OXIDATIVE MECHANISMS IN DIABETES RELATED URINARY BLADDER DYSFUNCTION

DISSERTATION

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Urinary bladder dysfunction is a common disorder associated with diabetes and is known to affect nearly 80% of this population. While this syndrome is typically not life threatening, it is associated with several debilitating symptoms including insidious onset of voiding, progressive bladder paralysis, urinary retention and incomplete voiding. In addition to functional impairment of bladder smooth muscle (e.g. decreased tone and contractility), chronic diabetes is also associated with a general increase in bladder size and capacity.

While the bladder clearly increases in size and capacity during diabetes a detailed account of the morphological changes in all tissue layers over time has not been previously conducted. We studied the morphological characteristics of the bladder using the well-established streptozotocin induced model of diabetes. We focused on earlier time points to define time dependent morphological events that occur in the bladder during diabetes using the highly automated digital imaging methods to quantify general size and composition of bladder tissue at various times during diabetes progression. We observed a near doubling of total
bladder tissue over the 5 weeks of investigation. Most notably time-dependent increases in smooth muscle and urothelium and reduction in collagen prevalence were observed at 2 weeks; changes consistent with the clinical symptoms of bladder dysfunction.

Given our findings of time dependent structural changes and an established view that neurological alterations play a major role in this setting we investigated the prevalence of the three major neurological controllers of bladder function; viz. Tyrosine hydroxylase (TH) and vesicular acetylcholine transporter protein (VACHT) as well established and commonly employed presynaptic markers of sympathetic and parasympathetic fibers respectively and Nitric Oxide Synthase I (NOS1) as an important controller of genitourinary function via its involvement in non-adrenergic non-cholinergic pathways. The major finding of this work is that the markers of innervation in the bladders from diabetic animals changed before significant alterations in bladder mass were observed (3 days vs. 2 weeks). Interestingly, markers for parasympathetic, sympathetic, and NANC all changed by three days after induction of diabetes. This suggests that substantial neuronal plasticity occurs in bladder early in the diabetic state and implies the possibility that neuronal plasticity drives structural plasticity. Thus a process of “neuronal remodeling” was detected in this setting in which there was an apparent shift toward pathways favoring bladder capacitance and smooth muscle relaxation; these changes preceded changes in structure (2 weeks). Furthermore the changes observed were not consistent with a global loss of neurons or nerve
terminals, as would be expected by neuropathic mechanisms. There was a significant increase in sympathetic terminals at blood vessels in the lamina propria and a decrease in parasympathetic terminals in the smooth muscle.

Growing evidence suggests that oxidative stress is a common occurrence associated with many of the complications of diabetes, although its role in diabetes related bladder disorders has not been investigated. Using immunohistochemical methods we studied the in vivo formation of 3-nitrotyrosine (biomarker of peroxynitrite and oxidative stress) (3NT) in control and diabetic bladders. Statistically significant increases in 3NT prevalence were observed in the urothelial and smooth muscle layers that were readily detectable at 3 days, suggesting that changes in reactive nitrogen species formation occurs in the very early phases of bladder alterations during diabetes. Since the formation of peroxynitrite is bimolecular, increased prevalence of either nitric oxide or superoxide anion can promote protein nitrination events. In addition to bladder-region-specific control levels, we observed selective increases in NOS isoforms in the early phase following diabetes induction. These changes illustrate independent control of each gene product in each layer of bladder tissue and suggest fundamentally different and independent responses of these cell types during the diabetic state. Early biochemical changes after the induction of diabetes likely include increased NO signaling and protein tyrosine nitration. As these changes precede measures of significant modeling, it is likely that such NO signaling is involved in the process of remodeling.
We downsized our techniques to study bladder morphology and function from STZ induced rat model to mouse model. We have successfully performed cystometric (functional) and morphological assessment of mouse bladders and investigated changes at 5 weeks post STZ injection. We employed invitro cystometry techniques to study changes in an intact bladder during diabetes. Our data showed significant decrease in plateau pressure, increase in structural capacity and compliance; similar to human and rat studies described previously. Thus we developed a STZ induced mouse model that mimics the human and classical STZ rat model of diabetes as shown by functional, structural and neuronal remodeling. These studies suggest that STZ induced mouse model has a likely value in learning more about bladder biology and diabetes influences and has great potential for disease studies and therapeutic evaluations.

Several settings of smooth muscle hypertrophy and proliferation have been linked to oxidant related signaling cascades, although such phenomena have not been detailed in the setting of diabetic bladder remodeling. For these reasons, and in light of some preliminary data in a rat model, herein we focused on potential oxidant related mechanisms in murine diabetic bladder. Previous studies in vascular smooth muscle suggest that activation of NADPH oxidase is an important source of oxidants especially superoxide. NADPH oxidase activation requires presence of active GTP bound Rac1. Since increased Rac1 is associated with increased superoxide formation; we investigated role of increased superoxide anion by measuring the levels of active Rac1 in the bladder
during diabetes. Our data showed significant increases in smooth muscle specific Rac1 immunoprevalence in diabetic mouse bladders. This suggests that an inducible oxidase could be a potentially important source of oxidants in this setting and not just glucose chemistry thru AGEs, as described previously, be the only source of oxidants in diabetic bladders. Of note is the important observation that regional distribution of protein nitration (urothelium and smooth muscle) did not generally follow regional patterns for Rac1 (smooth muscle); which has been most suggested as an important contributor of superoxide formation in vivo. Thus, under these conditions, not superoxide availability alone but changes in NOS isoform availability are apparently involved as well.

Recently Rac1 has been shown to be an important contributor in signaling pathways mainly thru increased reactive oxygen/nitrogen species and is shown to interact with serine threonine kinases especially the mitogen activated protein kinases and protein kinase B (Akt/PKB). Here we investigated the role of these kinases to study the Rac1 driven oxidant-signaling pathways in a diabetic mouse bladder. We observed increases in p-p-38 and p-Erk in urothelium during diabetes; these markers are consistent with cellular inflammation activation and growth in this region. In contrast we found that p-JNK and p-Akt were specifically increased in the smooth muscle layer; these responses are consistent with anti-apoptosis and proliferation signal pathways respectively. Thus, the general pattern of these signaling markers in each of these regions is consistent with structural and functional characteristics of the sites (e.g., urothelial inflammation
and growth, smooth muscle proliferation and hypertrophy). Later biochemical changes after the induction of diabetes (occurring after substantial remodeling is documented) suggest involvement of Rac1 and MAPK mediated signaling in either regulation or maintenance of remodeling. The changes that occur suggest that oxidative signaling pathways may be involved and that, especially in urothelium, signaling pathways having many elements in common with induction of inflammatory events are involved (even though there is no evidence for typical inflammatory responses). These data suggests that oxidant related events might play an important role in diabetic bladder remodeling and that Rac1 signaling pathways are involved. In the light of increased Rac1 in bladder smooth muscle during diabetes, we studied the effect of oxidative stress alone specifically in the smooth muscle of the bladder in absence of diabetes or glucose/volume related changes. Here we employed the Rac CA over expressed transgenic mouse model to study the role of oxidative stress alone on bladder morphology, function and neuronal remodeling in absence of diabetic condition. We have engineered (in collaboration) a transgenic mouse model that overexpresses the constitutively active mutant of human Rac1 (Rac CA) in FVB/N mice, using mouse smooth muscle \( \alpha \)-actin promoter containing all elements known to be required for optimal transcription of the SM \( \alpha \)-actin gene. Using the SM \( \alpha \)-actin we were able to express Rac1 specifically in bladder smooth muscle of the mice. We observed functional, structural and neuronal remodeling in the Rac1 CA mouse model, which mimics those of the well-established streptozotocin–induced diabetic rat
model even in absence of glucose or volume overload. These data demonstrate that oxidative phenomenon may play a key role in bladder dysfunction and remodeling during diabetes, especially thru Rac1. The Rac1 overexpression mouse model confirms that substantial bladder remodeling occurs in response to increased activity of Rac1 and MAPK mediated signaling, independently of changes in volume of urine.

Further investigations of oxidant signaling events and role of Rac1 during diabetes related bladder may provide new insight regarding adaptive and maladaptive processes in bladder physiology and enhanced opportunities for improved therapeutic strategies for an important diabetic complication.
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CHAPTER 1

INTRODUCTION AND THESIS OBJECTIVES

UROLOGICAL COMPLICATIONS IN DIABETES MELLITUS: NEW INSIGHTS TO COMMON PROBLEMS
INTRODUCTION

Diabetes mellitus currently afflicts more than 100 million people worldwide and it has been estimated that roughly 238 million will be affected by the year 2010 [1]. Diabetes is a chronic disorder of carbohydrate, fat and protein metabolism and is characterized by decreased production of insulin or developing resistance to its actions [2, 90]. Clinically diabetes is divided into two types: Type1 or insulin dependent diabetes mellitus (IDDM) or Type2 or non-insulin dependent diabetes mellitus (NIDDM) [2, 90]. Type 1 diabetes is most frequent in patients less than 50 years and as the name suggests requires exogenous administration of insulin to compensate for the body’s inability to produce sufficient quantities of the hormone. Type 2 diabetes occurs mainly in middle aged and elderly people and can be controlled by diet and/or oral hypoglycemic agents [2, 90]. This chronic and progressive condition is associated with many complicating illnesses, including cardiovascular and renal disease, neuropathy and urological dysfunction [2, 90].

Urinary bladder dysfunction is a common complication associated with diabetes [3-81]. While this syndrome is typically not life threatening, it is associated with several debilitating symptoms including insidious onset of voiding, progressive bladder paralysis, urinary retention and incomplete voiding [3-81]. In addition to functional impairment of bladder smooth muscle (e.g. decreased tone and contractility); it is also associated with a general increase in bladder size and capacity [3-81]. These changes are associated with a reduced
quality of life as well as an increased risk of other conditions, including urinary tract infections and bladder cancers [82]. These debilitating problems often progress throughout a patient’s lifespan and are irreversible. While the phenomenon of diabetes related bladder dysfunction is well recognized, its pathogenesis is not well understood and current therapies are not effective in completely preventing the damage. Furthermore, as the incidence of diabetes rises, and as patient’s age, the importance and associated medical costs of this syndrome are likely to escalate. Improved mechanistic understanding is necessary for the advancement of improved therapies.

NORMAL BLADDER STRUCTURE AND FUNCTION

**Structure:** Urinary bladder is a hollow muscular organ whose function is to store and expel urine. The urinary bladder is divided into two distinct regions: bladder body or dome which mainly contains detrusor smooth muscle and bladder neck or base region which is a smaller region from ureters to urethra [83,84,88-91]. As a vesicular organ the urinary bladder is comprised of three primary and concentric regions: **Urothelium:** It is a specialized stacked epithelial cell type at the lumen also called transitional epithelium and has a turnover time of ~6 weeks in mice [84]. The urothelium is composed of three cell layers viz. the basal cell layer, the intermediate layer and the superficial umbrella cells, which are joined together by tight junctions thus offering a physical barrier to the movement of substances between blood and urine [84]. **Lamina propria:** It is a
connective layer supporting urothelium rich in supply of blood vessels and nerve bundles and also making close contacts with the smooth muscle cells of the mucosa [85]. It is mainly composed of connective and elastic tissue. **Smooth muscle:** This layer consists of both inner and outer longitudinal muscle and intermediate circular muscles, resulting in formation of a meshwork of smooth muscle. The smooth muscle cells and bundles are enclosed in collagen which confer elasticity to the bladder and are responsible for tone generation and accommodating large volumes of urine during filling [83-91].

**Peripheral Bladder innervation:** The urinary bladder smooth muscle is innervated by peripheral nerves which include both efferent (motor) and afferent (sensory) nerves and coordination of both these nerves is required for successful continence and micturition [92-97].

Urinary bladder performs two main functions: continence or prolonged urine storage and micturition or brief period of evacuation [95]. During continence, urine is stored into the bladder body at low intravesical pressure and the bladder neck remains closed, while during micturition, there is contraction of detrusor muscle followed by relaxation of the muscles of the pelvic floor and urethra resulting in opening of the bladder neck [92,97]. Autonomic control of bladder expansion and contraction is predominantly governed by 3 set of peripheral nerves: parasympathetic, sympathetic and pudendal nerves. Apart from these, nonadrenergic noncholinergic (NANC) inputs have also been shown to play an important role in bladder function [88,89,97]. Stimulation of sacral
parasympathetic (pelvic nerve) activity causes a highly coordinated contraction of the bladder body and subsequent emptying of the bladder, whereas the thoracolumbar sympathetic activation through the hypogastric nerve enhances detrusor muscle relaxation [88,89,92-97].

**Parasympathetic innervation:** The major excitatory input to the urinary bladder is provided by the parasympathetic efferent pathway. The preganglionic parasympathetic axons originate in the intermediolateral column of the S2-S4 spinal cord [86-97]. The preganglionic parasympathetic axons terminate on postganglionic neurons in the bladder wall and pelvic plexus and release acetylcholine which activate the postjunctional nicotinic receptors [86-97].

The postganglionic parasympathetic nerve terminal release acetylcholine that activates cholinergic muscarinic receptors which are found throughout the bladder wall, greater in density in the bladder body than the base. There are two major subtypes which are found to be present in the bladder i.e. M2 and M3 receptors. Although the density of M2 receptors is 3 to 10 fold higher than M3 receptors, M3 receptors are responsible for acetylcholine–induced bladder contractions [88]. The mechanism for causing bladder contractions for the M3 receptors is by coupling to phospholipase C (PLC) and stimulating hydrolysis of phosphatidylinositol (PI) into inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG), while M2 receptors which are coupled to inhibitory G protein, inhibit adenylate cyclase and causes bladder contraction by preventing β-adrenergic induced formation of cyclic AMP and thus inhibiting norepinephrine induced
relaxation [88,92]. Recent studies using transgenic mouse models especially the M3 receptor knock out model shows distinct bladder changes distended bladders, urinary retention and poor/no responses of bladder strips to nonselective muscarinic agonist carbachol [98]. In contrast, M2 receptor knockout model showed similar responses of bladder strips to carbachol as the wild types [99]. These studies suggest that although the M2 receptors dominate in number the M3 receptors are mainly responsible for causing bladder contractions.

Apart from these, prejunctional facilitatory M1 and inhibitory M2 receptors are also present in the bladder. The facilitatory M1 are activated during high frequency nerve firing and amplify the parasympathetic excitatory input to the bladder and cause complete bladder emptying while the inhibitory M2 receptors inhibit the release of acetylcholine from postganglionic nerve terminals in the bladder [86-89,92, 97].

**Sympathetic innervation:** The preganglionic sympathetic neurons are present in the intermediolateral cell column of the T11 to L2 spinal cord. They synapse with postganglionic neurons in the inferior mesenteric ganglia, paravertebral ganglia and pelvic ganglia [86-89,92,97]. The sympathetic preganglionic transmission is mediated by acetylcholine acting on the nicotinic receptors. The postganglionic sympathetic neurons release norepinephrine which causes relaxation of the bladder body and contraction of the bladder base through β2 and α1 adrenoreceptors respectively [86-89,92, 97].
The urinary bladder body has a high density of β receptors and few α receptors while the bladder base and urethra have high α and fewer β receptors. The expression of α receptor subtypes in the bladder is not consistent since male rabbits and human bladder base show 80% α1 and 20% α2 [88] while RT-PCR studies show 60-70% of α1d and 30-40% of α1a, rat detrusor shows 33% of α1a, α1b and α1d [88], in-situ hybridization techniques show only α1a in bladder smooth muscle of rat, monkeys and humans [88]. α1 and α2 receptors are also prejunctional and are known to modulate release of acetylcholine and norepinephrine [86-89].

Classical view has been that β2 is the predominant receptor subtype responsible for bladder relaxation, however recent studies, both at molecular and pharmacological level, have shown presence of β1, β2 and β3 receptors in the bladder smooth muscle [86-88]. Although all the three subtypes have been expressed in the bladder there is no consensus on which one is responsible for functional responses to β agonists. The activation of β adrenoreceptors coupled to the stimulatory G proteins results in activation of adenylate cyclase and increased levels of cyclic AMP resulting in bladder relaxation [86-89].
**NANC innervation:** Apart from acetylcholine and norepinephrine various endogenous substances are known to elicit an inhibitory or excitatory effect on the bladder wall, some of which act as cotransmitters [86-89,92,97]. Most prominent among these are ATP, nitric oxide and cyclooxygenases which have been discussed below. The role of other endogenous substances in the bladder like serotonin, histamine is not clear and has been discussed in some reviews by deGroat WC etc [97]

**Purinergic System:** Stimulation of the pelvic nerve causes contraction of the detrusor muscle although this effect is partially blocked by atropine suggesting that acetylcholine might be the primary but not the only neurotransmitter that is responsible for causing bladder contraction. Various different neuropeptides have been located in the bladder wall such as substance P, CGRP, neuropeptide Y although some investigators believe that ATP is the excitatory neurotransmitter released by pelvic nerve stimulation as shown by studies in the guinea pigs, rabbit and dog bladders [86-89,92,97].

ATP produces concentration dependent biphasic response on bladder strips [88]. ATP causes both excitatory and inhibitory effects on bladder smooth muscle through P2 purinoreceptors, which are further subdivided into P2X and P2Y receptors. The contraction mediating P2X receptors act on the bladder through non-selective Ca channels, which upon activation increase calcium influx and mediate bladder contraction [86-89,92,97]. Thus ATP could be responsible for the initial rapid contraction followed by the slower maintained contraction
produced by acetylcholine. Other than contraction, ATP also causes relaxation of the bladder through the relaxation mediating P2Y receptors that are coupled with G proteins that activate adenylate cyclase and increase cyclic AMP levels to promote bladder relaxation [88]. Knock out mouse models especially the P2X3 knock out mice show marked urinary impairment suggesting their role in micturition while the P2X1 knockout have normal bladders [100-101]. Various different ATP receptor subtypes have been associated with bladder disease for example decrease in P2X3 and P2X5 in urinary incontinence [102] and more studies to evaluate the role of purinergic system in bladder function are warranted.

**Nitrergic system:** In addition to autonomic control, non-adrenergic non-cholinergic innervation and local nitric oxide production have been shown to have important physiological roles in the lower urinary tract [103-105]. For example, neuronal nitric oxide synthase (NOS1) plays a key role in modulating smooth muscle tone in the bladder detrusor muscle and in controlling sphincter tone for successful micturition [106]. In addition, urothelial expression of the high capacity NOS isoform (NOS2) has been shown to play a role in local host defense mechanisms [103-104]. Nitric oxide is synthesized by the enzyme family of nitric oxide synthases (NOS), which catalyze the conversion of L-arginine to L-citrulline [103-115]. Three distinct isoforms exist, NOS1, NOS2 and NOS3 [103-115] that have been shown to be important contributors in bladder physiology. Apparently the role of nitric oxide in the bladder detrusor has been controversial; with some
reports suggesting role of nitric oxide in the urethra but not in the detrusor while others showing partial mediation of relaxation of the detrusor strips by nitric oxide [103-109]. Perhaps the most convincing evidence of a critical role for nitric oxide in normal bladder function is the fact that NOS1 knock out mice developed enlarged bladders similar to those observed in other animal models of bladder outlet obstruction and/or diabetes [106]. However there is no general consensus on the role of NOS1 in the rat bladder since studies on bladder outlet obstruction have shown decrease or no change in NOS immunoreactivity [110-111]. In addition, NOS2, the inducible form of nitric oxide, is up regulated mainly in the urothelial cells and inflammatory cells after experimental urinary tract infections and bladder cancers [110-112]. Expression of NOS3 in the urinary bladder is controversial as Birder et al [113] have shown NOS3 immunoreactivity in the bladder urothelial cells while Fathian-Sabet et al [114] have shown localization of NOS3 in the bladder smooth muscle and Persson et al have reported no immunoreactivity for NOS3 in the rat and pig bladder [115].
**Prostaglandins:** Prostaglandins are potent spasmogens released from smooth muscle and epithelium of urinary bladder and are known to modulate afferent nerve activity and efferent neurotransmission [86-87, 97, 116]. PGE$_2$, PGF$_{2\alpha}$, TXA2 contract the bladder smooth muscle in human and animal studies while PGE$_1$, PGE$_2$ relax or have no effect on urethral smooth muscle [97]. Distension, increased intraluminal osmolarity, stimulation of specific receptors like muscarinic receptors, ATP-activated purinoreceptors, concentration of calcium and potassium ions are all known to modulate prostanoid release by the bladder [86-88, 97, 116].

Cyclooxygenases, one of the key enzymatic steps in prostaglandin synthesis might be important contributors in lower urinary tract function [117-120]. In the bladder cyclooxygenases are expressed in both the urothelium and smooth muscle of the bladder. Cyclooxygenases exist in two distinct isoforms, COX1, the constitutive isoform which is expressed in most tissues at a fairly stable level and COX2, the inducible form which is increased in response to inflammation, stretch and cancers in bladders [117-120]. Thus COX2 inhibitors may have value in treating bladder disorders.

**Neuropeptides:** Various different neuropeptides such as vasoactive intestinal polypeptide (VIP), tachykinins, Substance P (Sub P), Neuropeptide Y, Calcitonin gene related protein (CGRP), somatostatin, bradykinin, bombesin, endothelins, angiotensins, galanin, enkephalins, arginine vasopressin have been shown to affect bladder responses when administered both centrally and
Neuropeptides are capsaicin-sensitive primary afferents in the bladder and urethra that not only have a sensory function but also efferent function and can act as neurotransmitters or neuromodulators, potentiating or inhibiting adrenergic, cholinergic and purinergic activity and hence could be involved in maintaining micturition reflex, smooth muscle contraction, and change in vascular tone and permeability [86-87,97,121]. The discussion will be restricted to some of the neuropeptides that have also been shown to be altered in a diabetic bladder.

**Substance P:** Substance P is present in the suburothelial layer of the human bladder and also in the dorsal root ganglia and dorsal horn of the spinal cord [86-87,97,121]. It is a potent spasmogen of the bladder smooth muscle with an EC$_{50}$ of 0.1µM in the rat [86-87,97,121]. Substance P acts thru’ NK1 neurokinin receptors in the rat and guinea pig bladders and through NK2 receptors in the human and hamster bladders [86-87,97,121]. Substance P plays an important role as a sensory neurotransmitter since it is present in 22% of bladder efferents identified in feline sacral dorsal root ganglia and administration of capsaicin (substance that depletes Sub P and other peptides) results in depressed micturition reflex and urinary retention [86-87,97,121].

**VIP:** Vasoactive intestinal polypeptide or VIP is a 29 amino acid peptide found in all bladder layers, beneath urothelium, around blood vessels and in the smooth muscle although the highest concentration is in the urethra and trigone region [86-87,97,121]. VIP is known to cause relaxation of the rabbit bladder
smooth muscle and inhibits spontaneous activity in isolated detrusor while it causes a contractile effect on rat and guinea pig bladder and is inactive in the human bladder strips, suggesting that the actions of VIP vary from species to species [86-87,97,121]. 24% of the bladder afferents in the sacral dorsal root ganglia contain VIP suggesting a role of potential afferent neurotransmitter [86-87,97,121]. There is conflicting clinical data on the role of VIP in the bladder since some studies have reported decreased levels of VIP during detrusor instability while others do not support the role of VIP in bladder disorders [86-87,97,121].

**CGRP:** Calcitonin gene related peptide is present in the capsaicin sensitive nerves of the bladder and inhibits micturition reflex when applied topically in high concentrations to rat bladder [86-87,97,121]. CGRP produces different effects on the bladders of different species. For example it causes relaxation of rat bladder neck, no effect on rat, pig or guinea pig bladder body and inhibits neuromuscular transmission in the guinea pig detrusor [86-87,97,121]. It has been suggested to lay a neuromodulatory role in the bladder.

Distention of the bladder wall causes activation of sensory receptors, which convey information (feeling of bladder fullness) to the spinal cord via the afferent axons in the pelvic and hypogastric nerves [92, 94]. The afferent fibers carry impulses from tension receptors and nociceptors in the bladder wall to neurons in the dorsal horn of the spinal cord [97]. The pelvic nerve afferents are more numerous in the smooth muscle layers and are responsible for initiating the
micturition reflex while the hypogastric nerve afferents are more in mucosa and submucosa [95]. There are two different types of afferents in the bladder wall viz. the myelinated Aδ fibers and the unmyelinated C fibers [86-87,92-97]. Normal micturition reflex is mediated by Aδ fibers that respond to bladder distention as shown by electrophysiological studies in rats and cats while the C fibers also termed as the silent fibers do not respond to mechanical stimuli (bladder distention) but respond to chemical, noxious or cold stimuli [92-97]. The afferent neurons contain various neuropeptides like substance P (Sub P), calcitonin gene related protein (CGRP), vasoactive intestinal peptide (VIP), neurokinin A etc as shown by immunohistochemical studies and are known to trigger inflammatory responses.
URINARY BLADDER DYSFUNCTION IN DIABETES

**Clinical studies:** Urinary bladder dysfunction in diabetes has been well documented in humans, both males and females and is shown in Table 1. Recent studies suggest that bladder dysfunction affects around 40-85% of patients with diabetes, with no significant difference in age and sex [122-123]. Diabetes related bladder dysfunction in humans has been commonly attributed to altered neuronal control and/or neuronal injury [10,13,14,17,20-21,27]. For example, patients commonly experience impaired sensation of full bladder and loss of sphincter control (decreased relaxation during voiding and break-through leaks between voiding intervals) [10,13,14,17,20-21,27]. Increased bladder size and altered urodynamic properties such as 2-4 fold increase in capacity, increased urine output, decreased intravesical pressure, increased residual urine, and enlarged atonic bladders have also been well-documented [10-27]. These symptoms have been associated with impaired sympathetic skin response and decreased sympathetic and parasympathetic nerve conduction, suggesting that diabetic bladder dysfunction is a likely manifestation of peripheral nerve deficiencies, including either loss of nerve function and/or anatomical loss of neuromuscular nerve terminals [10,13,14,17,20-21,27]. Although many adaptive and maladaptive changes probably occur early during diabetes progression, most clinical studies have only reported bladder changes after months to year's duration of diabetes.
**Animal studies: Rodents:** Both chemically induced (streptozotocin or alloxan) and genetic models (BB rats) of diabetes have been used to study bladder dysfunctions during diabetes; although the streptozotocin induced diabetic rat model has been well-established and well-characterized to study bladder dysfunctions [90, 124]. Nearly all of the bladder changes found in humans are apparently recapitulated in this rat model of streptozotocin (STZ) induced diabetes [30-81]. For example, increased urine output, frequent voiding, and atonic bladder following at least five weeks of diabetes induction have been observed in this animal model [30-81]. Histochemical and functional studies have also shown altered bladder innervations of post-ganglionic sympathetic and parasympathetic fibers at 5 or more weeks post diabetes induction [30-81]. Review of the literature shows significant changes in the autonomic innervation of the bladder during diabetes however there is no consensus since studies have reported increase, decrease or no effect to various muscarinic and adrenergic agonists. Most of the characteristic changes in the bladder associated with STZ induced rat model of diabetes have been shown in Table 2.

Several previous reports [42,67] have demonstrated that this animal preparation is a well-known model of diabetes as well as polyuria. However it is important to note that that polyuria does not necessarily mimic bladder changes observed in the streptozotocin model of diabetes. For example, Eika et al [67] have previously shown that sucrose fed rats have pressure-volume responses that are not different from controls, whereas streptozotocin-induced diabetic
animals have significant passive function changes. Also changes in nerve densities were unaltered in the sucrose-fed model, whereas striking changes were observed in diabetic rats [42]. In more recent studies we have observed that mice selectively over-expressing pro-oxidant genes in bladder smooth muscle also develop bladder smooth muscle hypertrophy and decreased collagen content (identical to the remodeling phenomena observed here) in the absence of polyuria or glucose changes [7]. Collectively, these findings suggest that bladder remodeling may not be exclusively dictated by increased urine output per se.

Other than the rat model; mouse models (STZ, alloxan induced and db/db) have also been documented (Table3) but not well characterized to study bladder changes during diabetes. Most of the studies in the diabetic mice bladders have been focused on urinary tract infections in the bladders [125-129]. A detailed investigation of the changes in the bladder during diabetes has not been documented earlier. Developing a mouse model to systematically study bladder dysfunctions during diabetes would provide opportunities to do transgenic research in these animals in addition to being cost efficient.

**Other animal models:** Other than rodents, rabbits [107,130-133] (alloxan induced), guinea pig (spontaneously diabetic) [137] and Chinese hamsters (spontaneously diabetic) [134-136] have all been used to study bladder changes and have been documented in Table 3.
MECHANISMS OF BLADDER DYSFUNCTION IN DIABETES

Different mechanisms have been proposed that could be responsible for causing bladder dysfunctions during diabetes; however, the most prominent is the role of polyuria and neuropathy in causing bladder dysfunctions. Also there is a debate on whether polyuria precedes neuropathy or vice versa.

Polyuria: Most of the early studies suggest that polyuria is mainly responsible for bladder dysfunction associated with diabetes \[31,33,35-36\]. Diabetes through osmotic diuresis results in increased urine volume, increased frequency in turn resulting in hypertrophy, which has been, suggested as physiological adaptation of detrusor to increased load. Increased diuresis observed through cystometric measurements has been suggested as an important mechanism contributing to adaptive changes involving both sensory and motor control of the bladder \[31,33,35-36\]. Santicioli et al has reported that volume overload and not neuropathy, is largely if not solely responsible for altered detrusor properties at 7-9 weeks in diabetic rat bladders \[36\].

Diabetic Neuropathy: Diabetic bladder has been suggested as a manifestation of peripheral neuropathy \[Table1\]. Damage to sensory nerves or a sensory deficit shown by decreased intraluminal pressure results in distention of the bladder causing increased bladder capacity due to impaired sensation of bladder fullness and ultimately results in hypertrophy. Demyelination, decrease in nerve fibers, decrease in conduction velocity and impaired sympathetic skin response have all been previously documented \[Table 1,2,3\]. The classical view
has been denervation or frank loss of nerve terminals as an operable mechanism involved in loss of bladder tone and function. Diabetic neuropathy has been investigated as abnormalities in the efferent as well as afferent pathways of the bladder.

**Alteration in cholinergic/adrenergic receptors in smooth muscle:** The neurogenic response in bladders is composed of cholinergic, adrenergic and NANC components. A detailed report of the responses of diabetic bladder to muscarinic, adrenergic and electrical field stimulation has been shown in Table 2. Using radioligand binding, functional and histochemical studies either increases, decrease or no change in the response to agonists and antagonists has been reported suggesting alterations in the autonomic innervations in bladder during diabetes [Table 2]. Increase in muscarinic receptor density and activity; cholinergic potentiation or increased postjunctional responsiveness to acetylcholine have all been suggested [32,40,42,45,52,54,64,75]. There is still no consensus on role of postjunctional supersensitivity to acetylcholine in these diabetic bladders [40,45,54,75]. The role of adrenergic receptors in the bladders has not been studied extensively. Increased relaxation responses to isoproterenol, increased β-adrenergic receptor density, increased dopamine levels or increase in norepinephrine concentrations have been documented suggestive of adrenergic potentiation or adaptive over function of adrenergic nerve activity probably to maintain continence [39,54].
**Abnormalities in afferent innervation of bladder:** Apart from studying efferent innervation of the bladder, understanding the role of afferent innervations in bladder function is important. Previous studies have reported the role of capsaicin, a toxin specific for primary afferent nerves, as an important tool for investigating the neurochemical and functional characteristics of afferent neurons during diabetes [Table 2]. Impairment [65,72] or no changes [36,41,63] in capsaicin sensitive neurons have been documented in diabetic bladders. Various different neuropeptides such as Substance P, VIP, CGRP, neurokinin A have been studied in the diabetic bladder although Substance P has been the predominant one. Role of substance P in diabetic bladder is controversial since either increase or no changes in contractile response have been documented [36,41,48,59,65,72]. A role of capsaicin and other neuropeptides in the bladder during diabetes has been shown in Table 2.

**Myogenic component of smooth muscle:** The exact mechanism, which results in bladder hypertrophy seen during diabetes, is not clear. There is no consensus in the literature on whether volume overload or neurogenic defect leads to increased bladder mass. Alternatively, involvement of myogenic component of the bladder has been reported during diabetes [86-87], although its role is not clear. Longhurst et al have reported decreased responsiveness of the smooth muscle to KCL suggesting that decreased myogenic response in bladder body might result in functional obstruction and hypertrophy [34] while Uvelius et al have shown no smooth muscle impairment in high K solution [35].
EMERGING HYPOTHESIS:

Structural Remodeling: While the structural changes in the bladder such as bladder size, weight, mass, hypertrophy and cross sectional area have been well documented [table 2]; detailed bladder wall morphology during diabetes have not been previously conducted. The bladder wall consists of three well-demarcated layers [85]. The outermost layer consists of smooth muscle; middle is a connective tissue interface predominantly composed of extracellular collagen and innermost is a layer of stacked epithelial cells (urothelium) that borders the lumen. This epithelial cell layer plays an important role in normal bladder function (particularly barrier and host-defense mechanisms), but few studies have investigated its properties during diabetes. The only other report on urothelial cells from diabetic bladder showed increased DNA synthesis (measured by thymidine incorporation) [66]. Recent studies suggest that these layers interact during normal bladder development and that tissue composition is regulated via paracrine signaling pathways [138]. Understanding the changes that occur in these discreet regions of the bladder during diabetes could be important.
Again most of the previous studies have looked at changes in bladder wall during a single time point (around 5 weeks); however looking at the early and time dependent changes that occur in the bladder could be important in understanding bladder pathophysiology during diabetes. Investigating early events in the diabetic bladder that may provide new information regarding remodeling process and may lead to better identification of initiating events for therapeutic improvements.

**Neuronal Remodeling:** Many previous investigations have suggested that diabetic bladder dysfunction may be related to autonomic neuropathy, in which a global loss of neurons and/or nerve terminals has been documented [10,13,14,17,20-21,27]. Both structural and neurological changes are known to occur in bladder during diabetes, but their inter-relationships are not clear, especially at early time points and with respect to regional distributions.

**Oxidative stress in bladder dysfunction:** Oxidative stress is defined as serious imbalance between production of free radicals and antioxidant defense leading to tissue damage [139,141-143] and the free radicals formed during increased oxidative stress include both the reactive oxygen intermediates and reactive nitrogen species. Growing evidence suggests that oxidative stress via formation of reactive oxygen/nitrogen species is a common occurrence associated with most of the complications of diabetes [139,141-143]. Recent study has shown that that oxidative stress may play an important role in the development of the bladder dysfunction [140]. Furthermore, although there is
now convincing evidence that oxidative stress is an important component of several other diabetes related complications [139], the potential involvement of such phenomena in diabetic bladder dysfunction has not been previously investigated, particularly studies looking at the detailed region specific and time dependent changes in the bladder during diabetes are warranted.

Reactive oxygen/nitrogen species (ROS/RNS) are highly reactive, short lived and unstable chemical entities formed by the reaction of oxygen/nitrogen in aerobic conditions [139,141-143]. ROS/RNS are known to bind to proteins, break DNA strands, and react with vital cellular components of the body causing tissue damage [139,141-143]. ROS/RNS are produced in the body both intra and extracellularly by different enzymes [shown in Fig1, reproduced from Bomzon et al, Pharmacology and therapeutics, 2001 [141]. Some of these are discussed below
**Figure 1.1:** Sources of ROS/RNS in the body

**NADPH oxidase:** NADPH oxidase is the most important source of oxidants in the smooth muscle [142,144-146]. Until recently, NADPH oxidase was thought to be an important source of superoxide formation in the phagocytic cells for host defense but is now considered to be one of the key participants in ROS formation in the smooth muscle [142,144-146]. NADPH oxidase is a
membrane bound flavohemoprotein with cytochrome b558 comprising of the gp91 phox and p21phox and cytosolic proteins, p47phox, p6 phox, p40phox and rac1 [142,144-146]. The cytochrome b constitutes the redox core of NADPH oxidase and contains FAD group and heme sites while p4phox, p67phox and p40phox along with GTP protein rac1 bind to the membrane proteins on activation and in turn activate NADPH oxidase [142,144-146]. Electrons are then transferred from NADPH to FAD and then to heme, which serves as the terminal electron donor and finally to molecular oxygen resulting in generation of superoxide [142,144-146].

\[
2O_2 + \text{NADPH} \rightarrow \text{NADP}^+ + H^+ + 2O_2^-
\]

**Mitochondrial electron transport chain:** Damage to the electron transport chain such as hypoxia or ischemia followed by reperfusion results in increased mitochondrial ROS formation [141-142,144]. Reduced components in the NADPH dehydrogenase and coenzyme Q region [leakage of electrons in reactions mediated by coenzyme Q and ubiquinone and its complexes] of the electron transport chain also result in massive production of superoxide or ROS [141-142,144].

**Xanthine oxidase (XO):** Xanthine oxidase, the key enzyme in purine degradation pathway, generates uric acid [excreted in urine] and the byproduct superoxide anion by metabolizing hypoxanthine, xanthine and NADH. Induction of xanthine oxidase or increased concentration in plasma can contribute to increased superoxide production [141-142,144]. Xanthine oxidase is an important
contributor of ROS formation during ischemia/reperfusion, hypoxia and hypercholesterolemia [141-142,144]. Pretreatment with xanthine oxidase inhibitor, allopurinol has proved to be effective in cardiac dysfunctions [141-142,144] although the role of xanthine oxidase and its inhibitor in bladder dysfunction during diabetes awaits further studies.

**Nitric oxide synthase (NOS):** Nitric oxide synthase is the key enzyme involved in the conversion of L-arginine to L-citrulline and forming nitric oxide. Three different isoforms of NOS exist viz. NOS1 or neuronal NOS, NOS2 or inducible NOS and NOS3 or endothelial NOS. Deficiency of the tetrahydrobiopterin cofactor promotes superoxide formation by NOSs [141-142,144]. Separate from controllers of NO production, the actions of NO can be severely altered under conditions of oxidative stress [105,148]. Of particular importance is the interaction of NO with superoxide anion (O$_2^-$); this reaction is known to occur at a diffusion-limited, nearly instantaneous rate, forming the highly aggressive oxidant peroxynitrite (ONOO-) [149], a reactive nitrogen species known to nitrate protein tyrosine residues, forming 3-nitro-L-tyrosine (3NT) [150]. Thus, increased oxidative stress shunts intact NO towards formation of peroxynitrite and thus reduces its availability for signaling.

Nitric oxide is now recognized as an important regulator of many physiological processes, including functional aspects of the lower urinary tract [103-104]. For example, neuronal nitric oxide synthase (NOS1) plays a key role in modulating smooth muscle tone in the bladder detrusor muscle and in
controlling sphincter tone for successful micturition [106]. In addition, urothelial expression of the high capacity NOS isoform (NOS2) has been shown to play a role in local host defense mechanisms [103-104]. Despite the interest in nitric oxide as a neurotransmitter, a thorough investigation of the roles of nitric oxide synthase isoforms (NOS1, 2 and 3) and the potential changes in these 3 isoforms of NOS during a relevant setting of diabetic bladder dysfunction have not been previously investigated. Furthermore, although there is now convincing evidence that oxidative stress is an important component of several other diabetes related complications [139], the potential involvement of such phenomena in diabetic cystopathy has not been previously investigated, particularly with respect to reactive nitrogen species.

**Cyclooxygenases:** Cyclooxygenases, one of the key enzymatic steps in prostaglandin synthesis might be important contributors of superoxide through co-oxidation of the auto-oxidizable cofactors including NADPH [141-142,144]. Cyclooxygenases exist in two distinct isoforms, COX1, the constitutive isoform which is expressed in most tissues at a fairly stable level and COX2, the inducible form which is increased in response to inflammation, stretch etc. [116-120].

Cyclooxygenases play an important role in the in lower urinary tract function [116-120]. COX1, the constitutive isoform that is expressed in bladder tissues and COX2, the inducible form that is increased in response to inflammation, stretch and cancers in bladders [116-120]. COX2 expression has
been shown to be regulated by NOS2, both with positive and negative effects in the experimental systems and is highly dependent upon the cell types studied [147]. Most of the studies in the bladder have shown a positive effect of NO on COX2 activity or expression [117,147] suggesting a pathophysiological significance of the NO-COX cross-talk [147].

The different reactive oxygen/nitrogen species associated with increased oxidants in the body include nitric oxide, superoxide, peroxynitrite, hydrogen peroxide, hydroxyl radicals, carbonyl stress thru advanced glycation end products etc.[141-143] The discussion will be restricted to some of these oxidants that we have studied in the bladder setting during diabetes.

**Nitric oxide (NO):** Nitric oxide known as the ‘Molecule of the year’ by the end of 1992 was considered merely as an atmospheric pollutant and a byproduct of automobile exhaust, electric power stations and lightening. NO is now recognized as a ubiquitous molecule actively involved in host defense mechanisms, physiology and pathophysiology [142-143]. Nitric oxide is an uncharged molecule that can diffuse freely across the membrane with a half life of 2 to 30 seconds [142-143]. The main functions of NO is to activate cGMP, form peroxynitrite by reaction with superoxide, act as an signal transduction molecule, and cause destruction of oxy-hemoglobin to met-hemoglobin.
Most of the studies suggest that loss of NO in various organs can lead to pathologic conditions; however recent studies have shown that not only loss but also excess production of NO can be equally deleterious [142-143]. Role of NO in the bladder is controversial, with some studies suggesting its partial involvement in the relaxation of the detrusor while others showing no changes in bladder function with NO [108-109].

**Superoxide (O$_2^-_{}$):** Superoxide, the simplest of the peroxyl radicals, historically known to be an important bactericidal in host defense mechanisms, is generated by the reduction of molecular oxygen to water [141-143,151]. It is not permeable like NO and is restricted to the compartment where it is generated. It has a short life span of $10^{-6}$ sec [141-143,151]. It can act both as an oxidizing agent and get reduced to H$_2$O$_2$ or act as a reducing agent and donate electrons to form peroxynitrite with NO [141-143,151]. Under physiological conditions superoxide acts as a bactericidal, causes termination of lipid peroxidation and increases cell proliferation while with increased oxidative stress it produces paradoxical actions such as inflammation, initiation of lipid peroxidation and apoptosis of the cell [151].

**Peroxynitrite (ONOO$^-$):** Interaction of NO with superoxide anion (O$_2^-_{}$); is known to occur at a diffusion-limited, nearly instantaneous rate, forming the highly aggressive oxidant peroxynitrite (ONOO$^-$) [149], a reactive nitrogen species known to nitrate protein tyrosine residues, forming 3-nitro-L-tyrosine (3NT). Peroxynitrite is a powerful oxidant actively involved in inducing cellular
injury by DNA fragmentation, lipid peroxidation, damage to proteins and lipids, hydroxylating and nitrating aromatic compounds leading to tissue injury and organ dysfunction [148-150]. With a half-life of less than 1 sec, peroxynitrite is very stable due to its cis-conformation and is produced 6 times faster than superoxide dismutase (SOD) strength to dismutate superoxide. Formation of peroxynitrite is bimolecular and increased prevalence of either nitric oxide or superoxide anion can promote protein nitration events [148-150]. The reaction of formation of peroxynitrite is shown below.
Antioxidant defense mechanisms: Antioxidant is defined by B. Halliwell as ‘any substance that, when present at low concentrations compared to those of an oxidizable substrate, significantly delays or inhibits oxidation of this substrate’ while L. Pecker defines it as ‘a substrate which protects biological tissues from free radical damage, which is able to be recycled or regenerated by biological reductants’ [139]. Antioxidants present in the tissues neutralize the free radicals formed during increased oxidative stress under pathological conditions and decreased antioxidative defense has been shown previously in disease conditions [139]. However antioxidant defense in the bladder; for example activity and or content of antioxidant enzymes has not been investigated in the diabetic bladder settings and these studies would help to further understand the alterations in antioxidative defense mechanisms in the bladder during diabetes.

Antioxidant defense mechanisms fall into two categories the enzymatic defenses and the non-enzymatic defenses. Some of these systems are discussed below

Enzymatic defenses: Superoxide dismutase: Superoxide dismutase is an important enzyme that dismutates superoxide into oxygen and hydrogen peroxide. Three distinct isoforms of SOD are present viz. SOD1 or copper zinc form of SOD [Cu/Zn SOD] found in the cytoplasm, SOD2 or manganese form [MnSOD] found in the mitochondria and SOD3 or extracellular SOD [ECSOD] found extracellularly [141,152]. Although a detailed investigation of the SOD activities in the diabetic bladders is lacking; alterations in SOD activity have been

31
shown during diabetes and decreased SOD activity is found in the bladders from human cancer tissues [153].  **Catalase:** Catalase is actively involved in converting hydrogen peroxide into oxygen and water. Similar to SOD, studies on the activity of catalase in the bladder are few and have shown decreased activity during bladder cancers [141,153].  **Glutathione peroxidase:** Another important enzyme that is involved in detoxification of hydrogen peroxide into oxygen and water is glutathione peroxidase. Glutathione is a ubiquitous sulfhydryl antioxidant that is water soluble and present in millimolar concentrations in the cell, making it the most highly concentrated intracellular antioxidant [141,154]. Glutathione exists in two forms the reduced glutathione (GSH) and the oxidized glutathione (GSSG) [141,154]. Increased oxidative stress has been associated with depletion of glutathione and administration of N-acetyl cysteine (NAC) has shown beneficial results [141,154].

**Non-enzymatic defenses: Vitamins:** Vitamin supplements such as vitamin C (ascorbic acid), vitamin E (tocopherol) and vitamin A (β-carotene) have been suggested as important exogenous antioxidants and most of the animal studies and clinical trials have shown promising results [141,144]. Protective role of vitamin supplementation in bladder cancers [156] and partial outlet obstruction [155] is established however investigations of role of vitamin supplementation in diabetic bladder are lacking. Detailed studies on the role of vitamin supplements alone and in combination to maximize antioxidant defense are warranted.
**Vitamin C:** Vitamin C or ascorbic acid is a water-soluble antioxidant that can scavenge singlet oxygen, superoxide and hydroxyl radicals [144]. Based on the new clinical, epidemiological, molecular and chemical data, the daily recommended dose for vitamin C has been increased from 60 mg/day to 120 mg/day by The Food and Nutritional Academy of Sciences [144]. **Vitamin E:** The lipid soluble antioxidant inhibits lipid peroxidation [scavenging peroxyl radicals] and regenerates vitamin C and GSH [maintaining protein sulfhydryls in their reduced form] [141,144]. Protective effects of vitamin E have been recently documented in a rat model of partial outlet obstruction of the bladder [155]. **Vitamin A:** Vitamin A and beta-carotene have been suggested to be important antioxidants since they interrupt the generation of ROS at a very early stage [144].

**Oxidant driven signaling pathways in diabetic bladder:** Oxidant driven signaling pathways are known to be associated with smooth muscle hypertrophy, proliferation, cell growth, survival and death by activating the mitogen activated protein kinase pathway (MAPK) or Akt pathway or caspases. Here we have discussed the MAPK and Akt pathways as important contributors to smooth muscle proliferation and hypertrophy [157].

**Mitogen activated protein kinase pathway:** MAPKs are serine threonine kinases that are activated through dual phosphorylation at conserved threonine or tyrosine residues and are known to be involved in a diverse set of responses
affecting cell fate, cell proliferation, cell differentiation, adaptation to environmental stress and apoptosis [158-159]. MAPK pathways are known to alter gene expression of AP-1, CREB, SRE etc which are responsible for regulation of transcription of genes involved in cell growth, differentiation etc. [158-159]. The MAPK pathways are categorized into three major subfamilies viz. Erk, JNK and the p38 pathway [158-159]. There is large and accumulating evidence in the literature linking diabetes and oxidative stress with the activation of MAPKs [158]. Urinary bladders exposed to mechanical stretch are known to activate the MAPK pathways measured by the phosphorylated antibodies to Erk, JNK and p38; [160-161]. In addition, several settings of smooth muscle hypertrophy and proliferation have been linked to oxidant related signaling cascades [157], although such phenomena have not been detailed in the setting of diabetic bladder remodeling.

**Akt/protein kinase B:** Akt/protein kinase B is a serine threonine kinase and a downstream effector of PI3 kinase and has also been known to play a role in cell survival and protein synthesis [157,162-163]. Studies on the role of p-Akt in Type 2 diabetes suggest an increase [164], decrease [165] or no change [166] in the Akt status. ROS associated Akt activation has been previously documented [157]. NADPH oxidases thru Rac1 have been implicated in its activation [157]. Role of Akt activation in the diabetic bladders is not known.
An understanding of the source of reactive oxygen species, the type of reactive species and the signaling pathways that they modify in the diabetic bladder will enable us to gain insight on the physiological and pathophysiological changes that occur in the bladder during diabetes and help us devise important therapeutic targets.
Current therapeutic strategies and limitations:

The first step in managing diabetic cystopathy is the control of blood glucose levels in the body. Previous studies in the animal models have shown that insulin therapy can prevent or delay urodynamic and histopathological changes in diabetes [50,60,131]. The Diabetes Control and Complications Trial (DCCT), however, had disappointing results when intensive glycemic control for 5 years could not completely prevent diabetic neuropathy [139]. Although some studies have documented no specific treatments for diabetic bladders [91]; earlier treatments aimed at compensating deficient bladder sensation have been routinely used. None of the treatments available so far are effective in completely curing the dysfunction. Some of the commonly employed treatment strategies are discussed below.

**Conservative treatments:** The conservative treatments include educating the patients for scheduled or timed voiding every third hour during the daytime [166-168]. This helps to compensate for the lack of first desire to void. Repetitive voiding by double or triple voiding technique may be recommended to replace a deficient detrusor contraction enabling the patient to discharge almost all of the bladder volume [166-168]. Applying manual suprapubic pressure (Crede maneuver) has been commonly used for patients who find it difficult to maintain sufficient straining [166-168].
Pharmacologic treatment: Cholinergic drug treatment with bethanechol 10 to 20 mg orally three to four times a day has been commonly employed to compensate for the loss of the afferent part of the reflex arc triggering detrusor function. These drugs increase the intravesical pressure and decrease bladder capacity by stimulating the autonomic effector cells and postganglionic parasympathetic receptors [122-123,166-168]. However side effects include sweating, salivation, tachycardia and flushing limit their use and so use booster doses 30 to 60 mg orally twice weekly have proved to be effective. The urethral resistance is decreased by use of prazocin, an alpha1 adrenergic blocker [168].

Indwelling catheter: Intermittent catheterization or indwelling catheter is used in severe cases to decrease the overstretched detrusor and to eliminate the stasis of upper urinary tract [166-168]. The chances of increased urinary infections associated with catheter can be reduced by using silicone catheters or those supplemented with oral disinfectants.

Electrical stimulation and neuromodulation: Active contraction and voluntary control of the detrusor is achieved by intravesical electrical stimulation, which results in activation of mechanoreceptors within the bladder wall [168]. This technique can be performed at home using a catheter containing a stimulating electrode (pulse width 2ms. frequency 20 Hz and current 1 to 10 mA) that is introduced into the bladder. Each therapy session lasts for 90 minutes.
5 to 6 days a week for 2 weeks [168]. Although the mechanism is still unclear, neural modulation of sacral nerve roots has been implemented to stimulate the somatic portion of the sacral nerves and improve bladder voiding and filling [168].

**Surgery:** If none of the above treatments work then transurethral surgery of the bladder neck is considered and advocated. This is however associated with sexual disturbances and retrograde ejaculation [166-168].

**Emerging treatment: Gene therapy:** Recently the importance of gene therapy in diabetic bladder research has been increased mainly because of the awareness and significance of the disabilities and the potential market they represent [170-171]. Minimally invasive molecular medicine therapy with lower risk of systemic toxicity makes bladder the most suited organ for gene therapy [170-171].

Recently, Goins et have used herpes simplex virus mediated nerve growth factor expression in the bladder and afferent neurons as a potential treatment for diabetic bladder dysfunction [172]. Their studies showed improved bladder function in the 6 week STZ induced diabetic rats treated with nerve growth factor suggesting that gene therapy approach for diabetic bladder dysfunction is a powerful and promising treatment which is realistically within reach [170-172].
Although gene therapy is still in rudimentary stages and much work needs to be done to establish it as an approved therapeutic technique in the clinical setting, it still holds a great potential and promise to revolutionize treatments for diabetic bladder dysfunctions.

**Summary:** In summary, urinary bladder dysfunction in diabetes is a well-recognized phenomenon but the mechanisms involved and initiating events are not clear. Oxidative stress and oxidant driven signaling pathways have been shown to be important contributors in pathological conditions; however, their role in bladder dysfunction during diabetes has not been investigated earlier. For my dissertation research we used the well-established streptozotocin induced diabetic rodent models and transgenic Rac1 over expressed model to test the hypothesis that oxidants and oxidant signaling pathways might be important contributors in diabetic bladder dysfunction. We studied the functional, structural, neuronal and oxidative aspects of the bladder using immunohistochemistry and digital imaging methods to get detailed morphometric analysis of bladder tissues in a time dependent and region specific manner. The main goal of this research is to provide new insight regarding adaptive and maladaptive processes in bladder physiology and find enhanced opportunities for improved therapeutic strategies for an important diabetic complication.
Thesis Objectives

The central goal of my thesis project was to study oxidative mechanisms in diabetes related urinary bladder dysfunction. Chapter 2 focused on the using automated digital imaging methods as an unbiased, reproducible, and convenient method for detailed morphometric analysis of bladder tissues in the streptozotocin induced diabetic rat model and studying the early, time dependent structural remodeling in the three well demarcated layers of the rat urinary bladder. Early and time-dependent alterations in key neurological controllers of the bladder were investigated using the rat STZ model and immunohistochemical techniques, viz. the localization of tyrosine hydroxylase (TH, a classical marker of prejunctional sympathetic innervation), vesicular acetylcholine transporter (VACHT, classical marker of prejunctional parasympathetic innervation), and neuronal nitric oxide synthase (NOS1, a marker of non-adrenergic non-cholinergic innervation) as shown in Chapter 3.

Although there is now convincing evidence that oxidative stress is an important component of several other diabetes related complications, the potential involvement of such phenomena in diabetic cystopathy has not been previously investigated, particularly with respect to reactive nitrogen species. For these reasons we investigated time dependent changes in NOS isoforms and 3NT (a stable biomarker of reactive nitrogen species in the urinary bladder of the STZ induced diabetic rats in Chapter 4.
Chapter 5-6 we downsized our techniques from the rat to the STZ mouse model of diabetes and studied role of functional, structural, neuronal remodeling as well as alterations in oxidant species formation and oxidant signaling pathways, especially derived by Rac1 as an important activator of NADPH oxidase and superoxide. Thus the goals of Chapter 5 were to test the hypothesis that the mouse is an appropriate and relevant model for diabetic bladder investigations and to define the alterations in bladder structure, functional responses, and neuronal prevalence in this setting. In Chapter 6 we studied the role of oxidant and oxidant driven signaling pathways especially the pathways that have been associated with cell proliferation and cell differentiation and their effects on bladder during diabetes.

In Chapter 7 we worked on a transgenic mouse model that selectively overexpresses Rac1 in the smooth muscle using SM α-actin. We studied and compared the functional, structural and neuronal remodeling that occurs in this transgenic model with the STZ mouse model of diabetes demonstrating that oxidative phenomenon in the absence of glucose and volume overload mimics bladder changes seen during diabetes. Inflammatory responses in the urothelium and the smooth muscle of the bladder during diabetes and increased oxidant conditions were studied in Chapter 8 using NOS2 and cyclooxygenases as markers for inflammatory changes in the bladder.
Autonomic neuropathy is a serious, common, and often overlooked component of diabetic neuropathy. Frequent clinical symptoms include erectile dysfunction, bladder and gastrointestinal dysfunction and orthostatic hypotension. In Chapter 9 we studied the perineurium characteristics and alterations in connexin isoform expression and oxidant formation, in the dorsal penile nerve of the STZ rat model of diabetes, as an important event during diabetic neuropathy.

In Appendix 1, we demonstrated that detrusor tissue NO production may play a subtle but important role in bladder accommodation to increased volume and this is selectively involved in the initiation event mediating an initial component of the bladder auto relaxation response. Effects of 5% sucrose on the functional changes in the bladder during diabetes and volume overload were studied in Appendix 2 using the STZ mouse model of diabetes (Type 2 like).
<table>
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<th>YEAR</th>
<th>PATIENT</th>
<th>GENDER</th>
<th>AGE</th>
<th>DURATION</th>
<th>TREATMENT</th>
<th>METHOD</th>
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<td>20 men</td>
<td>17-73 years</td>
<td>1.9-10.1 years</td>
<td>net mentioned</td>
<td>Clinical exam</td>
<td>43% had ↑ urine frequency; women &gt; men</td>
<td>Indicate a neurogenic dysfunction of the bladder</td>
</tr>
<tr>
<td></td>
<td></td>
<td>47 women</td>
<td>avg. 65.3 years</td>
<td>avg. 8.9 years</td>
<td></td>
<td>Roentgenological exam</td>
<td>Large and abnormal bladders; men &gt; women</td>
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<td></td>
<td>Micturition</td>
<td>↑ urine retention; men &gt; women</td>
<td>type of sensory as well as motor</td>
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<td></td>
<td>Urethrocystography</td>
<td>Disturbance in internal sphincter; men &gt; women</td>
<td></td>
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<td></td>
<td></td>
<td>Roentgenological exam</td>
<td>↑ with neuropathy</td>
<td></td>
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<tr>
<td>1967</td>
<td>n = 36 diabetics with neuropathy</td>
<td>20 males</td>
<td>22-69 years</td>
<td></td>
<td>diet: 10%</td>
<td>Cystoscopy &amp; cystoscopy</td>
<td>83% of diabetics with neuropathy had: Progressive decapsulation of the incipient asymptomatic diabetic bladder is cause of increased infections in diabetics</td>
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<tr>
<td></td>
<td></td>
<td>16 females</td>
<td></td>
<td></td>
<td></td>
<td>↑ Intravesical pressure (450 to 600 cc)</td>
<td></td>
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<tr>
<td></td>
<td>n = 24 diabetics without neuropathy</td>
<td>10 males</td>
<td>16-73 years</td>
<td></td>
<td>diet: 8.3%</td>
<td>Cystoscopy &amp; cystoscopy</td>
<td>↑ initial response (900 to 1300 cc)</td>
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<tr>
<td></td>
<td></td>
<td>14 females</td>
<td></td>
<td></td>
<td></td>
<td>↑ Bladder capacity (1300 cc)</td>
<td></td>
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<tr>
<td></td>
<td>n = 25 non diabetic controls</td>
<td>10 males</td>
<td>35-71 years</td>
<td></td>
<td>diet: 33%</td>
<td>Cystoscopy &amp; cystoscopy</td>
<td>↑ residual urine (100 cc)</td>
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<td></td>
<td></td>
<td>16 females</td>
<td></td>
<td></td>
<td></td>
<td>↑ Large flabby bladders</td>
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<td></td>
<td></td>
<td></td>
<td>Advanced diabetic neurogenic bladder associated with:</td>
<td>residual urine, urine infection, renal infection, bacterinia</td>
<td></td>
</tr>
<tr>
<td>1970</td>
<td>n = 30 diabetic</td>
<td>30 male</td>
<td>20-50 years</td>
<td>avg 10 years</td>
<td></td>
<td>Cystoscopy</td>
<td>polyuria</td>
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<td></td>
<td></td>
<td>25 male</td>
<td></td>
<td>avg 35 years</td>
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<td>n = 25 controls</td>
<td>25 male</td>
<td></td>
<td>avg 30 years</td>
<td></td>
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<td></td>
<td></td>
<td>Cystoscopy</td>
<td>↑ Bladder capacity &gt; 600 ml</td>
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<td></td>
<td></td>
<td>Intravesical pressure (450 to 500 cm of water)</td>
<td>↑ Intravesical pressure</td>
<td></td>
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<td></td>
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<td>Hypertonic bladder shape</td>
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<td></td>
<td></td>
<td></td>
<td>Signs of infection</td>
<td></td>
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<td></td>
<td></td>
<td>Electromyography</td>
<td>↑ motor unit potential duration in spinthers</td>
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<td></td>
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<td></td>
<td></td>
<td></td>
<td>No neuronal damage or myopathy</td>
<td></td>
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<tr>
<td>1971</td>
<td>n = 31 type 1 diabetics</td>
<td>24 males</td>
<td>14-46 years</td>
<td>↑ residual urine (&gt; 50 ml)</td>
<td></td>
<td>Cystoscopy</td>
<td>↑ Bladder capacity (550 ml vs 960 ml)</td>
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<tr>
<td></td>
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<td>7 females</td>
<td></td>
<td></td>
<td></td>
<td>Intravesical pressure (&lt; 10 vs 5.0 cm of water)</td>
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<td></td>
<td></td>
<td>Enlarged acetic bladders</td>
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<td></td>
<td></td>
<td>67% had abnormal bladder lesions</td>
<td>Bladder lesions are due to neuropathy</td>
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<td></td>
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<td>8 controls</td>
<td>24 years</td>
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<td></td>
<td>Cystoscopy</td>
<td>67% had abnormal bladder lesions</td>
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<td></td>
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<td>5 males</td>
<td></td>
<td></td>
<td></td>
<td>↑ Intravesical pressure (&lt; 10 vs 5.0 cm of water)</td>
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<tr>
<td></td>
<td></td>
<td>3 females</td>
<td></td>
<td></td>
<td></td>
<td>6% with neck hypertrophy</td>
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<td></td>
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<td></td>
<td></td>
<td>Bio-electrical study</td>
<td>↑ Bladder capacity (550 ml vs 960 ml)</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>↑ residual urine (&gt; 50 ml)</td>
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<td></td>
<td>Urodynamic techniques</td>
<td>58% had acetic bladders. Low pressure curves and absence of detrusor contractions</td>
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<td>Grossly abnormal bladder capacity (avg 817 ml)</td>
<td>Higher incidence of abnormal detrusor function in asymptomatic diabetics</td>
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<td>↑ in bladder sensation</td>
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<td>24% with complete bladder loss</td>
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<tr>
<th>Study</th>
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<th>Sex</th>
<th>Finding</th>
<th>Notes</th>
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<tr>
<td>1973</td>
<td>n = 10</td>
<td>26-67 years</td>
<td>4-30 years</td>
<td>not mentioned</td>
<td>Histochemistry: Morphologic abnormalities in intraneuronal nerve axons with altered normal Vesicoureteral reflux bullets and beaded thickening of nerve fibre</td>
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<td></td>
<td>Autopsies: 5</td>
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<td>Cholinesterase activity ↓ activity in the muscle ↓ activity in the nerve axons</td>
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<tr>
<td></td>
<td>n = 5 controls</td>
<td></td>
<td></td>
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<tr>
<td>1974</td>
<td>n = 40</td>
<td>26 males</td>
<td>ages 26-75</td>
<td>0-39 years</td>
<td>not mentioned</td>
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<tr>
<td></td>
<td>14 females</td>
<td></td>
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<tr>
<td>1974</td>
<td>n = 102</td>
<td>not mentioned</td>
<td>not mentioned</td>
<td>not mentioned</td>
<td>Denervation supersensitivity: gp1-negative (avg pressure 4 cms of water)</td>
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<td></td>
<td>gp1-normal</td>
<td>n = 20</td>
<td></td>
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<td>↑ bladder capacity</td>
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<tr>
<td></td>
<td>gp2-cystitis</td>
<td>n = 24</td>
<td></td>
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<td>↑ bladder capacity</td>
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<tr>
<td></td>
<td>gp3-upper motor neuron lesion</td>
<td>n = 21</td>
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<td>↑ bladder capacity</td>
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<td>gp4-Lower motor neuron lesion</td>
<td>n = 16</td>
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<td>↑ bladder capacity</td>
</tr>
<tr>
<td></td>
<td>gp5-multiple sclerosis</td>
<td>n = 24</td>
<td></td>
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<td>↑ bladder capacity</td>
</tr>
<tr>
<td></td>
<td>gp6-diabetes</td>
<td>n = 8</td>
<td></td>
<td></td>
<td>↑ bladder capacity</td>
</tr>
<tr>
<td>1976</td>
<td>n = 50</td>
<td>not mentioned</td>
<td>not mentioned</td>
<td>not mentioned</td>
<td>T bladder capacity</td>
</tr>
<tr>
<td></td>
<td>(1 diabetic patient)</td>
<td></td>
<td></td>
<td></td>
<td>↑ bladder capacity</td>
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<td></td>
<td></td>
<td></td>
<td>↑ bladder capacity</td>
</tr>
<tr>
<td>1976</td>
<td>n = 60</td>
<td>35 males</td>
<td>20-60 years</td>
<td>dat: 11.8% Ophio: 18.6% insulin: 51.6%</td>
<td>Urodynamic studies: Cystometry</td>
</tr>
<tr>
<td></td>
<td>25 females</td>
<td></td>
<td></td>
<td></td>
<td>↑ bladder sensitivity</td>
</tr>
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<td>↑ bladder sensitivity</td>
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<td>↑ bladder sensitivity</td>
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<td></td>
<td></td>
<td></td>
<td>↑ bladder sensitivity</td>
</tr>
<tr>
<td>1976</td>
<td>n = 27</td>
<td>15 men</td>
<td>12-74 years</td>
<td>1-44 years</td>
<td>Insulin</td>
</tr>
<tr>
<td></td>
<td>type 1 and type 2</td>
<td>12 women</td>
<td>avg 46.9 years</td>
<td>avg 16.7 years</td>
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<table>
<thead>
<tr>
<th>Year</th>
<th>n</th>
<th>Gender</th>
<th>Age (years)</th>
<th>Duration</th>
<th>Studies</th>
<th>Findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>1976</td>
<td>30</td>
<td>17 men: 13 females</td>
<td>12-78</td>
<td>1-44</td>
<td>Bladder capacity (635 ml)</td>
<td>Diabetic vesical dysfunction is principally the result of detrusor reflex function (33%), normal detrusor function (23%), detrusor hyperreflexia (43%), and detrusor areflexia. Integrated sphincter electromyography: 10% had normal sphincter function, 10% had sphincter dysynergy, and 10% had detrusor sphincter dyssynergia. Reflex evoked-response latency measurement: 160% increase in neural conduction velocity (96.5 msec).</td>
</tr>
<tr>
<td>1976</td>
<td>124</td>
<td>67 males: 57 females</td>
<td>13-76</td>
<td>Avg: 45</td>
<td>Urodynamic studies: Bladder capacity (&gt; 500 ml)</td>
<td>Residual urine (50-1500 ml)</td>
</tr>
<tr>
<td>1981</td>
<td>50</td>
<td>12 men: 38 women</td>
<td>50 years</td>
<td>5-10</td>
<td>Cystoscopy: 52% suffered from bladder disorders (36% had enlarged bladders, 30% had residual urine (&gt;200 ml)</td>
<td>Micrrthecography: 24% with bladder distention and 76% with effective treatment of these 42%</td>
</tr>
<tr>
<td>1985</td>
<td>6 IDDM</td>
<td>3 males: 3 females</td>
<td>15-40</td>
<td>3 months</td>
<td>Cystoscopy: Bladder capacity (800 vs 582 ml before &amp; after)</td>
<td>Residual volume (254 vs. 47 ml)</td>
</tr>
<tr>
<td>1995</td>
<td>182</td>
<td>116 men: 68 women</td>
<td>48-83</td>
<td>58.4</td>
<td>Diet: 37%</td>
<td>Ophthalmic: 36%</td>
</tr>
<tr>
<td>1997</td>
<td>53 diabetic (unselected)</td>
<td>25 men: 26 women</td>
<td>24-67</td>
<td>3 months</td>
<td>Cystoscopy: Bladder capacity (avg 600 ml)</td>
<td>Diabetic bladder dysfunction is a likely manifestation of sympathetic skin response (52%), sympathetic skin response (52%), and sympathetic skin response (52%). Diabetic bladder dysfunction is a likely manifestation of peripheral neuropathy.</td>
</tr>
<tr>
<td>1997</td>
<td>10 controls</td>
<td>3 men: 7 women</td>
<td>22-60</td>
<td>50</td>
<td>Sympathetic activity: Autonomic neuropathy</td>
<td>Mean amplitude (1.40 vs 0.60 mV)</td>
</tr>
<tr>
<td>Year</td>
<td>Study</td>
<td>Gender</td>
<td>Age</td>
<td>Intensive Therapy</td>
<td>Urinary Symptoms</td>
<td>Intensive Therapy Effects</td>
</tr>
<tr>
<td>------</td>
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<tr>
<td>1999</td>
<td>DCCT</td>
<td>763 males 677 females</td>
<td>avg: 27 years avg: 15 years</td>
<td>Conventional insulin therapy: 1-2 inj/day</td>
<td>Urinary symptoms: Significant decrease in incomplete bladder emptying</td>
<td>Intensive therapy prevents or retards the development of abnormal R-R variation but does not affect majority of the diabetic bladder disorders</td>
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</tr>
<tr>
<td>1999</td>
<td>DCCT</td>
<td>21 men 8 women</td>
<td>13-78 years avg: 58 years avg: 13.2 years</td>
<td>Diet: 26% Hypoxic: 37% Insulin: 44%</td>
<td>Cystometry: First urge to void (avg 1900ml)</td>
<td>Vesicourethral dysfunction highly correlated with nerve conduction velocity</td>
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<td></td>
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<td></td>
<td>Denervation supersensitivity test of the bladder</td>
<td>Negative in all patients</td>
<td>No interruption in peripheral or distal spinal innervation of the bladder</td>
</tr>
<tr>
<td></td>
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<td></td>
<td>Nerve conduction velocity</td>
<td>Abnormal sensory nerve conduction was associated with bladder capacity and first desire to void</td>
<td>Abnormal motor nerve conduction was associated with voiding dysfunction</td>
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</tr>
<tr>
<td>1</td>
<td>Blood glucose/ urine glucose</td>
<td>Increase</td>
<td>30-81</td>
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<td>2</td>
<td>Urine output</td>
<td>Increase</td>
<td>30-32, 34-45, 48-55, 59, 60, 62, 66, 67, 69, 70, 73, 78</td>
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<td>Urine frequency</td>
<td>Increase</td>
<td>38, 41, 42, 45, 48, 50, 51, 60, 62, 66, 67, 69, 70, 73</td>
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<td>4</td>
<td>Body weight</td>
<td>Decrease</td>
<td>30-32, 35-40, 43-52, 54-67, 69, 72-78</td>
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<td>5</td>
<td>Bladder weight</td>
<td>Increase</td>
<td>30-81</td>
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<td>6</td>
<td>Serum Insulin</td>
<td>Decrease</td>
<td>40, 54, 69, 67, 74</td>
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<td>7</td>
<td>Protein weight/ bladder wt</td>
<td>Unchanged/Decrease/Increase</td>
<td>30, 31, 39, 50, 51, 59, 67, 69</td>
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<td>8</td>
<td>Water consumption/ polydipsia</td>
<td>Increase</td>
<td>36, 41, 42, 45, 48, 50, 51, 54, 60, 62, 67, 66, 69, 73, 77</td>
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**STRUCTURAL CHANGES**

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<td>1</td>
<td>Total diameter</td>
<td>Increase</td>
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<td>Muscle wall thickness</td>
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<td>Lumen area</td>
<td>Unchanged</td>
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<td>4</td>
<td>Muscle cell diameter</td>
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<td>5</td>
<td>Collagen concentration</td>
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<td>Cross-sectional area</td>
<td>Increase</td>
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<td>Hypertrophy/distension</td>
<td>Yes</td>
<td>31, 33, 44-48, 58, 62, 66, 69</td>
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<td>8</td>
<td>Bladder size/mass</td>
<td>Increase</td>
<td>31, 33-34, 39, 40, 43-52, 54, 58, 62, 66, 67, 69, 70, 72, 73, 76, 77</td>
</tr>
</tbody>
</table>

**TABLE 1.2:** Experimental evidence of urinary bladder dysfunction during diabetes in the rat model
| TABLE 1.2 CONTD... |
|------------------|------------------|------------------|------------------|------------------|------------------|
| **URODYNAMIC CHANGES** | **Bladder capacity** | **Increase** | **36-42, 44-47, 49-51, 62-62, 67** |
| 2 | **Bladder compliance** | **Increase** | **38, 39, 45, 49, 62, 67, 70** |
| 3 | **Intravesicular pressure** | **Decrease** | **37, 39, 45-47, 62-63, 67, 70** |
| 4 | **Voiding function** | **Unimpaired** | **36** |
| 5 | **Residual urine** | **Increase** | **32, 49, 52, 63** |
| **NEUROLOGICAL** | **Neurogenic lesion** | **No** | **35** |
| 2 | **Demyelination/ nerve fibers/axonopathy** | **Yes** | **48, 44** |
| 3 | **Parasympathetic (musca.) responsiveness/sensitivity** | **Increased** | **32, 40, 50, 51, 54, 55, 57, 62, 64, 67, 70, 73, 75** |
|  |  | **Decreased** | **34, 37, 41-42, 46-48, 50, 51** |
|  |  | **Unchanged** | **30-31, 43, 45-47, 50-52, 61, 67** |
|  | **Receptor density/ Content/ staining/ activity** | **Increase** | **40, 43, 50, 51, 54, 56-57, 61, 69, 79 81,** |
|  |  | **Decrease** | **30-31, 41-42,** |
| 4 | **Sympathetic (beta & alpha) responsiveness/sensitivity** | **Increased** | **32, 41, 42, 54** |
|  |  | **Decreased** | **34, 39** |
|  |  | **Unchanged** | **30-31, 50, 51, 61** |
|  | **Receptor density/ content/ activity** | **Increase** | **54, 61** |
|  |  | **Decrease** | **30, 41, 42** |
| 5 | **ATP responsiveness/sensitivity/ receptor density** | **Increased** | **37, 50, 51, 67, 75, 76** |
|  |  | **Decreased** | **34, 50, 51** |
|  |  | **Unchanged** | **41-42, 48, 50-51, 80** |
| 6 | **Electrical/nerve field stimulation contractile resp.** | **Increase** | **46-47, 50-52, 55, 67, 70, 73** |
|  |  | **Decrease** | **34, 36, 46-48, 62** |
|  |  | **Unchanged** | **35, 43, 45, 49-51, 67** |
### TABLE 1.2 CONTD.....

#### AFFERENT CHANGES

<p>| | | | |</p>
<table>
<thead>
<tr>
<th></th>
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<tbody>
<tr>
<td>1</td>
<td>Capsaicin</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Unchanged</td>
<td>36, 41, 42, 63</td>
<td></td>
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<tr>
<td></td>
<td>Decrease</td>
<td>65, 72</td>
<td></td>
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<tr>
<td>2</td>
<td>Afferent Conduction velocity</td>
<td>Decrease</td>
<td>49, 58</td>
</tr>
<tr>
<td>3</td>
<td>Sub P (Function)</td>
<td>Increase</td>
<td>63, 65</td>
</tr>
<tr>
<td></td>
<td>Unchanged</td>
<td>41, 42, 48, 59, 72</td>
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<tr>
<td></td>
<td>Sub P conc.</td>
<td>Decrease</td>
<td>36, 59, 65</td>
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<tr>
<td></td>
<td>Sub P content</td>
<td>Increase</td>
<td>36, 59</td>
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<tr>
<td>4</td>
<td>Neurokinin A content</td>
<td>Increase</td>
<td>36,</td>
</tr>
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<td>5</td>
<td>CGRP content</td>
<td>Increase</td>
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<td>6</td>
<td>Prostaglandin</td>
<td>Increase</td>
<td>33, 41, 42, 64</td>
</tr>
<tr>
<td></td>
<td>Decrease</td>
<td>80</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Unchanged</td>
<td>65</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>VIP function</td>
<td>Unchanged</td>
<td>59</td>
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<tr>
<td></td>
<td>VIP conc.</td>
<td>Decrease</td>
<td>59</td>
</tr>
<tr>
<td></td>
<td>VIP content</td>
<td>Increase</td>
<td>59</td>
</tr>
<tr>
<td>8</td>
<td>Bradykinin</td>
<td>Decrease</td>
<td>80</td>
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<td>9</td>
<td>NGF</td>
<td>Increase</td>
<td>77</td>
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<td>10</td>
<td>Calcium sensitivity/ force</td>
<td>Increase</td>
<td>55, 76</td>
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<tr>
<td></td>
<td>Calcium conc.</td>
<td>Unchanged</td>
<td>76</td>
</tr>
<tr>
<td></td>
<td>Increase</td>
<td>57</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>Gender differences</td>
<td>No</td>
<td>67</td>
</tr>
<tr>
<td>12</td>
<td>PI hydrolysis/ production</td>
<td>Increase</td>
<td>74</td>
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<tr>
<td>13</td>
<td>Insulin treatment</td>
<td>Reversed</td>
<td>33, 50, 51, 60</td>
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<tr>
<td></td>
<td>Not reversed</td>
<td>69</td>
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#### Myogenic component

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<td>1</td>
<td>KCL response/sensitivity</td>
<td>Unchanged</td>
<td>32, 35, 46, 47</td>
</tr>
<tr>
<td></td>
<td>Decreased</td>
<td>34</td>
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### Table 1.3: Experimental evidence of urinary bladder dysfunction during diabetes using other models.

<table>
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<th>Other Animals</th>
<th>Effects</th>
<th>Ref</th>
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<tr>
<td>Mice</td>
<td>blood glucose/ glucosuria, ↓ insulin&lt;br&gt;bladder weight, ↓ bodyweight&lt;br&gt;urine volume, urine frequency&lt;br&gt;bladder size, enlarged bladders&lt;br&gt;UTI, bladder infections&lt;br&gt;↓ urea in urine&lt;br&gt;Altered kinin receptors</td>
<td>[125-129]</td>
</tr>
<tr>
<td>Rabbits</td>
<td>blood glucose, ↓ insulin&lt;br&gt;bladder weight, ↓ bodyweight&lt;br&gt;capacity, compliance&lt;br&gt;smooth muscle cell proliferation, SMC count&lt;br&gt;Nerve injury: thickening, vacuole, beaded &amp; fragmentation&lt;br&gt;NOS,&lt;br&gt;↓ contractions to Cch&lt;br&gt;Insulin partly/completely reversed the effects</td>
<td>[107,130-133]</td>
</tr>
<tr>
<td>Chinese hamsters</td>
<td>blood glucose, ↓ insulin, Glycated Hb&lt;br&gt;bladder weight, frequency, incomplete voiding&lt;br&gt;VIP fibers/content, ↓ AChE&lt;br&gt;Nerve injury: aberrant myelination &amp; axonal degeneration&lt;br&gt;Unchanged contractions to Cch&lt;br&gt;Dilation and distension</td>
<td>[134-136]</td>
</tr>
<tr>
<td>Guinea pig</td>
<td>volume per void&lt;br&gt;Unchanged response to Ach, neurogenic stimulation &amp; intracellular Ca</td>
<td>[137]</td>
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</tbody>
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REFERENCES


5. Poladia DP and Bauer JA: Early changes during diabetes related bladder remodeling: cell specific changes in nitric oxide synthases, reactive nitrogen species formation, and ubiquitinylation. Diabetes metabolism research and reviews, in press.


39. Kudlacz EM, Chun AL, Skau KA, Gerald MC, Wallace LJ. Diabetes and

40. Latifpour J, Gousse A, Kondo S, Morita T, Weiss RM. Effects of
experimental diabetes on biochemical and functional characteristics of

41. Kudlacz EM, Gerald MC, Wallace LJ. Sensory nerves and urinary bladder
function: effects of diabetes, capsaicin and acrylamide treatment. Gen

42. Kudlacz EM, Gerald MC, Wallace LJ. Effects of diabetes and diuresis on
contraction and relaxation mechanisms in rat urinary bladder. Diabetes.

43. Lee CM, Wong CC. Effects of neonatal capsaicin treatment and
streptozotocin-induced diabetes on urinary bladder function in rats.

44. Paro M, Prosdocimi M, Zhang WX, Sutherland G, Sima AA. Autonomic
neuropathy in BB rats and alterations in bladder function. Diabetes.

45. Malmgren A, Andersson PO, Uvelius B. Bladder function in rats with short-
and long-term diabetes; effects of age and muscarinic blockade. J Urol.


84. Lewis SA. Everything you wanted to know about the bladder epithelium but were afraid to ask. Am J Physiol Renal Physiol. 2000;278(6):F867-74.


148. Freeman BA, Free radical chemistry of nitric oxide, Chest 1994: 105(3); 79S-83S.


150. Beckman JS. Oxidative damage and tyrosine nitration from peroxynitrite. Chemical Research in Toxicology 1996: 9; 836-44.


CHAPTER 2

TIME DEPENDENT URINARY BLADDER REMODELING IN THE 
STREPTOZOTOCIN-INDUCED DIABETIC RAT MODEL.

This chapter has been published in Acta Diabetologica [2002 Apr; 39(1): 23-7] 
and is presented in the style appropriate for the journal. The authors of the 
manuscript are Deepali Anant Pitre, Thong Ma, Lane J. Wallace, and John 
Anthony Bauer.
ABSTRACT

Aim/hypothesis: Urinary bladder dysfunction and remodeling are well-recognized phenomena in diabetes but detailed assessments of tissue morphological changes have not been conducted. Here we studied time dependent morphological changes in bladders from diabetic rats (streptozotocin model) and evaluated the usefulness of automated digital imaging technology as an unbiased, reproducible, and convenient method for the bladder morphometric analysis. Methods: Urinary bladders were isolated from diabetic (3 days, 2 weeks or 5 weeks after single injection of streptozotocin 65mg/kg) or control rats (0 or 5 weeks) and were processed for histochemical evaluations (hematoxylin/eosin and Mason’s trichrome staining). Digital image analysis was used to quantify equatorial cross-sectional areas of bladder tissue and lumen, as well as relative prevalence of the three primary tissue components viz. smooth muscle, urothelium, and extracellular matrix. Results: Digital imaging and color segmentation provided reliable and unbiased evaluations of the bladder tissue sections. Progressive increases in total bladder tissue and lumen area were observed in the diabetic animals relative to controls (p<0.05), demonstrating classical hypertrophy and dilation. Prevalence of smooth muscle and urothelium (% of total tissue) both increased significantly, but collagen content decreased. Average bladder wall thickness and urothelium thickness were unchanged. Conclusion: Bladder remodeling during experimental diabetes is associated with time dependent chamber dilation and increased tissue mass. Changes in bladder
wall composition also occurred in a time dependent manner, most notably increased smooth muscle and urothelium and decreased collagen prevalence. Furthermore, automated digital imaging technologies provide an unbiased, reproducible, and convenient method for detailed morphometric analysis of bladder tissues.
INTRODUCTION

Urinary bladder dysfunction is a common disorder associated with diabetes and is known to affect nearly 80% of this population [1-3]. While this syndrome is typically not life threatening, it is associated with several debilitating symptoms including insidious onset of voiding, progressive bladder paralysis, urinary retention and incomplete voiding [1]. In addition to functional impairment of bladder smooth muscle (e.g. decreased tone and contractility), chronic diabetes is also associated with a general increase in bladder size and capacity [2-4]. While these general changes in bladder properties are well documented, detailed investigations of bladder wall morphology during diabetes have not been previously conducted.

The present investigation was performed to define time dependent morphological changes in the rat urinary bladder during diabetes. We employed the well-characterized streptozotocin model of diabetes [5], which we and others have previously shown to mimic structural and functional changes seen in human bladders, including polyuria, urinary retention, increased bladder mass and capacity [3-6, 10]. Using an intact bladder preparation, we have also observed decreased norepinephrine content and uptake, and decreased contractile response to parasympathetic agonists [4]. This experimental model additionally exhibits a loss of sensory innervation in the detrusor region, a finding consistent with a loss of sensation often reported by diabetics and the inability to initiate micturition appropriately [7].
The bladder wall consists of three well-demarcated layers. A layer of stacked epithelial cells (urothelium) borders the lumen, behind which is a connective tissue interface predominantly composed of extracellular collagen. The outermost layer consists of smooth muscle. Recent studies suggest that these layers interact during normal bladder development and that tissue composition is regulated via paracrine signaling pathways [8]. An important component of our investigations was to develop a digital imaging methods to conduct detailed morphometric analysis of bladder tissues during diabetes progression. This approach allowed us, for the first time, to simultaneously evaluate general bladder morphology (bladder tissue and cavity dimensions) as well as specific prevalence of the three primary components of bladder wall; smooth muscle, urothelium and extracellular matrix. These methods provided a unique opportunity to evaluate time dependent changes in bladder wall composition during diabetes using quantitative methods.
MATERIALS AND METHODS

Animals: All animal handling protocols were approved by The Ohio State University Animal Care and Use Committee. Male Sprague Dawley rats (412±40g, Harlan, Indianapolis, IN) were dosed with streptozotocin (65mg/kg i.p., n=24, Sigma, St. Louis, MO) or vehicle (n=6) and were housed in a 12-hour light/dark facility with free access to food and water. Diabetic rats were sacrificed at 3 days, 2 weeks, or 5 weeks post STZ dosing (n=8 per group). Control animals were sacrificed at either 3 days (n=3) or 5 weeks (n=3) post vehicle injection. Animals were sacrificed by pentobarbital overdose, at which time blood was collected by cardiac puncture, and urinary bladder tissues were rapidly isolated. Blood glucose was measured by a clinical blood glucose monitor (Glucometer Encore, Miles Inc, IN). Glycated hemoglobin was measured using affinity resin column assay kit (Sigma, procedure #422, St. Louis, MO).

Histochemistry and Digital Image Analysis: Urinary bladder was rapidly isolated, sectioned at the equatorial midline and allowed to equilibrate in buffer (e.g., with no intralumenal pressure). Following 48hr fixation in 10% buffered formalin, tissues were dehydrated and paraffin embedded using standard procedures, as previously described [9]. Serial five micron tissue sections were placed on microscope slides, dewaxed, and rehydrated for routine histochemical staining (hematoxylin & eosin, Mason’s trichrome) for morphological studies. Photomicrographs were captured on a Polaroid DMC high-resolution digital camera, mounted on an Olympus research microscope. The images were
analyzed with Image Pro 5.0 image analysis software (Media Cybernetics, Silver Springs, MD). Hematoxylin and eosin stained slides were used to determine bladder tissue cross-sectional area (at the equatorial midline). Each bladder cross-section was captured in its entirety at 80x magnification and internal and external edges were traced (automated edge detection, Image Pro 5.0) for determination of total tissue and inner lumen cross-sectional areas. Mason’s trichrome stained slides were used to determine composition of bladder tissues. Images were captured at 400x magnification and color segmentation was employed to determine the % of the tissue area that was stained “red” (smooth muscle) or “blue” (collagen). A similar segmentation technique was also employed to measure the urothelial cell area with hemotoxylin and eosin stained slides. In all cases the capture and processing of images were performed by investigators unaware of treatment group assignments. For all parameters measured intra- and inter-observer variability was less than 5% (coefficients of variation for 3 daily measurements made by 3 different investigators evaluating 6 bladders).
Statistics: All data are expressed as the mean ± SEM. In all parameters measured the two groups of control animals (from 3 days and 5 weeks post injection) were not statistically different, therefore these animals were combined and are presented as a single group. Comparisons between groups were performed by one-way analysis of variance, followed by Student-Newman-Keuls posthoc tests (Sigma Stat 5.0, Jandel Scientific). Probability values of p< 0.05 were considered significant.
RESULTS

Similar to other investigations, hyperglycemia was observed at day 3 following STZ and was maintained throughout the study (control, 135±14mg/dl; 3 days, 502±27; 2 weeks, 548±19; 5 weeks, 586±14). Glycated hemoglobin, a marker of long term glycemic control, reached statistical significance at 5 weeks (control, 5.0±.5%; 3 days, 5.4±.2%; 2 weeks, 6.5±.3; 5 weeks, 14.2±.5%).

Representative photomicrographs of bladder cross-sections from control and diabetic bladders and image analysis methods are shown in Figure 1 (upper panels 1A-D). Entire cross-sections of trichrome stained tissues are shown at 80x magnification, (panels 1A-D; control, 3 day, 2 week, 5 weeks respectively). These representative images demonstrated progressive changes in bladder shape and size during diabetes in this animal model. Digital image analysis revealed statistically significant increases in total cross-sectional bladder wall area and lumen area (at the equatorial midline) as early as 2 weeks (Figure 1E). Despite these striking increases in bladder tissue mass and lumen dimension, no change in average wall thickness was observed (Figure 1F). These changes are consistent with organ accommodation of increased urine output associated with diabetes.

Shown in Figure 2A and 2B are representative images (400x magnification) demonstrating the color segmentation methods we employed for tissue composition analysis. Figure 2A shows an actual image (trichrome stain), providing red colored smooth muscle, blue colored collagen and red/pink colored...
urothelial layer. Using an appropriate color segmentation protocol, the digital image software has the ability to recognize these different areas and accurately measure each of their cross-sectional areas. Shown in Figure 2B (400x) is a pseudo-colored image demonstrating the accuracy of the color segmentation protocol. The orange color defines the smooth muscle area, the green shows the collagen area, and the yellow shows the urothelium, which was recognized and captured by the automated imaging system; the cross-sectional areas of these 3 regions were calculated automatically. Figure 2C shows the cross-sectional areas of the 3 primary components of bladder wall. As total wall mass nearly doubled (data shown in Figure 1E), the increase was primarily driven by increased smooth muscle and urothelium, rather than increased extracellular matrix. Figure 2D shows the relative prevalence as a percentage of total tissue. Smooth muscle content showed an increasing trend with a significant increase (p < 0.05) at 5 weeks in the diabetic animals as compared to controls. In contrast, the percent of bladder wall area composed of urothelium was not significantly altered during diabetes progression and percent of extracellular collagen steadily declined with the progression of bladder remodeling.
DISCUSSION

Changes in urinary bladder function and structure are well-recognized phenomena in diabetic patients [1,3]. Hallmark clinical features include increases in bladder volume and compliance, and decreased tone generation during micturition. This bladder stasis predisposes diabetic patients to urinary tract infections, which may in turn accelerate renal damage and dysfunction [1]. These complications of diabetes are typically not life threatening but can be very debilitating; they often progress throughout a patient’s life and are associated with significant health care costs. Despite their common occurrence, the mechanisms of diabetes-related urological problems are not well understood. Furthermore, while the bladder clearly increases in size and capacity during diabetes [2-6] a detailed account of the morphological changes in all tissue layers over time has not been previously conducted.

We studied the morphological characteristics of the bladder in the well-established streptozotocin-induced model of diabetes. Since several previous reports have shown that bladder function is already altered in this rat model by 5 weeks post-streptozotocin [4,6,8], we focused on earlier time points to define morphological events prior to this time and to gain mechanistic insight into the early events in this remodeling phenomenon. Several previous reports [4,6] have demonstrated that this animal preparation is a well-known model of diabetes as well as polyuria. However it is important to note that that polyuria does not necessarily mimic bladder changes observed in the streptozotocin model of
diabetes. For example, Eika et al [6] have previously shown that sucrose fed rats have pressure-volume responses that are not different from controls, whereas streptozotocin induced diabetic animals have significant passive function changes. We have also shown that changes in nerve densities were unaltered in the sucrose-fed model, whereas striking changes were observed in diabetic rats [4]. In more recent studies we have observed that mice selectively over-expressing pro-oxidant genes in bladder smooth muscle also develop bladder smooth muscle hypertrophy and decreased collagen content (identical to the remodeling phenomena observed here) in the absence of polyuria or glucose changes (Pitre and Bauer, unpublished). Collectively, these findings suggest that bladder remodeling may not be exclusively dictated by increased urine output per se.

Here we used highly automated digital imaging methods to quantify general size and composition of bladder tissue at various times during diabetes progression. These parameters were objectively and accurately assessed and throughput was facilitated by the creation of an automated subroutine. Further advantages include a high degree of reproducibility (inter- and intra-observer variabilities were <5%), and the generation of continuous parameters for statistical analysis.

We observed a near doubling of total bladder tissue over the 5 weeks of investigation. This increase in total bladder wall area correlates closely to the well-documented increase in bladder mass associated with experimental
diabetes [3,6]. The primary source of this increased tissue mass was the smooth muscle layer, which progressively increased to comprise about 85% of bladder wall over the 5 weeks investigated. Our quantitative analyses of smooth muscle cross-sectional area are consistent with a previous report of bladder smooth muscle hypertrophy in this model, in which smooth muscle cells were assessed using manual measurements [3]. We also observed a progressive increase in the total urothelium but no change in urothelial thickness. This epithelial cell layer plays an important role in normal bladder function (particularly barrier and host-defense mechanisms), but few studies have investigated its properties during diabetes. The only other report on urothelial cells from diabetic bladder showed increased DNA synthesis (measured by thymidine incorporation) [10]. Our results demonstrate that this was translated into cellular proliferation. The progressive increases in the urothelium paralleled changes in the smooth muscle layer, suggesting that there is close regulation between these two components of the bladder wall, at least within the time period we investigated.

It is interesting to note that despite a doubling of total tissue mass, no change in the total amount of collagen in the bladder wall was observed. Thus, as a percentage of total tissue area a statistically significant decrease in collagen was detected at the 2 and 5 week time points. This decrease in extracellular support in combination with smooth muscle proliferation may be important contributors to the changes in passive bladder function (increased compliance) and decreased contractile efficiency observed in this rat model and in diabetic
humans [6,8] and is consistent with other previous investigations that employed chemical measurement techniques from bladder homogenates at a single time point [8].

In summary, automated digital imaging methods provided an unbiased, reproducible, and convenient method for detailed morphometric analysis of bladder tissues. Using these methods we observed time-dependent bladder remodeling during experimental diabetes including chamber dilation and increased tissue mass. Furthermore, important changes in bladder wall composition also occurred in this animal model. Most notably time-dependent increases in smooth muscle and urothelium and reduction in collagen prevalence was observed; changes consistent with the clinical symptoms of bladder dysfunction. Further investigations of these early morphological changes during diabetes progression may provide new mechanistic insights and offer new strategies for therapeutic prevention.
FIGURE LEGENDS

**Figure 2.1:** Upper panel: Representative photomicrographs of Masson's trichome stained transverse sections (collage of images taken at 80X) of bladders obtained from C (control), 3D (3 day), 2W (2 week) and 5W (5 week) diabetic rats are shown in Fig 1A, 1B, 1C, and 1D respectively. Middle panel: Mean morphological assessments shows significant increases in bladder wall area, measured in mm$^2$, (■) and lumen area, measured in mm$^2$, (●) in CTL (control) and streptozotocin-induced diabetic rats at 3D (3 day), 2W (2 week) and 5W (5 week) post injection (Fig 1.5). Lower panel: No changes in the average bladder wall thickness (●) was observed in the CTL (control) and streptozotocin-induced diabetic rats at 3D (3 day), 2W (2 week) and 5W (5 week) post injection Fig (1.6). All data are mean ± S.E.M. *, statistically significant difference from control, $P < 0.05$.

**Figure 2.2:** Upper panel: Representative photomicrograph (400x) demonstrating the color segmentation methods for tissue composition analysis. The different layers of the bladder wall divided into the red colored smooth muscle area (SM), blue colored collagen area (CO) and pink colored urothelial layer (UR) are shown in Fig 2A. Software color segmentation performed on the Masson's stained slides (400X), shows the orange colored smooth muscle area (SM), the green colored collagen area (CO) and the yellow colored urothelium (UR), that was recognized and captured by the automated digital image analysis for the measurement of the % of total tissue cross-section (Fig 2B). Middle panel: Figure 2C shows the
cross-sectional areas of the 3 primary components of bladder wall. Significant increases in the smooth muscle, (▲) and urothelium, (■) were observed as compared to the collagen area, (●) in the streptozotocin-induced diabetic rats as compared to controls. Lower panel: The % of total tissue cross-section for the smooth muscle (▲), collagen (●) and urothelium (■) for the control and streptozotocin-induced diabetic rats at 3D (3day), 2W (2 week) and 5W (5 week) post injection are shown in Fig 2D. All data are mean ± S.E.M. *, statistically significant difference from control, P < 0.05.
**Figure 2.1:** Time dependent structural remodeling in the rat urinary bladder during diabetes.
Figure 2.2: Altered cross sectional areas in the three major regions of the rat bladder with the progression of diabetes.
REFERENCES


CHAPTER 3

INNERVATION AND CONNEXIN ISOFORM EXPRESSION DURING DIABETES RELATED BLADDER DYSFUNCTION: EARLY STRUCTURAL VERSUS NEURONAL REMODELING.

This chapter has been submitted to The Journal of Diabetes and its Complications and is presented in the style appropriate for the journal.
ABSTRACT

Background: Urinary bladder dysfunction is a major complication in diabetes mellitus whose mechanism has been attributed to altered neurological function (autonomic and/or peripheral neuropathy). Previous studies have demonstrated impaired nerve deficiencies, including either loss of nerve function and/or anatomical loss of neuromuscular nerve terminals. While the phenomenon of diabetes related neurological injury is well recognized, its pathogenesis is not well understood. Methods: Using a well-established rat model of diabetes (streptozotocin model) we investigated the prevalence of sympathetic and parasympathetic nerves and relative prevalence of connexin isoforms (gap junction proteins) during diabetes related bladder dysfunction. Immunohistochemistry and digital image analysis was used to detect the prevalence of postjunctional neuronal markers, NOS1 and connexin isoform expressions. Results: Immunohistochemistry showed significant increases in tyrosine hydroxylase (marker of sympathetic innervation) and decreased vesicular acetylcholine transporter (marker of parasympathetic innervation), predominantly in the smooth muscle layer, at 3d after diabetes induction when compared to age-matched controls. Time-dependent and cell specific decreases in the connexin 43 isoform, but transient increases in connexin 32 and 26, were also observed in diabetic rats vs. controls (p<0.05).
**Conclusion:** These data suggest that selective and time dependent expression of gap junction proteins and altered prevalence of sympathetic/parasympathetic innervation are early events during diabetes related bladder dysfunction and remodeling.
INTRODUCTION

Urinary bladder dysfunction is a major complication associated with diabetes [1-7, 14]. Typical symptoms include increased total urinary output, frequent and/or incomplete micturition, atonic bladder, and urinary retention [1-7, 9-14]. These changes are associated with a reduced quality of life as well as an increased risk of other conditions, including urinary tract infections and bladder cancers [8]. Although these structural and functional changes are well recognized, the mechanisms involved are not well defined, and treatment strategies are limited. Furthermore, as the incidence of diabetes continues to rise and as diabetic patients age, the importance and medical costs associated with this syndrome are likely to escalate.

Diabetes related urinary bladder dysfunction in humans has been commonly attributed to altered neuronal control and/or neuronal injury [9-13]. For example, patients commonly experience impaired sensation of full bladder and loss of sphincter control (decreased relaxation during voiding and break-through leaks between voiding intervals). Increased bladder size and altered urodynamic properties such as a 2-4 fold increase in capacity, increased urine output, decreased intravesical pressure, increased residual urine, and enlarged atonic bladders have also been well-documented [10-13]. These symptoms have been associated with impaired sympathetic skin response and decreased sympathetic and parasympathetic nerve conduction, suggesting that diabetic bladder dysfunction is a likely manifestation of peripheral nerve deficiencies, including
either loss of nerve function and/or anatomical loss of neuromuscular nerve terminals. Although many adaptive and maladaptive changes probably occur early during diabetes progression, most clinical studies have only reported bladder changes after many years of duration of diabetes [10-13].

Nearly all of the bladder changes found in humans are apparently recapitulated in the rat model of streptozotocin (STZ) induced diabetes [1-7]. For example, we and others have observed increased urine output, frequent voiding, and atonic bladder following at least five weeks of diabetes induction in this animal model [1-7]. Similar to some previous studies [28, 29] looking at early time points (2 weeks) in diabetic bladder dysfunction, our focus was to investigate early events in the diabetic bladder that may provide new information regarding remodeling process and may lead to better identification of initiating events for therapeutic improvements [14].

As a vesicular organ the urinary bladder is comprised of three primary and concentric regions: the urothelium (a specialized stacked epithelial cell type at the lumen), the lamina propria (a connective layer supporting urothelium rich in supply of blood vessels and nerve bundles) and the smooth muscle layer (responsible for tone generation) [15]. These regions are readily apparent using common microscopy methods, and changes in each of them may have specific consequences with respect to organ function and structure. We have recently documented early, time-dependent, and region-specific urinary bladder remodeling in the rat STZ diabetic model using a specially designed digital
imaging analysis approach. We were able to detect structural changes (hypertrophy and chamber dilation) in the urinary bladder as early as 2 weeks after the induction of diabetes. Selective changes in bladder wall composition also occurred in a time dependent manner (most notably increased smooth muscle and urothelium and decreased collagen prevalence) [14]. These previous studies suggested to us that important regional changes occur very early during diabetic bladder structural remodeling.

Both structural and neurological changes are known to occur in bladder during diabetes, but their inter-relationships are not clear, especially at early time points and with respect to regional distributions [1-7]. Here we tested the hypothesis that the early and time-dependent structural remodeling during diabetes is related to alterations in key neurological controllers of this organ. Using the rat STZ model and immunohistochemical techniques, we investigated the localization of tyrosine hydroxylase (TH, a classical marker of prejunctional sympathetic innervation) [16], vesicular acetylcholine transporter (VACht, classical marker of prejunctional parasym pathetic innervation) [17], and neuronal nitric oxide synthase (NOS1, a marker of non-adrenergic non-cholinergic innervation) [18-20]. Using digital imaging methods developed specifically for bladder tissue investigations, we investigated time and cell type dependent expression of these proteins, allowing us for the first time to determine distinct time dependencies relative to structural changes. An additional component of our study was to investigate the prevalence and distribution of connexin (Cx)
isoforms in diabetic bladder tissues. This family of gap junction proteins plays a critical role in intercellular communication, and alterations in their expression and/or function has been associated with several settings of peripheral neuropathy [21-22]. We have previously shown that dorsal nerve expression of isoforms 43, 32, and 26 are altered during diabetes related peripheral nerve injury and dysfunction [22], and changes in these isoforms have also been implicated in urinary bladder disorders [23-25]. We therefore tested the hypothesis that region specific alterations in connexin 43, 32, and 26 occur during diabetes related bladder dysfunction.
MATERIAL AND METHODS

Animals: All animal handling protocols were approved by the Institutional Laboratory Animal Care and Use Committee. Male Sprague Dawley rats (412±40g, Harlan, Indianapolis, IN) were dosed with streptozotocin (65mg/kg i.p., n=16, Sigma, St. Louis, MO) or vehicle (n=6) and were housed in a 12-hour light/dark facility with free access to food and water. Diabetic rats were sacrificed at 3 days and 2 weeks post STZ dosing (n=8 per group). Age matched vehicle injected controls showed no statistical significant differences and were combined as a single control group (n=6). Animals were sacrificed by pentobarbital overdose, at which time blood was collected by cardiac puncture, and urinary bladder tissues were rapidly isolated. Blood glucose was measured by a clinical blood glucose monitor (Glucometer Encore, Miles Inc, IN).

Histochemistry and Digital Image Analysis: Urinary bladders were rapidly isolated, sectioned at the equatorial midline and allowed to equilibrate in buffer (e.g., with no intralumenal pressure). Following 48hr fixation in 10% buffered formalin, tissues were dehydrated and paraffin embedded using standard procedures, as previously described [14,22]. Serial five micron tissue sections were placed on microscope slides, dewaxed, and rehydrated for routine histochemical staining (hematoxylin & eosin, Mason’s trichrome) for morphological studies. Photomicrographs were captured on a Polaroid DMC high-resolution digital camera, mounted on an Olympus research microscope. The images were analyzed with Image Pro 5.0 image analysis software (Media 102
Cybernetics, Silver Springs, MD). Hematoxylin and eosin stained slides were used to determine bladder tissue cross-sectional area (at the equatorial midline). Each bladder cross-section was captured in its entirety at 400x magnification, and internal and external edges were traced (automated edge detection, Image Pro 5.0) for determination of total tissue and inner lumen cross-sectional areas. Mason’s trichrome stained slides were used to determine composition of bladder tissues. Images were captured at 400x magnification and color segmentation was employed to determine the tissue area that was stained “red” (smooth muscle), “blue” (lamina propria), or red/pink (urothelium). The image analysis procedure converted these to pseudo-colored segments (see Fig 1C for an example) for measurement of cross-sectional areas. In all cases the capture and processing of images were performed by investigators unaware of treatment group assignments. For all parameters measured, intra- and inter-observer variability was less than 5% (coefficients of variation for 3 daily measurements made by 3 different investigators evaluating 6 bladders).

Immunohistochemical staining was performed using methods previously described [14,22], using a DAKO Autostaining system. Briefly, 5µM cross-sections of bladder tissue were dewaxed, rehydrated, and treated with preheated citrate buffer (pH 6.0) to recover the antigenicity. The endogenous peroxidase activity was blocked by the tissues with 3% hydrogen peroxide / methanol solution, followed by 10% goat serum / PBS blocking solution (Vector Lab, Burlingame, CA). The following primary antibodies were used in these
investigations: tyrosine hydroxylase (Rabbit polyclonal antibody, 1:800 dilution, Novus Biologicals Inc; Littleton, CO); vesicular acetylcholine transporter (Rabbit polyclonal antibody, 1:2000, Accurate Chemicals and Scientific Corp; Westbury, NY); connexin 26, 32 and 43 (Rabbit polyclonal antibodies, 1:100, Zymed Lab, San Francisco, CA), NOS1 (Rabbit polyclonal antibody, 1:200 dilution, Research Diagnostics Inc., Flanders, NJ). All primary incubations were conducted for 1 hour; tissues were then washed and exposed to biotinylated goat anti-rabbit secondary antibody (1:200) (Vector Labs, Burlingame, CA) for 20 minutes followed by Horseradish peroxidase-complex reagent (ABC Elite; Vector Labs, Burlingame, CA). Antigen prevalence and localization was visualized using diaminobenzidine (0.06% w/v) and counterstained with methyl green. Preliminary experiments were performed to verify the specificity of all antibodies used herein, by replacing primary antibody with pre-immune serum (isotypic staining controls, used for all antibodies) [14,22]. All staining controls showed no detectable immunoreactivity in any treatment group. The urinary bladders were visualized using light microscopy (Olympus BX40) and photomicrographs were captured using a calibrated digital camera (Polaroid, Cambridge, MA) and transferred into research-based digital image analysis software (Image Pro Plus, Media Cybernetics, Silver Spring, MD). Relative immunoreactivities in cross-sectional bladder regions were determined via integrated optical density measurements [14]. Image analysis was conducted by investigators who were unaware of treatment group assignments. For all parameters measured intra-observer
variability was less than 5% (coefficients of variation for 3 daily measurements) whereas the inter-observer variability was less than 7% (n=3 observers evaluating 6 bladders on three different days).

**Statistics:** All data are represented as mean ± SEM. Parameters between the diabetic and control group were evaluated for significance using ANOVA whereas statistical associations between pairs of measured variables were evaluated using Spearman’s nonparametric correlation analysis (SigmaStat, Jandel Scientific, San Rafel, CA). In all cases statistically significant differences were defined as p < 0.05.
RESULTS

Similar to previous studies, a single dose of STZ caused rapid induction of diabetes; hyperglycemia was observed at day 3 following STZ and was maintained throughout the study (control, 135±14mg/dl; 3 days, 502±27; 2 weeks, 548±19; p< 0.05).

Within two weeks after induction of diabetes, total bladder cross-sectional area (wall mass) and total luminal areas had nearly doubled (Fig 1A), whereas the total thickness of the bladder tissue was unchanged (Fig 1B). At the two week time point, the amount of smooth muscle and of urothelium had approximately doubled, while the lamina propria had not changed in size (Fig. 1D). These time dependent region specific changes in the diabetic rat bladders have been previously documented [14].

Shown in Figure 2 are representative photomicrographs of tyrosine hydroxylase (TH), vesicular acetylcholine transporter (VACHT), and neuronal nitric oxide synthase (NOS1) immunostaining in rat bladders. In control tissues, TH (sympathetic neuronal marker) was most prevalent in the lamina propria regions, especially in the nerve bundles and surrounding the blood vessels. VACHT (parasympathetic/neuromuscular junction marker) was most prevalent in the smooth muscle regions. NOS1 (NANC marker) was detectable in all three regions (urothelium, lamina propria, and smooth muscle).
Changes in markers of innervation were seen as early as three days after the induction of diabetes (Fig 3). TH in the lamina propria was nearly tripled by three days and remained at this level at two weeks. The small amount of this marker in the smooth muscle was not altered by diabetes. The smooth muscle content of VACHT decreased by nearly half at three days of diabetes and decreased even more at two weeks. Finally, NOS1 levels increased in all three areas as early as three days after induction of diabetes. The further increase to approximately three times the control level at two weeks of diabetes was particularly striking in the smooth muscle bladder region.

Shown in Fig 4 are representative photomicrographs for connexins 43, 32 and 26 in bladders from control and diabetic rats (each at 400X magnification). In control tissues connexin 43 and connexin 26 were present predominantly in smooth muscle and urothelium regions, whereas connexin 32 was present in all the three layers. Data from digital analysis of each tissue region are shown in Fig 5. Statistically significant decreases in connexin 43 were observed 2 weeks after the induction of diabetes in the smooth muscle of the diabetic bladders as compared to the controls, although urothelial levels remained unaltered (Figure 5A). Connexin 32 was found to increase significantly in 3-day diabetic rats but its prevalence was decreased at 2 weeks post induction of diabetes in all three regions of bladder tissues (Fig 5B). Similar to connexin 32, connexin 26 was significantly increased (P<0.05) at 3 days but significantly decreased at 2 weeks during diabetes when compared to controls (Fig 5C).
In an effort to relate the various measured parameters derived from identical bladder tissues of control and diabetic rats we employed nonparametric correlation analysis (Spearson’s correlation analysis). Using data for each analyte in each individual bladder region, each pair-wise association was tested for statistical significance using all available data; of these only three relationships were found to be statistically significant (shown in Figure 6). A statistically significant inverse relationship was observed between TH in the lamina propria vs. VACHT in smooth muscle (Figure 6A). In addition a positive association was observed for TH vs. NOS1 in the lamina propria region (6B), and an inverse association was detected for NOS1 and VACHT in the smooth muscle region (6C). No other statistically significant associations were identified.
DISCUSSION

Although structural and functional abnormalities of urinary bladder are well known during diabetes, the mechanisms involved and time dependent interrelationships are not ascertained [1-14]. We have previously shown that the streptozotocin-induced rat model of diabetes exhibits urodynamic alterations similar to humans [2-3], and more recently we have found that region-specific structural changes also occur in a time dependent manner [14]. Because of the importance of early events in remodeling we focused on the initial 14 days following diabetes induction in this model and further developed techniques to allow for detailed in situ measurements of changes in discrete tissue regions. In our initial investigations we detected changes in bladder wall size and composition, but only at the 2 week time point [14]. We found evidence of structural remodeling (increased tissue and lumenal dilation) and hypertrophy of the smooth muscle layer appeared to be the primary contributor to this increased bladder mass [14]. This finding is generally consistent with other published reports (e.g., that smooth muscle hypertrophy occurs in this model weeks after diabetes induction), but simultaneous measurements of all 3 regions have not been described [5,7]. Interestingly, other investigators [28] have shown in this same model that tissue weight are not changed at 2 weeks but increase by ~30% at 4 weeks. In contrast we were able to detect structural changes at two weeks, suggesting that our assessments of composition were more sensitive to the more standard tissue weight measurements. Thus, we feel that the data we provide in
this manuscript illustrates that early changes occur in the bladder that perhaps are not predicted by simple measurements of tissue weights, and that hypertrophy of some regions (smooth muscle) could be masked by changes in the other regions.

We have used the well-established streptozotocin induced diabetic rat model, which is a well-known model of diabetes as well as of polyuria. However, it is important to note that polyuria alone does not necessarily mimic bladder changes observed in the STZ model. For example, Eika et al [27] have previously shown that sucrose fed rats have pressure-volume responses that are not different from controls, whereas STZ treated animals have significant passive function changes. Kudlacz et al [3] also showed that changes in nerve densities were unaltered in the sucrose-fed model, whereas striking changes were observed in STZ diabetic rats. Furthermore, we have recently observed that mice selectively over-expressing pro-oxidant genes in bladder smooth muscle develop organ remodeling phenomena identical to those observed here in the absence of polyuria or glucose changes (Pitre and Bauer, unpublished). Collectively, these findings suggest that bladder remodeling is not exclusively dictated by increased urine output. Therefore, while many of the changes in the bladder are associated with polyuria, there are apparently distinct functional and morphological changes related to diabetes that are different from a simple diuresis model.
Autonomic control of bladder expansion and contraction is predominantly governed by parasympathetic, sympathetic and nonadrenergic noncholinergic inputs [18,26]. Stimulation of local parasympathetic nerve activity causes a highly coordinated contraction of the bladder body and subsequent emptying of the bladder, whereas sympathetic activation and NANC enhances detrusor muscle relaxation [18,26]. Given our findings of time dependent structural changes and an established view that neurological alterations play a major role in this setting we investigated the prevalence of the three major neurological controllers of bladder function. Tyrosine hydroxylase (TH) and vesicular acetylcholine transporter protein (VACHT) are well established and commonly employed prejunctional markers of sympathetic and parasympathetic fibers respectively [16-17]. Nitric Oxide Synthase I (NOS1) is also recognized as an important controller of genitourinary function via its involvement in non-adrenergic non-cholinergic pathways [18-20]. In our initial studies of control tissues we found these markers to be localized to certain rat bladder regions, in that TH was most prevalent in the lamina propria region, VACHT most prevalent in smooth muscle region and NOS1 was widely distributed. Although no detailed immunohistochemistry studies have been previously published, our findings of distribution are generally consistent with evidence from various species [16-20].
The major finding of this work is that the markers of innervation in the bladders from diabetic animals changed before significant alterations in bladder mass were observed. Interestingly, markers for the parasympathetic, sympathetic, and NANC all changed by three days after the induction of diabetes. This suggests that substantial neuronal plasticity occurs in urinary bladder early in the diabetic state and implies the possibility that neuronal plasticity drives structural plasticity.

The changes in bladder innervation markers that were observed at three days and two weeks after the induction of diabetes may be an adaptive process in an attempt to accommodate increased urine output in this setting. The observed general trend was one of reduced parasympathetic influence with simultaneous increases in sympathetic and NANC pathways (see Fig 3), consistent with a controlled physiological shift towards relaxation or capacitance processes. This apparent autonomic shift toward a predominantly smooth muscle relaxant state is consistent with the increased functional capacity, decreased tone, and dilated state observed. Many previous investigations have suggested that diabetic bladder dysfunction may be related to autonomic neuropathy, in which a global loss of neurons and/or nerve terminals has been documented [1-13]. However, neuropathy appears to be a complication associated with a long-term diabetic state. Our data are consistent with this concept in that within the early time frame we examined, markers for two of the three nerve pathways examined were increased – an observation that suggests that frank loss of nerve
terminals is not an operable mechanism involved in changes in bladder tone and function in the early stages of diabetes. These findings are in contrast to nerve terminal loss reported in other settings of diabetic neuropathy [1-13].

The locations of the markers for innervation provide some clues as to how bladder innervation changes in the very early diabetic state. Our data show that VACHT is located exclusively in the smooth muscle, consistent with this and other markers that indicate the parasympathetic nervous system dominates in neuronal control of smooth muscle in the bladder body. Our data indicate that in the early stages of diabetes, the density of parasympathetic innervation is decreasing, consistent with observations of decreased cholinergic activity in diabetic patients and streptozotocin induced 8 week old diabetic rat bladders as shown by reduced histochemical staining for acetylcholine esterase [5,9]. Historically, acetylcholine esterase has been used as a marker of cholinergic innervations in the bladder. However, recent studies have shown that acetylcholine esterase is not always a reliable marker of cholinergic innervation [17]. Hence, we used the most recently available antibody to vesicular acetylcholine transporter as a specific marker of cholinergic innervations.

Our data also show that TH was located primarily in the lamina propria, with a small amount also observed in the smooth muscle. The presence of sympathetic nerves near blood vessels in this bladder region suggests that a major function of sympathetic innervation in the bladder body is control of blood flow to the organ. The small amount of TH marker in the smooth muscle is
consistent with observations that β-adrenergic agonists are capable of stimulating relaxation in this tissue. Finally, NOS1 was observed throughout all regions of the bladder. This suggests either that NANC nerves innervate all regions or that nitric oxide functions in multiple roles, some of which are not related to NANC nerve function. Interestingly, this protein was found to be increased in all three tissue regions during diabetes.

Another major finding of this work is that the amount of connexin proteins in the bladder changes early in the diabetic animals. This connexin family of proteins is important for maintenance of gap junctions in virtually all cell types, but selective expression of certain isoforms in certain tissues and/or pathophysiologic conditions is apparent [21]. The X-linked form of Charcot-Marie-Tooth syndrome, a demyelinating neuropathy of the peripheral nervous system, associated with mutations in a connexin gene, suggests a possible role of gap junctions in human neurological diseases [21]. Using immunohistochemical techniques, we have also previously shown the importance of connexin isoform (43,32,26) expression during diabetes related neuropathy in the STZ induced diabetic rat dorsal penile nerve [22]. Separate from their involvement in nerve function, connexins are also known to play important roles in the initiation, maintenance and modulation of bladder detrusor tone [25]. In the urinary bladder, Cx 43 has been reported to be abundant in smooth muscle and to contribute to electrical coupling between myocytes [23, 25]. In contrast, Cx 26 was reported to be located only in the urothelium [23]. Grossman et al have also
shown that decreased connexin isoform expression and intercellular communication may drive urothelial cell proliferation and human bladder cancer [24]. However, the evaluation of location of connexins in the bladder is incomplete. Gene expression (mRNA) for six of the seven known connexins has been observed in urinary bladder [23], but regional distribution of most of these have not been reported.

Distributions of connexin proteins have also been studied in rat bladder outlet obstruction, in which Cx 43 increased markedly in the smooth muscle after eight hours of distension, whereas Cx 26 increased markedly in urothelium [23]. These changes were transient in that levels of these connexins decreased after the outlet obstruction was removed. These previous observations are dissimilar from our findings in diabetes.
Both connexin 32 and 26 increased markedly by three days of diabetes; however, in contrast to markers of innervation, these connexins returned nearly to control levels by two weeks of diabetes. The observation that the transient changes occur before structural changes are detectable and then return to control values by the time structural changes seem to have reached their maximum response suggests that connexin 32 and 26 might be important players in the process of structural, and possibly innervation, plasticity. Furthermore, the phenomena observed during diabetic bladder remodeling and outlet obstruction apparently involve different patterns of changes in connexin proteins, suggesting that the mechanisms involved in these settings are discrete.

Since each animal tissue throughout our studies was investigated for all measurements, we were able to investigate associative relationships among these parameters using nonparametric correlation analysis (e.g., on a per-tissue region basis). For each of these statistical tests a total of 30 data-points were available for each plot. Using this approach we detected only 3 statistically significant associations (shown in Fig 6), each of these is consistent with the view of a coordinated shift toward relaxant pathways and simultaneous reduction in contractile pathways. A statistically significant negative correlation observed for smooth muscle VACHT vs. TH and VACHT vs. NOS1. Furthermore the prevalence of TH was positively correlated with the prevalence of NOS1 in the lamina propria, suggesting that upregulation of these pathways may be linked.
In summary bladder dysfunction is a well-recognized feature of diabetes in which structural changes and neuropathy have been documented, especially during late time points. Using a specialized digital imaging approach we investigated specific changes in bladder wall composition and neuronal innervation in the early phases of bladder remodeling in the streptozotocin rat model. Similar to previous reports, we observed significant evidence of structural remodeling (dilation, smooth muscle hypertrophy) at 2 weeks post diabetes induction, but also observed early alterations in neuronal markers for the three prominent controllers of detrusor tone. Thus a process of “neuronal remodeling” was detected in this setting in which there was an apparent shift toward pathways favoring bladder capacitance and smooth muscle relaxation; these changes preceded changes in structure. Furthermore the changes observed were not consistent with a global loss of neurons or nerve terminals, as would be expected by neuropathic mechanisms. Time dependent and isoform specific alterations in connexins were also observed, and these also preceded structural changes. Further investigations of the early processes involved in these neuronal and structural changes may provide new insight regarding adaptive and maladaptive processes in bladder physiology and enhanced opportunities for improved therapeutic strategies for an important diabetic complication.
FIGURE LEGENDS

Figure 3.1: 1A: Mean morphological assessment using digital image parameters shows significant increases in bladder wall area (closed circle) and lumen area (open square), both measured in mm², in CTL (control) and streptozotocin-induced diabetic rats at 3D (3day) and 14D (14 days) post injection. Fig 1B shows no changes in the bladder wall thickness measured in µm. Fig1C: Representative photomicrograph (400x) demonstrating the color segmentation methods for tissue composition analysis. The different layers of the bladder wall divided into the red colored smooth muscle area (SM), blue colored lamina propria (LP) and pink colored urothelial layer (UR) are shown in the right panel. Software color segmentation performed on the Masson’s stained slides (400X), shows the orange colored smooth muscle area (SM), the green colored lamina propria (LP) and the yellow colored urothelium (UR), recognized and captured by the automated digital image analysis for the measurement of the cell-specific cross-sectional area, are shown in the left panel. Figure 1D shows the cross-sectional areas of the 3 primary components of bladder wall. Significant increases in the smooth muscle, (closed square) and urothelium, (closed circle) were observed as compared to the lamina propria, (open triangle) in the streptozotocin-induced diabetic rats as compared to controls. All data are mean ± S.E.M. *, statistically significant difference from control, P < 0.05.
**Figure 3.2:** Representative photomicrographs showing immunostaining for tyrosine hydroxylase (TH) at 800X magnification, vesicular acetylcholine transporter at 400X magnification and NOS1 at 200X magnification for control (CTL), 3 day diabetic (3D-DIA) and 14 day diabetic (14D-DIA) rat bladders.

**Figure 3.3:** Effect of diabetes on neuronal markers of innervation in the rat bladder are shown in Fig 3A, 3B and 3C for tyrosine hydroxylase, vesicular acetylcholine transporter and NOS1 respectively. Digital imaging parameters derived from control and diabetic photomicrographs showed alterations in the immunoprevalence of all the markers in the three discreet regions of the bladder viz. smooth muscle (closed square), lamina propria (open triangle) and urothelium (closed circle), as early as 3 days after the induction of diabetes and remained elevated till 14 days. All data are mean ± S.E.M. *, statistically significant difference from control, P < 0.05.

**Figure 3.4:** Representative photomicrographs for connexin 43, 32 and 26 taken at 400X magnification are shown in Fig 4A, 4B and 4C respectively; in the control (CTL) and streptozotocin induced 3 day diabetic (3D-DIA) and 14 day diabetic (14D-DIA) rat bladders.
**Figure 3.5:** Alterations in connexin isoform expressions were observed in the diabetic rat bladders as shown in Fig 5A, 5B and 5C for connexin 43, 32 and 26 immunostaining, respectively. Digital imaging parameters derived from control and diabetic photomicrographs showed alterations in the connexin isoform expression in the three discreet regions of the bladder viz. smooth muscle (closed square), lamina propria (open triangle) and urothelium (closed circle). All data are mean ± S.E.M. *, statistically significant difference from control, P < 0.05.

**Figure 3.6:** Pearson’s nonparametric correlation analysis showing statistically significant inverse relationship between TH in the lamina propria vs. VACHT in smooth muscle for control (closed circle), 3 day (open triangle) and 14 days (closed square) diabetic rat bladders. In addition a positive association was observed for TH vs. NOS1 in the lamina propria region (Fig 6B), and an inverse association was detected for NOS1 and VACHT in the smooth muscle region (Fig 6C). No other statistically significant associations were identified.
Figure 3.1: Morphological parameters showing structural remodeling as an early event in diabetes related bladder dysfunction.
**Figure 3.2:** Representative photomicrographs of tyrosine hydroxylase, vesicular acetylcholine transporter protein and nitric oxide synthase1.
**Figure 3.3:** Digital imaging parameters showing immunoprevalence of neuronal markers during diabetes related bladder dysfunction.
**Figure 3.4:** Representative photomicrographs showing Connexin 43, 32 and 26 immunoprevalance during bladder dysfunctions in diabetes.
Figure 3.5: Digital imaging parameters showed altered connexin expression during diabetes bladder dysfunction.
Figure 3.6: Spearman’s nonparametric correlation analysis showing significant correlation between different groups.
REFERENCES


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CHAPTER 4

EARLY CELL SPECIFIC CHANGES IN NITRIC OXIDE SYNTHASES,
REACTIVE NITROGEN SPECIES FORMATION AND UBIQUITINYLATION
DURING DIABETES RELATED BLADDER REMODELING.

This chapter has been published in Diabetes Metabolism Research and Reviews [2003, 19(4): 313-319] and is presented in the style appropriate for the journal. The authors of the manuscript are Deepali Anant Pitre and John Anthony Bauer.
ABSTRACT

Background: Urinary bladder dysfunction in diabetes is a well-recognized phenomenon but the mechanisms involved and initiating events are not clear. The physiological production of nitric oxide (NO) plays an important role in bladder tone and local immune defense and recent studies have shown that NO-derived reactive nitrogen species occur in many settings of chronic disease, including other diabetic complications. Here we investigated the early time dependent and cell specific changes in the nitric oxide synthase isoforms (NOS1, 2 and 3), peroxynitrite and ubiquitinylation in the well-documented streptozotocin induced rat model of diabetes. Methods: Immunohistochemical methods and automated digital imaging was used for the measurement of morphometric and histochemical analysis of the bladder tissue regions. Region-specific 3-nitrotyrosine (a biomarker of NO dysregulation and reactive nitrogen species formation) and ubiquitinylated protein prevalence (marker of proteasomal activity) were also investigated. Result: Immunohistochemistry revealed early, time dependent, and cell specific alterations in the three isoforms of NOS. We also observed region specific increases in protein nitration, demonstrating first-time evidence of reactive nitrogen species formation in this setting. The changes in nitration did not pattern changes in NOS2 induction or tissue ubiquitinylation, and these alterations preceded any detectable changes in bladder structure (3-days vs. 2 wks) in this same animal preparation. Conclusion: These data demonstrate that selective and regionally distinct changes in nitric oxide
production and impaired nitric oxide control are early events during diabetic
cystopathy and that mechanisms leading to increased oxidative stress and
proteosomal activation may be key participants leading to organ dysfunction in
this setting.
INTRODUCTION

Urinary bladder dysfunction is a major complication associated with diabetes, affecting roughly 80% of the diabetic population [1-4]. Typical symptoms include increased total urinary output, frequent and/or incomplete micturition, atonic bladder, and urinary retention [1-4]. These changes are associated with a reduced quality of life as well as an increased risk of other conditions, including urinary tract infections and bladder cancer [1]. While mechanistic understanding is incomplete, alterations in neuronal control (especially autonomic neuropathy), volume overload, and structural remodeling have all been implicated [2].

In addition to autonomic control, non-adrenergic non-cholinergic innervation and local nitric oxide production have been shown to have important physiological roles in the lower urinary tract [5-7]. For example, neuronal nitric oxide synthase (NOS1) plays a key role in modulating smooth muscle tone in the bladder detrusor muscle and in controlling sphincter tone for successful micturition [6]. In addition, urothelial expression of the high capacity NOS isoform (NOS2) has been shown to play a role in local host defense mechanisms [5]. Separate from controllers of NO production, the actions of NO can be severely altered under conditions of oxidative stress [8-9].
Of particular importance is the interaction of NO with superoxide anion ($O_2^-$); this reaction is known to occur at a diffusion-limited, nearly instantaneous rate, forming the highly aggressive oxidant peroxynitrite (ONOO-) [10], a reactive nitrogen species known to nitrate protein tyrosine residues, forming 3-nitro-L-tyrosine (3NT) [11]. Thus, increased oxidative stress shunts intact NO towards formation of peroxynitrite and thus reduces its availability for signaling.

Several recent reports have suggested that diabetes related bladder dysfunction involves changes in expression and/or content of NOS isoforms [5-7]. These previous studies have typically examined one or more NOS isoform, using histochemical or blotting techniques; at a single time-point well after severe bladder abnormalities are present [5-7]. Despite the interest in nitric oxide as a neurotransmitter, a thorough investigation of the roles of nitric oxide synthase isoforms (NOS1, 2 and 3) and the potential changes in these 3 isoforms of NOS (each of which have been detected in bladder tissues) during a relevant setting of diabetic bladder dysfunction have not been previously investigated. Furthermore, although there is now convincing evidence that oxidative stress is an important component of several other diabetes related complications [12], the potential involvement of such phenomena in diabetic cystopathy has not been previously investigated, particularly with respect to reactive nitrogen species. For these reasons we investigated time dependent changes in NOS isoforms and 3NT (a stable biomarker of reactive nitrogen species [10] in the urinary bladder of the STZ induced diabetic rats.
In addition to studies of NO production sources and protein nitration we further investigated changes in the ubiquitin-proteasome pathway. Ubiquitin is a 76 amino acid protein that is known to be a cofactor in the soluble ATP-dependent proteolysis of some short-lived proteins and ubiquitinylation apparently plays a role in targeting mildly oxidized or otherwise abnormal proteins for proteosomal digestion by forming ubiquitin protein conjugates [13-16]. Previous studies have reported elevated ubiquitin and ubiquitin protein conjugates during increased oxidative stress [14,17] and diabetes [13,15,18]. Immunoreactivity for ubiquitin was recently detected in rat and human bladder epithelial cells [19]; however, the role of ubiquitinylation in diabetes-related bladder dysfunction has not been investigated.

The bladder wall consists of three well-demarcated layers, namely the urothelium (a lumenal layer of stacked epithelium), the lamina propria (connective tissue interface predominantly composed of collagen and some recently recognized neuronal fibers) [20], and an outermost layer of smooth muscle. Recent studies suggest that these layers interact during normal bladder development and that tissue composition is regulated via paracrine signaling pathways [21]. In our previous studies we developed a digital imaging approach to allow quantitative and region-specific morphometric analysis of bladder tissues during diabetes progression. Using these methods we found that a well-defined rat model of diabetes (streptozotocin induced) mimics human phenomena with respect to bladder dysfunction and structural remodeling during diabetes [2].
Thus, our goals in the studies described herein were to define time and region dependent changes in NOS isoform prevalence, protein nitration, and ubiquitinylation status. Important elements of these studies were our focus on early time-points in which active structural remodeling occurs and our effort to simultaneously investigate discrete regions of the urinary bladder, in an attempt to further define early events during diabetes induced bladder remodeling and dysfunction.
MATERIAL AND METHODS

Animals: All animal handling protocols were approved by the Institutional Laboratory Animal Care and Use Committee. Male Sprague Dawley rats (412±40g, Harlan, Indianapolis, IN) were dosed with streptozotocin (65mg/kg i.p., n=24, Sigma, St. Louis, MO) or vehicle (n=6) and were housed in a 12-hour light/dark facility with free access to food and water. Diabetic rats were sacrificed at 3 days and 2 weeks post STZ dosing (n =8 per group). Age matched vehicle injected controls showed no statistical significant differences and were combined as a single control group (n=6). Animals were sacrificed by pentobarbital overdose, at which time blood was collected by cardiac puncture, and urinary bladder tissues were rapidly isolated. Blood glucose was measured by a clinical blood glucose monitor (Glucometer Encore, Miles Inc, IN).

Histochemistry and Digital Image Analysis: Urinary bladder was rapidly isolated, sectioned at the equatorial midline and allowed to equilibrate in buffer (e.g., with no intralumenal pressure). Following 48hr fixation in 10% buffered formalin, tissues were dehydrated and paraffin embedded using standard procedures, as previously described [2,22]. Serial five micron tissue sections were placed on microscope slides, dewaxed, and rehydrated for routine histochemical staining (hematoxylin & eosin, Mason’s trichrome) for morphological studies. Photomicrographs were captured on a Polaroid DMC high-resolution digital camera, mounted on an Olympus research microscope. The images were analyzed with Image Pro 5.0 image analysis software (Media...
Cybernetics, Silver Springs, MD). Hematoxylin and eosin stained slides were used to determine bladder tissue cross-sectional area (at the equatorial midline). Each bladder cross-section was captured in its entirety at 80x magnification and internal and external edges were traced (automated edge detection, Image Pro 5.0) for determination of total tissue and inner lumen cross-sectional areas. Mason’s trichrome stained slides were used to determine composition of bladder tissues. Images were captured at 400x magnification and color segmentation was employed to determine the tissue area that was stained “red” (smooth muscle) or “blue” (lamina propria). A similar segmentation technique was also employed to measure the urothelial cell area with hematoxylin and eosin stained slides. In all cases the capture and processing of images were performed by investigators unaware of treatment group assignments. For all parameters measured intra- and inter-observer variability was less than 5% (coefficients of variation for 3 daily measurements made by 3 different investigators evaluating 6 bladders).

Immunohistochemical staining was performed using methods previously described [22], using a DAKO Autostaining system. Briefly, 5µM cross-sections of bladder tissue were dewaxed, rehydrated, and treated with preheated citrate buffer (pH 6.0) to recover the antigenicity. The endogenous peroxidase activity was blocked by the tissues with 3% hydrogen peroxide/ methanol solution, followed by 10% goat serum / PBS blocking solution (Vector Lab, Burlingame, CA). The following primary antibodies were used in these investigations: Tyrosine
hydroxylase (Rabbit polyclonal antibody, 1:800 dilution, Novus Biologicals Inc; Littleton, CO); NOS1 (Rabbit polyclonal antibody, 1:200 dilution, Transduction Laboratories, Lexington, KY); NOS2 (Rabbit polyclonal antibody, 1:1000 dilution, Transduction Laboratories, Lexington, KY); NOS3 (Rabbit polyclonal, 1:200 dilution, Transduction Laboratories, Lexington, KY); 3NT (Rabbit polyclonal antibody, 1:1500, Transduction Laboratories, Lexington, KY) and Ubiquitin (Rabbit polyclonal, 1:400 dilution, Dako Corporation, Carpinteria, CA). All primary incubations were conducted for 1 hour; tissues were then washed and exposed to biotinylated goat anti-rabbit secondary antibody (1:200) (Vector Labs, Burlingame, CA) for 20 minutes followed by Horseradish peroxidase-complex reagent (ABC Elite; Vector Labs, Burlingame, CA). Antigen prevalence and localization was visualized using diaminobenzidine (0.06% w/v) and counterstained with methyl green. In preliminary experiments the specificity of each antibodies was verified through the use of isotypic staining controls (by replacing primary antibody with pre-immune serum) and/or demonstration of single protein bands of appropriate molecular weights from mouse tissue homogenates. [22]. All staining controls showed no detectable immunoreactivity in any treatment group. The urinary bladders were visualized using light microscopy (Olympus BX40) and photomicrographs were captured using a calibrated digital camera (Polaroid, Cambridge, MA) and transferred into research-based digital image analysis software (Image Pro Plus, Media Cybernetics, Silver Spring, MD). Relative immunoreactivity in the bladder layer
was determined via integrated optical density measurements. Image analysis was conducted by investigators who were unaware of treatment group assignments. For all parameters measured intra-observer variability was less than 5% (coefficients of variation for 3 daily measurements) whereas the inter-observer variability was less than 7% (n=3 observers evaluating 6 bladders on three different days).

All data are represented as mean ± SEM. Parameters between the diabetic and control group were evaluated for significance using ANOVA from SigmaStat (Jandel Scientific, San Rafel, CA). In all cases, significance was defined as p < 0.05.
RESULTS

Similar to our previous studies, a single dose of STZ caused rapid induction of diabetes; hyperglycemia was observed at day 3 following STZ and was maintained throughout the study (control, 135±14mg/dl; 3 days, 502±27, 2 weeks, 548±19 P< 0.05).

Representative photomicrographs of NOS1, NOS2 and NOS3 immunostaining are shown in Figure 1A-C, 1D-F and 1G-I at 200X, 800X and 800X magnification respectively. NOS1, the neuronal form of nitric oxide synthase, was expressed in all the tissue layers viz. urothelium, lamina propria and smooth muscle of the rat urinary bladder. NOS 2, the inducible form of nitric oxide synthase, was predominantly present in the urothelial layer of the diabetic urinary bladders as compared to the controls (Fig 1D-F). NOS3, the endothelial form of nitric oxide synthase, was present in the lamina propria layer mainly around the blood vessels.

Digital imaging parameters derived from control and diabetic photomicrographs are shown in Figure 2A, 2B and 2C for NOS1, 2 and 3 respectively. Statistically significant (P<0.05) increases in the NOS1 immunoprevalance were detected in all the three layers as early as 3 days after the induction of diabetes and remained elevated at 2 weeks (Fig 2A).
Figure 2B shows statistically significant (P < 0.05) increases in the urothelial NOS2 immunoprevalence in the diabetic vs. controls. Urothelial NOS2 induction was 3-4 fold higher in the 3-day diabetic rat bladders. NOS3 immunoprevalence was significantly increased (P < 0.05) 3 days post STZ induction and decreased in 2 week diabetic animals as compared to the controls (Fig 2C).

Representative photomicrographs of 3NT and ubiquitin immunostaining are shown in Figure 3A-C and 3D-F at 200X magnification respectively. 3-nitrotyrosine immunostaining was present predominantly in the smooth muscle and the urothelial layer in the diabetic rat bladders as compared to the controls. Ubiquitin immunostaining was observed in all the three layers of the control urinary bladder. However, the ubiquitin-protein conjugation was increased only in the lamina propria and urothelium during diabetes.

Digital imaging parameters showed statistically significant increases (P < 0.05) in 3NT immunoprevalence in the smooth muscle and urothelium of the diabetic bladders vs. controls (Figure 4A). A 4-fold increase in the prevalence of 3NT was seen in the smooth muscle as early as 3 days in the diabetic rat bladders vs. controls. The immunoprevalence of the ubiquitin-protein conjugation was significantly increased (P < 0.05) in the urothelium and lamina propria in the diabetic group as compared to the controls (Figure 4B). A 2 fold increases were evident in the urothelial layer of the diabetic rat bladders as early as 3 days after the induction of diabetes.
DISCUSSION

Diabetes associated urinary bladder dysfunction is a well recognized clinical problem but the mechanisms involved are poorly understood and optimal therapeutic strategies are not well defined. In previous studies several research groups have shown that the STZ-induced rat model of diabetes mimics humans with respect to increased urine output, frequent and/or incomplete micturition, atonic bladder, and urinary retention following at least five weeks of diabetes induction [3,4,21]. Previously, we have shown time dependent and cell specific urinary bladder remodeling in the streptozotocin induced diabetic rat model [2]. Using digital imaging we were able to detect structural changes (hypertrophy and chamber dilation) in the urinary bladder as early as 2 weeks after the induction of diabetes. Selective changes in bladder wall composition also occurred in a time dependent manner, most notably increased smooth muscle and urothelium and decreased collagen prevalence [2]. Our previous studies therefore suggested that additional investigations of the early phase of bladder remodeling might provide additional mechanistic insight and identify key early components. Urothelium, lamina propria, and smooth muscle regions were discretely investigated from the same bladder tissues, allowing detailed mapping of protein prevalence in control tissues and selective changes at these sites during diabetes.
We have used the well-established streptozotocin induced diabetic rat model, which is a well-known model of diabetes as well as of polyuria. However, it is important to note that polyuria alone does not necessarily mimic bladder changes observed in the STZ model. For example, Eika et al [4] have previously shown that sucrose fed rats have pressure-volume responses that are not different from controls, whereas STZ treated animals have significant passive function changes. Kudlacz et al [3] also showed that changes in nerve densities were unaltered in the sucrose-fed model, whereas striking changes were observed in STZ diabetic rats. Furthermore, we have recently observed that mice selectively over-expressing pro-oxidant genes in bladder smooth muscle develop organ remodeling phenomena identical to those observed here in the absence of polyuria or glucose changes (Pitre and Bauer, unpublished). Collectively, these findings suggest that bladder remodeling is not exclusively dictated by increased urine output. Therefore, while many of the changes in the bladder are associated with polyuria, there are apparently distinct functional and morphological changes related to diabetes that are different from a simple diuresis model.

Nitric oxide is now recognized as an important regulator of many physiological processes, including functional aspects of the lower urinary tract [5]. Although NOS isoform expression in bladder tissues is already established, their relative abundance, distributions, and potential interactions in bladder tissue during a relevant disease setting are not well described. In our studies we found that all 3 isoforms were detectable in control rat bladders, but that these tended
to have discrete distributions within bladder wall. For example, in control tissues NOS1 was most abundant in the smooth muscle region, NOS2 was mostly in urothelium, and NOS3 was mostly in lamina propria. These observations illustrate the selective cellular expression patterns of the NOS family of gene products, within the same bladder tissue sections, and demonstrate the value of in situ technologies for our studies since these observations would have been impossible using homogenate and blotting methods.

In addition to bladder-region-specific control levels, we observed selective increases in NOS isoforms in the early phase following diabetes induction. Again, the changes observed were dependent on the specific NOS isoform. For example, statistically significant increases in NOS1 were observed in all 3 layers (urothelium, lamina propria, and smooth muscle), whereas NOS2 induction was only observed in the urothelial layer, and NOS3 only changed in the lamina propria. Again, these changes illustrate the independent control of each gene product in each layer of bladder tissue and suggest fundamentally different and independent responses of these cell types during the diabetic state. Importantly, each of the changes in NOS isoform expression observed occurred at only 3 days after diabetes induction—this time point precedes any evidence in structural or morphological changes we observed in our previous studies (at 2 and 5 weeks post STZ) [2]. Interestingly, both isoforms typically associated with smooth muscle relaxation effects (the “constitutive” and calcium dependent isoforms NOS1 and NOS3) were found to increase at 3 days; this may be an attempt to
increase bladder compliance in response to increased urine volume following diabetes induction. In contrast, regulation of NOS2, classically referred to as “the inducible isoform”, takes place primarily at the transcriptional level. NOS2 induction typically occurs in settings of tissue injury and/or inflammation and is known to be increased by a variety of inflammatory stimuli, particularly cytokines [8-10]. We observed a rapid and striking induction of NOS2 within 3 days of diabetes induction and this activation was solely confined to the urothelial layer and was not associated with immune cell infiltrates. Thus, the increased and urothelial cell-specific expression of NOS2 in the diabetic bladders is consistent with a bladder inflammatory response in this setting and suggest that urothelial cell activation/ stress response is a very early event in diabetic bladder changes.

Separate from favorable physiological effects of local nitric oxide production, it is also known to interact with other molecules to form potentially toxic products [10-11]. Growing evidence suggests that oxidative stress is a common occurrence associated with many of the complications of diabetes, although its role in diabetes related bladder disorders has not been investigated [12]. Here we employed immunohistochemical methods to study the in vivo formation of 3NT in control and diabetic bladders. Statistically significant increases in 3NT prevalence were observed in the urothelial and smooth muscle layers, with no change in lamina propria. Again, these changes were readily detectable at 3 days, suggesting that changes in reactive nitrogen species formation occurs in the very early phases of bladder alterations during diabetes.
Since the formation of peroxynitrite is bimolecular, increased prevalence of either nitric oxide or superoxide anion can promote protein nitration events. Of note is the important observation that the regional distribution of protein nitration did not generally follow the regional patterns for NOS2; this high capacity isoform has been most often suggested as an important driving force for peroxynitrite formation in vivo. Rather, the general distribution of nitration more closely paralleled regional changes in NOS1 prevalence (Figure 3 vs. Figure 5). Thus, under the conditions of these studies NOS2 induction is not apparently an obligatory event for significant increases in tissue reactive nitrogen species formation; other NOS isoforms, as well as changes in superoxide anion availability are apparently involved as well. Further studies to identify key pathways and cell-specific controllers involved are underway in our laboratory but thus far our data demonstrate that alterations in NOS isoform prevalence and distribution and increased formation of reactive nitrogen species preceded any evidence of structural remodeling.

An important pathway for degradation of oxidized intracellular proteins is the non-lysosomal ubiquitin-proteasome pathway. In light of our observation of increased protein nitration in bladder regions, we tested the hypothesis that changes in tissue ubiquitinylation occurs in the rat diabetic bladder. Using immunohistochemical methods we observed ubiquitin immunoprevalence in all the three regions of control bladder. In addition, we found significant increases in the ubiquitinylation (as shown by increased ubiquitin protein conjugate formation)
in the urothelial layer following diabetes induction. The regional distribution pattern of ubiquitylation was not significantly correlated with regional patterns of protein nitration in the same tissues (see figure 5). Interestingly, in vitro studies suggest that mild oxidative stress can activate increases in the proteasome dependent proteolysis of oxidized protein substrates by the 20S core proteasome complex whereas more severe oxidative stress can cause extensive protein oxidation leading to cross-linked and aggregated proteins that are resistant to proteolytic degradation and can act as irreversible inhibitors by binding to the 20S core; they thus cause a vicious cycle of progressively worsening accumulation of cytotoxic protein oxidation products [16]. Further studies investigating relationships of reactive nitrogen species formation, protein nitration and proteosomal processing are warranted to determine their potential roles in diabetic cystopathy and structural remodeling. Further identification of these mechanisms may lead to new treatment strategies for this important medical problem.

In summary, urinary bladder dysfunction in diabetes is a well-recognized phenomenon but the mechanisms involved and initiating events are not clear. Using automated digital imaging methods we observed early, time dependent, and cell specific alterations in the three isoforms of NOS. We also observed region specific increases in protein nitration, demonstrating first-time evidence of reactive nitrogen species formation in this setting. The changes in nitration did not pattern changes in NOS2 induction or tissue ubiquitylation, and these
alterations preceded any detectable changes in bladder structure in this same animal preparation. These data demonstrate that selective and regionally distinct changes in nitric oxide production and impaired nitric oxide control are early events during diabetic cystopathy and that mechanisms leading to increased oxidative stress and proteosomal activation may be key early participants leading to organ dysfunction in this setting.
FIGURE LEGENDS

Figure 4.1: Representative photomicrographs showing immunostaining for NOS1 at 200X magnification (1A-C), NOS2 at 200X magnification (1D-F) and NOS3 at 800X magnification (1G-I) for control (CTL), 3 day diabetic (3D-DIA) and 2 week diabetic (2W-DIA) rat bladders. NOS1, the neuronal form of nitric oxide synthase, was expressed in all the tissue layers viz. urothelium (UR), lamina propria (LP) and smooth muscle (SM) of the rat urinary bladder. NOS2, the inducible form of nitric oxide synthase, was predominantly present in the urothelial layer of the diabetic urinary bladders as compared to the controls (Fig 1D-F). NOS3, the endothelial form of nitric oxide synthase, was present in the lamina propria layer mainly around the blood vessels.

Figure 4.2: Digital imaging parameters derived from control and diabetic photomicrographs are shown in Fig 2A, 2B and 2C for NOS1, NOS2 and NOS3 respectively. Immunoprevalence was expressed as integrated optical density demonstrating statistically significant (p < 0.05) increases in the NOS1 immunoprevalence in all the three layers as early as 3 days after the induction of diabetes and remained elevated till 2 weeks (Fig 2A).
Figure 2B shows statistically significant (P < 0.05) increases in the urothelial NOS2 immunoprevalance in the diabetic vs. controls. Urothelial NOS2 induction was 3-4 fold higher in the 3 day diabetic rat bladders. NOS3 immunoprevalance was significantly increased (p < 0.05) 3 days post STZ induction and decreased in 2 week diabetic animals as compared to the controls (Fig 2C). All data are mean ± S.E.M. *, statistically significant difference from control, p < 0.05.

**Figure 4.3:** Representative photomicrographs showing immunostaining for of 3-Nitrotyrosine (3-NT) (3A-C) and ubiquitin (3D-F) at 200X magnification respectively. 3-nitro tyrosine immunostaining was present predominantly in the smooth muscle and the urothelial layer in the diabetic rat bladders as compared to the controls. Ubiquitin immunostaining was observed in all the three layers of the control urinary bladder. However, the ubiquitin-protein conjugation was increased only in the lamina propria and urothelium during diabetes.

**Figure 4.4:** Digital imaging parameters derived from control and diabetic photomicrographs are shown in Fig 4A and 4B for 3-NT and Ubiquitin immunostaining respectively. Immunoprevalance expressed as integrated optical density showed statistically significant increases (P < 0.05) in 3-NT immunoprevalance in the smooth muscle and urothelium of the diabetic bladders vs. controls (Figure 4A). 4-fold increase in the formation of the reactive nitrogen
species was seen in the smooth muscle as early as 3 days in the diabetic rat bladders vs. controls. The immunoprevalance of the ubiquitin-protein conjugation was significantly increased (P < 0.05) in the urothelium and lamina propria in the diabetic group as compared to the controls. A 2 fold increases were evident in the urothelial layer of the diabetic rat bladders as early as 3 days after the induction of diabetes. All data are mean ± S.E.M. *, statistically significant difference from control, p < 0.05.
Figure 4.1: Representative photomicrographs of NOS1, NOS2 and NOS3 immunostaining in the rat urinary bladder during diabetes.
**Figure 4.2**: Digital imaging parameters showing increased NOS immunoprevalence in the rat bladders during diabetes.
Figure 4.3: Representative photomicrographs of 3-nitrotyrosine and ubiquitin immunostaining in the rat bladders.
Figure 4.4: Increased 3-nitrotyrosine and ubiquitin prevalence in the rat bladders during diabetes.
REFERENCES


9. Freeman BA, Free radical chemistry of nitric oxide, Chest 1994: 105(3); 79S-83S.


CHAPTER 5

FUNCTIONAL, STRUCTURAL, AND NEURONAL ALTERATIONS IN URINARY BLADDER DURING DIABETES: INVESTIGATIONS OF A MOUSE MODEL.

This chapter has been submitted to The British Journal Of Pharmacology and is presented in the style appropriate for the journal.
SUMMARY

1. Urinary bladder dysfunction is a common complication in diabetes but mechanisms involved are undefined. The use of murine models provides opportunity to utilize transgenic technologies for bladder research.

2. Here we investigated functional, structural and neuronal aspects of bladder in a mouse model of type1 diabetes, and compared these parameters to the more traditional rat model.

3. Mice were dosed with streptozotocin (150mg/kg) or vehicle and studied at 5 wks. Increases in blood glucose, urine glucose and total urine output were observed.

4. In-vitro cystometry showed a 2-fold increase in bladder capacity and compliance and a decrease in intravesical plateau pressure in diabetic vs. controls.

5. Bladder structure and composition was evaluated by digital imaging; region specific changes included increased smooth muscle and urothelium and no change in collagen content; changes that are identical to those observed in rats.

6. Significant alterations in cholinergic and adrenergic and N-nitro-L-arginine (NOS inhibitor) functional responsiveness were also observed.
7. Prevalence of cholinergic and adrenergic neuronal tracts was determined by immunohistochemistry: decreased vesicular acetylcholine transferase was observed in smooth muscle whereas tyrosine hydroxylase was increased in lamina propria, demonstrating a shift toward pro-relaxant neuronal pathways.

8. These studies demonstrate that mouse model of STZ induced diabetes exhibits important urinary bladder remodeling and has a likely value in learning more about bladder biology and diabetes influences.
INTRODUCTION

Urinary bladder dysfunction is major complication in diabetes [Ellenberg 1980, Pitre et al 2002, Poladia et al in press]. Symptoms include frequent micturition, enlarged and distended bladders, incomplete bladder emptying resulting in a atonic bladder, urinary retention, urinary tract infections and increased risk of bladder cancers [Ellenberg 1980, Kravchick et al 2001, Pitre et al 2002, Poladia et al in press] While mechanistic understanding is incomplete, alterations in neuronal control (especially autonomic neuropathy), volume overload, and structural remodeling have each been implicated [Pitre et al 2002]. At this time optimal therapies for this condition remain poorly defined, and as the incidence of diabetes continues to rise the importance and medical costs associated with this syndrome are likely to escalate.

For over 3 decades the streptozotocin-induced rat model of diabetes has been used as a chemically induced model of diabetes mellitus [Ozturk et al 1996]. Despite some important differences from human phenomena, the model has provided valuable insight regarding related complications, including cardiomyopathy [Mihm et al 2001], vascular complications [Ozturk et al 1996], neuropathies [Kolta et al 1985, Kudlacz et al 1989], and genitourinary alterations [Ozturk et al 1996]. Nearly all of the bladder changes found in diabetic humans are apparently recapitulated in the STZ rat model, including increased urine output, frequent voiding, and atonic bladder [Ellenberg 1980, Kolta et al 1985, Kudlacz et al 1989, Latifpour et al 1991, Lincoln et al 1984, Longhurst et al 1986,
Pitre et al 2002]. We have also recently documented time and region specific detrusor muscle remodeling using a specially designed digital image analysis approach [Pitre et al 2002]. With advances in transgenic mouse technologies, more recent studies have begun to employ this smaller species for diabetes research, and in “wild-type” mice streptozotocin dosing elicits a diabetic state similar to the rat model [Cheta et al 1998, Ozturk et al 1996]. Interestingly, two transgenic mouse models (NOS1 [Burnett et al 1997] and M3 [Matsui et al 2000] receptor knock outs have each been recently shown to have distinct bladder changes, suggesting that this species may be valuable for bladder research. For these reasons, we have investigated urinary bladder functional and structural changes in normal mice and in a mouse model of STZ induced diabetes; using methods we have already established as valuable in rat studies [Pitre et al 2002].
Diabetes related urinary bladder dysfunction in humans has been commonly attributed to altered neuronal control and/or neuronal injury [Faerman et al 1973, Kahan et al 1970, Kaplan et al 1995], and such changes have also been observed in several animal models (e.g., rat, guinea pig, hamster, rabbit) [Kolta et al 1985, Belis et al 1996, Itoh et al 1994, Mumtaz et al 1999]. However, a review of the literature shows no consensus since studies have reported increase, decrease or no effect to various muscarinic and adrenergic agonists [Kolta et al 1985, Longhurst et al 1986, Lincoln et al 1984], and there is a lack of such investigations in any mouse model during diabetes. Thus the goals of these studies were to test the hypothesis that the mouse is an appropriate and relevant model for diabetic bladder investigations and to define the alterations in bladder structure, functional responses, and neuronal prevalence in this setting.
MATERIALS AND METHODS

Animals: All animal handling protocols were approved by the Institutional Laboratory Animal Care and Use Committee. Male CF1 mice (5 weeks old, 30±2g, Harlan, Indianapolis, IN) were dosed with streptozotocin (150mg/kg i.p., n=18, Sigma, St. Louis, MO) or vehicle (n= 18) and were housed in a 12-hour light/dark facility with free access to food and water. Diabetic mice were sacrificed at 5 weeks post STZ dosing. Animals were sacrificed by pentobarbital overdose, at which time blood was collected by cardiac puncture, and urinary bladder tissues were rapidly isolated. Blood glucose was measured by a clinical blood glucose monitor (Glucometer Encore, Miles Inc, IN).

Urine output: The animals were placed in stainless steel metabolic cage for 24 hours. They were deprived of food and given free access to water. The urine was collected after 24 hours and the volume was determined. Urine glucose was determined by the Clinistix Reagent Strips for Urinalysis (Bayer Corp., Elkhart, IN). The amount of water consumed by the animals over 24 hours was measured.

In vitro cystometry: The animals viz. controls (n=18) and 5 week diabetic mice (n=18) were euthanized by an overdose of pentobarbital sodium (75 mg/kg i.p., Abbott Laboratories, Chicago, IL). A midline incision through the lower abdominal wall was made to expose the urinary bladder. The ureters were ligated with a suture and the urethra was isolated and cut distal to the bladder body (~ 5 mm). The bladder was gently emptied and cannulated with PE 10
tubing and infused with Tyrode solution. The rate of Tyrode infusion was 0.005 ml/min. The bladder was mounted on a tissue bath containing warm Tyrode solution (37°C) and oxygenated with 95% O₂ and 5% CO₂. The PE10 tubing was attached to a 3-way stopcock directly connected to a pressure transducer. The third connection of the stopcock was attached to an infusion pump (Harvard Apparatus) via a tygon tubing (3mm i.d.). After adjusting the bladder base to the same level of the pressure transducer, the bladder was allowed to equilibrate for 30 minutes. During this time the bladder was washed three times with warm Tyrode solution. Following equilibration, the intravesicular pressure was recorded in response to a continuous infusion of Tyrode solution into the bladder, until a pressure twice the plateau pressure was reached. The bladder was then emptied and allowed to equilibrate for 30-45 minutes following intermittent washings with Tyrode solution. The bladder was again infused and the above procedure will be repeated for 2 times. The initial run (run1) was to stabilize the bladder under the invitro conditions. The volume of Tyrode infused into the bladder associated with twice the plateau pressure was defined as the structural capacity of the bladder. The pressure–volume relationship (cystometrogram) was generated for each bladder. The structural capacity was measured for the normal and 5-week diabetic mice bladders. Concentration of Tyrode solution used was NaCl 125 mM, KCl 2.7 mM, NaH₂PO₄ 0.4 mM, CaCl₂ 1.8 mM, NaHCO₃ 23.8 mM and Glucose 11 mM.
The bladders were then divided into three groups (n=6, control) and (n=6, diabetic). Each group was treated with either Bethanechol (BTH) 100µM or Isoproterenol (ISO) 100µM or N-nitro-L-arginine 250µM, incubated for 20-30 mins, in the organ bath and also infused into the bladder, and cystometrograms were plotted for controls and diabetic bladders. The cystometrogram was then divided into three parameters viz plateau pressure: pressure at which the bladder maintains a relaxed tone, structural capacity: volume at twice the plateau pressure and compliance: innate ability of the bladder to expand to capacity with minimal changes in pressure [Dmochowski et al 1996]. We also measured the compliance at ½ capacity to study effect of drugs on volumetric filling phase and at structural capacity to study effect of stretch on bladder. Sensitivity or drug response was calculated as difference in compliance before and after the administration of the drug.

**Immunohistochemistry and Image Analysis:** After cystometry the bladders were blotted dry and weighed. They were cut through the equatorial midline and formalin fixed. After 48 hrs of fixation, tissues were dehydrated and embedded in paraffin as described previously [Pitre et al 2001, Pitre et al 2002, Poladia et al in press]. Immunohistochemical staining was performed using methods previously described [Pitre et al 2001, Pitre et al 2002, Poladia et al in press], using a DAKO Autostaining system. Briefly, 5µM cross-sections of bladder tissue were dewaxed, rehydrated, and treated with preheated citrate buffer (pH 6.0) to recover the antigenicity. The endogenous peroxidase activity
was blocked by the tissues with 3% hydrogen peroxide/methanol solution, followed by 10% goat serum/PBS blocking solution (Vector Lab, Burlingame, CA). The following antibodies were used: Tyrosine hydroxylase (Rabbit polyclonal antibody, 1:400 dilution, Novus Biologicals Inc; Littleton, CO), Vesicular acetylcholine transporter (Rabbit polyclonal antibody, 1:2000, Accurate Chemicals and Scientific Corp; Westbury, NY) and NOS1 (Rabbit polyclonal antibody, 1:200 dilution, Transduction Laboratories, Lexington, KY). All primary incubations were conducted for 1 hour; tissues were then washed and exposed to biotinylated goat anti-rabbit secondary antibody (1:200) (Vector Labs, Burlingame, CA) for 20 minutes followed by Horseradish peroxidase-complex reagent (ABC Elite; Vector Labs, Burlingame, CA). Antigen prevalence and localization was visualized using diaminobenzidine (0.06% w/v) and counterstained with methyl green. Preliminary experiments were performed to verify the specificity of the antibody used herein, by replacing primary antibody with pre-immune serum (isotypic staining controls), and initial experiments using western blotting demonstrated specific protein recognition for each analyte in mouse tissue homogenates.

Sections of tissue were also stained with Masson's Trichome for morphological evaluation and to evaluate collagen prevalence. Images were captured on a Polaroid DMC high-resolution digital camera, mounted on an Olympus research microscope. The images were analyzed with Image Pro 5.0 (Media Cybernetics, Silver Springs, MD) image analysis software as described
earlier [Pitre et al 2001, Pitre et al 2002, Poladia et al in press]. Relative immunoreactivity in the bladder was determined via integrated optical density measurements. Software color segmentation was performed on the trichrome stained slides, to measure the area of smooth muscle, extracellular collagen and urothelium present in the cross sections. Image analysis was conducted by investigators who were unaware of treatment group assignments. For all parameters measured intra- and inter-observer variability was less than 5% (coefficients of variation for 3 daily measurements and n=3 for inter-observers evaluating 6 bladders on three different days).

Statistics: Results were expressed as the mean ± SEM. Comparison between groups was performed by Student’s t test using Sigma Stat 5.0 software from Jandel Scientific. Statistical associations between pairs of measured variables were evaluated using Spearman’s nonparametric correlation analysis (SigmaStat, Jandel Scientific, San Rafel, CA). Probability values of P < 0.05 were considered significant.
RESULTS

Shown in Figure 1 are general characteristics from normal and STZ-dosed diabetic mice. Hyperglycemia (Fig 1A), glucosuria (Fig 1B) and weight loss (Fig 1G) was evident at 5 weeks after the induction of diabetes. Measurement of 24-hour urine output (Fig 1E) also showed a 3-fold increase in urine volume while water consumption (Fig 1F) was increased significantly at 5 weeks as compared to controls. Renal clearance of glucose (urinary excretion rate/blood glucose concentration) was also significantly increased (Fig 1D). On sacrifice, the bladders were found to be highly distended and total bladder weight to body weight was increased 3-fold in the diabetic mice versus controls (Fig 1H).

Representative photomicrographs of bladder cross-sections from control and diabetic mice are shown in Fig 2A and 2B respectively. Representative images (400X magnification) of trichome stained tissues showing the red colored smooth muscle; blue colored collagen and pink colored urothelium are shown. Total tissue area was significantly increased in diabetic animals as compared to controls (Fig 2C).
Using an appropriate color segmentation protocol as previously described [Pitre et al 2002], the cross sectional areas of the three primary components of bladder wall; were determined. Figure 2D shows the cross-sectional areas of the 3 primary components of bladder wall. Total wall mass nearly doubled (data shown in Figure 2C), whereas this increase was primarily driven by increased smooth muscle (a near 3-fold increase in this region was detected) and to a lesser extent urothelium, rather than increased extracellular matrix.

Representative cystometrograms for the control (solid curve) and diabetic mice (dotted curve) is shown in Figure 3A. These pressure-volume tracings were then used to derive three parameters: plateau pressure, structural capacity, and bladder compliance. Plateau pressure, volumetric filling phase of the bladder, was decreased in diabetic mice as compared to controls (Fig 3B). Figure 3C shows changes in structural capacity, measured at twice plateau pressure. Structural capacity was significantly increased in diabetic mice vs. controls. Compliance (Fig 3D) was also significantly increased at 5 weeks in diabetic mice as compared to controls.

Following baseline pressure-volume determinations, tissue responsiveness to specific agents were investigated. Representative cystometrograms before (solid curve) and after (dotted curve) infusion of classic cholinergic agonist, bethanechol (100 µM) is shown for control and diabetic mice in Figure 4A and 4B respectively. Administration of bethanechol resulted in a profuse contraction of the control bladders resulting in absence of the plateau
formation and continuous increase in the pressure as shown in figure 4A in dotted curve. Compliance was determined for control and diabetic group at ½ capacity to study the effect of bethanechol on volumetric filling phase and at structural capacity to study the effect on stretch. Bethanechol administration produced a decrease in both control and diabetic groups (Fig 4C-D). Sensitivity to bethanechol was calculated as difference in compliance before and after the administration of bethanechol in both control and diabetic groups. Figure 4E and 4F show increases in sensitivity or increased response to bethanechol in diabetic groups at both capacity and ½ capacity.

Figure 5A and 5B show representative cystometrograms for control and diabetic groups respectively before and after administration of classical adrenergic agonist, isoproterenol (100µM). The solid curve represents the cystometrogram with Tyrode infusion while dotted curve represents cystometrogram with isoproterenol infusion. Similar to bethanechol response the compliance was determined for isoproterenol at capacity and ½ capacity. Figure 5C-D show increased compliance in controls and diabetic mice upon isoproterenol administration. The response to isoproterenol is however decreased in diabetic mice at ½ capacity and unchanged at capacity as compared to controls (Fig 5E-F).
We investigated the potential role of basal NO in detrusor pressure-volume responses via the incubation with N-nitro-L-arginine (250 µM). Representative cystometrograms before (solid curve) and after the infusion (dotted curve) are shown for control and diabetic mice in Figure 6A and 6B respectively. Two parameters viz. peak pressure, plateau pressure and compliance were determined for control and diabetic group before and after N-nitro-L-arginine administration. In all control bladders a transient increase in vesicular pressure was observed in the presence of N-nitro-L-arginine, whereas the remaining portion of the cystometrogram was unaltered. In diabetic mice, this initial peak was absent but bladders showed increased plateau pressure as compared to run before N-nitro-L-arginine administration (Fig 6D). Figure 6C shows increased peak pressure in control group after treatment with N-nitro-L-arginine. Significant decreases (P<0.05) in plateau pressure were seen in diabetic group as compared to the controls while plateau pressure was slightly but significantly increased upon administration of N-nitro-L-arginine in the diabetic group (Fig 6D). Figure 6E-F shows significant increases in bladder compliance in diabetic group before and after administration of N-nitro-L-arginine.

Following cystometric investigations, bladder tissues were transversely cut at the equatorial midline and processed for determination of neuronal pathway prevalence and distribution, using classical prejunctional markers. Shown in Figure 7 are representative photomicrographs of tyrosine hydroxylase (TH) immunostaining (7A-B), vesicular acetylcholine transporter (VACht) (7D-E) and
neuronal nitric oxide synthase (NOS1) (7G-H) in mice bladders. In control tissues TH (sympathetic neuronal marker) was most prevalent in lamina propria regions, especially in nerve bundles and surrounding blood vessels and to a small extent in smooth muscle. VAChT (parasympathetic/neuromuscular junction marker) was most prevalent in smooth muscle regions of the urinary bladder. Images showed apparent increase in TH in diabetic mice group whereas VAChT was consistently reduced. NOS1, the neuronal form of nitric oxide synthase, was expressed in all the tissue layers viz. urothelium, lamina propria and smooth muscle of the rat urinary bladder. Using digital image analysis, changes in markers of innervation were seen in diabetic group as compared to controls. TH in lamina propria was nearly doubled at five weeks (Fig 7C). The small amount of this marker in smooth muscle was not altered by diabetes. Smooth muscle content of VAChT was significantly decreased in diabetic bladders vs. controls (Fig 7.F). Statistically significant (P < 0.05) increases in NOS1 immunoprevalance were detected in all the three layers at 5 weeks after the induction of diabetes (Fig 7I).

In an effort to relate various measured parameters derived from identical bladder tissues of control and diabetic rats we employed nonparametric correlation analysis (Spearman’s nonparametric correlation analysis). Using data for each analyte in each individual bladder region, each pair-wise association was tested for statistical significance using all available data; and the following relationships were found to be statistically significant (shown in Figure 8). A positive association was observed for urine output vs. blood glucose (8A), urine
output vs. compliance (8B), compliance vs. total tissue area (8C) and TH staining in the lamina propria region vs. compliance (8E). A statistically significant inverse relationship was observed between VACHT in the smooth muscle region and compliance (8F) and TH in the lamina propria vs. VACHT in smooth muscle (8D).
DISCUSSION


The streptozotocin induced mouse models have been used previously to study bladder changes [Cheta D et al 1998]; however a detailed investigation of the functional, structural and neuronal remodeling in the mouse bladder during diabetes has not been conducted earlier. The data presented herein provide evidence that the mouse model is similar if not identical to this well-documented rat preparation. We have downsized our techniques to study bladder morphology and function ( invitro cystometry) from STZ induced rat model to mouse model. We have successfully performed cystometric and morphological assessment of the mouse bladders and investigated changes at 5 weeks post STZ injection. Similar to human [Faerman et al 1973, Kahan et al 1970, Kaplan et al 1995] and STZ induced diabetic rat bladders [Ellenberg 1980, Pitre et al 2002, Pitre Poladia
et al in press Kolta et al 1985, Kudlac et al 1989, Longhurst et al 1986, Lincoln et al 1984, Latifpour et al 1991] we found significant hyperglycemia, glucosuria, and increased urine output in the diabetic mice bladders as compared to the age matched controls. The water consumed by the diabetic animals was almost tripled.

Since several previous reports have shown that bladder function is already altered in the streptozotocin induced rat model after 5 weeks [Kudlac et al 1989, Eika et al 1994], we focused on same time point to define morphological events in this remodeling phenomenon. They have also demonstrated that this animal preparation is a well-known model of diabetes as well as polyuria. However it is important to note that that polyuria does not necessarily mimic bladder changes observed in the streptozotocin model of diabetes. For example, Eika et al have previously shown that sucrose fed rats have pressure-volume responses that are not different from controls, whereas streptozotocin-induced diabetic animals have significant passive function changes [Eika et al 1994]. Also changes in nerve densities were unaltered in the sucrose-fed model, whereas striking changes were observed in diabetic rats [Kudlac et al 1989].
In more recent studies we have observed that mice selectively over-expressing pro-oxidant genes in bladder smooth muscle also develop bladder smooth muscle hypertrophy and decreased collagen content (identical to the remodeling phenomena observed here) in the absence of polyuria or glucose changes [Pitre and Bauer, unpublished]. Collectively, these findings suggest that bladder remodeling may not be exclusively dictated by increased urine output per se.

Here we used highly automated digital imaging methods to quantify general size and composition of bladder tissue during diabetes. These parameters were objectively and accurately assessed and throughput was facilitated by the creation of an automated subroutine. Further advantages include a high degree of reproducibility (inter- and intra-observer variabilities were <5%), and the generation of continuous parameters for statistical analysis. We observed a 2 fold increase of total bladder tissue over the 5 weeks of investigation. This increase in total bladder wall area correlates closely to the well-documented increase in bladder mass associated with experimental diabetes [Eika et al 1994, Lincoln et al 1984, Pitre et al 2002]. The bladder wall consists of three well-demarcated layers. A layer of stacked epithelial cells (urothelium) borders the lumen, behind which is a connective tissue interface predominantly composed of extracellular collagen called the lamina propria. The outermost layer consists of smooth muscle [Fowler et al 2002]. Recent studies suggest that these layers interact during normal bladder development and that
tissue composition is regulated via paracrine signaling pathways [Baskin et al
1996]. We used the digital image analysis as described previously [Pitre et al
2002] to study specific prevalence of the three primary components of bladder
wall; smooth muscle, urothelium and lamina propria and reported consistent
results as seen in the mouse model during diabetes. Our data showed that the
primary source of this increased tissue mass was the smooth muscle layer,
which progressively increased to comprise about 60% of bladder wall over the 5
weeks investigated. Our quantitative analyses of smooth muscle cross-sectional
area are consistent with a previous report of bladder smooth muscle hypertrophy
in this model, in which smooth muscle cells were assessed using manual
measurements [Lincoln et al 1984]. In addition, the observed increase of smooth
muscle region was more substantial (3-fold) than the total tissue cross-section or
total bladder weights (each an approximate 2-fold increase), suggesting that this
measurement may be a more sensitive index of early changes in bladder than
simple weight measurements. We also observed a progressive increase in total
urothelium. This epithelial cell layer plays an important role in normal bladder
function (particularly barrier and host-defense mechanisms), but few studies
have investigated its properties during diabetes. The only other report on
urothelial cells from diabetic bladder showed increased DNA synthesis
(measured by thymidine incorporation) [Eika et al 1993].
Our results demonstrate that this was translated into cellular proliferation. The progressive increases in the urothelium paralleled changes in the smooth muscle layer, suggesting that there is close regulation between these two components of the bladder wall; at least within the time period we investigated.

Functional changes in the bladder during diabetes have been previously documented using bladder strips [Longhurst et al 1986, Lincoln et al 1984]; however studies suggest that within a strip the orientation of the bundles is variable and difficult to predict. Hence, we employed invitro cystometry techniques to study changes in an intact bladder during diabetes. Our data showed significant decrease in plateau pressure, increase in structural capacity and compliance; similar to human and rat studies described previously [E llenberg 1980, Kolta et al 1985, Kudlacz et al 1989, Longhurst et al 1986, Lincoln et al 1984, Latifpour et al 1991, Faerman et al 1973, Kahan et al 1970, Kaplan et al 1995]. Our data thus suggests that the mouse model mimics the functional changes seen in the human and STZ induced rat model during diabetes.

Autonomic control of bladder expansion and contraction is predominantly governed by parasympathetic, sympathetic and non-adrenergic non-cholinergic (NANC) inputs [Longhurst et al 2000]. Stimulation of local parasympathetic nerve activity causes a highly coordinated contraction of the bladder body and subsequent emptying of the bladder, whereas sympathetic and NANC activation enhances detrusor muscle relaxation [Longhurst et al 2000]. Previous studies using either cystometry or bladder strips have shown significant changes in the
autonomic innervation of the bladder during diabetes [Ellenberg 1980, Kolta et al 1985, Kudlac et al 1989, Longhurst et al 1986, Lincoln et al 1984, Latifpour et al 1991] however there is no consensus since studies have reported increase, decrease or no effect to various muscarinic and adrenergic agonists [Kolta et al 1985, Longhurst et al 1986, Lincoln et al 1984]. Our data is consistent that changes occur in the stimulation of autonomic receptor of the bladder during diabetes. Bethanechol (100 µM) administration resulted in profuse contraction of the bladders and decrease in compliance in both control and diabetic groups (Fig 4). Post-junctional responsiveness to cholinergic stimuli was increased in the diabetic mice vs. controls consistent with other studies in STZ rat model [Kolta et al 1985, Hashitani et al 1996]. Since supersensitivity of post junctional muscarinic receptors has been associated with diminished or impaired prejunctional activity, we investigated the role of VACHT, the well established and commonly employed prejunctional markers of parasympathetic fibers [Dixon JS et al 1999, 2000]. Our data shows significant decrease (Fig 7) in VACHT prevalence in the smooth muscle of the bladders during diabetes which is consistent with increased post-junctional response that might compensate for diminished prejunctional activity.

Administration of the classical adrenergic agonist, isoproterenol (100µM) resulted in increased compliance in controls mice however, response to isoproterenol was decreased in diabetic mice at ½ capacity and unchanged at capacity as compared to controls (Fig 5). Upregulation of β-adrenergic receptors in the rat diabetic bladders using radioligand binding and increased [Kolta et al
1985] or no change [Nakamura et al 1992] in functional responsiveness of bladder strips to isoproterenol has been well documented. Our data shows decreased responsiveness to isoproterenol at ½ compliance and no change at compliance in the diabetic mice. However, investigations on role of prejunctional sympathetic marker, tyrosine hydroxylase, showed a significant increase in tyrosine hydroxylase staining in the lamina propria region of the bladder in the diabetic mice vs. controls. Although diabetic neuropathy has been considered as an important mechanism responsible for bladder dysfunction in diabetes, our studies suggest that autonomic dysfunction by itself is not enough to explain the discordance between functional responses we observed to isoproterenol (no change) vs. the prevalence of tyrosine hydroxylase positive sympathetic fibers (increase) in the urinary bladder during diabetes. Previously Latifpour et al have shown that the increased density of β receptors in the bladder did not correlate with the magnitude of isoproterenol induced relaxation thereby suggesting that the muscarinic and adrenergic receptors are under different regulatory mechanism [Latifpour et al 1991]. Also, recent studies have linked increased reactive oxygen/nitrogen species formation to increased oxidative damage of catecholamines as an important contributor in disease settings such as neurodegeneration, cardiovascular toxicity etc. Using STZ rat model we have recently demonstrated that increased oxidative stress is an important participant in diabetic bladder complications [Poladia et al 2003, in press]. Further studies on the role of catecholamine oxidation in the bladder during diabetes are warranted.
Apart from parasympathetic and sympathetic influence on the bladder we also studied the role of nitric oxide, which is now recognized as an important regulator of many physiological processes, including functional aspects of the lower urinary tract [Andersson et al 1995, 1996]. Thus far the role of nitric oxide in the bladder detrusor has been controversial; with some reports suggesting a role in urethral control but not in the detrusor [Persson et al 1992] while others showing partial mediation of relaxation of the detrusor strips [James et al 1993].

Using in vitro cystometry we found that NOS inhibition in normal isolated bladders caused a highly reproducible and transient increase in intravesicular pressure during initial filling. These findings suggest that some basal NO production may modulate an initial component of the auto relaxation process in normal tissues and this was apparently absent in the diabetic bladder tissues. We have also recently observed the same phenomenon using rat tissues; in light of the controversy regarding NO related detrusor effects additional mechanistic investigations of this phenomenon appear warranted.

We also investigated the prevalence of the three major neurological controllers of bladder function. Tyrosine hydroxylase (TH) and vesicular acetylcholine transporter protein (VACht) are well established and commonly employed prejunctional markers of sympathetic and parasympathetic fibers respectively [Dixon JS et al 1999, 2000]. Nitric Oxide Synthase I (NOS1) is also recognized as an important controller of genitourinary function via its involvement in non-adrenergic non-cholinergic pathways [Andersson et al 1995,1996; Burnett
et al 1997, Mumtaz et al 1999]. In our initial studies of control tissues we found these markers to be localized to certain mouse bladder regions, in that TH was most prevalent in the lamina propria region while VAChT most prevalent in smooth muscle region and NOS1 was widely distributed. Our findings of distribution are consistent with the previous studies done in the streptozotocin induced diabetic rat bladders [Pitre et al 2002] and with evidence from various species [Dixon JS et al 1999, 2000].

The observed general trend was one of reduced parasympathetic influence with simultaneous increases in sympathetic pathways (see Fig 7), consistent with a controlled physiological shift towards relaxation or capacitance processes. This apparent autonomic shift toward a predominantly smooth muscle relaxant state is consistent with the increased functional capacity, decreased tone, and dilated state observed. Many previous investigations have suggested that diabetic bladder dysfunction may be related to autonomic neuropathy, in which a global loss of neurons and/or nerve terminals has been documented [Ellenberg 1980, Kolta et al 1985, Kudlacz et al 1989, Longhurst et al 1986, Lincoln et al 1984, Latifpour et al 1991, Faerman et al 1973, Kahan et al 1970, Kaplan et al 1995]. However, neuropathy appears to be a complication associated with a long-term diabetic state. Our data are consistent with this concept in that within the time frame we examined, markers for two of the three nerve pathways examined were increased – an observation that suggests that frank loss of nerve terminals is not an operable mechanism involved in changes

Our data also show that TH was located primarily in the lamina propria, with a small amount also observed in the smooth muscle. The presence of sympathetic nerves near blood vessels in this bladder region suggests that a major function of sympathetic innervation in the bladder body is control of blood flow to the organ. The small amount of TH marker in the smooth muscle is consistent with observations that β-adrenergic agonists are capable of stimulating relaxation in this tissue.

Since each animal tissue throughout our studies was investigated for all measurements, we were able to investigate associative relationships among these parameters using nonparametric correlation analysis (e.g., on a per-tissue region basis). Using this approach we detected only the following statistically significant associations (shown in Fig 8), each of these is consistent with the view of a coordinated shift toward relaxant pathways and simultaneous reduction in contractile pathways. A positive correlation was observed between blood glucose and urine output suggesting increased osmotic diuresis during diabetes (Fig 8A). A positive correlation between urine output and compliance (function) suggested compensatory mechanism to accommodate increased work load (Fig 8B).
Increased compliance was highly correlated to structural remodeling (Fig 8C). A statistically significant negative correlation observed for smooth muscle VACHT vs. TH (Fig 8D) suggesting neuronal remodeling along with structural remodeling. Furthermore the compliance changes associated more strongly with VACHT than TH (Fig 8E-F) suggesting that the preganglionic parasympathetic dominate over the sympathetic. These studies suggest fundamental differences between the parasympathetic and sympathetic nervous system and their selective activation could be responsible for the bladder changes seen during diabetes.

In summary, urinary bladder dysfunction in diabetes is a well-recognized phenomenon but the mechanisms involved and initiating events are not clear. We developed a streptozotocin induced mouse model that mimics the human and classical STZ induced rat model of diabetes as shown by functional, structural and neuronal remodeling. These studies suggest that the streptozotocin induced mouse model has a likely value in learning more about bladder biology and diabetes influences and has a great potential for disease studies and therapeutic evaluations.
FIGURE LEGENDS

**Figure 5.1:** General characteristics of control mice (CTL) and 5 week diabetic mice (DIA) are shown in Figure 1. The solid bars represent the control mice (CTL) while the white bar represents the diabetic mice (DIA). Hyperglycemia, glucosuria, increased renal clearance, increased urine output, increased water consumption and increased bladder weight are evident. All data are mean ± S.E.M. *, statistically significant difference from control, P < 0.05.

**Figure 5.2:** Representative photomicrographs of Masson’s trichome stained transverse sections (collage of images taken at 40X) of bladders obtained from CTL (control) and 5W DIA (5 week) diabetic rats are shown in Fig 2A-B respectively. Mean morphological assessments shows significant increases in total bladder tissue area, measured in µ² in CTL (control) and DIA (streptozotocin-induced diabetic rats at 5 week) post injection (Fig 2C). The total tissue cross-section area for smooth muscle (SM), lamina propria (LP) and urothelium (UR) for control (C) and streptozotocin-induced diabetic rats at 5 week post injection (D) are shown in Fig 2D. All data are mean ± S.E.M. *, statistically significant difference from control, P < 0.05.
**Figure 5.3:** Representative cystometrogram of a control (CTL) and diabetic (DIA) mouse bladder after Tyrode infusion are shown in Fig 3A. The three parameters derived from cystometrogram viz plateau pressure, structural capacity and compliance are calculated for diabetic (DIA) and control (CTL) group. Plateau pressure was significantly decreased while structural capacity and compliance were significantly increased for diabetic bladders as compared to the controls. All data are mean ± S.E.M. *, statistically significant difference from control, P < 0.05.

**Figure 5.4:** Representative cystometrograms before (solid curve) and after (dotted curve) the administration of bethanechol (100 µM) for the control (CTL) and diabetic (DIA) mouse bladders are shown in Figure 4A and 4B. Compliance was calculated for basal Tyrode infusion (BAS) and after bethanechol (BTH) administration at capacity and ½ capacity for control and diabetic mice (Fig 4C-D). Bethanechol response was measured as difference in compliance and was significantly increased in diabetic (DIA) bladders vs. controls (CTL) (Fig 4E-F). All data are mean ± S.E.M. *, statistically significant difference from control, p<0.05 CTL vs. DIA; ^, p < 0.05 CTL vs. CTL-BTH; #, p < 0.05 DIA vs. DIA-BTH.
Figure 5.5: Representative cystometrograms before (solid curve) and after (dotted curve) the administration of isoproterenol (100 µM) for the control (CTL) and diabetic (DIA) mouse bladders are shown in Figure 5A and 5B. Compliance (Fig5C-D) was plotted before (BAS) and after administration of Isoproterenol (ISO) shown in closed circles for controls and closed squares for 5 week diabetic mouse bladders. Isoproterenol response was significantly decreased in diabetic (DIA) bladders vs. controls (CTL) at \( \frac{1}{2} \) compliance but was unchanged at compliance (Fig 5E-F). All data are mean \( \pm \) S.E.M. *, statistically significant difference from control, \( p < 0.05 \) CTL vs. DIA; ^, \( p < 0.05 \) CTL vs. CTL-ISO; #, \( p < 0.05 \) DIA vs. DIA-ISO.

Figure 5.6: Representative cystometrograms before (solid curve) and after (dotted curve) administration of N-nitro-L-arginine (250 µM) for control (CTL) and diabetic (DIA) mouse bladders are shown in Figure 6A and 6B respectively. Peak pressure (Fig 6C), plateau pressure (Fig 6D), and compliance (Fig 6E-F) were plotted for control (closed circles) and diabetic (closed squares) at basal (BAS) before administration of N-nitro-L-arginine and 5 week diabetic after administration of N-nitro-L-arginine (NO-ARG). All data are mean \( \pm \) S.E.M. *, statistically significant difference from control, *, \( p < 0.05 \) CTL vs. DIA; ^, \( p < 0.05 \) CTL vs. CTL-NO-Arg; #, \( p < 0.05 \) DIA vs. DIA-NO-Arg.
**Figure 5.7:** Effect of diabetes on neuronal markers of innervation in mouse bladder are shown in Fig 7A-B, 7D-E and 7G-H for tyrosine hydroxylase (TH), vesicular acetylcholine transporter (VAChT) and NOS1 at 400x magnification respectively. Digital imaging parameters derived from control (CTL) and 5 week diabetic (5W DIA) photomicrographs showed alterations in immunoprevalance of these markers; in smooth muscle for VAChT and in lamina propria for tyrosine hydroxylase and in all the three layers urothelium (UR), lamina propria (LP) and smooth muscle (SM) for NOS1 in diabetic (D) vs. controls (C). All data are mean ± S.E.M. *, statistically significant difference from control, P < 0.05.

**Figure 5.8:** Spearman's nonparametric correlation analysis showing statistically significant associations for control (closed circle) and 5 week diabetic mouse bladders (closed triangle) between urine output, blood glucose, compliance, total tissue area, TH staining and VAChT staining. No other statistically significant associations were identified. All data are mean ± S.E.M. *, statistically significant difference from control, P < 0.05.
Figure 5.1: General characteristic changes associated with diabetic mouse bladder
Figure 5.2: Region specific structural remodeling in diabetic mouse bladder
Figure 5.3: Functional remodeling in diabetic mouse bladders
Figure 5.4: Altered cholinergic response in diabetic mouse bladders
Figure 5.5: Altered adrenergic response in diabetic mouse bladders
Figure 5.6: Altered response to NOS inhibitor during diabetes
Figure 5.7: Neuronal remodeling in diabetic mouse bladders
Figure 5.8: Spearman’s nonparametric correlation analysis
REFERENCES


CHAPTER 6

OXIDANT DRIVEN SIGNALING PATHWAYS DURING DIABETES: ROLE OF RAC1 AND MODULATION OF PROTEIN KINASE ACTIVITY IN MOUSE URINARY BLADDER.

This chapter has been submitted to Diabetologia and is presented in the style appropriate for the journal.
ABSTRACT

Background: Urinary bladder dysfunction is a common complication in diabetes but the mechanisms involved are undefined. Using rat model we have previously shown time dependent structural remodeling and increased oxidative stress during diabetic bladder dysfunction. Here we investigated role of oxidative stress and oxidant driven signaling pathways in a murine model of diabetes. Methods: Mice were dosed with streptozotocin (150mg/kg) or vehicle and studied at 5 wks. We performed in vitro cystometry to study the functional changes in the bladders during diabetes. Immunohistochemical methods and automated digital imaging was used for measurement of morphometric and histochemical analysis of bladder tissue regions. Results: Using immunohistochemistry we detected significant increases in 3-nitrotyrosine (stable biomarker of reactive nitrogen species) in urothelium and smooth muscle, whereas increased Rac1 (coordinating protein of NADPH oxidase) was observed in smooth muscle only (diabetic vs. controls). In light of nitric oxide synthase (NOS) isoforms as important contributors to increased protein nitration, we looked at changes in all three NOS isoforms in mouse bladders and found region specific increases in NOS1 (urothelium and smooth muscle), NOS2 (urothelium) but no alterations in NOS3 isoform during diabetes. We also investigated phosphorylation of ERK, JNK, p38 and Akt using immunohistochemical techniques; each of these was increased during diabetes but with different distributions in the 2 major regions of bladder tissues viz the smooth muscle and urothelium.
**Conclusions:** These data suggests that oxidant related events may play an important role in diabetic bladder remodeling and that Rac1 and MAPK signaling pathways are involved.
INTRODUCTION

Diabetes mellitus is a costly disease with an incidence of 100 million people worldwide and this incidence is expected to double within the next decade [1]. Urinary bladder dysfunction is one of the most common complications in diabetes and is associated with debilitating consequences, including increased urinary output and frequency, bladder paralysis, incomplete voiding, urinary retention, urinary tract infections and increased risk of bladder cancers [2-10]. These symptoms often progress throughout a patient’s life and are associated with significant health care costs. Despite their widespread occurrence, the mechanisms of diabetes-related bladder dysfunctions are not well understood and treatment strategies are limited. While mechanistic understanding is incomplete, alterations in neuronal control (especially autonomic neuropathy), volume overload, and structural remodeling have all been implicated [2]. Thus further understanding of the underlying mechanisms is necessary to devise enhanced prevention strategies.

We have recently documented that the streptozotocin-induced diabetic mouse model mimics the structural, functional and neuronal changes in human as well as the well-documented streptozotocin-induced rat diabetic bladder [2-10]. Using highly automated digital imaging methods to quantify general size and regional composition of bladder tissue during diabetes, we observed a 2-fold increase of total bladder tissue over 5 weeks of investigation. This increase in total bladder wall area correlated closely to the well-documented increase in
bladder mass associated with experimental diabetes [2,8-9]. Our data showed that the primary increase in total bladder tissue mass occurred within the smooth muscle layer, which progressively increased to comprise about 60% of bladder wall over the 5 weeks investigated. In addition, the observed increase of smooth muscle region was more substantial (3-fold) than total tissue cross-section or total bladder weights (each an approximate 2-fold increase), suggesting that this measurement may be a more sensitive index of early changes in bladder than simple weight measurements. We also observed a progressive increase in total urothelium. This epithelial cell layer plays an important role in normal bladder function (particularly barrier and host-defense mechanisms), but few studies have investigated its properties during diabetes [10]. For this reason we were particularly interested in studying the changes in the smooth muscle vs. the urothelium during diabetes related bladder dysfunction.

Growing evidence suggests that oxidative stress is a common occurrence associated with many of the complications of diabetes, although its role in diabetes related bladder disorders has not been investigated [11]. In addition, several settings of smooth muscle hypertrophy and proliferation have been linked to oxidant related signaling cascades [12], although such phenomena have not been detailed in the setting of diabetic bladder remodeling. For these reasons, and in light of some preliminary data in a rat model [2-3], herein we focused on potential oxidant related mechanisms in murine diabetic bladder structural and functional changes. Originally recognized as host defense mechanism, Rac1 has
been shown to be a coordinating protein for the NADPH oxidase, catalyzing the production of superoxide anion and contributing to oxidant related signaling pathways [13]. In other organ systems the Rac1/NADPH system has been shown to contribute to smooth muscle tone and proliferation, and others have shown that Rac1 over expression in human and mouse endothelial, fibroblast and smooth muscle cells induces increased intracellular reactive oxygen species [14-18], but a role for this pathway in diabetic bladder changes has not been investigated. Hence, using immunohistochemistry we investigated a role for active p21-Rac1 in mouse bladder during diabetes. An additional goal of these studies was to evaluate a role for oxidant related signaling pathways. Rac1 dependent ROS formation is known to activate various different signal transduction pathways [13] particularly the mitogen activated protein kinase pathways (MAPK). These pathways have been associated with cell proliferation and cell differentiation and are affected in other tissues during diabetes [19]. Here we investigated smooth muscle vs. urothelial changes in three major MAPK pathways viz. Erk, JNK and p38 (by measuring the active phosphorylated forms) in mouse urinary bladder during diabetes.
An additional component of our investigation was to study the role of Akt/protein kinase B, a serine threonine kinase that is a downstream effector of PI3 kinase and an upstream regulator of Rac1 [20]. It has been known to play a role in insulin actions and proliferation [20-21] however its role in the diabetic mouse bladders is not known. Here we investigated the role of protein kinase B (Akt/PKB) in the mouse diabetic bladders using immunohistochemistry and measuring the active phosphorylated form of Akt.
MATERIALS AND METHODS

Animals: All animal handling protocols were approved by the Institutional Laboratory Animal Care and Use Committee. Male CF1 mice (5 weeks old, 30±2g, Harlan, Indianapolis, IN) were dosed with streptozotocin (150mg/kg i.p., n=8, Sigma, St. Louis, MO) or vehicle (n= 8) and were housed in a 12-hour light/dark facility with free access to food and water. Diabetic mice were sacrificed at 5 weeks post STZ dosing. Animals were sacrificed by pentobarbital overdose, at which time blood was collected by cardiac puncture, and urinary bladder tissues were rapidly isolated. Blood glucose was measured by a clinical blood glucose monitor (Glucometer Encore, Miles Inc, IN).

In vitro cystometry: The animals viz. controls (n=8) and 5 week diabetic mice (n=8) were euthanized by an overdose of pentobarbital sodium (75 mg/kg i.p., Abbott Laboratories, Chicago, IL). A midline incision through the lower abdominal wall was made to expose the urinary bladder. The ureters were ligated with a suture and the urethra was isolated and cut distal to the bladder body (~ 5 mm). The bladder was gently emptied and cannulated with PE 10 tubing and infused with Tyrode solution. The rate of Tyrode infusion was 0.005 ml/min. The bladder was mounted on a tissue bath containing warm Tyrode solution (37°C) and oxygenated with 95% O₂ and 5% CO₂. The PE10 tubing was attached to a 3-way stopcock directly connected to a pressure transducer. The third connection of the stopcock was attached to an infusion pump (Harvard Apparatus) via a tygon tubing (3mm i.d.). After adjusting the bladder base to the
same level of the pressure transducer, the bladder was allowed to equilibrate for 30 minutes. During this time the bladder was washed three times with warm Tyrode solution. Following equilibration, the intravesicular pressure was recorded in response to a continuous infusion of Tyrode solution into the bladder, until a pressure twice the plateau pressure was reached. The bladder was then emptied and allowed to equilibrate for 30-45 minutes following intermittent washings with Tyrode solution. The bladder was again infused and the above procedure will be repeated for 2 times. The initial run (run1) was to stabilize the bladder under the invitro conditions. The volume of Tyrode infused into the bladder associated with twice the plateau pressure was defined as the structural capacity of the bladder. The pressure–volume relationship (cystometrogram) was generated for each bladder. The structural capacity was measured for the normal and 5-week diabetic mice bladders. Concentration of Tyrode solution used was NaCl 125 mM, KCl 2.7 mM, NaH₂PO₄ 0.4 mM, CaCl₂ 1.8mM, NaHCO₃ 23.8 mM and Glucose 11mM.

**Immunohistochemistry and Digital Image Analysis:** Urinary bladders were rapidly isolated, blotted dry and weighed. They were sectioned at the equatorial midline and allowed to equilibrate in buffer (e.g., with no intralumenal pressure). Following 48hr fixation in 10% buffered formalin, tissues were dehydrated and paraffin embedded using standard procedures, as previously described [1-4]. Immunohistochemical staining was performed using methods previously described [1-4], using a DAKO Autostaining system. Briefly, 5µM
cross-sections of bladder tissue were dewaxed, rehydrated, and treated with preheated citrate buffer (pH 6.0) to recover the antigenicity. The endogenous peroxidase activity was blocked by the tissues with 3% hydrogen peroxide/methanol solution, followed by 10% goat serum / PBS blocking solution (Vector Lab, Burlingame, CA). The following primary antibodies were used: NOS1 (Rabbit polyclonal antibody, 1:200 dilution, Transduction Laboratories, Lexington, KY); NOS2 (Rabbit polyclonal antibody, 1:1000 dilution, Transduction Laboratories, Lexington, KY); NOS3 (Rabbit polyclonal, 1:200 dilution, Transduction Laboratories, Lexington, KY); p21-Rac1 (Rabbit polyclonal antibody, 1:100 dilution, Research Diagnostics Inc., Flanders, N.J.); 3NT (Rabbit polyclonal antibody, 1:1500, Transduction Laboratories, Lexington, KY); p-JNK (Goat polyclonal antibody, 1:100, Santacruz biotechnology, S’cruz, CA); p-ERK (Goat polyclonal antibody, 1:100, Santacruz biotechnology, S’cruz, CA); p-p38 (Rabbit polyclonal antibody, 1:100, Santacruz biotechnology, S’cruz, CA); p-Akt (ser) (Goat polyclonal antibody, 1:100, Santacruz biotechnology, S’cruz, CA). All primary incubations were conducted for 1 hour, tissues were then washed and exposed to biotinylated goat anti-rabbit secondary antibody (1:200) for rabbit polyclonal primary antibodies and in rabbit anti-goat secondary antibody (1:200) for goat polyclonal antibodies (Vector Labs, Burlingame, CA) for 20 minutes followed by Horseradish peroxidase-complex reagent (ABC Elite; Vector Labs, Burlingame, CA). Antigen prevalence and localization was visualized using diaminobenzidine (0.06% w/v) and counterstained with methyl green. Preliminary
experiments were performed to verify the specificity of all antibodies used herein, by replacing primary antibody with pre-immune serum (isotypic staining controls, used for all antibodies) [1-4]. All staining controls showed no detectable immunoreactivity in any treatment group. Sections of tissue were also stained with Masson’s Trichome for morphological evaluation and to evaluate collagen prevalence. Images were captured on a Polaroid DMC high-resolution digital camera, mounted on an Olympus research microscope. The urinary bladders were visualized using light microscopy (Olympus BX40) and photomicrographs were captured using a calibrated digital camera (Polaroid, Cambridge, MA) and transferred into research-based digital image analysis software (Image Pro Plus, Media Cybernetics, Silver Spring, MD). Relative immunoreactivities in cross-sectional bladder regions were determined via integrated optical density measurements [1-4]. Software color segmentation was performed on the trichome stained slides, to measure the area of smooth muscle, extracellular collagen and urothelium present in the cross sections. Image analysis was conducted by investigators who were unaware of treatment group assignments. For all parameters measured intra-observer variability was less than 5% (coefficients of variation for 3 daily measurements) whereas the inter-observer variability was less than 7% (n=3 observers evaluating 6 bladders on three different days).
Statistics: Results were expressed as the mean ± SEM. Comparison between groups was performed by Student’s t test using Sigma Stat 5.0 software from Jandel Scientific. Probability values of $P < 0.05$ were considered significant.
RESULTS

Similar to other studies, hyperglycemia was observed at 5 weeks post streptozotocin injection (5.85±0.22 vs. 16.82±2.01mM, p<0.05).

Shown in Figure1A-B is the representative photomicrograph of mouse bladder cross-sections from control and diabetic mice. Representative images (40X magnification) of trichome stained tissues showing the red colored smooth muscle; blue colored collagen and pink colored urothelium are shown. Total tissue area was significantly increased in diabetic animals as compared to controls (Fig 1C). Figure 1D shows the cross-sectional areas of the 2 primary components of bladder wall viz. smooth muscle (closed circles) and urothelium (closed triangles). Total wall mass nearly doubled, whereas this increase was primarily driven by increased smooth muscle (a near 3-fold increase in this region was detected) and to a lesser extent urothelium. Representative cystometrograms for the control (solid curve) and diabetic mice (dotted curve) is shown in Fig 1E. These pressure-volume tracings were then used to derive structural capacity (measured at twice plateau pressure). Structural capacity was significantly increased in diabetic mice vs. controls (Fig 1F).
Shown in Figure 2 (2A-2B) are representative photomicrographs of protein-3NT immunostaining from control and diabetic mouse bladder tissues at 5 weeks. Undetectable levels of basal nitration were apparent in control tissues in the urothelial or smooth muscle regions. Both of these regions demonstrated statistically significant increases in protein nitration during diabetes (Fig 2C), confirming the increased prevalence of reactive nitrogen species in this setting.

In light of evidence of NO dysregulation, we investigated relative prevalence and distributions of the 3 NOS isoforms in mouse bladder tissues. Representative photomicrographs of NOS1, NOS2 and NOS3 immunostaining are shown in Figure at 400X magnification respectively, and digital image analysis comparisons for average data are also shown. In control tissues NOS1 (the neuronal form of nitric oxide synthase) was detected in urothelium and smooth muscle regions but was most prominent in urothelium; these were significantly increased in the tissues from diabetic animals (Fig 3C). In contrast, induction of NOS2 (the inducible form of nitric oxide synthase) was predominantly present in the urothelial layer of diabetic urinary bladders as compared to controls (Fig 3F). NOS3 (the endothelial form of nitric oxide synthase) was present in both the tissue layers in control mouse bladders, but in contrast to the other isoforms no change was observed during diabetes (Fig 3I).
Shown in Fig 4 are representative photomicrographs of p21-Rac1 prevalence (400x). In control tissues p21-Rac1 was most prevalent in the smooth muscle region, and this was substantially and specifically increased in this region in the tissues from diabetic animals (p<0.05, Fig 4C).

Shown in Figure 5 are representative photomicrographs and digital imaging analysis data for p-Erk, p-JNK and p-p38, and p-Akt in bladder tissues from control and diabetic animals. Each of these were detectable in both the urothelial and smooth muscle regions in control tissues, but each was found to be discretely altered in diabetic tissues. For example p-Erk and p-p38 was significantly increased in the urothelial region only during diabetes (Fig 5C & 5F), whereas p-JNK and p-Akt were both found to be increased specifically in the smooth muscle region during diabetes (Fig 5I & 5L).
DISCUSSION

Urinary bladder dysfunction is a major complication in diabetes and is known to be associated with severe debilitating consequences such as frequent urination, enlarged bladders, urine retention and urinary tract infections [2-10]. Although these changes are well recognized, the mechanisms involved are not well defined, and treatment strategies are limited. Furthermore, as the incidence of diabetes continues to rise and as diabetic patients’ age, the importance and medical costs associated with this syndrome are likely to escalate.

Here we investigated the role of oxidants and oxidant driven pathways especially through Rac1 in a mouse model of diabetes. Previously, we have shown that the streptozotocin induced diabetic mouse model apparently recapitulates all the bladder changes seen in humans and the well-documented rat model of diabetes [2-10]. Our data suggested altered structural, functional and neuronal remodeling similar to the streptozotocin induced rat model of diabetes [4].
Of importance was the finding that the smooth muscle cross sectional area was 3-fold higher than the 2-fold increase seen in total tissue area. Also, there was a significant increase in the urothelial layer area and thickness during diabetes in mouse bladders. These results suggest region specific changes occur in urinary bladder specifically in the smooth muscle and urothelium during diabetes and necessitate the distinct evaluation of these layers in the bladder during diabetes. Hence we studied the oxidant related signaling events in bladder by comparing the changes that take place in smooth muscle vs. urothelium during diabetes.

Growing evidence suggests that oxidative stress is a common occurrence associated with many of the complications of diabetes, although its role in diabetes related bladder disorders has not been investigated [11]. In a rat model of diabetes we have shown oxidative stress is an early event in the bladder before structural remodeling [3]. Here we employed immunohistochemical methods to study the in vivo formation of 3NT in control and diabetic bladders. Statistically significant increases in 3NT prevalence were observed in the urothelial and smooth muscle layers. Since the formation of peroxynitrite is bimolecular, increased prevalence of either nitric oxide or superoxide anion can promote protein nitration events [22]. Of note is the important observation that the regional distribution of protein nitration did not generally follow the regional patterns for NOS2; this high capacity isoform has been most often suggested as an important driving force for peroxynitrite formation in vivo. Rather, the general
distribution of nitration more closely paralleled regional changes in NOS1 prevalence. Thus, under the conditions of these studies NOS2 induction is not apparently an obligatory event for significant increases in tissue reactive nitrogen species formation; other NOS isoforms, as well as changes in superoxide anion availability are apparently involved as well.

In the light of NOS isoforms as important contributors to increased protein nitration, we looked at changes in all the three NOS isoforms in the mouse bladders. Other than its role in protein nitration, nitric oxide is now recognized as an important regulator of many physiological processes, including functional aspects of the lower urinary tract [23]. Nitric oxide is synthesized by the enzyme family of nitric oxide synthases (NOS), which catalyze the conversion of L-arginine to L-citrulline. Three distinct isoforms exist, NOS1, NOS2 and NOS3 [23], and under normal physiologic conditions, NO is primarily produced in bladder tissue via the constitutive isoforms of NOS (NOS1 and NOS3), serving as a critical signal transduction agent for local relaxation of the smooth muscle via a cyclic GMP dependent pathway [23]. Of note is the observation that NOS1 deficient mice (transgenic knockouts) have severe bladder abnormalities, suggesting that this isoform is particularly important for normal bladder function [24]. However there is no general consensus on the role of NOS1 in urinary bladder since a decrease or no change in NOS1 immunoreactivity has been observed during bladder disorders such as bladder outlet obstruction [25-26]. In addition, NOS2, the inducible form of nitric oxide, normally not found in control
bladders [29] is up regulated mainly in the urothelial cells and inflammatory cells after experimental urinary tract infections and bladder cancers [25,27]. Expression of NOS3 in the urinary bladder is controversial as Birder et al [28] have shown NOS3 immunoreactivity in the bladder urothelial cells while Fathian-Sabet et al [29] have shown localization of NOS3 in the bladder smooth muscle and Persson et al have reported no immunoreactivity for NOS3 in the rat and pig bladder [30]. Previously in a streptozotocin induced diabetic rat bladder we have shown time dependent discreet early changes in the NOS isoform expression during diabetes [3]. In our studies we found that all 3 isoforms were detectable in control mouse bladders, but that these tended to have discrete distributions within bladder wall. For example, in control tissues NOS1 and NOS3 were widely distributed in both the regions of the bladder while NOS2 was mostly in urothelium. These observations illustrate the selective cellular expression patterns of the NOS family of gene products, within the same bladder tissue sections, and demonstrate the value of in situ technologies for our studies since these observations would have been impossible using homogenate and blotting methods.

In addition to bladder-region-specific control levels, we observed selective increases in NOS isoforms following diabetes induction. Again, the changes observed were dependent on the specific NOS isoform. For example, statistically significant increases in NOS1 were observed in urothelium and smooth muscle, whereas NOS2 induction was only observed in the urothelial layer, while no
changes in NOS3 were detected at 5 weeks post STZ injection. Again, these changes illustrate the independent control of each gene product in each layer of bladder tissue and suggest fundamentally different and independent responses of these cell types to the same stimuli during the diabetic state. Interestingly, the NOS1 isoform typically associated with smooth muscle relaxation effects (the “constitutive” and calcium dependent isoform) was increased; this may be an attempt to increase bladder compliance in response to increased urine volume following diabetes induction. In contrast, regulation of NOS2, classically referred to as “the inducible isoform”, takes place primarily at the transcriptional level. NOS2 induction typically occurs in settings of tissue injury and/or inflammation and is increased by a variety of inflammatory stimuli, particularly cytokines [23]. We observed a rapid and striking induction of NOS2 at 5 weeks of diabetes induction and this activation was solely confined to the urothelial layer and was not associated with immune cell infiltrates. Thus, the increased and urothelial cell-specific expression of NOS2 in the diabetic bladders is consistent with an urothelium bladder inflammatory response in this setting and suggest that specific urothelial cell activation/ stress response is an event in diabetic bladder changes.

Previous studies in the vascular smooth muscle suggest that activation of NADPH oxidase is an important source of oxidants especially superoxide [13]. NADPH oxidase activation requires the presence of the active GTP bound Rac1. Since increased Rac1 is associated with increased superoxide formation; we
investigated the role of increased superoxide anion by measuring the levels of active p21-Rac1 in the urinary bladder during diabetes. Our data showed significant increases in the smooth muscle specific p21-Rac1 immunoprevalence in the diabetic mouse bladders. Our data suggests that an inducible oxidase could be a potentially important source of oxidants in this setting and not just glucose chemistry thru AGEs as described previously could be the only source of oxidants in the diabetic bladder setting [11]. Of note is the important observation that the regional distribution of protein nitration (urothelium and smooth muscle) did not generally follow the regional patterns for Rac1 (smooth muscle); which has been most suggested as an important contributor of superoxide formation in vivo [14-15]. Thus, under the conditions of these studies, not superoxide availability alone but changes in NOS isoform availability are apparently involved as well.

Rac1 is a small GTP binding protein of the Ras superfamily that regulates a variety of cellular activities such as actin polymerization, transcriptional activation, cellular proliferation and superoxide generation [14-18]. Rac1 proteins are known to be activators of oxidative burst thru activation of NADPH oxidase. Activation of the Rac1 protein requires its binding to the GTP. The GTP bound Rac1 triggers the clustering of a multiprotein complex and in the presence of the cofactor NADPH, catalyzes the formation of superoxide [14-18]. Classically Rac1 has been shown to be an participant in host defense mechanisms [14-18], however, recently it has been shown to be an important contributor in signaling
pathways mainly thru increased reactive oxygen/nitrogen species [13]. Rac1 has been shown to interact with serine threonine kinases: mitogen activated protein kinases, diacylglycerol kinase–zeta, phosphatidylinositol phosphate kinases (PIP kinases), phosphatidylinositol-3-kinase (PI3K), and protein kinase B (Akt/PKB) [31]. Here we investigated the role of these different kinases specifically the MAPKs and phosphorylated Akt/PKB to study the Rac1 driven oxidant-signaling pathways in a diabetic mouse bladder.

MAPKs are serine threonine kinases that are activated through dual phosphorylation at conserved threonine or tyrosine residues and are known to be involved in a diverse set of responses affecting cell fate, cell proliferation, cell differentiation, adaptation to environmental stress and apoptosis [19,32] The MAPK pathways are categorized into three major subfamilies viz. Erk, JNK and the p38 pathway [19, 32]. There is large and accumulating evidence in the literature linking diabetes and oxidative stress with the activation of MAPKs [19]. Urinary bladders exposed to mechanical stretch are known to activate the MAPK pathways measured by the phosphorylated antibodies to Erk, JNK and p38; although their role in diabetic mouse bladders is undefined [33-34]. We found that diabetes related bladder dysfunction is associated with highly cell and region specific MAPK activation. Our studies are consistent with bladder structural changes and known MAPK roles since MAPK pathways are known to alter gene expression of AP-1, CREB, SRE etc which are responsible for regulation of transcription of genes involved in cell growth, differentiation etc. [32].
Apart from MAPKs, PI3K has been associated with Rac1 signaling pathway [35]. However the role of PI3K in Rac1 signaling has been controversial. There is data suggesting that PI3K is a downstream effector of Rac1; although most studies suggest that PI3K functions upstream of Rac1 [35]. Akt kinase has been characterized as the primary signal transducer of the PI3K pathway and has been used previously to study the relationship between the PI3K and Rac1. Recently Kwon et al have suggested that Akt kinase of the PI3K signal transduction pathway inhibits Rac1 thru its phosphorylation [35]; while Jiang et al suggest a critical role of Rac1 in mediating PI3K activation of Akt [36]. Akt/protein kinase B is a serine threonine kinase and a downstream effector of PI3 kinase and has also been known to play a role in insulin actions [20-21]. Studies on the role of p-Akt in Type 2 diabetes suggest an increase [37], decrease [38] or no change [39] in the Akt status. Recently using the p-Akt antibody Thirone et al have shown increase in the phosphorylation of Akt in the kidneys of the streptozotocin induced diabetic rats [37]. Similar to Thirone et al, we found significant increases in the p-Akt in the smooth muscle of the bladder. In our studies we observed increases in p-p-38 and p-Erk in urothelium during diabetes; these markers are consistent with cellular inflammation activation and growth in this region. In contrast we found that p-JNK and p-Akt were specifically increased in the smooth muscle layer; these responses are consistent with anti-apoptosis and proliferation signal pathways respectively. Thus, the general pattern of these signaling markers in each of these regions is consistent with structural and
functional characteristics of the sites (e.g., urothelial inflammation and growth, smooth muscle proliferation and hypertrophy). In addition, our region specific findings illustrate the liabilities of using whole homogenates for analysis of signal pathways in such tissues.

In summary, urinary bladder dysfunction in diabetes is a well-recognized phenomenon but the mechanisms involved and initiating events are not clear. Automated digital imaging methods provided an unbiased, reproducible, and convenient method for detailed histochemical analysis of the bladder tissue regions from a well-documented mouse model of diabetic cystopathy. Using these methods we observed cell specific alterations in two regions of the bladder viz urothelium, and smooth muscle. We observed region specific increases in protein nitration and Rac1 demonstrating first-time evidence of inducible oxidases in this setting. Further investigations of the signaling pathways altered by Rac1 during diabetes showed increased MAPK and Akt/PKB activation. These data suggests that oxidant related events might play an important role in diabetic bladder remodeling and that Rac1 signaling pathways are involved. Further investigations of the signaling events during diabetes related bladder may provide new insight regarding adaptive and maladaptive processes in bladder physiology and enhanced opportunities for improved therapeutic strategies for an important diabetic complication.
FIGURE LEGENDS

Figure 6.1: Representative photomicrographs of Masson’s trichome stained transverse sections (collage of images taken at 40X) of bladders obtained from CTL (control) and DIA (5 week) diabetic rats are shown in Fig 1A and 1B respectively. Mean morphological assessments shows significant increases in total bladder tissue area, measured in mm$^2$ in CTL (control) and DIA (streptozotocin-induced diabetic rats at 5 week) post injection (Fig 1C). The total tissue cross-section area for smooth muscle (closed circles) and urothelium (closed triangles) for control (CTL) and streptozotocin-induced diabetic rats at 5 week post injection (DIA) are shown in Fig 1D. Representative cystometrogram of a control (CTL) and diabetic (DIA) mouse bladder after Tyrode infusion are shown in Fig 1E. Significantly increased bladder capacity for control (CTL) and streptozotocin-induced diabetic rats at 5 week post injection (DIA) are shown in Fig 1D. All data are mean ± S.E.M. *, statistically significant difference from control, P < 0.05.
Figure 6.2: Representative photomicrographs of 3-nitrotyrosine (3-NT) immunostaining are shown in Figure 2A-B respectively for the control (CTL) and 5 week diabetic (DIA) mouse bladders. Digital imaging parameters derived from control and diabetic photomicrographs showed alterations in the 3-NT immunoprevalence in the smooth muscle (closed circles) and urothelium (closed triangles). All data are mean ± S.E.M. *, statistically significant difference from control, P < 0.05.

Figure 6.3: Representative photomicrographs show immunostaining for NOS1 (3A-B), NOS2 (3D-E) and NOS3 (3G-H) at 400X magnification for control (CTL) and 5 week diabetic (DIA) mouse bladders. Digital imaging parameters derived from control and diabetic photomicrographs are shown in Fig 3C, 3F and 3I for NOS1, NOS2 and NOS3 respectively. Immunoprevalence was expressed as integrated optical density demonstrating statistically significant (p < 0.05) increases in NOS1 and NOS2 immunoprevalence but no changes in NOS3 staining in STZ induced diabetic animals vs. controls (Fig 3C). All data are mean ± S.E.M. *, statistically significant difference from control, p < 0.05.
Figure 6.4: Representative photomicrographs of p21-Rac1 immunostaining are shown in Figure 4A-B respectively in the control (CTL) and 5 week diabetic (DIA) mouse bladders. Digital imaging parameters derived from control and diabetic photomicrographs showed alterations in the Rac1 expression predominantly in smooth muscle (closed circles) and no changes in urothelium (closed triangles). All data are mean ± S.E.M. *, statistically significant difference from control, P < 0.05.

Figure 6.5: Representative photomicrographs of p-ERK, p-JNK, p-p38 and p-Akt are shown in Figure 5A-B, 5D-E, 5G-H and 5J-K respectively for the control (CTL) and diabetic (DIA) mouse bladders. The phosphorylated antibodies of MAPK pathway and p-Akt were widely distributed in all the urothelium (closed triangles) and smooth muscle (closed circles) of the control mouse urinary bladder. Cell and region specific alteration in the urothelium (closed triangles) and smooth muscle (closed circles) were observed for the diabetic mouse bladders vs. controls as measured by digital imaging technique. All data are mean ± S.E.M. *, statistically significant difference from control, P < 0.05.
Figure 6.1: Structural and functional remodeling in diabetic mouse bladders.
Figure 6.2: Region specific increased oxidative stress in diabetic mouse bladders
Figure 6.3: Region specific alterations in NOS isoforms in diabetic mouse bladders
Figure 6.4: Smooth muscle specific Rac1 activation in the diabetic mouse bladders
Figure 6.5: Region specific alterations in MAPK and Akt pathways
REFERENCES


CHAPTER 7

OXIDATIVE STRESS AS AN IMPORTANT PARTICIPANT IN URINARY BLADDER DYSFUNCTION AND REMODELING: THE USE OF A TRANSGENIC MODEL.

This study was done in collaboration with Dr Goldschmidt-Clermont PJ and Hassanain H; who provided us the transgenic mouse model for bladder research.
ABSTRACT

Urinary bladder dysfunction is a major complication in diabetes and is associated with altered organ structure and function as well as increased bladder smooth muscle oxidative stress. Here we employed transgenic mice that selectively over express a pro-oxidant gene (Rac1, coordinator of NADPH oxidase) in bladder smooth muscle to test the hypothesis that oxidative stress is a key component of diabetes related bladder dysfunction and remodeling. Non-diabetic mice over-expressing constitutively active RAC1 (Rac CA) in bladder smooth muscle and wild-type (CTL) controls were investigated. Significant increase in RAC1 were observed in bladder smooth muscle in Rac CA vs. CTL mice. No differences in urine glucose concentrations were observed but 2-fold increase in bladder capacity and compliance, and increased bladder weight. Digital imaging also showed smooth muscle hypertrophy and decreased collagen (as % tissue composition). Neuronal remodeling and altered oxidant signaling pathways were seen. These data demonstrate that selective production of oxidants in bladder smooth muscle produces structural, functional and neuronal changes in urinary bladder that are identical to those observed in diabetes. Oxidative pathways may be major contributors in diabetes related bladder changes.
INTRODUCTION

Urinary bladder dysfunction is a common disorder with severe debilitating consequences, including increased urine output, increased frequency of micturition, bladder paralysis, incomplete voiding and urinary retention [1-5,7-8,17,25,27-28]. This syndrome often irreversibly progresses throughout a patient’s lifespan and is associated with significant health care costs. Despite their common occurrence, the mechanisms of diabetes related bladder dysfunctions are not well understood and treatment strategies are limited. Thus further understanding of the underlying mechanisms is necessary to devise enhanced prevention and treatment strategies.

An important aspect of diabetic bladder investigations is the potential of organ changes related purely to the increased urine volume handling (e.g., diuresis related remodeling) versus changes related to the diabetic state per se. While some investigators have reported that polyuria causes increased capacity and hypertrophy as a compensatory adaptation of bladder to increased volume overload others have suggested neuropathy through impaired sensation of bladder fullness resulting in bladder distention and remodeling [7-8,17,25,27]. In previous investigations, we and others have demonstrated that rodent models (both rat and mouse) of streptozotocin (STZ) induced diabetes recapitulate the functional, structural, and neurological changes associated with diabetic cystopathy observed in humans [1-5,7-8,17,25,27-28]. More recently, we have also documented increased oxidative stress in smooth muscle and urothelium of
diabetic animals as well as increased prevalence of the pro-oxidant Rac1 (a coordinating protein of the NADPH oxidase system), predominantly in the smooth muscle of the diabetic mouse bladders [3,5]. These changes were observed as early as 3 days post STZ injection, which preceded structural changes [3], suggesting an important role of oxidants in bladder dysfunction during diabetes, and that the structural changes may be driven primarily via oxidant related processes rather than volume changes. In addition our previous data suggested that this inducible oxidase system could be a potentially important source of oxidants in this setting [5], rather than the involvement of glucose autooxidation chemistries as the sole source of oxidants in the diabetic bladder setting [6].

We and others have previously demonstrated that Rac1 over expression in human and mouse endothelial and fibroblast cells induces increased intracellular reactive oxygen species [9-13]. Rac1, an important GTP binding protein, triggers the clustering of a multi-protein complex, which in the presence of the cofactor NADPH catalyzes the generation of superoxide. [9-13]. Rac1, which was historically known to be involved in host defense mechanisms, has been recently shown to be an important contributor in signaling pathways mainly thru increased reactive oxygen/nitrogen species [14]. Rac1 dependent ROS formation is known to activate mitogen activated protein kinase pathways (MAPK) especially the JNK and p38 pathways and protein kinase B (PKB/Akt) pathways [14]. The MAPK pathways are known to be involved in cell hypertrophy, cell proliferation and cell growth [15,16]. Structural remodeling
especially hypertrophic and proliferative growth in the smooth muscle of the urinary bladder during diabetes is well documented [2,17]; however, the oxidant driven signaling mechanism downstream from bladder muscle cell activation during diabetes are not defined. We have shown discreet and region specific alterations in the MAPK and Akt pathways during diabetes, suggesting that these signaling pathways participate in the structural alterations observed [5].

In an effort to investigate the consequences of RAC1 induction with respect to bladder function and structure, here we employed a transgenic mouse model of Rac1 constitutively over expressed (CA) via a selective smooth muscle promoter. We investigated the role of oxidative stress alone as an important participant in altering the bladder morphology, function and neuronal remodeling, in the absence of diabetes (e.g. in both absence of increased glucose or volume related changes). In addition to functional and structural investigations we also evaluated three major MAPK pathways viz ERK, JNK and p38 and Akt pathways by measuring the active phosphorylated form of ERK, JNK, p38 and Akt respectively using immunohistochemical techniques, determining their prevalence in three discrete regions of the urinary bladder viz. urothelium, lamina propria and smooth muscle.
MATERIALS AND METHODS

**Generation of transgenic mice:** The cDNA coding for the constitutively active mutated form of human Rac1 (Rac CA), with glycine 12 to valine substitution and myc-tagged (a gift from Alan Hall), was subcloned into the EcoR1 site of Bluescript II KS+, to create new restriction sites. Smp8 plasmid was cut with Xho1 and blunt ends produced by filling, then cut with Kpn1. The cDNA of human Rac CA including its polyadenylation tail were cloned within the blunt Xho1 and Kpn1 ends of the Smp-8 plasmid that contain a 3.6 kb segment of the 5’-region of the mouse smooth muscle (SM) α-actin promoter containing all the elements known to be required for optimal transcription. This promoter was used to induce selective overexpression of Rac CA in smooth muscle cells. A Sph1/Kpn1 fragment of this plasmid was isolated and microinjected into mouse fertilized eggs of FVB/N females as described earlier [18]. Single cell embryos derived from superovulated FVB/N females were used for the microinjection procedure. Surviving microinjected embryos were implanted into pseudopregnant CBA/B6 foster mothers [19]. Founder mice were identified using southern blot analysis and PCR.

**PCR:** Transgenic mice were screened using a polymerase chain reaction (PCR) assay. Genomic DNA was isolated from tail clips and incubated in 500 µl of lysis buffer overnight at 60°C. The lysis buffer contains 50 mM tris pH 8.0, 50 mM NaCl, 25 mM EDTA and proteinase K (0.7 mg/ml). The DNA was further cleaned with three cycles of phenol/chloroform (1:1) and once with chloroform.
The supernatant was recovered and DNA was precipitated with 2 volume of ethanol. The PCR reaction was performed using primers for human Rac CA: forward primer 5'TCTCTGCAGAACC TGAGAC3' (derived from SM α-actin promoter), and reverse primer 3'GAGGTCGACGGTATCGATAAGCT TG5' derived from Bluescript II ks+ complementary sequence located at 3' end of Rac CA.

**Southern blot analysis:** Founder mice were identified using southern blot analysis. Genomic DNA isolated from tail clips of transgenic mice and a radiolabeled human-Rac CA cDNA probe were used to confirm the proper recombination of the human Rac CA gene into the mouse genome. DNA was isolated as mentioned above and 20 g of each DNA sample was digested with EcoR1 to release the human Rac CA cDNA. The blot was hybridized with radiolabeled human-Rac CA cDNA probe according to standard protocols [20]. The positive mice are expected to show a band of size 0.65 kb.

**RNA detection by RT-PCR:** Tissues isolated from mice were immediately frozen in liquid nitrogen. The frozen tissue was pulverized in liquid N₂, and total cellular RNA was extracted using RNeasy Mini Kit (QIAGEN) according to the manufacturer's instructions. The cDNA template was obtained utilizing Superscript II (Gibco-BRL) then amplified using Taq-DNA polymerase (Gibco BRL) with 35 cycles of denaturation (94°C, 45 s), primer annealing (55°C, 30 s) and extension (72°C, 2 min). The primers used for this assay were a c-myc primer, which recognize the c-myc tag sequence that was attached to the 5' end
of the cDNA 5'GAGCAGAAGCTGATCT CCGAGGAG3', and a reverse primer corresponding to a 3' end of the Rac CA cDNA 3'TTACAACAGCAGGCACTTTCTCTT5'.

All animal handling protocols were approved by The Ohio State University Animal Care and Use Committee. These animals were housed in a 12-hour light/dark facility and were allowed free access to food and water.

**Urine output:** The animals were placed in stainless steel metabolic cages for 24 hours. They were deprived of food and given free access to water. The urine was collected after 24 hours and the volume was determined. Urine glucose was determined by the Clinistix Reagent Strips for Urinalysis (Bayer Corp., Elkhart, IN).

**Invitro-cystometry:** The animals viz. normal controls (n=7) and Rac CA (n=7) were euthanized by an overdose of pentobarbital sodium (75 mg/kg i.p., Abbott Laboratories, Chicago, IL). A midline incision through the lower abdominal wall was made to expose the urinary bladder. The ureters were ligated with a suture and the urethra was isolated and cut distal to the bladder body (~ 5 mm). The bladder was gently emptied and cannulated with a PE 10 tubing and infused with Tyrode solution. The rate of Tyrode infusion was 0.005 ml/min. The bladder was mounted on a tissue bath containing warm Tyrode solution (37°C) and oxygenated with 95% O₂ and 5% CO₂. The PE10 tubing was attached to a 3-way stopcock directly connected to a pressure transducer. The third connection of the stopcock was attached to an infusion pump (Harvard Apparatus) via a tygon
tubing (3mm i.d.). After adjusting the bladder base to the same level of the pressure transducer, the bladder was allowed to equilibrate for 30 minutes. During this time the bladder was washed three times with warm Tyrode solution.

**Tyrode infusion:** The stopcock was open to the pressure transducer, bladder and infusion pump during the equilibration period and infusion. Following infusion, the intravesicular pressure was recorded in response to a continuous infusion of Tyrode solution into the bladder, until a pressure twice the plateau pressure was reached. The bladder was then emptied and allowed to equilibrate for 30-45 minutes following intermittent washings with Tyrode solution. The bladder was again infused and the above procedure will be repeated for 2 times. The initial run (run1) was to stabilize the bladder under the invivo conditions. The volume of Tyrode infused into the bladder that correlated with twice the plateau pressure was defined as the structural capacity of the bladder. The pressure–volume relationship (cystometrogram) was generated for each bladder. The structural capacity was measured for the normal and Rac CA bladders.

Concentration of Tyrode solution used was NaCl 125 mM, KCl 2.7 mM, NaH$_2$PO$_4$ 0.4 mM, CaCl$_2$ 1.8mM, NaHCO$_3$ 23.8 mM and Glucose 11mM.

**Immunohistochemistry and Image Analysis:** After cystometry the bladders were dried and weighed. They were cut through the equatorial midline and formalin fixed. After 48 hrs of fixation, tissues were dehydrated and embedded in paraffin as described previously [2-5]. Immunohistochemical staining was performed using methods previously described [2-5], using a DAKO
Autostaining system. Briefly, 5µM cross-sections of bladder tissue were
dewaxed, rehydrated, and treated with preheated citrate buffer (pH 6.0) to
recover the antigenicity. The endogenous peroxidase activity was blocked by the
tissues with 3% hydrogen peroxide/ methanol solution, followed by 10% goat
serum / PBS blocking solution (Vector Lab, Burlingame, CA). The following
antibodies were used:p21-Rac1 (Rabbit polyclonal antibody, 1:100 dilution,
Research Diagnostics Inc., Flanders, N.J.); 3NT (Rabbit polyclonal antibody,
1:1500, Transduction Laboratories, Lexington, KY);, Tyrosine hydroxylase
(Rabbit polyclonal antibody, 1:800 dilution, Novus Biologicals Inc; Littleton, CO);
Vesicular acetylcholine transporter (Rabbit polyclonal antibody, 1:2000, Accurate
Chemicals and Scientific Corp; Westbury, NY); p-JNK (Goat polyclonal antibody,
1:100, Santacruz biotechnology, S’cruz, CA); p-ERK (Goat polyclonal antibody,
1:100, Santacruz biotechnology, S’cruz, CA); p-p38 (Rabbit polyclonal antibody,
1:100, Santacruz biotechnology, S’cruz, CA); p-Akt ser (Goat polyclonal
antibody, 1:200, Santacruz biotechnology, S’cruz, CA). All primary incubations
were conducted for 1 hour, tissues were then washed and exposed to
biotinylated goat anti-rabbit secondary antibody (1:200) for rabbit polyclonal
primary antibodies and in rabbit anti-goat secondary antibody (1:200) for gat
polyclonal antibodies (Vector Labs, Burlingame, CA) for 20 minutes followed by
Horseradish peroxidase-complex reagent (ABC Elite; Vector Labs, Burlingame,
CA). Antigen prevalence and localization was visualized using dianinobenzidine
(0.06% w/v) and counterstained with methyl green.
Preliminary experiments were performed to verify the specificity of the antibody used herein, by replacing primary antibody with pre-immune serum (isotypic staining controls).

Sections of tissue from the same block were also stained with Mason’s Trichome for morphological studies and to evaluate collagen deposition. Images were captured on a Polaroid DMC high-resolution digital camera, mounted on an Olympus research microscope. The images were analyzed with Image Pro 5.0 (Media Cybernetics, Silver Springs, MD) image analysis software. Relative immunoreactivity in the bladder was determined via integrated optical density measurements. Software color segmentation was performed on the Massan’s stained slides, to measure the area of smooth muscle, extracellular collagen and urothelium present in the cross sections. Image analysis was conducted by investigators who were unaware of treatment group assignments. For all parameters measured intra- and inter-observer variability was less than 5% (coefficients of variation for 3 daily measurements and n=3 for inter-observers evaluating 6 bladders on three different days).

**Statistics:** Results were expressed as the mean ± SEM. Comparison between groups was performed by Student’s t test using Sigma Stat 5.0 software from Jandel Scientific. Probability values of P < 0.05 were considered significant.
RESULTS

Founder mice were identified by Southern blot analysis of tail genomic DNA. Three transgenic founders were selected on the basis of strongly positive Southern blot analysis, and the one confirmed assumed to have the highest number of human Rac CA gene copies (35) was used to establish a stable transgenic line by breeding it with nontransgenic FVB/N mates. Further Southern blot analysis of genomic DNA from tails of F1 founder offsprings indicated that nearly 50% of them were transgenic as shown in Figure 1A. Extracted DNA was digested with EcoR1 restriction enzyme to release the human Rac CA cDNA. The arrow indicates the position of human Rac CA (0.65 kb). In addition to the 0.65 kb fragment corresponding to Rac CA transgene, the probe also hybridized to a 4.4 kb DNA fragment that has the size of the fragment of microinjected DNA used to generate the transgenic mice. RT-PCR analysis confirmed the selective transcription of Rac CA in smooth muscle, including blood vessels, spleen and intestine as shown in Fig 1B, while the transgene transcript was not detected in control mice (data not shown), nor in the heart, liver and brain of transgenic mice. The kidney was also positive for Rac CA mRNA as the mesangial cells of glomeruli express SM α-actin [21]. Shown in Fig 1C-1D is the representative photomicrograph of the bladder cross-sections from the control and Rac CA mice stained with Rac1 antibody at 400X magnification. Extensive and smooth muscle specific Rac1 staining was observed in the smooth muscle of the Rac CA mice as compared to age-matched non-transgenic controls. Statistically significant
increases (P< 0.05) in Rac1 immunoprevalance were observed in Rac CA mice as compared to the control mice, as measured by the digital image parameters, as shown in Fig 1E. Representative photomicrographs of 3NT, a biomarker of peroxynitrite and oxidative stress, immunostaining are shown in Figure 1F-1G at 200X magnification. 3-nitrotyrosine immunostaining was present predominantly in the smooth muscle in the Rac CA mice bladders as compared to the controls. Digital imaging parameters showed statistically significant increases (P < 0.05) in 3NT immunoprevalance in the smooth muscle of the Rac CA mice bladders vs. controls (Figure 1H). A 3-fold increase in the prevalence of 3NT was seen in the smooth muscle in the Rac CA mice bladders vs. controls.

No changes in the urine output were observed in the Rac CA mice and age-matched controls as shown in Fig 2A. The urine glucose was negative in both the groups of animals suggesting that the mice were not diabetic. Fig 2B shows the changes in the bladder weight, measured as bladder weight to body weight; which was significantly increased (P < 0.05) in the Rac CA mice as compared to the controls.
Shown in Fig 2C is a representative cystometrogram of the control and the
Rac CA mice. The cystometrogram records the pressure to volume changes that
take place in the bladder during filling. The figure shows a longer
cystometrogram for the Rac CA mice as compared to the controls. Fig 2D and 2E
measure the two classical parameters viz. the structural capacity and bladder
compliance of the control and Rac CA mice. Both these parameters were found
to be significantly increased (P< 0.05) in the Rac CA mice as compared to the
controls.

Representative photomicrographs of bladder cross-sections from control
and Rac CA mice are shown in Fig 3A and 3B respectively. The representative
images (400X magnification) were stained with Massan’s trichome stain showing
the red colored smooth muscle, blue colored collagen and pink colored
urothelium, Using an appropriate color segmentation protocol as previously
described (2-5), the relative prevalence of the three primary components of the
bladder wall; as a percentage of the total tissue; was determined. Fig 3C, 3D and
3E show the relative prevalence of the smooth muscle, collagen and urothelium
respectively. Smooth muscle content showed a significant increase (P< 0.05) in
the Rac CA mice as compared to the controls. In contrast, the percent of bladder
wall area composed of the urothelium was not significantly altered and percent of
the extracellular collagen declined in the Rac CA mice as compared to the
controls.
Shown in Figure 4 are representative photomicrographs of tyrosine hydroxylase (TH) (4A-4B) and vesicular acetylcholine transporter (VChAT) (4D-4E) immunostaining in mice bladders. In control tissues TH (sympathetic neuronal marker) was most prevalent in the lamina propria regions, especially in the nerve bundles and surrounding the blood vessels. VChAT (parasympathetic/neuromuscular junction marker) was most prevalent in the smooth muscle regions of the urinary bladder. Images showed apparent increase in TH in the Rac CA mice group whereas VChAT was consistently reduced. Region specific and time dependent measurements from control and Rac CA mice bladders are shown in Figures 4C & 4F. Using digital image analysis each of the three regions were separately analyzed for relative analyte prevalence as described in the methods section above. Significant increase in TH was observed in the Rac CA mice as compared to controls and a simultaneous decrease in VChAT was also observed in Rac CA mice tissues (Fig4C & 4F). The distribution of these neuronal markers was not significantly altered in the Rac CA mice (e.g., there was no apparent “recruitment” of TH or VChAT into other regions).

Representative photomicrographs of p-ERK, p-p38, p-JNK and p-Akt are shown in Figure 5A-B, 5D-E, 5G-H and 5J-K respectively. In control tissues p-JNK and p-ERK was present in both the layers viz. urothelium and smooth muscle of the urinary bladder, while p-p38 was present only in the urothelium. Images showed apparent increase of p-JNK and p-Akt in smooth muscle of the Rac CA mice and no changes in the p-ERK activation, while p-p38 was
upregulated in urothelium of *Rac CA* mice bladders. Using digital image analysis, the immunoprevalance of each of the MAPKs and Akt in the bladder was quantitated.
DISCUSSION

Urinary bladder dysfunction is a common complication in diabetes mellitus and has been recognized for over hundred years. Bladder dysfunction is characterized by increased bladder volume and compliance and decreased tone. Additionally, bladder stasis predisposes diabetic patients to urinary tract infections, which in turn may accelerate the dysfunction [1-8]. While the phenomenon of diabetes related bladder dysfunction is well recognized, its pathogenesis is not well understood and no therapies are effective in completely preventing the damage.

We have previously shown that the streptozotocin-induced rat model of diabetes exhibits urodynamic alterations similar to humans, and more recently we have found that region-specific structural changes also occur in a time dependent manner [2-3]. Apart from structural remodeling, we have also recently shown that oxidative stress could an important participant in diabetic bladder [2-5]. Using immunohistochemical techniques and automated digital image analysis, we have shown increased oxidative stress; using 3-nitrotyrosine as a biomarker; specifically in the smooth muscle and urothelium of the STZ-induced rat urinary bladder; precedes structural remodeling found in this model.
Our data suggests that increased oxidative stress especially in smooth muscle of the bladder may be key early participant leading to organ dysfunction in this setting [3,5]. Also, growing evidence suggests that oxidative stress via formation of reactive oxygen/nitrogen species is a common occurrence associated with most of the complications of diabetes [6]. For these reasons, we were interested in studying the effect of oxidative stress alone specifically in smooth muscle of the urinary bladder in the absence of diabetes or glucose/volume related changes. Here we employed the Rac CA over expressed transgenic mouse model to study the role of oxidative stress alone on the bladder morphology, function and neuronal remodeling in the absence of diabetic condition. We have engineered a transgenic mouse model that overexpresses the constitutively active mutant of human Rac1 (Rac CA) in FVB/N mice, using the mouse smooth muscle α-actin promoter containing all elements known to be required for optimal transcription of the SM α-actin gene [22,23]. Using the SM α-actin we were able to express Rac1 specifically in the smooth muscle of the mice.
RT-PCR analysis confirmed the selective transcription of Rac CA in smooth muscle, including blood vessels, spleen and intestine as shown in Fig 1B, while the transgene transcript was not detected in control mice (data not shown), nor in the heart, liver and brain of transgenic mice. We detected extensive and smooth muscle specific Rac1 immunostaining in the bladder smooth muscle of the Rac CA mice as compared to age-matched non-transgenic controls (Fig 1C-D). Statistically significant increases (P<0.05) in Rac1 immunoprevalance were observed in Rac CA mice as compared to the control mice, as measured by the digital image parameters (Fig 1E).

We and others have already demonstrated that Rac1 over expression in the human and mouse endothelial and fibroblast cells induces increased intracellular reactive oxygen species [9-13]. Rac1, an important GTP binding protein, triggers the clustering of a multi-protein complex, which in the presence of the cofactor NADPH catalyzes the generation of superoxide [9-13]. Since Rac1 is associated with increased superoxide formation we measured increased oxidative stress in the Rac CA mice bladders using 3-nitrotyrosine, a stable biomarker of peroxynitrite and oxidative stress. We found smooth muscle specific 3-NT formation with a 3-fold increase in the Rac CA mice as compared to controls (Fig 1F-H)
Increase in bladder capacity and compliance measured by in-vitro cystometry is well-documented in diabetes associated bladder dysfunction [24]. Cystometry is a method that measures the pressure-volume relationship of the bladder. We and others have shown increases in bladder capacity and compliance as measured by in-vitro cystometry in diabetic rats as compared to the controls [4, 25]. We were successfully able to recapitulate similar changes in the mouse bladder using invitro cystometry. Our results showed striking two fold increases in the bladder capacity and compliance in the Rac CA over expressed mice as compared to the controls (Fig 2D-E). We observed changes in the bladder weight, measured as bladder weight to body weight; which was significantly increased (P < 0.05) in the Rac CA mice as compared to the controls (Fig 2B).

No changes in the urine output were observed in the transgenic Rac CA mice and age-matched controls. The urine glucose was negative in both the groups of animals suggesting that the mice were not diabetic. Thus for the first time we have shown that oxidative stress by itself can produce functional remodeling in the mouse bladders. Our study did not shown any decrease in the intravesical pressure, another commonly documented feature associated with diabetic bladders, suggesting that oxidative stress might not be involved in maintaining the tone of the urinary bladder.
Previously others have documented altered bladder morphology characterized by increased bladder size, and hypertrophy of the smooth muscle in the well defined streptozotocin induced diabetic rat models [17]. Recently we have documented early, time-dependent, and region specific urinary bladder remodeling in the rat STZ diabetic model using a specially designed digital imaging analysis approach [2,4]. We were able to detect structural changes (hypertrophy and chamber dilation) in the urinary bladder as early as 2 weeks after the induction of diabetes and selective changes in bladder wall composition also occurred in a time dependent manner (most notably increased smooth muscle and urothelium and decreased collagen prevalence) [2]. These previous studies suggested to us that important regional changes occur very early during diabetic bladder structural remodeling. Our quantitative analyses demonstrate an increase in the fraction of the bladder wall composed of the smooth muscle, decrease in the collagen content and no changes in the urothelial layer in the \textit{Rac CA} mice as compared to the controls.
Our data is consistent to the structural remodeling associated with STZ rat and mouse bladders during diabetes. We and others have previously shown bladder smooth muscle hypertrophy in the streptozotocin–induced diabetic rat model [2,5,17]. The fraction of the bladder wall composed of collagen was decreased and is consistent with other previous investigations that employed chemical measurement techniques from rat bladder homogenates [26]. The selective reduction of collagen prevalence may play a role in the enhanced bladder compliance and reduced stiffness known to occur in this organ during diabetes.

Diabetic neuropathy is a likely manifestation of urinary bladder dysfunction in diabetes. Diabetes related urinary bladder dysfunction has been commonly attributed at least in part to altered neuronal control and/or neuronal injury [8,27]. Previously, we have shown neuronal remodeling in streptozotocin–induced diabetic rat and mouse model to precede structural remodeling (3day vs. 2 weeks) using digital image analysis.
Using classical prejunctional neuronal markers viz TH (sympathetic) and VACHT (parasympathetic); we documented a decrease in VACHT staining and increase in tyrosine hydroxylase staining [4,28]. Recent evidence suggests that oxidative stress could be an important participant of diabetic neuropathy. We found neuronal remodeling in the Rac CA mice as shown by increase in TH immunoprevalance and decrease in VACHT immunoprevalance. Our data suggests that oxidative stress by itself can produce neuronal remodeling in the urinary bladder consistent with an apparent shift toward pathways favoring bladder capacitance and smooth muscle relaxation (decrease in VACHT staining and increase in tyrosine hydroxylase staining.

Originally Rac1 was described to play an important role on cytoskeleton in cells; however recently Rac1 has been associated in signaling events [14]. Particularly important is the role of Rac1 in regulating the mitogen activated protein kinase pathways (MAPK) especially the JNK and p38 pathway. MAPKs are a group of serine/threonine kinases that are involved in a diverse set of responses affecting cell fate, growth, differentiation, apoptosis and adaptation to stress [15,16]. However role of Rac1 dependent ROS formation in urinary bladder during diabetes has not been studied. We found region and cell specific activation of JNK and p38 pathways in the urinary bladder from the Rac CA mice and no changes in the ERK activation.
Apart from MAPKs, PI3K has been associated with Rac1 signaling pathway [29]. However the role of PI3K in Rac1 signaling has been controversial. There is data suggesting that PI3K is a downstream effector of Rac1; although most studies suggest that PI3K functions upstream of Rac1 [29]. Akt kinase has been characterized as the primary signal transducer of the PI3K pathway and has been used previously to study the relationship between the PI3K and Rac1. Akt/ protein kinase B is a serine threonine kinase and a downstream effector of PI3 kinase and has also been known to play a role in insulin actions [30-31]. Studies on the role of p-Akt in Type 2 diabetes suggest an increase [32], decrease [33] or no change [34] in the Akt status. Recently we have shown region specific alterations in MAPK and Akt pathways [5]. In Rac CA mice we observed increases in p-p-38 and p-Erk in urothelium during diabetes; these markers are consistent with cellular inflammation activation and growth in this region. In contrast we found that p-JNK and p-Akt were specifically increased in the smooth muscle layer; these responses are consistent with anti-apoptosis and proliferation signal pathways respectively. Thus, the general pattern of these signaling markers in each of these regions is consistent with structural and functional characteristics of the sites (e.g., urothelial inflammation and growth, smooth muscle proliferation and hypertrophy). In addition, our region specific findings illustrate the liabilities of using whole homogenates for analysis of signal pathways in such tissues.
In summary, we observed functional, structural and neuronal remodeling in the Rac1 over expressed mouse model, which mimic those of the well-established streptozotocin–induced diabetic rat model. Oxidative phenomenon may play a key role in bladder dysfunction and remodeling during diabetes.
FIGURE LEGENDS

Figure 7.1: Founder mice identified by Southern blot analysis of tail genomic DNA. The arrow indicates the position of human Rac CA (0.65 kb). In addition to 0.65 kb fragment corresponding to Rac CA transgene, the probe also hybridized to a 4.4 kb DNA fragment that has the size of the fragment of microinjected DNA used to generate the transgenic mice. RT-PCR analysis confirmed the selective transcription of Rac CA in smooth muscle, including blood vessels, spleen and intestine as shown in Fig 1B, while the transgene transcript was not detected in control mice (data not shown), nor in the heart, liver and brain of transgenic mice. Representative photomicrographs of p21-Rac1 and 3-nitrotyrosine (3-NT) immunostaining are shown in Figure 1C-D and 1F-G respectively for the control (CTL) and Rac1 overexpressed transgenic (Rac CA) mouse bladders. Digital imaging parameters derived from control and diabetic photomicrographs showed alterations in the p21-Rac1 and 3-NT immunoprevalance in the smooth muscle (closed circles) and urothelium (closed triangles). All data are mean ± S.E.M. *, statistically significant difference from control, P < 0.05.

Figure 7.2: General characteristics of control mice (CTL) and Rac1 overexpressed transgenic (Rac CA) mice are shown in Figure2. The black bars represent the control mice (CTL) while the white bar represents the Rac1 overexpressed transgenic (Rac CA) mice. No changes in urine output but increased bladder weight are evident. Representative cystometrogram of a
control (CTL) and Rac1 overexpressed transgenic (Rac CA) mouse bladder after Tyrode infusion are shown in Fig 2C. The two parameters derived from cystometrogram viz structural capacity and compliance are calculated for Rac1 overexpressed transgenic (Rac CA) and control (CTL) group. Structural capacity and compliance were significantly increased for Rac CA mouse bladders as compared to the controls. All data are mean ± S.E.M. *, statistically significant difference from control, P < 0.05.

**Figure 7.3**: Representative photomicrographs of Masson’s trichome stained transverse sections of bladders obtained from CTL (control) and Rac1 overexpressed transgenic (Rac CA) mice are shown in Fig 3A-B respectively. Mean morphological assessments shows significant increases in % smooth muscle area, decrease in % collagen and no change in urothelium for control (CTL) and Rac CA mice as shown in Fig 3C, 3D and 3E respectively. All data are mean ± S.E.M. *, statistically significant difference from control, P < 0.05.
**Figure 7.4:** Effect of oxidative stress on neuronal markers of innervation in mouse bladder are shown in Fig 4A-B and 4D-E for tyrosine hydroxylase (TH) and vesicular acetylcholine transporter (VAChT) at 400x magnification respectively. Digital imaging parameters derived from control (CTL) and Rac1 overexpressed transgenic (Rac CA) photomicrographs showed alterations in immunoprevalence of these markers; in smooth muscle for VAChT and in lamina propria for tyrosine hydroxylase. All data are mean ± S.E.M. * , statistically significant difference from control, P < 0.05.

**Figure 7.5:** Representative photomicrographs of p-ERK, p-JNK, p-p38 and p-Akt are shown in Figure 5A-B, 5D-E, 5G-H and 5J-K respectively for the control (CTL) and Rac1 overexpressed transgenic (Rac CA) mice bladders. The phosphorylated antibodies of MAPK pathway and p-Akt were widely distributed in all the urothelium (closed triangles) and smooth muscle (closed circles) of the control mouse urinary bladder. Cell and region specific alteration in the urothelium (closed triangles) and smooth muscle (closed circles) were observed for the Rac1 overexpressed transgenic (Rac CA) mice bladders vs. controls as measured by digital imaging technique. All data are mean ± S.E.M. * , statistically significant difference from control, P < 0.05.
Figure 7.1: Rac1 overexpression and increased oxidative stress in Rac CA bladder smooth muscle
Figure 7.2: Functional remodeling in the Rac CA bladders
Figure 7.3: Structural remodeling in the Rac CA bladders
Figure 7.4: Neuronal remodeling in the Rac CA bladders
Figure 7.5: Altered MAPK and Akt signaling pathways in the *Rac CA* bladders
REFERENCES


CHAPTER 8

CELL SPECIFIC CHANGES IN NITRIC OXIDE SYNTHASE, CYCLOOXYGENASES AND TLR4 DURING DIABETIC BLADDER REMODELING: ROLE OF RAC1
ABSTRACT

Urinary bladder dysfunction is a major complication in diabetes but the mechanisms involved are not well defined and the treatment strategies are limited. Previously using a STZ mouse model of diabetes we have shown structural, functional remodeling and increased oxidative stress in diabetic bladders. Also using the transgenic Rac1 over expressed mouse model we observed identical functional and structural remodeling in bladders as seen during diabetes suggesting role for oxidants in bladder dysfunction. Here we investigated the role of nitric oxide and cyclooxygenases as important mediators of inflammation during diabetes and increased oxidative stress using immunohistochemistry and digital imaging specifically in the smooth muscle and urothelium of the mouse bladders. We found urothelium specific NOS2 induction and urothelium and smooth muscle specific COX2 induction with no changes in the COX1 immunoprevalance either in the diabetic or Rac CA animals. We also observed a significant increase in the TLR4 immunoprevalance in the urothelium of the diabetic and Rac CA mouse bladders. These studies suggest inflammatory responses could be associated with diabetes and may occur through increased oxidative stress.
INTRODUCTION

Urinary bladder dysfunction is a major complication in diabetes and is associated with severe debilitating consequences such as frequent urination, enlarged bladders, urine retention and urinary tract infections [1-4]. These conditions although not typically life threatening are highly debilitating affecting the quality of life of individuals. Although these changes are well recognized, the mechanisms involved are not well defined, and treatment strategies are limited. Furthermore, as the incidence of diabetes continues to rise and as diabetic patients age, the importance and medical costs associated with this syndrome are likely to escalate.

Recently using the streptozotocin induced diabetic rat model we have shown structural and neuronal remodeling in the diabetic bladders and increased oxidative stress, as an important contributor to bladder dysfunctions during diabetes [2,3]. An important feature of our studies was the use of immunohistochemical techniques and digital image analysis, which allowed us to investigate changes in analyte prevalence in discrete tissue regions. Urothelium, lamina propria and smooth muscle, the three well-demarcated regions of the bladder were discretely investigated from the same bladder tissues, allowing detailed mapping of protein prevalence in control tissues and selective changes at these sites during diabetes. Our data showed that the primary increase in total bladder tissue mass occurred within the smooth muscle layer and urothelium [2,3]. This epithelial cell layer plays an important role in normal bladder function.
(particularly barrier and host-defense mechanisms), but few studies have investigated its properties during diabetes [4]. For this reason we were particularly interested in studying the changes in the smooth muscle vs. the urothelium during diabetes related bladder dysfunction.

Both nitric oxide and prostaglandins are important mediators of inflammation and other physiological and pathophysiological processes [5]. Previously various investigators have shown that the urothelium and/or smooth muscle is associated with increased inflammatory changes as shown by increased nitric oxide synthase 2 (NOS2) and/or cyclooxygenases (COX) expressions during bladder dysfunctions [6-13]. Using the STZ induced diabetic rat model we have shown urothelium specific NOS2 expression as early as 3 days in the diabetic rat bladders [3]. Here we investigated the role of NOS2 and cyclooxygenases 1 and 2 in the diabetic mouse bladders as important markers of inflammation in the bladder.
An important component of our investigation was to study the role of Toll-like receptor 4 in diabetic mouse bladders. Toll-like receptor 4 (TLR4) is the lipopolysaccharide (LPS) receptor and biochemical evidence suggests that TLR4 confers LPS responsiveness as determined by activation of NF-kB and expression of inducible cyclooxygenase 2 [14]. Previously, Backherd et al have shown that the TLR4 receptor is found to be present in the epithelial cells of the human bladder during urinary tract infections [15]. Since bladder dysfunction in diabetes is associated with urine retention and urinary tract infections we investigated the role of TLR4 signaling in the diabetic mouse bladder.

Recent studies suggest that oxidative stress might play an important role in inflammatory changes and TLR signaling pathways. Previously using a transgenic mouse model that selectively overexpresses Rac1 we have found increased structural, functional and neuronal remodeling similar to those seen during diabetes suggesting that oxidants alone in the absence of glucose or increased urine output might produce diabetic bladder remodeling [16]. Hence we studied the changes that occur in the inflammatory markers specifically the NOS2 and COX and TLR4 signaling in the Rac1 overexpressed animals to study the role of oxidants alone as important regulators of inflammation.
MATERIALS AND METHODS

Animals: All animal handling protocols were approved by the Institutional Laboratory Animal Care and Use Committee. Male CF1 mice (5 weeks old, 30±2g, Harlan, Indianapolis, IN) were dosed with streptozotocin (150mg/kg i.p., n=8, Sigma, St. Louis, MO) or vehicle (n=8) and were housed in a 12-hour light/dark facility with free access to food and water. Diabetic mice were sacrificed at 5 weeks post STZ dosing. Animals were sacrificed by pentobarbital overdose, at which time blood was collected by cardiac puncture, and urinary bladder tissues were rapidly isolated. Blood glucose was measured by a clinical blood glucose monitor (Glucometer Encore, Miles Inc, IN).

Generation of transgenic mice: The transgenic mice were generated as described previously. Briefly, the cDNA coding for the constitutively active mutated form of human Rac1 (Rac CA), with glycine 12 to valine substitution and myc-tagged (a gift from Alan Hall), was subcloned into the EcoR1 site of Bluescript II KS+, to create new restriction sites. Smp8 plasmid was cut with Xho1 and blunt ends produced by filling, then cut with Kpn1. The cDNA of human Rac CA including its polyadenylation tail were cloned within the blunt Xho1 and Kpn1 ends of the Smp-8 plasmid that contain a 3.6 kb segment of the 5'-region of the mouse smooth muscle (SM) α-actin promoter containing all the elements known to be required for optimal transcription. This promoter was used to induce selective overexpression of Rac CA in smooth muscle cells. A Sph1/Kpn1 fragment of this plasmid was isolated and microinjected into mouse fertilized...
eggs of FVB/N females as described earlier [16]. Single cell embryos derived from superovulated FVB/N females were used for the microinjection procedure. Surviving microinjected embryos were implanted into pseudopregnant CBA/B6 foster mothers. Founder mice were identified using southern blot analysis and PCR.

**Immunohistochemistry and Digital Image Analysis:** Urinary bladders were rapidly isolated, blotted dry and weighed. They were sectioned at the equatorial midline and allowed to equilibrate in buffer (e.g., with no intralumenal pressure). Following 48hr fixation in 10% buffered formalin, tissues were dehydrated and paraffin embedded using standard procedures, as previously described [2,3,16]. Immunohistochemical staining was performed using methods previously described [2,3,16], using a DAKO Autostaining system. Briefly, 5µM cross-sections of bladder tissue were dewaxed, rehydrated, and treated with preheated citrate buffer (pH 6.0) to recover the antigenicity. The endogenous peroxidase activity was blocked by the tissues with 3% hydrogen peroxide/methanol solution, followed by 10% goat serum / PBS blocking solution (Vector Lab, Burlingame, CA). The following primary antibodies were used: NOS2 (Rabbit polyclonal antibody, 1:1000 dilution, Transduction Laboratories, Lexington, KY); COX1 (Rabbit polyclonal antibody, 1:100, Cayman Chemical, Ann Arbor, MI); COX2 (Rabbit polyclonal antibody, 1:150, Cayman Chemical, Ann Arbor, MI); TLR4 (Goat polyclonal antibody, 1:100, Santacruz biotechnology, S’cruz, CA). All primary incubations were conducted for 1 hour; tissues were
then washed and exposed to biotinylated goat anti-rabbit secondary antibody (1:200) for rabbit polyclonal primary antibodies and in rabbit anti-goat secondary antibody (1:200) for goat polyclonal antibodies (Vector Labs, Burlingame, CA) for 20 minutes followed by Horseradish peroxidase-complex reagent (ABC Elite; Vector Labs, Burlingame, CA). Antigen prevalence and localization was visualized using diaminobenzidine (0.06% w/v) and counterstained with methyl green. Preliminary experiments were performed to verify the specificity of all antibodies used herein, by replacing primary antibody with pre-immune serum (isotypic staining controls, used for all antibodies) [2,3,16]. All staining controls showed no detectable immunoreactivity in any treatment group. Images were captured on a Polaroid DMC high-resolution digital camera, mounted on an Olympus research microscope. The urinary bladders were visualized using light microscopy (Olympus BX40) and photomicrographs were captured using a calibrated digital camera (Polaroid, Cambridge, MA) and transferred into research-based digital image analysis software (Image Pro Plus, Media Cybernetics, Silver Spring, MD). Relative immunoreactivities in cross-sectional bladder regions were determined via optical density measurements [2,3,16].
Software color segmentation was performed on the trichome stained slides, to measure the area of smooth muscle and urothelium present in the cross sections. Image analysis was conducted by investigators who were unaware of treatment group assignments. For all parameters measured intra-observer variability was less than 5% (coefficients of variation for 3 daily measurements) whereas the inter-observer variability was less than 7% (n=3 observers evaluating 6 bladders on three different days).

Statistics: Results were expressed as the mean ± SEM. Comparison between groups was performed by Student's t test using Sigma Stat 5.0 software from Jandel Scientific. Probability values of P < 0.05 were considered significant.
RESULTS

Similar to other studies, hyperglycemia was observed at 5 weeks post streptozotocin injection (5.85±0.22 vs. 16.82±2.01mM, p<0.05).

Shown in Fig 1 are representative photomicrographs of NOS2 (1A-B), COX1 (1D-E), COX2 (1G-H) and TLR4 (1J-K) at 400x magnification respectively and digital image analysis comparisons for average data are also shown. NOS2 (the inducible form of nitric oxide synthase) was absent in the control bladders but its induction was predominantly present in the urothelial layer of diabetic urinary bladders as compared to controls (Fig 1C). COX1 was expressed in both the tissue layers viz urothelium and smooth muscle of control mouse bladder. COX2, the inducible form of cyclooxygenase, was significantly increased in diabetic bladders in urothelium and smooth muscle. Digital imaging parameters showed no changes in the prevalence of COX1 in both the layers of urinary bladder during diabetes as compared to controls (Fig 1F). Statistically significant increases (P<0.05) in COX2 immunoprevalance were seen in urothelium and smooth muscle of diabetic mouse bladders vs. controls (Fig 1I). TLR4 immunoprevalance was significantly increased (P<0.05) in the urothelium of diabetic mouse bladders vs. controls (Fig 1L).

Shown in Fig 2 are representative photomicrographs of NOS2 (2A-B), COX1 (2D-E), COX2 (2G-H) and TLR4 (2J-K) at 400x magnification for the wild type and Rac1 overexpressed (Rac CA) animals. NOS 2, the inducible form of nitric oxide synthase, was predominantly present in urothelial layer of Rac CA
urinary bladders as compared to controls (Fig 2C). Similar to diabetic bladder studies, COX1 was expressed in both the urothelium and smooth muscle of the control bladders but was not changed in Rac CA mice (Fig 2F) while COX2 was significantly increased in the smooth muscle and urothelium in the transgenic animal bladders (Fig 2I). TLR4 showed faint staining in the urothelium of wild type controls but was significantly increased in the Rac CA mice (Fig 2L).
DISCUSSION

Urinary bladder dysfunction is a common complication and has been recognized for over hundred years. Bladder dysfunction is characterized by increased bladder volume and compliance and decreased tone. Additionally, bladder stasis predisposes patients to urinary tract infections, which in turn may accelerate the dysfunction [1-4]. While the phenomenon of bladder dysfunction is well recognized, its pathogenesis is not well understood and therapeutic strategies are limited. Improved mechanistic understanding is necessary for improvement of therapies.

Here we employed two different mouse models to compare the inflammatory changes that occur in the bladder using the STZ mouse model and the Rac1 overexpressed transgenic mouse model. Previously using the STZ mouse model we have shown altered functional, structural and neuronal remodeling that mimics the human and rat diabetes [17]. Also recently using the Rac1 overexpressed transgenic mouse model we have shown that oxidative stress via an inducible oxidase can produce diabetes bladder like changes in the absence of glucose and volume overload [16].

Here we employed nitric oxide synthase 2 and cyclooxygenases as important markers for inflammation and compared the smooth muscle to urothelium changes that occur in the bladder using immunohistochemistry as previously described [2,3,16,17]. The urinary bladder is composed of three well demarcated layers viz. urothelium, lamina propria and smooth muscle [18].
Previously we have shown that structural remodeling in the bladder is primarily driven by urothelium and smooth muscle [2,17]. For this reason we were particularly interested in studying the changes that occur in these two regions of the bladders.

Nitric oxide is synthesized by the enzyme family of nitric oxide synthases (NOS), which catalyze the conversion of L-arginine to L-citrulline. Three distinct isoforms exist, NOS1, NOS2 and NOS3 [10], and under normal physiologic conditions, NO is primarily produced in bladder tissue via the constitutive isoforms of NOS (NOS1 and NOS3), serving as a critical signal transduction agent for local relaxation of the smooth muscle via a cyclic GMP dependent pathway [10]. NOS2, the inducible form of nitric oxide, is up regulated mainly in the urothelial cells and inflammatory cells after experimental diabetes, urinary tract infections and bladder cancers [3,11-13]. Previously using the streptozotocin induced diabetic rat model we have shown urothelial specific NOS2 induction in diabetic bladder as early as 3 days suggesting inflammatory changes as early events during diabetes [3]. Similar to previous studies our data shows that NOS2 is upregulated predominantly in the urothelium of the diabetic and transgenic mouse bladders. Kuncewicz et al have shown that Rac1 physically interacts with NOS2 suggesting a role of Rac1 in NOS2 induction [19]. Our data shows urothelial specific NOS2 induction was evident in the Rac CA mouse bladders as compared to the controls.
Cyclooxygenases, one of the key enzymatic steps in prostaglandin synthesis might be important contributors in lower urinary tract function [5-9]. Cyclooxygenases exist in two distinct isoforms, COX1, the constitutive isoform which is expressed in most tissues at a fairly stable level and COX2, the inducible form which is increased in response to inflammation, stretch and cancers in bladders [5-9]. COX2 is also expressed constitutively in the normal bladders. Using immunohistochemistry with affinity purified antibodies against murine COX1 and COX2 that did not cross react with each other we found COX1 expression in the urothelium and smooth muscle of the control urinary mouse bladder. Also similar to other studies [6-9], COX2 expression was present in the smooth muscle and urothelium of the control mouse bladder. During diabetes we found no significant changes in the COX1 expression while COX2 was significantly increased in the urothelium and smooth muscle. COX2 expression is shown to be regulated by NOS2, both with positive and negative effects in the experimental systems and is highly dependent upon the cell types studied [5]. Most of the studies in the bladder have shown a positive effect of NO on COX2 activity or expression [5,6] suggesting a pathophysiological significance of the NO-COX cross-talk [5]. We also found a significant increase in NOS2 expression in the mouse urothelial cells while COX2 was up regulated in the urothelium and smooth muscle. These studies suggest that region and cell specific changes in the mouse bladder could be important. Interestingly we found similar changes in the diabetic mouse bladders and Rac CA mice (Urothelial specific NOS2
induction and COX2 in smooth muscle and urothelium) suggesting that Rac1 dependent ROS formation could be responsible for at least in part to mediate these effects.

An additional component of our investigation was to study the role of Toll-like receptors 4 (TLR4) signaling in diabetic bladders. Urinary bladder dysfunction in diabetes is associated with urine retention and severe urinary tract infections. [1-4] Uvelius et al have shown that approximately 40% of diabetic patients with bladder dysfunctions have urinary tract infections [20]. Mammalian Toll-like receptors are a growing family of molecules involved in innate immunity and are involved in Gram positive and Gram negative bacteria recognition and signaling mainly through the TLR2 and TLR4 receptors respectively [14]. TLR2/4 are shown to be involved in the activation of the transcription factor NF-Kb. Recent studies suggest that Rac1 could be an important participant in the TLR signaling pathway [21-22]. Previously, Backhed et al have shown that the TLR4 receptor is found to be present in the epithelial cells of the human bladder during urinary tract infections [15]. We investigated the role of TLR4 signaling in the diabetic mouse bladder during diabetes. We observed a significant increase in the TLR4 immunoprevalence in the urothelium of the diabetic and Rac CA mouse bladders. Further studies to define the role of TLR2/4 and its signaling in the bladder during diabetes are warranted.
FIGURE LEGENDS

Figure 8.1: Representative photomicrographs showing immunoprevalance for NOS2 (1A-B), COX1 (1D-E), COX2 (1G-H) and TLR4 (1J-K) at 400x magnification respectively for control (CTL) and 5 week diabetic (DIA) mouse bladders. Digital imaging parameters derived from control (CTL) and diabetic (DIA) mice bladders for urothelium (closed triangles) and smooth muscle (closed circles) are plotted as optical density (OD) for NOS2 (1C), COX1 (1F), COX2 (1I) and TLR4 (1L) respectively. All data are mean ± S.E.M. *, statistically significant difference from control, P < 0.05.

Figure 8.2: Representative photomicrographs showing immunoprevalance for NOS2 (2A-B), COX1 (2D-E), COX2 (12G-H) and TLR4 (2J-K) at 400x magnification respectively for wild type control (CTL) and Rac1 over expressed transgenic (Rac CA) mouse bladders. Digital imaging parameters derived from control (CTL) and Rac1 over expressed transgenic (Rac CA) mice bladders for urothelium (closed triangles) and smooth muscle (closed circles) are plotted as optical density (OD) for NOS2 (2C), COX1 (2F), COX2 (2I) and TLR4 (2L) respectively. All data are mean ± S.E.M. *, statistically significant difference from control, P < 0.05.
Figure 8.1: Region specific alterations in NOS2, COX and TLR4 in diabetic mouse bladders.
Figure 8.2: Region specific alterations in NOS2, COX and TLR4 immunoprevalence in Rac CA mice bladders.


18. Fowler CJ Bladder afferents and their role in the overactive bladder. Urology 2002: 59 (Supp 5A); 37-42


CHAPTER 9

PERINEURIOUM INFLAMMATION AND ALTERED CONNEXIN ISOFORM EXPRESSION DURING DIABETES RELATED PERIPHERAL NEUROPATHY.

This chapter has been published in Neuroscience Letters [2001 Apr 27; 303(1): 67-71] and is presented in the style appropriate for the journal. The authors of the manuscript are Deepali Anant Pitre, Jennifer L. Seifert, and John Anthony Bauer.
ABSTRACT

Diabetes related peripheral neuropathy involves both somatic and autonomic nerves and leads to an array of debilitating abnormalities. Mechanisms may include decreased neuronal conductance, reactive oxygen species, and decreased performance of the perineurium blood-nerve barrier. Here we studied the perineurium characteristics of the dorsal penile nerve in a rat model of diabetes related peripheral neuropathy. Immunohistochemistry showed extensive and perineurial cell-specific NOS2 staining in diabetic animals as compared to age matched controls (p<0.05); however no apparent difference in immunostaining pattern was observed for 3-nitrotyrosine (a stable biomarker of peroxynitrite formation). Significant reductions in connexins 32 and 26 were seen in the diabetic perineurium with no detectable levels of connexin 43 in either control or diabetic dorsal nerve. These data provide new evidence of perineurial cell inflammatory responses and altered gap junction protein expression during diabetes related neuropathies and suggests that strategies to protect this cell type may have therapeutic value.
INTRODUCTION

Diabetes mellitus currently afflicts more than 100 million people worldwide and it has been estimated that roughly 238 million will be affected by the year 2010 [19]. Diabetes related peripheral neuropathy is a common complication; its incidence is clearly related to duration of diabetes and is associated with increased morbidity and mortality in this patient group [7,19]. This neuropathy involves both somatic and autonomic nerves and leads to an array of debilitating abnormalities including sensory deficits, palsies, cardiovascular, gastrointestinal, and genitourinary dysfunction [7, 19]. While the phenomenon of diabetes related neurological injury is well recognized, its pathogenesis is not well understood and no therapies are effective in completely preventing the nerve damage. Furthermore, as the incidence of diabetes rises, and as patient’s age, the importance and associated medical costs of this syndrome are likely to escalate.

The mechanisms of peripheral nerve injury are not clear but nerve terminal destruction, decreased neuronal conductance, demyelination, and microvascular insufficiencies have all been implicated [7,19]. Recent reports also suggest that increased inflammatory responses and/or reactive oxygen species such as nitric oxide or peroxynitrite may participate in peripheral nerve injury and dysfunction [9,11,19]. An important feature of a healthy peripheral nerve is the maintenance of a blood-nerve barrier (analogous to a blood-brain barrier in the central nervous system), and perturbation of this barrier may contribute to diabetes related neuropathies. This barrier is provided by the perineurium, a
layer of tight-junctioned endothelium-like cells separated by layers of collagen [13]. While the perineurium was first described over 100 years ago, its potential significance in diabetes related neuropathies has more recently been suggested [6,8,17]. For example, increased perineurium permeability has been demonstrated in diabetic animal models, which may contribute to decreased peripheral nerve conductance and subsequent neuronal injury [17]. Pathological studies of perineurium in diabetic patients have also shown increased basement membrane thickening and perineurium cell necrosis [6, 8].

Here we studied the perineurium characteristics of the dorsal penile nerve in a well-established rat model of diabetes (streptozotocin induced model) [14]. Several previous reports have demonstrated that this animal preparation develops peripheral nerve deficits that mimic those observed in humans, including neurogenic erectile dysfunction [5] and associated decreases in penile nerve conductance [10]. We investigated the roles of NOS 2 (the inducible isoform of nitric oxide synthase) and peroxynitrite formation as markers of inflammatory events and protein oxidation.
An important component in the maintenance of the perineurium blood-nerve barrier is the expression of gap junction proteins, or connexins [2]. The connexin family of proteins (particularly isoforms 43, 32, and 26) have been implicated in perineurium cell function and diffusion barrier maintenance in vivo [1,12,16] but their relative expression in diabetes related peripheral neuropathy is not known. Therefore an additional aspect of our investigation was to test the hypothesis that the relative prevalence of these connexin isoforms is altered during diabetes related peripheral neuropathy.
MATERIALS AND METHODS

Animals: Male Sprague-Dawley rats (300-325g, Harlan, Indianapolis, IN), dosed with streptozotocin (65mg/kg i.p., n=8, Sigma, St. Louis, MO) or vehicle (n=6) via a single intraperitoneal injection were used for the study. All animal handling protocols were approved by The Ohio State University Animal Care and Use Committee. These animals were housed in a 12-hour light/dark facility and were allowed free access to food and water. At 8 weeks animals were sacrificed by pentobarbital overdose (75 mg/kg i.p., Abbott Laboratories, Chicago, IL) and blood and tissues were rapidly harvested. Blood glucose was determined by a clinical blood glucose monitor (Glucometer Encore, Miles Inc, IN). The penile shaft and glans was dissected, and a 5 mm proximal portion of the shaft was fixed immediately in 10% formalin. The sciatic nerve was also dissected from both thighs of one control rat and processed identically. After 48 hrs of fixation tissues were then dehydrated and embedded in paraffin as described previously [20].

Immunohistochemistry and digital imaging: Immunohistochemical staining was performed using methods previously described [20], using a DAKO Autostaining system. Briefly, 5µM cross-sections of cavernous tissue were dewaxed, rehydrated, and treated with preheated citrate buffer (pH 6.0) to recover the antigenicity. The endogenous peroxidase activity was blocked by the tissues with 3% hydrogen peroxide/ methanol solution, followed by 10% goat serum / PBS blocking solution (Vector Lab, Burlingame, CA). The following
primary antibodies were used in these investigations: NOS2 (Rabbit polyclonal antibody, 1:400 dilution, Upstate Biotechnology, Lake Placid, NY); 3NT (Rabbit polyclonal antibody, 1:200, Transduction Laboratories, Lexington, KY); Connexin 26, 32 and 43 (Rabbit polyclonal antibody, 1:100, Zymed Lab, San Francisco, CA). All primary incubations were conducted for 1 hour, tissues were then washed and exposed to biotinylated goat anti-rabbit secondary antibody (1:200) (Vector Labs, Burlingame, CA) for 20 minutes followed by Horseradish peroxidase-complex reagent (ABC Elite; Vector Labs, Burlingame, CA). Antigen prevalence and localization was visualized using diaminobenzidine (0.06% w/v) and counterstained with methyl green. Preliminary experiments were performed to verify the specificity of all antibodies used herein, by either pre-exposing primary antibody to excess antigen in solution (pre-adsorbed staining controls, used for NOS2 and 3NT), and/or by replacing primary antibody with pre-immune serum (isotypic staining controls, used for all other antibodies) [20]. All staining controls showed no detectable immunoreactivity in any treatment group.

Cavernous dorsal nerve bundles were visualized using light microscopy (Olympus BX40) and photomicrographs were captured using a calibrated digital camera (Polaroid, Cambridge, MA) and transferred into research-based digital image analysis software (Image Pro Plus, Media Cybernetics, Silver Spring, MD). For each tissue cross-section 5-6 nerve bundles were investigated. As previously described by others, we observed that several branches of the dorsal nerve were apparent in the proximal penile cross-sections we investigated, and that these
appeared to have a range of sizes (cross-sectional areas $3880\pm667 - 53071\pm118 \mu m^2$). In preliminary studies we found that the distribution of nerve sizes were not different in diabetic vs. control animals (NS) and all other digital imaging parameters were found to be unrelated to nerve size (non-parametric correlation analyses, NS). Thus all dorsal nerve branches available were included from each penile cross-section and were used in statistical analyses (e.g. a total of 75 dorsal nerve cross-sections were investigated in these studies). Perineurium layer thickness and total area was determined by careful tracing of the inner and outer interface (auto-trace feature of Image Pro Plus) and digital area integration. Relative immunoreactivity in this perineurium layer was determined via integrated optical density measurements. Image analysis was conducted by investigators who were unaware of treatment group assignments. For all parameters measured intra-observer variability was less than 5% (coefficients of variation for 3 daily measurements) whereas the inter-observer variability was less than 7% ($n=3$ observers evaluating 6 nerve bundles on three different days).

**Statistics:** All data are represented as mean ± SEM. Parameters between the diabetic and control group were evaluated for significance using the Students $t$ test (SigmaStat, Jandel Scientific, San Rafel, CA). In all cases, significance was defined as $P < 0.05$. 

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RESULTS

Blood glucose was measured in diabetic and age-matched control rats at 8 weeks. As expected, statistically significant increases in blood glucose were observed (520±28.19 vs. 120±8.49, p< 0.001). The general anatomical structure of the proximal penile cross-section investigated is shown in Figures 1A and 1B. Low power magnification (250x) demonstrates a high degree of homology relative to human tissue organization (Figure 1A and reference 18). At higher magnification (400x, Figure 1B) several branches of the dorsal nerve are found; each is composed of the outer epineurium, middle perineurium and inner endoneurium.

Representative photomicrographs of NOS2 and 3NT immunostaining are also shown in Figure 1 (at 500x magnification, NOS2, panels 1C and 1D; 3NT, panels 1E and 1F). Extensive and cell-specific NOS2 staining was observed in the perineurium of the dorsal nerve from diabetic animals as compared to age matched controls. In contrast, there were no apparent differences in immunostaining pattern for 3NT prevalence. Digital imaging parameters derived from control and diabetic photomicrographs are shown in Figure 2. Statistically significant increases in perineurium NOS2 were observed in diabetic vs. control (Figure 2A), whereas no statistically significant difference in 3NT immunoprevalence was observed. Statistically significant increases in perineurium wall thickness and total perineurium area were also observed in diabetic animals compared to age matched controls (Figures 2C and 2D).
Perineurium immunostaining (1000x magnification) for connexin gap junction proteins (connexins 43, 32 and 26) are shown in Figure 3. No detectable levels of connexin 43 were observed in either control or diabetic dorsal nerve, whereas detectable levels of this isoform were found in control sciatic nerve (thus, absence of this isoform was apparently not due to analytical difficulties). In contrast, connexins 32 and 26 were selectively expressed in control perineurial cells (panels 3A, 3B). Immunoreactivity for these two isoforms was also significantly reduced in diabetic perineurium relative to age matched control (panels 3E, 3F, and lower panels).
DISCUSSION

Neuropathy is a primary complication in diabetes mellitus, leading to significant morbidity and mortality and a huge economic burden for diabetes care [7,19]. Neurologic complications occur equally in Type I (insulin dependent) and Type II (non-insulin dependent) forms and additionally in various forms of acquired diabetes [7]. Recent studies show that these complications can be delayed, but not completely abrogated, by intensive glucose monitoring and control [4]. Thus, further understanding of underlying mechanisms involved are necessary to devise enhanced prevention strategies.

Autonomic neuropathy is a serious, common, and often overlooked component of diabetic neuropathy. Frequent clinical symptoms include erectile dysfunction, bladder and gastrointestinal dysfunction and orthostatic hypotension [19]. These autonomic abnormalities may be present in early stages of neuropathy, even before the onset of other sensory symptoms [7]. In addition to having effects on quality of life, diabetes autonomic neuropathy may participate in increased cardiovascular disease risk in this population (the number one cause of diabetes-related death) [4].

Interestingly, the streptozotocin-induced rat model of diabetes mimics nearly all clinical features of autonomic neuropathy, including decreased heart rate variability, reduced intestinal tone, bladder abnormalities, and erectile dysfunction [14]. Here we employed this Type I model of diabetes, focusing on characteristics of the penile dorsal nerve as a model of peripheral neuropathy. In
humans this nerve is directly involved in all aspects of the male sexual response (libido/sensation, erection and ejaculation), and reduced nerve conductance has been implicated in more than 80% of diabetic impotence cases [5]. Others have already demonstrated that erectile dysfunction also occurs in the rat streptozotocin model, and that this impairment is associated with reduced dorsal nerve conductance [10]. In addition the tissue regions we investigated had anatomical characteristics that were almost identical to human tissues [18].

Several recent studies have suggested that the perineurium layer is important in maintaining nerve function, and that in this layer is perturbed in animal and human diabetes [6,8,17]. Since diabetic neuropathies involve most or all types of peripheral nerve processes (sensory, motor, and autonomic), it is possible that alterations in the barrier layer participate in, or perhaps initiate, the decreased nerve conductance associated with diabetic neuropathy. An important challenge in this line of enquiry is the sparse abundance of perineurial cells in relevant tissues, making in situ detection methods necessary for reliable conclusions. Thus far the most common observation has been that of increased thickening of perineurium basement membrane, particularly in large fibers such as the sciatic nerve [6, 8].
We similarly detected increased perineurium thickness, which coincided with perineurium cell-specific prevalence of NOS2. Regulation of this NOS isoform takes place primarily at the transcriptional level, and it is known to be induced by a variety of inflammatory stimuli, particularly cytokines [9]. Thus, the increased and cell-specific expression of NOS2 is consistent with a perineurium inflammatory response in this setting.

How increased local production of NO (via NOS2 induction) may play a role in perineurium performance is uncertain, but either neuronal signaling effects or oxidative biochemistry may be involved. In addition to being a participant in host-defense reactions (primarily through the NOS2 isoform), other NOS isoforms play an important role as a modulator of erectile responses and neurotransmission [3]. Thus increased perineurium production of NO may alter nerve cells via its signaling properties. Alternatively, NO rapidly reacts with superoxide anion to form peroxynitrite, a potent cellular oxidant known to cause nitration of protein tyrosine residues, DNA damage, and apoptosis [15, 20]. In these studies we also assessed the in situ presence of nitrated protein tyrosyl residues and observed no statistically significant changes, suggesting that local peroxynitrite formation may not be involved in this setting. We and others have demonstrated that protein 3NT is a stable biomarker of in vivo peroxynitrite formation, but this biochemical pathway is not exclusively driven by NOS2 induction [15,20]. Further studies investigating perineurium specific NOS2 induction and its potential impact on nerve conductance are clearly warranted.
The connexin family of proteins is important for maintenance of gap junctions in virtually all cell types, but selective expression of certain isoforms in certain tissues and/or pathophysiologic conditions is apparent [2]. Several studies have demonstrated the importance of connexins in the maintenance of the blood-nerve barrier, and a specific mutation of connexin 32 in humans is strongly associated with a syndrome of peripheral neuropathy (X-linked Charcot-Marie-Tooth disease) [1]. Connexin 43 knockout mice show a vast reduction in the junctional conductance, dye coupling and their ability to propagate Ca2+ waves [16]. Nagaoka et al [12] recently showed that connexins 43, 32, and 26 are present in rat sciatic nerve, and that their relative expression is altered following nerve injury. We therefore looked for these isoforms in the dorsal nerve during diabetes. Interestingly, we found no evidence of the connexin 43 isoform in control tissues, but the 32 and 26 isoforms were readily apparent. This site-specific expression suggests that regional changes (rather than global) may play a role in neuropathy progression, and underscores the importance of selecting relevant tissue sites for mechanistic studies. We also observed significant reductions in connexins 32 and 26 in the dorsal nerve in this diabetic model. It is possible (or perhaps likely) that these reductions in gap junction protein expression play a role in decreased perineurium barrier function in this setting.
In summary, peripheral neuropathies are well-established complications observed in diabetes but the mechanisms involved and initiating events are not clear. Alteration of perineurium barrier function may be a unifying event in these settings. In a relevant site of peripheral nerve dysfunction we observed selective perineurium NOS2 induction and increased thickness as well as reduced expression of gap junction proteins, all of which may contribute to blood-nerve barrier leakage. These data provide new evidence of perineurium cell inflammatory responses during diabetes related neuropathies and suggest that strategies to protect this cell type may have therapeutic value.
FIGURE LEGENDS

Figure 9.1: Representative photomicrographs. (1A) Anatomical structure of proximal penile cross-section (control rat, 250x). U, urethra; CS, corpus spongiosum; CC, corpus cavernosum; DV, dorsal vein; DNT, dorsal nerve tract. (1B) Dorsal nerve tract (400x) showing several branches of the dorsal nerve. Each dorsal nerve branch is composed of outer (EPI, epineurium), middle (PERI, perineurium) and inner (ENDO, endoneurium) layers. (1C & 1D) Representative NOS2 immunohistochemistry on the perineurium of the dorsal nerve in the control and diabetic rats (500x). NOS2 immunoreactivity was observed in the perineurium of the dorsal nerve in diabetic rats only. (1E & 1F) Representative photomicrographs of the 3NT immunoreactivity. No difference in the immunoprevalence for 3NT in the control vs. diabetic was observed. CTRL: Control; DIAB: diabetic.

Figure 9.2: Digital image analysis of the perineurium immunostaining. Upper panels: 2A: Statistically significant increases in NOS2 prevalence (IOD, integrated optical density) was observed in diabetic vs. control (*, p<0.05) 2B: No statistically significant difference observed for 3NT immunoprevalence.
Lower panels: 2C & 2D: Perineurium layer thickness and total perineurium area was determined by careful tracing of the inner and outer interface and digital area integration (see methods). Both average thickness and cross sectional area were statistically increased in diabetic vs. control tissues. All data presented as mean ± SE, n=32 control, n=43 diabetes nerve tracts investigated (*, p < 0.05, Student t-test).

**Figure9.3:** Representative photomicrographs of connexin immunoreactivity (isoforms 43, 32 and 26, 1000x magnification). Tissue sections were immunostained with rabbit polyclonal connexin 43 (3A & 3D) connexin 32 (3B & 3E) and with connexin 26 (3C & 3F) respectively. Connexin 43 was not found to be present in the perineurium of the dorsal penile nerve in both the control and 8 week STZ-induced diabetic rats. The insert shows connexin 43 immunoprevalance in the sciatic nerve of the control rat thus confirming the absence of this isoform in the perineurium of the dorsal penile nerve in the rat. Connexin 32 (3B & 3E) immunoreactivity was apparent by the distinct brown colored immunostaining in the perineurium of the dorsal penile nerve in the control rats and decreased in 8 week STZ-induced diabetic rats. Similar distinct immunoreactivity was seen for connexin 26 in the perineurium of the dorsal penile nerve in the control rats as compared to the in 8 week STZ-induced diabetic rats. The lower panel shows the digital image analysis of connexin 32 (3F) and connexin 26 (3G) immunoprevalance expressed as integrated optical
density demonstrating positive staining for both connexin 32 and 26 in the perineurium of the dorsal penile nerve in the control rats as compared to the 8 week STZ-induced diabetic rats. All data presented as mean ± SE, n=32 control, n=43 diabetes nerve tracts investigated (*, p < 0.05, Student t-test).
**Figure 9.1:** Representative photomicrograph showing anatomical structure of penile cross-section and NOS2 and 3-NT immunostaining in control and diabetic rat perineurium.
Figure 9.2: Digital imaging analysis to study perineurium characteristics in control and diabetic rats.
Figure 9.3: Representative photomicrographs and digital image analysis showing Connexin 43, 32 and 26 immunoprevalance in control and diabetic rat perineurium.
REFERENCES


CHAPTER 10

CONCLUSIONS

Urinary bladder dysfunction is a common complication associated with diabetes and is known to affect nearly 80% of this population. While this syndrome is typically not life threatening, it is associated with several debilitating symptoms including insidious onset of voiding, progressive bladder paralysis, urinary retention and incomplete voiding. In addition to functional impairment of bladder smooth muscle (e.g. decreased tone and contractility); chronic diabetes is also associated with a general increase in bladder size and capacity. These changes are associated with a reduced quality of life as well as an increased risk of other conditions, including urinary tract infections and bladder cancers. These debilitating problems often progress throughout a patient’s lifespan and are irreversible.
While the phenomenon of diabetes related bladder dysfunction is well recognized, its pathogenesis is not well understood and no therapies are effective in completely preventing the damage. Furthermore, as the incidence of diabetes rises, and as patient’s age, the importance and associated medical costs of this syndrome are likely to escalate. Improved mechanistic understanding is necessary for the advancement of improved therapies.

While the bladder clearly increases in size and capacity during diabetes a detailed account of the morphological changes in all tissue layers over time has not been previously conducted. We studied the morphological characteristics of the bladder in the well-established streptozotocin induced model of diabetes. Since several previous reports have shown that bladder function is already altered in this rat model by 5 weeks post-streptozotocin, we focused on earlier time points to define morphological events prior up to this time and to gain mechanistic insight into the early events in this remodeling phenomenon. Here we used highly automated digital imaging methods to quantify general size and composition of bladder tissue at various times during diabetes progression. These parameters were objectively and accurately assessed and throughput was facilitated by the creation of an automated subroutine.
We observed a near doubling of total bladder tissue over the 5 weeks of investigation. Most notably time-dependent increases in smooth muscle and urothelium and reduction in collagen prevalence were observed at 2 weeks; changes consistent with the clinical symptoms of bladder dysfunction. These data demonstrate that automated digital imaging methods provided a comprehensive, reproducible, and convenient method for detailed morphometric analysis of bladder tissues.

Autonomic control of bladder expansion and contraction is predominantly governed by parasympathetic, sympathetic and nonadrenergic noncholinergic inputs. Given our findings of time dependent structural changes and an established view that neurological alterations play a major role in this setting we investigated the prevalence of the three major neurological controllers of bladder function. Tyrosine hydroxylase (TH) and vesicular acetylcholine transporter protein (VACHT) are well established and commonly employed prejunctional markers of sympathetic and parasympathetic fibers respectively. Nitric Oxide Synthase I (NOS1) is also recognized as an important controller of genitourinary function via its involvement in non-adrenergic non-cholinergic pathways. Although no detailed immunohistochemistry studies have been previously published, our findings of distribution are generally consistent with evidence from various species. The major finding of this work is that the markers of innervation in the bladders from diabetic animals changed before significant alterations in bladder mass were observed (3 days vs. 2 weeks). Interestingly, markers for the
parasympathetic, sympathetic, and NANC all changed by three days after the induction of diabetes. This suggests that substantial neuronal plasticity occurs in urinary bladder early in the diabetic state and implies the possibility that neuronal plasticity drives structural plasticity. Thus a process of “neuronal remodeling” was detected in this setting in which there was an apparent shift toward pathways favoring bladder capacitance and smooth muscle relaxation; these changes preceded changes in structure (2 weeks). Furthermore the changes observed were not consistent with a global loss of neurons or nerve terminals, as would be expected by neuropathic mechanisms. There was a significant increase in sympathetic terminals at blood vessels in the lamina propria and a decrease in parasympathetic terminals in the smooth muscle.

The central goal of my thesis project was to study oxidative mechanisms in diabetes related urinary bladder dysfunction. Growing evidence suggests that oxidative stress is a common occurrence associated with many of the complications of diabetes, although its role in diabetes related bladder disorders has not been investigated. Here we employed immunohistochemical methods to study the in vivo formation of 3-nitrotyrosine (biomarker of peroxynitrite and oxidative stress) (3NT) in control and diabetic bladders. Statistically significant increases in 3NT prevalence were observed in the urothelial and smooth muscle layers, with no change in lamina propria. Again, these changes were readily detectable at 3 days, suggesting that changes in reactive nitrogen species formation occurs in the very early phases of bladder alterations during diabetes.
Since the formation of peroxynitrite is bimolecular, increased prevalence of either nitric oxide or superoxide anion can promote protein nitration events. In addition to bladder-region-specific control levels, we observed selective increases in NOS isoforms in the early phase following diabetes induction. Again, the changes observed were dependent on the specific NOS isoform. These changes illustrate the independent control of each gene product in each layer of bladder tissue and suggest fundamentally different and independent responses of these cell types during the diabetic state. Importantly, each of the changes in NOS isoform expression observed occurred at only 3 days after diabetes induction - this time point precedes any evidence in structural or morphological changes we observed in our previous studies (at 2 and 5 weeks post STZ). Early biochemical changes after the induction of diabetes likely include increased NO signaling and protein tyrosine nitration. As these changes precede measures of significant modeling, it is likely that such NO signaling is involved in the process of remodeling.

We downsized our techniques to study bladder morphology and function (invitro cystometry) from STZ induced rat model to mouse model. We have successfully performed cystometric (functional) and morphological assessment of the mouse bladders and investigated changes at 5 weeks post STZ injection. Functional changes in the bladder during diabetes have been previously documented using bladder strips; however studies suggest that within a strip the orientation of the bundles is variable and difficult to predict. Hence, we employed invitro cystometry techniques to study changes in an intact bladder during
diabetes. Our data showed significant decrease in plateau pressure, increase in structural capacity and compliance; similar to human and rat studies described previously. We developed a streptozotocin induced mouse model that mimics the human and classical STZ induced rat model of diabetes as shown by functional, structural and neuronal remodeling. These studies suggest that the streptozotocin induced mouse model has a likely value in learning more about bladder biology and diabetes influences and has a great potential for disease studies and therapeutic evaluations.

Several settings of smooth muscle hypertrophy and proliferation have been linked to oxidant related signaling cascades, although such phenomena have not been detailed in the setting of diabetic bladder remodeling. For these reasons, and in light of some preliminary data in a rat model, herein we focused on potential oxidant related mechanisms in murine diabetic bladder. Previously data on structural remodeling in mouse bladders suggest region specific changes occur in urinary bladder specifically in smooth muscle and urothelium during diabetes and necessitate the distinct evaluation of these layers in diabetic bladder. Hence we studied the oxidant related signaling events in bladder by comparing the changes that take place in smooth muscle vs. urothelium.

Previous studies in the vascular smooth muscle suggest that activation of NADPH oxidase is an important source of oxidants especially superoxide. NADPH oxidase activation requires presence of active GTP bound Rac1. Since increased Rac1 is associated with increased superoxide formation; we
investigated the role of increased superoxide anion by measuring the levels of active Rac1 in the bladder during diabetes. Our data showed significant increases in smooth muscle specific Rac1 immunoprevalence in diabetic mouse bladders. Our data suggests that an inducible oxidase could be a potentially important source of oxidants in this setting and not just glucose chemistry thru AGEs, as described previously, be the only source of oxidants in diabetic bladders. Of note is the important observation that the regional distribution of protein nitration (urothelium and smooth muscle) did not generally follow the regional patterns for Rac1 (smooth muscle); which has been most suggested as an important contributor of superoxide formation in vivo. Thus, under the conditions of these studies, not superoxide availability alone but changes in NOS isoform availability are apparently involved as well.

Classically Rac1 has been shown to be a participant in host defense mechanisms, however; recently it has been shown to be an important contributor in signaling pathways mainly thru increased reactive oxygen/nitrogen species. Rac1 has been shown to interact with serine threonine kinases: mitogen activated protein kinases, phosphatidylinositol phosphate kinases (PIP kinases), phosphatidylinositol-3-kinase (PI3K), and protein kinase B (Akt/PKB). Here we investigated the role of these different kinases specifically the MAPKs and phosphorylated Akt/PKB to study the Rac1 driven oxidant-signaling pathways in a diabetic mouse bladder. We observed increases in p-p-38 and p-Erk in urothelium during diabetes; these markers are consistent with cellular
inflammation activation and growth in this region. In contrast we found that p-JNK and p-Akt were specifically increased in the smooth muscle layer; these responses are consistent with anti-apoptosis and proliferation signal pathways respectively. Thus, the general pattern of these signaling markers in each of these regions is consistent with structural and functional characteristics of the sites (e.g., urothelial inflammation and growth, smooth muscle proliferation and hypertrophy). In addition, our region specific findings illustrate the liabilities of using whole homogenates for analysis of signal pathways in such tissues. Later biochemical changes after the induction of diabetes (occurring after substantial remodeling is documented) suggest involvement of Rac1 and MAPK mediated signaling in either regulation or maintenance of remodeling. The changes that occur suggest that oxidative signaling pathways may be involved and that, especially in urothelium, signaling pathways having many elements in common with induction of inflammatory events are involved (even though there is no evidence for typical inflammatory responses).

In the light of increased Rac1 in the bladder smooth muscle during diabetes, we were interested in studying the effect of oxidative stress alone specifically in the smooth muscle of the bladder in absence of diabetes or glucose/volume related changes. Here we employed the Rac CA over expressed transgenic mouse model to study the role of oxidative stress alone on bladder morphology, function and neuronal remodeling in absence of diabetic condition. We have engineered (in collaboration) a transgenic mouse model that
overexpresses the constitutively active mutant of human Rac1 (Rac CA) in FVB/N mice, using mouse smooth muscle α-actin promoter containing all elements known to be required for optimal transcription of the SM α-actin gene. Using the SM α-actin we were able to express Rac1 specifically in bladder smooth muscle of the mice. We observed functional, structural and neuronal remodeling in the Rac1 CA mouse model, which mimics those of the well-established streptozotocin–induced diabetic rat model even in absence of glucose or volume overload. This data demonstrates that oxidative phenomenon may play a key role in bladder dysfunction and remodeling during diabetes, especially thru Rac1.

Previous studies of the remodeling induced by diabetes have conclusively shown that some aspects of the remodeling are simply a response to handling a large volume of urine and that some aspects result form metabolic events associated with diabetes. There is a well-established model for studying volume-induced changes; however, there has been no model for studying changes occurring independently of increased urine volume. The Rac1 overexpression mouse model in which remodeling occurs in the absence of volume changes, making this the first model for studying non-volume induced remodeling.

Our data clearly suggests that oxidative stress is not only an important participant in bladder dysfunction but is involved early and is associated with most of the functional, structural and neuronal remodeling associated with diabetic bladder dysfunction. These data suggests that oxidant related events
might play an important role in diabetic bladder remodeling and that Rac1 signaling pathways are involved. The Rac1 overexpression mouse model confirms that substantial bladder remodeling occurs in response to increased activity of Rac1 and MAPK mediated signaling, independently of changes in volume of urine.

Further investigations of oxidant signaling events and role of Rac1 during diabetes related bladder may provide new insight regarding adaptive and maladaptive processes in bladder physiology and enhanced opportunities for improved therapeutic strategies for an important diabetic complication.


Beckman JS. Oxidative damage and tyrosine nitration from peroxynitrite. Chemical Research in Toxicology 1996: 9; 836-44.


Biochimie 2001: 83; 301-10.


Davies KJ. Degradation of oxidized proteins by the 20S proteasome.


346


Freeman BA, Free radical chemistry of nitric oxide, Chest 1994: 105(3); 79S-83S.


Lewis SA. Everything you wanted to know about the bladder epithelium but were afraid to ask. Am J Physiol Renal Physiol. 2000;278(6):F867-74.


Longhurst PA, Kauer J, Levin RM. The ability of insulin treatment to reverse or prevent the changes in urinary bladder function caused by streptozotocin-induced diabetes mellitus. Gen Pharmacol. 1991;22(2):305-11.


Perez-Sala D and Lamas S. Regulation of cyclooxygenase-2 expression by nitric oxide in cells. Antioxid Redox Signal 2001: 3(2); 231-48.


Poladia DP and Bauer JA: Early changes during diabetes related bladder remodeling: cell specific changes in nitric oxide synthases, reactive nitrogen species formation, and ubiquitylation. Diabetes metabolism research and reviews, in press.


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APPENDIX A

NITRIC OXIDE IN THE URINARY BLADDER: INVOLVEMENT OF AUTO-RELAXATION

This chapter has been submitted to Nitric Oxide and is presented in the style appropriate for the journal.
The mammalian urinary bladder is composed of three distinct regions (urothelial lining, lamina propria and smooth muscle) and is functionally regulated by both local and central autonomic controllers [1-2]. As a vesicular organ, the bladder also possesses several short term and long-term adaptive capabilities (e.g. autorelaxation, dilation, hypertrophy) stimulated by urinary filling, other stimuli, or disease states. Although not typically life threatening, bladder dysfunction is highly debilitating and represents significant health care costs, and the incidence of these conditions will to increase as the U.S. population ages.

Autonomic neurotransmission of the urinary bladder is primarily composed of parasympathetic cholinergic and sympathetic nor-adrenergic innervation. Sympathetic nerve activity results in stimulation of the beta-adrenergic receptors in the bladder body causing relaxation. Interactions with the alpha-adrenergic receptors in bladder neck and urethra results in contraction and closure of smooth muscle sphincter mechanism. This, in combination with auto-relaxation of detrusor muscle results in urine storage by the bladder, whereas parasympathetic nerve activity causes coordinated contraction of the bladder body and subsequent emptying [2].

Within the last decade it has been shown that nitric oxide (NO) plays several important roles in the lower genitourinary tract [3-4]. For example, regulation of cavernous smooth muscle tone and blood flow, modulation of sphincter tone, and host defense mechanisms have all been well-established
effects of locally produced NO via three distinct NO synthase (NOS) isoforms [3-4]. In contrast, the actual role of NO in bladder detrusor muscle function has been less well established and remains somewhat controversial. For example, some investigators suggest a role for NO in urethral relaxation but not in detrusor; while others observed partial relaxation of the detrusor strips by nitric oxide [3-6]. One potential explanation for these inconsistencies is the widely variant methods employed; many of the previous studies have used bladder strips, but within a strip the orientation of the bundles is variable and difficult to predict [3-6]. Of note is the observation that NOS1 deficient mice (transgenic knockouts) have severe bladder abnormalities, suggesting that this isoform is particularly important for normal bladder function [7].

Here we employed cystometry techniques to evaluate pressure-volume relationships in the isolated rat bladder (e.g. in the absence of neuronal control), as previously described [8]. Shown in Figure 1A is a typical pressure-volume relationship during infusion of Tyrode solution at a slow flow rate (25µL/min), demonstrating an initial rise in intravesicular pressure, a plateau during continuous filling (an auto-relaxation response), and an eventual rise in pressure as volume capacity is reached. In a separate group of bladders a faster flow rate (50µL/min or higher) caused an elevated response pattern, with a transient peak in pressure during the first 20% of the filling time (Figure 1B). This pattern was consistently observed at higher flow rates and is consistent with an altered response for auto-relaxation. Shown in Figure 1C is the pressure volume
relationship at the low flow rate in the presence of the non-selective NOS inhibitor N-nitro-L-arginine (LNA, 250µM). Under these conditions we consistently observed a transient peak in pressure during the initial onset of infusion. This data suggests the role of nitric oxide on the initial phase of bladder auto relaxation. No changes were seen in plateau pressure and structural capacity (defined as volume at twice plateau pressure, [9]) was not altered. All of these responses were also observed in mouse tissues (data not shown). These observations suggest that detrusor tissue NO production may play a subtle but important role in bladder accommodation to increased volume and this is selectively involved in the initiation event mediating an initial component of the bladder auto relaxation response. In contrast it apparently does not participate in maintained relaxation during filling. Further studies to identify the initial mechanoreceptor stimuli responsible for this phenomenon and signaling mechanisms involved are warranted.
Figure Legend

Figure A.1: Representative cystometrograms showing pressure to volume relationships for control rat bladders with LFR (low flow rate 25µL/min), HFR (high flow rate 50µL/min) and LNA (low flow rate 25µL/min + 250µL N-nitro-L-arginine) are shown in Fig 1A, 1B and 1C respectively. The lower panel shows altered peak pressure, plateau pressure and structural capacity for LFR (low flow rate 25µL/min), HFR (high flow rate 50µL/min) and LNA (low flow rate 25µL/min + 250µL N-nitro-L-arginine) in Fig 1D, 1E and 1F respectively.
Figure A.1: Representative cystometrograms showing pressure to volume relationships in control rat bladders (upper panel) and altered peak pressure, plateau pressure and structural capacity (lower panel).
REFERENCES


APPENDIX B

FUNCTIONAL ALTERATIONS IN URINARY BLADDER DURING DIABETES:
EFFECT OF SUCROSE
ABSTRACT

Urinary bladder dysfunction is common complication in diabetes but mechanisms involved are undefined. The use of murine models provides opportunity to utilize transgenic technologies for bladder research. Here we investigated functional aspects of bladder in a mouse model of diabetes. Mice were divided into four groups, control water dosed with vehicle and administered water for 15 weeks, control sugar dosed with vehicle and administered 5% sucrose in drinking water for 15 weeks, diabetic water dosed with streptozotocin (75mg/kg i.p.) and diabetic sugar dosed with streptozotocin (75mg/kg i.p.) and administered 5% sucrose in drinking water for 15 weeks. Increases in blood glucose, urine glucose and total urine output were observed in the diabetic sugar group as compared to others. In-vitro cystometry showed significant increase in bladder capacity and compliance in diabetic vs. controls. Significant increase in adrenergic functional responsiveness was also observed. No changes were seen in the cholinergic responsiveness. These studies demonstrate that mouse model of low dose STZ induced and fed on sucrose diet exhibits important urinary bladder remodeling.
INTRODUCTION

Diabetes mellitus is a costly disease with an incidence of 100 million people worldwide and has been estimated to affect around 238 millions by the year 2010 [1]. Type 2 or non-insulin dependent diabetes mellitus is characterized by lack of insulin secretion and/or insulin resistance, which in turn results in hyperglycemia and other complications [2]. Urinary bladder dysfunction is a common disorder in diabetes [3]. Although not typically life threatening, bladder dysfunction is associated with severe debilitating consequences such as increased urinary output, increased frequency of micturition, bladder paralysis, incomplete voiding and urinary retention [3]. Despite their occurrence, the mechanisms of diabetes related bladder dysfunctions are not well understood and treatment strategies are limited. Thus further understanding of the underlying mechanisms is necessary to devise enhanced prevention strategies.

Previously we have developed a Type1 mouse model of diabetes using a higher dose (150 mg/kg) of streptozotocin and shown that this model recapitulates most of the changes seen in human diabetic bladders [4,5]. Non-insulin dependent diabetes mellitus (NIDDM) and related bladder dysfunctions [6] have been studied extensively using genetic models of obesity like the ob/ob and db/db mice [7]. However, these mice models differ from the patients with NIDDM in many respects [8]. Previous studies have used streptozotocin, which was administered to neonatal rats at birth or 2-5 days later to produce animal models of NIDDM [9,10]. Recently, Luo et al have shown that the use of nongenetic
mouse models by administering a relatively low dose of streptozotocin and fructose diet develop characteristics similar to NIDDM patients [8]. Role of sucrose/fructose fed diets in the literature is controversial [11-13]. Consumption of a moderate amount of sucrose has been shown to be associated with hyperglycemia, hyperinsulinemia, hypercholesterolemia etc. suggesting that diets rich in sucrose should be avoided in patients with NIDDM [12,13].

Sucrose (5%) in drinking water has been extensively used in urinary bladder dysfunctions in diabetes as diuresis control model [14,15]. However, it is important to note that polyuria alone does not necessarily mimic bladder changes observed in the STZ model. For example, Eika et al [14] have previously shown that sucrose fed rats have pressure-volume responses that are not different from controls, whereas STZ treated animals have significant passive function changes. Kudlacz et al [15] also showed that changes in nerve densities were unaltered in the sucrose-fed model, whereas striking changes were observed in STZ diabetic rats. Furthermore, we have recently observed that mice selectively over-expressing pro-oxidant genes in bladder smooth muscle develop organ remodeling phenomena identical to those observed here in the absence of polyuria or glucose changes [16]. Collectively, these findings suggest that bladder remodeling is not exclusively dictated by increased urine output. Therefore, while many of the changes in the bladder are associated with polyuria, there are apparently distinct functional and morphological changes related to diabetes that are different from a simple diuresis model.
Here we investigated the effect of a low dose of streptozotocin in combination with sucrose and studied its effects on changes in functional parameters in the mouse bladder. Similar to the studies by Luo et al [8] we administered a low dose of streptozotocin (75mg/kg) and then administered 5% sucrose water and studied functional changes in the bladder in four different groups described below.
MATERIALS AND METHODS

Animals: All animal handling protocols were approved by the Institutional Laboratory Animal Care and Use Committee. Male CF1 mice (5 weeks old, 30±2g, Harlan, Indianapolis, IN) were divided into four groups, control water (n=18) dosed with vehicle and administered water for 15 weeks, control sugar (n=18) dosed with vehicle and administered 5% sucrose in drinking water for 15 weeks, diabetic water (n=18) dosed with streptozotocin (75mg/kg i.p., Sigma, St. Louis, MO) or diabetic sugar (n=18) dosed with streptozotocin (75mg/kg i.p., Sigma, St. Louis, MO) and administered 5% sucrose in drinking water for 15 weeks. The animals were housed in a 12-hour light/dark facility with free access to food (chow-fed) and water or 5% sucrose. All the animals were sacrificed at 15 weeks post STZ dosing. Animals were sacrificed by pentobarbital overdose, at which time blood was collected by cardiac puncture, and urinary bladder tissues were rapidly isolated. Blood glucose was measured by a clinical blood glucose monitor (Glucometer Encore, Miles Inc, IN).

Urine output: The animals were placed in stainless steel metabolic cages for 24 hours. They were deprived of food and given free access to water. The urine was collected after 24 hours and the volume was determined. Urine glucose was determined by the Clinistix Reagent Strips for Urinalysis (Bayer Corp., Elkhart, IN). The amount of water/sugar water consumed by the animals over 24 hours was measured. The food consumed by each animal was measured per day.
**Invitro-cystometry:** The animals were euthanized by an overdose of pentobarbital sodium (75 mg/kg i.p., Abbott Laboratories, Chicago, IL). A midline incision through the lower abdominal wall was made to expose the urinary bladder. The ureters were ligated with a suture and the urethra was isolated and cut distal to the bladder body (~ 5 mm). The bladder was gently emptied and cannulated with PE 10 tubing and infused with Tyrode solution. The rate of Tyrode infusion was 0.005 ml/min. The bladder was mounted on a tissue bath containing warm Tyrode solution (37°C) and oxygenated with 95% O₂ and 5% CO₂. The PE10 tubing was attached to a 3-way stopcock directly connected to a pressure transducer. The third connection of the stopcock was attached to an infusion pump (Harvard Apparatus) via a tygon tubing (3mm i.d.). After adjusting the bladder base to the same level of the pressure transducer, the bladder was allowed to equilibrate for 30 minutes. During this time the bladder was washed three times with warm Tyrode solution.

**Tyrode infusion:** The stopcock was open to the pressure transducer, bladder and infusion pump during the equilibration period and infusion. Following infusion, the intravesicular pressure was recorded in response to a continuous infusion of Tyrode solution into the bladder, until a pressure twice the plateau pressure was reached. The bladder was then emptied and allowed to equilibrate for 30-45 minutes following intermittent washings with Tyrode solution. The bladder was again infused and the above procedure will be repeated for 2 times. The initial run (run1) was to stabilize the bladder under the invitro conditions. The
volume of Tyrode infused into the bladder that correlated with twice the plateau pressure was defined as the structural capacity of the bladder. The pressure–volume relationship (cystometrogram) was generated for each bladder. The structural capacity and compliance was measured for the normal water and sugar administered control and diabetic mice bladders. Concentration of Tyrode solution used was NaCl 125 mM, KCl 2.7 mM, NaH₂PO₄ 0.4 mM, CaCl₂ 1.8 mM, NaHCO₃ 23.8 mM and Glucose 11 mM.

**Drug infusion:** The bladders were then divided into three groups (n=6, control water, diabetic water, control sugar and diabetic sugar each). Each group was treated with either Bethanechol (BTH) 100µM, Isoproterenol (ISO) 100µM or N-nitro-L-arginine (NO-ARG) 250µM, incubated for 40 mins, in the organ bath and also infused into the bladder, and cystometrograms were plotted. The cystometrogram was then divided into four parameters viz peak pressure, plateau pressure: pressure at which the bladder maintains a relaxed tone, structural capacity: volume at twice the plateau pressure and compliance: innate ability of the bladder to expand to capacity with minimal changes in pressure. [17] We also measured the sensitivity of the bladders to bethanechol or isoproterenol measured at changes in pressure at the structural capacity.

**Statistics:** Results were expressed as the mean ± SEM. Comparison between groups was performed by Student’s t test using Sigma Stat 5.0 software from Jandel Scientific. Probability values of P < 0.05 were considered significant.
RESULTS

General characteristic changes associated with diabetes and sugar is shown in Figure 1. Hyperglycemia (Fig 1A) was evident and significantly higher in the diabetic sugar group as compared to any other group. Glucosuria (Fig 1B) was seen in the control sugar and diabetic sugar group and significantly higher urine glucose was seen in the diabetic sugar as compared to the control sugar group. Urine output (Fig 1C) and water consumed (Fig 1E) by the animals was significantly higher in the sugar treated controls and diabetics however the diabetic sugar group was significantly higher than the control sugar animals. Similarly renal clearance (Fig 1D), measure of the concentration of glucose in urine to that in blood, was significantly increased in the diabetic sugar mice as compared to the controls. The body weight of the animals was not significantly different through out the study (data not shown) and the food consumed was actually decreased for the diabetic sugar group as compared to diabetic water group.

Representative cystometrogram with Tyrode infusion for the control water (solid curve) and control sugar mice (dotted curve) is shown in Figure 2A while that for the diabetic water (solid curve) and diabetic sugar mice (dotted curve) is shown in Figure 2B. The cystometrogram was then used to derive three parameters viz. plateau pressure, structural capacity and bladder compliance for the control and diabetic water/sugar animals. Plateau pressure, the volumetric filling phase of the bladder, was not significantly altered though the diabetic sugar
group showed a decreasing trend as compared to the control sugar group (Fig 2C). Figure 2D shows the changes in the structural capacity, measured at twice the plateau pressure. The structural capacity was significantly increased in the diabetic and control sugar groups as compared to their respective controls. The compliance (Fig2E) was however significantly increased only in the diabetic sugar group as compared to the diabetic water animals. After cystometry the bladders were highly distended and the dried bladder weight to body weight was increased significantly in the diabetic and control sugar groups as compared to their controls (Fig 2F).

Representative cystometrograms before (solid curve) and after the infusion of bethanechol (100 µM) (dotted curve) are shown for the control water, control sugar, diabetic water and diabetic sugar treated mice in Figure 3A, 3B, 3C and 3D respectively. These pressure-volume relationships were then used to measure the compliance, which was determined for the control and diabetic water/sugar group before and after the administration of bethanechol.
Administration of bethanechol resulted in a profuse contraction of the bladders resulting in absence of the plateau formation and continuous increase in the pressure as shown in figure 3A, 3B, 3C and 3D in dotted lines. The sensitivity to bethanechol was calculated as difference in compliance at capacity before and after the administration of bethanechol in all four groups as shown previously [4]. Figure 3E and 3F show decrease in compliance in all four groups upon bethanechol administration with no significant change in bethanechol response in either group compared.

Shown in Figure 4A, 4B, 4C and 4D are representative cystometrograms for the control water, control sugar, diabetic water and diabetic sugar groups respectively before (solid curve) and after the administration of Isoproterenol (100µM) (dotted curve). Similar to studies shown above the compliance and difference in compliance was plotted for the four groups. Figure 4E shows no change in the compliance but significant increase in sensitivity to isoproterenol in the diabetic sugar group was seen as compared to the control sugar group (Figure 4F).

Representative cystometrograms before (solid curve) and after the infusion of N-nitro-L-arginine (250 µM) (dotted curve) are shown for the control water, control sugar, diabetic water and diabetic sugar treated mice in Figure 5A, 5B, 5C and 5D respectively. Two parameters viz. peak pressure and compliance were determined for control and diabetic group before and after N-nitro-L-arginine administration. In all control bladders a transient increase in vesicular
pressure was observed in the presence of N-nitro-L-arginine, whereas the remaining portion of the cystometrogram was unaltered. In diabetic mice, this initial peak was absent. Figure 5E shows increased peak pressure in control group after treatment with N-nitro-L-arginine. No changes in the compliance were observed (Fig 5F).
DISCUSSION

Urinary bladder dysfunction is a common complication in diabetes and is known to be associated with severe debilitating consequences including frequent micturition, atonic bladder, increased bladder capacity, urinary retention, urinary tract infections and even bladder cancers [3,5,14-15,18-23]. Diabetes associated urinary bladder dysfunction is a well recognized clinical problem but the mechanisms involved are poorly understood and optimal therapeutic strategies are not well defined.

Type2 diabetes or NIDDM is the non-insulin dependent diabetes mellitus, which is characterized by increased insulin resistance or decreased ability of the cells to secret insulin. NIDDM has been linked to obesity, normal glucose tolerance and hyperglycemia that occurs later after the compensatory response fails [2,7-13]. Genetic rodent models have been commonly employed to study NIDDM and the complications associated with it [7]. Studies on the ob/ob and db/db models, the most frequently used to study NIDDM pathophysiology, differ from the NIDDM patients in the extreme degree of obesity, extreme degree of hyperinsulinemia found in these animal models [7,8]. Recent studies have used the non-genetic mouse models wherein a low dose of streptozotocin is administered followed by a diet rich in fructose [8-10]. Luo et al have shown that a low dose (100 mg/kg) of streptozotocin and a fructose rich diet produces changes similar to those seen during NIDDM, making it a good model to study the pathophysiology of NIDDM [8].
Growing evidence suggests that increased intake of sugar results in increased incidence of type2 diabetes mellitus and its complications especially the cardiovascular complications [12-13]. Previous studies have shown that not short term but long-term consumption of sucrose/fructose can result in hyperglycemia and other conditions associated with NIDDM [12]. Sucrose (5%) in drinking water has been used extensively as a diuresis control model to study bladder changes during diabetes [14,15]. Eika et al [14] have previously shown that sucrose fed rats have pressure-volume responses that are not different from controls, whereas STZ treated animals have significant passive function changes. Kudlac et al [15] also showed that changes in nerve densities were unaltered in the sucrose-fed model, whereas striking changes were observed in STZ diabetic rats. Furthermore, we have recently observed that mice selectively over-expressing pro-oxidant genes in bladder smooth muscle develop organ remodeling phenomena identical to those observed here in the absence of polyuria or glucose changes [16]. However, the role of a low dose of STZ and sucrose administration for a long-term study in the urinary bladder of a mouse has not been investigated before.

Here we employed a CF1 mouse model which was administered a low dose (75 mg/kg) of streptozotocin, which would result in hyperglycemia in the sucrose fed animals (Fig 1A) but not produce significant hyperglycemia in the rodents fed with chow and water. We divided the animals into four different groups viz control water (C+W), control sugar (C+S), diabetic water (D+W) and
diabetic sugar (D+S) to study and compare diuresis, control and diabetic changes in the urinary bladders of these animals at 15 weeks after on 5% sucrose water. We performed a detailed study of the bladder function changes in this animal model using bethanechol (cholinergic agonist), isoproterenol (adrenergic agonist) and N-nitro-L-arginine (non-selective nitric oxide inhibitor) as important regulators of bladder function.

Functional changes in the bladder during diabetes have been previously documented using bladder strips [19,20]; however studies suggest that within a strip the orientation of the bundles is variable and difficult to predict. Hence, we employed invitro cystometry techniques to study changes in an intact bladder during diabetes. Our data showed increase in structural capacity and compliance, similar to human and rat studies described previously [3, 5, 14, 15, 19, 20, 22, 23]. We saw a decreasing trend in the plateau pressure however didn’t see significant decreases as classically described.

Autonomic control of bladder expansion and contraction is predominantly governed by parasympathetic, and sympathetic inputs [21]. Stimulation of local parasympathetic nerve activity causes a highly coordinated contraction of the bladder body and subsequent emptying of the bladder, whereas sympathetic activation enhances detrusor muscle relaxation [21]. Previous studies using either cystometry or bladder strips have shown significant changes in the autonomic innervation of the bladder during diabetes [3, 5, 14, 15, 19, 20, 22, 23] however there is no consensus since studies have reported increase, decrease
or no effect to various muscarinic and adrenergic agonists [19,22,23]. Our data is consistent that changes occur in the autonomic innervation of the bladder during diabetes. Administration of isoproterenol (100µM) produced significant increase in isoproterenol sensitivity in the diabetic sugar bladders as compared to the controls. However no significant changes in sensitivity to bethanechol were seen in any of the groups.

Apart from the autonomic control, nitric oxide is now recognized as an important regulator of many physiological processes, including functional aspects of the lower urinary tract [24,25]. Nitric oxide is synthesized by the enzyme family of nitric oxide synthases (NOS), which catalyze the conversion of L-arginine to L-citrulline. Three distinct isoforms exist, NOS1, NOS2 and NOS3 [24,25], and under normal physiologic conditions, NO is primarily produced in bladder tissue via the constitutive isoforms of NOS (NOS1 and NOS3), serving as a critical signal transduction agent for local relaxation of the smooth muscle via a cyclic GMP dependent pathway [24]. Of note is the observation that NOS1 deficient mice (transgenic knockouts) have severe bladder abnormalities, suggesting that this isoform is particularly important for normal bladder function [26]. Apparently the role of nitric oxide in the bladder detrusor has been questionable; with some reports suggesting role of nitric oxide in the urethra but not in the detrusor while others showing partial mediation of relaxation of the detrusor strips by nitric oxide [27-28]. Here we employed invitro cystometry techniques to study the functional changes in the mouse bladder. Previously, using type1 mouse model
we have shown altered responses to autonomic agents in the bladder during diabetes suggesting a role of nitric oxide in the initial and relaxation maintenance phase of the bladder [29]. Using the non-specific NOS inhibitor, N-nitro-L-arginine (250µM) we found significant increases in the peak pressure suggesting that in control mouse bladders nitric oxide might be involved only in the initial phase of bladder relaxation; similar to what we had seen before [4].

In summary diabetes associated urinary bladder dysfunction is a well-recognized clinical problem but the mechanisms involved are poorly understood and optimal therapeutic strategies are not well defined. Increased intake of sugar results in increased incidence of type2 diabetes mellitus and its complications. Here we studied a mouse model administered with low dose streptozotocin and 5% sucrose water for 15 weeks. We found significant changes in the diabetic sugar group as compared to the controls viz hyperglycemia, glucosuria, increased capacity, compliance and increased sensitivity to isoproterenol. These studies suggest that the low dose STZ with sucrose is a good model to study bladder dysfunctions associated with NIDDM. Further studies on the morphological and neuronal alterations are warranted.
FIGURE LEGENDS

Figure B.1: General characteristics of the control (closed circles) and diabetic (closed squares) mice administered with water (BAS) and 5% sucrose (SUG) are shown in Figure 1. Hyperglycemia, glucosuria, increased renal clearance, increased urine output, increased water consumption are evident. All data are mean ± S.E.M. *, statistically significant difference in diabetic water vs. diabetic sugar group, P < 0.05; ^, statistically significant difference in control water vs. control sugar group, P < 0.05; #, statistically significant difference in control water vs. diabetic water group, P < 0.05; +, statistically significant difference in control sugar vs. diabetic sugar group, P < 0.05.

Figure B.2: Representative cystometrogram of a control (left) and diabetic (right) mouse bladder after Tyrode infusion are shown in Fig 2. The black curve represents the run in control water (2A) and diabetic water (2B) group while the dotted curve is Tyrode infusion in the control sugar (2A) and diabetic sugar (2B) group. The three parameters derived from the cystometrogram viz plateau pressure, structural capacity and compliance are calculated for the diabetic (closed squares) and control (closed circles) mice administered with water (BAS) and 5% sucrose (SUG).
All data are mean ± S.E.M. *, statistically significant difference in diabetic water vs. diabetic sugar group, P < 0.05; ^, statistically significant difference in control water vs. control sugar group, P < 0.05; #, statistically significant difference in control water vs. diabetic water group, P < 0.05; +, statistically significant difference in control sugar vs. diabetic sugar group, P < 0.05.

**Figure B.3:** Representative cystometrograms for the four different groups viz control water (C+W BTH), control sugar (C +S BTH), diabetic water (D+W BTH) and diabetic sugar (D+S BTH) are shown in Fig 3. The black curve represents the runs with Tyrode before the administration of bethanechol (100µM) and the dotted curve represents the run after the administration of bethanechol. Representative changes in the compliance are plotted for the control water (closed circles), control sugar (closed triangles), diabetic water (closed squares) and diabetic sugar (closed diamonds) groups administered before (BAS) and after (BTH) the administration of bethanechol. Bethanechol sensitivity was plotted for the control water (C+W), control sugar (C+S), diabetic water (D+W) and diabetic sugar (D+S) groups.

**Figure B.4:** Representative cystometrograms for the four different groups viz control water (C+W ISO), control sugar (C +S ISO), diabetic water (D+W ISO) and diabetic sugar (D+S ISO) are shown in Fig 3. The black curve represents the runs with Tyrode before the administration of isoproterenol (100µM) and the
dotted curve represents the run after the administration of isoproterenol. Representative changes in the compliance are plotted for the control water (closed circles), control sugar (closed triangles), diabetic water (closed squares) and diabetic sugar (closed diamonds) groups administered before (BAS) and after (ISO) the administration of isoproterenol. Isoproterenol sensitivity was plotted for the control water (C+W), control sugar (C+S), diabetic water (D+W) and diabetic sugar (D+S) groups. All data are mean ± S.E.M. *, statistically significant difference in control sugar vs. diabetic sugar group, P < 0.05.

**Figure B.5:** Representative cystometrograms for the four different groups viz control water (C+W NO-ARG), control sugar (C+S NO-ARG), diabetic water (D+W NO-ARG) and diabetic sugar (D+S NO-ARG) are shown in Fig 3. The black curve represents the runs with Tyrode before the administration of N-nitro-L-arginine (250µM) and the dotted curve represents the run after the administration of N-nitro-L-arginine. Representative changes in the peak pressure and compliance are plotted for the control water (closed circles), control sugar (closed triangles), diabetic water (closed squares) and diabetic sugar (closed diamonds) groups administered before (BAS) and after (NO-ARG) the administration of N-nitro-L-arginine.
All data are mean ± S.E.M. *, statistically significant difference in diabetic water vs. diabetic sugar group, P < 0.05; ^, statistically significant difference in control water vs. control sugar group, P < 0.05; #, statistically significant difference in control water vs. diabetic water group, P < 0.05; +, statistically significant difference in control sugar vs. diabetic sugar group, P < 0.05.
Figure B.1: General characteristic changes in the mouse bladders in four treatment groups
Figure B.2: Functional remodeling in the mouse bladders
Figure B.3: Altered responses to cholinergic agonist, bethanechol
Figure B.4: Increased isoproterenol response in diabetic sugar mouse bladders
Figure B.5: Altered response of mouse bladders to NOS inhibitor
REFERENCES


