PROGRESS REPORT – NIH AMDCC Pilot & Feasibility Award 2010-2011
NCE through 5/31/2012

A. Specific Aims

AIM #1a: Measure ALDH2 activity and 4-HNE levels in glomeruli from diabetic vs. non-diabetic mice by immunohistochemistry. Correlate levels of 4-HNE with histologic (podocyte number, mesangial matrix accumulation, TGFβ1, fibronectin, and type IV collagen) and clinical markers of disease (albuminuria).

AIM #1b: Test whether the newly developed activator (Alda1) of carbonyl-degrading enzyme, ALDH2, will prevent injury in diabetic mice. Treat diabetic mice with Alda1 and quantify decreases in reactive carbonyls by immunohistochemistry, and reduction of histologic and clinical markers of DKD.

AIM #2a: Quantify increases in reactive carbonyls and activity of ALDH2 in mesangial cells under hyperglycemic conditions.

AIM #2b: Elucidate the mechanisms of carbonyl stress-induced glomerular injury. (1) Treat mesangial cells directly with 4-HNE and (2) Treat cells under hyperglycemic conditions with Alda1, and measuring for increases or decreases respectively, in mediators of mesangial cell injury.

B. Studies and Results

During the past year, we have been delayed in completion of AIM #1a due to a change of personnel in the laboratory due to health reasons for our prior postdoctoral fellow. A No-Cost extension has been granted to complete these experiments.

B1a. We have measured ALDH2 activity in diabetic and non-diabetic samples. These data are shown below. Mitochondrial ALDH activity (measured kinetically with acetaldehyde as a substrate and therefore a surrogate for ALDH2 activity) does not change after 8 weeks of diabetes in this mouse model of diabetic kidney disease.

Mitochondrial ALDH Activity ("ALDH2")- kidney; STZ/Vehicle - 8wks; Sacrifice – 16 wks	
Diabetic DBA/2 mice	2.73 +/- 0.30
Non-Diabetic DBA/2 mice	2.96 +/- 0.94

- Units (μMoles of NADH/min/mg of protein, normalized to mitochondrial ALDH2 expression)
- $P > 0.05$

To quantify 4-HNE in kidney cortex, we originally proposed to use quantitative immunohistochemistry but due to technical issues this results in imprecise measurements. Therefore, we have initiated two new collaborations (Drs. Sanjay Srivastava-Louisville; and Allis Chien-Stanford) to provide internal standards and expertise with quantitation of 4-HNE by LC/MS. These results are currently pending.

The dose of STZ was adequate as evidenced by adherence to the AMDCC protocol and that diabetic DBA/2 mice yield significantly increased urine albumin:creatinine ratios relative to non-diabetic controls.

Further histologic evaluation will also be completed.

B1b. Our preliminary data suggested that Alda1 treatment would decrease 4-HNE and urine albumin:creatinine ratio after 8 weeks of diabetes mellitus, however, a validation control showed that the decrease in albuminuria was due mainly to the vehicle administered rather than the active compound.

Urine ACR; STZ/Vehicle - 8wks; Pump – 12 wks; Sacrifice – 16 wks		
Treatment	Diabetes?	μg urine albumin/gm creatinine
Alda1	Diabetic	25.01 +/- 3.30 (n=4)
Vehicle	Diabetic	31.56 +/- 5.97 (n=4)
Alda1	Non-Diabetic	37.30 +/- 8.28 (n=8)
Vehicle	Non-Diabetic	16.90 +/- 4.63 (n=4)
None	Diabetic	53.13 +/- 2.67 (n=3)
None	Non-Diabetic	32.51 +/- 13.7 (n=2)

Interestingly, the ALDH2 activity was higher in diabetic and non-diabetic mice given Alda1 vs. vehicle. This could indicate a number of possible conclusions: (1) that a larger cohort of mice is needed to detect the decrement in Urine ACR seen with Alda1 in diabetic mice; (2) a longer duration of treatment might bear out a significant difference between diabetic mice treated with Alda1 vs. vehicle; or (3) modulation of ALDH2 does not significantly affect albuminuria in this mouse model of diabetic kidney disease.

ALDH2 activity; STZ/Vehicle - 8wks; Pump – 12 wks; Sacrifice – 16 wks		
Treatment	Diabetes?	μMoles of NADH/min/mg of protein, normalized to mitochondrial ALDH2 expression
Alda1	Diabetic	10.08 +/- 0.57
Vehicle	Diabetic	5.66 +/- 0.53
Alda1	Non-Diabetic	6.62 +/- 0.79
Vehicle	Non-Diabetic	5.07 +/- 0.30
None	Diabetic	2.73 +/- 0.30
None	Non-Diabetic	2.96 +/- 0.94

We are currently tabulating the histologic data to refine our conclusions. We have also move forward with broader experiments to study the possible role of other detoxification enzymes that regulate 4-HNE.

We have also noted that non-diabetic mice showed increased albuminuria with Alda1 treatment. With quantification of 4-HNE we will be able to determine if reduction in low levels of endogenous 4-HNE removes a protective mechanism which results in albuminuria. This could confound the data in diabetic mice and suggests that a longer study duration to precisely determine the optimal timing of Alda1 treatment relative to the duration of diabetes after STZ injection.

B2a. Our quantification of 4-HNE adducts by western blot in mesangial cell cultures has shown that hyperglycemia increases 4-HNE at 96 but not 24 hours. We are confirming the amount of free 4-HNE and 4-HNE metabolites by LC/MS and GC/MS as described in B1a. ALDH2 activity measurements in mesangial cells have been delayed due to technical issues with the quality of mitochondrial preps when transitioning from the prior to current postdoctoral fellow.

B2b. Our mesangial cell culture model (MES-13 cells) shows increases in TGF-beta and fibronectin at 24 hours of hyperglycemic conditions by real-time PCR (data not shown), confirming intermediate end-points of mesangial cell injury in diabetic kidney disease. We will be adding Alda1 vs. vehicle to test whether modulation of ALDH2 activity changes these end-points *in vitro*.

C. Significance

These ongoing experiments will establish the role of both 4-HNE adducts and ALDH2 activation in the pathogenesis of diabetic kidney disease. These aims address a fundamental shift in the paradigm for treatment of oxidative stress by using a drug compound which catalyzes removal of reactive carbonyls rather than mass action (the current mechanism of general antioxidants, e.g. Vitamin C, E). These findings will have implications for the pathophysiology of all microvascular complications of diabetes mellitus.

D. Plans

D1. We will continue to tabulate our *in vivo* data for 4-HNE quantification and glomerular histology. The unexpected difference in albuminuria with Alda1 treatment of non-diabetic mice necessitates a more thorough characterization of 4-HNE levels post-STZ injection.

D2. Upon completion of the in vivo studies, we will treat cells *in vitro* with Alda1 vs. vehicle to determine the effects of Alda1 on 4-HNE levels, TGF-beta, and fibronectin in cells treated with high vs. low (25 vs. 5 mM) glucose. We will also prepare abstracts and a manuscript providing our results and conclusions generated due to the generous funding of the Pilot & Feasibility award.

Other Activities Planned for the Next Year

During the no-cost extension, Dr. Bhalla will continue to work with the multiple collaborators which this funding mechanism has inspired. He will also attend and participate in campus-wide and international scientific conferences. Dr. Bhalla presented at the “Epithelial Physiology and Cell Biology” conference in Telluride, Colorado in July 2010, and presented at the American Society of Nephrology conference in November 2010. Dr. Bhalla will also continue as a contributing member of the editorial boards for the *American Journal of Physiology-Renal Physiology*, *Frontiers in Physiology – Renal and Epithelial Physiology*, and the *Journal of Diabetes Mellitus*. He also serves on an American Heart Association study section and reviewed grants for the DCC-Pilot & Feasibility Awards for 2011. The awardee will maintain similar clinical commitments (4-6 weeks / year of inpatient nephrology consult attending and one weekly nephrology clinic) and continue to mentor the postdoctoral fellow within his independent laboratory.

In the coming year the awardee will complete the aims during the no-cost extension. This will form the basis of preliminary data for a competitive R01 application likely to be submitted in Fall 2012. Furthermore, the data will assist the postdoctoral fellow to write a stronger application for his independent funding.

E. Publications

The awardee has trained one postdoctoral fellow in the pathophysiology of carbonyl stress and its effects on the diabetic kidney. We have two manuscripts in preparation from this work on Aims #1 and #2.

F. Project-Generated Resources
We have collected and stored tissue and urine samples at week 16, 20, and 24 in diabetic and non-diabetic DBA/2J mice treated with vehicle or Alda1. These samples will be available for future tests as needed or requested by colleagues upon acceptance of our manuscript on correlation of 4-HNE and its metabolism in these mouse models of diabetic kidney disease.

Collaborator/Consultant Update
The two consultants for the principal investigator (Drs. Mochly-Rosen and Tim Meyer) are still actively involved in the project. Additionally, we have added Drs. Sanjay Srivastava and Allis Chien to assist with quantification of 4-HNE from tissues and cells.

Vertebrate Animals
Our animal protocol has been renewed for the duration of the no-cost extension, and there are no anticipated changes to the protocol.

Select Agent Research
Not applicable