Diabetic Complications Consortium

Application Title: The Impact of Diabetes Mellitus on Antimicrobial Peptide Production and Renal Bacterial Defense

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1. Project Accomplishments:

Over the past 12 months, our application resulted in the following accomplishments:
(**A**) In regards to Aim 1, funding from Diacomp's Pilot and Feasibility Program allowed us to expand the pilot data presented in our initial proposal and demonstrate that urinary antimicrobial peptide concentrations (AMP) are suppressed in patients with new-onset insulin deficient diabetes mellitus (DM). A subset of these results, which evaluate the urinary expression of an AMP called Ribonuclease 7 (RNase 7) were recently published in *Kidney International* (see section #3 below for full reference).
(**B**) In regards to Aim 2, our lab successfully deleted the insulin receptor (IR) from the kidney's intercalated cells and demonstrates that IR deletion increases urinary tract infection (UTI) risk (see section #2 below).
(**C**) To date, data generated from this project provided key preliminary data for a NIH/NIDDK R01 proposal (application ID 1R01DK114035, submitted October 2016, review pending) regarding the role of insulin in renal bacterial defense. Moreover, some of the data generated from this project was presented as an oral presentation at the 2015 American Society of Nephrology Kidney Week national conference (San Diego, CA) as well as at the 2016 Midwest Society of Pediatric Research Regional Conference (MWSPR, Chicago, IL) where it was awarded the Cleveland Clinical Outstanding Abstract award. Once our remaining studies are complete, we anticipate 1-2 additional manuscript submissions in the next 6 months.

2. Specific Aims:

Specific Aims of Original Proposal: Diabetes Mellitus (DM) is a systemic disease associated with a deficiency of insulin secretion or action. DM is associated with many complications, including increased infection risk. With DM, the most common site of infection is the urinary tract.[1] To date, the mechanisms that predispose diabetics to urinary tract infection (UTI) have not been elucidated. Recent interest in the molecular defenses of the urinary tract suggests that altered antimicrobial peptides (AMP) production predisposes patients to developing UTI.[2-5] Data from our laboratory show that DM patients have decreased urinary AMP levels. Our data also suggest that insulin induces AMP production via the phosphatidylinositide 3-kinase (PI3K) signaling pathway to shield the uroepithelium from uropathogenic *E. coli* (UPEC). Thus, we hypothesize that insulin bioavailability and PI3K/AKT signaling are essential for AMP production. We also hypothesize that DM patients have increased UTI risk due to altered AMP regulation. Expanding on our preliminary studies, we propose the following aims:

Specific Aim 1: Evaluate how insulin therapy and PI3K/AKT signaling affect AMP production in DM patients.

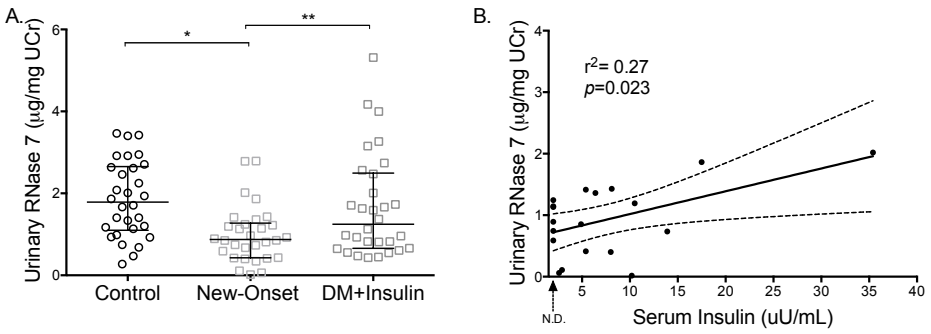
Specific Aim 2: Identify how insulin receptor deletion in the renal intercalated cells alters bacterial defense.

The proposed research will (A) develop a novel model to evaluate how insulin and downstream PI3K signaling regulate renal AMP production and (B) identify a specific and essential regulatory mechanism that shields the uroepithelium from uropathogens. We will apply the knowledge generated from this proposal to future studies evaluating how modulation of the insulin-signaling pathway can enhance AMP production, decrease UTI susceptibility in high-risk DM populations, and translate these strategies to other DM-infectious complications. Ultimately, these findings will reduce healthcare costs and enhance the quality of health in DM patients.

Progress on Aim 1: Evaluate how insulin therapy and PI3K/AKT signaling affect AMP production in DM patients.

Results: To evaluate how insulin regulates AMPs, we evaluated how insulin impacts RNase 7 expression. RNase 7 is a member of the antimicrobial RNase A Superfamily. RNase 7 rapidly kills Gram-positive and Gram-negative uropathogens at low micromolar concentrations, and it has been described as the most potent *human* AMP.[6-8] Our research group has identified RNase 7 as a front-line human AMP that is produced by the bladder urothelium and renal ICs.[7, 9] RNase 7 is one of the most abundant AMPs in urine.[3] When it is neutralized in human urine, urinary bacterial growth increases – suggesting that deficient urinary RNase 7 production increases UTI susceptibility.[9, 10]

Figure 1. Insulin Increases Urinary RNase 7 Concentrations

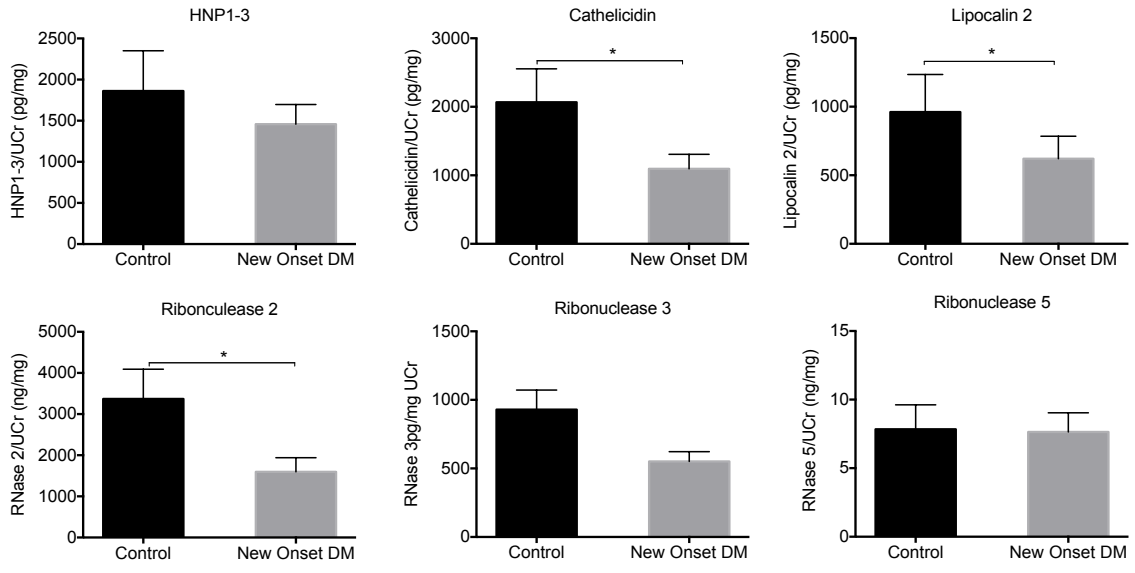


(**A**) Urinary RNase 7 in control and new-onset T1DM patients (*n*=30) before and after starting insulin. Horizontal bars denote median RNase 7/UCr concentrations and the 25th/75th percentiles. * indicates significant p-values <0.05 (Mann-Whitney U test). (**B**) Pearson correlation shows urinary RNase 7 correlates with serum insulin in patients with new-onset T1DM.[57]

Because people with DM have an increased risk to develop infection, we hypothesized that they have suppressed urinary RNase 7 production – rendering them more susceptible to UTI. Also, because DM is associated with insufficient insulin action, we hypothesized that insulin regulates RNase 7. To begin to investigate how insulin affects RNase 7, we quantitated urinary RNase 7 levels from children who presented to the Emergency Department with new-onset, insulin-deficient T1DM vs. healthy children. From our recently published manuscript, ELISA shows that RNase 7/urine creatinine (UCr) levels are significantly lower in T1DM children (**Figure 1A**).[11] Also, serial urine samples were collected from T1DM patients 30 days after starting insulin. ELISA indicates that median RNase 7 levels increased two-fold with insulin (**Figure 1A**). In the new-onset T1DM patient cohort, insulin levels were measured in 19/30 children at ED presentation. In these 19 children, we observed a moderate positive correlation between urinary RNase 7 and serum insulin levels – suggesting that insulin induces RNase 7 (**Figure 1B**). No correlation was found between RNase 7 and serum/urine glucose, hemoglobin A1C, or glomerular filtration rate.[11] There results were recently published in *Kidney International*.[11]

In addition to RNase 7, we also quantitated the expression of several other urinary AMPs with ELISA using the same samples outlined above. Our results show that urinary AMPs like cathelicidin, lipocalin 2, and Ribonuclease 2 (RNase 2) are also significantly suppressed in states of insulin deficiency/new onset T1DM (**Figure 2**).

Figure 2. Urinary AMPs Concentrations



Urinary AMP concentrations in control and new-onset T1DM patients (n=25) normalized to urine creatinine.
* indicates significant p-values <0.05 (Mann-Whitney U test).

These results address the primary outcome objective in **Specific Aim 1** of our initial proposal. In our initial application submission, we proposed to evaluate cellular AKT/Protein Kinase B activity from mononuclear cells collected from people with DM before and after starting insulin therapy. Unfortunately, the method in which we processed the mononuclear cells was insufficient to develop high-quality protein lysates to assess AKT activity. Therefore, we recently revised our protocol to complete this study. However, using the the above cellular fractions, we were able to isolate RNA and perform qRT-PCR on mononuclear cells from healthy control patients and patients with new-onset insulin deficient DM. Our results show that mRNA expression of AMPs, including Ribonuclease 6 (RNase 6), alpha defensin-1, and cathelicidin are significantly lower in patients with DM compared to healthy controls (not shown).

Work in Progress: Once sample collection above is complete, we will continue to assess AKT activity and serum insulin levels as outlined in our initial proposal. We will also continue to collect serial blood samples from healthy controls and DM patients after starting insulin to assess if AMPs increase in the mononuclear cell fractions at both the transcript and peptide levels.

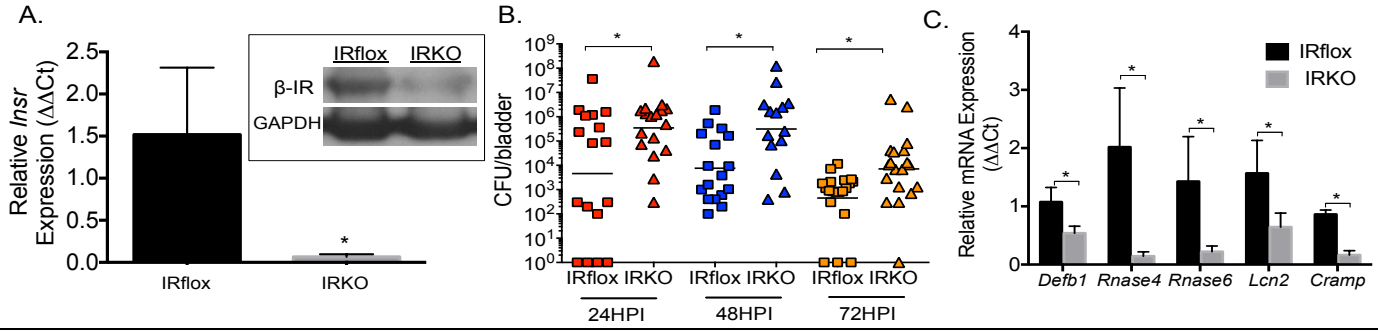
Progress on Aim 2: Identify how insulin receptor deletion in the renal intercalated cells alters bacterial defense.

Results: To evaluate insulin's impact on host defense, we selectively deleted IR from the kidney's intercalated cells (IC) using a Cre-loxP approach. Escalating evidence suggests that ICs have key roles in maintaining urine sterility. Mice homozygous for the floxed IR gene ($IR^{fl/fl}$), which possess *loxP* sites flanking exon 4 of *Insr* gene, were crossed with V-ATPase-Cre mice. Successful deletion of the loxP-flanked exon 4 by V-ATPase-Cre recombinase causes a

frameshift mutation that stops IR translation at amino acid 308 of the mature protein.[12] V-ATPase-Cre mice express Cre-recombinase under the IC-specific *Atp6v1b1* promoter. V-ATPase-Cre(+);$IR^{fl/+}$ progeny were bred with $IR^{fl/fl}$ mice, generating V-ATPase-Cre(+);$IR^{fl/fl}$ offspring that lack IR in the ICs ($IR^{IC/IC}$, which will be referred to as IRKO). In addition, we incorporated the Cre-dependent tdTomato (tdT) fluorescent protein variant reporter strain into our breeding scheme to allow us to detect Cre-mediated recombination by immunofluorescence and to isolate ICs by fluorescent activated cell sorting (FACS). All mice are on a C57BL/6J genetic background. One of the benefits of using the IRKO model is that it allows us to evaluate the effects of insulin without the confounding effects of hyper/hypoglycemia.

When compared to V-ATPase-Cre(-) littermates (IRflox), IRKO mice exhibit a normal phenotype with normal serum insulin levels, normal serum/urine pH, and no evidence of impaired glucose homeostasis. qRT-PCR and Western blot (WB) confirmed IR mRNA and protein knockdown in FACs-isolated ICs (**Figure 3A**). To evaluate how IR deletion impacts host defense, female mice were transurethrally challenged with 10^7 CFU UPEC (strain UTI89). IRKO mice had significantly greater UPEC bladder burden 24, 48, and 72 hrs post-infection (HPI; **Figure 3B**). *These results suggest that intact IC insulin signaling is critical for UTI defense. Moreover, they indicate that the hyperglycemic environment alone does not explain DM-associated UTI risk.* These findings support evidence, from our research team and others, that alterations in IC function impact lower urinary tract UPEC clearance.

Figure 3. Insulin Receptor Deletion Increases UTI Risk

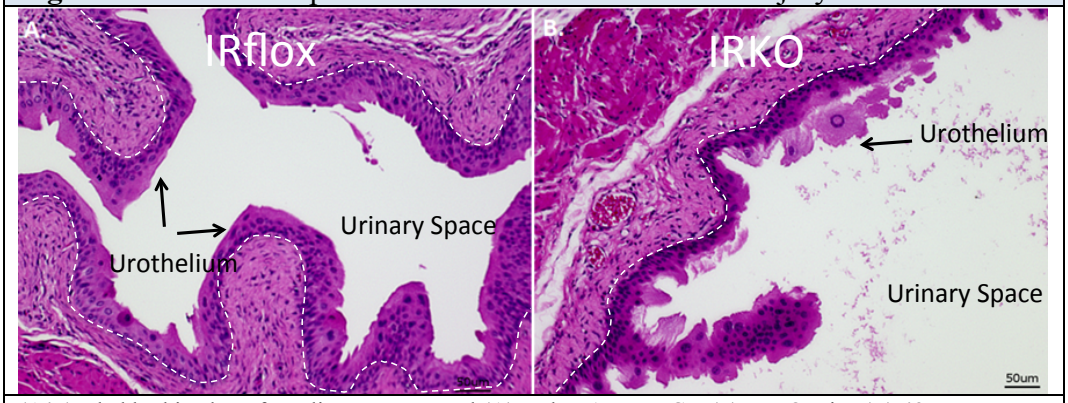


(**A**) qRT-PCR and WB (inset) confirm IR mRNA and protein knockout in FACS-isolated ICs from IRKO mice. (**B**) UPEC burden was significantly greater in IRKO mouse bladders at the indicated time points. The horizontal line indicates the geometric mean. (**C**) AMP mRNA expression was suppressed in FACs-isolated ICs from non-infected IRKO mice. * indicates significant *p*-values < 0.05.

To begin to investigate why IRKO mice have increased UPEC susceptibility, we performed targeted qRT-PCR on AMPs produced by ICs. qRT-PCR on FACS-isolated ICs shows that IRKO mice have decreased transcript expression of several AMPs, including defensins, ribonucleases, lipocalin 2, and cathelicidin (**Figure 3C**). We are in the process of confirming that AMP peptide expression is also suppressed in FACs-isolated ICs and in murine urine. The data presented above help address the hypothesis presented in our initial application and address the primary (and part of the secondary outcome measures) in our grant submission. In addition, although not proposed in our initial application, we also selectively deleted IR in the bladder urothelium using a tamoxifen inducible uroplakin 2-Cre (UPK2, Jackson Stock #024768). Emerging data from our laboratory show that deletion of IR in the bladder urothelium also increased UTI susceptibility similar to that shown above (**Figure 3B**).

Work in Progress: Currently, we are in the process of completing a histopathological evaluation from the kidneys and bladders from our Cre(+) and Cre (-) mice. Preliminary studies show that kidney and bladder morphology is similar in non-infected Cre(+) and Cre (-) animals. However, when Cre(+) animals are infected, there is significant urothelial injury (**Figure 4**).

Figure 4. Insulin Receptor Deletion Increases Urothelial Injury with UTI



(**A/B**) Bladder histology from littermate control (A) and V-ATPase Cre (+) IRKO mice (B) 48 HPI. Results show significant urothelial injury in IRKO mice compared to controls. Dashed lines demarcate the urothelium from the bladder stroma. Magnification 20X.

We are also continuing to generate IRKO mice using both the V-ATPase-Cre and UPK2-cre. As outlined above, we are in the process of confirming that AMP peptide expression is suppressed in FACs isolated ICs. We are also in the process of determining if AMPs are suppressed in the urothelium of mice in which IR is deleted from the bladder urothelium.

Finally, in **Specific Aim 2** of our grant application, we proposed to assess PI3K/AKT activity in these mice. Western blot did not show suppressed basal PI3K/AKT activity in FACs-isolated ICs or bladder urothelium. Moreover, WB did not identify suppressed activity of the RAF/MEK/ERK pathway (not shown). Thus, we are in the process of determining if activity of these classical insulin-mediated pathways is altered in response to systemic insulin therapy. Results from treating IRKO mice with insulin are currently pending.

3. Publications generated from this proposal:

Eichler TE, Becknell B, Easterling RS, Ingraham SE, Cohen DM, Schwaderer AL, Hains DS, Li B, Cohen A, Metheny J, Tridandapani S, Spencer JD. Insulin and the phosphatidylinositol 3-kinase signaling pathway regulate Ribonuclease 7 expression in the human urinary tract. Kidney Int. 2016 Sep;90(3):568-79. doi: 10.1016/j.kint.2016.04.025. PubMed PMID: 27401534.

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1. Korbel L, Spencer JD. Diabetes mellitus and infection: an evaluation of hospital utilization and management costs in the United States. *Journal of diabetes and its complications* 2014.
2. Zasloff M. Antimicrobial peptides, innate immunity, and the normally sterile urinary tract. *J Am Soc Nephrol* 2007; **18:** 2810-2816.
3. Spencer JD, Schwaderer AL, Becknell B*, et al.* The innate immune response during urinary tract infection and pyelonephritis. *Pediatr Nephrol* 2014; **29:** 1139-1149.
4. Chromek M. The role of the antimicrobial peptide cathelicidin in renal diseases. *Pediatr Nephrol* 2014.
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6. Boix E, Nogues MV. Mammalian antimicrobial proteins and peptides: overview on the RNase A superfamily members involved in innate host defence. *Mol Biosyst* 2007; **3:** 317-335.
7. Spencer JD, Schwaderer AL, Wang H*, et al.* Ribonuclease 7, an antimicrobial peptide upregulated during infection, contributes to microbial defense of the human urinary tract. *Kidney Int* 2013; **83:** 615-625.
8. Wang H, Schwaderer AL, Kline J*, et al.* Contribution of Structural Domains to the Activity of Ribonuclease 7 against Uropathogenic Bacteria. *Antimicrob Agents Chemother* 2013; **57:** 766-774.
9. Spencer JD, Schwaderer AL, Dirosario JD*, et al.* Ribonuclease 7 is a potent antimicrobial peptide within the human urinary tract. *Kidney Int* 2011; **80:** 174-180.
10. Spencer JD, Schwaderer AL, Eichler T*, et al.* An endogenous ribonuclease inhibitor regulates the antimicrobial activity of ribonuclease 7 in the human urinary tract. *Kidney Int* 2014; **85:** 1179-1191.
11. Eichler TE, Becknell B, Easterling RS*, et al.* Insulin and the phosphatidylinositol 3-kinase signaling pathway regulate Ribonuclease 7 expression in the human urinary tract. *Kidney Int* 2016; **90:** 568-579.
12. Bruning JC, Michael MD, Winnay JN*, et al.* A muscle-specific insulin receptor knockout exhibits features of the metabolic syndrome of NIDDM without altering glucose tolerance. *Mol Cell* 1998; **2:** 559-569.