A. Specific Aims

Specific Aim 1: To determine if correction of DM2 in a genetic animal model will restore the bladder function.

Specific Aim 2: To determine the effect of high-glucose concentration on inflammatory mediators in DKO mice.

B. Studies and Results

Type 2 diabetes mellitus (DM2) stems from insulin resistance and affects 7% of the US population. Lower urinary tract dysfunction is one of the major urologic complications associated with DM2, and the patients present with detrusor overactivity or underactivity. Although diabetic bladder dysfunction affects up to 80% of patients with DM2, its underlying pathophysiology is poorly understood. With the support of AMDCC, we investigated a genetic mouse model with DM2 with conditional knockout of insulin receptor substrates 1 & 2 (Irs1, Irs2), and demonstrated that the Irs1/Irs2 double knockout (DKO) mice mimic many of the signs and symptoms seen in human patients with diabetic lower urinary tract dysfunction.

We first systematically examined the diabetic bladder dysfunction in the DKO mice. We found that the bladder of the DKO mice exhibited higher amplitudes of muscle tension at age 6 and 12 weeks relative to age-matched controls. However, 16 and 20 week old DKO mice developed lower amplitudes than age-matched controls. These data suggests that spontaneous bladder contractions in the DKO mice with DM2 are different from normal controls, and the type of spontaneous activity is different in early/mid life than compared to later in adulthood. Although the amplitude of the spontaneous tension generated in the bladder muscle varied between the DKO and control mice, the frequency of spontaneous activity in the bladders did not vary, suggesting that abnormal tensions generated within the bladder, and not the frequency of contraction, is associated with the bladder dysfunction in DM2. Carbachol (10^{-9}M~10^{-5}M), KCl (120 mM) stimulation and electrical field stimulation (EFS) tests on bladder muscle strips revealed similar findings as that of spontaneous activity.

We next evaluated the urine voiding behavior in the DKO mice in vivo by performing cystometry. We found that the frequency of bladder contractions in the bladder of the intact animals were higher in the DKO mice than the age matched controls. In addition, the voided urinary volumes were lower in the DKO mice than the age-matched controls. These findings are commonly found in many patients with DBD, and are consistent with previous streptozotocin-induced diabetic rat bladder models where the diabetic bladders go through a compensated followed by a decompensated state in a time-dependent manner. Therefore, our findings suggest that the DKO model, which mimics DM2, is a rational model to evaluate the pathophysiology and molecular alterations associated with diabetic cystopathy.

Gene expression profile by microarray analysis demonstrated that in the bladder of 12 week old DKO animals, the gene expression of ATPase, Rho GTPase, Rho kinase, IRS1 and Foxo1, which are known important regulators of cellular metabolism and contractility, are upregulated. In addition, apoptotic response related genes Gspt2, Bik, Casp3, are upregulated. In 20 week old DKO mice, however, ATPase, Rho GTPase and Rho kinase expression were decreased relative to age-matched control animal – findings which are in accordance with our bladder functional experimental studies: i.e. hyperactivity in early stage and hypoactivity in late stage.

Mitochondrial dysfunction is closely associated with insulin resistance and might contribute to the progression of diabetes. Our previous data showed that the DKO animal livers contained twofold larger and 50% fewer mitochondria per hepatocyte than control liver. In order to evaluate the secondary complications associated with DM2, we determined whether there is any evidence of mitochondrial dysfunction in DKO animals. Different from that in liver, unexpectedly, we found that the mitochondria in the DKO BSMCs were significantly shrunken compared to the age matched controls, particularly in older mice (20 weeks of age), suggesting abnormal energy metabolism in the mice suffering from late stage diabetic cystopathy.

Because of alterations in the mitochondrial ultrastructure of BSMC in DKO animals and the close association between mitochondrial function and apoptotic pathways, we examined for degree of apoptosis in the BSMC of control and DKO animals. We found by TUNEL staining that there was significantly increased apoptotic cells in 20 week and 44 week old DKO bladders compared to the floxed-control animals.

The DKO mice develop hyperglycemia and dyslipidemia on the fifth week of life and the metabolic abnormalities last beyond 30 weeks of life. Because the Irs1 and Irs2 genes regulate the Foxo1 gene, the TKO (Irs1, Irs2 & Foxo1 knockout) were generated, and previous data has demonstrated that the metabolic abnormalities associated with DM2 are corrected in the TKO mice. So we systematically determined if the diabetic bladder dysfunction that we observed in the DKO mice is reversible by the genetic modification in the TKO animal. We found that normalizing blood glucose in TKO mice not only restored the metabolic defects associated with DM2 such as hyperglycemia, but also corrected the hypoactivity that is found in the 20-week old DKO mice by electrical field stimulation studies. Therefore, the DKO & TKO mice serve as a powerful modifiable genetic animal model for DM2.

Inflammation is known to play an important role in the pathogenesis of diabetic organ damages. With respect to bladder, we believe that the inflammatory molecules are significantly altered during hyper-glucosuria and hyperglycemia, which can lead to hyper- and hypo-stimulated states that are observed in the compensated and decompensated states of bladder function in DM2. Therefore, we examined the expression of inflammatory regulated genes in bladder tissues with Affymetrix gene chip. Evaluation of 45,000 genes revealed over 20 inflammatory genes which were up-regulated 1.5 folds or more in 12w old DKO animals relative to age-matched control, and were persistently elevated in 20w old mice, as well. It is noteworthy that most of the identified inflammatory mediated genes belong to the TNF superfamily. These data suggest that inflammatory mediators, particularly the TNF superfamily members, may play an important role in the pathogenesis of DBD.

We further investigated the levels of circulating and localized bladder inflammatory mediators by other assays. While the protein levels of ten cytokines in urine and homogenized bladder tissue were below the limit of detection by the Multiplex kit assay, the serum levels of TNFα was higher in DKO animals as compared to age-matched control. Western blot result showed that TNFα and TNFR1 were significantly upregulated in DKO bladder, although TNFR2 did not have any significant change. To investigate whether specific inflammatory cells infiltrate the bladder in diabetic DKO mice, we performed histological and immunohistochemical analyses. We did not find any detectable histologic differences between the control and DKO bladders by H&E analysis. However, immunohistochemical analysis with staining for macrophage specific marker F4/80 antibody showed that $F4/80^{+}$ macrophages significantly increased in DKO animal bladders in an age-dependent manner. More interestingly, close ultrastructural histologic evaluations revealed that the macrophages were mainly located in the lamina propria (i.e. junction between the bladder smooth muscle and urothelium).

To determine whether inflammatory mediators directly contribute to the bladder dysfunction, we treated primary cultured rat BSMC with TNFα and cell contraction was evaluated with collagen gel retraction assay. Treatment with 1~30ng/mL TNFα for 4hrs dramatically promoted the contraction of BSMC in a dose-dependent manner. Although high glucose (22 mM) alone did not affect the cell contraction, combined treatment with high glucose amplified the effect of TNFα on cell contraction. Cells treated with Rho kinase inhibitor Fasudil, MLCK inhibitor peptide 18 (p18) or anti-inflammatory agent indomethacin inhibited the effect of TNFα-induced bladder contractions, suggesting that TNF-α may play a direct or indirect role in activation of the Rho-kinase pathway.

C. Significance

The significance of these results is that we have identified a DM2 mouse model without obesity which demonstrates many of the secondary urologic signs and symptoms that we see in human patients. The DKO animal model is a genetically reversible system (i.e. TKO animals) which enables us to evaluate the molecular alterations and its correction associated with bladder dysfunction in type 2 insulin resistant diabetes. To our knowledge, this is the first attempt to use a reversible genetically modifiable animal model to evaluate the prevention/correction of DBD in DM2. Our study also discovered that inflammatory mediators are associated with the bladder dysfunction in DM2. In general, the present and subsequent studies will allow us to gain further insights into the molecular alterations that are responsible for diabetic bladder dysfunction, which may help identify diabetic patients at risk of developing bladder dysfunction prior to presentation of the late stages of the disease.

D. Plans

Based on our preliminary findings, we will be working on a series of subsequent studies to elucidate the underlying molecular mechanism of DM2 diabetic bladder dysfunction in DKO/TKO animal model. We will:

1. Examine if systemic inhibition of TNFR can correct diabetic bladder dysfunction;
2. Determine whether simultaneous inhibition of TNFR and correction of hyperglycemia will normalize the TNF levels and bladder dysfunction associated with DM2;
3. Determine whether the onset of bladder dysfunction occurs before or after hyperglycemia;
4. Investigate the mechanism of the correction of bladder dysfunction in TKO animal;
5. Determine if TNFα activates the Rho-Rho kinase-MLC signaling pathway in order to induce abnormal contraction of bladder smooth muscle.

E. Publications

Manuscripts in preparation

F. Project-Generated Resources

None.

G. Research Development

- Laboratory techniques: During this study, we have developed and further perfected our techniques in studying the bladder function, which include small animal cystometric test system and muscle strip contraction signal collecting system.

- I have attended three national meetings thus far and presented our progress upon this project. All these meetings have served to further define my research career path, and refine our own on-going research projects.

H. Other Activities

I continue to serve as an expert reviewer for many of the basic science and urology journals.

I. Research Development and Other Activities Planned for the Next Year

I am submitting an NIH/R01 grant – which is a direct followup to the current project that was funded by AMDCC. The title of the grant that will be submitted on October 5, 2010 is "Mechanisms of bladder dysfunction in type 2 diabetes".