Animal Models of Diabetic Complications Consortium
(U01 DK076133)

Annual Report
(2010)

"Adiponectin and Nox4 in Diabetic Kidney Disease"
University of California, San Diego

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Part A:

Principal Investigator's Summary

1. Program Accomplishments:

Our original proposal sought to determine the role for adiponectin and Nox4 in diabetic kidney disease. **Our hypothesis was that adiponectin and Nox4 are key modifier genes for diabetic nephropathy and diabetic cardiovascular complications.** As described in the last annual report, the two new mouse models of Diabetic Nephropathy and Vascular Complications that we proposed were the:

a. diabetic adiponectin KO mouse
b. an inducible tissue specific Nox4 transgenic diabetic mouse

Recent Progress and Major Accomplishments
Project a. Over the past year, we have completed studies evaluating the effects of adiponectin deficiency on chronic type 1 diabetes induced by the low dose strep protocol in C57Bl6J male mice. The data was presented at the last steering committee meeting and as a free communication in the 2009 ASN meeting in San Diego. We found that the *Apn* KO mouse mimics several features of diabetic kidney disease in the absence of diabetes and hyperglycemia. This appears to be largely via suppression of AMPK, the energy sensing pathway. There is a reduction of AMPK with established diabetes and in the Apn KO mouse without diabetes. With the induction of diabetes in the *Apn* KO mouse, there is no further reduction in AMPK although there is an enhancement of glomerular changes with diabetes. The diabetic *Apn* KO mouse has an exaggerated increase in albumin/creatinine ratio, glomerular enlargement and glomerular matrix expansion with 4 months of diabetes. With diabetes as long as 9 months there is a persistent increase in the glomerular parmeters, however there is a reduction in albuminuria.

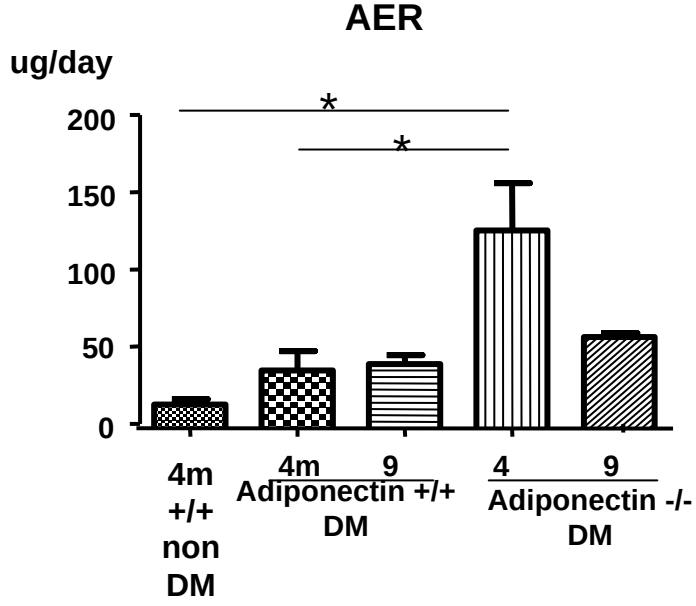

Figure 1. The increased albuminuria in the diabetic adiponectin KO mouse is reduced at 9 months of diabetes.

The surprising reduction in albuminuria is likely not due to a major impairment in renal function as the plasma creatinine by HPLC only showed a modest increase with duration of diabetes (Figure 2). With early diabetes (4 months) there was a significant increase in glomerular surface area and mesangial matrix accumulation (Figure 3), however there was no further progression at 9 months of diabetes in the *Apn* KO diabetic mouse. The basis for the reduction in albuminuria may be due to a partial restoration of AMPK activation with chronic diabetes, even in the absence of adiponectin (data not shown). The restoration of AMPK activation may be part of an overall compensatory response in the Bl6 mouse that attenuates a

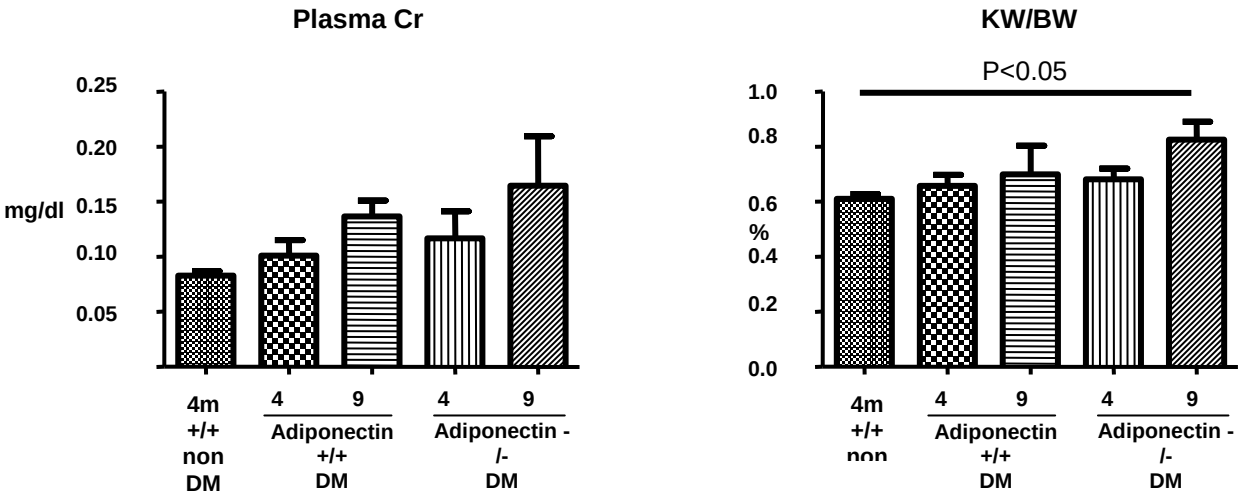

Figure 2. Parameters of diabetic kidney disease in early and advanced diabetes in *Apn* KO mice. There is a modest increase in plasma creatinine in the Apn KO diabetic mouse at 9 months (not significant) and an increase in diabetic kidney hypertrophy at 9 m (vs control mice).

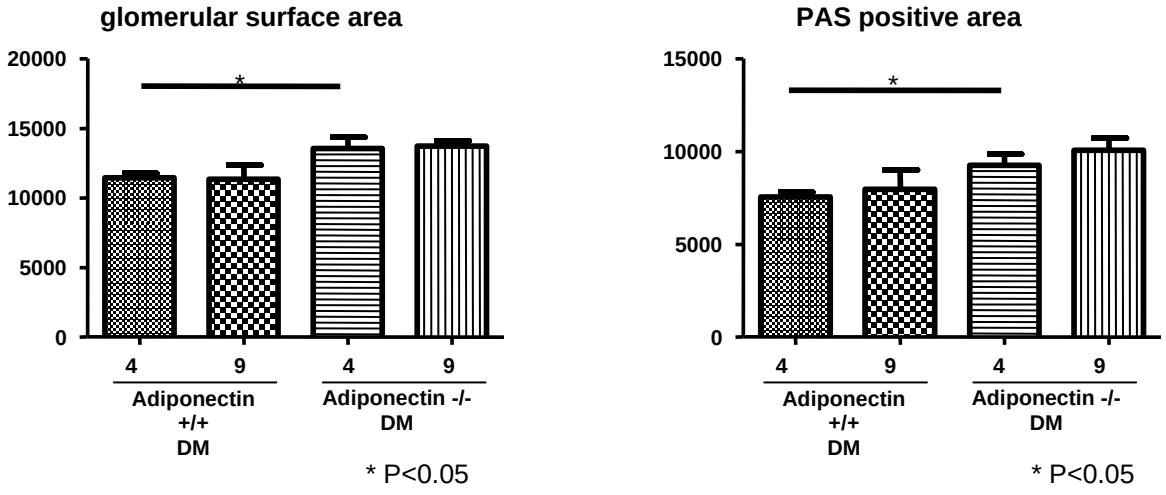

Figure 3. Glomerular histology in early and advanced diabetes in Apn KO mice. Glomerular surface area and mesangial matrix area is increased at 4 months of diabetes in Apn KO mice (vs WT diabetic), however there is no further increase at 9 months of diabetes.

further decline in glomerular podocyte function. The degree of hyperglycemia remained at 400-500 mg/dl and insulin levels are pending. The relevance of adiponectin in human diabetic kidney disease is indicated as studies in biopsy samples reveal that adiponectin is reduced in deposition in the kidney with advanced diabetic nephropathy vs healthy control kidneys. Immunostaining with antibody to adiponectin demonstrates staining is reduced in glomeruli of patients with

advanced diabetic nephropathy (Figure 4). The data is being incorporated into a manuscript that will be submitted by August 2010.

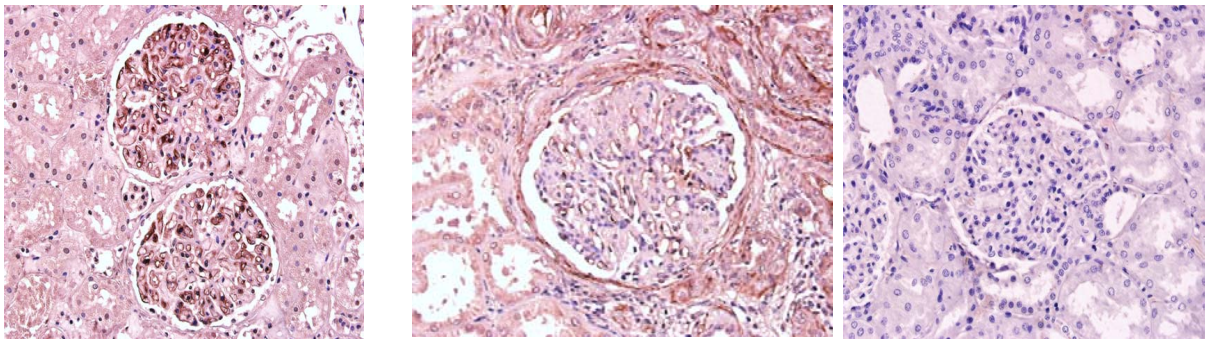

Figure 4. Adiponectin staining of normal human kidney (left), diabetic kidney (center), negative control (right). There is presence of adiponectin in glomerular capillary loops and podocytes in normal human glomeruli with a marked reduction with established nephropathy.

The data with the *Apn* KO mouse kidneys has been mimicked in relation to heart disease as well. Studies performed independently by the Walsh group has demonstrated that the Apn KO mouse develops worse cardiac hypertrophy and fibrosis with aortic banding. We hope to involve other AMDCC investigators (Dale Abel, Ira Goldman) to study heart and nerve (Feldman, Calcutt) with the next round of diabetic studies with Akita-induced diabetes.

We have begun to characterize albuminuria and AMPK in two models that are commonly used by the AMDCC investigators, i.e. the Akita mouse and the high fat fed mouse on C57Bl6J background. Our data with the high fat fed mice is shown below. The mice gain weight more rapidly with high fat feeding as compared to standard chow (Figure 5), develop hyperglycemia (Figure 5), and kidney and heart enlargement (Figure 6).

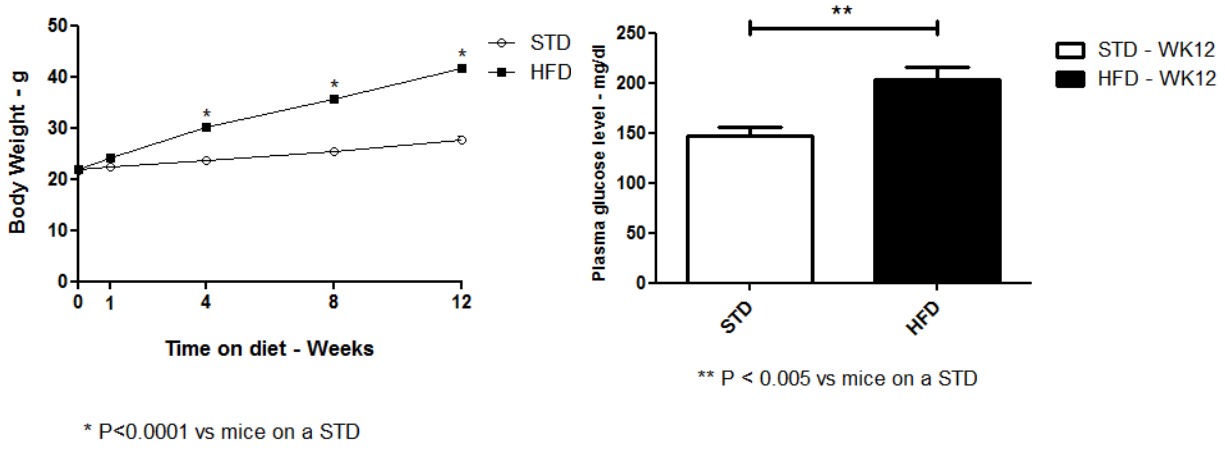

Figure 5. Pregressive weight gain and significant hyperglycemia with high fat feeding. C57Bl6J mice were given standard chow (5% fat) vs high fat diet (60%) nfor a period of 12 weeks. There is progressive weight gain and significant non-fasting blood glucose levels at the end of the dietary intervention.

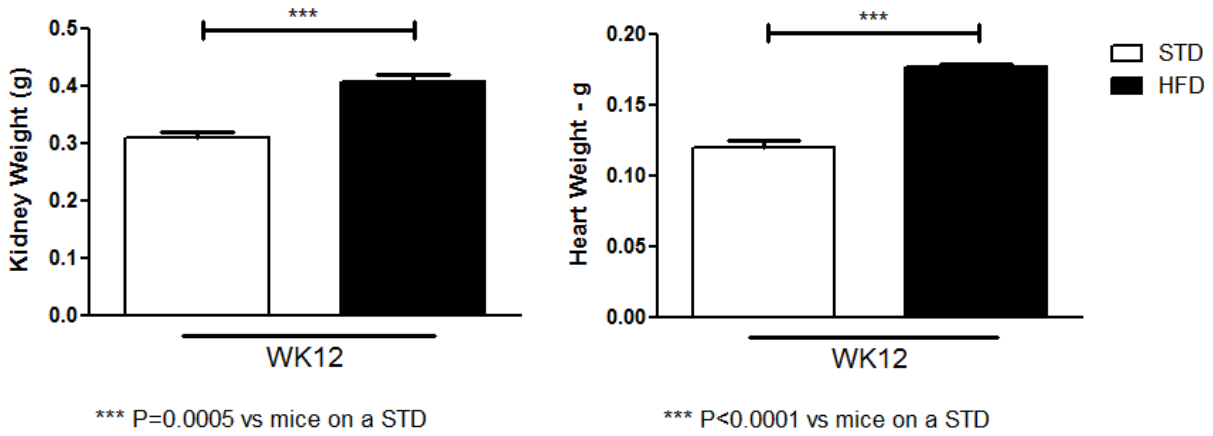

Figure 6. Significant renal and cardiac weight gain with high fat feeding. Kidneys and hearts were weighed after 12 weeks on high fat diet (n=6 per group).

Of great interest is that the urine albumin excretion was markedly increased (>5-fold) after just 12 weeks on the high fat diet (Figure 7, left panel). The increase in albuminuria in the Bl6J mouse with high fat feeding is much greater than with 4-9 months of diabetes (compare with Figure 1). Also of great interest is that there is a very early increase in the inflammation marker (hydrogen peroxide) in the urine with just 1 week of high fat feeding (Figure 7 right panel).

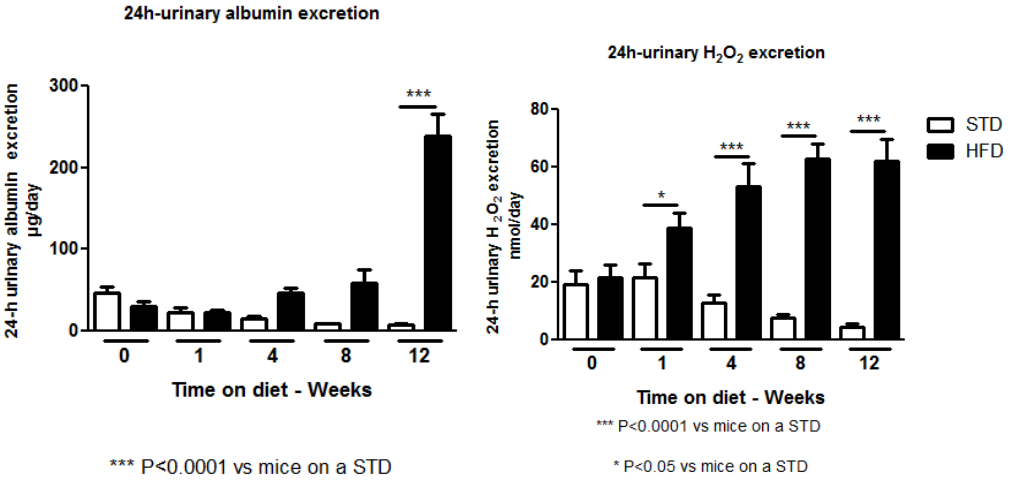

Figure 7. Urine albumin and urine hydrogen peroxide are increased following high fat feeding in Bl6J mice. The increase in urine hydrogen peroxide precedes the increase in urine albumin excretion in the high fat (60%) fed mouse.

The increased urine hydrogen peroxide may indicate stimulation of NADPH oxidase function in the kidney. As we had previously shown that AMPK regulates Nox activity, we evaluated AMPK activity with immunostaining with a P-AMPK antibody using double staining

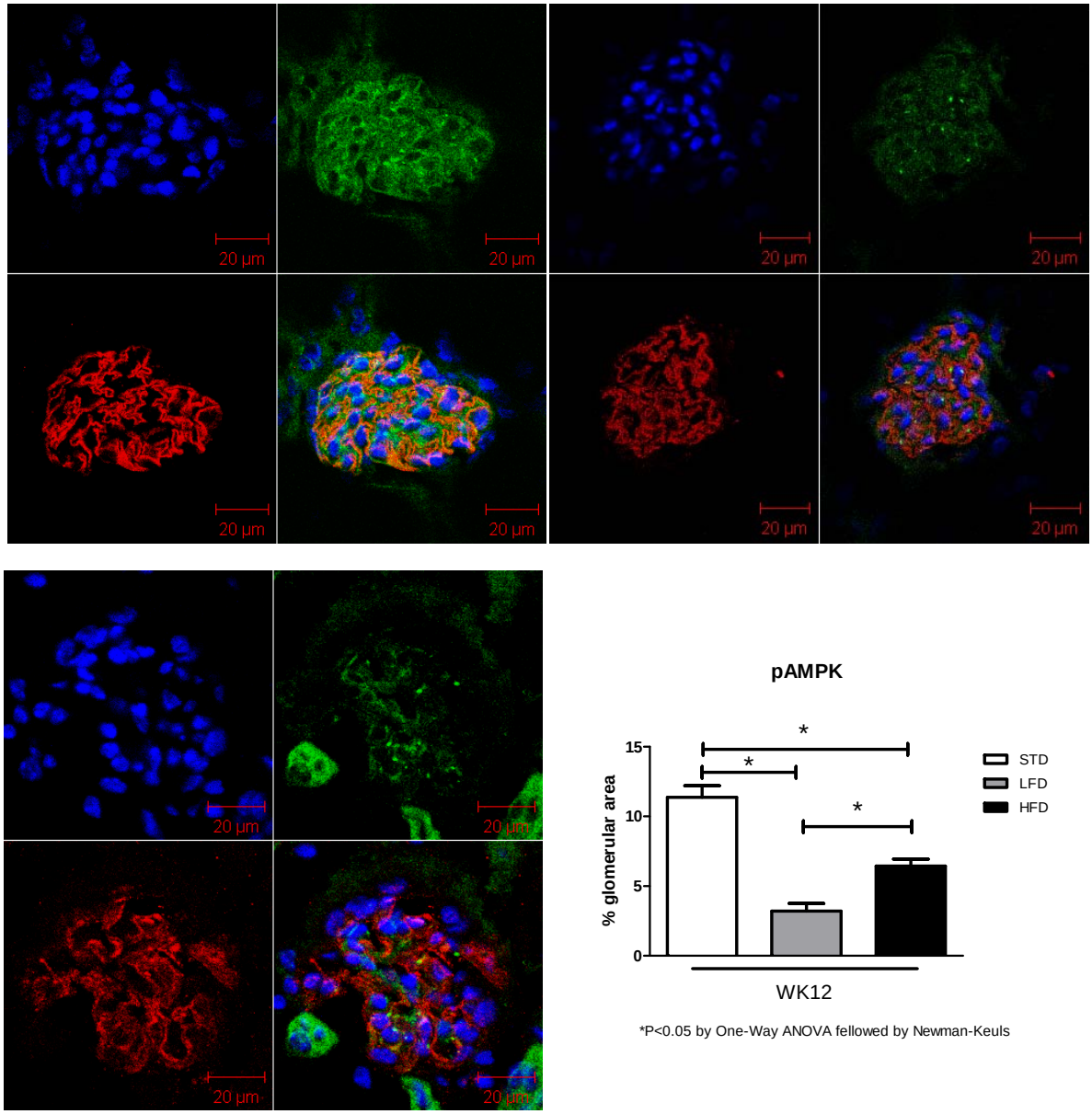

Figure 8. Confocal analysis of immunofluorescent staining for P-AMPK (green) and podocin (red). Mice were fed a standard diet (<5% fat, upper left panels), a low fat/high carb diet (<5% fat, 70% carbs, upper right panels), and a high fat diet (60% fat, lower left panels) for 12 weeks. Quantitative data derived from the fluoresecent images is depicted in the lower right panel.

A key discovery that emanted from the prior study of adiponectin in kidney disease was the identification that podocytes are a target cell for adiponectin, and via its AdipoR1 receptor responds to adiponectin via stimulation of the AMPK. The further study of AMPK has been enormously fruitful. Based on our initial studies with the *Apn* KO mice where AICAR was able to inhibit the albuminuria in these mice, we have examined the AMPK pathway in models of caloric excess, such as high fat and Akita diabetes. With both models there is a reduction of glomerular PAMPK. Ongoing studies will evaluate the role of AMPK activation in these models.

Further study on the role of adiponectin, the adipo receptors and AMPK are being pursued with new grant funding from a VA merit award. We have published several reviews on this topic (see publication list) and will be submitting a manuscript on the role of AMPK in obesity related and diabetic kidney disease this summer. The current data will be presented at the steering committee meeting in August, 2010.

Project b. To understand the role of NAPDH oxidase in diabetic kidney disease, we evaluated the gene expression of the various Nox isoforms and their membrane and cytosolic partners with multi-low dose STZ in Bl6J mice. Gene expression in wild type and Nox2 KO kidneys revealed that Nox2 and Nox4 are the most highly expressed in the normal kidney (Figure 9).

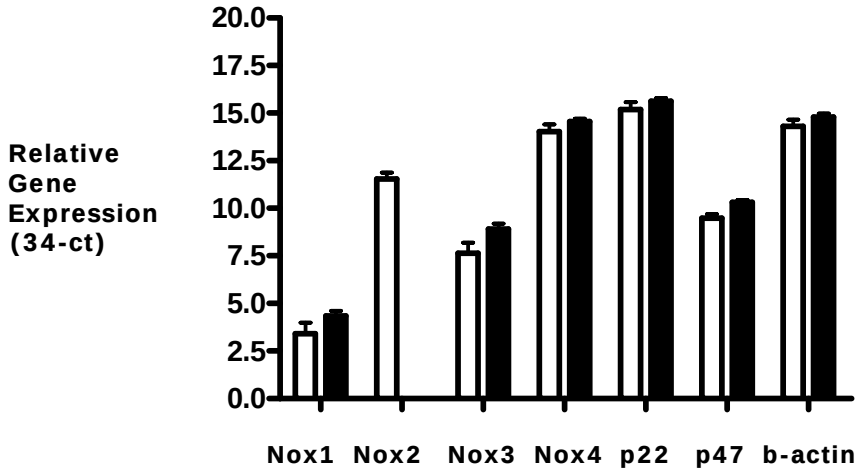

Figure 9. Real time PCR analysis of Nox and Nox subunits in the non-diabetic kidney in WT (white bars) and Nox2 KO mice (black bars). The most prominent Nox isoforms that are expressed in the kidney is Nox2 and Nox4

To evaluate the role of Nox2, the Nox2 KO mice (from Jackson Labs, on the C57Bl6J background) was made diabetic with low dose STZ and studied after 2 months of diabetes. There was no significant difference between blood glucose, kidney hypertrophy, urine albumin excretion, and urine hydrogen peroxide in the two groups of diabetic mice (Figure 10). There was a marked stimulation of glomerular Nox4 in the Nox2 KO diabetic mice may have compensated for the reduction in Nox2.

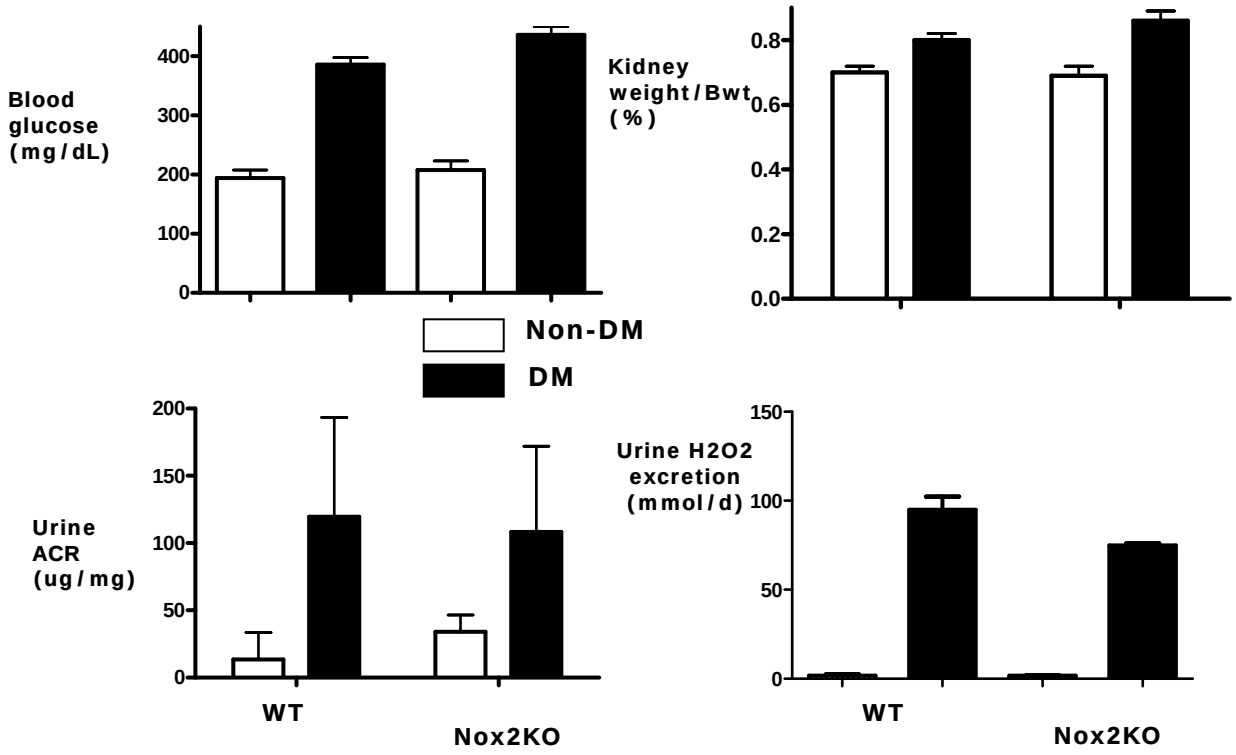

Figure 10. Wild type and Nox2 KO (gp91 KO) mice were made diabetic with multi low dose STZ protocol. There is a similar increase in blood glucose (upper left), kidney hypertrophy (upper right). Urine albumin (left bottom) and urine hydrogen peroxide levels were increased with diabetes but similar in WT and Nox2 KO mice.

To evaluate the functional role of Nox4 we recently created the Nox4 podocyte specific inducible tg mouse. We have begun the characterization of several lines that have robust transmission. We have identified 3 lines (46-high expressors, 56-moderate expressor, and 69-low expressors) that we are expanding. Initial studies with the high and moderate expressor mice demonstrate stimulation of the HA-tagged human Nox4 in the glomerular podocytes after 10 days of doxycycline administration (Figure 11). Of great interest is that there was an increase in albumin/creatinine ratio and a significant thickening of the GBM with just 10 days of doxycycline administration to induce podocyte Nox4 (Figure 12). **As GBM thickening is a characteristic feature of diabetes and not well developed in other models, we consider this a major advance in the field.** Furthermore, there appears to be subtle alterations of the podocyte foot processes and glomerular enlargement (data not shown). Thus, a single gene stimulation in podocytes recapitulates many of the cardinal features of diabetic nephropathy, without diabetes. Ongoing studies will evaluate the effect of diabetes on the Nox4 tg mouse. We expect that with persistent upregulation of Nox4 coupled with hyperglycemia there will be a rapid progression of podocyte dysfunction leading to marked albuminuria, GBM thickening, and mesangial matrix expansion. The initial studies in the Nox4 tg mouse will be presented at an international Nexus meeting on Renal Fibrosis in Geneva and further studies will be presented at the ASN in Denver as well as the AMDCC Steering committee meeting.

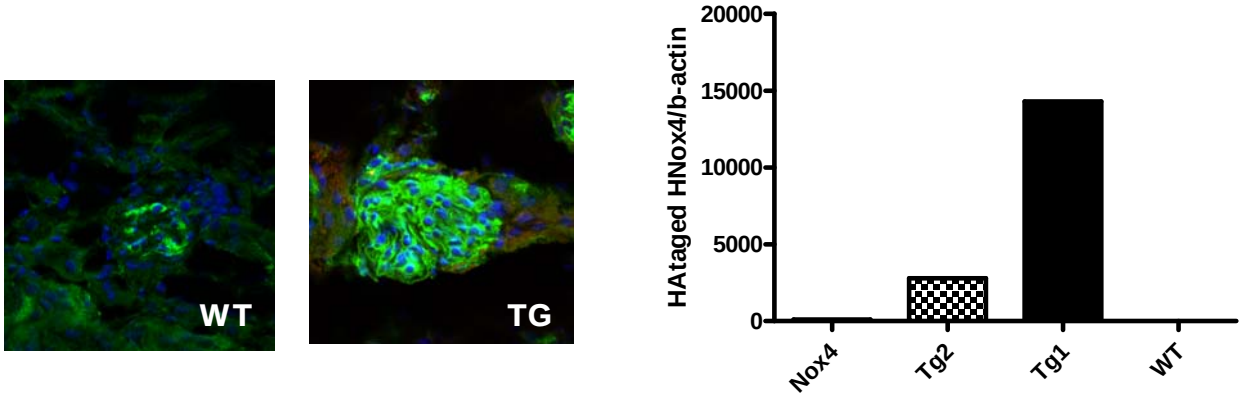

Figure 11. HA Tagged Human Nox4 transgene expression in glomerulus (A), Gene expression analysis of HA transgene by QRT-PCR in isolated GM from kidney by Dynabeads (B) 2 weeks after Doxycycline treatment in wild-type and RT/HAHNox4 bi-TG mice.

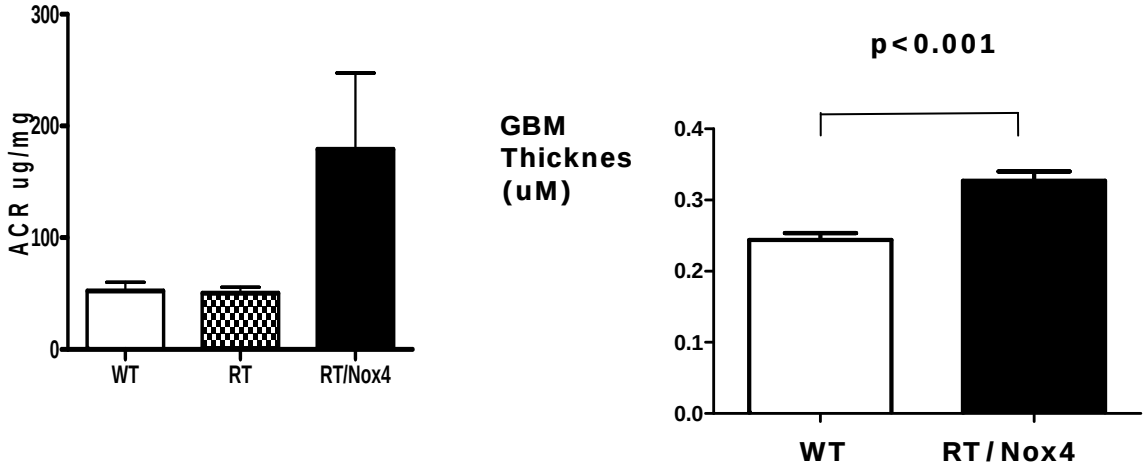

Figure 12. Two lines of high and moderate expressors for podocyte Nox4 tg mouse demonstrate an increase in urine ACR and GBM thickening. The urine ACR was measured in 2 mice per line and averaged together after 10 days of doxycycline. The GBM thickness was measured in coordination with EM analysis of glomeruli that was performed in collaboration with Dr. Marilyn Farquhar (UCSD).

Plans for the Upcoming Year (2010-11)

We will characterize the Akita diabetic *Apn* KO mouse. Initial studies were halted due to severe abdominal pathology that developed in the Akita/Apn +/- mice. Due to loss of this line, additional mice were obtained from Jackson Labs and renewed studies are ongoing with putting the ApnKO mice onto Akita. The studies with the ApnKO/Akita diabetic mice will be completed by the end of the funding period. Both sexes will be studied as it is possible that adiponectin deficiency will lead to a more robust diabetes in females as well as male Akitas.

For the tgNox4 mice, we are quite excited regarding the intial data with two lines of the tg mice. With increased podocyte Nox4 there is impressive GBM thickening and glomerular enlargement, even without diabetes. We have already developed a few Akita mice carrying the double transgene and will be inducing Nox4 with doxycycline to determine if enhanced podocyte Nox4 will lead to progressive diabetic glomerulopathy with a rapid time course. These studies with sufficient mice and at least two separate lines will be completed before the funding period. We have discussed further plans with Racheal Wallace and have set up the following strategy. The 3 lines will be expanded and made homozygous to enhance the development of the Akita mice. The 3 lines will be placed onto the Bl6 background in order to study its role in this strain and determine if the strain is protective. This will also allow us to develop F1 crosses with DBA. Additional studies with the inducible Nox4 tg mice can be studied in other organs and cell types with cell specific promoters on the Bl6 background. We have already received requests from several groups to study the role of Nox4 in liver and heart.

Preliminary Milestones for 2010 and Beyond
By end of 2010 we will have completed the first round of phenotyping of the diabetic inducible Nox4 mice and the Akita Apn KO diabetic mice. In 2011 and 2012 the Apn and Nox4 transgenic mice will be studied in the Bl6 background and renal and cardiovascular complications will be investigated. The mice will be available for additional studies that will likely include retinopathy and neuropathy. Additional studies with fat feeding will determine the role of adiponectin, AMPK and Nox4 in development of obesity related complications.

2. Collaboration:

With other AMDCC PIs
We will make available several new methods for phenotyping diabetic mice. These methods will include mtDNA analysis, ROS imaging analysis, and urine metabolomic analysis. We are continuing to run HPLC creatinines for consortial and non-consortial members.

With Jax We are working closely with Racheal Wallace to identify the best lines to expand of our Nox4 tg lines and to backcross the tg to the Bl6 background.

With the MMPCs We will be submitting the samples from our latest Nox4 tg mice to Dr. Alpers for validation of our findings with light and EM analysis.

With other non-AMDCC PIs In collaboration with Dr. Robert Naviaux, who is a leading member of the Mitochondrial Medicine Society, we have developed methodology for the accurate and quantitative characterization of mitochondrial functional and mtDNA damage indices. In a joint collaboration we have begun a metabolomic analysis of urines in patients with diabetes and have identified several new biomarkers. We will apply these analyses to our mouse studies to determine if they mimic the human condition. We have also begun a collaboration with Dr. Laura Dugan to comprehensively evaluate ROS formation in vivo using DHE labeling and paramagnetic spin resonance techniques. We have now optimized methods to measure ROS in the kidneys of live mice. As these methods are published they will be made available to the AMDCC.

3. Address previous EAC comments:

EAC comments 2009

- Dr. Sharma continues to study aberrant regulation of AMPK in diabetic podocytes. Since publication of JCI paper, most of studies have been qualitative immunofluorescence. Images in slide set do not appear identical technique (different luminosity with DAPI staining). Will need to develop quantitative system to be convincing. Hypothesis is intriguing. If true, may make sense to do metformin trials in human DN, especially since the drug is currently stopped with onset of renal insufficiency [Are we denying a drug where benefit might outweigh risk if appropriately monitored?; Could metformin be a lead compound for drug without the acidosis complication?]. Studies with Nox4 overexpression are less convincing. He should justify the cost of generating the proposed mouse lines (multiple background; significant husbandry costs).

- The data is rather qualitatively provided in the report, especially glycemia, weight gain/food intake and kidney weight.

Response:

a. We are encouraged by the EAC comments regarding the potential role of AMPK as a key pathway in diabetic complications. These studies arose as a result of the evaluation of the signaling pathway by which adiponectin conferred benefit to podocytes. The wild type mice had robust AMPK activity in podocytes whereas Apn KO mice had reduced glomerular AMPK activation. To further evaluate this pathway we have performed additional AMPK studies in mice with diabetes and high fat feeding. In both models we find reduced AMPK activity in glomeruli and restoration with AICAR. We have quantitated these studies and find them to be significant. As the glomeruli have a more consistent pattern with regards to AMPK the studies are limited to immunostaining. We will perform additional studies with glomerular isolates, although the rapid nature of AMPK phosphorylation may be altered with the process of glomerular isolation. Importantly, we now show that stimulation of AMPK with AICAR confers substantial benefit by reducing albuminuria, hydrogen peroxide in the urine and TGF-b upregulation in glomeruli. We agree with the EAC that metformin may theoretically be beneficial for patients with kidney disease. However, the life threatening adverse event of lactic acidosis will need to be carefully considered before clinical trials are instituted to study the effect of metformin in patients with established nephropathy. Other agents that may be more potent and

direct to stimulate AMPK are being evaluated by several pharmaceutical companies and should be evaluated in pre-clinical studies in mice with diabetic kidney disease. Additional studies will evaluate the role of AMPK KO mice and we have already obtained these mice.

b. We have completed analysis of two additional series of studies. 1. The diabetic adiponectin KO mice and 2. The Nox2 KO diabetic mice. The data is included in the report and manuscripts are in final preparation. Both these studies demonstrate that Nox4 is upregulated in the glomerular podocytes and thus further establishes the rationale for the study of Nox in diabetic kidney disease. We have now evaluated 3-5 mice from two separate lines of inducible double transgenic after doxycylcine administration. The data as described in the report shows a significant increase in GBM thickness in the transgenic mice and an increase in glomerular enlargement. Ongoing studies are further characterizing the degree of matrix expansion and podocyte dysfunction in these mice. As no other models have demonstrated GBM thickening and glomerular enlargement from AMDCC models as yet we feel this is a valuable new model that is exciting and potentially could demonstrate severe and progressive disease when coupled with diabetes. The studies with Akita diabetes in the FVB background will be completed by the end of the final funding year.

4. Publications 2009-10:

1. Meijering, B. Els A. van der Wouden, Vincent Pelgröm, Robert H. Henning, **Kumar Sharma,** and Leo E. Deelman. TGF-ß inhibits Ang II-induced MAPK signaling in vascular smooth muscle cells through downregulation of Ang II type 1 receptors. ***Journal of Vascular Research***, 10:459-468, 2009

2. Burnt-out diabetes: impact of chronic kidney disease progression on the natural course of diabetes mellitus. Kalantar-Zadeh K, Derose SF, Nicholas S, Benner D, **Sharma K**, Kovesdy CP. ***J Ren Nutr***. 2009 Jan;19(1):33-7

3. Mechanisms of kidney fibrosis and the role of antifibrotic therapies. Deelman L, **Sharma K.** ***Curr Opin Nephrol Hypertens***. 2009 Jan;18(1):85-90.

4. Bakris GL, Fonseca VA, **Sharma K**, Wright EM. Renal sodium-glucose transport: role in diabetes mellitus and potential clinical implications. ***Kidney Int.*** 2009 Jun;75(12):1272-7.

5. Sanjoy Ghosh, Majid Khazaei, Farzad Moien Afshari, Lisa S. Ang, David J. Granville, Bruce Verchere, Stephen R. Dunn, Peter McCue, Andrew Mizisin, **Kumar Sharma**, and Ismail Laher. Moderate exercise attenuates caspase-3 activity, oxidative stress, and inhibits progression of diabetic renal disease in db/db mice ***Am J Physiol Renal Physiol*** 2009 Apr;296(4):F700-8.

6. Sanchez AP, **Sharma K**. Transcription factors in the pathogenesis of diabetic nephropathy. ***Expert Rev Mol Med***. 2009 Apr 28;11:e13.

7. **Sharma K**. the Link between Obesity and Alkbuminuria: Adiponectin and Podocyte Dysfunction. ***Kidney Int.*** 2009 Jul;76(2):145-8. Epub 2009 Apr 29.

8. Ramachandrarao SP, Zhu Y, Ravasi T, McGowan TA, Toh I, Dunn SR, Okada S, Shaw MA, **Sharma K.** Pirfenidone Is Renoprotective in Diabetic Kidney Disease**. *J Am Soc Nephrol.*** 2009 Jul 2. [Epub ahead of print]

9. Brosius FC 3rd, Alpers CE, Bottinger EP, Breyer MD, Coffman TM, Gurley SB, Harris RC, Kakoki M, Kretzler M, Leiter EH, Levi M, McIndoe RA, **Sharma K,** Smithies O, Susztak K, Takahashi N, Takahashi T; for the Animal Models of Diabetic Complications Consortium. Mouse Models of Diabetic Nephropathy. ***J Am Soc Nephrol*** 2009 Sept 3 [Epub ahead of print]

10. Ix JH, Sharma K. Mechanisms Linking Obesity, Chronic Kidney Disease, and Fatty Liver Disease: The Roles of Fetuin-A, Adiponectin, and AMPK. ***J Am Soc Nephrol*** 2010 [Epub ahead of print]

11. Rossini M, Naito T, Yang H, Freeman M, Donnert E, Ma LJ, Dunn SR, Sharma K, Fogo AB. Sulodexide ameliorates early but not late kidney disease in models of radiation nephropathy and diabetic nephropathy.. **Nephrol Dial Transplant.** 2010 Jan 7. [Epub ahead of print]

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(U01 XX#####)

Part B:

Update by Individual Project Leaders
(not applicable)