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Analysis of Changes in Nitric Oxide Synthase Signaling in BB/WOR Diabetic Prostate

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Abstract

Purpose: Diabetes is associated with increased lower urinary tract symptom (LUTS) severity. An underlying cause of LUTS is increased prostate smooth muscle tone. A critical regulator of prostate innervation and tone is nitric oxide (NO), produced by nitric oxide synthase (NOS). Since NO regulates proliferation and relaxation, and NOS signaling is altered in patients with LUTS, we propose that decreased NO in diabetic patients leads to increased LUTS. We examined this hypothesis by quantifying changes in NOS signaling in the BB/WOR diabetic rat prostate.

Materials and Methods: Protein and RNA abundance and localization of NOS I, -II and -III were examined in control and diabetic BB rat prostate by Real time RT-PCR, Western, immunohistochemical analysis and *in situ*. Morphological changes were examined by electron microscopy (EM), and TUNEL.

Results: *NosIII* is the most abundant isoform in ventral and dorsal prostate. NOS I, -II and -III protein and RNA localize to ductal epithelium. NOS III protein and RNA were significantly decreased in diabetic prostate. Apoptosis was increased in diabetic dorsal prostate. EM of the diabetic dorsal prostate showed abundant protein filled vacuoles and abnormal cytoplasmic morphology indicative of apoptosis.

Conclusions: Since NOS III is the most abundant form of NOS in the prostate diabetes may contribute to LUTS severity by down regulating NO, which may lead to increased proliferation in ductal epithelium

Keywords: NOS; Prostate; Diabetes; Apoptosis

Introduction

Male lower urinary tract symptoms (LUTS) encompass a variety of urologic symptoms including increased obstructive and storage symptoms. The prevalence of LUTS increases with age [1]. In the United States in 2000, 4.5 million visits to physicians for LUTS diagnosis occurred with total treatment costs of 1.3 billion dollars [2]. In spite of the high number of affected individuals and the cost of their health care, the underlying cause(s) of age related male LUTS remains controversial and is often attributed to benign prostatic hyperplasia (BPH) [3]. Previous studies have focused on age and androgen fluctuation as potential risk factors, however there is increasing evidence that a diverse group of co-morbidities including diabetes mellitus (DM), obesity and components of the metabolic syndrome, contribute to LUTS severity [4,5]. Several important studies support an association between diabetes and LUTS [4,6,7]. Diabetes is associated with a 1.78 fold higher risk of developing LUTS [8] and a recent review concluded that diabetes has a profound influence on risk of LUTS development [6]. Emphasis has been placed on BPH as the underlying risk factor for LUTS, ignoring the potential for pelvic/prostate ischemia and alteration in prostatic smooth muscle tone as contributing factors. A critical regulator of prostate innervation, smooth muscle tone and pelvic perfusion is nitric oxide (NO) derived from nitric oxide synthase (NOS). NOS is the enzyme responsible for synthesis of NO from L-arginine in non-adrenergic/non-cholinergic nerve terminals and in endothelium. NO is a widely distributed neurotransmitter that has been shown to regulate apoptosis, tissue remodeling, cellular proliferation, angiogenesis, and maintenance of smooth muscle tone [9]. Several isoforms of NOS exist including neuronal NOS (NOS I or nNOS, found in nerve terminals), inducible NOS (NOS II or iNOS, involved in the inflammatory response), and endothelial NOS (NOS III or eNOS, present in endothelial cells). In prostate, NOS I has been identified in the epithelium, stroma, and nerve fibers [9]. NOS II is present in stroma of BPH patients but not in normal prostate [9], and NOS III has been identified in basal cells of the glandular epithelium [9]. In human prostate, NOS plays a role in smooth muscle tone and relaxation, neuronal innervation and the regulation of vascular, secretory and hormonal pathways [10,11]. If NOS abundance is decreased in diabetic prostate, as was shown in other genitourinary organs such as the penis [12], this reduction in associated smooth muscle tone may manifest as obstructive and/or irritative voiding and thus present a putative mechanism of how LUTS severity is increased in diabetic patients. Although changes in NOS abundance and localization have been observed in patients with LUTS, the influence that DM has on NOS signaling in the prostate has not been previously examined. Thus in this study we have examined the localization and quantified changes in NOS protein and RNA abundance in the BB/WOR rat prostate a model that mimics type I diabetes in humans. We hypothesize that decreased NOS signaling in diabetic rats will cause morphological changes in the prostate that may contribute to increased symptom severity of LUTS in diabetic men.

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Materials and Methods

Animals

Postnatal day 120 (P120) Sprague-Dawley rats were obtained from Charles River. BB/WOR diabetes resistant (control) and diabetic rats were obtained from Biomedical Research Models, Inc., Worcester, MA. The presence of diabetes was determined by measuring blood glucose levels (Biomedical Research Models), with onset developing between P60 and P120. Rats were P160 to P190 when sacrificed, with 70 to 100 days of diabetes duration. Control BB rats were age matched. All animals were cared for according to the National Institute of Health *Guidelines for the Care and Use of Laboratory Animals* and protocols received institutional approval.

Overview of *Nos* expression in the prostate by RT-PCR

RNA was isolated from adult Sprague-Dawley rat ventral, dorsal and lateral (n=4) prostate with TRIzol (Invitrogen, Carlsbad, CA) as previously described [12]. RT-PCR was performed on 150 ng RNA, assaying for *NosIII, NosII, NosIa, NosIb,* and *NosIc* expression using the Gene Amp RNA PCR Core kit (Perkin-Elmer, Branchburg, NJ) as described previously [12]. Primers (Table 1) were synthesized at the Northwestern University Biotechnology Facility and products were restriction digested to confirm they represented the sequence of interest.

Quantification of *Nos* expression using Real time RT-PCR

RNA was isolated and DNase treated as stated previously [12] on control (n=4) and diabetic (n=4) BB/WOR ventral, dorsal and lateral prostate. cDNA was synthesized from 300 ng of RNA using the Gene Amp RNA PCR Core kit (Perkin-Elmer, Branchburg, NJ, USA). Real-time RT-PCR was performed on the Opticon Monitor system (Bio-Rad, Irvine, CA, USA) using SYBR Green Super UDG mix (Invitrogen) according to manufacturer's recommendations as previously described [13]. Primers are outlined in Table 2 (Integrated DNA Technologies, Coralville, IA). Negative controls without cDNA were run to ensure the absence of contamination. Specificity of primers was verified by melt curve analysis and sequencing. *NosI, NosII,* and *NosIII* were normalized to *Ribosomal subunit L-19 (Rpl19)*, by the $2^{-\Delta\Delta C_T}$ method [14]. Assays were performed in triplicate on individual tissue specimens, the results averaged and the product ratios reported as the mean plus or minus the standard error of the mean.

Gene		Product size
Noslas:	5′-ACT TAG CAC AGA GAC TGC TC-3′	401
Noslbs:	5′-ATT AAG GCC TTG AGT CGC TC-3′	215
Noslcs:	5′-AGA CAC AGA AGA TTC AGG GC-3′	216
Nosas:*	5′-TGT GAC TCA CAA GCA TAG GG-3′	
NosIIs:	5′-GCC TCC CTC TGG AAA GA-3′	500
NosIIas:	5′-TCC ATG CAG ACA ACC TT-3′	
NosIIIs:	5′-AGG CTG CTG CCC GAG ATA TCT TCA-3′	261
NosIIIas:	5′-TTG GGT GGG CAC ACA CCT ATG TGG-3′	

*Nosas primer was used as the reverse primer for *NosIa, NosIb* and *NosIc*.

Table 1: Primers used for RT-PCR.

Gene	Primer sequence
NosIs:	5′-ACCTCGATGGCAAATCGCACAAAG-3′
Noslas:	5′-ACGGGTTGTTAAGGATCACAGGAA-3′
NosIIs:	5′-AGGTGCTGGAAGAGTTCCCATCAT-3′
NosIIas:	5′-TGGGTCTTCGGGCTTCAGGTTATT-3′
NosIIIs:	5′-TATTTGATGCTCGGGACTGCAGGA-3′
NosIIIas:	5′-GCTGAACGAAGATTGCCTCGGTTT-3′
Rpl19s:	5′-TGATCTCCTCCTTCTTGGCTTGGA-3′
Rpl19as:	5′-TGATCTCCTCCTTCTTGGCTTGGA-3′

Table 2: Real time RT-PCR primers.

In situ hybridization

In situ hybridization was performed as described previously [12] on control (n=5) and diabetic (n=5) dorsal and ventral prostates. Riboprobes for *Nos-II* and *Nos-III* were synthesized as described previously [15].

Immunohistochemical analysis (IHC)

IHC was performed on control (n=5) and diabetic (n=5) ventral, dorsal and lateral prostate as described previously [16] using the Dako LSAB + System, HRP. Sections were incubated with mouse monoclonal antibodies for NOS I, NOS II and NOS III (250µg/ml, Transduction Laboratories, Lexington, Kentucky). Sections were stained with DAB and mounted using Crystal Mount (Biomedia).

TUNEL

TUNEL assay for apoptosis was performed according to manufacturer's instructions using the ApopTag kit (Intergen, Purchase, NY) on control (n=5) and diabetic (n=5) dorsal prostate as previously described [13].

Electron microscopy (EM)

EM was performed as described previously [17] on control (n=3) and diabetic (n=3) dorsal prostate.

Western

Western analysis was performed as described previously [16] on control (n=4) and diabetic (n=4) ventral, dorsal and lateral prostates assaying for NOS I and NOS III proteins. Membranes were incubated with mouse monoclonal NOS I and NOS III (Transduction Laboratories, Lexington, KY), and β-actin (Sigma, St Louis, MO) antibodies for 18 h at 4°C. Membranes were washed and incubated with goat-anti-mouse IgG conjugated to horseradish peroxidase (Jackson Immuno Labs; 1:20000 dilution) for 4 h at 37°C. Protein bands were visualized using enhanced chemi luminescence detection reagent (ECL Western Blotting Analysis System, Amersham) according to manufacturers directions and exposed to Kodak (Eastman Kodak) X-AR2 film for 1-5 min. Protein bands were quantified by densitometry using Kodak 1D software (Rochester, NY). Quantification was performed by comparing the density of NOS I and NOS III bands to β-actin in order to eliminate differences in protein loading.

Statistics

Statistics were performed using the Excel program and the results are reported ± the standard error of the mean (SEM). A t-test was performed to determine significant differences. P-values ≤0.05 were considered significant.

Results

Nos expression in prostate

RT-PCR was performed to determine the distribution of *Nos* isoforms in Sprague-Dawley rat prostate (n=4). *NosIII* was the most abundant isoform in the ventral, dorsal and lateral prostate (Figure 1). *NosII* and *NosIa* were also abundant in the dorsal prostate (Figure 1B) and *NosIb* in the lateral prostate (Figure 1C).

Real time RT-PCR of *Nos* expression in the prostate

Real time RT-PCR was performed to quantify changes in *Nos* abundance in control (n=4) and diabetic (n=4) rat prostate. *NosIII* was significantly decreased 62% in diabetic ventral prostate (p=0.0001), 75% in diabetic dorsal prostate (p=0.0001) and 37% in diabetic lateral prostate (p=0.008) (Figure 2). *NosI* was unaltered in control and diabetic ventral (p=0.38), dorsal (p=0.19), and lateral (p=0.21) prostate (Figure 2). *NosII* was unaltered in diabetic ventral prostate (p=0.60), however *NosII* expression was too low to quantify in dorsal and lateral prostate.

NOS protein localization by IHC in control and diabetic prostate

IHC analysis was used to localize NOS I, -II and -III proteins in control (n=5) and diabetic (n=5) BB/WOR rat ventral, dorsal and lateral prostate. NOS I protein was localized in the epithelium of all three lobes of control and diabetic prostate (Figure 3). The diabetic ventral prostate displayed abnormal morphology with less stroma apparent between ducts by visual observation and the ducts appeared to have lost their circular architecture (Figure 3). NOS I staining in the diabetic dorsal prostate appeared to have gaps in the epithelium that was not apparent in controls (Figure 3). NOS II protein was identified in ductal epithelium of control and diabetic ventral and dorsal prostates but was not present in control or diabetic lateral prostate (Figure 4).

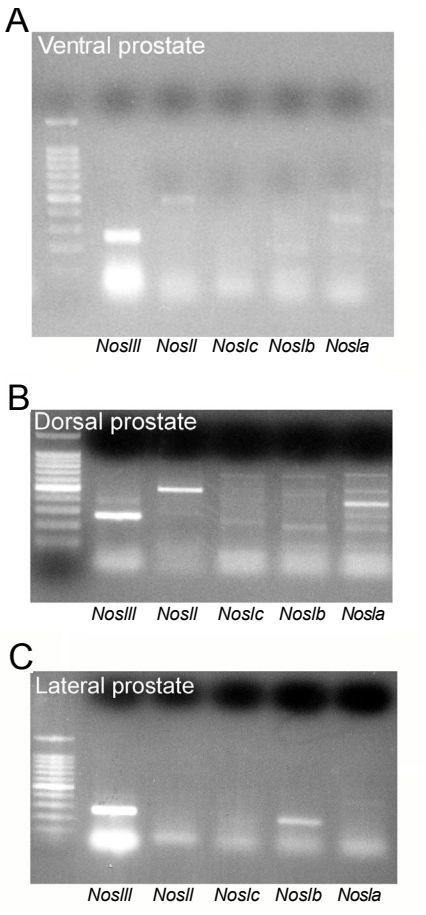

Figure 1: Survey of *Nos* isoform expression in Sprague Dawley rat ventral (A), dorsal (B) and lateral (C) prostates. *NosIII* is most abundant in all prostate lobes. 100 base pair marker (Left) was used to determine band size.

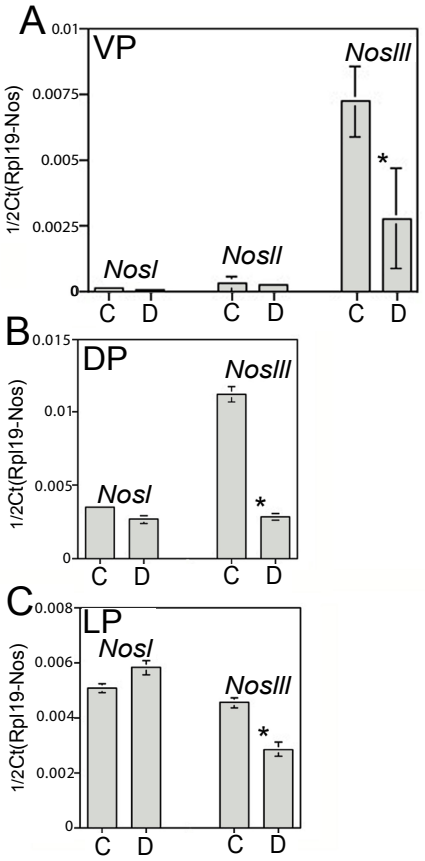

Figure 2: Quantification of *NosI, NosII and NosIII* RNA expression in control and diabetic ventral (A), dorsal (B) and lateral (C) prostates using Real time RT-PCR. *NosIII* is significantly decreased in all diabetic prostate lobes while *NosI* remains unchanged with diabetes.

NOS III protein was abundant in control and diabetic ventral and dorsal prostate ductal epithelium (Figure 5). NOS III protein also had gaps in staining in the diabetic dorsal prostate ductal epithelium that was not apparent in controls (Figure 5). NOS III protein was identified in diabetic lateral prostate but not control lateral prostate (Figure 5).

In situ hybridization of *NosII* and *NosIII* in control and diabetic prostate

In situ hybridization was performed on control (n=5) and diabetic (n=5) ventral and dorsal prostate assaying for *NosII* and *NosIII* localization. *NosII* and *NosIII* were localized in the ductal epithelium of control and diabetic ventral and dorsal prostate (Figure 6).

Quantification of NOS I and NOS III proteins in prostate by western analysis

Western analysis was performed on control (n=4) and diabetic (n=4) ventral, dorsal and lateral prostate assaying for NOS I/β-actin and NOS III/β-actin. NOS III/β-actin was significantly decreased 45% in diabetic ventral prostate (p-value=0.002), but remained unaltered in diabetic dorsal (p-value=0.20) and in diabetic lateral (p-value=0.48) prostate. NOS I/β-actin was increased 95% (p=2.82 E-5) in diabetic ventral prostate and 90% (p=0.02) in diabetic lateral prostate (Figure 7). NOS I/β-actin protein was increased 79% in the diabetic dorsal prostate (p=0.079), however in light of the large standard deviations, statistical significance was not reached (Figure 7).

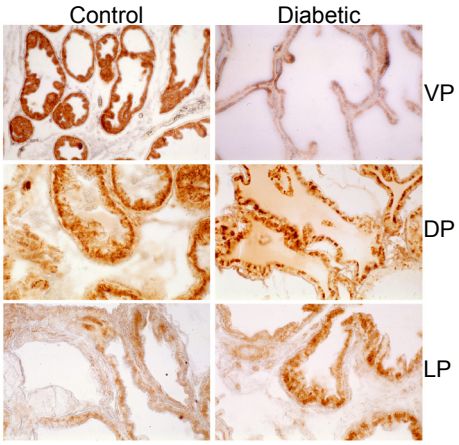

Figure 3: IHC analysis showing localization of NOS I protein in control and diabetic ventral (100X), dorsal (200X) and lateral (200X) prostates. Morphology changes were observed in diabetic VP and DP.

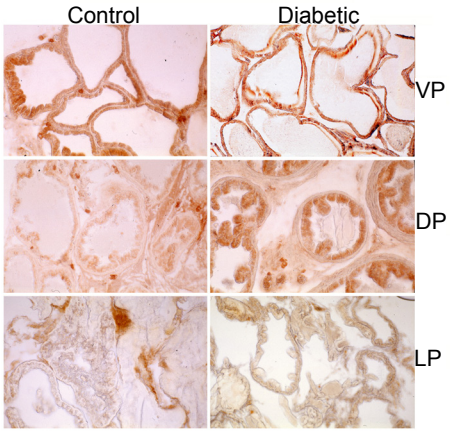

Figure 4: IHC analysis showing localization of NOS II protein in control and diabetic ventral, dorsal and lateral prostates. 100-200X. Abnormal morphology was observed in the diabetic VP.

EM of control and diabetic prostate

EM was performed on control (n=3) and diabetic (n=3) dorsal prostate. Many protein filled vacuoles were identified in diabetic dorsal prostate however in control dorsal prostates the vacuoles were empty (Figure 8). Abnormal cytoplasmic morphology was also observed in diabetic dorsal prostate that was not present in controls (Figure 8). The presence of condensed chromatin that is detached from the nuclear membrane, large vacuoles containing altered cell organelles and dilated cisternae of the endoplasmic reticulum in the diabetic are suggestive of apoptosis [18,19]. Apoptosis in the diabetic dorsal prostate was confirmed by TUNEL (Figure 9).

Discussion

In this study the effect of type I diabetes mellitus was examined on NOS isoform localization and abundance in the BB/WOR rat model. NOS III is the most abundant isoform in the rat prostate. NOS I and -III are abundant in ductal epithelium of all three prostate lobes while NOS II is present in the ventral and dorsal but not lateral prostate. The localization of NOS is consistent with past studies [20,21] except for NOS II, which was not previously detected in normal human prostate but was identified in prostate tissue from LUTS/BPH patients [9]. The localization of NOS isoforms did not change with DM, however the staining was non-uniform for NOS I and -III in ductal epithelium of diabetic dorsal prostate (Figure 3 and 5). We identified apoptosis taking place in the diabetic dorsal prostate by TUNEL and EM, and the tissue morphology is suggestive of gaps in NOS I and -III staining where the tissue had been compromised. The diabetic ventral prostate also displayed abnormal morphology in many of the rats examined (Figure 3 and 5). The ductal architecture lost its characteristic uniform circular structure. The ductal epithelium appeared thin and the stromal layer reduced between ducts by visual observation. Similar findings were noted in an animal model of prostate denervation suggesting impact on neural function [22]. Changes in *Nos* RNA and protein abundance were striking in the diabetic prostate. *NosIII* RNA expression decreased 62%

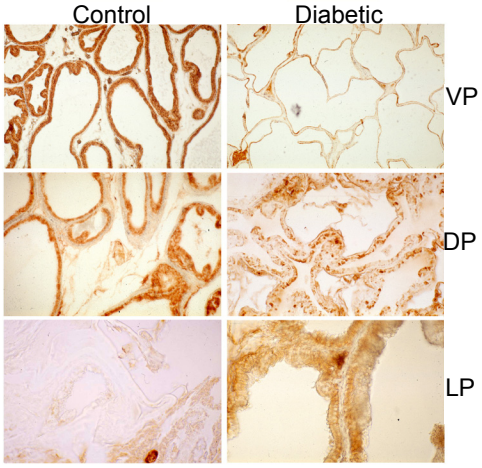

Figure 5: IHC analysis showing localization of NOS III protein in control and diabetic ventral (100X), dorsal (200X) and lateral (400X) prostates. Abnormal morphology was observed in the diabetic VP and DP.

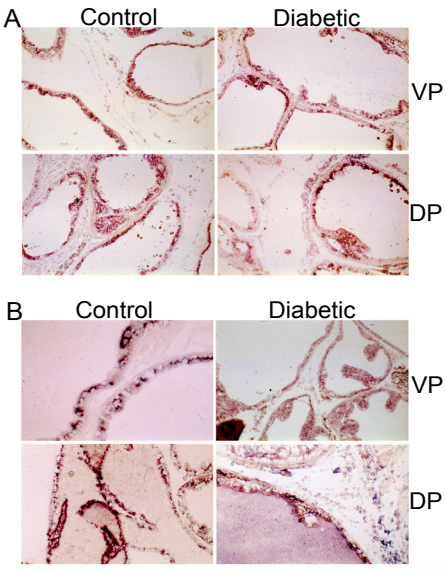

Figure 6: In situ hybridization showing localization of *NosII* (A) *and NosIII* (B) RNA expression. 200-400X.

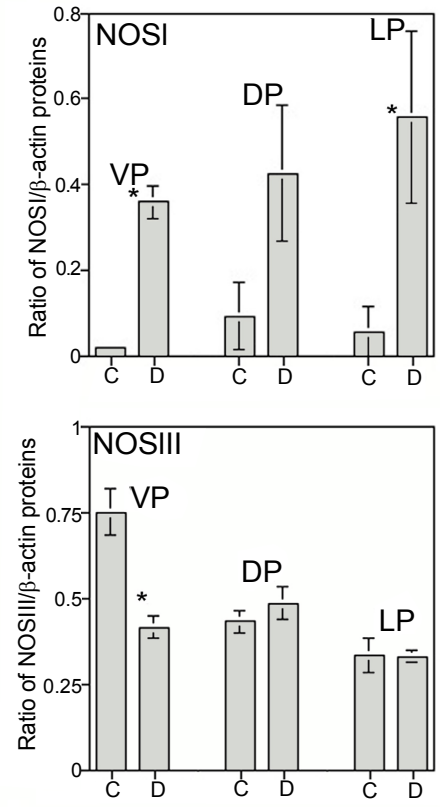

Figure 7: Western analysis quantifying NOS I (A) and NOS III (B) proteins in control and diabetic ventral, dorsal and lateral prostate. NOS III protein was decreased in the VP while NOS I protein was increased in all three diabetic prostate lobes.

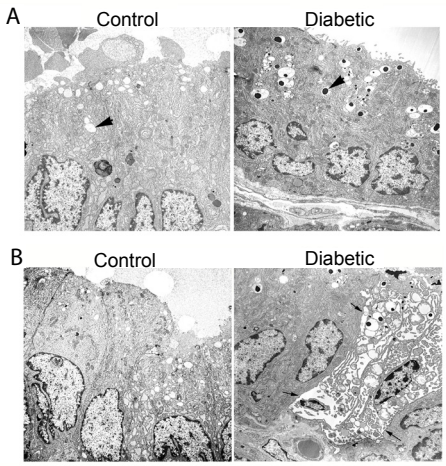

Figure 8: EM of control and diabetic dorsal prostate shows abundant protein filled vacuoles in the diabetic dorsal prostate (A). 30,000X. Abnormal cytoplasmic morphology was observed in cells of the diabetic dorsal prostate indicative of apoptosis (B). Arrows indicate vacuoles. 30,000X.

in diabetic ventral, 75% in diabetic dorsal and 37% in diabetic lateral prostate by Real time RT-PCR. This finding was supported by decreased NOS III protein in the ventral prostate. *NosI* RNA expression was unaltered in diabetic prostate however NOS I protein was significantly increased 95% in diabetic ventral, 79% in diabetic dorsal, and 90% in

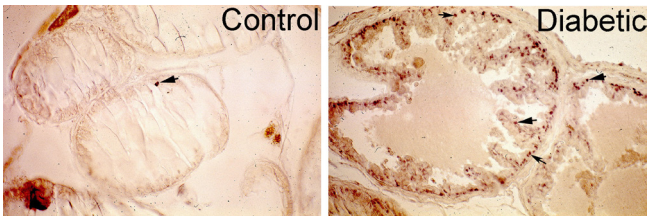

Figure 9: Apoptosis was identified by TUNEL assay in the diabetic DP. (200X).

diabetic lateral prostate. *NosI* RNA expression is so low by comparison to *NosIII* in all three prostate lobes, that it's possible that small changes are difficult to quantify. *NosI* is not highly expressed in the penis since it is primarily synthesized and translated in the cavernous nerve. A potential mechanism of how altered NOS signaling can impact LUTS is discussed in detail below.

Several lines of evidence indicate a role for NO in the regulation of smooth muscle tone of bladder, prostate and urethra [23]. Following NO release by NOS, cGMP formation elicits smooth muscle relaxation by decreasing intracellular calcium and the activity of downstream proteins, such as cGMP-specific protein kinase (PKG-1). Calcium-dependent NOS (NOS I and NOS III) has been identified in the lower urinary tract. NOS I is present in nerve terminals, interspersing the prostatic peripheral and transition zones, in the stroma and in the glandular epithelium [24]. The bladder urothelium expresses NOS and releases NO in response to several stimuli [25]. Since NOS III is localized in endothelial cells of the prostate, it has been hypothesized that its role is primarily in regulating local vascular perfusion, whereas NOS I regulates smooth muscle tone and glandular activity [23]. The demonstration of antiproliferative and pro-apoptotic effects of NO donors on cultured prostate smooth muscle cells further suggests a role for the nitrergic pathway in LUTS [26]. Clinical and pre-clinical studies have demonstrated that pelvic ischemia/hypoxia closely correlate with alteration of lower urinary tract tissues through the induction of fibrosis and reduced NOS [27,28]. These findings suggest a mechanism of how pathological conditions characterized by the deterioration of nerves and endothelium with impairment of NO production, such as hypertension, diabetes and metabolic syndrome are associated with LUTS.

In our study, NOS III protein was significantly decreased. Since NOS III is the most abundant form of NOS in the prostate, diabetes may contribute to LUTS severity by down regulating the available NO, which leads to increased proliferation in ductal epithelium [29]. A second mechanism of how LUTS may result is due to increased tone of prostate smooth muscle [2], resulting from an increase in constricting forces while relaxation forces are reduced. These dynamic changes in smooth muscle tone are mediated by the autonomic nervous system. As the nitrinergic innervation of the prostate is distinctly reduced with BPH, we propose that prostatic innervation is altered in diabetes such that relaxation mechanisms that regulate prostate tone become skewed [30]. It is proposed that total NO is reduced, thereby reducing prostate tone and relaxation. Our results in this study show that NOS III protein is significantly down regulated in the ventral prostate while NOS I is significantly up-regulated. Although NOS I is increased NOS III is the predominant form of NOS in the prostate so decreased NOS III would result in an overall reduction in available NO despite the increase in NOS I. We can speculate as to why NOS I increases when overall NO is reduced. One possibility is prostatic neural hyperactivity which has been previously noted in male LUTS [31]. Perturbations

of this neural regulatory mechanism may lead to altered prostate innervation and autonomic nervous system hyperactivity in prostate tissue resulting in increased growth and worsening of LUTS. Given that metabolic syndrome, found in diabetes is characterized by autonomic hyperactivity, it is possible that increased NOS I may be one such manifestation. To confirm this will require additional studies looking at the sympathetic supply of the rat prostate using the same model. Such experiments were beyond the scope of this investigation.

Studies of patients with diabetes report that up to one third have urodynamically abnormal bladder function [32], with voiding affected more than storage function [10]. No difference in prostate volume or PSA were found in diabetic patients, suggesting that the presence of diabetes may be less directly associated with prostate growth and more closely associated with dynamic components of lower urinary tract function. It is also possible that diabetes precipitates LUTS through neurologic mechanisms that are independent of BPH bulk changes.

Conclusions

We propose that decreased NOS in the prostate may account for some of the increased risks of LUTS in men with DM. Our results show decreased NOS III in the diabetic prostate. Since NOS III is the most abundant NOS isoform in the prostate, diabetes may contribute to LUTS severity by down regulating NO, which leads to increased proliferation in ductal epithelium.

Summary Sentence

Changes in NOS signaling were examined in control and diabetic BB/WOR prostate in order to examine if altered NOS signaling and decreased nitric oxide may contribute to increased LUTS severity in diabetics.

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References

1. Armstrong N, Vale L, Deverill M, Nabi G, McClinton S, et al. (2009) Surgical treatments for men with benign prostatic enlargement: cost effectiveness study. BMJ 338: b1288.
2. Sarma AV, Parsons JK (2009) Diabetes and benign prostatic hyperplasia: emerging clinical connections. Curr Urol Rep 10: 267-275.
3. Chapple CR, Wein AJ, Abrams P, Dmochowski RR, Giuliano F, et al. (2008) Lower urinary tract symptoms revisited: a broader clinical perspective. Eur Urol 54: 563-569.
4. Kupelian V, McVary KT, Kaplan SA, Hall SA, Link CL, et al. (2009) Association of lower urinary tract symptoms and the metabolic syndrome: results from the Boston Area Community Health Survey. J Urol 182: 616-624.
5. Kasturi S, Russell S, McVary KT (2006) Metabolic syndrome and lower urinary tract symptoms secondary to benign prostatic hyperplasia. Curr Urol Rep 7: 288-292.
6. Sarma AV, Parsons JK, McVary K, Wei JT (2009) Diabetes and benign prostatic hyperplasia/lower urinary tract symptoms-what do we know? J Urol 182: S32-S37.
7. Hammarsten J, Hogstedt B (2001) Hyperinsulinaemia as a risk factor for developing benign prostatic hyperplasia. Eur Urol 39: 151-158.
8. Wang CC, Chancellor MB, Lin JM, Hsieh JH, Yu HJ (2009) Type 2 diabetes but not metabolic syndrome is associated with an increased risk of lower urinary tract symptoms and erectile dysfunction in men aged <45 years. BJU Int 105: 1136-1140.
9. Gradini R, Realacci M, Ginepri A, Naso G, Santangelo C, et al. (1999) Nitric oxide synthases in normal and benign hyperplastic human prostate: immunohistochemistry and molecular biology. J Pathol 189: 224-229.
10. Bloch W, Klotz T, Loch C, Schmidt G, Engelmann U, et al. (1997) Distribution of nitric oxide synthase implies a regulation of circulation, smooth muscle tone, and secretory function in the human prostate by nitric oxide. Prostate 33: 1-8.
11. McVary K (2006) Lower urinary tract symptoms and sexual dysfunction: epidemiology and pathophysiology. BJU Int 97: 23-28.
12. Podlasek CA, Zelner DJ, Bervig TR, Gonzalez CM, McKenna KE, et al. (2001) Characterization and localization of nitric oxide synthase isoforms in the BB/WOR diabetic rat. J Urol 166: 746-755.
13. Angeloni NL, Bond CW, Monsivais D, Tang Y, Podlasek CA (2009) The role of hedgehog-interacting protein in maintaining cavernous nerve integrity and adult penile morphology. J Sex Med 6: 2480-2493.
14. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25: 402-408.
15. Podlasek CA, Gonzalez CM, Zelner DJ, Jiang HB, McKenna KE, et al. (2001) Analysis of NOS isoform changes in a post radical prostatectomy model of erectile dysfunction. Int J Impot Res 13: S1-S15.
16. Takeda M, Tang R, Shapiro E, Burnett AL, Lepor H (1995) Effects of nitric oxide on human and canine prostates. Urology 45: 440-446.
17. Tang Y, Rampin O, Calas A, Facchinetti P, Giuliano F (1998) Oxytocinergic and serotonergic innervation of identified lumbosacral nuclei controlling penile erection in the male rat. Neuroscience 82: 241-254.
18. Gomez MI, de Castro CR, Fanelli SL, Quintans LN, Costantini MH, et al. (2007) Biochemical and ultrastructural alterations in the rat ventral prostate due to repetitive alcohol drinking. J Appl Toxicol 27: 391-398.
19. Sesso A, Marques MM, Monteiro MM, Schumacher RI, Colquhoun A, et al. (2004) Morphology of mitochondrial permeability transition: morphometric volumetry in apoptotic cells. Anat Rec A Discov Mol Cell Evol Biol 281: 1337-1351.
20. Burnett AL, Lowenstein CJ, Bredt DS, Chang TS, Snyder SH (1992) Nitric oxide: a physiologic mediator of penile erection. Science 257: 401-403.
21. Limanowski A, Miskowial B, Partyka M, Konwerska A (2004) Immunohistochemical studies on brain nitric oxide synthase (bNOS) in the male genital accessory glands of the rat during postnatal development. Folia Morphol (Warsz) 63: 189-194.
22. Wang JM, McKenna KE, McVary KT, Lee C (1991) Requirement of innervation for maintenance of structural and functional integrity in the rat prostate. Biol Reprod 44: 1171-1176.
23. Kedia GT, Uckert S, Jonas U, Kuczyk MA, Burchardt M (2008) The nitric oxide pathway in the human prostate: Clinical implications in men with lower urinary tract symptoms. World J Urol 26: 603-609.
24. Dixon JS, Jen PY (1995) Development of nerves containing nitric oxide synthase in the human male urogenital organs. Br J Urol 76: 719-725.
25. Gillespie JI, Markerink-van Ittersum M, De Vente J (2006) Endogenous nitric oxide/cGMP signalling in the guinea pig bladder: evidence for distinct populations of sub-urothelial interstitial cells. Cell Tissue Res 325: 325-332.
26. Fibbi B, Morelli A, Vignozzi L, Filippi S, Chavalmane A, et al. (2010) Characterization of phosphodiesterase type 5 expression and functional activity in the human male lower urinary tract. J Sex Med 7: 59-69.
27. Ghafar MA, Anastasiadis AG, Olsson LE, Chichester P, Kaplan SA, et al. (2002) Hypoxia and an angiogenic response in the partially obstructed rat bladder. Lab Invest 82: 903-909.
28. Tarcan T, Azadzoi KM, Siroky MB, Goldstein I, Krane RJ (1998) Age-related erectile and voiding dysfunction: the role of arterial insufficiency. Br J Urol 82: 26-33.
29. Guh JH, Hwang TL, Ko FN, Chueh SC, Lai MK, et al. (1998) Antiproliferative effect in human prostatic smooth muscle cells by nitric oxide donor. Mol Pharmacol 53: 467-474.
30. Brown JS, Wessells H, Chancellor MB, Howards SS, Stamm WE, et al. (2005) Urologic complications of diabetes. Diabetes Care 28: 177-185.

31. McVary KT, Rademaker A, Lloyd GL, Gann P (2005) Autonomic nervous system overactivity in men with lower urinary tract symptoms secondary to benign prostatic hyperplasia. J Urol 174: 1327-1433.

32. Beck R, Fowler CJ, Mathias CJ (1994) Genitoruinary dysfunction in disorders of the autonomic nervous system. In Handbook of Neuro Urology, Edited by Rushton DN, New York: Marcel Dekker: 281.