associated with DM, BPH and prostatic cancer. An increase in the expression of ET receptors in the ureter and prostate of experimentally induced diabetic rats (Saito et al. 1995, 1996) has also been observed. The increase in ET receptors in the diabetic prostate did not correlate with serum testosterone levels. This is of interest because low testosterone concentrations have been implicated as a cause for a reduced prostatic growth and down-regulation of muscarinic receptors in diabetic animals (Saito et al. 1995, 1996). The influence of ET-1 on cellular proliferation and its synergism with several other peptide growth factors suggests that it
may have a role in bladder and prostatic smooth muscle hyperplasia associated with DM and bladder outflow obstruction. These findings support a possible role of ET in the control of bladder wall structure. Alterations in the density of ET receptors in the bladder and prostate have also been observed secondary to bladder outflow obstruction (Kondo et al 1993). The density of the ET receptors was found to decrease in the bladder and increase in prostates of patients with BPH (Kondo et al 1995). This increase in prostatic ET receptor density may contribute to the raised intraurethral pressure associated with bladder outflow obstruction. These results also support the rationale for developing selective ET antagonists for the treatment of prostatic outflow obstruction and bladder dysfunction.

Several studies have also suggested that ET-1 may exert a potentially significant role in the pathophysiology of prostatic cancer. Human seminal fluid contains the highest concentration of ET-1 in any body fluid studied, approximately 500 times more than plasma (Battistini et al 1993). Plasma ET-1 concentrations are significantly increased in men with metastatic prostatic cancer (Nelson et al 1995). ET-1 also increases alkaline phosphatase activity in new bone formation, indicating that it may be a mediator of the osteoblastic response of bone to metastatic prostate cancer (Nelson et al 1995). In addition, ET-1 attenuates apoptosis of SMC in BPH (Wu-Wong et 1997), whereas on prostate cancer cells it acts both as an independent mitogen as well as a stimulator of the mitogenic effects of other important growth mediators (Nelson et al 1996). The mitogenic effect of ET-1 was $ET_A$ receptor-mediated. Interestingly, $ET_B$ binding sites that are present in benign prostatic epithelial tissues have not been demonstrable in both prostate cancer cell lines and at metastatic prostate cancer sites (Nelson et al 1996). Thus, it
appears that during prostate cancer progression to metastases, ET-1 and ET$_A$ expression are retained, whereas ET$_B$ receptor expression is reduced. ET-1, as an independent mitogen, may provide a mechanism by which androgen-independent prostate cancer progression occurs (Nelson et al 1996). Indeed clinical trials are underway investigating the effect of ET$_A$ receptor antagonists in hormone refractory metastatic prostatic cancer progression.

1.4. NO IN THE LOWER URINARY TRACT

1.4.1 The discovery of NO

The identification that mammals release large amounts of nitrates in the urine indicated that NO may be produced in the urinary tract (Mitchell et al 1916). In 1980, Furchgott and Zawadzki reported the existence of a labile endothelium-derived relaxing factor (EDRF) (Furchgott et al 1980), that elicited the relaxation of blood vessels. Although, it was soon recognised that the endothelium dependent relaxation required Ca$^{2+}$ (Griffith et al 1986) and cGMP formation (Rapoport et al 1983), the identity of EDRF remained elusive for 7 years. In 1987, it became clear that the EDRF synthesised by cultured endothelial cells was in fact NO and was dependent on the presence of L-arginine (Palmer et al 1987, Ignarro et al 1987). It was later demonstrated that there were many similarities between the EDRF and the neurotransmitter involved in NANC nerve-mediated smooth muscle relaxation (Garthwaite et al 1991, Gillespie 1986). The definitive evidence that NO was the neurotransmitter mediating this effect came from studies on the rat anococcygeus muscle (Gillespie et al 1989). Subsequently, the role of
NO as a neuronal messenger in the urinary tract was established in the rabbit urethra (Andersson et al 1992a). Since then, a considerable amount of information has accumulated on the biochemistry and the role of this ubiquitous messenger in the urogenital tract.

1.4.2 Synthesis, release and degradation of NO

NO is neither pre-stored nor packed in vesicles; instead, it is produced on demand. It then diffuses from its site of production in a random manner, being highly membrane permeable. Endogenous NO is formed by the hydroxylation of L-arginine to citrulline (Palmer et al 1989). The reaction is catalysed by one of the three isoforms of NOS (Forstermann et al 1994). Due to the short half-life of NO (0.1-6s) and its high reactivity, NO physiology has largely been investigated indirectly by techniques that have identified the distribution and activity of the NOS isoforms. These distinct isoforms of NOS have been named after the cells in which they were first isolated, purified and cloned (Forstermann et al 1994). Each NOS isoform, endothelial (eNOS), neuronal (nNOS) and macrophage inducible (iNOS) varies considerably in subcellular location, structure, kinetics, regulation and function (Forstermann et al 1994). Each of the enzymes is a product of a unique gene, which is located on human chromosomes 7 (eNOS), 12 (nNOS) and 17 (iNOS) (Forstermann et al 1994). Both nNOS and eNOS are normal constituents of cells and are termed constitutive. The activity of both eNOS and nNOS is transient (minutes) and is triggered by Ca$^{2+}$ elevating agonists (Moncada et al 1991). In contrast, iNOS is not present in resting cells, but rather induced by certain cytokines or bacterial endotoxins (Moncada et al 1991). Under certain circumstances the expression of eNOS
and nNOS is also inducible. For example, fluid flow (causing endothelial shear stress) up-regulates the expression of eNOS; indeed, six shear stress-responsive elements have been identified in the promotor region of eNOS (Cooke et al 1997). Unlike the constitutive NOS isoforms, iNOS activity is sustained (lasting many days) and is independent of Ca\(^{2+}\) elevation. Structurally, the eNOS NH\(_2\) terminus contains a consensus site for N-myristoylation that plays a significant role in the membrane localisation of eNOS. This attribute explains the observation that eNOS is membrane-associated, whereas iNOS and nNOS are cytosolic. Although each of these isoforms contains a calmodulin-binding site, iNOS binds to it with a higher affinity so that calmodulin forms a constitutive subunit to this isoform. Thus, eNOS and nNOS are dependent on exogenous Ca\(^{2+}\) and calmodulin for activation, whereas iNOS is less so. However, common to all three isoforms are heme containing enzymes that catalyse the nicotinamide adenine dinucleotide phosphate (NADPH) and oxygen (O\(_2\))-dependent oxidation of L-arginine to NO and citrulline (Cooke et al 1997). All three isoenzymes have been identified in the lower urinary tract of several animal species including man (Andersson et al 1994, Ehen et al 1994a, Dokita et al 1994, Burnett et al 1997).

1.4.3 Distribution of NO synthase in the lower urinary tract.

Immunohistochemical techniques identified putative NO synthesis sites in the lower urinary tract. Neuronal NADPH-diaphorase (NADPH-d) is used as a specific marker for neurons producing NO (Dawson et al 1991). NOS activity has been identified in the urothelium, smooth muscle, blood vessels and more importantly in the nerves supplying the lower urinary tract.
The origin of nerves producing NO in the lower urinary tract is not fully established, however, they appear to originate in close proximity to the parasympathetic sacral nucleus. The presence of NOS in the postganglionic parasympathetic and preganglionic sympathetic nerves supplying the rat lower urinary tract support the functional evidence that NO may have a role as an inhibitory NANC transmitter (McNeill et al 1992). As such, stimulation of the parasympathetic input to the urethra elicits NO-dependent urethral smooth muscle relaxation (Fraser et al 1995, Bennett et al 1995, Persson et al 1992). The major pelvic ganglia supplying the urinary tract also exhibit NADPH-d activity and NOS-immunoreactivity (NOS-ir) (Vizzard et al 1994). Double immunolabelling studies also show that NOS-ir cell bodies in the major pelvic ganglia display choline acetyltransferase (Persson et al 1995, Alm et al 1995). Furthermore, the removal of the major pelvic ganglia in the rat completely abolishes NO-dependent urethral smooth muscle relaxation (Persson et al 1998), whilst hypogastric nerve transection has no effect. These observations, therefore, support the concept of a parasympathetic origin for NO. In addition, the neurons of the major pelvic ganglia innervating the urethra exhibit significantly greater NADPH-d activity and NOS-ir than those supplying the bladder (Vizzard et al 1994, Keast et al 1994). NADPH-d and NOS-ir positive nerves have also been demonstrated in and around muscular bundles of the entire lower urinary tract of several animal species (Keast et al 1994, Persson et al 1993, Triguero et al 1993, Smet et al 1996) and man (Smet et al 1994, 1996, Ehem et al 1994b). Despite minor interspecies variations in the distribution of NOS-containing nerves, the density of these nerves is significantly greater in the bladder neck and urethra, compared to the detrusor. Similar regional differences in NOS activity as measured by the citrulline
formation assay were also observed; higher NOS activity in the urethra compared to the
detrusor (Persson et al 1993, Ehem et al 1994a). Functionally, the inhibitory effects of
NO correlate with the density and distribution of NOS-containing nerves in the lower
urinary tract. As such, smooth muscle relaxation mediated by NO is significantly greater
in the urethra compared to the detrusor in most species (Garcia-Pascual et al 1996, Ehem
et al 1994b, Saffrey et al 1996). NADPH-d positive neuronal bodies are present in the
intramural ganglia as well as in the outer layer of the bladder wall (Vizzard et al 1994).

Primary afferent nerves seen beneath and penetrating the urothelium are also
considered as a possible source of NO, since these nerves exhibit NADPH-d activity
(Garcia-Pascual et al 1996, Ehem et al 1994a, Saffrey et al 1996). In addition, the bladder
afferent neurons in the 6th lumbar and 1st sacral dorsal root ganglia and in the dorsal horn
of the spinal cord exhibit NADPH-d activity, although NOS-ir was not identified
(Vizzard et al 1994, 1996). However, following chronic irritation of the bladder, NOS-ir
in the spinal cord and dorsal root ganglia becomes increasingly recognised (Vizzard et al
1996). This suggests that up-regulation of NOS in response to chronic inflammation may
represent an adaptive mechanism to enhance the spinal nociceptive mechanisms or reflex
responses elicited by nociceptive afferent input from the bladder. In this context, NO is
thought to play a role in the bladder hyperreflexia following chronic irritation of sensory
neurons in the lower urinary tract. Indeed, intravesical capsaicin, due to its destructive
effect on sensory nerves (Holzer et al 1991), has been used in the treatment of detrusor
hyperreflexia in humans (Fowler et al 1994). Although, capsaicin stimulates the release
of neuropeptides such as calcitonin gene related peptide (CGRP), substance P and VIP,
recent evidence suggests that it also stimulate the release of NO from the bladder (Brider
et al 1998a). Furthermore, stimulation by capsaicin produces an endogenous NO-dependent relaxation in the isolated dog urethra (Nishizawa et al 1997). At the spinal level, the impairment of capsaicin-induced micturition reflex has been shown to be independent of NO (Pandita et al 1997), suggesting that capsaicin-induced release of NO, may exert its effects locally by inhibiting the short loop reflexes within the bladder. In addition, the close proximity of NADPH-d stained afferent nerve fibers to other neuropeptide-containing, capsaicin-sensitive afferent neurons (Zhou et al 1998) would suggest that NO released locally may also exert an indirect influence on the sensory neural pathways in the urinary bladder.

Double staining techniques have shown that NADPH-d positive neurons also stain for choline acetyltransferase (a marker for parasympathetic neurones), tyrosine hydroxylase (TH) (a marker for sympathetic neurons) and VIP in the lower urinary tract of several species including man (Zhou et al 1998). In a subpopulation of human bladder neck (Dixon et al 1997) and urethral (Ho et al 1999a) ganglionic cell bodies, both NOS-ir and TH-ir have been demonstrated. In fact, recent studies suggest that blocking the prejunctional α-adrenergic receptors influences NO-mediated smooth muscle relaxation in the rabbit urethra (Yoshida et al 1998). The NO donor, sodium nitroprusside (SNP), has also been shown to modulate the detrusor contractile responses to electrical field stimulation (Liu et al 1997, Moon et al 1997). These findings indicate that apart from acting as a primary inhibitory neurotransmitter, NO may also serve as a neuromodulator by virtue of its close association with the cholinergic and sympathetic system.

It is now clear that like the endothelium, the urothelial lining of the lower urinary tract also exhibits NOS and produces NO (Brider et al 1998a). Both eNOS and iNOS
isoforms have been identified within this layer (Burnett et al 1997, Lemack et al 1999). Equally, the epithelial lining of the prostate expresses eNOS. The urothelial cells following exposure to NO donors also express cGMP-ir (Smet et al 1996). NO synthesis by the urothelium can be measured using a porphyrinic microsensor placed on the surface of the bladder, following stimulation by capsaicin (an afferent neurotoxin) and a NE α/β-adrenergic agonist (Brider et al 1998a). The physiological role of NO, produced by the bladder urothelium, is not yet clear. However, it may serve as a source of peroxynitrite, especially in conditions of excess production (Cooke et al 1997). In this context NO may play a role in oxidative injury following bladder outlet obstruction.

There is no direct evidence that NO is produced by the detrusor smooth muscle. However, NO generated by electrically stimulated human detrusor smooth muscle strips mediates relaxation via mechanism(s) that are nerve independent (James et al 1993). In both the guinea pig and human proximal urethra, there is a prominent induction of cGMP-ir in the urethral smooth muscle by NO (Smet et al 1996). Despite the presence of significant NOS-ir in both human and guinea pig detrusor, cGMP-ir could not be induced in the detrusor smooth muscle cells following stimulation with SNP. Instead, the interstitial cells within the detrusor expressed an intense cGMP-ir. These interstitial cells appear, therefore, to be the predominant target for NO in the detrusor.

NOS-ir has been detected in the striated muscle of several mammalian species (Grozdanovic et al 1995, Kobzik et al 1994). Recent observations suggest that NO has a role in the control of intramural striated muscle of the human male membranous urethra as well as in the female urethral striated muscle (Ho et al 1998, 1999b). Both NOS-ir and NADPH-d activity are evident in the sarcolemma of the intramural striated muscle fibers.
as well as in the nerve trunks and fine nerve fibers present in the human male and female urethral striated muscle (Ho et al 1998, 1999b). Whether the nerve fibres actually innervate the striated muscle remains to be elucidated. The association of NO with the sarcolemma suggests that it may have an inhibitory role, mediating relaxation of the striated sphincter of the membranous urethra during the voiding phase of micturition.

NADPH-d staining is also observed in the connective tissue and in the endothelium of small and large arteries supplying the lower urinary tract. It is therefore conceivable that there are several sources of putative NO production in the urinary tract: the NANC neurons, the urothelium, SMC and the blood vessels.

1.4.4 Role of NO in the prenatal development of the lower urinary tract

During human fetal development, the NOS-expressing nerves are mainly localised to the developing prostate gland/bladder neck with minimal expression around the bladder (Dixon et al 1995). As gestational age increases, NOS-containing nerves also increase in number, but they continue to be predominant around the bladder neck and urethra. NOS has been identified in the paraganglionic cells within the bladder musculature of the developing human male fetus (Dixon et al 1998). NADPH-d and NOS-ir are also expressed in the majority of intramural neurons in the neonatal guinea pig urinary bladder (Saffrey et al 1994). Intrauterine inhibition of NO production increases bladder capacity as well as postvoiding residual volume despite normal bladder pressures, suggesting inadequate sphincter relaxation (Mevorach et al 1994). These effects were reversed following the administration of L-arginine. Regional variations in the density of NOS containing nerves and the differences in their temporal development in the lower urinary
tract suggest that NO may play a role in the autonomic control of the lower urinary tract during fetal development.

1.4.5 Evidence of NO-dependent smooth muscle relaxation.

(a) Bladder outlet

Before the discovery of NO, NANC-nerve mediated relaxation was first reported in the female rabbit (Andersson et al 1983), porcine urethra (Klarskov et al 1983) and human bladder neck (Speakman et al 1988). Evidence that NO was involved came by blocking the relaxation with N^G-nitro-L-arginine, a NOS inhibitor (Andersson et al 1992b, Dokita et al 1991). These results were further confirmed using a selective cGMP phosphodiesterase inhibitor, which potentiates relaxation, whereas methylene blue (a guanylyl cyclase inhibitor) reduced it (Persson et al 1994). NANC-mediated relaxation of the bladder neck and urethral smooth muscle is associated with increased intracellular cGMP levels (Dokita et al 1994). These studies provided compelling evidence that NO is an important mediator in the relaxation of isolated urethral and bladder neck smooth muscle. In vivo studies also demonstrated that NO mediates urethral smooth muscle relaxation in the rat following stimulation of parasympathetic efferent neurons (Fraser et al 1995, Bennett et al 1995, Persson et al 1992). Thus, NOS inhibitors abolished somato-urethral smooth muscle reflexes mediated by the parasympathetic efferent pathways following spinal cord injury in the female rat (Kakizaki et al 1997). Furthermore, the inhibitory component of the somato-pelvic parasympathetic reflex utilises NO as a neurotransmitter or neuromodulator at the level of the brain stem (Morrison et al 1996). Also stimulation of afferent nerve fibers in the bladder initiate a reflex that results in
urethral smooth muscle relaxation, which is thought to be mediated by NO (Kakizaki et al 1997). In vivo studies indicate that both baseline urethral pressure and maximum urethral relaxation are decreased by the systemic infusion of NO donors. Topical intraurethral NO donors have been shown to induce urethral smooth muscle relaxation without affecting bladder smooth muscle function (Fraser et al 1998). The intraurethral application of NO donors may, therefore, be clinically effective in cases of urethral smooth muscle sphincter spasticity and obstruction. Interestingly, the systemic administration of L-\textsuperscript{N\textsuperscript{G}}-nitroarginine (L-NOARG) stimulates detrusor hyperactivity (Persson et al 1991) and inhibits urethral smooth muscle relaxation during micturition (Bennett et al 1995). These findings further suggest that detrusor instability secondary to bladder outlet obstruction may be due to the lack of NO bioactivity at the bladder outlet.

(b) Detrusor

Unlike the bladder neck and urethra, there is as yet no convincing evidence that nerve-mediated relaxation of the detrusor muscle involves NO as a neurotransmitter. However, exogenous NO, as well as endogenous locally produced NO does induce detrusor smooth muscle relaxation (James et al 1993, Chung et al 1996). Inhibiting NOS activity, either by the systemic or intravesical administration of NOS inhibitors, decreases the bladder capacity as well as increasing the magnitude of bladder contractions (Theobald et al 1996). The exact role of NO in the detrusor physiology is not clear, but it may be a factor keeping the bladder relaxed during the filling phase of the micturition process. In fact, NO is thought to have a similar function in the stomach, i.e. as a mediator of adaptive relaxation to accommodate food or fluid (Desai et al 1991). Furthermore, NO has recently been shown to regulate the blood supply to the detrusor
during the filling and voiding phase of the micturition cycle (Kozlowski et al 1999). In experimentally-induced bladder inflammation, both NOS activity and basal blood flow to the bladder were reduced (Kozlowski et al 1999). Further studies are therefore needed to investigate if alterations in NO bioactivity play a role in the bladder ischaemia associated with distension following bladder outflow obstruction.

(c) Prostate

The nerves supplying the ventral and peripheral zone of the rat and human prostate, respectively, express NOS-ir (Burnett et al 1995a, 1995b). NOS activity appeared to be greater in the peripheral zone relative to the transition zone. In human prostatic tissue, dense NADPH-d staining and NOS-ir is present in the glandular epithelium, stroma and blood vessels (Bloch et al 1997). An intense NADPH-d activity is also demonstrated in the glandular and stromal cells of seminal vesicles (Machtens et al 1998). NOS activity was found to be greater in the secretory epithelium compared to the stromal component. Functionally, NO modulates prostatic and seminal vesicle smooth muscle tone (Machtens et al 1998, Takeda et al 1995). The magnitude of the prostatic smooth muscle relaxation to NO is significantly greater in the human prostate compared to the canine (Takeda et al 1995). Although further studies are needed to understand the physiological role of NO in the prostate, castration in the rat leads to a reduction in NOS activity and cGMP formation by the prostate (Shabsigh et al 1999). These effects were thought to be associated with apoptosis of prostatic endothelial cells. NO is, therefore, thought to play a role in supporting prostatic growth, regulating prostatic smooth muscle tone as well as possibly having a neuroregulatory function in the male reproductive tract.

The exact mechanism by which NO mediates smooth muscle relaxation is not fully understood, however, it is associated with an increase in the intracellular production of cGMP (Moncada et al 1991, Persson et al 1993, Persson et al 1994, Smet et al 1996, Garcia-Pascual et al 1996). This is achieved by binding NO to the heme moiety of guanylyl cyclase following its local production and diffusion. This step activates guanylyl cyclase which then catalyses the formation of cGMP from guanosine 5' triphosphate. Activation of a cGMP-dependent protein kinase may hyperpolarise the cell membrane, possibly by activating potassium (K⁺) channels (Robertson et al 1993, Waldeck et al 1995, Zygmunt et al 1996). However, application of NO had no effect on the membrane potential of the rabbit urethral smooth muscle (Waldeck et al 1998). Other mechanisms of NO-induced relaxation, mediated by cGMP, may involve reduced intracellular Ca²⁺ levels by sequestration or reduced sensitivity to Ca²⁺ (Peng et al 1996). NO can also act independent of cGMP pathway. For example, the cytotoxic action of macrophage-derived NO on tumour cells and other tissue components is the result of nitrosylation and subsequent inactivation of iron-containing enzymes of the respiratory cycle and DNA synthesis (Mirvish et al 1995). Additional biochemical roles for NO include interactions with superoxide anions leading to the production of peroxynitrite (Billair et al 1995). This cytotoxic oxidant is thought to contribute to tissue injury in a number of pathophysiological situations, such as inflammation and ischaemic-reperfusion injury.
1.4.7 Pathophysiological role of NO.

Alterations in the NO pathway are increasingly recognised in the lower urinary tract following bladder outflow obstruction. Insight into the pathophysiological role of NO has been provided from mice with targeted deletion of nNOS. The bladders from these mice become hypertrophic and markedly dilated (Burnett et al. 1997). Furthermore, the bladder outlet failed to relax in response to neuronal and exogenous NO (Burnett et al. 1997). Others have found that despite the disruption of the main pathway for the synthesis of neuronal NO, nNOS knockout mice voided normally and demonstrated normal in vitro smooth muscle responses to various agonists (Sutherland et al. 1997). In the latter study, the absence of any impairment in bladder function may be due to the earlier recruitment of compensatory mechanisms to overcome the lack of neuronal NO.

In a mouse model with partial bladder outflow obstruction, enhanced expression of iNOS is seen soon after obstruction (Lemack et al. 1999). Reverse transcriptase-polymerase chain reaction studies also demonstrated iNOS in the majority of mice subjected to obstruction, with almost complete absence in controls (Zhou et al. 1997).

Intramural ganglia in the bladder show evidence of degeneration and cell death following acute complete outlet obstruction (Saito et al. 1998). It is suggested that NO may be involved in neuronal death since the NADPH-d activities as well as iNOS-ir in such neurones is increased (Saito et al. 1998). Increased iNOS-ir may also be involved in excess NO production, leading to the formation of reactive nitrogen species such as peroxynitrite. These may in turn contribute to the ultimate development of bladder dysfunction.
Interestingly, NO inhibitors reverse the effects of ischaemic injury on the bladder (Saito et al 1998). It is, therefore, conceivable that NO may also play a role in bladder ischaemic injury associated with acute retention. Furthermore, NO may regulate bladder mucosal and muscle perfusion, which appears to be inversely related to intravesical pressure (Kozlowski et al 1999). Since NO, the product of iNOS, is a vasodilator, an increase in iNOS may be an initial compensatory response to overcome the effects of ischaemia generated by bladder outflow obstruction (Lemack et al 1999). Although alterations in NOS activity were not observed following bladder outlet obstruction in a rat model (Lin et al 1999), inhibiting NOS activity had different effects in the obstructed group compared to controls. There was an increased bladder capacity and maximum detrusor pressure in control animals; in contrast, no changes were noted in the obstructed group. This suggests that NO may have varying biological roles in different pathophysiological bladder states.

Inactivation of the cGMP-dependent protein kinase 1 gene in mice results in vascular and intestinal smooth muscle dysfunction. In the same animal model, NO/cGMP-dependent relaxation of urethral smooth muscle was also abolished (Persson et al 1999). Furthermore, these animals developed bladder hyperactivity suggesting that bladder instability may be associated with impaired NO/cGMP-dependent protein kinase 1 signaling (Persson et al 1999). These findings strongly suggest that manipulating the NO pathway may play a role in the treatment of bladder outflow obstruction. This concept is strengthened by the fact that oral administration of nitrates has been shown to improve urinary flow rates and reduce residual urine volume in patients with symptomatic bladder outflow obstruction (Mathers et al 1999). Interestingly, in rats,
using myoblast-based iNOS gene therapy, NO-mediated urethral smooth muscle relaxation is also enhanced (Yokoyama et al 1999). This approach may open the opportunity for gene therapy in the treatment of bladder outlet obstruction. In addition, modulating cyclic nucleotide-dependent signal transduction pathways in human detrusor smooth muscle by selective phosphodiesterase-inhibitors may also play a role in the treatment of detrusor hyperactivity (Seeman et al 1999).

The production of NO in response to inflammation following chronic irritation or infection is increased (Nussler et al 1993). Urinary NO levels, as well as expression of iNOS-ir and cGMP levels are markedly increased in bacterial cystitis (Smith et al 1996, Lundberg et al 1996b). Measurement of urinary levels of NO has therefore been shown to differentiate bladder dysfunction due to inflammation from neurogenic disorders or outflow obstruction (Ehern et al 1999). Up-regulation of iNOS-ir is also evident in the afferent and efferent neurons within the bladder and pelvic ganglions in response to chronic irritation of the bladder (Olsson et al 1998) and cyclophosphamide-induced haemorrhagic cystitis (Souza-Fiho et al 1997). These bladders become hyper-reflexic following chronic inflammation. Alterations in iNOS and cGMP levels have also been described in interstitial cystitis (Nussler et al 1993). NOS activity in bladder biopsy and urine samples is lower in patients with interstitial cystitis than in controls (Smith et al 1996, Lundberg et al 1996b). A reduction in NOS may be secondary to either an inhibition of induction or an inability to express NOS. The cytokine interleukin 6 (an inhibitor of NOS) is elevated in urine from patients with interstitial cystitis (Lotz et al 1994). In animal models of interstitial cystitis, decreased NOS activity is associated with alterations in bladder perfusion during the micturition process (Baselli et al 1999).
Intravesical administration of dimethylsulphoxide, used for the symptomatic relief of interstitial cystitis, stimulates the release of NO from bladder afferents (Brider et al 1997). This suggests that NO may be involved in the desensitisation of nociceptive pathways in the lower urinary tract. Oral L-arginine has also been shown to improve symptoms in some patients with interstitial cystitis (Ehem et al 1998), thereby further strengthening this view.

Interruption of the sacral parasympathetic outflow to the lower urinary tract following damage to the sacral spinal cord or spinal roots results in bladder areflexia and urinary retention. Alterations in adrenergic and cholinergic innervation to the lower urinary tract following spinal cord injury are well known (Atta et al 1984). Recently, NO has been implicated in voiding dysfunction after spinal cord injury (Vizzard et al 1997). Up-regulation of nNOS-ir in bladder afferents and spinal neurons of the sacral parasympathetic nucleus is observed following chronic spinal cord injury (Vizzard et al 1997). However, NO release by the rat bladder following stimulation by capsaicin is significantly reduced in chronic spinal cord injury (Brider et al 1998b). These early findings indicate that NO may play a pathophysiological role in voiding disorders associated with spinal cord injury.

NO is involved in regulating cell growth and cell differentiation (Mirvish et al 1995, Maeda et al 1998, Lala 1998). At low concentrations, NO can stimulate cell growth whereas high concentrations result in cytostatic effects. High iNOS-ir is identified in prostatic cancer cells in contrast to the complete absence in benign prostatic epithelial cells (Klotz et al 1998). Further studies also indicate that by inhibiting NOS activity, neovascularisation and proliferation of human prostate cancer cell lines were shown to be
reduced (Moy et al 1999). Both human prostate cancer tissues and prostate cancer cell lines also express eNOS as the active isoform. Changes in its expression are associated with increased growth of prostate cancer and angiogenesis. These studies suggest that NO may influence the progression of prostate cancer.

Following the intravesical administration of Bacille Calmette-Guerin (BCG), for the treatment of bladder carcinoma in situ and recurrent bladder cancer, there is a marked increase in NOS activity (Swana et al 1999). In the rat bladder, BCG up-regulates the gene and protein expression of iNOS, but eNOS and nNOS are also induced albeit to a lesser extent (Jansson et al 1998, Oh et al 1999). The expression of Ca\textsuperscript{2+}-independent NOS activity by bladder tumour cell lines (MBT2 and T24) are also induced by treatment with cytokines (Jansson et al 1998). This effect is reversed by L-nitroarginine. As a result of increased iNOS, high concentrations of NO are produced, which are thought to modulate the growth of bladder cancer cell lines. Thus, the tumourocidal effects of BCG in the treatment of bladder cancer may involve NO-dependent mechanisms.

As alterations in the NO pathways are being increasingly recognised in the pathophysiology of lower urinary tract disorders, manipulating the NO-cGMP axis may form the basis for new therapeutic intervention.
1.5 PROSTAGLANDINS IN THE LOWER URINARY TRACT

1.5.1 Synthesis and distribution of prostaglandins in the lower urinary tract

The synthetic pathway for prostaglandin production involves the release of a 'parent' fatty acid from membrane phospholipids by the phospholipase enzymes followed by the oxygenation by cyclooxygenase to form unstable endoperoxides. The endoperoxides are then converted into various prostanoids spontaneously or by specific synthases or isomerases. The urinary bladder tissue synthesises several PGs of the E and F (PGE, PGF) series (Brown et al 1980, Abrams et al 1979, Khalaf et al 1979, Alkondon et al 1980) as well as I₂ (Leslie et al 1984, Jeremy et al 1984, Kasakov et al 1985). In the rabbit, it has been demonstrated that the detrusor produces significantly more PGs than the base (Leslie et al 1984) and that both the outer layer and the inner mucosal layer are capable of PGE₂ synthesis (Brown et al 1980). Both the bladder smooth muscle and the transitional cell epithelium have the capacity to synthesize PGE₂. Mucosal biopsies taken from healthy areas of the human bladder during cystoscopy are also shown to produce PGs in the following quantitative order: PGI₂ > PGE₂ > PGF₂α (Zwergel et al 1991, Jeremy et al 1987). In a study involving the measurement of urinary concentrations of prostanoids in children the excretion of 6-oxo-PGF₁α was higher in males, whereas PGE₂ excretion was comparable in both sexes (Mikhailidis et al 1987). Combined multiple regression analysis revealed that age is the only significant factor that influences PGs secretion (Barden et al 1985).
1.5.2. Factors modulating prostaglandins release by the bladder

Distension (with buffer solutions) of isolated whole rat bladders increases the production of PGI$_2$, PGE$_2$, and TXA$_2$ intraluminally even in the absence of exogenous arachidonic acid (Jeremy et al 1984). This response to distension appears to be common to several organs since it has also been observed in portal and peripheral veins (Mikhailidis et al 1987). Increased intraluminal osmolarity also enhances bladder prostanoid synthesis (Jeremy et al 1984). The optimum pH for PGs production, established using rat bladder 'minces' is 7.0 – 8.0 (Jeremy et al 1984). PGs release and synthesis is also mediated by the stimulation of specific receptors. For example, significant amounts of PGI$_2$ are produced in response to muscarinic agonists but not following adrenergic receptor stimulation (Jeremy et al 1984). Adenosine triphosphate (ATP)-activated purinoceptors (P$_2$) (Kasakov et al 1985) also stimulates the release of PGs by the rat bladder detrusor muscle (Kasakov et al 1985). PGE$_2$ and 6-oxo-PGF$_2\alpha$ are the main PGs released. Furthermore, it is also known that the concentration of certain ions (Ca$^{2+}$ and K$^+$) can influence PGs synthesis in other tissues (Jeremy et al 1988). Urinary PGs concentrations are also influenced by the urinary pH (Haylor et al 1984), urinary sodium (Na$^+$) and K$^+$ concentrations (Reyes et al 1990, Nasjletti et al 1985), level of exercise (Vesterqvist et al 1984) and variations in fluid intake. Dietary Na$^+$ intake also influences PGs levels in the urine (Reyes et al 1990). Na$^+$ depletion in normal man is associated with increased excretion whereas acute replenishment of Na$^+$ (physiological saline intravenously) results in decreased excretion. Dietary K$^+$ intake also influences the urinary concentrations of PGs in the rat (Nasjletti et al 1985). Glutathione regulates PG synthesis in microsomes of the porcine urinary bladder epithelium (Mimata et al 1988). At a glutathione
concentration of less than $10^{-5}$M, microsomes produced more PGI$_2$ and PGF$_{2\alpha}$ than PGE$_2$. At higher glutathione concentrations, PGE$_2$ synthesis was enhanced but PGI$_2$ and PGF$_{2\alpha}$ synthesis was inhibited. This reciprocal effect of glutathione is also observed in rabbit and bovine urinary bladder epithelium (Mimata et al 1988).

1.5.3. Effects of prostaglandins on smooth muscle tone

PGs are thought to contribute to the maintenance of bladder tone and the process of micturition (Bultitude et al 1976). PGE$_2$ elicits dose-related contractions of detrusor muscle strips in vitro (Bultitude et al 1976). Incubation with indomethacin (an inhibitor of PGs synthesis) inhibits smooth muscle tone and spontaneous activity in the rat. Furthermore, the addition of PGE$_2$ or PGF$_{2\alpha}$ in the presence of indomethacin restores bladder strip tone and spontaneous activity (Bultitude et al 1976). In vivo work using normal conscious rats has shown that intravesically instilled PGE$_2$ facilitated micturition and increased intravesical pressure (Ishizuka et al 1995). PGE$_2$ given intra-arterially produces a distinct increase in bladder pressure before initiating a micturition reflex, indicating that PGE$_2$ has a direct contracting effect on the detrusor smooth muscle (Ishizuka et al 1995). SC-19220, a competitive receptor antagonist of PGE$_2$, increases bladder capacity and impairs the voiding efficiency of micturition in anaesthetized rats. The effect of SC-19220 was also prevented by indomethacin pretreatment (Maggi et al 1988). The spontaneous contractile force of muscle strips isolated from rabbit detrusor and bladder base is also increased, in a dose-dependent manner, by the administration of
PGE\(_1\), PGE\(_2\) or PGF\(_{2\alpha}\). Isolated muscle strips from the detrusor respond to PGs more markedly than those from the bladder base (Hanawa et al 1991). The rank order of potency (to induce contractile responses) is PGF\(_{2\alpha}\) > PGE\(_2\) > PGE\(_1\) in both regions (Hanawa et al 1991). These effects are significantly inhibited by pretreatment with verapamil (a Ca\(^{2+}\) antagonist) suggesting that these smooth muscle contractions in response to PGs are dependent on Ca\(^{2+}\) influx. These findings support the concept that endogenous PGE\(_2\) and other PGs are physiologically involved in the regulation of vesicourethral motility.

PGI\(_2\) also elicits a dose-dependent contraction of the strips from the rabbit detrusor, base and urethra (Gotoh et al 1986). The contractile activity of PGI\(_2\) was greatest in the bladder body. PGI\(_2\) was less potent than PGE\(_2\) or PGF\(_{2\alpha}\), and contractions induced by this PG were slower in onset and short in duration (Gotoh et al 1986). This phenomenon is also Ca\(^{2+}\)-influx dependent (Jeremy et al 1986, 1988). In the rabbit urinary bladder PGE\(_1\), PGE\(_2\) and PGF\(_{2\alpha}\) have all been shown to increase adenylyl cyclase activity, dose-dependently, in the detrusor and base (Kondo et al 1993). Hence, it is thought that PGs act on the urinary tract via cAMP as well as Ca\(^{2+}\) influx.

1.5.4 Prostaglandins and diabetes mellitus

In a rat model of streptozotocin-induced DM, the increased contractile response to substance P and electrical field stimulation (Kamata et al 1993) was attributed to increased synthesis of PGs. This change is also accompanied by a marked increase in bladder weight (Jeremy et al 1986). The increased capacity to release PGI\(_2\) may be due to distension or to a raised urinary osmolarity secondary to glycosuria (Jeremy et al 1986).
The actual distension of the bladder appears to be a consequence of diabetic neuropathy, since experimental bladder denervation in the non-diabetic rat is also associated with gross bladder distension and hypertrophy (Ekstrom et al 1984). The increased synthesis of PGI₂ by bladder tissue obtained from diabetic rats (Jeremy et al 1986) is compatible with the increase in urinary 6-oxo-PGF₂α excretion. The absence of such an increase in urinary excretion in insulin-treated diabetic animals (Quilley et al 1985) further supports a link between DM and abnormal PGI₂ production by the bladder. It is tempting to speculate that the increased release of PGI₂ in long-term diabetic rats is a compensatory response to neurogenic distension.

1.6 PATHOPHYSIOLOGY OF LOWER URINARY TRACT DYSFUNCTION IN DIABETES MELLITUS.

The changes in bladder function in DM have been attributed to autonomic neuropathy, changes in autonomic receptors and alterations in detrusor muscle structure and function.

1.6.1 Autonomic neuropathy

Diabetic neuropathy affects spinal nerve roots, sympathetic ganglia, peripheral autonomic nerves, intramural ganglia and nerve fibers of the bladder wall (Jordan et al 1935, Bartley et al 1966, Ellenberg et al 1967, Faerman et al 1973, Mastri 1980). The pathological changes described include nerve loss, axonal regeneration and segmental demyelination (Brown et al 1984). Although the exact aetiology of diabetic neuropathy is unclear, it is likely to be multifactorial. Early observations indicated that it was the result of
atherosclerotic vascular disease (Woltman et al 1929). This hypothesis has now largely been discarded, although microvascular occlusion and infarction does appear to be related to some diabetic mononeuropathies (Raff et al 1968). More recent studies have implicated changes in the axonal blood supply (Low 1987, Cameron et al 1994), abnormal myoinositol metabolism (Low 1987) and alterations in the intracellular pathways induced by chronic hyperglycaemia (Von-Poppel et al 1988, Green et al 1985) as important factors contributing to the pathogenesis of diabetic neuropathy. The presence of enhanced sorbitol concentration within nerves as a consequence of increased aldose reductase activity may also contribute to the development of diabetic autonomic neuropathy (Gabbay et al 1966). This is because sorbitol induces structural damage of nerves and hence produces neuropathy (Green et al 1985). Not surprisingly, the aldose reductase inhibitor, sorbinil has been shown to enhance axonal transport of glucose (Tomlinson et al 1984) and improve nerve conduction velocity (Yue et al 1987).

Impairment of nerve blood flow is now considered to be an important factor in the pathogenesis of diabetic neuropathy. The endoneural hypoxia is thought to be sufficient enough to cause alterations in neuronal conduction velocity. ET-1 has been shown to cause a significant reduction in blood flow to peripheral sensory nerves (Zochodone et al 1992) in rats, similar to that observed after induction of DM (Cameron et al 1994). The ischemic/hypoxic-induced neuropathy is similar in DM and ET-1-treated animals, suggesting the enhanced local production of ET-1 (or elevated circulating levels of ET-1 that is known to occur in DM (Takahashi K et al 1990)) can contribute to the pathogenesis of diabetic neuropathy. Changes in both afferent and efferent innervation to the bladder in conjunction with alterations in conduction velocity of afferent fibers have
been described in diabetic rats (Steers et al 1994). The sensory nerves are thought to be affected first, leading to impaired sensation of bladder filling (Smith et al 1917, Buck et al 1974, Ellenberg et al 1980). Alteration in bladder emptying in DM is thought to be due to abnormalities in the conduction of afferent Aδ-fibers, which are involved in both the spinal and supraspinal micturition reflexes (Steers et al 1990). These abnormalities correlate well with the structural changes in the ganglia and neurons innervating the bladder (Medori et al 1988). Interestingly, indirect evidence has also come from studies using vasodilator agents, indicating that diabetic neuropathy could also result from a deficit in the blood flow to nerves. Thus, in diabetic rats, vasodilator agents, such as angiotensin II and ET receptor antagonists, corrected defects in nerve conduction velocity (Cameron et al 1996). Furthermore, the NO donor, isosorbide dinitrate also restored conduction and nerve blood flow abnormalities in DM (Cameron et al 1995). These findings suggest that alterations in ET and NO bioactivity may contribute to the pathogenesis of diabetic neuropathy leading to bladder dysfunction.

1.6.2 Autonomic receptor and functional changes
Alterations in the expression and function of various type of receptors has been described in both clinical (Faerman et al 1973, Buck et al 1976) and experimental DM (Lincoln et al 1984,b Kolta et al 1985, Longhurst et al 1986, Moss et al 1987, Luhamish et al 1990b, Morita et al 1991, Nakamura et al 1992). For example, sensitivity to purinergic agonists is high in 8-week diabetic animals and markedly reduced in 16-week diabetic animals (Moss et al 1987). However, the responsiveness of diabetic smooth muscle strips to cholinergic and adrenergic agonists may increase (Kolta et al 1985), decrease (Longhurst
et al 1986) or remains unchanged (Lincoln 1984a) compared to controls. Potentiation of
the cholinergic motor transmission is thought to be secondary to enhanced release and
activity of ACh in the diabetic detrusor (Luheshi et al 1990a, Dail et al 1977, Lincoln et
al 1984a).

1.6.3 Structural changes of the bladder smooth muscle.

Increased urine output in DM causes bladder wall distension. The bladder
responds to distension with a rapid and substantial increase in bladder mass (Lincoln et al
1984b, Uvelius et al 1986, Eika et al 1993) with concomitant alterations in the smooth
muscle contractile responses to various stimuli (Longhurst et al 1986, Santicioli et al
1987, Latifpour et al 1989). In the rat DM model, both hyperplasia (increased in cell
number as a result of increased cell division) as well as hypertrophy (increase in cell size)
is evident in the urinary bladder. An increase in DNA synthesis and [\(^{3}\)H]-thymidine
uptake (Levin et al 1994) has been clearly demonstrated in the DM rat bladder. However,
there are no histological studies available that describe the structural changes in the
diabetic human and rabbit bladder.

Studies of growth factor expression during the early period of bladder
hypertrophy have also proven to be interesting. These have identified changes in the
expression of several growth factors (e.g. heat-shock protein-70, basic fibroblast growth
factor (bFGF) and transforming growth factor-beta (TGF-\(\beta\)), which may play a
significant role in the development of detrusor hypertrophy in response to bladder
distension (Levin et al 1994). Apoptosis and restoration of growth factor expression
accompany regression of bladder hypertrophy and hyperplasia following the reversal of bladder distension (Levin et al 1994). These findings imply that bladder hypertrophy/hyperplasia and apoptosis are directly opposing processes that are controlled by different effects on the gene expression of growth factors (Santarosa et al 1994). In this context, several reports indicate that ET stimulates mitogenesis (Muldoon et al 1989, 1990, Takuwa et al 1989, Walden et al 1998) and that NO enhances apoptosis and inhibits cellular proliferation (Guh et al 1998). Indeed ET-1 is also thought to enhance the mitogenic effects of several growth factors. In addition, the increased production of PGI2, by the STZ-induced diabetic rats is also associated with a significant increase in bladder weights.

Several animal models have been used to investigate the pathophysiology of diabetic bladder dysfunction. These have included dogs (Shishito et al 1964), Chinese hamsters (Dail et al 1977), streptozotocin-(Lincoln et al 1984a,b) or alloxan-induced diabetic rats (Uvelius et al 1986, Paro et al 1990), spontaneous diabetic rats (Marliss et al 1982) and alloxan-induced diabetic rabbits (Gupta et al 1996). Although, the rat has been the most widely used animal model for the study of the effect of DM on bladder function, it suffers from severe metabolic effects of starvation leading to marked growth retardation and loss of weight. Furthermore, in the rat model there is marked hypertrophy of the bladder, which at two months after induction of DM is three to five times greater in diabetic animals compared to age-matched controls (Lincoln et al 1984b, Miller et al 1994). Since, clinical DM is not associated with such extremes the validity of the rat model for the investigation of the effects of DM on the urinary bladder is debatable. In the rabbit model, however, there is only a modest decrease in body weight and minimal
hypertrophy of the urinary bladder. Furthermore, unlike the rat, which suffers from tissue availability, the bladder and urethra of the rabbit are large enough to carry out both receptor and functional analysis in the same group of animals. Finally, due to the similarity of distribution and function of most autonomic receptors in the rabbit and human, we selected the rabbit model to investigate the role of ET, NO and PGs in experimental DM.
2.1. METHODS

2.1.1 Animals, diet and animal weights

Experiments presented in this thesis were performed on 6 month old male NZW rabbits (3 – 4.5 kg).

Thirty-six rabbits were used in two groups:

In the first group 18 rabbits were used for receptor and organ bath studies: 12 rabbits were injected with alloxan and 6 rabbits acted as controls. Of those who received alloxan, one died from ketoacidosis and three were resistant to alloxan. These four animals were not included as diabetic animals. The three alloxan-treated rabbits that did not develop diabetes were used as an additional category of controls.

In the second group 18 rabbits were used for biochemical and tissue culture experiments: 12 rabbits were injected with alloxan and the remaining 6 acted as controls. Of those who received alloxan, two died (one from ketoacidosis and the other from severe hypoglycemia) and four were resistant to alloxan. These six animals were not included as diabetic animals. The four alloxan-treated rabbits that did not develop diabetes were used as an additional category of controls. All animals were housed individually in a temperature-controlled (25°C) room, with a 12 h dark/ 12 h light cycle. Animals were maintained with standard plain diet (Whitam, UK) and given fresh tap water ad libitum.

2.1.2 Alloxan administration and validation of diabetic state

DM was induced by a single intravenous injection of alloxan monohydrate 65 mg/kg in 5 ml vehicle (0.9% NaCl) into an ear vein. Control animals were injected with the vehicle
alone. The alloxan-injected rabbits required subcutaneous injection (scruff of neck) of 10 ml of 50% dextrose, at 6 hourly intervals for the first 24 h, to counteract the hypoglycaemia resulting from alloxan-induced pancreas β cell necrosis. Thereafter they were given free access to food and water ad libitum.

Confirmation of the severity of DM was obtained by testing for glycosuria (Multistix, Ames division, Miles Laboratories Ltd, Stoke Poges, Berks, UK) and measurement of serum glucose (Hitachi 747 Automatic Autoanalyser, Boehringer Mannheim, Lewes, Sussex, UK).

Rabbits were classified as diabetic if their serum glucose concentrations were 16 mmol/l or greater throughout the study. Alloxan-treated rabbits with glucose concentrations less than 10 mmol/l were considered nondiabetic. These alloxan-treated nondiabetic rabbits (n = 6) were used as a second control group along with vehicle rabbits.

2.1.3 Blood collection and analysis of samples

Blood was taken prior to alloxan administration, 5 days after alloxan and at monthly intervals thereafter. The blood was taken from an ear vein (5 ml) or from the central ear artery (5 ml) and placed into serum gel bottles for urea and electrolytes, glucose, lipids and liver function tests and K⁺-ethylenediamine tetra-acetic acid bottles for haematological analysis.

Serum concentrations of urea and electrolytes, bicarbonate (HCO₃⁻) phosphate (PO₄²⁻), magnesium (Mg²⁺), Ca²⁺, creatinine, glucose, urate, amylase, triglycerides, creatine kinase, lactate dehydrogenase, liver function tests and haematological indices were determined using standard methodology.
2.1.4. Preparation of tissues

Following cervical dislocation, urinary bladders (divided at the level of the ureteric orifice into detrusor and bladder neck) and urethrae were excised from rabbits. Part of the detrusor (taken from the dome), bladder neck (taken approximately 1 cm below the ureteric orifice) and proximal urethra (taken below the striated external sphincter) were used for the experiments.

Part of the detrusor, bladder neck and proximal urethra were stored immediately at -70°C in air tight containers and the rest stored in cold Tyrode's buffer solution (see Tyrode's composition) and taken directly to the laboratory for in vitro organ bath, biochemical and tissue culture studies. The tissues stored at -70°C were then mounted in AMES OCT embedding compound (BDH Laboratory Supplies, Poole, UK). Transverse 10μm sections were cut in a cryostat (Bright Instruments Co. Ltd, Huntingdon, UK) at approximately -20°C and thaw mounted onto gelatinised microscope slides. These sections were used for autoradiographic and histochemical experiments. The same procedure was performed on six age matched, healthy control NZW rabbits and alloxan induced non-diabetic rabbits.

2.1.5 Materials

The following drugs and other materials were supplied by Sigma Chemical Co. (Poole, Dorset UK): L-arginine, TTX, atropine sulphate, L-NOARG, α,β, methylene ATP, ACh, antisera against 6-oxo-prostaglandin F₁₀, arachidonic acid (sodium salt), Dulbecco's
minimum essential medium (DMEM), forskolin, gelatin, isobutylmethylxanthine, Norit activated charcoal, phorbol ester dibutyrate, 6-oxo-prostaglandin F$_{1\alpha}$ and SNP.

Fetal calf serum (FCS), L-glutamine, penicillin G, streptomycin sulphate, trypsin, ethylenediamine tetra-acetic acid were supplied by Gibco (Paisley, UK).

ET-1, IRL1620, BQ123, BQ788, PD156707 and L-arginine were obtained from Bachem Fine Chemicals, Switzerland.

The following radiochemicals, kits and other materials were obtained from Amersham Radiochemicals (Amersham International, Aylesbury, Bucks, UK): $[^{125}\text{I}]$-endothelin-1, $[^{125}\text{I}]$-PD151242, $[^{125}\text{I}]$-BQ3020, $[^{3}\text{H}]$-L-$\text{N}^\text{G}$-nitroarginine, $[^{3}\text{H}]$-prostaglandin E$_2$, $[^{3}\text{H}]$-6-oxo-prostaglandin F$_{1\alpha}$, $[^{125}\text{I}]$-cAMP and $[^{125}\text{I}]$-cGMP radioimmunoassay kits. $^{125}\text{I}$ and $^{3}\text{H}$ microscales, Hyperfilm 3H, LM-1 nuclear emulsion

The Tyrode solution used had the following composition (mM) NaCl 118, KCl 4.0, NaHCO$_3$ 24.0, NaH$_2$PO$_4$ 0.4, MgCl$_2$ 1.0, CaCl$_2$ 1.8, Glucose 6.1, Na Pyruvate 5.0. The Tyrode solution was gassed with 95% O$_2$ and 5% carbon dioxide (CO$_2$). The Krebs transporting solution had a composition (mM) of NaCl 115, NaHCO$_3$ 24.5, KCl 4.0, NaH$_2$PO$_4$ 0.5, CaCl$_2$ 0.7.
Microtitre tissue culture plates (Falcon, Becton Dickinson, Oxford, UK). 5-bromo-2' deoxy-uracil (BrdU), phosphate buffered saline (PBS) an enzyme-linked immunosorbant assay plate-reader were obtained from Metertech, Watford, UK.

2.1.5. **Statistical analysis**

The results are expressed as mean and standard error of the mean (s.e.m.). Data was analysed using ANOVA for multiple comparisons. Paired comparisons between two groups were performed using paired Student’s t tests where ANOVA indicated significance for the multiple comparison. Statistical significance was accepted when $p < 0.05$. 
2.2 RESULTS

The methodology involved in setting up this experimental diabetic rabbit model had a high success rate with 84% of the rabbits responding to alloxan. Mortality secondary to the diabetic state was also low (8%); 1 rabbit dying from severe hypoglycaemia and 1 from diabetic ketoacidosis.

The range of several haematological and biochemical variables, at two time intervals was also established in this diabetic rabbit model. The values reported here are very similar to those expected in non-insulin dependent diabetic patients.
Table 1. Starting and finishing weights in the control (CT) and diabetic (DM) groups studied. Results are expressed as means ± s.e.m are given (n = 6 in each study group).

<table>
<thead>
<tr>
<th></th>
<th>Starting</th>
<th>Final</th>
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<tbody>
<tr>
<td></td>
<td>Body weight (kg)</td>
<td></td>
</tr>
<tr>
<td>3 months</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CT</td>
<td>3.0 ± 0.1</td>
<td>4.0 ± 0.2</td>
</tr>
<tr>
<td>DM</td>
<td>3.1 ± 0.1</td>
<td>3.9 ± 0.1</td>
</tr>
<tr>
<td>6 months</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CT</td>
<td>2.9 ± 0.1</td>
<td>4.1 ± 0.1</td>
</tr>
<tr>
<td>DM</td>
<td>3.1 ± 0.1</td>
<td>3.6 ± 0.2*</td>
</tr>
</tbody>
</table>

* p < 0.05 (Student’s t test vs corresponding control group)

There was no significant reduction in body weight in 3 months DM animals when compared to age-matched CT. However, after 6 months of DM there had been a significant decrease in body weight in 6 months DM animals compared to age matched CT. The weight loss at 6 month in the DM animals was compatible with the energy production associated with non-ketotic DM.
Table 2. Changes in haematological variables in control (CT) and diabetic (DM) rabbits. Results are expressed as means ± s.e.m are given (n = 6 in each study group).

The haematological profile was not significantly different between 3 and 6 month DM rabbits and age matched controls, except for a reduction in the mean corpuscular volume in the DM rabbits compared to age-matched controls.

<table>
<thead>
<tr>
<th></th>
<th>3 months</th>
<th></th>
<th>6 months</th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>CT</td>
<td>DM</td>
<td>CT</td>
<td>DM</td>
</tr>
<tr>
<td>Hb (g/dl)</td>
<td>12 ± 0.4</td>
<td>12 ± 0.4</td>
<td>11 ± 0.5</td>
<td>12 ± 0.9</td>
</tr>
<tr>
<td>Hct (%)</td>
<td>44 ± 1.8</td>
<td>41 ± 1.3</td>
<td>38 ± 1.1</td>
<td>40 ± 1.5</td>
</tr>
<tr>
<td>MCV (fl)</td>
<td>74 ± 1.4</td>
<td>68 ± 1.3</td>
<td>67 ± 1.4</td>
<td>65 ± 1.2</td>
</tr>
<tr>
<td>MCH</td>
<td>20 ± 0.4</td>
<td>20 ± 0.3</td>
<td>21 ± 0.6</td>
<td>19 ± 1.0</td>
</tr>
<tr>
<td>MCHC</td>
<td>28 ± 0.4</td>
<td>29 ± 0.7</td>
<td>30 ± 0.1</td>
<td>30 ± 1.2</td>
</tr>
<tr>
<td>RBC (x 10¹²/l)</td>
<td>6 ± 0.3</td>
<td>6 ± 0.2</td>
<td>5 ± 0.1</td>
<td>6 ± 0.2</td>
</tr>
<tr>
<td>WBC ((x 10¹²/l)</td>
<td>8 ± 1.1</td>
<td>8 ± 0.9</td>
<td>7 ± 1.0</td>
<td>10 ± 1.4</td>
</tr>
<tr>
<td>Plt (x 10¹²/l)</td>
<td>385 ± 34</td>
<td>390 ± 46</td>
<td>446 ± 47</td>
<td>539 ± 68</td>
</tr>
</tbody>
</table>

Hb = Haemoglobin, MCHC = mean corpuscular haemoglobin concentration
MCV = mean corpuscular volume, RBC = red blood cells
MCH = mean corpuscular haemoglobin, WBC = white blood cells
Plt = platelet count
Table 3. Changes in the serum urea and electrolytes, bicarbonate \((\text{HCO}_3^-)\) phosphate \((\text{PO}_4^{2-})\), magnesium \((\text{Mg}^{2+})\), \(\text{Ca}^{2+}\), creatinine and glucose of the control (CT) and diabetic (DM) rabbits. Results are expressed as means ± s.e.m are given (n = 6 in each study group).

<table>
<thead>
<tr>
<th></th>
<th>3 Month</th>
<th>6 Month</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CT</td>
<td>DM</td>
</tr>
<tr>
<td>(\text{Na}^+) (mmol/l)</td>
<td>145.3 ± 0.8</td>
<td>135.2 ± 1.3*</td>
</tr>
<tr>
<td>(\text{K}^+) (mmol/l)</td>
<td>4.5 ± 0.1</td>
<td>5.4 ± 0.2*</td>
</tr>
<tr>
<td>Urea (mmol/l)</td>
<td>6.3 ± 0.1</td>
<td>10.5 ± 0.2*</td>
</tr>
<tr>
<td>(\text{HCO}_3^-) (mmol/l)</td>
<td>25.0 ± 1.4</td>
<td>27.2 ± 1.0</td>
</tr>
<tr>
<td>(\text{PO}_4^{2-}) (mmol/l)</td>
<td>1.5 ± 0.1</td>
<td>1.4 ± 0.1</td>
</tr>
<tr>
<td>(\text{Mg}^{2+}) (mmol/l)</td>
<td>0.8 ± 0.02</td>
<td>0.8 ± 0.05</td>
</tr>
<tr>
<td>(\text{Ca}^{2+}) (mmol/l)</td>
<td>3.7 ± 0.1</td>
<td>3.6 ± 0.1</td>
</tr>
<tr>
<td>Creatinine (µmol/l)</td>
<td>89.0 ± 3.0</td>
<td>118.0 ± 3.0*</td>
</tr>
<tr>
<td>Glucose (mmol/l)</td>
<td>7.6 ± 0.3</td>
<td>33.4 ± 2.1**</td>
</tr>
</tbody>
</table>

* \(p < 0.05\), ** \(p < 0.001\) (Student's t test vs corresponding control group)
Table 4. Changes in the serum urate, amylase and liver function tests of control (CT) and diabetic (DM) rabbits. Results are expressed as means ± s.e.m are given (n = 6 in each study group).

<table>
<thead>
<tr>
<th></th>
<th>3 month</th>
<th>6 month</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CT</td>
<td>DM</td>
</tr>
<tr>
<td>Urate (mmol/l)</td>
<td>0.01 ± 0</td>
<td>0.1 ± 0</td>
</tr>
<tr>
<td>Amylase (mmol/l)</td>
<td>890 ± 82</td>
<td>921 ± 84</td>
</tr>
<tr>
<td>Albumin (g/l)</td>
<td>43.3 ± 0.7</td>
<td>41.2 ± 0.7</td>
</tr>
<tr>
<td>TP (g/l)</td>
<td>63 ± 1</td>
<td>59 ± 1</td>
</tr>
<tr>
<td>Tbil (µmol/l)</td>
<td>5 ± 1</td>
<td>8 ± 1</td>
</tr>
<tr>
<td>AST (U/l)</td>
<td>18 ± 4</td>
<td>*126 ± 38</td>
</tr>
<tr>
<td>ALT (U/l)</td>
<td>43 ± 6</td>
<td>98 ± 24</td>
</tr>
<tr>
<td>ALP (U/l)</td>
<td>68 ± 11</td>
<td>61 ± 5</td>
</tr>
<tr>
<td>GGT (U/l)</td>
<td>8 ± 1</td>
<td>16 ± 2</td>
</tr>
</tbody>
</table>

TP = total protein, ALT = alanine transaminase
Tbil = total bilirubin, ALP = alkaline phosphatase
AST = aspartate transaminase, GGT = gamma glutamyl transferase
* p < 0.001 (Student’s t test vs corresponding control group)
A number of biochemical abnormalities were seen in the diabetic groups, with the more significant change occurring after 3 months. The fall in serum Na\(^{2+}\) and rise in serum K\(^+\) concentration has been attributed to hyporenin-hypoaldosteronism occurring in DMs with mild renal insufficiency (D’Elia et al 1985). This electrolyte pattern was observed in our diabetic rabbits after 3 months, though the K\(^+\) had returned to control levels at 6 months of DM.

The increase in serum urea is probably a consequence of impaired renal function and catabolic state, since deterioration in renal function is a well-documented feature of DM (D’Elia et al 1985). The occurrence of this phenomenon in our experimental model is supported by the observed increase in serum creatinine concentration.

Serum glucose concentrations were significantly (p < 0.001) higher in both 3 and 6 month’s diabetic rabbits, although the levels were slightly lower in the 6-month diabetic rabbits. Increased sensitivity to insulin has been documented in man when renal function deteriorates (Weinrauch et al 1978). A similar phenomenon may be occurring in the 6-month diabetic rabbit.

Serum AST activity was significantly (p < 0.001) elevated in the 3 and 6 month diabetic rabbits. These changes were paralleled by an increase, though not significant, in ALT activity. Elevations of AST and ALT have also been documented in diabetic patients (Averbuch et al 1991, Bell et al 1988, Maxwell et al 1986). The reason for these rises is not clear, although increases in liver enzymes has previously been attributed to elevated plasma glucose concentrations (Averbuch et al 1991)
Table 5. Changes in serum lipid, creatine kinase and lactate dehydrogenase values in 3 and 6 month control (CT) and diabetic (DM) groups. Results are expressed as means ± s.e.m are given (n = 6 in each study group).

<table>
<thead>
<tr>
<th></th>
<th>3 Month</th>
<th></th>
<th>6 Month</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CT</td>
<td>DM</td>
<td>CT</td>
<td>DM</td>
</tr>
<tr>
<td>Triglyceride (mmol/l)</td>
<td>0.9 ± 0.1</td>
<td>1.2 ± 0.1</td>
<td>0.8 ± 0.1</td>
<td>0.9 ± 0.1</td>
</tr>
<tr>
<td>Cholesterol (mmol/l)</td>
<td>0.9 ± 0.2</td>
<td>0.7 ± 0.1</td>
<td>1.3 ± 0.3</td>
<td>0.9 ± 0.1</td>
</tr>
<tr>
<td>Creatine kinase (U/l)</td>
<td>498 ± 116</td>
<td>504 ± 143</td>
<td>343 ± 30</td>
<td>263 ± 33</td>
</tr>
<tr>
<td>LDH (U/l)</td>
<td>146 ± 17</td>
<td>176 ± 23</td>
<td>132 ± 24</td>
<td>132 ± 23</td>
</tr>
</tbody>
</table>

LDH = lactate dehydrogenase

Lipids, creatine kinase and lactate dehydrogenase (Table 5)

No significant changes were seen in the cholesterol, creatine kinase activity and lactate dehydrogenase activity in the 3 and 6 month diabetic rabbits when compared to age-matched controls. Thus, hypercholesterolaemia, which can adversely influence detrusor function, is unlikely to have a pathophysiological role in our study. Serum triglycerides were increased, but not significantly, in the 3 and 6 month diabetic rabbits when compared to age-matched controls. These differences mimic to some extent the well-established pattern of hypertriglyceridaemia in clinical DM (Elkeles 1991).
LOCALISATION OF $\text{ET}_A$ ($[^{125}\text{I}]\text{PD151242}$), $\text{ET}_B$ ($[^{125}\text{I}]\text{BQ3020}$) BINDING SITES
AND PHARMACOLOGICAL CHARACTERISATION OF ENDOTHELIN
RECEPTORS IN THE RABBIT LOWER URINARY TRACT: EFFECT OF
EXPERIMENTAL DIABETES
3.1 INTRODUCTION

The extensive distribution of ET-1 synthesis in the urinary bladder, occurring in almost all cell types, suggests that this peptide could play a role in bladder wall modeling, the control of bladder smooth muscle tone and the regulation of local blood flow (Saenz de Tejada et al 1992). To date two ET receptor subtypes, ET\textsubscript{A} and ET\textsubscript{B}, have been characterised and cloned from bovine (Arai et al 1990) and rat lung (Sakurai et al 1992), respectively. These receptors have been demonstrated in the rabbit lower urinary tract using SDS polyacrylamide gel electrophoresis and characterised based upon differential binding affinities to sarafotoxin 6c (S6c) and a cyclic pentapeptide, BQ123, at \(^{125}\text{I}-\text{ET-1}\) binding sites (Traish et al 1992, Latifpour et al 1995). Regional differences in the density and distribution of ET receptors in the rabbit urinary tract were identified, suggesting that the specific functions of ET-1 depend on the region of the urinary tract. However, the exact function of these receptor subtypes is unclear. In the vascular bed, ET\textsubscript{A} receptors mediate vasoconstriction and cellular proliferation, whereas ET\textsubscript{B} receptors, predominantly found on endothelial cells, mediate vasodilatation via the production of NO and prostacyclin (PGI\textsubscript{2}) (although ET\textsubscript{B}-mediated vasoconstriction is also well established) (Clozel et al 1992).

The ETs exert their effects on ET\textsubscript{A} and ET\textsubscript{B} receptors via several second messenger systems, among which the phosphatidyl inositol-specific phospholipase C activation via various G proteins seems to play a major role. Both second messengers inositol 1,3,5-triphosphate and diacylglycerol are responsible for transmitting the signal via an increase in intracellular Ca\textsuperscript{2+}. ETs are also thought to cause an accumulation of cGMP in the cell by activating the Ca\textsuperscript{2+}-dependent NOS through the activation of the ET\textsubscript{B}
receptors. This pathway is considered to be ultimately responsible for the smooth muscle relaxant effect. The intracellular mechanisms of signal transduction triggered by the interaction between ETs and their receptors continue to be under intensive scientific investigation.

We investigated if ET receptor subtypes are altered numerically or functionally in the diabetic urinary bladder. Using in vitro ligand-based autoradiography, the effect of DM on the density and distribution of ET receptor subtypes (ET\textsubscript{A} and ET\textsubscript{B}) in the rabbit urinary bladder was determined. The functional properties of these ET receptors were also characterised using isometric tension studies.
3.2 MATERIALS AND METHODS

3.2.1 Saturation binding analysis

Preliminary binding studies (saturation analysis) were performed, where consecutive 10 μm transverse sections were preincubated in 50 mM tris hydrochloric acid (HCl) buffer, pH 7.4, for 15 min at 22°C in order to reduce endogenous peptide levels. Incubations were then carried out (2 h at 22°C) in buffer containing 5 mM MgCl₂, 1% bovine serum albumin and 100 kiu/ml aprotonin in the presence of 0.003-1 nM [¹²⁵I]-ET-1 (Amersham International, Amersham, UK). ETₐ and ETₐ binding sites were identified using the selective radioligands [¹²⁵I] PD151242 (ETₐ) (Davenport et al 1994) and [¹²⁵I] BQ3020 (ETₐ) (Molenaar et al 1992) (Amersham International). Non-specific binding was established by incubating adjacent sections in the presence of 1μM unlabelled ET-1 (Bachem Fine Chemicals, Switzerland). Sections were then washed (two times) and binding determined by wiping sections from microscope slides and measuring the ¹²⁵I bound using a gamma counter. Receptor density (B_max) and affinity (K_D) were then calculated using GraphPad Inplot Software (Graph Pad, San Diego, California, USA).

3.2.3 Quantitative assessment of ¹²⁵I-ET-1, ¹²⁵I-PD151242 (ETₐ) and ¹²⁵I-BQ3020 (ETₐ) receptors.

Consecutive 10 μm sections from the detrusor and bladder neck were preincubated, as described above, and then incubated for 120 min, at 22°C, in buffer containing a fixed concentration of 0.15 nM [¹²⁵I]-ET-1, [¹²⁵I]-PD151242 and [¹²⁵I]-BQ3020 (concentration at the approximate K_D values established from the saturation studies described above).
The degree of non-specific binding was established by incubating alternate sections in the presence of 1 µM unlabelled ET-1. The slides were washed twice in buffer for 10 min, dipped in 4°C distilled water and then dried in a stream of cold air. Low resolution autoradiography was carried out by exposing the sections to Hyperfilm 3H (Amersham International) in X-ray cassettes for 1-3 days. Photodensitometric analysis was performed using a Bio-Rad GS-700 imaging densitometer (Bio-Rad Laboratories, Hemel Hempstead, UK). Data was analysed using Molecular Analyst software. Binding was expressed in terms of radioligand bound (disintegrations per min; dpm) per unit area (mm²), calculated from standard curves generated by ¹²⁵I microscales (Amersham International) that were co-exposed with tissue sections. Microscopic localisation (high-resolution autoradiography) of binding sites was performed by post-fixing tissue in paraformaldehyde vapour (2h at 80°C) and coating slides in molten nuclear emulsion (LM-1, Amersham International). Slides were then stored in light proof boxes for up to 8 days at 4°C, after which they were processed in D19 high contrast developer (Kodak, UK) and fixed (Hypam, Ilford, UK). Underlying tissue was stained with haematoxylin and eosin and high-resolution autoradiographs were viewed on an Olympus Vanox microscope; selected sections were photographed under dark field and bright field illumination, where appropriate.
3.2.3 In-vitro organ bath studies

Detrusor strips were taken from the anterior wall of the dome, and bladder neck strips were taken just below the trigone in an oblique direction from the internal urethral orifice towards one of the orifices of the ureters. The strips measured 1 x 1 x 5mm.

Contractile studies were performed on the same day that the tissue was obtained. The strips were mounted vertically in 1.5ml organ baths, containing Tyrode’s solution maintained at 37°C by a thermoregulated circuit. The Tyrode solution was bubbled with a mixture of 95% O₂ and 5% CO₂, maintaining pH at 7.4. An initial tension of 2g (detrusor) and 1g (bladder neck) was applied to the suspended tissue strips. The tension was recorded with a force displacement transducer (FT-03, Grass Instruments, Quincy, Massachusetts, USA) on a Grass Polygraph (model 7D). All strips were equilibrated for at least 30 min. At the end of the equilibration period, the strips were challenged with KCl (124 mM). Two reproducible contractions varying in magnitude by less than 10% were consistently obtained.

Cumulative response curves (CRCs) were recorded (0.5 log unit steps, one curve for each smooth muscle strip). CRCs were performed for ET-1 and IRL 1620 (a selective ET_b agonist) on detrusor and bladder neck from both control and diabetic rabbits.

Further CRCs to ET-1 were constructed in the presence of ET_a (BQ123) and ET_b (BQ788) selective antagonists to identify the receptor mediating the contractile responses.
3.2.4 Statistical Analysis

Animal weights and blood glucose concentrations are shown as median and range. The results of radioligand binding were expressed as \text{d.p.m./mm}^2 (median and range). Statistical analysis was by the two-tailed Mann Whitney U test (unpaired values). \( P < 0.05 \) was accepted as significant. \( N_A \) denotes the number of animals and \( N_{SM} \) the number of smooth muscle strips.

The effects of ET-1 and IRL1620 are expressed in millinewtons (mN). The contractile responses to \( 10^{-6}\text{M} \) of ET-1 and the mean \( EC_{50} \) (concentration of ET-1 eliciting half the contractile response) values were estimated by regression analysis and compared by an unpaired t test. Results are expressed as mean and s.e.m. ANOVA was used to compare CRCs. \( p < 0.05 \) was accepted as significant.
3.3 RESULTS

(A) Autoradiography

Receptor binding studies confirmed that $^{125}$I-ET-1, $^{125}$I-PD151242 and $^{125}$I-BQ3020 all bound in a concentration-dependent fashion to detrusor and bladder neck sections. Saturation analysis showed that binding was to high affinity sites with $K_D$ values in the subnanomolar range ($^{125}$I-ET-1 = 0.11nM, $^{125}$I-PD151242 = 0.071nM, and $^{125}$I-BQ3020 = 0.091 nM). Fixed concentrations of 0.15nM (based on these $K_D$ values) were used for subsequent experiments.

There was dense $[^{125}\text{I}]-\text{PD151242}$ (ET$_a$) and $[^{125}\text{I}]-\text{BQ3020}$ (ET$_b$) binding to all sections of the detrusor (fig 1) and bladder neck (fig 2) in control and diabetic animals. The nonspecific binding was undetectable in the presence of 1μM unlabeled ET-1 for $[^{125}\text{I}]$PD151242 and was reduced by approximately 90% for BQ3020 (ET$_b$).

High-resolution autoradiographs revealed that ET receptor binding sites were associated with smooth muscle and urothelial cells in both the detrusor and bladder neck (fig 3 & 4). ET$_a$ receptor binding sites were also identified on the blood vessels within the bladder (fig 5).

The results of densitometric analysis of film images are presented in table 6.

Regional differences in ET receptor distribution

In both the control and diabetic tissue sections, the density of ET$_a$ receptors was significantly greater in the detrusor compared to the bladder neck. There were no
differences in the density of ET\textsubscript{B} receptor binding sites between the detrusor and bladder neck of both control and diabetic animals.

**DM-induced differences in ET receptors**

There was a significant increase in ET\textsubscript{B} receptor density in the diabetic detrusor and bladder neck sections compared to controls (fig 4). This increase in the density of ET\textsubscript{B} receptor binding sites was also reflected in a significant increase in ET-1 binding sites in the diabetic detrusor and bladder neck compared to controls (table 6).

No significant changes were observed in the density of ET\textsubscript{A} receptor binding sites between control and diabetic animals of either the detrusor or bladder neck.

The high resolution autoradiographs indicated that the increase in ET\textsubscript{B} receptor density occurred in both the urothelium and smooth muscle. A representative sections from the control and diabetic bladder neck is shown in fig 4.

**\textit{(B) In vitro organ bath studies}**

There were no significant differences in the weights and lengths of smooth muscle strips used from either control or diabetic animals or between detrusor and bladder neck. The results of smooth muscle contractile responses to ET-1 and IRL1620 in the control and diabetic detrusor and bladder neck are presented in table 7.

**Regional differences.**

CRCs to ET-1 in the control detrusor and bladder neck smooth muscle strips are presented in figure 6. ET-1 elicited a potent long-lasting contractile response in the detrusor and bladder neck.
The smooth muscle contractile responses to ET-1 in the detrusor were significantly greater than the bladder neck.

CRCs to ET-1 in the presence of ET\textsubscript{A} and ET\textsubscript{B} antagonists in the detrusor are shown in figure 7. The ET-1-mediated contractile responses were significantly inhibited in a competitive fashion by the ET\textsubscript{A} antagonist BQ123 but not by the ET\textsubscript{B} antagonist (BQ788). The contractile responses to ET-1 at 10\textsuperscript{-6}M were not significantly enhanced in the presence of BQ123.

The ET\textsubscript{B} selective agonist (IRL1620) had very little effect on the tone of either detrusor or bladder neck smooth muscle strips even at the highest concentration used (10\textsuperscript{-6}M) (table 7).

**Diabetes-associated differences**

CRCs to ET-1 in control and diabetic detrusor and bladder neck are shown in figures 8 & 9, respectively.

The mean contractile response to ET-1 at 10\textsuperscript{-6}M and its EC\textsubscript{50} values in the detrusor were not significantly different between control and diabetic animals (table 2). In contrast, mean contractile response to ET-1 at 10\textsuperscript{-6}M was significantly decreased and its EC\textsubscript{50} value was significantly increased in the diabetic bladder neck compared to control (table 7).

No significant changes were identified in the smooth muscle responses to IRL1620 (ET\textsubscript{B} agonist) in the detrusor and bladder neck.
Figure 1.
Low-resolution images generated from Hyperfilm $^3$H autoradiographs of $\text{ET}_A$ and $\text{ET}_B$ radioligand binding sites in the control (left panel) and diabetic (right panel) detrusor. Binding is evident as dark grain accumulation on a light background. The $\text{ET}_B$ radioligand binding is significantly increased in the diabetic detrusor compared to control. Bar = 2mm.
Figure 2.
Low-resolution images generated from Hyperfilm \(^3\)H autoradiographs of ET\(_A\) and ET\(_B\) radioligand binding sites in the control (left panel) and diabetic (right panel) bladder neck. Binding is evident as dark grain accumulation on a light background. The ET\(_B\) receptor binding is significantly increased in the diabetic bladder neck compared to control. Bar = 2mm.
Figure 3.
High-resolution images generated on nuclear emulsion. Left panel - Dark field illumination autoradiographs where $ET_A$ receptor binding sites in the control detrusor are evident as white grains on a black background. Right panel – Haematoxylin and eosin stained tissue-underlying autoradiograph. Urothelial (UR) and smooth muscle (SM). Bar $= 100\mu$m.
Figure 4.

High-resolution images generated on nuclear emulsion identifying ET$_B$ receptor binding sites in the control (CT) and diabetic (DM) bladder neck. ET$_B$ receptor binding sites evident as white grains on a black background appear denser in the urothelium (UR) and smooth muscle (SM) in the DM compared to CT section. The adjacent photograph is the haematoxylin and eosin stained tissue-underlying autoradiograph. Bar = 100μm.
Figure 5.

High-resolution autoradiograph of ETₐ receptor binding to the blood vessels within the detrusor. ETₐ receptor binding sites evident as white grains on a black background Bar = 200µm. Blood vessel (BV) and smooth muscle (SM)
CRC to ET-1 in the control (CT) detrusor ($N_{SM} = 12$) and bladder neck ($N_{SM} = 12$) smooth muscle. Tension is expressed in mN. Each point represents the mean ± s.e.m.

* $p < 0.01$ for detrusor vs bladder neck smooth muscle strips by ANOVA.
Figure 7.

CRC to ET-1 in the control detrusor ($N_{SM} = 12$) in the presence of BQ123 and BQ788.

Tension is expressed in mN. Each point represents the mean ± s.e.m.

* $p < 0.01$ for ET-1 alone vs ET-1 with BQ123 ($E_{T_A}$ receptor antagonist) in detrusor smooth muscle strips by ANOVA.
Figure 8.

CRC to ET-1 in the control (CT) ($N_{SM} = 12$) and diabetic (DM) ($N_{SM} = 12$) detrusor.

Tension is expressed in mN. Each point represents the mean ± s.e.m.
Figure 9.

CRC to ET-1 in the control (CT) ($N_{SM} = 12$) and diabetic (DM) ($N_{SM} = 12$) bladder neck. Tension is expressed in mN. Each point represents the mean ± s.e.m.

* $p < 0.01$ for CT vs DM bladder neck smooth muscle strips by ANOVA.
Table 6. Photodensitometric analysis of regional $[^{125}\text{I}]$ET-1, $[^{125}\text{I}]$PD151242 (ET$_A$ ligand) and $[^{125}\text{I}]$BQ3020 (ET$_B$ ligand) binding in the control (CT), diabetic (DM) and alloxan-non DM (A-NDM) rabbit urinary bladder. Receptor binding was measured as dpm x 1000/mm$^2$ and values are expressed as median and (range).

<table>
<thead>
<tr>
<th></th>
<th>ET-1</th>
<th>ET$_A$ ligand</th>
<th>ET$_B$ ligand</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Detrusor</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CT</td>
<td>98 (69-174)*</td>
<td>70 (43-79)</td>
<td>34 (10-49)</td>
</tr>
<tr>
<td>A-NDM</td>
<td>85 (74-126)</td>
<td>64 (38-66)</td>
<td>30 (16-44)</td>
</tr>
<tr>
<td>DM</td>
<td>166 (140-226)**</td>
<td>66 (57-89)</td>
<td>48 (39-69)#</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Bladder neck</strong></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>CT</td>
<td>73 (50-89)</td>
<td>19 (12-39)</td>
<td>25 (15-28)</td>
</tr>
<tr>
<td>A-NDM</td>
<td>68 (43-78)</td>
<td>16 (11-34)</td>
<td>22 (12-26)</td>
</tr>
<tr>
<td>DM</td>
<td>119 (78-203)</td>
<td>22 (7-36)</td>
<td>44 (28-93)##</td>
</tr>
</tbody>
</table>

Statistical analysis using the Mann-Whitney U test identified significant regional and DM-associated differences.

**Regional differences:**
*CT detrusor ET$_A$ vs CT bladder neck ET$_A$: p < 0.001
**DM detrusor ET$_A$ vs DM bladder neck ET$_A$: p < 0.001

**DM-associated differences:**
#CT detrusor ET$_B$ vs DM detrusor ET$_B$: p < 0.02
##CT bladder neck ET$_B$ vs DM bladder neck ET$_B$: p < 0.002
Table 7. The contractile properties of ET-1 and IRL1620 (ET<sub>B</sub> receptor agonist) in control (CT) and diabetic (DM). Values are expressed as mean and s.e.m.

<table>
<thead>
<tr>
<th></th>
<th>Detrusor</th>
<th>Bladder neck</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ET-1</td>
<td>IRL1620</td>
</tr>
<tr>
<td><strong>CT</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Response</td>
<td>39.8 (8.2) *</td>
<td>1.0 (0.5)</td>
</tr>
<tr>
<td>EC&lt;sub&gt;50&lt;/sub&gt; (nmol/L)</td>
<td>42.3 (37.0)</td>
<td>-</td>
</tr>
</tbody>
</table>

| **DM** |          |              |           |            |
| Response | 47.8 (3.9) ** | 1.0 (0.5) | 13.2 (1.04) | 1.0 (0.5) |
| EC<sub>50</sub> (nmol/L) | 28.3 (7.19) | - | 29.8 (1.70) | |

Tension is expressed as mean ± s.e.m. in millinewtons (mN).

EC<sub>50</sub> is expressed in molar concentrations and relates to the responses to 10^-6 of ET-1.

Statistical analysis: Mann-Whitney U test

ET-1 responses: *CT Detrusor vs CT bladder neck: p < 0.001

**DM Detrusor vs DM bladder neck: p < 0.001

### CT bladder neck vs DM bladder neck: p < 0.001
3.4 DISCUSSION

This study demonstrates that in both control and diabetic animals, the density of ET_A receptor binding sites was significantly greater in the detrusor compared to the bladder neck. Also the contractile responses to ET-1 were significantly greater in the detrusor compared to the bladder neck of both control and diabetic animals. The contractile responses to ET-1 are largely ET_A receptor-mediated since the ET_A selective receptor antagonist (BQ123) caused a competitive inhibition (Eguchi et al 1992) of contractile responses to ET-1 while the ET_B antagonist (BQ788) failed to inhibit these responses. Furthermore, the ET_B selective receptor agonist (IRL1620) did not elicit contractions, suggesting that ET_B receptor activation does not initiate contraction.

The findings of a significant increase in ET_B receptor binding sites in the diabetic urinary bladder are not clear, but are certainly provocative. These ET_B changes were not due to a toxic effect of alloxan since no differences in receptor expression were identified (at six months) in animals given alloxan who did not develop DM (table-6). The possible mechanisms contributing to a significant decrease in the contractile responses to ET-1 in the diabetic bladder neck associated with an increase in the ET_B receptor density certainly merits further investigation. Since ET_B receptors have been implicated in the release of endogenous NO (Clozel et al 1992), the activation of ET_B receptors may oppose ET-1 contractile responses in the bladder neck as NO is thought to play a prominent role in bladder neck smooth muscle relaxation (Persson et al 1994). Whether the increased intracellular Ca^{2+} levels observed in DM (Levy J et al 1993) contribute to NO production (via activation of Ca^{2+}-dependent NOS) remains unclear (Razmjouei et al 1997).
In contrast to the bladder neck, the contractile responses to ET-1 were not decreased in the diabetic detrusor compared to controls, despite a similar increase in ET\(_B\) receptor binding sites. This difference in ET-1-induced contractile responses between diabetic detrusor and bladder neck may be associated with the lack of responsiveness of detrusor smooth muscle to NO (Persson et al 1994).

Experimental DM may also precipitate a nonspecific decrease in the responsiveness of bladder neck smooth muscle to vasoactive agents. Alterations in responses to ET-1 and NOS inhibitors have been reported in the cutaneous microcirculation of diabetic rats (Kiff et al 1991). We have also shown that despite an increase in NOS in the diabetic bladder neck/urethra, NO-mediated smooth muscle relaxation was impaired (Chapter 4). It is therefore possible that an increase in ET\(_B\) receptors and NOS in the diabetic urinary bladder neck could represent an attempted compensatory response to altered NO bioactivity. Overproduction of NO, though beneficial in many systems, could also be potentially toxic due to the increased production of free radicals (Ceriello et al 1991, Nussler et al 1993). It is conceivable, therefore, that a similar increase in free radicals in the diabetic urinary bladder can cause cytotoxic damage and thus alter bladder function. Alterations in the synthesis of another potent vasodilator, PGI\(_2\), have been well documented in several diabetic tissues including the urinary bladder (Weisbrod et al 1993, Jeremy et al 1986 & 1987, Mikhailidis et al 1987). Hence, the interactions between NO, PGI\(_2\) and ET, which are thought to be important in maintaining vascular tone (Luscher et al 1993), may also contribute to the regulation of urinary tract smooth muscle tone. The precise relationship between NO,
PGI₂ and ET-1 in bladder function, however, has not been elucidated. We are currently investigating this relationship in the pathogenesis of diabetic cystopathy.

ET may also influence other aspects of bladder function. For example, ET is synthesised by SMC and it stimulates mitogenesis (Bobik et al 1990, Clozel et al 1992). Therefore, the DM-associated detrusor smooth muscle hyperplasia may be related to the significant increase in ET₉ receptor density. The proliferation of vascular SMC in response to ET-1 has been shown to correlate with ET-receptor density (Kanse et al 1995). Both ET₉ and ET₉ receptors have been implicated in this response. For example, ET₉ receptors participate in the development of intimal hyperplasia after endothelial injury (Azuma et al 1995) while human airway smooth muscle cell proliferation is ET₉ receptor-dependent (Panettieri et al 1996). ET-1 is also reported to enhance detrusor cholinergic and NANC responses (Saenz de Tejada et al 1992, Donso et al 1994), presumably by autocrine (release of ET-1 by SMC) or by paracrine mechanisms. Thus, alterations in circulating ET levels in DM (Takahashi et al 1990) and expression of ET receptors in the diabetic urinary bladder may contribute to changes in bladder smooth muscle contractility. Furthermore, the dense ET₉ binding to the blood vessels within the bladder may also be involved in the control of the blood supply to the detrusor and hence its function. In this context, it is of interest to note that, bladder vascular insufficiency has recently been shown to alter bladder smooth muscle contractile responses to ET-1 (Azadzoi et al 1997).

In conclusion, this study has demonstrated that alloxan-induced DM in the rabbit is associated with a significant increase in the density of ET₉ receptors in the urinary bladder and a significant decrease in the contractile responses to ET-1 in the bladder.
neck. These findings may contribute to the pathophysiologic changes in the bladder, which are associated with DM. The manipulation of ET\textsubscript{B} receptor-mediated responses may be of benefit in the treatment of bladder dysfunction associated with DM.
CHAPTER 4

LOCALISATION OF NITRIC OXIDE SYNTHASE AND IN VITRO FUNCTIONAL RESPONSES TO NITRIC OXIDE IN THE RABBIT LOWER URINARY TRACT:
IDENTIFICATION OF DIABETES-INDUCED CHANGES
4.1 INTRODUCTION

Morphological, histological and functional alterations in autonomic neurotransmission have been reported in DM, for noradrenergic, cholinergic and purinergic nerves in the urinary bladder, using experimental animal models (Longhurst et al 1986, Luheshi et al 1991, Latifpour et al 1988). Although, NANC nerves and neurotransmission in the bladder and urethra has been demonstrated in various species including man (Garcia-Pascual et al 1991; Andersson et al 1992a; Persson et al 1992), the effects of DM on this pathway has not been investigated. Particularly relevant are the inhibitory effects of NO in the bladder neck and urethra, mediating the decrease in outlet resistance, which accompanies micturition (Bennet et al 1995). The NO pathway may also be of importance in the detrusor, in coordinating the micturition process (James et al 1991, Chung et al 1996).

Abnormalities in the NO-mediating mechanisms in the cavernosal (Azadzoi et al 1992), gastric (Jenkinson et al 1996), duodenum (Martinez-Cuesta et al 1995) and anococcygeus (Way et al 1994) smooth muscle have been identified in DM. Since NO plays an important role in modulating bladder neck and urethral smooth muscle tone, we investigated its role in the pathogenesis of the urinary bladder dysfunction associated with DM.

Several techniques have been developed to study the distribution and activity of NOS, as an indirect measure of NO bioactivity. This has previously been studied in the urinary tract using the NADPH-d technique (Smet et al 1994, Hope et al 1991, Keast et al 1994) and anti-NOS antibodies (Persson et al 1993, Crowe et al 1995). These techniques allow the distribution of NOS to be determined, but cannot quantify the activity of the L-
arginine-NO pathway. Previous autoradiographic studies have described the localisation of NOS binding sites in both brain (Kidd et al 1995, Michel et al 1993) and peripheral tissues (Sullivan et al 1996), using $[^3H]$-L-$\text{NO}^0$-nitroarginine ($[^3H]$-L-NOARG) as a radioligand. This allows a quantitative assessment of receptor binding sites, an important factor when attempting to compare control and experimental tissue. Using in vitro ligand-based autoradiography and NADPH diaphorase activity, we investigated the effect of DM (6 months after onset), on the density and distribution of NOS binding sites in the rabbit lower urinary tract. Furthermore, the effect of DM on smooth muscle strip responses to NANC nerve stimulation and to exogenous NO was also assessed in the same group of control and diabetic rabbits.
4.2 MATERIALS AND METHODS

4.2.1 Autoradiographic localisation and quantification of NO synthase binding sites

Localisation of NOS was carried out essentially as previously described (Kidd et al 1995, Michel et al 1993) Slide mounted tissues were preincubated in 50mM Tris HCl, pH 7.2 for 15 min at 22°C. Consecutive sections were then incubated in Tris buffer containing 3mM CaCl₂ and 10nM [³H]-L-NOARG (specific activity 55 Ci/mmol; Amersham International, Amersham, UK) for 60 min at 4°C. The degree of non-specific binding was established by incubating alternate sections in the presence of 10μM unlabelled L-arginine. After incubation the slides were washed in buffer (4 times for 2 min) to reduce non-specific binding, dipped in glass distilled water (4°C) and dried in stream of cold air. Low-resolution autoradiography was carried out by exposing sections to Hyperfilm ³H (Amersham) in X-ray cassettes for 12 weeks. Photodensitometric analysis was performed on film images on a VIDAS imaging system (Kontron, Thame, UK) and the degree of binding determined from curves generated by ³H microscales (Amersham International) that were co-exposed with slide mounted tissue. Specific binding was calculated by substracting non-specific from total binding and expressed in terms of radioligand bound per unit area (i.e. dpm per mm²).

4.2.2 Histochemical localisation of NO synthase.

Localisation of putative NO-producing cells was performed by NADPH diaphorase histochemistry as described by Smet et al (1994). Briefly, sections of the detrusor, bladder neck and urethra, as used for autoradiographic analysis, were fixed for 30 min in 3% paraformaldehyde at 4°C, rinsed and incubated for 1 h at 37°C with 1 mg/ml NADPH
and 0.2 mg/ml nitro blue tetrazolium dissolved in 0.1M phosphate buffer (pH 7.6) containing 0.2% Triton X-100. The sections were then rinsed under running tap water and stained with eosin. The NADPH diaphorase-reactive cells (blue staining) were observed by using an Olympus Vanox microscope. In control experiments in which NADPH was excluded, no staining occurred. Selected sections were photographed.

4.2.3 In vitro organ bath studies

All detrusor, bladder neck and urethral smooth muscle strips measured approximately 1 x 1 x 5 mm. The urethral strips were cut transversally from the proximal part of the urethra. The sizes and the weights of these strips were similar in the control and diabetic animals. Tissue preparations were investigated on the same day as the tissue was obtained. The strips were mounted as described in Chapter 3. An initial tension of 2g (detrusor) and 1g (bladder neck and urethra) was applied to the suspended tissue strips. All strips were equilibrated for 30 mins. At the end of the equilibration period, the strips were challenged with KCl (124 mM). Two reproducible contractions varying in magnitude by less than 10% were consistently obtained.

Transmural stimulation of nerves was performed with a Grass S48 or S88 stimulator delivering single square waves (duration 0.8 ms) at a frequency of 5 to 20Hz in 5s trains at 2 min intervals on a supramaximal voltage.

Relaxant responses to electrical field stimulation (EFS) and to authentic solutions of NO were studied on precontracted preparations. The smooth muscle strips were precontracted with 10^{-7} M ET-1. Authentic solutions of NO with a range of concentrations were prepared as previously described (Palmer et al 1987). First, EFS (5 - 20Hz) was
performed and then concentration-response curves to NO (10^{-4} to 10^{-6} M) were constructed. Next, L-NOARG, 10^{-6} M, tetrodotoxin (TTX) or the guanylyl cyclase inhibitor, oxadiazoloquinoxalin-1-one (ODQ) 10^{-6} M were added at least 15 min before the preparations were once again subjected to EFS or exposed to NO.

The effects of EFS and NO were investigated in the presence of αβ-methylene ATP (10^{-5} M), atropine (10^{-6} M) and guanethedine (10^{-6} M) to block the effects of purinergic, cholinergic and adrenergic receptors, respectively.

4.2.4 Statistical analysis

The results of radioligand binding were expressed as d.p.m./mm² and values are given as mean ± s.e.m. The effects of EFS and NO are expressed as percentage relaxation of the agonist-induced tension. Statistical determinations were performed by the use of Mann-Whitney U test. N_A denotes the number of animals and N_SM the number of smooth muscle strips. ANOVA was used to compare concentration response curves; p < 0.05 was accepted as significant.
4.3. RESULTS

(A) Autoradiographic Analysis

$[^3H]-L$-NOARG binding sites were localised around the urothelium and submucosa of the detrusor, bladder neck and urethra (fig 10, 11 and 12, respectively). The specificity of $[^3H]-L$-NOARG binding to tissue sections was confirmed by the significant reduction (> 90%) in binding in the presence of L-arginine.

NOS binding sites were significantly greater in the bladder neck compared to the detrusor in both control (p < 0.03) and diabetic rabbits (p < 0.004) (Table - 8). In the diabetic rabbits there was a significant (p < 0.04) increase in NOS binding sites in the bladder neck compared to the controls (fig 11).

NOS binding sites also appeared denser in the diabetic urethra compared to the control (Fig 12). Although the NOS binding sites were increased in the detrusor of the diabetic rabbits, this difference was not significant.

(B) NADPH diaphorase histochemistry

Tissue sections from all regions of the lower urinary tract (detrusor, bladder neck and urethra) exhibited positive NADPH-d activity. This activity was evident in and around the urothelium of all regions, similar to that seen with the $[^3H]-L$-NOARG binding sites (fig 13).

At low magnification, NADPH-d activity appeared greater (as assessed by two independent histopathologists) in the urothelium and smooth muscle of diabetic detrusor and bladder neck sections when compared to the controls (fig 13). This difference is clearly demonstrated at higher magnification (fig 14). This is a representative photograph.
of the NADPH-d activity in diabetic bladder neck section compared to control indicating an obvious increase in the NADPH-d activity in both the urothelium and smooth muscle in DM.

Neuronal NADPH-d was identified in nerve fibers of various sizes in and/or around muscular bundles. The nerve fibers in the bladder neck and urethra were visibly thinner and more dispersed within the muscular tissue than in the detrusor. Neuronal-NADPH-d activity appeared greater in the diabetic bladder and urethra compared to control as shown in a representative section of control and diabetic urethra (fig 15).

(C) In vitro organ bath studies

The detrusor

Only 4 out of 14 detrusor strips pre-contracted with ET-1 10^{-7} M showed relaxations to EFS and NO (10^{-4} - 3 \times 10^{-3} M). The maximum relaxation to EFS at 10 Hz was 9% of the ET-1 induced contraction in the control and 12% in the diabetic rabbits. L-NOARG and TTX partially abolished the relaxation to EFS. At the maximum concentration of NO, the maximal relaxation achieved was 12% of the induced contraction in the controls and 14% in the diabetic rabbits. There was no difference in the relaxations achieved in both the control and the diabetic animals.

The bladder neck

Bladder neck strips, precontracted by ET-1 (10^{-6} M), exhibited TTX-sensitive relaxations in response to EFS. Maximum relaxation was obtained at 10 Hz in both the control (n = 10) and diabetic (n = 10) animals and amounted to 44.3 \pm 2.5\% and 43.7 \pm 3.4\%
respectively. Exposure to L-NOARG reduced the relaxant response to EFS by 64% in controls and by 56% in the diabetic rabbit bladder neck. The same strips were also exposed to NO (10^{-4} - 10^{-6} M). The mean IC_{50} in control vs diabetics in the bladder neck (1.03 x 10^{-4} M vs 9.8 x 10^{-5} M) did not differ significantly (fig 16).

The Urethra

The mean IC_{50} for exogenously administered NO in control vs diabetics were not statistically different (8.1 x 10^{-5} M vs 8.8 x 10^{-5} M), but the relaxations to low concentration (5 x 10^{-6} M) of NO were significantly impaired (p < 0.04) in the diabetic urethral smooth muscle (fig 16).

The urethral smooth muscle strips (n = 10) were also precontracted with ET-1 10^{-6} M. The NANC-induced smooth muscle relaxation to all frequencies of EFS were significantly impaired (P < 0.001) in the diabetic rabbits compared to controls (fig 17); at 5 Hz, maximal relaxation in the diabetic group was 23.2% ± 2.7% compared to 61.7% ± 5.6% of the induced contractions in the control group.

L-NOARG and the guanylyl cyclase inhibitor inhibited the relaxation induced by EFS and NO solutions. L-NOARG inhibited NANC relaxations in the control and diabetic urethra by 72% and 63%, respectively at 10Hz. ODQ inhibited NANC relaxations in the control and diabetic urethra by 80% and 74%, respectively.

The relaxation to both exogenous NO and NANC nerve stimulation was significantly greater in the urethral smooth muscle strips compared to the bladder neck (NO - p < 0.02; NANC - p < 0.03) and detrusor (NO - p < 0.001; NANC - p < 0.001) smooth muscle strips.
Figure 10.
Low resolution autoradiographs of $[^3]$H-L-NOARG binding to a transverse section from the control detrusor. (A) Haematoxylin and eosin stained tissue-underlying autoradiograph. (B) Total radioligand binding, and, (C) nonspecific binding to an adjacent section incubated in the presence of 10 µM L-arginine. SM - smooth muscle, URO- urothelium. Bar = 2mm.
Figure 11.

Low resolution autoradiographs of \( ^3\text{H}\)-L-NOARG binding to a transverse section from the control (top panel) and diabetic (bottom panel) bladder neck. (A & D) Haematoxylin and eosin stained tissue-underlying autoradiograph. (B & E) Total radioligand binding and (C & F) nonspecific binding to an adjacent section incubated in the presence of 10 \( \mu\text{M} \) L-arginine. SM = smooth muscle, URO = urothelium. Bar = 2mm.
Figure 12.

Low resolution autoradiographs of $[^3H]$-L-NOARG binding to a transverse section from the control (top panel) and diabetic (bottom panel) urethra. (A) Haematoxylin and eosin stained tissue-underlying autoradiograph. (B) Total radioligand binding and (C) nonspecific binding to an adjacent section incubated in the presence of 10 μM L-arginine. CC = corpous cavernosum, URE = urethra. Bar = 2mm.
Figure 13.

Photograph showing the distribution of NADPH diaphorase activity in detrusor (top panel) and bladder neck (bottom panel) of control (A & C) and diabetic (B & D) transverse sections. SV = seminal vesicles. Bar = 2mm.
Figure 14.

Photograph taken at a higher magnification (x 4) demonstrates increased NADPH diaphorase activity in the diabetic (right panel) bladder neck compared to control (left panel). Bar = 0.1mm.
Figure 15.
Photographs showing neuronal NADPH-d activity in control (left panel) and diabetic (right panel) urethra. Bar = 0.01mm.
Figure 16.

CRC to exogenous application of NO in control (open circle) and diabetic (solid circle) urethral ($N_{SM} = 12$) and bladder neck control (open square) and diabetic (solid square) smooth muscle ($N_{SM} = 12$). Diabetic urethral smooth muscle responses to $5 \times 10^{-6}$M of NO were significantly impaired compared to controls; $p < 0.04$. No significant differences in relaxation to NO were observed in the bladder neck.
Figure 17. EFS-induced relaxation of control (triangle) and diabetic (square) urethral smooth muscle (n = 8); ANOVA *p < 0.001.
Table 8. Photodensitometric analysis of NOS binding sites in the control (CT) and diabetic (DM) detrusor and bladder neck. Data is expressed as dpm x 1000/mm². Values were derived from curves generated from ³H microscales.

<table>
<thead>
<tr>
<th></th>
<th>Detrusor</th>
<th>Bladder neck</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SEM</td>
</tr>
<tr>
<td>CT</td>
<td>1.45*</td>
<td>0.18</td>
</tr>
<tr>
<td>DM</td>
<td>2.93**</td>
<td>0.62</td>
</tr>
</tbody>
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Statistical analysis: Mann Whitney U test (unpaired values).

*Control detrusor vs DM detrusor; p < 0.1

**DM detrusor vs DM bladder neck; p < 0.004

***Control bladder neck vs control detrusor; p < 0.03

****DM bladder neck vs control bladder neck; p < 0.04
4.4 DISCUSSION

Using a specific radioligand, [³H]-L-NOARG, differences in the density and distribution of NOS binding sites were identified in the rabbit lower urinary tract. NOS binding sites were significantly denser in the bladder neck compared to the detrusor in both control and diabetic rabbits. NOS activity, identified by NADPH-d (in nerves, endothelium and urothelium), was also more prominent in the urethra compared to the detrusor, as previously described in other species (Persson et al 1993, Keast et al 1994). This preferential localisation of NOS was reflected by the significantly greater NO-mediated smooth muscle relaxation in the bladder outlet compared to the detrusor, suggesting that NO plays a role in the micturition process. NO production is likely to occur in the urothelium since NADPH-d activity was co-located with NOS radioligand binding. This conclusion is supported by studies which show that cGMP-ir is induced in urothelial cells following exposure to NO donors (Dokita et al 1994). It has also been proposed that the epithelial lining of the urethra can convert arginine into citrulline (Smet at al 1996). Thus, the localisation of NOS (an index of NO production) in the urothelium suggests that NO, if it can diffuse into the muscle layer, may play a role in detrusor and urethral function.

NOS and neuronal NADPH-d are identical in peripheral tissues (Hope et al 1991). Therefore, our observation of increased neuronal NADPH-d activity in the diabetic bladder and urethra, suggests that despite an increase in one of the NOS isoforms there is an impairment of NANC-nerve mediated relaxation in the diabetic urethra. As [³H]-L-NOARG binding has been shown by us and others to be Ca²⁺-dependent (Michel et al 1993), the increase in NOS may be either endothelial NOS (eNOS) or nNOS, since both
are Ca\textsuperscript{2+}-requiring isoforms. Under certain conditions, the expression of nNOS and eNOS is inducible. For example, endothelial shear stress up-regulates the expression of eNOS (Cooke et al 1997). It is therefore possible that DM-induced diuresis contributed to the up-regulation of both epithelial (equivalent to eNOS) and nNOS in our study. It has also been suggested that increased cytokine availability in DM can induce the production of iNOS (Raymond et al 1996). Thus, the overall increase in NOS may be due to a variable effect on all NOS isoforms. Further work is needed to clarify this point.

While there was a significant increase in the NOS-binding sites, and increased neuronal NADPH-d activity in both the diabetic rabbit bladder neck and urethra compared to controls, there was no enhancement of relaxation to NANC nerve stimulation or to exogenous NO. In fact, diabetic urethral smooth muscle relaxation was significantly impaired to NANC nerve stimulation (and lower concentration of exogenous NO), as previously reported in other diabetic tissues (Way et al 1994). The disparity between NOS receptor density and functional responses in the diabetic bladder neck and urethra could be due to the reduction in the production of cGMP in the diabetic bladder neck and urethra compared to controls (Bucala et al 1991). Alterations in the synthesis, release and/or quenching of NO, by increased levels of advanced glycosylation end products (AGEs) (Kiff et al 1991) may also be important factors that would contribute to the reduction in the urethral smooth muscle responses to NANC nerve stimulation in DM. The concept of NO quenching is further supported by the fact that urethral relaxations in DM were significantly impaired only to low concentrations of exogenous NO. At higher concentrations, the presence of excess NO may mask the phenomenon of quenching. The increase in NOS may therefore be a compensatory response to a lack of adequate NO.
Furthermore, hyperglycaemia per se, through the production of AGEs, inhibits NO activity in numerous vascular beds either directly or indirectly causing an increased release of ET-1 (Getz et al 1993, Weisbrod et al 1991), contractile prostanoids (Ceriello et al 1991) and superoxide radicals (Jeremy et al 1986). This situation could be analogous to the increased prostacyclin production associated with hypertrophic and distended bladders in DM (Mikhailidis et al 1987, Jeremy et al 1987) and bladder outlet obstruction (Calver et al 1993). Further work is needed to clarify the contribution of each of these putative mechanisms.

Since DM is associated with voiding dysfunction, the impairment of NO-mediated urethral smooth muscle relaxation in DM suggests that NO plays a role during micturition. This interpretation is supported by experiments in vivo in which bladder hyperactivity could not be induced by NOS inhibitors in bladders that were surgically separated from the urethra, unlike those that remained ‘connected’ (Bennett et al 1995). This view is also consistent with our functional and histochemical observations in vitro, and with the evidence that the urethra receives more innervation by NO-containing nerves than the remainder of the bladder (Vizzard et al 1994).

Dysfunction of the NANC pathway has been described at other sites in experimental DM (Azadzoi et al 1992, Jenkinson et al 1996, Martinez-Cuesta et al 1995, Way et al 1994). A similar dysfunction has also been reported in the forearm vasculature (Calver et al 1993) and corpous cavernosum of patients with DM, despite NO overproduction (Saenz de Tejada et al 1989). These findings suggest that NO may be functionally inactive and/or unavailable in DM. It is, therefore, tempting to speculate that a lack of NO may contribute to bladder outlet obstruction (not related to benign prostatic
hyperplasia) reported to occur in 35 - 40% of patients with long-term DM (Lincoln et al 1984b, Uvelius et al 1986). This hypothesis also suggests that detrusor instability, prevalent in patients with DM (Kaplan et al 1995) may be secondary to a lack of NO bioactivity in the bladder outlet region.

Although NOS binding density was not significantly increased, NADPH-d activity was greater in the detrusor of diabetic rabbits when compared to controls. The functional relevance of this change is unclear, since the role of NO in the detrusor has not been established. If NO plays a physiological role in the relaxation of the detrusor (Chung et al 1996), an increase in NOS could be a compensatory response to bladder distension caused by polyuria associated with DM. Alternatively, if NO inhibits the afferent activity of the detrusor (Andersson et al 1994), an increase in NO may contribute to an impaired sensation of bladder fullness in DM. Since NO can influence cell viability and proliferation, alterations in the NO pathway may be of relevance to the detrusor smooth muscle hyperplasia evident in diabetic animals (Lincoln et al 1984b, Uvelius et al 1986). In conclusion, this study has demonstrated that DM is associated with an increase in NOS binding sites in the bladder neck and urethra and impaired smooth muscle relaxation in response to NANC nerve stimulation in the urethra. Alterations in the NO pathway may, therefore, play a role in the pathogenesis of bladder outlet disorders associated with DM. Manipulating NO bioactivity, may form the basis of new therapies for urinary bladder dysfunction.
CHAPTER 5

PROSTACYCLIN AND CYCLIC NUCLEOTIDE SYNTHESIS BY THE RABBIT

LOWER URINARY TRACT: DIABETES-INDUCED CHANGES
5.1 INTRODUCTION

In addition, to autonomic neuropathy, bladder dysfunction has also been attributed to a direct effect of DM on endogenous mediators that modulate smooth muscle function (Jeremy et al 1986). For example, cAMP and cGMP are recognised as second messengers mediating detrusor and urethral smooth muscle relaxation, respectively (Andersson et al 1992a, Morita et al 1986). The formation of cGMP is regulated by NO via activation of guanylyl cyclase. Whereas PGs or forskolin increases intracellular levels of cAMP, via activation of adenylyl cyclase. PGs also elicit their effects via activation or modulation of cAMP (Andersson et al 1993).

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. For example, PGE$_2$ elicits detrusor smooth muscle contractions, whereas it relaxes the urethral smooth muscle: both actions are necessary for bladder emptying (Gotoh et al 1986, Ueda et al 1985). In addition, putative interactions between the arachidonic acid (a precursor of arachidonic acid) and the NO pathways have emerged recently (Salvemini et al 1995).

Alterations in the production of PGI$_2$ in the diabetic rat bladder (Jeremy et al 1986), as well as changes in the formation of cyclic nucleotides in the diabetic rabbit cavernosa (Sullivan et al 1998) have been documented.

The effect of DM on the formation of cyclic nucleotides and PGs in the rabbit 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.
5.2 MATERIALS AND METHODS

5.2.1 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 DMEM pregassed with 95%O₂/5%CO₂. 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 2mm². 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.

5.2.2 PG\textsubscript{I₂} and PG\textsubscript{E₂} formation

Following preincubation of detrusor, bladder neck and urethral discs for four hours, with frequent changes of medium, one disc, in duplicate for each stimulant dose was placed in DMEM containing ACh (receptor agonist) and arachidonate (substrate), which stimulate the synthesis of PGE\textsubscript{2} and PG\textsubscript{I₂}. Two discs were used for each dose of stimulator. Tissues were then incubated for one hour at 37°C. Supernatants were then removed and 6-oxo-PGF\textsubscript{1α} concentrations (the stable spontaneous hydrolysate of PG\textsubscript{I₂}) and PGE\textsubscript{2} measured by radioimmunoassay. Briefly, aliquots were diluted with Tris HCl-(1%) gelatin buffer, pH 7.4. To these 6-oxo-PGF\textsubscript{1α} or PGE\textsubscript{2} standards (0-10ng) was added 200 µl diluted 6-oxo-PGF\textsubscript{1α} or PGE\textsubscript{2} antisera containing 1 (Ci [³H] 6-oxo-PGF\textsubscript{1α} or [³H]-PGE\textsubscript{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 2,500 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.

5.3.3 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 1M perchloric acid and the tissues sonicated (3 x 30 sec; Soniprep, MSE, Bucks, UK), which extracts the cyclic nucleotides. Following centrifugation at 1000 g for 15 min, supernatants were taken and neutralised with 1M K₃PO₄. 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 μl diluted cAMP or cGMP antisera containing [¹²⁵I] cAMP or [¹²⁵I] 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.
5.2.4 Statistical analysis

The measurements of cAMP, cGMP, PGE$_2$ and 6-oxo-PGF$_{1\alpha}$ formation were expressed as the mean ± s.e.m. value per mg of tissue per min (wet weight) from 6 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.
5.3. RESULTS

(A) Formation of PGI₂, and PGE₂,

Both PGI₂ and PGE₂ formation were inhibited in response to arachidonic acid in the detrusor, bladder neck and urethra (fig 18) of the diabetic rabbit compared to age-matched controls; the degree of inhibition was similar for both PGs. In contrast, in response to ACh, the formation of both PGI₂ and PGE₂ was significantly enhanced in the detrusor, bladder neck and urethra (fig 19) of the diabetic rabbit compared to age-matched controls; the degree of increase was similar for both PGs.

Formation of cAMP and cGMP

In response to forskolin, cAMP formation was significantly reduced in the detrusor, bladder neck and urethra (fig 20) of the diabetic rabbit compared to age-matched controls.

In response to sodium nitroprusside, cGMP formation was also significantly reduced in the detrusor, bladder neck and urethra (fig 21) of the diabetic rabbit compared to age-matched controls.
Figure 18.
Arachidonic acid-stimulated prostaglandin E2 formation by the detrusor, bladder neck and urethra from diabetic (circle) and control (triangle) rabbits 6 months after the induction of DM. Each point = mean ± s.e.m., n = 6. *p < 0.05.
Figure 19.
Acetylcholine-stimulated prostaglandin E\textsubscript{2} formation by the detrusor, bladder neck and urethra from diabetic (circle) and control (triangle) rabbits 6 months after the induction of DM. Each point = mean ± s.e.m. n = 6. *p < 0.05.
Figure 20.

Forskolin-stimulated cAMP formation by the detrusor, bladder neck and urethra from diabetic (circle) and control (triangle) rabbits at 6 months after the induction of DM. Each point = mean ± s.e.m. n =6. *p < 0.05.
Figure 21.

Sodium nitroprusside-stimulated cGMP formation by the detrusor, bladder neck and urethra from diabetic (circle) and control (triangle) rabbits at 6 months after the induction of DM. Each point = mean ± s.e.m. n =6. *p < 0.05.
5.4. DISCUSSION

The results of the present study demonstrate significant differential changes in the formation of cGMP, cAMP, PGI$_2$ and PGE$_2$ 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 was similar 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 (Jeremy et al 1986).

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 (Andersson et al 1993). 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 (Gotoh et al 1986, Ueda et al 1985). 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 an atonic bladder with poor flow rate.

In contrast to arachidonic acid, the formation of both PGI$_2$ and PGE$_2$ was significantly enhanced in response to ACh in the detrusor, bladder neck and urethra of the diabetic rabbit. In the detrusor muscle, ACh elicits contraction and the concomitant of release of PGs through an increase in cytosolic Ca$^{2+}$ (Borda et al 1982). The increase in Ca$^{2+}$ triggers excitation-contraction coupling as well as the activation of phospholipase
A2, which release arachidonic acid from endogenous phospholipid stores (Irvine et al 1982). 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 ACh and phenylephrine are enhanced in the diabetic rabbit detrusor and urethra, respectively, (unpublished results). Thus, although basal cyclooxygenase activity is reduced, the increased responsiveness to contractile agonists (e.g. ACh and phenylepherine) 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 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 the diabetic bladder may be a compensatory response to the failing detrusor, as it gradually distends to become atonic in DM.

It is often overlooked that PGs probably have several roles in the urinary tract other than the control of contraction-relaxation cycles. Firstly, PGI2 and PGE2 are produced in large quantities by the urothelium (Jeremy et al 1986). Since PGs promote the secretion of mucus via activation of adenylyl cyclase in the gastrointestinal tract (Rampton et al 1986) 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 PGs production leads to an increased risk of ulceration (Rampton et al 1986). 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. DM is associated with polyuria and increased micturition rate and as such the bladder adapts by cellular hypertrophy and remodeling (Mikhailidis et al 1987). In this context, dividing SMC increases PGs production. It is not known what role PGI\(_2\) and PGE\(_2\) play in this adaptive process in bladder/urethral SMC. The investigation of this possibility again is warranted.

Since the action of PGs is mediated through 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 activity of adenylyl cyclase since forskolin activates this enzyme directly (Morita et al 1986). This supports our results (unpublished), which demonstrated that relaxation in response to forskolin is impaired in the detrusor and urethra of diabetic rabbits.

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 activity directly, this indicates a generalised reduction in the activity of this enzyme. Interestingly, cGMP-dependent urethral smooth muscle relaxation was impaired in DM despite an increase in NOS in the bladder neck and urethra. Thus, an up-regulation of NOS may be a compensatory response to a reduction in cGMP production. The functional relevance of impaired formation of cGMP by the detrusor is not clear, since the role of NO in the
detrusor has not been established. Since the NO-cGMP pathway influences cell viability and proliferation, alteration in this axis may also be of relevance to the detrusor smooth muscle hyperplasia evident in diabetic animals.

In conclusion, the present study demonstrates a reduction in the formation cAMP and cGMP and increased production of PGI$_2$ and PGE$_2$ in response to cholinergic stimulation. These changes would result in a tendency toward enhanced contractility and reduced relaxation of the urinary tract in DM. These data may be useful in devising pharmacological strategies for the treatment of urinary tract smooth muscle dysfunction in DM.
INHIBITION OF DIABETIC BLADDER SMOOTH MUSCLE CELL PROLIFERATION BY ENDOTHELIN RECEPTOR ANTAGONISTS.
6.1 INTRODUCTION

The bladder responds to stress, for example in DM and bladder outflow obstruction, with compensatory alterations in micturition frequency and volume, bladder mass, capacity and compliance and significant alterations in the contractile response to various forms of stimulation (Longhurst et al 1990, Levin et al 1990). The significant increase in bladder mass associated with DM has been attributed to polyuria and autonomic neuropathy (which leads to prolonged distension as a result of impaired bladder sensation) (Longhurst et al 1990). This augmentation in bladder mass involves an increase in smooth muscle cell volume (hypertrophy) as well as cell division (hyperplasia) in the urothelium, lamina propria and connective tissue elements (Levin et al 1990). The genetic signals that are stimulated (or inhibited) in the rabbit bladder by partial bladder outflow obstruction involve changes in bFGF, TGF-β1, nerve growth factor, and epidermal growth factor (Buttyan et al 1992).

Since ET-1 has potent mitogenic properties, its synthesis by SMC and by fibroblasts within the urinary bladder indicates that it may have a role in the detrusor hyperplasia associated with DM (Saenz de Tejada et al 1992). ET has been shown to stimulate vascular and non-vascular smooth muscle hypertrophy and hyperplasia in cell culture systems (Alberts et al 1994). Furthermore, in vivo studies also demonstrate that following acute and chronic exogenous administration of ET-1 neointimal formation is increased (Douglas et al 1993). ET-1 is thought to exert its mitogenic effects through the activation of protein kinase C. In addition ET-1 acts in concert with other mitogenic factors such as TGF-β1 and epidermal growth factor (Yeh et al 1991). These observations suggest that ET-1 may have a significant role in the initiation and
progression of cellular pathways leading to bladder hyperplasia and hypertrophy. As we have demonstrated alterations in the function and distribution of ET receptor subtypes in the diabetic rabbit bladder, we investigated the potential role of ET-1 and its receptor subtypes in bladder SMC proliferation.
6.2. MATERIALS AND METHODS

6.2.1 Proliferation assay with detrusor and bladder neck smooth muscle cells

Following cervical dislocation, the diabetic and control urinary bladders were excised and weighed and then divided into detrusor and bladder neck at the level of ureteric orifice. The detrusor (n=6) and bladder necks (n=6) were then used for cell culture studies. The SMC from the detrusor and bladder neck were obtained in a similar manner to previously established methods (Shukla et al 1997). Detrusor and bladder neck smooth muscle segments were dissected from the urothelium and the SMC were grown using standard explant methods. The segments were then placed in DMEM supplemented with 10% heat-inactivated fetal 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\textsubscript{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 diabetic rabbit sera (DRS) (n=6) and left for a further 96 h in a 5% CO\textsubscript{2} humidified incubator. Subsequently, the selective ET\textsubscript{A} antagonist BQ123 (Sakamoto et al 1994) or the ET\textsubscript{B} antagonist BQ788 (Hamilton et al 1994) (10, 30 or 100nM) or vehicle were dissolved in 2.5% of the appropriate serum (FCS, CRS or DRS, respectively) and added to the culture.

SMC proliferation was measured 24h later with 5-bromo-2‘deoxy-uracil (BrdU), a thymidine analogue (Hamilton et al 1994). Cells actively synthesizing DNA take up this
substance. Hence, BrdU gives an accurate indication of cell proliferation. BrdU measurement was carried out as previously described by our group (Shukla et al 1997). In brief, 10 Mol/L of BrdU was added to each of the wells for 24 h in the presence of either $\text{ET}_A$ or $\text{ET}_B$ antagonists. The supernatant was then discarded and the cells fixed with ethanol. The fixative was then removed and the cells washed with 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. 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).

6.2.2. Cell counts

For cell counts, the supernatant was discarded at 48 h and the cells washed with PBS free of $\text{Ca}^{2+}$ 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.

6.2.3. Statistical analysis

All the results are presented as mean ± s.e.m. Statistical analysis was carried out using Student’s (unpaired and paired) t test.
6.3 RESULTS

(A) Bladder weights

There was a significant increase (p = 0.03) in the bladder weights (g) of the 6 month DM rabbits [4.8 (2.9-5.4)] compared to age-matched controls [2.5 (1.6-2.8)].

(B) 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 or CRS (table 9). In the presence of DRS the incorporation of BrdU was significantly greater in the diabetic detrusor and bladder neck SMC compared to that in the controls (table 9).

BQ123 and BQ788 at concentrations of 30 and 100nmol/L significantly inhibited BrdU incorporation in the presence of DRS by the diabetic detrusor (p < 0.03) (fig 22) and bladder neck (p < 0.03) (fig 23) SMC. Whereas, BQ123 and BQ788 had no effect on BrdU incorporation in the control detrusor (fig 22) and bladder neck (fig 23) 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).

(C) Cell counts

Diabetic detrusor and bladder neck SMC counts were significantly greater than control in the presence of DRS (table 10). In the presence of BQ123 and BQ788 (30 and 100nmol/L) diabetic detrusor and bladder neck SMC counts were significantly reduced following 48 h incubation in the presence of DRS (table 11). 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 (data not shown). Also, these antagonists had no
effect on the control detrusor and bladder neck SMC counts in the presence of DRS (data not shown).
Table 9: 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. Results are expressed as mean ± s.e.m.

<table>
<thead>
<tr>
<th></th>
<th>Detrusor</th>
<th>Bladder neck</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CT</td>
<td>DM</td>
</tr>
<tr>
<td>DRS</td>
<td>1.2 ± 0.1</td>
<td>2.8 ± 0.09(^1)</td>
</tr>
<tr>
<td>FCS</td>
<td>0.52 ± 0.05(^3)</td>
<td>0.46 ± 0.04(^3)</td>
</tr>
<tr>
<td>CRS</td>
<td>0.48 ± 0.03(^4)</td>
<td>0.35 ± 0.04(^4)</td>
</tr>
</tbody>
</table>

Statistical analysis: Mann Whitney U test

\(^1\)CT vs DM detrusor in the presence of DRS < 0.001

\(^2\)CT vs DM bladder neck in the presence of DRS p < 0.01

\(^3\)FCS vs DRS p < 0.01

\(^4\)CRS vs DRS p < 0.01
Table 10: Effect of control rabbit sera (CRS), Fetal calf sera (FCS) and diabetic rabbit sera (DRS) on the control (CT) and diabetic (DM) detrusor and bladder neck SMC counts. Results are expressed as mean ± s.e.m.

<table>
<thead>
<tr>
<th></th>
<th>Detrusor</th>
<th>Bladder neck</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CT</td>
<td>DM</td>
</tr>
<tr>
<td>DRS</td>
<td>1.1 ± 0.04</td>
<td>2.6 ± 0.05¹</td>
</tr>
<tr>
<td>FCS</td>
<td>0.5 ± 0.01³</td>
<td>0.3 ± 0.02</td>
</tr>
<tr>
<td>CRS</td>
<td>0.4 ± 0.02⁴</td>
<td>0.4 ± 0.03</td>
</tr>
</tbody>
</table>

Statistical analysis: Mann Whitney U test

¹CT vs DM detrusor in the presence of DRS < 0.001
²CT vs DM bladder neck in the presence of DRS p < 0.01
³FCS vs DRS p < 0.03
⁴CRS vs DRS p < 0.03
Table 11: Effect of $\text{ET}_A$ (BQ123) and $\text{ET}_B$ (BQ788) antagonists on 6 month diabetic detrusor and bladder neck SMC in the presence of diabetic rabbit sera (DRS). The results are expressed as median and range ($x \times 10^6$ cell/ml).

<table>
<thead>
<tr>
<th></th>
<th>DRS only</th>
<th>DRS + BQ123 (10 nMol)</th>
<th>DRS + BQ123 (30 nMol)</th>
<th>DRS + BQ123 (100 nMol)</th>
<th>DRS + BQ788 (10 nMol)</th>
<th>DRS + BQ788 (30 nMol)</th>
<th>DRS + BQ788 (100 nMol)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Detrusor</strong></td>
<td>2.6 ± 0.02</td>
<td>1.2 ± 0.03</td>
<td>0.7 ± 0.02$^1$</td>
<td>0.5 ± 0.03$^1$</td>
<td>1.3 ± 0.03</td>
<td>0.8 ± 0.02$^2$</td>
<td>0.6 ± 0.02$^2$</td>
</tr>
<tr>
<td><strong>Bladder neck</strong></td>
<td>1.9 ± 0.04</td>
<td>0.7 ± 0.02</td>
<td>0.5 ± 0.03$^1$</td>
<td>0.3 ± 0.03$^1$</td>
<td>0.7 ± 0.05</td>
<td>0.4 ± 0.01$^4$</td>
<td>0.3 ± 0.01$^4$</td>
</tr>
</tbody>
</table>

Statistical analysis: Mann Whitney U test

**Detrusor**

$^1$DRS vs DRS + BQ123 (30 and 100 nMol) $p < 0.01$

$^2$DRS vs DRS + BQ788 (30 and 100 nMol) $p < 0.01$

**Bladder neck**

$^3$DRS vs DRS + BQ123 (30 and 100 nMol) $p < 0.03$

$^4$DRS vs DRS + BQ788 (30 and 100 nMol) $p < 0.03$
Figure 22: Effect of ET\textsubscript{A} (BQ123) and ET\textsubscript{B} (BQ788) antagonists on the level of BrdU incorporation by the SMC in the presence of diabetic rabbit sera (DRS). The results are expressed as mean ± s.e.m. (Absorbance at 450nm). * p < 0.03, **p < 0.01. Control = CT and diabetic = DM detrusor SMC.
Figure 23: Effect of ET$_A$ (BQ123) and ET$_B$ (BQ788) antagonists on the level of BrdU incorporation by the SMC in the presence of diabetic rabbit sera (DRS). The results are expressed as mean ± s.e.m. (Absorbance at 450nm).

*p < 0.03, **p < 0.01

Control = CT and diabetic = DM bladder neck SMC.
6.4 DISCUSSION

This study has demonstrated, using BrdU and cell count techniques, that at concentrations of 30 and 100nmol/L, ET\textsubscript{A} and ET\textsubscript{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 since at similar concentrations these antagonists had no effect on SMC proliferation in the presence of CRS or FCS. Furthermore, the inhibition of SMC proliferation in other cell culture models has been reported at similar concentrations of ET antagonists (Harada et al 1997, Hasselblatt et al 1998, Spatz et al 1997).

Interestingly, plasma ET-1 levels are increased in patients with DM (Takahashi et al 1990). 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\textsubscript{A} and ET\textsubscript{B} receptors are constitutively expressed in the rabbit bladder SMC, 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 suggests that the local synthesis of ET-1 by the diabetic urinary bladder may have been increased. This suggestion would be compatible with the demonstration of a significant increase in ET-1 binding sites in the diabetic detrusor and bladder neck as described in Chapter 3. Thus, in DM 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 (Kanse et al 1995). 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 (Azuma et al 1995), while human airway smooth muscle cell proliferation is ET_a receptor-dependent (Panettieri et al 1996). Thus, the inhibition of bladder SMC proliferation by both ET_a and ET_b antagonists in this study implies that both ET receptor subtypes play a role in detrusor hyperplasia associated with DM.

One of the most striking features of diabetic cystopathy is the increase in bladder mass (Lincoln et al 1984b). 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 (Gray 1997, Monson et al 1992). However, it is not known what triggers the proliferative process. It has been postulated that the initial signal for DNA synthesis might be the primary distension of the bladder associated with diabetic autonomic neuropathy (Gray 1976). It is not clear from the present study if ET is the primary stimulator of SMC proliferation in the diabetic bladder. The effect of urinary diversion (e.g. by cutaneous vesicostomy) from the bladder on ET receptor density and ET-mediated SMC proliferation needs to be investigated since this procedure produces a decrease in bladder mass, bladder capacity and compliance (Chun et al 1989).

Bladder outflow obstruction is another pathological state where an increase in bladder weight occurs (Monson et al 1992). Interestingly, our group has recently demonstrated an increase in the density of ET receptors in a rabbit model with partial
bladder outflow obstruction (Khan et al 1999). 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 (Khan et al in press). It would, therefore, be of interest to also investigate if ET-1 has a role in bladder hypertrophy following polyuria associated with diabetes insipidus (Malmgren et al 1992).

The functional significance of detrusor SMC proliferation in patients with DM and/or bladder outflow obstruction is not clear. However, it may be involved in the development of altered detrusor pressures (O’Connor et al 1997) 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.

In conclusion, we suggest that ET may play a role in modeling the detrusor structure in response to the pathophysiological effects of DM on the urinary bladder. Therefore, ET receptor antagonists could play a therapeutic role in the treatment of bladder dysfunction associated with DM.
CHAPTER 7

GENERAL DISCUSSION
The experimental findings described in this thesis provide evidence for a pathophysiological role of ET-1, NO and PGs in DM-induced lower urinary tract dysfunction. This discussion combines the information generated from this thesis, into an explanation of the possible role of these mediators in the pathogenesis of diabetic cystopathy.

Several significant findings were noted with regard to the density and distribution of ET-1 receptor subtypes and NOS binding sites in both healthy and diabetic rabbits. In addition, the functional impact of these changes was also assessed.

ET-1 and ET\textsubscript{B} receptor binding sites were increased in the urothelium and smooth muscle of the diabetic detrusor and bladder neck. Activation of ET\textsubscript{B} receptors in the detrusor or bladder neck smooth muscles did not elicit a contractile response. This finding suggests that these receptors may mediate other effects of ET-1 such as mitogenesis and/or the smooth muscle relaxant effects of ET-1. The smooth muscle relaxant effects of ET-1 have been shown to involve mechanisms such as the activation of K\textsuperscript{+} channels (Hasunuma et al 1990), release of inhibitory prostanoids such as PGE\textsubscript{2} and PGI\textsubscript{2} (Albertini et al 1998) and/or the ET\textsubscript{B} receptor-dependent release of NO (Moritoki et al 1993). These effects of ET-1 may explain the impairment of bladder neck smooth muscle responses to ET-1 in DM. Relaxant effects of ET-1 mediated by the ET\textsubscript{B} receptor-dependent release of NO via cGMP formation have been reported in pre-contracted rabbit and guinea pig tracheal smooth muscle (Grunstein et al 1991, Filep et al 1993, El-Mowafy et al 1996). Further work is needed to determine if ET contributes to the formation of cyclic nucleotides in the lower urinary tract. As no alterations in the ET-1 induced contractile responses were observed in the detrusor, it is possible that ET
receptors via may act via different signal transduction pathways in different regions of the bladder. This interpretation is supported by the evidence showing that detrusor smooth muscle relaxation is mediated via cAMP, whereas bladder neck relaxation is cGMP-dependent (Levin et al 1994). The reduced contractility of the bladder neck smooth muscle to ET-1 may reduce bladder outlet resistance to assist the hypotonic bladder to empty effectively. In this context, an increase in ET$_B$ receptors in the DM bladder neck could be a compensatory response to a hypotonic bladder.

ET-1 and NO have also been shown to inhibit cholinergic neurotransmission of the rabbit detrusor (Saenz de Tejada et al 1992). As such, the increased ET$_B$ and NOS binding sites in DM detrusor may play an indirect role in the impairment of the diabetic detrusor contractility. As ET-1 binding sites have not been not identified on the bladder nerves, neuromodulation by ET-1 may occur through either a paracrine mechanism or by circulating plasma ET-1 that has been shown to be elevated in DM (Takahashi et al 1990). However, NOS has been colocalised to acetylcholinestrase containing nerves (Persson et al 1995), thus indicating that NO may also modulate cholinergic neurotransmission either directly or in a paracrine manner. In this context, ET$_B$ receptor-mediated NO production may also indirectly influence detrusor contractility. An up-regulation of ET$_B$ receptors could therefore be a compensatory response to the impaired NO bioactivity in the DM bladder, as NO-induced cGMP formation was also found to be impaired. A compensatory response would also be consistent with a reciprocal regulation of ET-1 synthesis and/or secretion of NO and PGI$_2$.

As ET$_A$ binding sites were evident in the blood vessels within the bladder, it may play a role in the regulation of its blood supply during the micturition process.
Interestingly, ET-1 is considered to play a role in the development of atherosclerosis (Hocher et al 1997). In this context, it has been shown that atherosclerosis and hypercholesterolaemia-induced chronic ischaemia of the bladder alters TGF-β expression, which in turn leads to alterations in the smooth muscle structure and function (Azadzoi KM et al 1999). Similar changes have been observed in various other non-vascular smooth muscle beds such as intestine, stomach and lungs (Murray et al 1994). Alterations in the expression of ET<sub>B</sub> receptors in the corpus cavernosum of a hypercholesterolaemic rabbit model have also been demonstrated (Sullivan et al 1998). It will therefore be interesting to investigate the effect of chronic ischaemia (secondary to distension) and hypercholesterolaemia on the density and distribution of ET receptor subtypes in the bladder.

DM has been shown to induce changes in bladder morphology akin to furosemide- and sucrose-induced diuresis as well as bladder outflow obstruction; these conditions lead to bladder wall distension (Tammela et al 1993). It is thought that the bladder responds to distension with rapid and substantial increases in bladder mass and concomitant alterations in the smooth muscle function (Lincoln et al 1984b). In the rat DM model there is probably a hyperplasia (increased in cell number as a result of increased cell division), which is accompanied by hypertrophy (increase in cell size) (Lincoln et al 1984b). It is thought that DM induces the activation bFGF in association with a decrease in TGF-β resulting in stimulation of DNA synthesis and increased cellular proliferation within both the urothelium and smooth muscle (Eika et al 1993). Since DM is associated with raised serum ET-1 levels (Takahashi et al 1990) as well as increased ET-1 binding sites within the bladder, the enhanced diabetic SMC proliferation
in the presence of DRS would favour the hypothesis that the diabetic rabbit SMC releases more ET-1. As both ET$_A$ and ET$_B$ receptor antagonists inhibited SMC proliferation in the presence of DRS, both ET$_A$ and ET$_B$ receptors may play a role in the prevention of detrusor hyperplasia associated with DM. These antagonists may, therefore, play a role in the treatment of detrusor hyperplasia associated with DM. Further studies are needed to assess if insulin treatment can influence ET-1-mediated responses in the diabetic lower urinary tract.

NOS binding sites and NADPH-d activity (an index of NO synthesis) were significantly increased in the diabetic bladder neck and urethra. This implies that there may be an increase in NO production by the diabetic tissues. Despite this, NO-mediated urethral smooth muscle relaxation by NANC nerves in the urethra was impaired. This impairment can be explained by:

1. a decrease in the release of NO,
2. the release of a constricting factor (e.g. from the cyclooxygenase pathway),
3. decreased sensitivity of DM urethral smooth muscle to NO,
4. increased quenching of NO by O$_2$ derived free radicals, and/or,
5. increased formation of AGEs.

Since the smooth muscle responses to exogenous NO were impaired only at low concentrations, quenching of NO by O$_2$ derived free radicals (by forming peroxynitrite) may be an important factor. Furthermore, quenching of NO is only likely to be seen at low concentrations of NO since at higher concentrations, the presence of an excess of NO may mask this phenomenon. In addition, impairment in the formation of cGMP in response to SNP (NO donor) by the diabetic smooth muscles is indicative of a defect in
the NO-cGMP pathway. The reduction in intracellular cGMP levels in diabetic tissues may therefore be due to abnormalities in the synthesis/release or quenching of NO and/or a decreased ability to synthesise cGMP. Thus, the significant increase in NOS (an index of NO synthesis) may also be a compensatory response to a decrease in NO bioavailability as well as decreased production of cGMP in diabetic tissues.

The reactive $\mathit{O}_2^\mathit{-}$ species, known to modulate the biological actions of NO are greatly increased in DM (Honing et al 1998). Superoxide ions, which are produced by all aerobic cells, react with NO and reduce its biological activity (Beckman et al 1990). This process may contribute to the impairment of NO-mediated urethral smooth muscle relaxation and hence play a role in the pathogenesis of the alterations in the micturition process in DM. Under physiological conditions $\mathit{O}_2^\mathit{-}$ is rapidly converted to hydrogen peroxide by the enzyme superoxide dismutase, which is present both intracellularly and extracellularly (Murphy et al 1998). Superoxide dismutase enhances, while $\mathit{O}_2^\mathit{-}$ impairs the effects of NO. The mechanism underlying this enhancement has not been fully elucidated. It has been proposed that the removal of $\mathit{O}_2^\mathit{-}$ would prolong the activity of NO (Murphy et al 1998). Alternatively, hydrogen peroxide may have an enhancing effect on smooth muscle reactivity to NO (Naseem et al 1995). We investigated the effect of physiological concentrations of hydrogen peroxide on NO-mediated smooth muscle relaxation in the rabbit's genito-urinary tract (Naseem et al 2000). In the presence of hydrogen peroxide, both cavernosal and urethral smooth muscle relaxations to NO were significantly increased. This provides indirect evidence that $\mathit{O}_2^\mathit{-}$ may be involved in the pathogenesis of DM-associated smooth muscle dysfunction. The exact mechanism for this effect needs to be clarified. However, the evidence points towards an enhanced
activation of guanylyl cyclase leading to increased formation of cGMP since this potentiation was blocked by ODQ (a guanylyl cyclase inhibitor).

There is now a growing body of evidence indicating that the urothelium influences bladder function rather than solely acting as a barrier protecting the underlying smooth muscle (Hawthorn et al 2000). Thus, its removal has been shown to significantly alter the responses of the smooth muscle to various contractile agents (Hawthorn et al 2000). The urothelium is considered to be the first bladder component to respond to the stresses of bladder filling and distension by altering its structure and metabolic activity (Chen et al 1994). Thus, alterations in ET-1 and its receptor binding sites as well as NOS radioligand binding sites and NADPH-d activity identified in the urothelium of the DM rabbit bladder could be considered as a pathophysiological response to distension of the DM bladder. The consequent changes in the synthesis/release of ET-1 and NO by the urothelium may, therefore, influence the structure and function of the underlying smooth muscle in a paracrine manner analogous to other vascular and non-vascular smooth muscle beds (Rubanyi et al 1994, Cooke et al 1997). Although urothelium is considered to be watertight, it is thought that bladder distension in disease states such as DM and bladder outlet obstruction may alter the structure of the urothelium leading to increased permeability. In this context, it has been proposed that the urothelial NO may form stable products which following diffusion into the smooth muscle may form a reactive NO metabolite such as peroxynitrite (Ostad et al 1998). In increasing concentrations, peroxynitrite causes oxidative injury and hence smooth muscle dysfunction (Billair 1995). For example, its formation is increased in the muscle layers of obstructed animal bladders compared with controls (Ostad et al 1998). Thus, under pathological conditions
urothelial NO may modulate smooth muscle function. Furthermore, ET-1 and NO, as in vascular smooth muscle beds may influence each other’s activity due to their opposing actions (Rubanyi et al 1994, Cooke et al 1997).

The increase in NOS in the diabetic bladder outlet could represent any of the NOS isoforms. $^3$[H]-L-NOARG binding is Ca$^{2+}$-dependent, indicating that the increase in NOS may be either endothelial NOS (eNOS) or neuronal (nNOS), as both are Ca$^{2+}$-requiring isoforms (Moncada et al 1991). Furthermore, under certain conditions, the expression of nNOS and eNOS is inducible (Billair 1995). For example, fluid flow (causing endothelial shear stress) ‘up-regulates’ the expression of eNOS (Forstermann et al 1994). In the urinary bladder, it is therefore likely that DM-induced diuresis and bladder distension could contribute to the up-regulation of both epithelial (equivalent to eNOS and nNOS). The increased neuronal NADPH-d activity suggests that NOS activity is also increased in the nerves, which may be detrimental to neuronal function. Since the production of cytokines is enhanced in DM this may also induce the production of iNOS. Thus, the overall increase in NOS may be due to a combination of an increase in all NOS isoforms. Further studies are needed to clarify which NOS isoforms are involved. Identification of the isoform(s) will require the use of monoclonal antibodies, which are presently unavailable.

Hyperglycaemia and hyperinsulinaemia produces deleterious changes in nerve perfusion leading to diabetic neuropathy (Cameron et al 1994). Hyperglycaemia increases the release of ET and decreases the availability of NO via the actions of O$_2^-$ free radicals and AGEs (Cameron et al 1997). This effect may lead to a reduction in nerve blood flow
and hence endoneural hypoxia within the bladder. This may ultimately be responsible for bladder neuronal and hence smooth muscle dysfunction associated with DM.

The fact that ACh stimulates the formation of PGE$_2$ and PGJ$_2$ in the rabbit lower urinary tract is consistent with previous reports that the production of these PGs is stimulated by the parasympathetic drive in the urinary bladder of both the rat and man (Mikhailidis et al 1987). As to the impact of DM, there appears to be a defect in the basal cyclooxygenase activity, since the production of PGJ$_2$ and PGE$_2$ was significantly decreased in response to the addition of the substrate arachidonic acid. The overproduction of PGJ$_2$ and PGE$_2$ by muscarinic receptor stimulation by the diabetic rabbit lower urinary tract smooth muscle may, therefore, be a compensatory response to a decrease in cyclooxygenase activity. This differential effect appears to be secondary to a specific increase in muscarinic receptor number/activity linked to PGJ$_2$ and PGE$_2$ synthesis. These findings are in agreement with the marked increase in the ACh-stimulated contractions, which may be a compensatory response to chronic distension of the diabetic bladders. As PGE$_2$ shows a dual effect, contractile on the detrusor and relaxant on the urethra, a compensatory increase in the DM lower urinary tract will support the failing detrusor to empty efficiently. It is also notable that in a rabbit model of partial BOO, where there is marked bladder distension and hyperplasia, alterations in the synthesis of PGJ$_2$ and PGE$_2$ are also observed (Khan et al 1999). It is therefore, reasonable to suggest alterations in the synthesis of PGs may also relate to the production of hyperplasia.

In conclusion, the findings in this thesis support the concept that ET, NO and PGs play a role in the pathogenesis lower urinary tract dysfunction associated with DM.
Future work, investigating the underlying pathophysiological mechanisms, may help to discover novel therapeutic agents to treat the bladder dysfunction associated with DM. These findings may also be relevant to cystopathy attributed to other causes.
Further work completed

Since the completion of the work described in this thesis we have investigated the role of ET-1 and NO in bladder outflow obstruction, which like DM is associated with bladder hypertrophy. These studies have provided further insight into the role of ET-1 and NO in the pathophysiology of lower urinary tract dysfunction.

1. Alterations in the ET-1 and NO pathways were identified in the urinary bladder following partial bladder outflow obstruction, thus supporting my observations in the diabetic bladder.

2. The action of peroxynitrite and hydrogen peroxide was identified in the rabbit lower urinary tract smooth muscle.

3. Changes in the expression of ET receptor subtypes and NOS were also observed in the diabetic and obstructed kidneys.

Future work

1. The effect of long-term DM (one year) on the ET and NO pathways in the urinary bladder should be investigated.

2. DM could be combined with other risk factors such as hypercholesterolaemia to determine if the 'changes' previously observed are aggravated.

3. The effect of ‘treating’ DM with insulin should be evaluated to determine whether the changes reported in this thesis could be reversed or slowed down.

4. To assess the clinical relevance of the present results, bladder biopsies could be taken from diabetic patients with lower urinary tract dysfunction. These samples could be used to repeat the studies performed in this thesis. The findings of such a patient-based study may justify clinical trials involving NO donors and ET antagonists.
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Increased expression of endothelin B receptors in the diabetic rabbit urinary bladder: functional relevance

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Objectives To determine the effect of diabetes mellitus on the density and distribution of endothelin A (ET_A) and endothelin B (ET_B) receptor subtypes in the rabbit urinary bladder, and to assess the in vitro functional properties of endothelin-1 (ET-1) receptors in bladder smooth muscle strips from diabetic and healthy rabbits.

Materials and methods Diabetes mellitus was induced in six male New Zealand White rabbits with alloxan and their urinary bladders excised 6 months after the induction of diabetes. On serial detrusor and bladder neck sections, low- and high-resolution autoradiography was performed using radioligands for ET-1, ET_A and ET_B receptors; these sections were then analysed densitometrically. The results were compared with those from six age-matched healthy control rabbits. Functional responses were investigated using isometric tension studies.

Results ET_A and ET_B receptor binding sites were localized to both the urothelium and smooth muscle of the detrusor and bladder neck. There were significantly more ET_B receptor binding sites in the diabetic detrusor and bladder neck sections than in controls. ET-1 smooth muscle contractile responses were ET_A receptor-mediated. The smooth muscle contractile responses to ET-1 were unaltered in the detrusor, but significantly impaired in the bladder neck of diabetic animals compared with controls.

Conclusion Alteration in the expression of ET_B receptors and in vitro contractile smooth muscle responses to ET-1 in the diabetic rabbit urinary bladder neck may play a role in the pathophysiology of diabetic cystopathy.

Keywords Endothelin A, endothelin B, rabbit, urinary bladder, diabetes mellitus

Introduction

Alterations in bladder function (e.g. atomic or hyper-reflexic bladders) are increasingly recognized in diabetic patients [1,2]. These complications are thought to be due to abnormal function of sensory and autonomic innervation to the bladder [3,4], detrusor hypertrophy [5,6] and alterations in the density of various receptors [7].

Endothelin-1 (ET-1) (a 21 amino-acid peptide, originally identified in the culture medium of porcine endothelial cells) [8], has been shown to elicit smooth muscle contraction in the lower urinary tract of various animal species including humans [9,10]. In addition, ET-1 is mitogenic and has a growth promoting effect on different cell types [11,12]. Two ET receptor subtypes, ET_A and ET_B, have been characterized and recently cloned from bovine [13] and rat lung [14], respectively. These receptors have been detected in the rabbit lower urinary tract using SDS-PAGE and characterized based upon differential binding affinities of sarafotoxin 6c (S6c) and a cyclic pentapeptide, BQ123, at 125I-ET-1 binding sites [15,16]. These studies indicated that there are regional differences in the density and distribution of ET receptors in the rabbit urinary tract, suggesting that the specific functions of ET-1 depend on the region of the urinary tract. Whilst these studies provide a possible physiological role for ET-1 in the urinary bladder, the exact function of its receptor subtypes is unclear. In the vascular bed, ET_A receptors mediate vasoconstriction and cellular proliferation, whereas ET_B receptors, predominantly found on endothelial cells, mediate vasodilatation via the production of nitric oxide (NO) and prostacyclin (PGI_2) (although ET_B-mediated vasoconstriction is also well established) [17].

Recently, the enhancing effects of glucose and insulin on ET-1 release from endothelial cells and mesenteric arteries has been described in diabetic animals [18]. Up-regulation of ET receptor expression has been shown...
In the corpus cavernosum [19] and prostate [20] in experimental diabetes. In contrast, bladder outlet obstruction secondary to BPH significantly down-regulates the expression of ET receptors in the urinary bladder [21].

The aim of the present study was to investigate if ET receptor subtypes have a pathophysiological role in diabetic cystopathy. Using in vitro ligand-based autoradiography, we investigated the effect of diabetes on the density and distribution of ET receptor subtypes (ET\(_A\) and ET\(_B\)) in the rabbit urinary bladder. The functional properties of these ET receptors were also characterized using isometric tension studies.

Materials and methods

Induction of diabetes mellitus

Age-matched male New Zealand White rabbits (12, body weight 3 kg) were selected; six were injected intravenously (via the lateral ear vein) with alloxan (Sigma Chemical Co., Poole, UK) in a single dose of 65 mg/kg body weight, to induce nonketonuric, hyper-glycaemic diabetes mellitus. All animals were fed *ad libitum* with standard plain diet (SDS, Witham, UK) and allowed free access to water. Blood was sampled at monthly intervals for serum urea and electrolytes, cholesterol, triglycerides and glucose. Urine was also analysed for glucose, ketone bodies and proteins using a Multistix (Ames Division, Miles Laboratories Ltd, Stoke Poges, UK).

Preparation of tissues

After cervical dislocation, the urinary bladders were excised 6 months after the induction of diabetes. The bladders were then divided into detrusor and bladder neck at the level of ureteric orifice. Part of the detrusor and bladder neck was stored immediately at −70°C in airtight containers and the rest stored in cold Krebs buffer solution. The tissues stored at −70°C were mounted in Ames OCT embedding compound (BDH Laboratory Supplies, Poole, UK) and transverse 10 μm sections cut in a cryostat at −20°C, then thaw-mounted onto gelatinized microscope slides. The same procedure was performed on six age-matched, healthy control rabbits.

Autoradiographic studies

Preliminary binding studies (saturation analysis) were performed, where consecutive 10 μm transverse sections were pre-incubated in 50 mmol/L Tris HCl buffer, pH 7.4, for 15 min at 22°C to reduce endogenous peptide levels. Sections were then incubated (2 h at 22°C) in buffer containing 5 mmol/L MgCl\(_2\), 1% BSA and 100 kIU/mL aprotinin in the presence of 0.003–1 nmol/L \(^{125}\text{I}-\text{ET-1}\) (Amersham International, Amersham, UK). ET\(_A\) and ET\(_B\) binding sites were identified using the selective radioligands \(^{125}\text{I}\)-PD151242 (ET\(_A\)) [22] and \(^{125}\text{I}\)-BQ3020 (ET\(_B\)) [23] (Amersham International). Non-specific binding was established by incubating adjacent sections in the presence of 1 μmol/L unlabelled ET-1 (Bachem Fine Chemicals, Switzerland). Sections were then washed twice and binding determined by wiping sections from microscope slides and measuring bound \(^{125}\text{I}\) in a gamma counter. Receptor density (B\(_{\text{MAX}}\)) and affinity (K\(_{\text{D}}\)) were then calculated using GraphPad Inplot Software (Graph Pad, San Diego, California, USA).

Quantitative assessment of \(^{125}\text{I}-\text{ET1}, \(^{125}\text{I}\)-PD151242 (ET\(_A\)) and \(^{125}\text{I}\)-BQ3020 (ET\(_B\)) binding to rabbit urinary bladder

Consecutive 10 μm sections from the detrusor and bladder neck were pre-incubated, as described above, and then incubated for 120 min, at 22°C, in buffer containing a fixed concentration of 0.15 nmol/L \(^{125}\text{I}\)-ET-1, \(^{125}\text{I}\)-PD151242 and \(^{125}\text{I}\)-BQ3020 (concentration at the approximate K\(_{\text{D}}\) values established from saturation studies above). The degree of nonspecific binding was established by incubating alternate sections in the presence of 1 μmol/L unlabelled ET-1. The slides were washed twice in buffer for 10 min, dipped in 4°C distilled water and then dried in a stream of cold air. Low-resolution autoradiography was carried out by exposing the sections to Hyperfilm 3H (Amersham International) in X-ray cassettes for 1–3 days. Photodensitometric analysis was performed using a Bio-Rad GS-700 imaging densitometer (Bio-Rad Laboratories, Hemel Hempstead, UK). Data were analysed using Molecular Analyst software. Binding was expressed in terms of radioligand bound per unit area (d.p.m./mm\(^2\)), calculated from standard curves generated by \(^{125}\text{I}\) microscales (Amersham International) that were co-expressed with tissue sections. Microscopic localization (high-resolution autoradiography) of binding sites was performed by postfixing tissue in paraformaldehyde vapour (2 h at 80°C) and coating slides in molten nuclear emulsion (LM-1, Amersham International). Slides were then stored in light-proof boxes for up to 8 days at 4°C, after which they were processed in D19 high-contrast developer (Kodak, UK) and fixed (Hypam, Ilford, UK). Underlying tissue was stained with haematoxylin and eosin, and the high-resolution autoradiographs viewed on an Olympus Vanox microscope: selected sections were photographed under dark-field and bright-field illumination where appropriate.
Functional studies

Detrusor strips (1 x 1 x 5 mm) were taken from the anterior wall of the dome and bladder neck strips taken just below the trigone in an oblique direction from the internal urethral orifice towards one of the orifices of the ureter. Contractile studies were performed on the same day that the tissue was obtained. The strips were mounted vertically in 1.5 mL organ baths, containing Tyrode’s solution maintained at 37°C by a thermostatically regulated circuit. The Tyrode solution was bubbled with a mixture of 95% O₂ and 5% CO₂, maintaining the pH at 7.4. An initial tension of 2 g (detrusor) and 1 g (bladder neck) was applied to the suspended tissue strips. The tension was recorded with a force displacement transducer (FT-O3, Grass Instruments, Quincy, MA, USA) on a Grass Polygraph (model 7D). All strips were equilibrated for at least 30 min. At the end of the equilibration period, the strips were challenged with KCl (124 mmol/L). Two reproducible contractions, varying in magnitude by <10%, were consistently obtained.

Cumulative response curves (CRCs) were recorded (0.5 log unit steps, one curve for each smooth muscle strip). CRCs were obtained for ET-1 and IRL 1620 (a selective ETₐ agonist). Further CRCs to ET-1 were constructed in the presence of BQ123 (ETₐ) and BQ788 (ET₇) selective antagonists to identify the receptor mediating the contractile responses.

Drugs and solutions

ET-1, IRL1620, BQ123 and BQ788 were obtained from Bachem Fine Chemicals, Switzerland. The Tyrode solution used had the following composition (mmol/L): NaCl 118, KCl 4.0, NaHCO₃ 24.0, NaH₂PO₄ 0.4, MgCl₂ 1.0, CaCl₂ 1.8, glucose 6.1, Na pyruvate 5.0; and the Krebs transporting solution had a composition (mmol/L) of NaCl 115, NaHCO₃ 24.5, KCl 4.0, NaH₂PO₄ 0.5, CaCl₂ 0.7.

Analysis

Animal weights and blood glucose concentrations are shown as the median and range. Other results were assessed statistically using the two-tailed Mann–Whitney U-test (unpaired values), with P < 0.05 accepted as indicating significance. The effects of ET-1 and IRL1620 are expressed in milliNewtons. The contractile responses to 1 μmol/L of ET-1 and the mean EC₅₀ (concentration of ET-1 eliciting half the contractile response) values were estimated by regression analysis and compared using an unpaired t-test. Results are expressed as the mean (SEM). ANOVA was used to compare CRCs, with P < 0.05 accepted as significant.

Results

The starting weights in both the control and diabetic rabbit groups were similar (controls 3.0 [2.7–3.5] kg, n = 6; diabetics 3.1 [2.8–3.6], n = 6). At the end of the 6-month study, the diabetic rabbits were significantly lighter (P < 0.03) than the control group (controls 4.1 [3.5–4.35], Naₓ = 6; diabetic 3.6 [3.0–3.9], n = 6). Serum glucose concentrations (nonfasting) were significantly (P < 0.009) higher in the diabetic group, at 32.2 (18.3–41.9) mmol/L (n = 6) than in controls, at 6.4 (6.1–7.5) mmol/L (n = 6). Serum cholesterol and triglyceride concentrations did not differ significantly in the control and diabetic groups.

Autoradiography

Receptor binding studies confirmed that ¹²³I-ET-1, ¹²⁵I-PD151242 and ¹²⁵I-BQ3020 all bound in a concentration-dependent manner to detrusor and bladder neck sections. Saturation analysis showed that binding was to high-affinity sites, with Kᵦ values in the subnanomolar range (¹²⁵I-ET-1 = 0.11 nmol/L, ¹²⁵I-PD151242 = 0.071 nmol/L and ¹²⁵I-BQ3020 = 0.091 nmol/L). Fixed concentrations of 0.15 nmol/L (based on these Kᵦ values) were used for subsequent experiments.

ETₐ and ET₇ receptor binding sites

There was dense ¹²³I-PD151242 (ETₐ) and ¹²⁵I-BQ3020 (ET₇) binding to all sections of the detrusor (Fig. 1a) and bladder neck (Fig. 1b) in control and diabetic animals. The nonspecific binding was undetectable in the presence of 1 μmol/L unlabelled ET-1 for ¹²⁵I-PD151242 and was reduced by <90% for BQ3020 (ET₇). On high-resolution autoradiographs, binding sites were associated with smooth muscle and urothelial cells in both the detrusor and bladder neck (Figs 2 and 3). ETₐ receptor binding sites were also identified on the blood vessels within the bladder (Fig. 4). The results of densitometric analysis of film images are presented in Table 1.

In both the control and diabetic tissue sections, the density of ETₐ receptors was significantly greater in the detrusor than in the bladder neck. There were no differences in the density of ET₇ receptor binding sites between the detrusor and bladder neck of both control and diabetic animals. There were no significant changes in the density of ETₐ receptor binding sites between control and diabetic animals of either the detrusor or bladder neck. There was significantly greater ET₇ receptor density in the diabetic detrusor and bladder neck sections than in controls. This increase in the density of ET₇ receptor binding sites was also reflected...
in a significant increase in ET-1 binding sites in the diabetic detrusor and bladder neck compared with controls (Table 1).

The high-resolution autoradiographs indicated that the increase in ET₆ receptor density occurred in both the urothelium and smooth muscle. A representative section from the control and diabetic bladder neck is shown in Fig. 4a and 4b, respectively.

Functional studies

There were no significant differences in the weights and lengths of smooth muscle strips used from either control or diabetic animals, or between detrusor and bladder neck. The results of smooth muscle contractile responses to ET-1 and IRL1620 in the control and diabetic detrusor and bladder neck are presented in Table 2.
CRCs to ET-1 in the control detrusor and bladder neck smooth muscle strips are presented in Fig. 5a. ET-1 elicited a potent durable contractile response in the detrusor and bladder neck. The smooth muscle contractile responses to ET-1 in the detrusor were significantly greater than in the bladder neck. CRCs to ET-1 in the presence of ET<sub>A</sub> and ET<sub>B</sub> antagonists in the detrusor are shown in Fig. 5b. The ET-1 mediated contractile responses were significantly and competitively inhibited by the ET<sub>A</sub> antagonist BQ123 but not by the ET<sub>B</sub> antagonist (BQ788). The contractile responses to ET-1 at 1 μmol/L were not significantly enhanced in the presence of BQ123. Similar responses were observed in the bladder neck (data not shown). The ET<sub>B</sub> selective agonist (IRL1620) had very little effect on the tone of either detrusor or bladder neck smooth muscle strips even at the highest concentration used (1 μmol/L; Table 2).

**Diabetes-associated differences**

CRCs to ET-1 in control and diabetic detrusor and bladder neck are shown in Fig. 5c and 5d, respectively. The mean contractile response to ET-1 at 1 μmol/L and its EC<sub>50</sub> values in the detrusor were not significantly different between control and diabetic animals (Table 2). In contrast, the mean contractile response to ET-1 at 1 μmol/L was significantly decreased and its EC<sub>50</sub> value was significantly increased in the diabetic bladder neck compared with the control (Table 2). No significant changes were identified in the smooth muscle responses to IRL1620 in the detrusor and bladder neck.

**Discussion**

This study shows that in both control and diabetic animals, the density of ET<sub>A</sub> receptor binding sites and the contractile responses to ET-1 was significantly greater in the detrusor than in the bladder neck. The contractile responses to ET-1 in the presence of ET<sub>A</sub> and ET<sub>B</sub> antagonists in the detrusor are shown in Fig. 5b. The ET-1 mediated contractile responses were significantly and competitively inhibited by the ET<sub>A</sub> antagonist BQ123 but not by the ET<sub>B</sub> antagonist (BQ788). The contractile responses to ET-1 at 1 μmol/L were not significantly enhanced in the presence of BQ123. Similar responses were observed in the bladder neck (data not shown). The ET<sub>B</sub> selective agonist (IRL1620) had very little effect on the tone of either detrusor or bladder neck smooth muscle strips even at the highest concentration used (1 μmol/L; Table 2).
responses to ET-1 in the diabetic bladder neck associated with an increase in the ET$_A$ receptor density certainly merits further investigation. Because ET$_A$ receptors have been implicated in the release of endogenous NO [17], the activation of ET$_A$ receptors may oppose ET-1 contractile responses in the bladder neck, as NO is thought to play a prominent role in bladder neck smooth muscle relaxation [25]. Whether the increased intracellular Ca$^{2+}$ levels observed in diabetes [26] contribute to NO production (via activation of Ca$^{2+}$-dependent NO-synthase, NOS) remains unclear [27].

In contrast to the bladder neck, the contractile responses to ET-1 were not lower in the diabetic detrusor than in the controls, despite a similar increase in ET$_A$ receptor binding sites. This difference in ET-1-induced contractile responses between diabetic detrusor and bladder neck may be associated with the lack of responsiveness of detrusor smooth muscle to NO [25].

Experimental diabetes may also precipitate a non-specific decrease in the responsiveness of bladder neck smooth muscle to vasoactive agents. Alterations in responses to ET-1 and NOS inhibitors have been reported in the cutaneous microcirculation of diabetic rats [28]. We have also shown that despite an increase in NOS in the diabetic bladder neck/urethra, NO-mediated smooth muscle relaxation was impaired [29]. It is therefore possible that an increase in ET$_A$ receptors and NOS in the diabetic urinary bladder neck could represent an attempted compensatory response to altered NO bioactivity. Overproduction of NO, although

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Fig. 4. High-resolution autoradiograph of ET<sub>a</sub> receptor binding to the blood vessels within the detrusor. ET<sub>a</sub> receptor binding sites are evident as white grains on a black background. Bar = 200 μm.

### Table 1 Photodensitometric analysis of regional [¹²⁵I]ET-1, [¹²⁵I]PAC1242 (ET<sub>a</sub> ligand) and [¹²⁵I]BQ3020 (ET<sub>β</sub> ligand) binding in the control (CT) and diabetic (DM) rabbit urinary bladder

<table>
<thead>
<tr>
<th></th>
<th>Median (range) receptor binding (10^4 dpm/mm&lt;sup&gt;2&lt;/sup&gt;)</th>
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<tr>
<td></td>
<td>ET-1</td>
</tr>
<tr>
<td>Detrusor</td>
<td>CTR: 98 (69–174)</td>
</tr>
<tr>
<td></td>
<td>DM: 166 (140–226)</td>
</tr>
<tr>
<td>Bladder neck</td>
<td>CT: 73 (50–89)</td>
</tr>
<tr>
<td></td>
<td>DM: 119 (78–203)</td>
</tr>
</tbody>
</table>

Mann–Whitney U-test: regional differences: CT detrusor ET<sub>a</sub> vs CT bladder neck ET<sub>a</sub>; P < 0.001. DM detrusor ET<sub>a</sub> vs DM bladder neck ET<sub>a</sub>; P < 0.001. DM-associated differences: CT detrusor ET<sub>β</sub> vs DM detrusor ET<sub>β</sub>; P < 0.022. CT bladder neck ET<sub>β</sub> vs DM bladder neck ET<sub>β</sub>; P < 0.002.

### Table 2 The contractile properties of ET-1 and IRL1620 in control (CT) and diabetic (DM) rabbit urinary bladder

<table>
<thead>
<tr>
<th></th>
<th>Detrusor</th>
<th>Bladder</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ET-1</td>
<td>IRL1620</td>
</tr>
<tr>
<td>CT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Response</td>
<td>39.8 (8.2)</td>
<td>1.0 (0.5)</td>
</tr>
<tr>
<td>EC&lt;sub&gt;50&lt;/sub&gt; (nmol/L)</td>
<td>42.3 (37.0)</td>
<td>–</td>
</tr>
<tr>
<td>DM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Response</td>
<td>47.8 (3.9)</td>
<td>1.0 (0.5)</td>
</tr>
<tr>
<td>EC&lt;sub&gt;50&lt;/sub&gt; (nmol/L)</td>
<td>28.3 (71.9)</td>
<td>–</td>
</tr>
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*At 1 μmol/L (mN).

Beneficial in many systems, could also be potentially toxic because of the increased production of free radicals [30,31]. It is conceivable therefore that a similar increase in free radicals in the diabetic urinary bladder can cause cytotoxic damage and thus alter bladder function. Alterations in the synthesis of another potent vasodilator, PGI<sub>2</sub>, have been well documented in several diabetic tissues, including the urinary bladder [32–35]. Hence, the interactions between NO, PGI<sub>2</sub> and ET, which are thought to be important in maintaining vascular tone [36], may also contribute to the regulation of urinary tract smooth muscle tone. However, the precise relationships between NO, PGI<sub>2</sub> and ET-1 in bladder function has not been elucidated. We are currently investigating this relationship in the pathogenesis of diabetic cystopathy.

ET may also influence other aspects of bladder function. For example, ET is synthesized by smooth muscle cells and it stimulates mitogenesis [11,17]. Therefore, the diabetes-associated detrusor smooth muscle hyperplasia may be related to the significant increase in ET<sub>β</sub> receptor density. The proliferation of vascular smooth muscle cells in response to ET-1 has been shown to correlate with ET-receptor density [37]. Both ET<sub>a</sub> and ET<sub>β</sub> receptors have been implicated in this response. For example, ET<sub>β</sub> receptors participate in the development of intimal hyperplasia after endothelial injury [38], while human airway smooth muscle cell proliferation is ET<sub>a</sub> receptor-dependent [39]. ET-1 is also reported to enhance detrusor cholinergic and nonadrenergic, noncholinergic responses [40,41], presumably by autocrine (release of ET-1 by smooth muscle cells) or by paracrine (circulating ET-1) mechanisms. Thus, alterations in circulating ET levels in diabetes [42] and expression of ET receptors in the diabetic urinary bladder may contribute to changes in bladder smooth muscle contractility. Furthermore, the
dense ET\textsubscript{a} binding to the blood vessels within the bladder may also be involved in the control of the blood supply to the detrusor and hence its function. In this context, it is of interest that bladder vascular insufficiency has recently been shown to alter bladder smooth muscle contractile responses to ET-1 \cite{43}.

In conclusion, this study shows that alloxan-induced diabetes in the rabbit is associated with a significant increase in the density of ET\textsubscript{a} receptors in the urinary bladder and a significant decrease in the contractile responses to ET-1 in the bladder neck. These findings may contribute to the pathophysiological changes in the bladder which are associated with diabetes. The manipulation of ET\textsubscript{a} receptor-mediated responses may be of benefit in the treatment of bladder dysfunction associated with diabetes.

Acknowledgements

We thank Dr A. Naylor, Pfizer Ltd, Discovery Biology, for providing the necessary equipment and drugs. This study was also supported by a Charles Wolsan Research Grant. M.R. Dashwood is supported by the British Heart Foundation.

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© 1999 \textit{BJU International} 83, 113–122


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ALTERNATIONS IN THE NITRIC OXIDE SYNTHASE BINDING SITES AND NON-ADRENERGIC, NON-CHOLINERGIC MEDIATED SMOOTH MUSCLE RELAXATION IN THE DIABETIC RABBIT BLADDER OUTLET: POSSIBLE RELEVANCE TO THE PATHOGENESIS OF DIABETIC CYSTOPATHY


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ABSTRACT

Purpose: To investigate the effect of diabetes mellitus (DM) on the density and distribution of nitric oxide synthase (NOS) and the smooth muscle responses to non-adrenergic, non-cholinergic (NANC) nerve stimulation and exogenous nitric oxide (NO) in the rabbit lower urinary tract.

Materials and Methods: Transverse sections of detrusor, bladder neck and urethra, from control and six months alloxan-induced DM New Zealand White rabbits were incubated with a radioligand for NOS (\(^{3}H\)-L-N\(^{G}\)-nitroarginine). Densitometric analysis was performed on the autoradiographs. NADPH diaphorase histochemistry was also used as a marker for NOS activity. Responses to NANC nerve stimulation (5 to 20 Hz) and to NO (10\(^{-6}\) to 3 \times 10\(^{-4}\) M) on smooth muscle strips from detrusor, bladder neck and urethra were measured in organ baths.

Results: NOS binding sites were significantly (p <0.03) more dense in the bladder neck than in the detrusor in both DM and control groups. In DM bladder neck, NOS binding sites were significantly (p <0.04) increased compared with the controls. NADPH diaphorase activity appeared markedly increased in the detrusor, bladder neck and urethra of DM animals compared with controls. The mean IC\(_{50}\) for exogenous NO in control versus DM were not statistically different in the bladder neck (1.03 \times 10\(^{-4}\) M versus 9.8 \times 10\(^{-6}\) M) and urethra (8.1 \times 10\(^{-6}\) M versus 8.8 \times 10\(^{-6}\) M), but the relaxations to 5 \times 10\(^{-6}\) M of NO were significantly impaired (p <0.04) in the DM urethral smooth muscle. NANC nerve-mediated relaxations were significantly impaired (p <0.001) in the DM urethral smooth muscle.

Conclusions: Alterations of both the NOS binding sites and functional responses to NANC nerve stimulation suggest that NO may have a pathophysiological role in the urinary bladder dysfunction associated with DM.

Key Words: diabetes mellitus, nitric oxide, autoradiography, lower urinary tract

Bladder dysfunction is a recognized complication of diabetes mellitus (DM) and has been attributed in part to diabetic-induced peripheral autonomic neuropathy.\(^{6,7}\) Morphological, histological and functional alterations in autonomic neurotransmission have been reported in DM, for noradrenergic, cholinergic and purinergic nerves in the urinary bladder, using experimental animal models.\(^{8-10}\) Although non-adrenergic, non-cholinergic (NANC) nerves and neurotransmission in the bladder and urethra have been demonstrated in various species including man,\(^{11-14}\) the effects of DM on this pathway have not been investigated before. Particularly relevant are the inhibitory effects of nitric oxide (NO) in the bladder neck and urethra, mediating the decrease in outlet resistance, which accompanies micturition.\(^{15}\) The NO pathway could also be of importance in the detrusor, in coordinating the micturition process.\(^{16-17}\)

NO is synthesized from L-arginine by NO synthase (NOS) and acts as a primary messenger that stimulates soluble guanylyl cyclase activity. The subsequent increase in cyclic GMP concentration lowers calcium concentration in target cells to cause smooth muscle relaxation, or alteration in neurotransmission.

Abnormalities in the NO-mediated mechanisms in the cavernosal,\(^{18}\) gastric,\(^{19}\) duodenum\(^{20}\) and anococcygeus\(^{21}\) smooth muscle have been identified in DM. Since NO plays an important role in modulating bladder neck and urethral smooth muscle tone, it is clearly of interest to investigate its role in the pathogenesis of the urinary bladder dysfunction associated with DM.\(^{1-7}\)

Several techniques have been developed to study the distribution and activity of NOS, as an indirect measure of NO. This has previously been studied in the urinary tract using the NADPH-diaphorase technique\(^{22-25}\) and anti-NOS antibodies\(^{24,26}\). These techniques allow the distribution of NOS to be determined with greater sensitivity, but cannot quantify the activity of the L-arginine: NO pathway. Previous autoradiographic studies have described the localization of NOS binding sites in both brain\(^{27-28}\) and peripheral tissues,\(^{29}\) using \(^{3}H\)-L-N\(^{G}\)-nitroarginine (\(^{3}H\)-L-NOARG) as a radioligand. This allows a quantitative assessment of receptor binding sites, an important factor when attempting to compare control and experimental tissue. Using in vitro ligand-based autoradiography and NADPH diaphorase activity, we investigated the effect of DM, 6 months after onset, on the density and distribution of NOS binding sites. Furthermore, the effect of DM on smooth muscle strip responses to...
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NANC nerve stimulation and to exogenous NO was assessed in the rabbit lower urinary tract.

MATERIALS AND METHODS

Induction of diabetes mellitus. Age-matched 3 kg. male New Zealand White (NZW) rabbits (n = 12) were selected, 6 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 non-ketonuric, hyperglycaemic DM. All animals were fed ad libitum with SDS standard plain diet (SDS, Witham, UK) and allowed free access to water. Blood was sampled at monthly intervals for serum urea and electrolytes, cholesterol, triglycerides and glucose. Urine was also analyzed for glucose and ketone bodies and proteins using a Multistix (Ames Division, Miles Laboratories Ltd, Stoke Poges, UK).

Preparation of tissues. Following cervical dislocation, urinary bladders (divided at the level of ureteric orifice into the body and bladder neck) and urethrae were excised from rabbits, six months after the induction of DM. Part of the detrusor, bladder neck and proximal urethra were stored immediately at −70 C in air tight containers and the rest stored in cold Krebs buffer solution (see Krebs composition) and taken directly to the laboratory. The tissues stored at −70C were then mounted in AMES OCT embedding compound (BDH Laboratory Supplies, Poole, UK) and transverse 10 μm. sections were cut in a cryostat (Bright Instruments Co. Ltd, Huntingdon, UK) at approximately −20C and thaw mounted onto gelatinised microscope slides. The same procedure was performed on six age-matched, healthy control NZW rabbits.

Autoradiographic technique. Localization of NOS was carried out essentially as described by Kidd et al.27 and Michel et al.28 Slide mounted tissues were preincubated in 50 mM Tris HCl, pH 7.2 for 15 minutes at 22C. Consecutive sections were then incubated in Tris buffer containing 3 mM CaCl₂ and 10 nM [³H]-L-NOARG (specific activity 55 Ci./mmol.; Amersham International, Amersham, UK) for 60 minutes at 4C, the degree of non-specific binding being established by incubating alternate sections in the presence of 10 μM unlabeled L-arginine. After incubations the slides were washed in buffer (4 times for 2 minutes) to reduce non-specific binding, dipped in glass distilled water (4C) and dried in stream of cold air. Low resolution autoradiography was carried out by exposing sections to Hyperfilm ³H (Amersham) in X-ray cassettes for 12 weeks. Photodensitometric analysis was performed on film images on a VIDAS imaging system (Kontron, Thame, UK) and the degree of binding determined from curves generated by ³H microscales (Amersham International) that were co-exposed with slide mounted tissue.
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Fig. 2. Low resolution autoradiographs of [³H]-L-NOARG binding to transverse section from control (top panel) and diabetic (bottom panel) bladder neck. A & D, hematoxylin and eosin stained tissue underlying autoradiograph. B & E, total radioligand binding. C & F, nonspecific binding to adjacent section incubated in presence of 10 μM L-arginine. SM - smooth muscle, URO - urothelium. Bar = 2mm.

Fig. 3. Low resolution autoradiographs of [³H]-L-NOARG binding to transverse section from control (top panel) and diabetic (bottom panel) urethra. A, hematoxylin and eosin stained tissue underlying autoradiograph. B, total radioligand binding. C, nonspecific binding to adjacent section incubated in presence of 10 μM L-arginine. CC - corpous cavernosum, UR - urethra. Bar = 2mm.
cific binding was calculated by subtracting non-specific from total binding expressed in terms of radioligand bound per unit area (that is dpm per mm²).

[NADPH diaphorase histochemistry]. Localization of putative NO-producing cells was performed by NADPH diaphorase histochemistry as recently described. Briefly, sections of the detrusor, bladder neck and urethra, as used for autoradiographic analysis, were fixed for 30 minutes in 3% paraformaldehyde at 4°C. They were then rinsed and incubated for 1 hour at 37°C with 1 mg/ml β-NADPH and 0.2 mg/ml nitro blue tetrazolium dissolved in 0.1 M phosphate buffer (pH 7.6) containing 0.2% Triton X-100. The sections were then rinsed under running tap water and stained with eosin. The NADPH diaphorase-reactive cells (blue staining) were observed by using an Olympus Vanox microscope. In control experiments in which NADPH was excluded, no staining occurred. Selected sections were photographed.

**Functional studies.** The bladder and urethra were transported to the laboratory in cold Krebs solution (for composition, see below). The bladder was transected at the level of the ureteric orifice. Detrusor strips were taken from the anterior wall of the dome, and bladder neck strips were taken just below the trigone in an oblique direction from the bladder neck toward one of the orifices of the ureter. The urethral strips were cut transversally from the proximal part of the urethra. All strips measured approximately 1 x 1 x 5 mm. The sizes and the weights of these strips were similar in the control and diabetic animals.

Tissue preparations were investigated on the same day as the tissue was obtained. The strips were mounted vertically in 1.5 ml organ baths, equipped with two parallel platinum electrodes, containing Tyrode's solution (see Tyrode's composition) maintained at 37°C by a thermostatically regulated circuit. The Tyrode solution was bubbled with a mixture of 95% O₂ and 5% CO₂, maintaining pH at 7.4. An initial tension of 2 gm. (detrusor) and 1 gm. (bladder neck and urethra) was applied to the suspended tissue strips. The tension was recorded with a force displacement transducer (PT-03, Grass Instruments, Quincy, Massachusetts) on a Grass Polygraph (model 7D). All strips were equilibrated for at least 30 minutes. At the end of the equilibration period, the strips were challenged with KCl (124 mM). Two reproducible contractions varying in magnitude by less than 10% were consistently obtained.

Transmural stimulation of nerves was performed with a Grass S48 or 888 stimulator delivering single square waves (duration 0.8 ms) at a frequency of 5 to 20 Hz in 5 s trains at 2 minutes interval on a supramaximal voltage.

Relaxant responses to electrical field stimulation (EPS) and to authentic solutions of NO were studied on smooth muscle preparations precontracted with 10⁻⁷ M endothelin-1 (ET-1). Authentic solutions of NO with a range of concentrations were prepared as previously described. First, EPS (5 to 20 Hz) was performed and then concentration-response curves to NO (10⁻⁷ to 10⁻⁵ M) were constructed. Next, L-NOARG, 10⁻⁶ M, tetrodotoxin (TTX) or guanylate cyclase inhibitor, oxadiazoloquinoxalin-1-one (ODQ) 10⁻⁶ M were given at least 15 minutes before the preparations were once again subjected to EPS or exposed to NO.

The effects of EPS and NO were investigated in the presence of α-β methylene ATP (10⁻⁸ M), atropine (10⁻⁶ M) and guanethidine (10⁻⁶ M) to block the effects of purinergic, cholinergic and adrenergic receptors, respectively.

**[¹H]-L-NOARG binding (Amersham).** L-arginine, TTX, atropine sulfate, N⁰-nitro-L-arginine (L-NOARG) and α-β methylene ATP were from Sigma and endothelin-1 from BACHEM. The Tyrode solution used had the following composition (mM) NaCl 118, KCl 4.0, NaHCO₃ 24.0, NaH₂PO₄ 0.4, MgCl₂ 1.0, CaCl₂ 1.8, glucose 6.1, Na pyruvate 5.0, and the Krebs transporting solution had NaCl 115, NaHCO₃ 24.5, KCl 4.0, NaH₂PO₄ 0.5, CaCl₂ 0.7 mM.

**Analysis of data.** Animal weights and blood glucose are given as median and range. The results of radioligand binding were expressed as disintegrations per minute (d.p.m.)/mm² mean and S.E.M. The effects of EPS and NO are expressed as percentage relaxation of the agonist-induced tension. Statistical determinations were performed by the use of Welch's unpaired t test. N denotes the number of animals and n the number of smooth muscle preparations. Results are given as mean value ± standard error of mean (S.E.M.). An analysis of variance (ANOVA) was used to compare concentration response curves. A probability level < 0.05 was accepted as significant.

**RESULTS**

**Animal weights and serum glucose concentration.** The starting weights (kg) in both the control and diabetic rabbit groups were similar (control: range 3.8 to 3.5; diabetic: range 3.2 to 3.6; n = 6). At the end of the 6 months study, the diabetic rabbits were significantly lighter (p < 0.03) than the control group (control: median 4.1; range 3.5 to 4.35, n = 6; diabetic: median 3.6; range 3.0 to 3.9, n = 6). Serum glucose concentrations (non-fasting) (mmol/l) were significantly (p < 0.009) elevated in the 6 months diabetic group (median 32.2; range 18.3 to 41.9, n = 6) when compared with controls (median 6.4; range 6.1 to 7.5, n = 6).

Serum cholesterol and triglycerides were not significantly different between control and diabetic groups.

**Autoradiographic analysis.** [¹H]-L-NOARG binding sites were localized around the urothelium and submucosa of the detrusor, bladder neck and urethra (figs. 1, 2, 3). The specificity of [¹H]-L-NOARG binding to tissue sections was confirmed by the significant reduction (>90%) in binding in the presence of L-arginine.
NOS binding sites were significantly greater in the bladder neck compared with the detrusor in both control (p < 0.03) and diabetic rabbits (p < 0.004) (fig. 4). In the DM rabbits there was a significant (p < 0.04) increase in NOS binding sites in the bladder neck compared with the controls (figs. 2, 4). NOS binding sites also appeared denser in the DM urethra compared with the control (fig. 3). Although the NOS binding sites were increased in the detrusor of the DM rabbits, the difference was not significant.

NADPH diaphorase histochemistry. Tissue sections from all regions of the lower urinary tract (detrusor, bladder neck and urethra) exhibited positive NADPH diaphorase activity. The NADPH-diaphorase activity was evident in and around the urothelium of all regions, similar to that seen with the [H]-L-NOARG binding sites (fig. 5). At low magnification, NADPH diaphorase activity appeared greater (as assessed by two independent histopathologists) in the urothelium and smooth muscle of DM detrusor and bladder neck sections when compared with the controls (fig. 5). This difference is clearly demonstrated at higher magnification (fig. 6). This is a representative photograph of the NADPH-diaphorase activity in DM bladder neck section compared with control indicating an obvious increase in the NADPH-diaphorase activity in both the urothelium and smooth muscle in DM. Neuronal NADPH diaphorase was identified in nerve fibers of various sizes in and/or around muscular bundles. The nerve fibers in the bladder neck and urethra were visibly thinner and more dispersed within the muscular tissue than in the detrusor. Neuronal-NADPH diaphorase activity appeared greater in the DM bladder and urethra compared with control as shown in a representative section of control and DM urethra (fig. 7).

Functional studies: the detrusor. Only 4 out of 14 detrusor strips pre-contracted with ET-1 10^-7 M showed relaxations to EFS and NO (10^-4 - 3 × 10^-3 M). The maximum relaxation to EFS at 10 Hz was 9% of the ET-1 induced contraction in the control and 12% in the DM rabbits of the induced contraction. L-NOARG and TTX partially abolished the relaxation to EFS. At the maximum concentration of NO, the maximal relaxation achieved was 12% of the induced contraction in the controls and 14% in the DM rabbits. There was no difference in the relaxations achieved in both the control and the DM animals (data not shown).

The bladder neck. Bladder neck strips, precontracted by endothelin (10^-6) exhibited TTX-sensitive relaxations in response to EFS. Maximum relaxation was obtained at 10 Hz in both the control (n = 10) and DM (n = 10) animals and amounted to 44.3 ± 2.5% and 43.7 ± 3.4% respectively. Exposure to L-NOARG reduced the relaxant response to electrical stimulation by 64% in controls and by 56% in the diabetics. The same strips were also exposed to NO (10^-4 - 10^-6 M). The mean IC50 in control versus DM in the bladder

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Fig. 6. Photograph demonstrating increased NADPH diaphorase activity in the diabetic bladder neck (B) at a higher magnification (x 4) compared with control (A). Bar = 0.1 mm.

neck (1.03 × 10^-4 M versus 9.8 × 10^-5 M) were not statistically different (fig. 8).

The urethra. The urethral smooth muscle strips (n = 10) were also precontracted with endothelin 10^-6 M. The NANC-induced smooth muscle relaxation to all frequencies of EFS was significantly impaired (p <0.001) in the DM rabbits compared with controls (fig. 9). At 5 Hz, maximal relaxation in the DM group was 23.2% ± 2.7% compared with 61.7% ± 5.6% of the induced contractions in the control group. The mean IC50 for exogenously administered NO in control versus DM was not statistically different (8.1 × 10^-5 M versus 8.8 × 10^-5 M), but the relaxations to low concentration (5 × 10^-6 M) of NO were significantly impaired (p <0.04) in the DM urethral smooth muscle (fig. 8). L-NOARG and guanylate cyclase inhibitor inhibited the relaxation induced by EFS and NO solutions. L-NOARG inhibited NANC relaxations in the control and diabetic urethra by 72% and 63%, respectively at 10Hz. ODQ inhibited NANC relaxations in the control and diabetic urethra by 80% and 74%, respectively.

The relaxation to both exogenous NO and NANC nerve stimulation was significantly greater in the urethral smooth muscle strips compared with the bladder neck (NO - p <0.02; NANC - p <0.03) and detrusor (NO - p <0.001; NANC - p <0.001) smooth muscle strips.

DISCUSSION

Using a specific radioligand, [3H]-L-NOARG, differences in the density and distribution of NOS binding sites were identified in the rabbit lower urinary tract. NOS binding sites were significantly denser in the bladder neck compared with the detrusor in both control and DM rabbits. NOS activity, identified by NADPH diaphorase (in nerves, endothelium and urothelium), was also more prominent in the urethra compared with the detrusor, as previously described in other species. This preferential localization of NOS was reflected by the significantly greater NO-mediated smooth muscle relaxation in the bladder outlet compared with the detrusor, suggesting that NO has a role in the micturition process. NO production is likely to occur in the urothelium since NADPH diaphorase activity was co-located with NOS radioligand binding. This conclusion is supported by studies which show that cGMP-immunoreactivity is induced in urothelial cells following exposure to NO donors. It has also been proposed that the epithelial lining of the urethra can convert arginine into citrulline. Thus, the localization of NOS (an index of NO production) in the urothelium suggests that NO, if it can diffuse into the muscle layer, may play a role in detrusor and urethral function.

NOS and neuronal NADPH-diaphorase are identical in peripheral tissues. Therefore, our observation of increased neuronal NADPH-diaphorase activity in the DM bladder and urethra, suggests that despite an increase in one of the NOS isoforms there is an impairment of NANC-nerve mediated relaxation in the DM urethra. As [3H]-L-NOARG binding has been shown by us and others to be calcium-dependent, the increase in NOS may be either endothelial NOS (eNOS) or nNOS, since both are calcium-requiring isoforms. Under cer-
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Fig. 7. Photograph showing neuronal NADPH-diaphorase activity in control (CON) and diabetic (DM) urethra. Bar = 25 μm.

While there was a significant increase in the NOS-binding sites, and increased neuronal NADPH-diaphorase activity in both the DM rabbit bladder neck and urethra compared with controls, there was no enhancement of relaxation to NANC nerve stimulation or to exogenous NO. In fact, DM urethral smooth muscle relaxation was significantly impaired to NANC nerve stimulation (and lower concentration of exogenous NO), as previously reported in other DM tissues. The disparity between NOS receptor density and functional responses in the DM bladder neck and urethra could be due to the reduction in the production of cGMP in the DM bladder neck and urethra compared with controls. Alterations in the synthesis, release and/or quenching of NO, by increased levels of advanced glycosylation end products (AGEs) may also be important factors that would contribute to the reduction in the urethral smooth muscle responses to NANC nerve stimulation in DM. The concept of NO quenching is further supported by the fact that urethral relaxations in DM were significantly impaired only to low concentrations of exogenous NO. At higher concentrations, the presence of excess NO may mask the phenomenon of quenching. The increase in NOS may therefore be a compensatory response to lack of adequate NO. Furthermore, hyperglycaemia per se, through the production of AGEs, inhibits NO activity in numerous vascular beds either directly or indirectly causing an increased release of endothelin, contractile prostanoids and superoxide radicals. This situation could be analogous to the increased prostacyclin production associated with hypertrophic and distended bladders in DM and bladder outlet obstruction. Further work is needed to clarify the contribution of each of these putative mechanisms.

Since DM is associated with voiding dysfunction, the impairment of NO-mediated urethral smooth muscle relaxation in DM suggests that NO plays a role during micturition. This interpretation is supported by experiments in vivo in which bladder hyperactivity could not be induced by NOS inhibitors in bladders that were surgically separated from the urethra, unlike those that remained 'connected'. This view is also consistent with our functional and histochemical observa-
tions in vitro, and with the evidence that the urethra receives more innervation by NO-containing nerves than the remainder of the bladder. 37

Dysfunction of the NANC pathway has been described at other sites in experimental DM. 18-21 A similar dysfunction has also been reported in the forearm vasculature 22 and cavernosum of patients with DM, despite NO overproduction. 23,24 These findings suggest that NO may be functionally inactive and/or unavailable in DM. It is, therefore, tempting to speculate that a lack of NO may contribute to bladder outlet obstruction (not related to benign prostate hyperplasia) reported to occur in 35 to 40% of patients with longterm DM. 25 This hypothesis also suggests that detrusor instability, (prevalent in patients with DM 26) may be secondary to a lack of NO bioactivity in the bladder outlet region.

NOS binding density was increased and NADPH diaphorase activity was obviously more prominent in the detrusor of DM rabbits when compared with controls. The functional relevance of this change is unclear, since the role of NO in the detrusor has not been established. If NO plays a physiological role in the relaxation of the detrusor, 27 an increase in NOS could be a compensatory response to bladder distension caused by polyuria in DM. Alternatively, if NO inhibits the afferent activity of the detrusor, 28 an increase in NO may contribute to an impaired sensation of bladder fullness in DM. Since NO can influence cell viability and proliferation, alterations in the NO pathway may be of relevance to the detrusor smooth muscle hyperplasia evident in diabetic animals. 29-32

In conclusion, this study has demonstrated that DM is associated with an increase in NOS binding sites in the bladder neck and urethra and impaired smooth muscle relaxation in response to NANC nerve stimulation in the urethra. Alterations in the NO pathway may, therefore, play a role in the pathogenesis of bladder outlet disorders associated with DM. Manipulating NO bioactivity, may form the basis of new therapies for urinary bladder dysfunction.

Acknowledgment. The authors thank Dr. A. Naylor, Pfizer Ltd., Discovery Biology, for providing the reagents and equipment.

References

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

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

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 adenylly 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 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 (PGI2) in the DM rat bladder [12], as well as changes in the formation of cyclic nucleotides in the DM rabbit cavnosa [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, GMP-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 M 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

Aged-matched 3 kg male New Zealand white rabbits (n = 12) were used, six of which were injected intraocularly with alloxan (via the orbital ear vein) with a single dose of 65 mg/kg. The diabetic rabbits were fed ad libitum with SDS standard rabbit plain chow (SDS, Lipton Ltd., 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 period of diabetes for glucose, ketone bodies and proteins with kittest (Ames Division, Miles Laboratories Ltd., Stoke Poges, Buckinghamshire, UK).

Preparation of bladder tissue

Following cervical dislocation, bladders and urethrae were excised in the diabetic rabbits at 6 months (n = 6) together with the age-matched controls (n = 6). The tissues were immediately placed in Bicecco's Minimum Essential Medium (DMEM) pregassed with 95% O2/5% CO2. 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. 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 F1α, arachidonic acid (sodium salt), Dulbecco's minimum essential medium, forskolin, gelatin, isobutylmethylxanthine. Norit activated charcoal, phorbol ester dibutyrate, 6-oxo-prostaglandin F1α 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): [3H]-prostaglandin E2, [3H]-6-oxo-prostaglandin F1α and [125I]-labelled cAMP and [125I]-labelled cGMP radioimmunoassay kits.

PGI2 and PGE2 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 PGE2 and PGI2 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-PGF1α concentrations (the stable spontaneous hydrolylate of PGI2) and PGE2 measured by radioimmunoassay [11]. Briefly, aliquots were diluted with Tris HCl(1%) gelatin buffer, pH 7.4. To these and 6-oxo-PGF1α or PGE2 standards (0–10 ng) was added 200 μl diluted 6-oxo-PGF1α or PGE2 antisera containing 1 μCi [3H] 0-oxo-PGF1α or [3H]-PGE2. 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 x 30 s; Soniprep, MSE, Bucks, UK), which extracts the cyclic nucleotides. Following centrifugation at 1000 g for 15 min, supernatants were 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 fmole) was added 200 μml diluted 6-oxo-PGF1α or PGE2 antisera containing 1 μCi [3H] 6-oxo-PGF1α or [3H]-PGE2. 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). 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, PGE2 and 6-oxo-PGF1α, 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

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

Fig. 1 Arachidonic acid-stimulated prostaglandin E2 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

Discussion

The results of the present study demonstrate significant differential changes in the formation of PG12, PGE2, 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 PG12 and PGE2 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 PG12 and PGE2 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).
Fig. 2 Acetylcholine-stimulated prostaglandin E₂ 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

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 PGI₂ and PGE₂, the dominant PGs in the lower urinary tract of the rabbit, promote detrusor contractility and urethral smooth muscle relaxation [3, 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 PGI₂ and PGE₂ 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 A₂ 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 (Δ) 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, PGI₂ and PGE₂ are produced in large quantities by the urothelium [12]. Since PGs promote the secretion of mucus via activation of adenyl 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 PGI₂ and PGE₂ 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 PGI₂ and PGE₂ 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 adenyl 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 adenyl 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 PGI₂ and PGE₂ 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.

**References**


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

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 (ETα and ETβ) 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 ETα-antagonist (BQ123) or ETβ-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.

Key words Endothelin-1 · Rabbit · Bladder · Diabetes mellitus · Smooth muscle cell proliferation

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: ETα and ETβ [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 (PCS) (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 (CO2) 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% CO2 humidified incubator. Subsequently, the selective ETα antagonist BQ123 [29] or the ETβ 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), an 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 ETα or ETβ 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) (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 ETα or ETβ 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; Meter-tech, Watford, UK) at 450 nm (reference wavelength 690 nm).

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).

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 the controls (Table 2).

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.

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>+6 Months</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Body weight (kg)</strong></td>
<td></td>
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</tr>
<tr>
<td>Control</td>
<td>3.0 ± 0.2</td>
<td>4.1 ± 0.5</td>
</tr>
<tr>
<td>Diabetic</td>
<td>3.1 ± 0.3</td>
<td>3.6 ± 0.4</td>
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<tr>
<td><strong>Bladder weight (g)</strong></td>
<td></td>
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</tr>
<tr>
<td>Control</td>
<td>2.5 ± 0.04</td>
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<tr>
<td>Diabetic</td>
<td>4.8 ± 0.03</td>
<td></td>
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<tr>
<td><strong>Glucose (mmol/l)</strong></td>
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<td></td>
</tr>
<tr>
<td>Control</td>
<td>7.5 ± 0.2</td>
<td>6.4 ± 0.3</td>
</tr>
<tr>
<td>Diabetic</td>
<td>7.6 ± 0.3</td>
<td>33.0 ± 0.4</td>
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<tr>
<td><strong>Cholesterol (mmol/l)</strong></td>
<td></td>
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<tr>
<td>Control</td>
<td>0.7 ± 0.02</td>
<td>1.2 ± 0.02</td>
</tr>
<tr>
<td>Diabetic</td>
<td>0.7 ± 0.03</td>
<td>0.9 ± 0.04</td>
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</table>
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).

**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).

**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

<table>
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<th>Detrusor</th>
<th>Bladder neck</th>
</tr>
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<tr>
<td></td>
<td>CT</td>
<td>DM</td>
</tr>
<tr>
<td>DRS</td>
<td>1.2 ± 0.1</td>
<td>2.8 ± 0.09^a</td>
</tr>
<tr>
<td>FCS</td>
<td>0.52 ± 0.05^b</td>
<td>0.46 ± 0.04^a</td>
</tr>
<tr>
<td>CRS</td>
<td>0.48 ± 0.03^c</td>
<td>0.35 ± 0.04^d</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th></th>
<th>Detrusor</th>
<th>Bladder neck</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CT</td>
<td>DM</td>
</tr>
<tr>
<td>DRS</td>
<td>1.1 ± 0.04</td>
<td>2.6 ± 0.05a</td>
</tr>
<tr>
<td>FCS</td>
<td>0.5 ± 0.01c</td>
<td>0.3 ± 0.02</td>
</tr>
<tr>
<td>CRS</td>
<td>0.4 ± 0.02a</td>
<td>0.4 ± 0.03</td>
</tr>
</tbody>
</table>

Table 4 Effect of ET (BQ123) and ETB (BQ788) antagonists on SMC counts (×10^6 cell/ml) in diabetic detrusor and bladder neck in the presence of diabetic rabbit sera (DRS). Data are expressed as mean ± SEM.

<table>
<thead>
<tr>
<th></th>
<th>DRS + BQ123 (10 nMol)</th>
<th>DRS + BQ123 (30 nMol)</th>
<th>DRS + BQ123 (100 nMol)</th>
<th>DRS + BQ788 (10 nMol)</th>
<th>DRS + BQ788 (30 nMol)</th>
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<tr>
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<td>0.5 ± 0.03a</td>
<td>0.8 ± 0.02b</td>
<td>0.6 ± 0.02b</td>
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<tr>
<td>Bladder neck</td>
<td>1.5 ± 0.04</td>
<td>0.9 ± 0.02</td>
<td>0.5 ± 0.03</td>
<td>0.3 ± 0.03a</td>
<td>0.7 ± 0.05</td>
<td>0.4 ± 0.01d</td>
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</tbody>
</table>

Discussion

This study has demonstrated, using BrdU and cell count techniques, that at concentrations of 30 and 100 nmol/l, ETα and ETβ 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 ETα and ETβ 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 ETα and ETβ receptors have been implicated in this ET-mediated proliferative response. For example, ETβ receptors participate in the development of intimal hyperplasia after endothelial injury [4], while human airway smooth muscle cell proliferation is ETα receptor-dependent [27]. Thus, the inhibition of bladder SMC proliferation by both ETα and ETβ 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 hyperton-
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.

Acknowledgement Mr. FH. Muntaz is supported by the Charles Wolfson Charitable Trust.

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Nitric oxide in the lower urinary tract: physiological and pathological implications

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Introduction

The collection and storage of urine by the detrusor and its periodic expulsion through the bladder neck and urethra (bladder outlet) are dependent upon complex neural pathways [1]. The neurotransmitters and the mechanisms that regulate these pathways are not only of research interest but may also provide a basis for therapeutic intervention in patients with voiding dysfunction. A decade ago, the study of amine mediators such as noradrenaline and acetylcholine (ACh) released by the sympathetic and parasympathetic nervous systems, respectively, dominated pharmacological research in the lower urinary tract. Recently, several nonadrenergic, noncholinergic (NANC) mediators have been recognized to play a significant role in the physiology of the lower urinary tract [2]. Over the last decade, nitric oxide (NO) has increasingly gained recognition as an important cell mediator with a broad range of functions in the lower urinary tract. It is considered to be an important inhibitory NANC neurotransmitter as well as a modulator of the cellular immune response to invading microorganisms and tumour cells in the lower urinary tract of different animal species, including humans.

This review examines the possible mechanisms by which NO may influence the function of the lower urinary tract, emphasising the source and synthesis of NO, its mechanisms of action and its evolving role in the pathophysiology of lower urinary tract disorders.

The discovery of NO

In 1916, Mitchell et al. [3] first identified that mammals release large amounts of nitrates in their urine. Almost 70 years later, Iyengar et al. [4] showed that activated murine macrophages released nitrates. In 1980, Furchgott and Zawadzki reported the existence of a labile endothelium-derived relaxing factor (EDRF) [5] that elicited relaxation of blood vessels. Although it was soon recognized that the endothelium-dependent relaxation required calcium (Ca²⁺) [6] and cGMP formation [7], the identity of EDRF remained elusive for 7 years. In 1987, it became clear that the EDRF synthesized by cultured endothelial cells was in fact NO and was dependent on the presence of l-arginine [8,9]. It was later shown that there were many similarities between the EDRF and the neurotransmitter involved in NANC nerve-mediated smooth muscle relaxation [10,11]. The definitive evidence that NO was the neurotransmitter mediating this effect came from studies on the rat anococcygeus muscle [12]. Subsequently, the role of NO as a neuronal messenger in the urinary tract was established in the rabbit urethra [13]. Since then, much information has been accumulated on the biochemistry and the role of this ubiquitous messenger in the urogenital tract.

Synthesis, release and degradation of NO

NO is neither pre-stored nor packed in vesicles but is produced on demand. It then diffuses randomly from its site of production, being highly membrane-permeable. Endogenous NO is formed by the hydroxylation of l-arginine to citrulline [14], a reaction catalysed by one of the three isoforms of NO synthase (NOS) [15]. Because NO has a short half-life (0.1–6 s) and is very reactive, NO physiology has largely been investigated indirectly by techniques that identify the distribution and activity of the NOS isoforms (Table 1). These distinct isoforms of NOS have been named after the cells in which they were first isolated, purified and cloned [15]. Each NOS isoform, i.e. endothelial (eNOS), neuronal (nNOS) and macrophage inducible (iNOS), varies considerably in subcellular location, structure, kinetics, regulation and function [15]. Each of the enzymes is a product of a unique gene, located on human chromosomes 7 (eNOS), 12 (nNOS) and 17 (iNOS) [15]. Both nNOS and eNOS are normal constituents of cells and are termed constitutive. The activity of both eNOS and nNOS is transient (minutes) and is triggered by Ca²⁺-elevating agonists [16]. In contrast, iNOS is not present in resting
cells, but is induced by certain cytokines or bacterial endotoxins [16]. Under certain circumstances the expression of eNOS and nNOS is also inducible. For example, fluid flow (causing endothelial shear stress) upregulates the expression of eNOS; indeed, six shear stress-responsive elements have been identified in the promoter region of eNOS [17]. Unlike the constitutive NOS isoforms, iNOS activity is sustained (lasting many days) and is independent of Ca^{2+} elevation. Structurally, the eNOS NH2 terminus contains a consensus site for N-myristoylation that plays a significant role in the membrane localization of eNOS. This attribute explains the observation that eNOS is membrane-associated, whereas iNOS and nNOS are cytosolic. Although each of these isoforms contains a calmodulin-binding site, iNOS binds to it with a higher affinity, so that calmodulin forms a constitutive subunit of this isofom. Thus, eNOS and nNOS are dependent on exogenous calcium and calmodulin for activation, whereas iNOS is less so. However, common to all three isoforms are heme-containing enzymes that catalyse the NADPH and O2-dependent oxidation of L-arginine to NO and citrulline [17]. All three isoenzymes have been identified in the lower urinary tract of several animal species, including man [18–22].

The distribution of NOS in the lower urinary tract (fig. 1)

Using immunocytochemistry and NADPH-d histochemistry, putative NO synthesis sites have been identified in the lower urinary tract. Neuronal NADPH-d is used as a specific marker for neurones producing NO [23]. NOS activity has been identified in the urothelium, smooth muscle, blood vessels and, more importantly, in the nerves supplying the lower urinary tract.

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Table 1 Techniques used to determine the NO pathway

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Fig. 1. A diagrammatic representation of the putative sites of NO production, based on immunohistochemical and pharmacological studies. NOS immunoreactivity is localised to the MPG and pelvic nerves supplying the detrusor, bladder neck and urethra. Transection of the MPG abolishes NO dependent urethral smooth muscle relaxation. NOS receptor density is greater in the bladder outlet than in the detrusor.
The origin of nerves producing NO in the lower urinary tract is not fully established, but they appear to originate close to the parasympathetic sacral nucleus. The presence of NOS in the postganglionic parasympathetic and preganglionic sympathetic nerves supplying the rat lower urinary tract support the functional evidence that NO may have a role as an inhibitory NANC transmitter [24]. As such, stimulation of the parasympathetic input to the urethra elicits NO-dependent urethral smooth muscle relaxation [25-27]. The major pelvic ganglia (MPG) supplying the urinary tract also show NADPH-d activity and NOS-immunoreactivity (NOS-ir) [28]. Double-immunolabelling studies also show that NOS-ir cell bodies in the MPG have choline acetyltransferase (ChAT) [29,30]. Furthermore, removal of the MPG in the rat completely abolishes NO-dependent urethral smooth muscle relaxation [31], whilst hypogastric nerve transaction has no effect. These observations therefore support the concept of a parasympathetic origin for NO. In addition, the neurones of the MPG innervating the urethra have significantly greater NADPH-d activity and NOS-ir than those supplying the bladder [28,32]. NADPH-d and NOS-ir positive nerves have also been detected in and around muscular bundles of the entire lower urinary tract of several animal species [32-35], and man [35-37]. Despite minor interspecies variations in the distribution of NOS-containing nerves, the density of these nerves is significantly greater in the bladder neck and urethra than in the detrusor. There are similar regional differences in NOS activity, as measured by the L-[14C]-citrulline formation assay, i.e. a higher NOS activity in the urethra than in the detrusor [33,37]. Functionally, the inhibitory effects of NO correlate with the density and distribution of NOS-containing nerves in the lower urinary tract. As such, smooth muscle relaxation mediated by NO is significantly greater in the urethra than in the detrusor in most species [38-41]. NADPH-d positive neuronal bodies are present in the intramural ganglia and in the outer layer of the bladder wall [42].

Primary afferent nerves seen beneath and penetrating the urothelium are also considered as a possible source of NO, as these nerves have NADPH-d activity [39-41]. In addition, the bladder afferent neurones in the L6 and S1 dorsal root ganglia, and in the dorsal horn of the spinal cord, have NADPH-d activity, although NOS-ir was not identified [42,43]. However, after chronic irritation of the bladder, NOS-ir in the spinal cord and dorsal root ganglia becomes increasingly detectable [44]. This suggests that the up-regulation of NOS in response to chronic inflammation may represent an adaptive mechanism to enhance the spinal nociceptive mechanisms or reflex responses elicited by nociceptive afferent input from the bladder. In this context, NO is thought to play a role in bladder hyper-reflexia after chronic irritation of sensory neurones in the lower urinary tract [44]. Indeed, intravesical capsaicin, because it destroys sensory nerves [45], has been used to treat detrusor hyper-reflexia in humans [46]. Although, capsaicin stimulates the release of neuropeptides such as calcitonin-gene related peptide (CGRP), substance P and VIP, recent evidence suggests that it also stimulates the release of NO from the bladder [47]. Furthermore, stimulation by capsaicin produces an endogenous NO-dependent relaxation in the isolated dog urethra [48]. At the spinal level, the impairment of the capsaicin-induced micturition reflex has been shown to be independent of NO [49], suggesting that capsaicin-induced release of NO may exert its effects locally by inhibiting the short loop reflexes within the bladder. In addition, the close proximity of NADPH-d-stained afferent nerve fibres to other neuropeptide-containing, capsaicin-sensitive afferent neurones [50] would suggest that NO released locally may also have an indirect influence on the sensory neural pathways in the urinary bladder.

Double-staining techniques have shown that NADPH-d-positive neurones also stain for ChAT (choline acetyl transferase) (a marker for parasympathetic neurones), tyrosine hydroxylase (TH, a marker for sympathetic neurones) and VIP in the lower urinary tract of several species, including man [29,50]. In a subpopulation of human bladder neck [51] and urethral [52] ganglionic cell bodies, both NOS-ir and TH-ir have been detected. Indeed, recent studies suggest that blocking the prejunctional α-adrenergic receptors influences NO-mediated smooth muscle relaxation in the rabbit urethra [53]. The NO donor sodium nitroprusside (SNP) also modulates the detrusor contractile responses to electrical field stimulation [54,55]. These findings indicate that apart from acting as a primary inhibitory neurotransmitter, NO may also serve as a neuro-modulator because it is closely associated with the cholinergic and sympathetic system.

NOS in the urothelium (fig. 2)

It is now clear that like the endothelium, the urothelial lining of the lower urinary tract also has NOS and produces NO [47]. Both eNOS and iNOS isoforms have been identified within this layer [21,22]. NOS in the rabbit urothelium has also been detected using a specific NOS radioligand ([3H]-N^-nitro-l-arginine, l-NOARG) [56]. Equally, the epithelial lining of the prostate expresses eNOS [21]. After exposure to NO donors urothelial cells also express cGMP-ir [35]. NO synthesis by the urothelium can be measured using a porphyrinic microsensor placed on the surface of the bladder, after stimulation by capsaicin (an afferent neurotoxin) and...
noradrenaline (an α/β-adrenergic agonist) [47]. The physiological role of NO, produced by the bladder urothelium, is not yet clear but it may serve as a source of peroxynitrite, especially in conditions of excess production [17]. In this context NO may play a role in oxidative injury after bladder outlet obstruction.

**NOS in smooth muscle**

There is no direct evidence that NO is produced by urinary tract smooth muscle. However, NO generated by electrically stimulated human detrusor smooth muscle strips mediates relaxation via mechanism(s) that are nerve-independent [57]. In both guinea pig and human proximal urethra, there is a prominent induction of cGMP-ir in the urethral smooth muscle by NO [35]. Despite the presence of significant NOS-ir in both human and guinea pig detrusor, cGMP-ir could not be induced in the detrusor muscle cells after stimulation with SNP. Instead, the interstitial cells within the detrusor expressed an intense cGMP-ir. These interstitial cells appear therefore to be the predominant target of NO in the detrusor.

**NOS in the external urethral sphincter**

NOS-ir has been detected in the striated muscle of several mammalian species [58,59]. Recent observations suggest that NO has a role in the control of intramural striated muscle of the human male membranous urethra and in the female urethral striated muscle [60,61]. Both NOS-ir and NADPH-d activity are evident in the sarcolemma of the intramural striated muscle fibres, and in the nerve trunks and fine nerve fibres in human male and female urethral striated muscle [60,61]. Whether the nerve fibres actually innervate the striated muscle remains to be elucidated. The association of NO with the sarcolemma suggests that it may have an inhibitory role, mediating relaxation of the striated sphincter of the membranous urethra during the voiding phase of micturition.

**NOS in blood vessels**

MNADPH-d staining is also detectable in the connective tissue and in the endothelium of small and large arteries supplying the lower urinary tract [18,33,56]. It is therefore conceivable that there are several sources of putative NO production in the urinary tract: the NANC neurones, the urothelium, smooth muscle cells and the blood vessels.

**Role of NO in the prenatal development of the lower urinary tract**

During human fetal development, the NOS-expressing nerves are mainly localized to the developing prostate gland/bladder neck, with minimal expression around the bladder [62]. During gestation there are more NOS-containing nerves, but they continue to predominate around the bladder neck and urethra [62]. NOS has been identified in the paraganglion cells within the bladder musculature of the developing human male fetus [63]. NADPH-d and NOS-ir are also expressed in most intramural neurones in the neonatal guinea pig urinary bladder [41]. Intrauterine inhibition of NO production increases the bladder capacity and postvoid residual volume, despite normal bladder pressures, suggesting inadequate sphincter relaxation [64]; these effects were reversed after administering L-arginine. Regional variations in the density of NOS-containing nerves and the differences in their temporal development in the lower urinary tract suggest that NO may play a role in the autonomic control of the lower urinary tract during fetal development.
Evidence of NO-dependent smooth muscle relaxation

Bladder neck and urethra

Before the discovery of NO, NANC-nerve mediated relaxation was first reported in the female rabbit [65], porcine urethra [66] and human bladder neck [67]. Evidence that NO was involved came by blocking the relaxation with L-NOARG, a NOS inhibitor [13,20]. These results were further confirmed using a selective cGMP phosphodiesterase inhibitor, which potentiated the relaxation, whereas methylene blue (a guanylyl cyclase inhibitor) reduced it [68]. NANC-mediated relaxation of the bladder neck and urethral smooth muscle is associated with increased cGMP levels [69]. These studies provided compelling evidence that NO is an important mediator in the relaxation of isolated urethral and bladder neck smooth muscle. In vivo studies also showed that NO mediates urethral smooth muscle relaxation in the rat after stimulation of parasympathetic efferent neurons [25-27]. Thus, NOS inhibitors abolished somato-urethral smooth muscle reflexes mediated by the parasympathetic efferent pathways after spinal cord injury in the female rat [70]. Furthermore, the inhibitory component of the somato-pelvic parasympathetic reflex uses NO as a neurotransmitter or neuromodulator at the level of the brain stem [71]. Also, stimulation of afferent nerve fibres in the bladder initiate a reflex that results in urethral smooth muscle relaxation, which is thought to be mediated by NO [72]. In vivo studies indicate that both baseline urethral pressure and maximum urethral relaxation are decreased by the systemic infusion of NO donors. Topical intraurethral NO donors have been shown to induce urethral smooth muscle relaxation without affecting the bladder smooth muscle function [73]. The intraurethral application of NO donors may therefore be clinically effective in cases of urethral smooth muscle sphincter spasticity and obstruction. Interestingly, the systemic administration of L-NOARG stimulates detrusor hyperactivity [74] and inhibits urethral smooth muscle relaxation during micturition [26]. These findings further suggest that detrusor instability secondary to BOO may be caused by the lack of NO bioactivity at the bladder outlet.

Detrusor

Unlike the bladder neck and urethra, there is as yet no convincing evidence that nerve-mediated relaxation of the detrusor muscle involves NO as a neurotransmitter. However, exogenous NO, as well as endogenous NO produced by the smooth muscle cells, induces detrusor smooth muscle relaxation [57,74]. Inhibiting NOS activity, either by the systemic or intravesical administration of NOS inhibitors, decreases the bladder capacity and increases the magnitude of bladder contractions [27,75]. Whilst the exact role of NO in detrusor physiology is unclear, it may be a factor in keeping the bladder relaxed during the filling phase of micturition. Indeed, NO is thought to have a similar function in the stomach, i.e. as a mediator of adaptive relaxation to accommodate food or fluid [76]. Furthermore, NO has recently been shown to regulate the blood supply to the detrusor during the filling and voiding phase of the micturition cycle [77]. In experimentally induced bladder inflammation, both NOS activity and basal blood flow to the bladder are reduced. Further studies are therefore needed to investigate if alterations in NO bioactivity play a role in the bladder ischaemia associated with distension after BOO.

Prostate and seminal vesicle

The nerves supplying the ventral and peripheral zone of the rat and human prostate, respectively, express NOS-ir [78,79]. NOS activity appeared to be greater in the peripheral zone than in the transition zone. In human prostatic tissue, dense NADPH-d staining and NOS-ir is present in the glandular epithelium, stroma and blood vessels [80]. An intense NADPH-d activity is also detectable in the glandular and stromal cells of seminal vesicles [81]. NOS activity was greater in the secretory epithelium than in the stromal component. Functionally, NO modulates prostatic and seminal vesicle smooth muscle tone [81,82]. The magnitude of prostatic smooth muscle relaxation to NO is significantly greater in human than in canine prostate. Although further studies are needed to understand the physiological role of NO in the prostate, castration leads to a reduction in NOS activity and cGMP formation by the prostate [83]. These effects were thought to be associated with apoptosis of prostatic endothelial cells. NO is therefore thought to play a role in supporting prostatic growth, regulating prostatic smooth tone and possibly having a neuroregulatory function in the male reproductive tract.

Mechanisms of NO-mediated effects in the lower urinary tract (fig. 3)

The exact mechanism by which NO mediates smooth muscle relaxation is not fully understood, but it is associated with an increase in the intracellular production of cGMP [16,33,35,38,40,68]. NO binds to the heme moiety of guanylyl cyclase after local production and diffusion. This step activates guanylyl cyclase which then catalyses the formation of cGMP
from GTP. The activation of a cGMP-dependent protein kinase has been suggested to hyperpolarize the cell membrane, possibly by activating K⁺ channels [84–86]. However, application of NO had no effect on the membrane potential of the rabbit urethral smooth muscle [87]. Other mechanisms of NO-induced relaxation, mediated by cGMP, may involve reduced intracellular Ca²⁺ levels by sequestration or reduced sensitivity to Ca²⁺ [88]. NO can also act independently of the cGMP pathway, e.g. the cytotoxic action of macrophage-derived NO on tumour cells and other tissue components is the result of nitrosylation and subsequent inactivation of iron-containing enzymes of the respiratory cycle and DNA synthesis [89]. Additional biochemical roles for NO include interactions with superoxide anions leading to the production of peroxynitrite [90]. This cytotoxic oxidant is thought to contribute to tissue injury in several pathophysiological situations, e.g. inflammation and ischaemic-reperfusion injury [90].

**NO and the pathophysiology of the lower urinary tract**

Alterations in the NO pathway are increasingly recognized in the lower urinary tract after BOO. Some insight into the pathophysiological role of NO has been provided from mice with a targeted deletion of nNOS. The bladders from nNOS-deleted mice became hypertrophic and markedly dilated [21]. Furthermore, the bladder outlet failed to relax in response to neuronal and exogenous NO [21]. Others have found that despite the disruption of the main pathway for the synthesis of neuronal NO, nNOS-‘knockout’ mice voided normally and had normal *in vitro* smooth muscle responses to various agonists [91]. In that study, there may have been some impairment in bladder function because there was an earlier recruitment of compensatory mechanisms to overcome the lack of neuronal NO. In a mouse model with partial BOO, the expression of iNOS was enhanced soon after obstruction [22]. RT-PCR studies also detected iNOS in most mice subjected to obstruction, with almost complete absence in controls [92].

Intramural ganglia in the bladder showed evidence of degeneration and cell death after acute complete outlet obstruction [93]. It is suggested that NO may be involved in neuronal death, as the NADPH-d activity and iNOS-ir in such neurones are increased [93]. Increased iNOS-ir may also be involved in excess NO production, leading to the formation of reactive nitrogen species such as peroxynitrite. These may in turn contribute to the ultimate development of bladder dysfunction.

Interestingly, the effects of ischaemic injury on the bladder have been shown to be reversed by NO inhibitor [94]. It is therefore conceivable that NO may also play a role in bladder ischaemic injury associated with acute retention. Furthermore, NO may also regulate bladder mucosal and muscle perfusion, which appears to be inversely related to intravesical pressure [77]. As NO, the product of iNOS, is a vasodilator, an increase in iNOS may be an initial compensatory response to overcome the effects of ischaemia generated by BOO [95]. Although alterations in NOS activity were not detected after BOO in a rat model, inhibiting NOS activity had different effects in the obstructed group compared with controls. There was an increased bladder capacity and maximum detrusor pressure in control animals; in contrast, there were no changes in the obstructed group [95]. This suggests that NO may have varying biological roles in different pathophysiological bladder states.

Inactivation of the cGMP-dependent protein kinase 1 (cGKI) gene in mice results in vascular and intestinal smooth muscle dysfunction. In the same animal model, NO/cGMP-dependent relaxation of urethral smooth muscle was also abolished. Furthermore, these animals developed bladder hyperactivity, suggesting that bladder instability may be associated with impaired NO/cGKI signalling [96]. These findings strongly suggest that manipulating the NO pathway may have a role in treating BOO. This concept is strengthened in that the

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orally administered nitrites has been shown to improve urinary flow rates, and reduce residual urine volume and the IPSS in patients with symptomatic BOO [97]. Interestingly, in rats, using myoblast-based iNOS gene therapy, NO-mediated urethral smooth muscle relaxation is also enhanced [98]. This approach may open the opportunity for gene therapy in the treatment of BOO. In addition, modulating cyclic nucleotide-dependent signal-transduction pathways in human detrusor smooth muscle by selective phosphodiesterase-inhibitors may also have a role in the treatment of detrusor hyperactivity [99].

**Diabetic cystopathy**

Alterations in bladder capacity, detrusor contractility and urinary flow rates are well recognized in diabetes mellitus [100]. Functional and morphological alterations in cholinergic and adrenergic nerves are also well documented. Recently, our group has identified increased NOS radioligand ([3H]-l-NOARG) binding sites and NADPH-d activity in the bladder neck and urethra of diabetic rabbits [56]. There was an increase in NOS in the urothelium, smooth muscle and intramural neurones. Despite this increase in NOS levels, NO-dependent urethral smooth muscle relaxation was impaired. This disparity between NOS receptor density and functional responses in the bladder outlet could be caused by the lower cGMP formation by the diabetic bladder neck and urethra than in the control [101]. Furthermore, alterations in the synthesis, release and/or quenching of NO by increased levels of advanced glycosylation end-products in diabetic tissues may contribute to decreased NO-mediated urethral relaxation. As a result of reduced activity of the NO pathway, the ability of the bladder outlet to relax may be impaired during the voiding phase. It will therefore be interesting to explore the therapeutic role of drugs that enhance the NO-cGMP signal in diabetic bladder dysfunction.

**Inflammatory disorders**

The production of NO is increased in response to inflammation after chronic irritation or infection [102]. Urinary NO levels, as well as expression of iNOS-ir and cGMP levels, are markedly increased in bacterial cystitis [103,104]. The measurement of urinary levels of NO has therefore been shown to differentiate bladder dysfunction caused by inflammation from neurogenic disorders or BOO [105]. The up-regulation of iNOS-ir is also evident in the afferent and efferent neurones within the bladder and pelvic ganglia in response to chronic irritation of the bladder [106] and cyclophosphamide-induced haemorrhagic cystitis [107]; these bladders become hyper-reflexic after chronic inflammation. Alterations in iNOS and cyclic GMP levels have also been described in interstitial cystitis [102]. NOS activity in bladder biopsy and urine samples is lower in patients with interstitial cystitis than in controls. A reduction in NOS may be secondary to either an inhibition of induction or an inability to express NOS. The cytokine interleukin 6 (an inhibitor of NOS) is elevated in urine from patients with interstitial cystitis [108]. In animal models of interstitial cystitis, decreased NOS activity is associated with alterations in bladder perfusion during the micturition process [109]. The intravesical administration of DMSO, used for the symptomatic relief of interstitial cystitis, stimulates the release of NO from bladder afferents [110]. This suggests that NO may be involved in the desensitization of nociceptive pathways in the lower urinary tract. Oral L-arginine has also been shown to improve symptoms in some patients with interstitial cystitis [111], thereby further strengthening this view.

**Spinal cord injury (SCI)**

 Interruption of the sacral parasympathetic outflow to the lower urinary tract after damage to the sacral spinal cord or spinal roots results in bladder areflexia and urinary retention. Alterations in adrenergic and cholinergic innervation to the lower urinary tract after SCI are well known [112]. Recently, NO has been implicated in voiding dysfunction after SCI. There is an up-regulation of nNOS-ir in bladder afferents and spinal neurones of the sacral parasympathetic nucleus after chronic SCI [113], but NO release by the rat bladder after stimulation by capsaicin is significantly reduced in chronic SCI [114]. These early findings indicate that NO may play a pathophysiological role in voiding disorders associated with SCI.

**Urinary tract cancer**

NO is involved in regulating cell growth and cell differentiation [89,115,116]. At low concentrations, NO can stimulate cell growth, whereas high concentrations result in cytostatic effects. High iNOS-ir was identified in prostatic cancer cells, in contrast to their complete absence in benign prostatic epithelial cells [117]. Further studies also indicate that by inhibiting NOS activity, neovascularization and proliferation of human prostate cancer cell lines were reduced [118]. Both human prostate cancer tissues and prostate cancer cell lines also express eNOS as the active isofrom [118]. Changes in its expression are associated with increased growth of prostate cancer and of angiogenesis. These studies suggest that NO may influence the progression of prostate cancer.
After the intravesical administration of BCG, to treat bladder carcinoma in situ and recurrent bladder cancer, there is a marked increase in NOS activity [119]. In the rat bladder, BCG up-regulates the gene and protein expression of iNOS, but eNOS and nNOS are also induced, albeit to a lesser extent [120,121]. The expression of Ca^{2+}-independent NOS activity by bladder tumour cell lines (MBT2 and T24) are also induced by treatment with cytokines [122]. This effect is reversed by L-nitroarginine. As a result of increased iNOS, high concentrations of NO are produced, which are thought to modulate the growth of bladder cancer cell lines. Thus, the tumoricidal effects of BCG in the treatment of bladder cancer may involve NO-dependent mechanisms.

Conclusion

This review has highlighted both the physiological and pathophysiological roles of NO in the lower urinary tract. As alterations in the NO pathways are being increasingly recognized in the pathophysiology of lower urinary tract disorders, manipulating the NO-cGMP axis may form the basis for new therapeutic interventions.

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Potential Role of Endothelin and Nitric Oxide in Physiology and Pathophysiology of the Lower Urinary Tract

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(Received 03 February, 1999; In final form 03 February, 1999)

Endothelium-derived vasoactive mediators (endothelin-1 with its vasoconstrictive and mitogenic properties and nitric oxide with its vasodilatory and antiproliferative properties) play an important role in the regulation of vascular smooth muscle tone and cellular proliferation. Several recent studies have now demonstrated the presence of these vasoactive agents in the urinary tract where they are thought to play a prominent role in urinary tract physiology and disease. This article reviews the synthesis, localisation and actions of endothelin and nitric oxide in the lower urinary tract and examines the possible role of these mediators in disease.

ENDOTHELIN-1

(a) Synthesis and localisation of receptors

Endothelin-1 (ET-1) is a 21 amino acid peptide formed from a precursor, proET-1 (big endothelin), by the action of ET-1 converting enzyme (ECE-1) whose cDNA has been cloned and functionally expressed (Yanagisawa et al., 1988; Arai et al., 1990; Ikura et al., 1994; Schmidt et al., 1994). The presence of ET-like immunoreactivity and ET-1 mRNA in the epithelium, smooth muscle and fibroblasts of the urinary bladder provides evidence that ET is synthesised locally (Saenz de Tejada et al., 1992). ET-like immunoreactivity and ECE-1 mRNA has also been detected in the prostatic epithelium (Langenstroer et al., 1993; Walden et al., 1998). Furthermore, genes coding for ET-1 biosynthesis and its receptors are expressed in the human prostate indicating that this peptide is being produced in this organ (Prayer-Galletti et al., 1997). These results suggest that ET-1 may be a paracrine mediator of bladder and prostatic smooth muscle contraction and proliferation. This proposed paracrine mechanism is similar to that observed in vascular tissues where ET-1 is synthesised in the endothelium and exerts several physiological and pathophysiological effects on the vascular smooth muscle (Rubanyi et al., 1994).

Autoradiographic and radioligand binding studies have demonstrated the presence of ET_A and ET_B
receptors in the epithelium and smooth muscle of the bladder and prostate in humans and animal species (Kondo et al., 1993; Kobayashi et al., 1994; Traish et al., 1995; Le Burn et al., 1996; Mumtaz et al., 1997 and 1999). ET$_{A}$ and ET$_{B}$ receptor densities vary between different regions of the urinary bladder. (Latifpour et al., 1995; Mumtaz et al., 1999). The density of both ET receptors in the bladder dome is greater than in the bladder base and urethra. The predominant receptor subtype in the bladder dome is the ET$_{A}$ whilst both ET$_{A}$ and ET$_{B}$ receptors are approximately of equal proportion in the bladder base and urethra (Latifpour et al., 1995; Mumtaz et al., 1999). Regional variations in the distribution of ET receptor subtypes have also been described in the human prostate (Prayer-Galetti et al., 1997). For example, within the peripheral zone, where prostate cancer is initiated, the ET$_{A}$ and ET$_{B}$ receptor binding sites are found predominantly in the glandular epithelium and smooth muscle (Prayer-Galetti et al., 1997). In contrast, in the zone where benign prostatic hyperplasia (BPH) predominates, the ET$_{A}$ and ET$_{B}$ receptor subtypes are found only in the smooth muscle (Prayer-Galetti et al., 1997). This suggests that the function of ET-1 may vary depending on the region and the distribution pattern of its receptors in the lower urinary tract.

(b) Effects of ET on smooth muscle tone

ET-1 elicits potent and long-lasting receptor-dependent contractions in smooth muscle strips from the urinary tract and prostate in humans and several animal species (Maggi et al., 1989 and 1990; Gra西亚-Pascual et al., 1990; Saenz de Tejada et al., 1992; Langenstroer et al., 1993 and 1997; Kobayashi et al. 1994; Mumtaz et al., 1997 and 1999). The contractile responses to ET-1 in the rat and rabbit bladder are predominantly mediated via the ET$_{A}$ receptor subtype (Donoso et al., 1994; Mumtaz et al., 1999). The exact role of ET$_{B}$ receptors in the bladder is not clear, since its activation has not been shown to elicit either contraction or relaxation. The activation of the nitric oxide (NO) pathway via ET$_{B}$ receptors has been demonstrated in vascular tissues (Hirita Y et al., 1993; Moritoki et al., 1993). However, we can only specu-

late that a similar response occurs in the urinary tract smooth muscle. In vitro studies on human and canine prostatic smooth muscle strips indicate that both receptors mediate contraction (Kobayashi et al., 1994; Langenstroer et al., 1994). The magnitude of ET-1-induced contractions was at least 80% of the contraction elicited by the stimulation of alpha-adrenergic receptors. In comparison to in vitro studies, in vivo experiments indicate that although prostatic urethral pressure is raised following the intravenous administration of ET-1, the magnitude of this change is only 30% of that elicited by alpha-adrenergic receptors (Imajo et al., 1997). The discrepancy between the in vitro and in vivo studies leads to the hypothesis that the systemic administration of ET-1 may stimulate reverse compensatory mechanisms such as NO release by the prostatic epithelium. This additional effect may result in prostatic smooth muscle relaxation (Takeda et al., 1995).

(c) Effects of ET on cell proliferation

In addition to its contractile properties, ET-1 is also a potent mitogen of prostatic smooth muscle cells (Nelson et al., 1996; Walden et al., 1998). The proliferation of smooth muscle cells in response to ET-1 has been shown to correlate with ET-receptor density (Kanse et al., 1995). Both ET$_{A}$ and ET$_{B}$ receptors have been implicated in this response. For example, ET$_{B}$ receptors participate in the development of intimal hyperplasia after endothelial vascular injury (Azuma et al., 1995). In contrast, in the human airway smooth muscle cell proliferation is ET$_{A}$ receptor-dependent (Panettieri et al., 1996). However, both ET$_{A}$ and ET$_{B}$ receptors have been shown to have mitogenic properties on prostatic smooth muscle cells (Nelson et al., 1996; Walden et al., 1998).

(d) Mechanisms responsible for ET-1-mediated smooth muscle contraction

The calcium dependence of ET-1-mediated contractile responses in the urinary tract is tissue and species specific. For example, ET-1 induced contractile
responses in the rabbit urinary bladder smooth muscle can be antagonised by dihydropyridine calcium channel blockers, whilst human prostate smooth muscle contractions to ET-1 are independent of these calcium channels. ET-1 elicits its effects by binding to and activating its receptors, which subsequently leads to an increase in intracellular Ca^{2+} concentration via either transmembrane Ca^{2+} influx and/or Ca^{2+} liberation from intracellular storage sites (Maggi et al., 1989; Gracia-Pascual et al., 1990). Despite the fact that the peak elevations in intracellular Ca^{2+} are known to be transient, ET-1 is able to elicit long-lasting and sustained contractions. This may be mediated via Ca^{2+} sensitisation mechanisms, which have been described in diverse smooth muscles (Kitazawa et al., 1991).

(e) Pathophysiological role of ET-1 in the urinary bladder and prostate

Recent studies have implicated alterations in ET receptor density and distribution in the pathophysiology of lower urinary tract disorders such as diabetic cystopathy, benign prostatic hyperplasia (BPH) and prostatic cancer. An increase in the expression of ET receptors in the ureter and prostate of experimentally induced diabetic rats (Saito et al., 1995 and 1996) has been observed. The increase in ET receptors in the diabetic prostate did not correlate with serum testosterone levels. This is of interest because low testosterone concentrations have been implicated as a cause for a reduced prostatic growth and down-regulation of muscarinic receptors in diabetic animals. We have recently demonstrated an up-regulation of ET\(_B\) receptors in both the dome and bladder neck of the urinary bladder in alloxan-induced diabetic rabbit (Mumtaz et al., 1999). ET-1 induced contractile responses were impaired in the bladder neck whilst no changes were evident in the dome of diabetic animals. The impairment of ET-1 responses in the bladder neck could be due to the release of NO by the activation of ET\(_B\)-receptors. The lack of inhibition in the detrusor is presumably due to a low sensitivity of detrusor smooth muscle to NO. Alterations in the density of ET receptors in the bladder and prostate have also been observed secondary to bladder outflow obstruction (Kondo et al., 1993). The density of the ET receptors was found to decrease in the bladder and increase in prostates of patients with BPH (Kondo et al., 1995). This increase in prostatic ET receptor density may contribute to the raised intraurethral pressure associated with bladder outflow obstruction. These results support the rationale for developing selective endothelin antagonists for the treatment prostatic outflow obstruction.

The influence of ET-1 on cellular proliferation and its synergism with several other peptide growth factors suggests that it may have a role in bladder and prostatic smooth muscle hyperplasia associated with diabetes and bladder outflow obstruction. Our observations (unpublished) indicate that both ET\(_A\) and ET\(_B\) receptors may be involved in the proliferation of bladder smooth muscle cells in the presence of diabetic sera. These findings support a possible role of ET in the control of bladder wall structure.

Several studies have now also suggested that ET-1 may exert a potentially significant role in the pathophysiology of prostatic cancer. Human seminal fluid contains the highest concentration of ET-1 in any body fluid studied, approximately 500 times more than plasma (Battistini et al., 1993). Plasma ET-1 concentrations are significantly increased in men with metastatic prostatic cancer (Nelson et al., 1995). ET-1 also increases alkaline phosphatase activity in new bone formation, indicating that it may be a mediator of the osteoblastic response of bone to metastatic prostate cancer (Nelson et al., 1995). In addition, ET-1 attenuates apoptosis of smooth muscle cells in BPH (Wu-Wong et., 1997), whilst on prostate cancer cells it acts both as an independent mitogen as well as a stimulator of the mitogenic effects of other important growth mediators (Nelson et al., 1996). The mitogenic effect of ET-1 was ET\(_A\) receptor-mediated. Interestingly, ET\(_B\) binding sites that are present in benign prostatic epithelial tissues have not been demonstrable in both prostate cancer cell lines and at metastatic prostate cancer sites. Thus, it appears that during prostate cancer progression to metastases, ET-1 and ET\(_A\) expression are retained, whereas ET\(_B\) receptor expression is reduced. ET-1, as an indepen-
dent mitogen, may provide a mechanism by which androgen-independent prostate cancer progression occurs (Nelson et al., 1996). Indeed clinical trials are underway investigating the effect of ETA receptor antagonists in hormone refractory metastatic prostatic cancer progression.

NITRIC OXIDE

(a) Localisation of putative NO production sites in the urinary tract

The field of NO research has expanded beyond endothelium-dependent relaxation (Palmer et al. 1987). It has now become clear that many different cell types synthesise NO from L-arginine. NO is a short acting free radical, which is produced in humans and other mammals via the catalytic action of the enzyme NO synthase (NOS). Three isoforms of NOS have been isolated and cloned; endothelial, neuronal and inducible (macrophage) isoforms (Forstermann et al., 1994). Endothelial (eNOS) and neuronal (nNOS) isoforms are normal constituents of their respective cells. The inducible isoform (iNOS) is not a normal constituent of healthy cells, but is induced within endothelial cells, smooth muscle cells and immune cells, in response to stimulation by certain cytokines or bacterial endotoxins (Moncada et al., 1991; Nussler et al., 1996). All three isoenzymes have been identified in the lower urinary tract (Andersson et al., 1994; Ehren et al., 1994; Dokita et al., 1994; Burnett et al., 1997).

NO production is also thought to occur in the urothelium and smooth muscle. Staining with eNOS antiserum in the bladder has demonstrated its presence in the urothelial lining (Burnett et al., 1997). The association of eNOS with the urothelium has demonstrated that this isoform of NOS is not restricted to the endothelium. In addition, eNOS has also been identified in several other epithelial linings such as the bronchus (Schmidt et al., 1992), epididymis (Burnett et al. 1995) and prostate (Burnett et al., 1995). Our group, has demonstrated the presence of NOS in the rabbit urothelium using a specific NOS radioligand ([3H]-L-NOARG) and nicotinamide adenine dinucleotide phosphate diaphorase (NADPH-d) histochemistry (Mumtaz et al., 1999). Cyclic GMP-immunoreactivity has been identified in urothelial cells following exposure to NO donors (Smet et al., 1996). It has also been proposed that that the epithelial lining of the urethra can convert arginine into citrulline (Dokita et al., 1994). Interestingly, stimulation by capsaicin (an afferent neurotoxin) as well as norepinephrine (alpha/beta-adrenergic agonist) has been shown to release NO by the urothelium (Brider et al., 1998). These findings therefore suggest that the urothelium is a potential source of NO and, if it can diffuse into the muscle layer, it may play a role in detrusor and urethral smooth muscle function. To date, no detailed studies have been performed to evaluate this possible role of urothelial NO in the lower urinary tract. Detrusor relaxation is also mediated by NO produced from the smooth muscle rather than the nerves (James et al., 1993). It is therefore conceivable that at least four putative sources of NO production are found in the urinary tract: the NANC neurons, the urothelium, smooth muscle cells and the blood vessels.

Neuronal NO produced by the nonadrenergic, noncholinergic (NANC) nerves in the lower urinary tract in both humans and several animals species is considered to play an important role in the integration of the micturition process (Andersson & Persson, 1994; Burnett et al., 1997). These NO-producing nerves are predominant in the region of the bladder neck and urethra compared to the detrusor (Smet et al., 1994 and 1996). NADPH-d positive neuronal bodies are evident in the intramural ganglia as well as in the outer layer of the bladder wall (McNeill et al., 1992; Keast et al., 1994). NOS containing afferent and efferent postganglionic neurons supplying the urinary bladder has been demonstrated (Vizzard et al., 1994). These neurons originate from the major pelvic ganglia. They are seen to preferentially project to the bladder neck and urethra suggesting that NO plays a predominant role in the bladder outlet region compared to the detrusor. The role of postganglionic afferents is not completely clear since the prevalent NADPH-d activity in the bladder and urethral affer-
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ents in the L₅ and S₁ dorsal root ganglia was not paralleled by the presence NOS-immunoreactivity (Vizzard et al., 1994).

(b) Evidence of NO-dependent smooth muscle relaxation: in vitro studies

NANC-mediated relaxation was first reported in female rabbit urethra (Andersson K-E, 1983) and female porcine urethra and bladder neck (Klarskov et al., 1983), but the transmitters involved in mediating these responses were not established. Evidence that NO was involved came by blocking the relaxation with N⁭-nitro-L-arginine, a NOS inhibitor (Andersson et al., 1991 & 1992). These results were further confirmed using a selective cyclic GMP phosphodiesterase inhibitor which potentiated the relaxation whilst methylene blue reduced it (Dokita et al., 1991). Subsequently, several investigators have demonstrated the involvement of NO in the relaxation of isolated urethral and bladder neck smooth muscle in various animal species (Garcia-Pascual et al., 1991; Thornbury et al., 1992; Triguero et al., 1993; Persson et al., 1992 and 1993; Bridgewater et al. 1993) and humans (Leone et al., 1994). Unlike the bladder neck and urethra, there is as yet no convincing evidence that nerve-mediated relaxation of the detrusor muscle involves NO as a transmitter. However, exogenous as well as endogenous NO produced by the smooth muscle does induce a relaxation effect on detrusor muscle (James et al., 1993; Chung et al., 1996). Therefore, the role of NO in the detrusor is not clear. It has been suggested that NO could be a factor maintaining bladder relaxation during filling. In fact, NO is thought to have such a function in the stomach, i.e. as a mediator of adaptive relaxation to accommodate food or fluid (Desai et al., 1991).

In addition NO has also been shown to cause relaxation of human and canine prostate smooth muscle (Takeda et al., 1995). Further studies are needed to understand the physiological role of NO in prostatic tissue.

(c) Mechanism of NO-mediated smooth muscle relaxation

Although the mechanism by which NO mediates smooth muscle relaxation is not fully understood, a general observation is that this response is linked to an increase in intracellular cyclic GMP levels (Persson et al., 1994). Activation of a cyclic GMP-dependent protein kinase has been suggested to hyperpolarise the cell membrane, possibly by activating K⁺ channels, (Robertson et al., 1993, Peng et al., 1996). There have also been studies suggesting that NO might act directly on the K⁺ channels (Waldeck et al., 1995). Other mechanisms for NO-induced relaxation, mediated by cyclic GMP, may involve reduced intracellular Ca²⁺ levels by sequestration or reduced sensitivity to Ca²⁺ (Warner et al., 1994). Both mechanisms probably act without changing the membrane potential.

(d) In vivo role of NO

The exact role of NO in the micturition process is not clearly understood. In vivo experiments have demonstrated that urethral smooth muscle relaxation in the female rat is mediated by NO released by the parasympathetic postganglionic efferent neurons (Fraser et al., 1997). Systemic administration of L-NOARG has been shown to stimulate detrusor hyperactivity (Persson et al., 1991) and inhibit urethral smooth muscle relaxation during micturition (Bennett et al., 1995). These findings suggest that the detrusor hyperactivity secondary to bladder outlet obstruction may be due to the lack of NO bioactivity. Intrathecal injections of NOS inhibitors do not alter the cystometrogram or electromyographic parameters, suggesting that spinal NO pathways may not play a role in the normal micturition process. However, the facilitation of the micturition reflex by nociceptive bladder afferents activated by noxious chemical irritation of the bladder does involve NO at the spinal level (Kakizaki & de Groat 1996). Interestingly, it has also been proposed that afferents in the bladder stimulate a pelvic nerve-mediated urethral relaxation reflex mediated by
NO (Bennett et al., 1995). Further work is needed to clarify these issues.

(e) Role of nitric oxide in vesicourethral dysfunction

Insight into the pathophysiological role of NO has been provided from mice with targeted deletion of nNOS. The bladders from nNOS-deleted mice become hypertrophic and markedly dilated. More interestingly, the bladder outlet fails to relax in response to field stimulation and to NO (Burnett et al., 1997). Others have found that despite the disruption of the main pathway for the synthesis of neuronal NO, nNOS knockout mice voided normally, demonstrated normal organ bath responses and have a normal number of all nerves studied (except those staining for NO) (Sutherland et al., 1997). The absence of any impairment in bladder function in this study may have been due to the presence of compensatory mechanisms to overcome the lack of neuronal NO. It is also interesting to note those animals that showed alterations in the voiding pattern weighed more than those without any bladder dysfunction. This observation implies that animals with greater body weights were more likely to be older and hence had a more prolonged absence of nNOS, that may have led to bladder dysfunction.

NOS-immunoreactivity is also up-regulated in bladder afferent neurons in response to neurotropic factors released following chronic irritation. These bladders become inflamed and demonstrate hyperalgesia as well as hyper-reflexia, thus, implicating NO (Vizzard et al., 1996). Interestingly, it has been shown that NO is involved in the facilitation of the micturition reflex by noxious chemical irritation of the bladder (Kakizaki & de Groat 1996). Alterations in iNOS and cyclic GMP levels have also been described in interstitial cystitis and urinary tract infections (Smith et al., 1996). We have also demonstrated an up-regulation of NOS binding sites and NADPH-d activity in the bladder neck and urethra of diabetic rabbits (Mumtaz et al., 1999). The increase in NOS was evident in the urothelium, smooth muscle and intramural neurons. Despite this increase in NOS levels, urethral smooth muscle relaxations to NANC nerve stimulation involving NO as a neurotransmitter was impaired. The disparity between NOS activity and the functional responses to NO in the bladder neck and urethra could be due to the reduction in the production of cyclic GMP in diabetes mellitus by these tissues (Mumtaz et al., 1998). These alterations in the NO pathway may have a pathophysiological role in the urinary bladder dysfunction associated with diabetes mellitus.

CONCLUSIONS

This review provides an up-date on the potential role of ET-1 and NO in the lower urinary tract. It also raises questions from which further research could be initiated to enhance our understanding of the role of these mediators in the physiology and pathology of the lower urinary tract.

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Role of prostaglandins in the urinary bladder: an update

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Summary  Our knowledge of prostanoids is rapidly increasing. In this review we survey the factors governing the synthesis of prostanoids by the urinary bladder, their role in the maintenance of normal bladder function, the pattern of their secretion in bladder disease and the possible use of prostanoids in the treatment of bladder pathology.

INTRODUCTION

Prostaglandins (PGs) are found in virtually all tissues where they exert a wide variety of functions including modulating smooth muscle activity, haemostasis and cytoprotection. The urinary bladder is no exception.

The urinary tract provides a highly adapted system of conduits, which allow the conversion of a continuous involuntary production of urine by the kidneys into the intermittent, consciously controlled, voiding of urine (micturition). The urinary bladder has two main functions; the collection and low-pressure storage of urine and its subsequent voluntary expulsion. Disruption of the normal function of the bladder may produce symptoms that exert major effects on the quality of life. This abnormality may either be the consequence of local pathological disorders affecting the bladder and its outflow tract or of disordered neural control of the detrusor muscle.

THE URINARY BLADDER AS A SITE OF PROSTAGLANDIN SYNTHESIS

Most early studies reported that the urinary bladder tissue can synthesize several prostanoids of the E and F (PGE, PGF) series. Bladder tissue also synthesizes substantial amounts of prostacyclin (PGI2). Animal work (rabbit) has demonstrated that the body of the bladder produces significantly more PGs than the base and that both the outer layer and the inner mucosal layer are capable of PGE2 synthesis. Both the bladder smooth muscle and the transitional cell epithelium have the capacity to synthesize PGE2.

In isolated rat whole bladder preparations there is a progressive build-up of PGI2, PGE2 and thromboxane A2 (TXA2) intraluminally. This finding is compatible with the view that transitional epithelium can synthesize these prostanoids or that, they can 'pass' through this epithelial layer.

Mucosal biopsies taken from healthy areas of the human bladder during cystoscopy produced prostanoids in the following quantitative order: PGI2>PGE2>PGF2α>TXA2. However, specific radioimmunoassays and gas chromatography/mass spectrometry demonstrated a significant quantitative predominance of PGF2α and TXA2 over PGE2. In normal rats, the excretion of TXB2 and 6-oxo-PGF2α (the stable, spontaneous breakdown product of PGI2) was significantly greater in the urine collected from the bladder than in urine collected from the ureters leading into the bladder. These findings suggest that the bladder contributed significantly to the amount of eicosanoids present in the urine.

In a study involving the measurement of urinary concentrations of prostanoids in children (age 5-15 years) the excretion of 6-oxo-PGF2α was higher in males, whereas PGE2 excretion was comparable in both sexes. Combined multiple regression analysis revealed that age was the only significant influence on prostanoid secretion.
FACTORS MODULATING PROSTANOID RELEASE BY THE BLADDER

Distension (with buffer solutions) in isolated whole rat bladders resulted in an increase in the production of PGI₂, PGE₂, and TXA₂ intraluminally even in the absence of exogenous substrate (arachidonic acid). This response to distension appears to be common to several organs since it has also been observed in portal and peripheral veins. Increased intraluminal osmolarity also resulted in enhanced bladder prostanoïd synthesis in vitro with PGI₂ remaining the main product quantitatively. The optimum pH for prostanoïd production, using rat bladder 'minces', was established as 7.0–8.0.

The expected inhibition of prostanoïd synthesis by non-steroidal anti-inflammatory agents (NSAIDs), like indomethacin, has been demonstrated. It is of interest that some of these workers reported incomplete inhibition (approximately 80–90%) of in vitro prostanoïd synthesis by bladder tissue in the presence of indomethacin. This observation is in agreement with findings in other organs. The residual production of PGE₂ despite the administration of NSAIDs is consistent with the presence of prostanoïds, albeit in reduced amounts, in the urine following the administration of these drugs. There is also evidence that prostanoïds produced locally are further metabolized by bladder tissue, or alternatively that the re-uptake of prostanoïds occurs.

It would appear that prostanoïd release by bladder tissue may be mediated by the stimulation of specific receptors. We incubated rat bladder tissue until the trauma-stimulated surge of PGI₂ was reduced to a minimal amount. Although this tissue no longer spontaneously produced significant amounts of PGI₂, it responded to muscarinic agonists by releasing substantial amounts of this prostanoïd. In the same model, adrenergic stimuli were without effect and did not antagonize muscarinic-stimulated PGI₂ release.

ATP-activated purinoceptors (P₂ purinoceptors) can also stimulate the release of eicosanoids by the rat bladder detrusor muscle. PGE₂ and 6-oxo-PGF₁α were the main prostanoïds released. TXB₂ was only present in minimal amounts. The effects of drugs administered systemically does not necessarily exclusively reflect direct actions on the bladder. For example, the administration of indomethacin (or other NSAID) exerts variable inhibition on different aspects of renal function (haemodynamics; sodium; calcium and magnesium excretion). Such effects could in turn influence local prostanoïd synthesis by the bladder (by altering urine osmolarity, pH or volume). Furthermore, it is known that the concentration of certain ions (calcium and potassium) in incubates can influence prostanoïd synthesis in other tissues. Urinary prostanoïd concentrations are influenced by urinary pH, urinary sodium and potassium concentrations, level of exercise and variations in fluid intake. Dietary sodium intake also influences PG levels in the urine. Sodium depletion in normal man is associated with increased excretion whereas acute replenishment of sodium (physiological saline intravenously) resulted in decreased excretion. Dietary prostanoïd intake also influences the urinary concentrations of PGs in the rat. In this context, it is of interest that the potassium concentration in incubates influences PGI₂ synthesis in other systems. Glutathione (GSH) regulates PG synthesis in microsomes of the porcine urinary bladder epithelium. At a GSH concentration of less than 10⁻⁵M, microsomes produced more PGI₂ and PGF₁α than PGE₂. At higher GSH concentrations, PGE₂ synthesis was enhanced but PGI₂ and PGF₁α synthesis was inhibited. This reciprocal effect of GSH was also observed in rabbit and bovine urinary bladder epithelium.

URINARY BLADDER PROSTAGLANDINS AND BLADDER TONE

PGs are thought to contribute to the maintenance of bladder tone and the process of micturition. This conclusion is based on the observation that PGE₂ can cause dose-related contractions of detrusor muscle strips in vitro. Incubation with indomethacin (an inhibitor of prostanoïd synthesis) caused a reduction in tone and loss of spontaneous activity in this in vitro model. Furthermore, the addition of PGE₂ or PGF₁α in the presence of indomethacin restored bladder strip tone and spontaneous activity. In vivo work using normal conscious rats has shown that intravesically instilled PGE₂ facilitated micturition and increased intravesical pressure. PGE₂ given intra-arterially produced a distinct increase in bladder pressure before initiating a micturition reflex, indicating that PGE₂ had a direct contractant effect on the detrusor smooth muscle.

SC-19220, a competitive receptor antagonist of PGE₂, can increase bladder capacity and reduce the voiding efficiency of micturition of anaesthetized rats. The effect of SC-19220 was prevented by indomethacin pretreatment. The spontaneous contractile force of muscle strips isolated from rabbit urinary bladder dome and base increased in a dose-dependent manner, by the administration of PGE₂, PGE₁, or PGF₁α. Isolated muscle strips from the bladder dome responded to PGs more markedly than those from the bladder base. The rank order of potency (to induce contractile responses) was PGF₁α > PGE₁ > PGE₂ in both dome and base muscles. These effects were significantly inhibited by pretreatment with verapamil (a calcium antagonist) suggesting that these smooth muscle contractions in response to PGs are dependent on calcium influx. These findings also suggest that calcium may be an important component of PG-induced bladder smooth muscle contraction.
support the concept that endogenous PGE$_2$ and other PGs are physiologically involved in the regulation of vesicourethral motility.

PGI$_2$ caused a dose-dependent contraction of the strips from the rabbit bladder body, base and urethra.$^{37}$ The contractile activity of PGI$_2$ was greatest in the bladder body. PGI$_2$ was less potent than PGE$_2$ or PGF$_{2\alpha}$ and contractions induced by this prostanooid were slower in onset and short in duration.$^{37}$ This phenomenon is also calcium influx dependent.$^{38-41}$ In the rabbit urinary bladder PGE$_{1\alpha}$, PGE$_2$ and PGF$_{2\alpha}$ have all been shown to increase adenylate cyclase activity; dose-dependently, in the dome and base.$^{42}$ Hence, it is thought that PGs act on the urinary tract via cyclic AMP (cAMP) as well as calcium influx.

**BENEFICIAL EFFECTS OF PROSTAGLANDINS**

1. **Postoperative urinary retention**

Postoperative urinary retention is a common complication that may affect either sex and all age groups after any operation (especially pelvic surgery).$^{43}$ In studies involving women who had undergone vaginal hysterectomy,$^{43-45}$ or operations for stress incontinence,$^{46}$ the intravesical administration of PGE$_2$ enhanced bladder function and was associated with less postoperative urinary retention.$^{43-45}$ Among similar lines, intravesical PGF$_{2\alpha}$ instillation reduced the frequency of urinary retention after vaginal hysterectomy$^{47}$ and after operations for stress incontinence.$^{48,49}$

2. **Local cytoprotection by prostaglandins**

Certain prostanooids appear to play a cytoprotective role in several organs, possibly through the control of local mucous secretion.$^{50-56}$ For example, in the stomach, the inhibition of prostanooid synthesis is associated with an increased incidence of peptic ulceration.$^{53-55}$ Furthermore, certain prostanooids prevent the development of peptic ulcers in experimental animal models and may be of therapeutic benefit in man.$^{53-55}$ Analogies with these findings are now available for the bladder, since the urothelium possesses protective systems similar to those in the gut$^{57}$ and the local instillation of PGE$_2$ or a PGF$_{2\alpha}$ derivative improved the symptoms from schistosomal bladder ulcers.$^{58,59}$

3. **Haemorrhagic cystitis**

Haemorrhagic cystitis is a major complication of cyclophosphamide therapy.$^{58-60}$ Intravesical instillation of PGE$_2$, PGF$_{2\alpha}$ and PGF$_{2\alpha}$ have all been shown to be beneficial in the treatment of haemorrhagic cystitis.

4. **Bacterial cystitis**

Certain prostanooids raise intracellular cAMP levels, which in turn increases local glycosaminoglycan production.$^{52}$ It is not surprising, therefore, that NSAIDs (inhibitors of prostanooid synthesis) impair glycosaminoglycan synthesis.$^{53}$ This prostanooid-glycosaminoglycan link may be of clinical relevance since glycosaminoglycans appear to inhibit the direct contact and adherence of chemicals and bacteria to the bladder mucosal surface.$^{54}$ However, we are not aware of any study showing that patients taking NSAIDs have an increased risk of developing cystitis. It is of some interest, however, that desquamation of the bladder mucosa and other histological changes have been reported in patients taking an excess of analgesics (phenacetin, propylphenazone).$^{34,48}$ In a recent review, Bramble and Morley$^{65}$ emphasized that NSAIDs, especially tiaprofenic acid, can cause cystitis. Other surveys are also in broad agreement with their views.$^{66-69}$ This mechanism was predicted by our group almost a decade ago.$^{70}$ The reason why tiaprofenic acid may cause more cystitis than some other NSAIDs may relate to the fact that 90% of the ingested dose is excreted unchanged in the urine. This property would result in more extensive inhibition of urothelial PG formation.$^{71}$ We have also observed (unpublished results) some similarity in the histological changes in the bladder in reported cases of tiaprofenic acid-related cystitis and following the administration of this NSAID to rats. Clinically, it is important to always consider that the cause of 'cystitis' in some patients may be related to the treatment they are receiving (i.e. iatrogenic cystitis). Changes in urinary PGE$_2$ concentrations during acute bacterial cystitis have been demonstrated,$^{72}$ with raised levels in the early stage of infection. These levels remain elevated even when the symptoms decrease.$^{72}$ It is possible that this PG response is either a defence mechanism against infection or the result of local tissue damage. These findings also suggest that an impaired PG response to infection may be one of the factors responsible for recurrent cystitis.

5. **Interstitial cystitis**

PGE$_2$ excretion is also increased in interstitial cystitis.$^{73}$ Thus, as in bacterial cystitis, PGE$_2$ may play a role in the pathogenesis of interstitial cystitis.

6. **Radiation cystitis**

Local radiotherapy to the bladder can be associated with radiation cystitis.$^{74-77}$ Intravesical instillation of PGF$_{2\alpha}$ in patients with cervical carcinoma has beneficial effects on vesical haemorrhage due to radiation cystitis.$^{76-77}$
Carcinoma and Prostaglandins

Diminished cytoprotection is implicated in carcinogenesis. In this context it is of interest that β-naphthylamine (a bladder carcinogen present in cigarette smoke) and cigarette smoke extracts (but not nicotine) inhibit rat bladder PG synthesis, in vitro. These findings offer a potential explanation for the reported increased incidence of bladder malignancy in smokers. It is also possible that the association between cystitis and an increased incidence of bladder cancer is mediated by one of two mechanisms. Firstly, recurrent episodes of cystitis result in progressive impairment of local cytoprotective prostacyclin synthesis as a consequence of tissue damage. Secondly, recurrent cystitis is itself commoner in bladders with an inherent diminished ability to synthesize cytoprotective prostanooids.

It is also of interest that both cyclophosphamide-induced cystitis and schistosomal involvement of the bladder are associated with an increased incidence of bladder cancer and that both these conditions are improved by intravesical PG administration.

As well as exerting a cytoprotective effect, some prostanooids are also thought to be carcinogenic. A major effect of NSAIDs is the inhibition of cyclooxygenase. This is the rate-limiting enzyme for the conversion of arachidonic acid to important signal molecules, including PGs. In this context, it is of interest that PGH synthase, an arachidonic acid-dependent peroxidase, is implicated in the peroxidative activation of carcinogenic aromatic amines in extrahepatic carcinogen target tissues, catalyzes the first step in the synthesis of PGs from arachidonic acid. This process can be accompanied by the co-oxidation of xenobiotics resulting in extrahepatic and local tissue production of reactive carcinogenic products. Furthermore, the end product PGs, especially PGE2, can significantly affect cell proliferation. High levels of PGE2 stimulate growth of certain tumour cell lines while inhibition of PG synthesis with indomethacin or piroxicam can cause suppression. As such, it has been demonstrated that PGE2 inhibited interleukin-2 (IL-2)-mediated proliferation of lymphokine-activated killer (LAK) cells in a concentration-dependent manner. Pretreatment of LAK cells with indomethacin significantly enhanced cytotoxicity against bladder cancer cell lines. Others have also suggested that PGE2 may be involved in the promotion of bladder carcinogenesis. Finally, it has been shown that bradykinin and tissue plasminogen activator (tPA) appear to increase PGE2 levels by enhancing arachidonic acid availability through separate phospholipase pathways.

Prostanooids and Diabetes Melliitus

In man, diabetes mellitus (DM) can be associated with cystopathy with bladder dilatation and incomplete voiding. These effects, which in turn, may lead to an increased risk of local infection. In a rat model of streptozotocin-induced DM, the increased contractile response to substance P and electrical field stimulation was markedly decreased by the administration of indomethacin. These observations suggest that the enhanced contractile responses were due to increased synthesis of PGs. This conclusion is compatible with the finding that in long term non-ketonuric DM, in the same model, there was enhanced PGI2 release by the bladder. This change was accompanied by a marked increase in bladder weight. This increased capacity to release PGI2 may be due to distension or to a raised urinary osmolarity secondary to glycosuria. The actual distension of the bladder appears to be a consequence of diabetic neuropathy, since damage to local nerves has been histologically demonstrated in the diabetic rat. Furthermore, experimental bladder denervation in the non-diabetic rat is associated with gross bladder distension and hypertrophy.

The sustained enhanced PGI2 synthesis, in vitro, by bladder tissue obtained from DM rats is compatible with the reported increase in urinary 6-oxo-PGF1α excretion in experimental DM in the same animal. The absence of such an increase in urinary excretion in insulin-treated diabetic animals further supports a link between DM and abnormal PGI2 production by the bladder. It is tempting to speculate that the increased release of PGI2 in long-term diabetic rats is a compensatory response to neurogenic distension.

In contrast to the findings in the DM rat model we found a significant reduction in PGI2 production using a rabbit model of alloxan-induced DM. This apparently paradoxical finding requires clarification. However, it may be relevant that, in our hands, DM in the rat model is associated with a three-fold increase in bladder weight. This change is accompanied by a fall in body weight when compared with control animals. These changes are much less marked in the rabbit model of DM. Future studies should also investigate if other causes of bladder distension/hypertrophy (such as outflow obstruction) are associated with enhanced PGI2 release.

Prostaglandins in Detrusor Instability

Detrusor instability is a urodynamic diagnosis made when the detrusor is shown objectively to contract, spontaneously or on provocation, during the filling phase of a cystometrogram while the patient is attempting to inhibit micturition. Detrusor instability is often responsible for symptoms of urgency, frequency, nocturia, urge incontinence and nocturnal enuresis, but is not synonymous with any of them. The influence of intravenous administration of PGE2 and sulprostone, a PGE2 derivative, on urodynamic indices of the lower urinary tract in healthy
women demonstrated that these significantly decrease urethral closure pressure at rest.\textsuperscript{99} PGE\textsubscript{2} increased the detrusor opening pressure as well as the detrusor pressure during maximum flow.\textsuperscript{99} In other words, the bladder became overactive, thereby, causing a strong urgency sensation resulting in a reduced bladder capacity and measurable bladder instability.\textsuperscript{99} Hence, as PGE\textsubscript{2} is thought to play a role in bladder instability it has been proposed that the use of PG synthetase inhibitors in women with perimenstrual exacerbation of their symptoms may be useful.\textsuperscript{98}

**PROSTAGLANDINS AND ENURESIS**

Enuresis (bed-wetting) is a common problem in children.\textsuperscript{100-102} Urinary PGE\textsubscript{2} before and during desmopressin treatment in patients with monosymptomatic primary enuresis (PME) demonstrated that children who did not respond to this treatment had an overnight urinary PGE\textsubscript{2} level that was greater than those who responded or were normal subjects.\textsuperscript{103} These differences suggest that the pathogenesis of PME may be related to an increase in PGE\textsubscript{2} production in the bladder.\textsuperscript{99}

**AGEING AND THE ROLE OF PROSTAGLANDINS IN THE URINARY BLADDER**

PGs produce contractile responses in elderly rabbit bladders\textsuperscript{104} and the magnitude of these responses varies with age.\textsuperscript{104} Experiments on the effects of ageing on the intravesical pressure in female rats have demonstrated that PGF\textsubscript{2a} causes an increase in the intravesical pressure.\textsuperscript{104} However, a study investigating the possible aetiology of unstable bladder in the elderly by comparing aged and young rat bladders demonstrated no difference in PGF\textsubscript{2a}-mediated responses.\textsuperscript{105} Thus, PGF\textsubscript{2a} is unlikely to contribute to the aetiology of bladder instability in the elderly.\textsuperscript{106-108}

**PROSTAGLANDINS AND PROSTATIC DISEASE**

Although it is not within the remit of this review, it is nonetheless important to consider that the prostate has an intimate functional and pathological relationship with the bladder.\textsuperscript{109-111} This organ is also a source of prostanooid production.\textsuperscript{112} Hence the prostate may influence urinary prostanooid levels. There is also evidence that PGE\textsubscript{2} may play a significant role in the proliferation of prostate cancer cell lines.\textsuperscript{113,114} In a population-based study, the regular use of NSAIDs was associated with a reduced risk of developing advanced prostate cancer,\textsuperscript{113} possibly because these drugs (e.g. flurbiprofen) inhibit cell growth.\textsuperscript{114} PGF\textsubscript{2a} and PGE\textsubscript{1} have also been found to exert a growth promoting effect in the 'benign' prostate.\textsuperscript{115} Hence, prostanooids may also play a role in the development of benign prostatic hyperplasia (BPH).

**CONCLUSIONS**

It is evident that the urinary bladder has the capacity to produce substantial amounts of several prostanooids. These 'mediators' may play a role in modulating normal bladder function (e.g. voiding and the sensation of fullness). Prostanoids, as well as contributing to the defence against infection and carcinogens, have been found to be beneficial in the treatment of a wide variety of diseases including haemorrhagic and radiation cystitis. However, as well as having beneficial effects, prostanooids have also been associated carcinogenesis. Unfortunately, measuring prostanooid levels is unlikely to be of use as a diagnostic tool because of technical limitations. However, controlling the effects of prostanooids may prove to be of therapeutic benefit.

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Effects of prostaglandins on histopathology of male
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**Upergulation of Endothelin A Receptor Sites in the Rabbit Diabetic Kidney: Potential Relevance to the Early Pathogenesis of Diabetic Nephropathy**

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**Key Words**
Diabetes mellitus • Kidney • Endothelin • Diabetic nephropathy

**Abstract**

**Background/Aim:** Nephropathy is an important complication of diabetes mellitus (DM). The plasma endothelin 1 (ET-1) levels are increased in DM, and ET-1 may cause deleterious effects on renal function. We, therefore, investigated whether changes in ET receptors occur in the DM rabbit kidney.

**Methods:** Nine adult New Zealand White rabbits were injected with alloxan, of which 6 became diabetic; the other 3 acted as alloxan-treated controls. Six age-matched healthy rabbits served as controls. At 6 months, following cervical dislocation, the kidneys were removed, and sections (cortex and medulla) were incubated with ET\(_A\) and ET\(_B\) radioligands to produce low- and high-resolution autoradiographs. Immunohistochemical localization of ET-1 immunoreactivity was also performed. **Results:** There was greater ET\(_A\) and ET\(_B\) receptor binding in the control (ET\(_A\) \(p = 0.0003\); ET\(_B\) \(p < 0.0001\)) and DM (ET\(_A\) \(p = 0.001\); ET\(_B\) \(p < 0.0001\)) rabbits in the medulla as compared with the cortex. DM kidneys showed a significant increase in ET\(_A\), but not ET\(_B\), binding in the cortex (\(p < 0.0001\)) and in the medulla (\(p < 0.0001\)). High-resolution autoradiographs revealed striking \([\text{125I}]\)-ET-1 receptor binding predominantly to the glomeruli. Immunohistochemistry revealed dense ET-1 immunoreactivity associated with the renal tubules, but the glomeruli exhibited no staining. Alloxan-treated controls had similar results to age-matched controls. **Conclusion:** There are regional differences in both ET\(_A\) and ET\(_B\) binding in control and DM kidneys. ET\(_A\) receptor binding sites are increased in the DM kidney (cortex and medulla). ET-1 may act in a paracrine fashion on the glomeruli. These changes may contribute to the pathogenesis of diabetic nephropathy.

**Introduction**

Endothelin 1 (ET-1) belongs to a family of potent vasoconstrictor peptides consisting of 21 amino acids [1, 2]. To date two major ET receptors have been identified and...
cloned: ET$_A$ and ET$_B$ [3-5]. Endothelin is produced by, and binds to, most renal cell types [6]. ET-1 is known to cause a profound and prolonged renal vasoconstriction [7, 8]. ET-1 is also known to cause mesangial cell contraction [9, 10] and inhibition of sodium and water reabsorption by the nephron [11-14], to enhance glomerular cell proliferation [15, 16], and to stimulate extracellular matrix accumulation [17]. Previous studies have indicated that the vasoconstriction is mediated primarily by ET$_A$ receptors located on smooth muscle cells [18, 19].

Plasma ET-1 levels are elevated in patients with diabetes mellitus (DM) [20, 21] and in experimental DM [22]. ET-1 is also thought to play a role in progressive renal disease [23], and the plasma ET-1 levels are elevated in diabetic nephropathy [24]. We, therefore, used a combination of immunohistochemistry (to identify ET-1) and autoradiography to study the role of ET receptors in the pathogenesis of DM nephropathy. We used a rabbit model, since it has been previously demonstrated that it shares many similarities with human DM [25].

**Materials and Methods**

**Induction of Diabetes**

Age-matched 3-kg male New Zealand White rabbits ($n = 15$) were selected, 9 of which were injected intravenously (via the lateral ear vein) with alloxan (Sigma Chemical, Poole, UK) at a single dose of 65 mg/kg to induce non-ketotic DM. Six of these rabbits became diabetic, and the other 3 acted as alloxan-treated controls. All rabbits were fed ad libitum with SDS standard rabbit plain (SDS, Witham, UK) and allowed free access to water.

**Blood Sampling**

Blood was sampled at monthly intervals for serum urea and electrolyte, cholesterol, triglyceride, and glucose determinations. Urine was analyzed for glucose, ketone bodies, and protein using Multistix (Ames Division, Miles Laboratories, Stoke Poges, UK) [26]. In addition, we pooled the renal function results from the present study with those of our previous experiments involving control and diabetic rabbits sampled after 6 months.

**Preparation of Kidney Tissue**

Following cervical dislocation, the kidneys were excised from age-matched controls ($n = 6$), from the diabetic rabbits 6 months ($n = 6$) after induction of DM, and from the alloxan-treated controls ($n = 3$). Kidney tissues were stored immediately at $-70^\circ$C in airtight containers. The kidney blocks containing cortex and medulla were dissected and subsequently mounted in AMES OCT embedding compound (BDH Laboratory Supplies, Poole, UK) and transverse 10-µm sections cut in a cryostat at approximately $-20^\circ$C and thaw mounted onto gelatinized (for autoradiography) or aminopropyltriethoxysilane-coated (for immunohistochemistry) microscope slides. The slides were stored at $-70^\circ$C in airtight containers until use.

**Autoradiographic Studies: Quantitative Assessment of $[^{125}I]_P$D151242 (ET$_A$) and $[^{125}I]_B$Q3020 (ET$_B$) Binding to Rabbit Kidney**

Consecutive serial 10-µm rabbit kidney sections were initially preincubated in 50 mmol/l Tris-HCl buffer (pH 7.4) for 15 min at 22°C in order to reduce endogenous peptide levels. Slide-mounted sections were then incubated for 120 min at 22°C in buffer (plus 5 mmol/l MgCl$_2$, 1% bovine serum albumin, and 100 kIU aprotinin/ml) containing 0.15 mmol/l $[^{125}I]_P$D151242 (ET$_A$, receptor specific radioligand) [27] and 0.15 mmol/l $[^{125}I]_B$Q3020 (ET$_B$ receptor specific radioligand) [28]. Both radioligands had a specific activity 2,000 Ci/mmol (Amersham International, Amersham, UK). These concentrations were at the approximate Ke$_T$ values established from previous saturation studies [29]. The degree of non-specific binding was established by incubating alternate sections in the presence of 1 mmol/l unlabelled ET-1 - non-specific binding for $[^{125}I]_A$ET-1 was <10% of the total binding, undetectable for $[^{125}I]_B$PDI51242, and approximately 15% for $[^{125}I]_B$BQ3020. The slides were washed (two times for 10 min at 4°C) in buffer, dipped in distilled water at 4°C, and dried in a stream of cold air. Low-resolution autoradiography was carried out by exposing sections to Hyperfilm 3H (Amersham International) in X-ray cassettes for up to 4 days.

Densitometric analysis was performed using an imaging system (model GS-700 imaging densitometer; Bio-Rad, Hemel-Hempstead, UK). Binding was finally expressed in terms of radioligand bound (disintegrations/min; dpm) per unit area (mm$^2$), calculated from standard curves generated by microscales (Amersham International) that were co-exposed with tissue sections.

Microscopic localization (high-resolution autoradiography) of binding sites was performed by post-fixing tissue in paraformaldehyde vapour (2 h at 80°C) and coating slides in molten nuclear emulsion (LM-1, Amersham International) [30]. The slides were then stored in lightproof boxes for up to 8 days at 4°C, after which they were processed in D19 high-contrast developer (Kodak Pathé, Chalon-sur-Saône, France) and fixed (Hypam; Ilford, Mobberley, UK). The underlying tissue was stained with haematoxylin and eosin, and the high-resolution autoradiographs were viewed using an Olympus Vanox microscope; selected sections were photographed where appropriate.

**Immunohistochemical Localization of ET-1**

Immunohistochemical localization of ET-1 immunoreactivity was performed using the avidin-biotin-peroxidase complex technique [31] on acetone fixed 10-µm rabbit kidney tissue sections. The procedure involved sequential application of a primary antibody (1:100 dilution, anti-ET-1 monoclonal antibody; Peninsula Laborato­ries Europe, Merseyside, UK) followed by biotin-labelled secondary antibody (1:500 dilution, goat antimouse IgG antibody; Dako Laboratories, High Wycombe, UK) and then the avidin-biotin-peroxidase complex (Vector Laboratories, Peterborough, UK).

**Statistics**

The results are expressed as median and range. Two-tailed Mann-Whitney U test (unpaired values) and Wilcoxon two-tailed test (paired values) were used for the statistical analyses.
Table 1. Urea, electrolyte, and serum glucose levels in control and DM rabbits

<table>
<thead>
<tr>
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<th>Reference values</th>
<th>Control median (range)</th>
<th>Diabetic median (range)</th>
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<tbody>
<tr>
<td>Glucose, mmol/l</td>
<td>7.0 (5.2–7.8)</td>
<td>6.7 (6.0–8.4)</td>
<td>33.4* (32.7–35.0)</td>
</tr>
<tr>
<td>Urea, mmol/l</td>
<td>5.9 (5.1–6.5)</td>
<td>5.8 (4–7.5)</td>
<td>8.3** (6.5–12.4)</td>
</tr>
<tr>
<td>Creatinine, μmol/l</td>
<td>83 (77–128)</td>
<td>92 (77–125)</td>
<td>113** (97–153)</td>
</tr>
<tr>
<td>Na+, mmol/l</td>
<td>143 (139–145)</td>
<td>144 (141–148)</td>
<td>137** (131–147)</td>
</tr>
<tr>
<td>K+, mmol/l</td>
<td>4.7 (3.6–5.2)</td>
<td>4.4 (3.6–5.2)</td>
<td>4.5 (3.5–5.7)</td>
</tr>
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</table>

Combined results of the present and previous studies (n = 14). * p < 0.001; ** p < 0.0001.

Based on 12 healthy animals.

Results

Animal Weights, Urea, Electrolytes, and Serum Glucose

The starting weights in both the control and diabetic rabbit groups were similar [control: median 3.0 (range 2.9–3.1) kg; DM: 3.2 (range 3.0–3.4) kg, n = 6]. At the end of 6 months, the diabetic rabbits were significantly (p < 0.001) lighter than the control group animals [control: 4.3 (range 3.9–4.8) kg; DM: 3.5 (range 3.0–3.8) kg]. When the pooled renal function values were considered (n = 14), the serum urea and creatinine values were significantly (p < 0.0001 and p = 0.0008, respectively) elevated in the DM rabbits, whereas the serum sodium content was significantly (p = 0.0006) lower. There was no significant change in the serum potassium levels (table 1).

When the results of the present study were considered alone (n = 6), the same pattern was observed, but only the urea was significantly (p = 0.003) raised in the DM animals (results not shown). Despite the significant differences in serum urea, creatinine, and sodium between the control and DM groups, the median creatinine value (113 μmol/l) in the DM group was well within the reference range (77–128 μmol/l). Therefore, there was no significant renal failure. The median serum urea level (8.3 mmol/l) was outside the reference range (5.0–6.5 mmol/l). The serum sodium level (137 mmol/l) was marginally below the reference range (139–145 mmol/l). None of the animals developed ketonuria.

The serum glucose concentrations (non-fasting) were significantly (p = 0.002) elevated in the 6-month diabetic group (median 33.4, range 32.7–35.0 mmol/l) when compared with the control group (median 6.7, range 6.0–8.4 mmol/l).

Autoradiography

Receptor-binding studies indicated [125I]-PD151242 (ET-A-specific) and [125I]-BQ3020 (ET-B-specific) binding to kidney tissue sections of control and diabetic rabbits.

ET_A and ET_B Receptor Binding Sites

There was dense [125I]-PD151242 binding to tissue sections; less marked [125I]-BQ3020 binding to tissue sections was seen. ET_A- and ET_B-binding sites exhibited regional variations in both the control and diabetic kidney tissues (fig. 1, table 2). There was significantly greater binding to the medulla as compared with the cortex in control tissues for both ET_A (p = 0.0003) and ET_B (p < 0.0001) selective radioligands. A similar binding pattern was also seen in diabetic tissue (ET_A p = 0.001, ET_B p < 0.0001; fig. 1).

Densitometric analysis of film images indicated that [125I]-PD151242 binding (ET_A sites) showed a significant increase in both medulla (p < 0.0001) and cortex (p < 0.0001) of diabetic kidney tissues. [125I]-BQ3020 (ET_B receptor binding sites) did not exhibit a significant increase in these regions of diabetic kidney tissues (table 2).

Examination of the high-resolution autoradiographs revealed striking [125I]-ET-1 receptor binding that was predominantly to the glomeruli (fig. 2).

Densitometric analysis of kidney sections from alloxan-treated controls showed similar results to age-matched controls (results not shown).

Immunohistochemical Localization of ET-1

The immunohistochemical studies revealed dense positive ET-1 immunoreactivity that was associated with the renal tubules. Interestingly, the glomeruli exhibited no staining whatever (fig. 2, 3).
Table 2. Photodensitometric analysis of ET\(_{A}\) and ET\(_{B}\) receptor binding in kidney cortex and medulla of control and 6-month diabetic rabbits

<table>
<thead>
<tr>
<th>Receptor binding</th>
<th>Control median (range)</th>
<th>Diabetic median (range)</th>
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<tr>
<td>Cortex ET(_{A})</td>
<td>6.5 (3.0–15.6)</td>
<td>26.0* (17.6–43.7)</td>
</tr>
<tr>
<td>ET(_{B})</td>
<td>0.17 (0.01–1.30)</td>
<td>0.21 (0.09–0.70)</td>
</tr>
<tr>
<td>Medulla ET(_{A})</td>
<td>16.4 (4.9–39.4)</td>
<td>46.4* (22.9–68.8)</td>
</tr>
<tr>
<td>ET(_{B})</td>
<td>8.6 (7.1–12.1)</td>
<td>9.3 (7.4–12.3)</td>
</tr>
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</table>

\* p < 0.0001.

In the DM rabbit kidney, there was significantly increased ET\(_{A}\) receptor binding in both cortex and medulla as compared with control tissues. High-resolution autoradiographs revealed striking \(^{125}\)I-ET-1 receptor binding, predominantly to the glomeruli. However, ET-1 immunoreactivity was absent in the glomeruli, although dense staining was associated with the surrounding tubules.

Hence, it appears that ET-1 is synthesized by the tubules and acts on receptors present in the glomeruli. These results were present in the absence of any significant renal failure, since the median creatinine level remained within the reference range. The high urea levels in the DM rabbits may be attributed to polyphagia and the catabolic state associated with DM [32]. It is unlikely that the DM-induced changes in ET receptors or renal function indices were drug related, since the alloxan-treated controls showed similar results to age-matched controls.

Diabetic nephropathy is characterized by hypertrophy of both glomerular and tubulo-epithelial structures, thickening of the glomerular and tubular basement membranes, progressive accumulation of the extracellular matrix component in the glomerular mesangium and in the interstitium, and vascular ischaemic changes [33–35]. Glomerulosclerosis and tubulo-interstitial fibrosis may eventually occur. These changes progress through a number of stages; from an inflammatory to a proliferative response and then finally to scarring [36]. Endothelial injury is thought to be an early event initiating an inflammatory process within the glomeruli [36]. Glomerular endothelial, epithelial, and mesangial cells synthesize, bind, and respond to ET-1 [6, 9–10]. As plasma ET-1 levels are elevated in diabetics and in animal models of this disease [20–22], glomerular endothelial injury may play a role in the increased synthesis of ET-1. Glomerular endothelial injury results in pro-inflammatory, prothrombotic, and mitogenic changes [37]. ET-1, predominantly through the stimulation of its ET\(_{A}\) receptors, is mitogenic [38]. Hence, the upregulation of ET\(_{A}\) receptors may play a role in endothelial mitogenesis. The damaged endothelium releases factors, such as platelet-activating factor and platelet-derived growth factor (PDGF), that mediate the aggregation of platelets and endothelial proliferation.
Fig. 2. ET-1 binding and ET-1 immunoreactivity on diabetic rabbit kidney sections. A High-resolution autoradiograph (dark-field illumination, where binding sites are evident as white grains on a black background) of $[^{125}I]^{-}$ET-1 binding to a diabetic kidney section (cortex). B Haematoxylin and eosin stained tissue underlying A (gl = glomeruli). C Adjacent section used for immunohistochemistry. Positive ET-1 immunoreactivity is indicated by brown stain (note absence of positive staining at glomeruli). Section counterstained with haematoxylin (blue). Scale bar = 250 μm.

Fig. 3. ET-1 immunoreactivity in rabbit renal tubules. A Positive ET-1 immunostaining (brown reaction product) of renal tubules from a section of a diabetic rabbit kidney. B Negative control from an adjacent section incubated in the absence of primary antibody. Scale bar = 100 μm.
This leads to the formation of microthrombi in the glomerular capillaries of experimental animals and humans with chronic renal disease [39]. ET-1 causes a profound renal vasoconstriction (both afferent and efferent glomerular arterioles) via ETA receptors [40]. The upregulation of ETA receptors may, therefore, enhance this vasoconstriction. This, along with the formation of microthrombi, could compromise the renal medullary circulation.

ET-1 potently stimulates mesangial cell mitogenesis and has been shown to partially mediate the proliferative response to PDGF [41]. Therefore, the upregulation of ETA receptors may play a role in glomerular mesangial cell proliferation in DM. With further progression, scarring is produced which then proceeds to mesangiosclerosis and fibrosis. Here, transforming growth factor beta (TGFB-β) appears to be one of the most fibrogenic growth factors. TGFB-β may also initiate glomerulosclerosis through the transdifferentiation of mesangial cells into myofibroblasts [42]. Since ET-1 can induce the synthesis of TGFB-β and PDGF [17], it is not surprising that in diabetic patients there is an increased production of TGFB-β [43]. The treatment of diabetic rats with an ETA receptor antagonist can lead to a reduction in the TGFB-β expression in glomeruli as well as a decrease in albuminuria and matrix gene expression [44]. Hence, the ET-1-induced expression of TGFB-β may be mediated via ETA receptor stimulation. The upregulation of ETA receptors in a rabbit model of DM may, therefore, be involved in the increased production of TGFB-β. Proteinuria is thought to be nephrotoxic and a very good predictor of the rate of progression of chronic renal failure [45, 46]. Since ETA receptor antagonists can reduce proteinuria [44], the upregulation of ETA receptors may increase proteinuria, leading to further renal damage.

Tubulo-interstitial scarring also starts with inflammation. Proximal tubular cells when injured can synthesize and release PDGF and TGFB-β [47] which then attract inflammatory cells. This can lead to tubulo-interstitial necrosis, oedema, and ultimately to fibrosis. The synthesis of TGFB-β stimulates tubular synthesis of collagen [47]. PDGF is thought to stimulate fibroblast proliferation [48]. Since ET-1 can induce, probably via ETA receptor stimulation, the synthesis of TGFB-β and also PDGF [17] in DM, it may play a role in collagen synthesis and fibroblast proliferation. Hence, the upregulation of ETA receptors may be associated with increased TGFB-β bioavailability, resulting in increased collagen synthesis.

The progression of tubulo-interstitial fibrosis is characterized by the appearance of interstitial myofibroblasts expressing anti-smooth-muscle antigen [49]. The interstitial, peritubular, and periglomerular accumulation of myofibroblasts is an early event in renal fibrogenesis [49]. The intensity of the myofibroblastic infiltrate is a strong predictor of subsequent renal fibrosis and decline in renal function [50, 51]. Since TGFB-β and PDGF active myofibroblasts [42, 52], the upregulation of ETA receptors may play a role in the activation of interstitial myofibroblasts.

In summary, DM can lead to progressive renal disease. We have demonstrated, in a rabbit model of DM, that there is an upregulation of ETA receptors in cortex and medulla. ET-1, via its ETA receptor, may play a role in the development of DM nephropathy. The ETA receptor changes occur in the presence of 'normal' renal function or a very early stage of DM nephropathy. Hence, it is worth considering that ET antagonists, administered at this stage, may prevent/retard the occurrence of further changes leading to nephropathy. Whether these early changes are reversible remains to be established.

**Acknowledgements**

We thank Roe Gibbins for her help with the immunoreactivity work. We also thank the British Heart Foundation for supporting Dr. Dashwood. Dr. Khan is supported by the Charles Wolfson Charitable Trust.

**References**


Endothelin A Receptors in the Rabbit
Diabetic Kidney


Autoradiographic Localisation and Contractile Properties of Prostatic Endothelin Receptors in Patients with Bladder Outlet Obstruction


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Key Words
Endothelin receptors (ET$_A$ and ET$_B$) · Benign prostatic hyperplasia

Abstract

Objectives: Previous studies have used endothelin (ET) receptor agonists and antagonists to localise ET receptor subtypes in prostatic tissue. We have utilised high affinity ET$_A$ ([$^{125}$I]PD151242) and ET$_B$ ([$^{125}$I]BQ3020) receptor-specific radioligands to determine the density and distribution of ET receptor subtypes in prostatic tissues obtained from patients with symptomatic benign prostatic hyperplasia (BPH). The contractile properties of the ET receptor subtypes as well as the effect of ET-1 on $\alpha_1$-adrenergic receptor-mediated prostatic smooth muscle contraction were assessed.

Patients and Methods: Saturation binding and quantitative autoradiographic studies were performed using specific radioligands for ET$_A$ and ET$_B$ receptors on prostate sections obtained from patients with bladder outflow obstruction secondary to BPH. In vitro isometric tension studies were carried out to characterise the ET receptor subtypes in prostatic smooth muscle strips from the same group of patients. In addition, the effect of ET-1 on $\alpha_1$-adrenergic receptor-induced prostatic smooth muscle contraction was also investigated.

Results: There were dense ET$_A$ and ET$_B$ receptor-binding sites in the prostatic stroma. ET$_A$ receptor-binding sites were also prominent on the prostatic epithelium. ET-1 and sarafotoxin 6c (ET$_B$ receptor agonist) elicited prostatic smooth muscle contraction ($\log EC_{50}$ 8.31 ± 0.15 and 8.22 ± 0.22 M, respectively). Both BQ123 (ET$_A$ antagonist) and BQ788 (ET$_B$ antagonist) significantly inhibited ET-1- and S6c-mediated prostatic smooth muscle contractile responses, respectively. ET-1 at sub-threshold concentrations significantly enhanced $\alpha_1$-adrenergic receptor-mediated prostatic smooth muscle contractile responses.

Conclusions: ET$_A$ receptor-binding sites are prominent in both prostatic stroma and epithelium, whereas ET$_B$ receptor-binding sites were predominantly seen in the prostatic stroma in symptomatic BPH. Both ET$_A$ and ET$_B$ receptors mediate prostatic smooth muscle contraction. ET-1 en-
hances α₁-adrenergic receptor-mediated contractile responses, suggesting that ET may play a pathophysiological role in bladder outlet obstruction associated with BPH.

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Introduction

Symptoms associated with bladder outflow obstruction in patients with benign prostatic hyperplasia (BPH) may result from either progressive enlargement of the prostate gland and/or from prostatic smooth muscle contraction mediated by prostatic α₁-adrenergic receptors. Recent studies suggest that endothelin-1 (ET-1), a 21-amino acid peptide [1], mediates prostatic smooth muscle concentration [2], and also has growth-promoting effects on prostatic cells [3-4].

Two ET receptor subtypes, ETₐ and ETₐ, have been characterised and cloned [5, 6]. Pharmacological and molecular studies indicate that both receptor subtypes are present in the human prostate [7]. ETₐ receptors were thought to predominate in the prostatic smooth muscle and ETₐ receptors in the glandular epithelium [8]. However, recently, the distribution of ET receptor subtypes in different zones of the prostate gland has been characterised in greater detail [9]. For example, in the transition zone, involved in development of BPH, both ET receptor subtypes were identified only in the stroma. While in the peripheral zone, associated with the initiation of prostatic cancer, ETₐ receptors were predominantly localised to the stroma and ETₐ to the glandular epithelium. Indeed, using membrane homogenates, an increase in ETₐ receptors has been identified in BPH tissues compared to normal [10, 11], suggesting that ET may have a pathophysiological role in prostatic disease. Such regional and disease-induced alterations in ET receptor subtypes have also been observed in the urinary tract [12, 13].

The distribution of ET receptor subtypes in symptomatic BPH has not been clearly defined since previous studies appear to have had several limitations. Firstly, prostatic tissue was obtained from patients undergoing radical prostatectomy for prostatic cancer with no evidence of clinical BPH [8]. Secondly, the analysis of ET receptor-binding sites in BPH tissues was carried out using membrane homogenates, limiting the information on the tissue distribution of ET receptor subtypes [10]. Finally, the distribution of ET receptors was based on differential binding affinities of sarafotoxin 6c (S6c) and cyclic pentapeptide BQ123 at ¹²⁵I-ET₁-binding sites. Since ET receptor-specific radioligands are now available [14, 15], we investigated the density and distribution of ETₐ and ETₐ receptors in BPH tissues obtained from patients with clinical evidence of bladder outlet obstruction. Low- and high-resolution autoradiographs were generated using specific ET receptor radioligands to localise and quantify ET receptor density in prostatic stromal and epithelial tissues. ET-1 antagonists and S6c (ETₐ agonist) were used to assess the functional role of ET-1 in BPH using prostatic tissue from these patients. The effect of ET-1 on α₁-adrenergic receptor-mediated prostatic smooth muscle contraction was also investigated.

Patients and Methods

Prostatic tissue was obtained from 10 patients undergoing transurethral resection of the prostate for bladder outlet obstruction secondary to BPH. The mean age of the patient was 68 (range 58-79) years. All patients had normal serum prostatic specific antigen levels (range 1.8-3.9 ng/ml) and urodynamic studies that confirmed bladder outlet obstruction (mean maximum voiding detrusor pressure of 78 cm H₂O and mean maximum flow rate of 7.8 ml/s). The mean prostatic volume was 53.3 (range 28-68) cm³. All patients and histological evidence of BPH. The possible coexistence of cancer or any other prostatic disease was ruled out, both clinically and histologically. The prostatic tissue obtained represented the transition zone of the prostate, since it is the site at which BPH occurs.

Preparation of Tissues

Following collection, some of the prostatic chips were stored immediately at −70°C in air-tight containers and the rest stored in cold Krebs' buffer solution. The tissues stored at −70°C were then mounted in AMES OCT embedding compound (BDH Laboratory Supplies, Poole, UK) and transverse 10-μm sections were cut in a cryostat, at approximately −20°C, and thaw-mounted onto gelatinised microscope slides.

Autoradiographic Studies

Saturation Binding Analysis of [¹²⁵I]-ET₁, [¹²⁵I]PD151242 (ETₐ) and [¹²⁵I]BQ3020 (ETₐ). Preliminary binding studies (saturation analysis) were performed, where competitive 10-μm transverse sections were preincubated in 50 mM Tris-HCl buffer (pH 7.4) for 15 min at 22°C in order to reduce endogenous peptide levels. Incubations were then carried out (2 h at 22°C) in buffer containing 5 mM MgCl₂, 1% bovine serum albumin and 100 kallikrein international units/ml aprotonin in the presence of 0.003-1 nM [¹²⁵I]-ET₁, [¹²⁵I]PD151242 (ETₐ) and [¹²⁵I]BQ3020 (ETₐ) (Amersham International, Amersham UK). Non-specific binding was established by incubating adjacent sections in the presence of 1 μM unlabelled ET₁ (Bachem Fine Chemicals, Switzerland). Receptor distribution was assessed by exposing incubated tissue to Hyperfilm 3H (Amersham International) and receptor density was determined on a Bio-Rad GS-700 imaging densitometer (Bio-Rad Laboratories, Hemel Hempstead, UK) from curves generated by ¹²⁵I microscales (Amersham International) that were co-exposed with the tissue sections. Receptor density (Bmax) and

Autoradiographic Localisation and Contractile Properties of Prostatic Endothelin Receptors

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affinity (K<sub>D</sub>) were then calculated using GraphPad Inplot Software (Graph Pad, San Diego, Calif., USA).

**Quantitative Assessment of [125I]-ET-1, [125I]PD151242 (ET<sub>a</sub>) and [125I]BQ3020 (ET<sub>b</sub>) Binding.** Consecutive prostate sections (10 µm) were preincubated, as described above, and then incubated for 120 min, at 22°C, in buffer containing 0.20 nM [125I]-ET-1, and 0.15 nM of [125I]PD151242 and [125I]BQ3020 (concentration at the approximate K<sub>D</sub> values established from the saturation studies described above). The degree of non-specific binding was established by incubating alternate sections in the presence of 1 µM unlabelled ET-1. The slides were washed twice in buffer for 10 min, dipped in distilled water at 4°C and then dried in a stream of cold air. Low-resolution autoradiographs were generated by exposing the sections to Hyperfilm 3H (Amersham International) X-ray cassettes for 1–3 days. Photodensitometric analysis was performed on these autoradiographs using a Bio-Rad GS-700 imaging densitometer (Bio-Rad Laboratories, Hemel Hempstead, UK), which allowed the identification of prostatic stromal and epithelial areas. At least 6 sections from each patient were analysed for ET receptor binding. From each section, at least six measurements of ET receptor binding were performed on both epithelial and stromal areas. Data were analysed using Molecular Analyst software. Specific binding was calculated by subtracting non-specific binding from total binding. Binding was expressed in terms of radioligand bound (disintegrations per minute; dpm) per unit area (mm<sup>2</sup>), calculated from standard curves generated by [125I]microscales (Amersham International) that were co-exposed with tissue sections. Microscopic localisation (high-resolution autoradiography) of the binding sites was performed by post-fixing tissue in paraformaldehyde vapour (2 h at 80°C) and coating slides in molten nuclear emulsion (LM-1, Amersham International). Slides were then stored in light proof boxes for up to 8 days at 4°C, after which they were processed in D19 high contrast developer (Kodak, Ilford, UK) and fixed (Hypam, Ilford, UK). Underlying tissue was stained with haematoxylin and eosin and high-resolution autoradiographs were viewed on an Olympus Vanox microscope; selected sections were photographed under dark field and bright field illumination.

**Functional Studies**

Isolated prostatic chips were transported to the laboratory in cold Krebs’ solution. Strips measuring 2×2.5 mm of approximately equal weights were prepared and mounted in 1.5-ml organ baths. The strips were superfused with Krebs’ solution maintained at 37°C by a thermoregulated circuit and bubbled with a mixture of 95% O<sub>2</sub> and 5% CO<sub>2</sub>, maintaining pH at 7.4.

Contractile studies were performed within 1–2 h of collection of prostatic tissue. An initial tension of 1 g was applied to the suspended tissue strips. The tension was recorded with a force displacement transducer (FT-03, Grass Instruments, Quincy, Mass., USA) on a Grass Polygraph (model 7D). All strips were equilibrated for at least 60 min. At the end of the equilibration period, the strips were challenged with KCl (124 mM). Two reproducible contractions varying in magnitude by <10% were consistently obtained.

Concentration response curves (CRCs) were performed for phenylephrine (PE), ET-1 and S6c (a selective ET<sub>b</sub> agonist). Further CRCs to ET-1 and S6c were constructed in the presence of ET<sub>a</sub> (BQ123) and ET<sub>b</sub> (BQ788) selective antagonists (10<sup>-6</sup> and 10<sup>-3</sup> M) to identify the ET receptor mediating contractile responses.

A fixed molar ratio (FMR) method was utilised to determine the interactions following coactivation of prosthetic ET-1 and α<sub>1</sub>-receptors by ET-1 and PE respectively, as previously described in human caver-
Fig. 1. Low resolution autoradiographs of ET\textsubscript{A} and ET\textsubscript{B} radioligand-binding sites in BPH. Binding is evident as dark grain accumulation on a light background. Top panel: Haematoxylin and eosin-stained tissue-underlying autoradiograph. Prostatic epithelium (dark arrowheads) and smooth muscle (SM). TOT = Total binding; NSB = non-specific binding. Bar = 2 mm.

<table>
<thead>
<tr>
<th></th>
<th>K\textsubscript{D} (n = 20)</th>
<th>B\textsubscript{max} (n = 20)</th>
<th>PSM (n = 60)</th>
<th>PEP (n = 60)</th>
</tr>
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<tbody>
<tr>
<td>[^{125}\text{I}ET-1]</td>
<td>0.23±0.05</td>
<td>66.3±2.3</td>
<td>31±2.5</td>
<td>18.7±3.2</td>
</tr>
<tr>
<td>[^{125}\text{IPD}151242]</td>
<td>0.15±0.04</td>
<td>32.3±3.2</td>
<td>14.6±4.1</td>
<td>11.3±2.6</td>
</tr>
<tr>
<td>[^{125}\text{IBQ}3020]</td>
<td>0.16±0.02</td>
<td>28.7±2.7</td>
<td>12.5±2.4</td>
<td>4.2±2.5</td>
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</table>

Receptor binding affinity (K\textsubscript{D}) is expressed in nanomoles per liter. B\textsubscript{max} and receptor density at PSM and PEP are measured as dpm x1,000/mm\textsuperscript{2}. Values are expressed as mean ± SEM. The numbers in parentheses are the number of experiments.

Statistical analysis (Student’s t test):
- PSM ET\textsubscript{A} vs. PEP ET\textsubscript{A}: p = NS.
- PSM ET\textsubscript{B} vs. PEP ET\textsubscript{B}: p < 0.001.
- PE ET\textsubscript{A} vs. PEP ET\textsubscript{B}: p < 0.002.

Table 1. Saturation binding and densitometric analysis of \[^{125}\text{I}ET-1\], \[^{125}\text{IPD}151242\] (ET\textsubscript{A} ligand) and \[^{125}\text{IBQ}3020\] (ET\textsubscript{B} ligand) binding to prostatic smooth muscle (PSM) and prostatic epithelium (PEp) in BPH.

that there was no difference between the density of ET\textsubscript{A} and ET\textsubscript{B} receptor-binding sites on the prostatic stroma (table 1). However, the density of ET\textsubscript{A} receptors was greater than ET\textsubscript{B} in the prostatic epithelium (table 1). High-resolution autoradiographs confirm the presence of dense ET\textsubscript{A} and ET\textsubscript{B} receptor-binding sites to the prostatic stroma (fig. 2). ET\textsubscript{A} receptor-binding sites were prominent on the prostatic epithelium (fig. 2a), whereas ET\textsubscript{B} receptor-binding sites appeared to have a patchy distribution (fig. 2b).

**Functional Studies**

**Contractile Responses to PE, ET-1 and S6c.** PE, ET-1 and S6c produced a concentration-dependent contraction in human prostatic strips. The maximal contractile response to PE was 3.8±0.4 g. The maximal contractile response to ET-1 was 52±3% and to S6c was 42±3% of that obtained with PE (fig. 3).

**Effects of ET\textsubscript{A} and ET\textsubscript{B} Receptor Antagonists on the Contractile Responses to ET-1.** ET-1 and S6c (ET\textsubscript{B} receptor-specific agonist) elicited prostatic smooth muscle contraction with -log EC\textsubscript{50} of 8.31±0.15 and 8.22±0.22 M (n =
Fig. 2. a Left panel: High-resolution (dark field illumination) autoradiographs of ET<sub>a</sub> receptor-binding sites evident as white grains on a black background. Right panel: Haematoxylin and eosin-stained tissue-underlying autoradiograph. PE = Prostatic epithelium; SM = smooth muscle. Bar = 100 μm. b Left panel: High-resolution (dark field illumination) autoradiographs of ET<sub>b</sub> receptor-binding sites evident as white grains on a black background. Right panel: Haematoxylin and eosin-stained tissue-underlying autoradiograph. PE = Prostatic epithelium; SM = smooth muscle. Bar = 100 μm.
Fig. 3. CRC to ET-1, PE and S6c. Results are expressed as percent of maximum PE contraction. Each point represents the mean ± SEM.

Fig. 4. CRC to ET-1 in the presence of ET₄ (BQ123) antagonist (10⁻³ to 10⁻⁶ M). Tension is expressed in millinewtons. Each point represents the mean ± SEM. ET-1 alone vs. ET-1 with BQ123 (10⁻⁵ M): "p<0.01; ET-1 alone vs. ET-1 with BQ123 (10⁻⁶ M): 'p<0.05.

15), respectively (fig. 4, 5). BQ123 (10⁻⁶ and 10⁻⁵ M) significantly shifted the ET-1 CRC to the right with no reduction in the maximum response (fig. 4; "p<0.05 and "'p<0.01). The shift was greater at lower concentrations of ET-1, resulting in a steepening of ET-1 CRC as indicated by the Hill slopes in table 2. Estimation of the magnitude of the shift from −log EC₅₀ values (table 2) gives a 13-fold shift in the presence of BQ123 (10⁻⁶ M) with an apparent PA₂ value of 6.9. The shift in the presence of BQ123 (10⁻⁵ M) was 15-fold with a PA₂ value of 7.8. The previous mean cited value of PA₂ for BQ123 in the literature is 6.84 [7].

BQ788 (10⁻⁶ and 10⁻⁵ M) also significantly shifted the S6c CRC to the right with no reduction in the maximum response (fig. 5; "p<0.05 and "''p<0.01). The −log EC₅₀
values for S6c (table 2) were significantly reduced (p<0.05 and p<0.01) in the presence of BQ788 (10^-6 and 10^-5 M), respectively. The slope of the curve was only increased in the presence of BQ788 10^-5 M (table 2). The apparent PA2 values for BQ788 were 8.5 and 8.8. S6c-mediated prostatic smooth muscle contractile responses were not inhibited by BQ123 (10^-6 and 10^-5 M; data not shown).

**Effects of Simultaneous Activation of α1-Adrenergic and ET Receptors.** The ability of ET-1 to enhance prostatic smooth contraction in response to PE is shown in figure 6. The partial substitution of PE with ET-1 resulted in a leftward shift in the CRC using PE alone. The –log EC50 values for PE alone, PE:ET (70:30) and PE:ET (80:20) CRC were 6.99 ± 0.13 (n = 14), 7.78 ± 0.14 (8-fold shift) and
the previous studies, our group of patients had both clinical homogenates of BPH tissue [10, 11] to determine the density of ET receptors, the mitogenic activity of ET-1 is evident only in the smooth muscle [4]. The mitogenic effects of ET-1 on benign prostatic smooth muscle cells is mediated by both ET receptors [4, 17]. In contrast, the effect of ET-1 on prostate cancer cell proliferation is ETA receptor-dependent. Interestingly, in prostatic cancer cells lines there is an absence of ETB receptor expression [3]. Furthermore, ET-1 also reduces apoptosis in prostatic smooth cells [18]. These findings therefore, suggest that alteration in the activity of the ET pathway influences prostatic growth.

Despite the identification of ET-converting enzyme-1 mRNA [9] and secretion of ET-1 by prostatic epithelial cells [4], the exact function of ET-1 and epithelial ETA and ETB receptors in the human prostate remains unknown. However, it has been suggested that prostatic epithelial ET receptors may be involved in physiological functions such as feedback regulation of ET synthesis [4]. For example, ETB receptors exert an indirect influence on vascular smooth muscle tone by their ability to regulate the secretion and circulating levels of ET-1 [19].

The inhibition of ET-1- and S6c-induced prostatic smooth muscle contractile responses by both BQ123 (ETA antagonist) and BQ788 (ETB antagonist), respectively, suggests that both receptors mediate contractile responses. This finding is in agreement with previous work. Our observations indicate that the ET-1-induced prostatic smooth muscle contractions are mediated by at least by two receptor subtypes, since the slope of ET-1 CRC and the PA2 values for BQ123 increased with the increasing concentration of the antagonist. This is to be expected, since ET-1 is a non-specific agonist, i.e. it acts on both receptor subtypes. The S6c-induced CRCs were shifted to the right without changing the maximal response in the presence of increasing concentrations of BQ788. Although, the slope of the S6c CRC increased slightly in the presence of BQ788 (10^{-5} M), the PA2 values for BQ788 were similar between antagonist concentrations.

ET-1 significantly enhanced the sensitivity of prostatic smooth muscle to PE without altering the maximum contractile responses. A similar pattern of enhancement was observed with human cavernosal smooth muscle [20]. In addition, the simultaneous coactivation of α1-adrenergic and ET-1 receptors in vascular smooth muscle has also been described. For example, it has been demonstrated that ET-1 potentiates α1-adrenergic receptor-induced contractions in the rabbit aorta by increasing tissue sensitivity to norepinephrine [21]. Furthermore, Gondrè et al. [22] reported similar alterations in tissue responses to α1-adrenergic-receptor-mediated contractions in the rabbit aorta, femoral and mesenteric arteries as well as rabbit corpus cavernosum. The ET-1-induced enhancement of α1-adrenergic-

Table 2. The -log EC50 (M) and Hill slopes for ET-1 and S6c in the absence and presence of ETα (BQ123) and ETβ (BQ788) receptor antagonists

<table>
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<th>-log EC50 (M) (n = 15)</th>
<th>Hill slope (n = 15)</th>
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<tr>
<td>ET-1</td>
<td>8.31±0.15</td>
<td>0.58±0.05</td>
</tr>
<tr>
<td>BQ123 10^{-6} M</td>
<td>7.02±0.14*</td>
<td>0.71±0.07*</td>
</tr>
<tr>
<td>BQ123 10^{-5} M</td>
<td>6.99±0.09b</td>
<td>0.95±0.08b</td>
</tr>
<tr>
<td>S6c</td>
<td>8.22±0.22</td>
<td>0.72±0.04</td>
</tr>
<tr>
<td>BQ788 10^{-6} M</td>
<td>7.13±0.11*</td>
<td>0.79±0.06</td>
</tr>
<tr>
<td>BQ788 10^{-5} M</td>
<td>6.81±0.21b</td>
<td>0.88±0.04*</td>
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The numbers in parentheses are the number of experiments. A significant change in -log EC50 and slope of the agonist CRC in the presence of the antagonist: *p<0.05; **p<0.01.

8.11±0.13 (11-fold shift), respectively. The -log EC50 values were significantly (*p<0.01) increased in the presence of ET-1. There was no significant change in the maximal contractile response to PE in the presence of ET-1.

Discussion

Our findings demonstrate that both ETA and ETB receptor-specific radioligands bind to their respective receptor sites with KD values in the subnanomolar range. There were dense ETA and ETB receptor-binding sites in the prostatic stroma as previously reported [3, 8, 9]. However, in contrast to previous studies [7, 8], ETA receptor-binding sites were also prominent on the prostatic epithelium compared to ETB receptors. This apparent discrepancy in ET receptor distribution may be due to variations in the growth of stromal and epithelial components in BPH, which are regulated by a complex interaction between various growth factors [16]. Furthermore, specific ET receptor subtype radioligands allow the exact localisation of the ET receptors on slide-mounted sections whereas displacement techniques rely on specific ET receptor antagonists to delineate the distribution of ET receptor subtypes [7, 8]. Others have used membrane homogenates of BPH tissue [10, 11] to determine the density of ET receptors in hyperplastic prostates; this technique does not describe their distribution pattern. Finally, unlike the previous studies, our group of patients had both clinical and urodynamic evidence of bladder outflow obstruction secondary to BPH. Whether these factors influence the distribution of ET receptors needs to be established.

Although, prostatic smooth muscle and epithelial cells express ET receptors, the mitogenic activity of ET-1 is evident only in the smooth muscle [4]. The mitogenic effects of ET-1 on benign prostatic smooth muscle cells is mediated by both ET receptors [4, 17]. In contrast, the effect of ET-1 on prostate cancer cell proliferation is ETA receptor-dependent. Interestingly, in prostatic cancer cells lines there is an absence of ETB receptor expression [3]. Furthermore, ET-1 also reduces apoptosis in prostatic smooth cells [18]. These findings therefore, suggest that alteration in the activity of the ET pathway influences prostatic growth.

Despite the identification of ET-converting enzyme-1 mRNA [9] and secretion of ET-1 by prostatic epithelial cells [4], the exact function of ET-1 and epithelial ETA and ETB receptors in the human prostate remains unknown. However, it has been suggested that prostatic epithelial ET receptors may be involved in physiological functions such as feedback regulation of ET synthesis [4]. For example, ETB receptors exert an indirect influence on vascular smooth muscle tone by their ability to regulate the secretion and circulating levels of ET-1 [19].

The inhibition of ET-1- and S6c-induced prostatic smooth muscle contractile responses by both BQ123 (ETA antagonist) and BQ788 (ETB antagonist), respectively, suggests that both receptors mediate contractile responses. This finding is in agreement with previous work. Our observations indicate that the ET-1-induced prostatic smooth muscle contractions are mediated by at least by two receptor subtypes, since the slope of ET-1 CRC and the PA2 values for BQ123 increased with the increasing concentration of the antagonist. This is to be expected, since ET-1 is a non-specific agonist, i.e. it acts on both receptor subtypes. The S6c-induced CRCs were shifted to the right without changing the maximal response in the presence of increasing concentrations of BQ788. Although, the slope of the S6c CRC increased slightly in the presence of BQ788 (10^{-5} M), the PA2 values for BQ788 were similar between antagonist concentrations.

ET-1 significantly enhanced the sensitivity of prostatic smooth muscle to PE without altering the maximum contractile responses. A similar pattern of enhancement was observed with human cavernosal smooth muscle [20]. In addition, the simultaneous coactivation of α1-adrenergic and ET-1 receptors in vascular smooth muscle has also been described. For example, it has been demonstrated that ET-1 potentiates α1-adrenergic receptor-induced contractions in the rabbit aorta by increasing tissue sensitivity to norepinephrine [21]. Furthermore, Gondrè et al. [22] reported similar alterations in tissue responses to α1-adrenergic-receptor-mediated contractions in the rabbit aorta, femoral and mesenteric arteries as well as rabbit corpus cavernosum. The ET-1-induced enhancement of α1-adrenergic-

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Eur Urol 2001;39:48-56
mediated contractions might be related to concomitant increases in the influx of Ca$^{2+}$ or changes in the sensitivity of cells to Ca$^{2+}$ [22]. Since threshold or subthreshold concentrations of ET-1 exist in plasma [23], the modulation of intracellular Ca$^{2+}$ and prostatic smooth muscle tone by ET-1 may be of physiological and/or pathological importance. Whether such mechanisms are involved in the human prostate smooth muscle requires further detailed investigation.

In conclusion, this study demonstrates the use of high-affinity ET receptor-specific radioligands to localise and quantify ET receptor subtypes in prostatic tissue. ETA receptor binding was evident on both prostatic stroma and epithelium, whereas ETB receptor binding was predominant in the prostatic smooth muscle. Both ETA and ETB receptors mediated prostatic smooth muscle contraction. The study also shows that ET-1 enhances PE contractions. Thus, ET receptors may have a pathophysiological role in the development of bladder outlet obstruction associated with BPH.

ET receptor antagonists, in combination with α1-blockers, may prove useful in the treatment of BPH.

References