Animal Models of Diabetic Complications Consortium
(U01 DK060905-09)

Annual Report
(2008)

"Role and Mechanisms of Epithelial Injury in Diabetic Nephropathy"

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Part A:

Principal Investigator's Summary

1. Program Accomplishments:

Hypotheses

Observations from our laboratories provide a compelling rationale to focus our research program on investigating the emerging role and mechanisms of epithelial cell injury in diabetic nephropathy (DN). Specifically, **we will test two novel hypotheses:** 1. The peroxisomal membrane proteins Mpv17l and/or Mpv17 are essential regulators of antioxidant defenses in glomerular podocytes and protect against diabetes-induced podocyte apoptosis and podocyte depletion (Bottinger, Mount Sinai); and 2. CD36 scavenger receptor for AGE and/or FFA is an essential mediator of AGE and/or FFA-induced tubular epithelial injury/apoptosis and tubulointerstitial progression of DN (Susztak, AECOM).

Progress towards stated milestones

A. Studies of Mpv17-family proteins in DN (Aims 1 and 2; PI: Bottinger)

Aim 1: whether peroxisomal membrane proteins Mpv17 and Mpv17l regulate antioxidant defense mechanisms in podocytes and protect against ROS-mediated podocyte injury/apoptosis induced by diabetes

New directions for in vitro studies (Bottinger lab):

Focus on proximal tubular epithelial cells for Mpv17l, and podocytes for Mpv17:

- Characterize cell-type distribution of Mpv17 family mRNA and protein expression in kidney

Our in vitro and in vivo data demonstrate that Mpv17 and FKSG24 proteins are expressed predominantly in podocytes with modest expression of Mpv17 in tubular epithelia. In contrast, Mpv17l mRNA and protein is not expressed in glomeruli, but is expressed prominently in proximal tubular epithelia.

- Identify mechanisms of downregulation of Mpv17 family mRNA and protein in response to high glucose and tgfb

Our ongoing studies suggest that loss of Mpv17l protein in response to high glucose and tgfb is mediated through proteasome-dependent degradation.

- Identify mechanism of regulation of Htra2/Omi protease function by Mpv17l interaction in mitochondria
- Identify mechanisms of antioxidant and antiapoptotic function of Htra2/Omi protease in mitochondria

The interaction between Htra2 and Mpv17l is mediated by a PDZ domain and induces protease activation of HtrA2 which limits mitochondrial superoxide generation, stabilizes mitochondrial membrane potential, and prevents apoptosis at baseline and in response to extracellular inducers of mitochondrial stress.

Focus on conditional deletion of Mpv17l in mice

- Continuation of development and characterization of mice with conditional Mpv17^{flex3} allele

We generated Mpv17^{flex3} allele carrying mice through targeted allele replacement. However, we noticed difficulties in PCR based genotyping strategies and confirmed by Southern analysis that the 5' arm of the targeting vector was apparently rearranged. Thus, although we achieved germline transmission, we decided to terminate this line and start the process of generating mice with conditional Mpv17^{flex3} allele.

The following milestones depend on successful completion of the previous milestone

- Matings with PTEC-selective Cre transgenic mice for targeted deletion of Mpv17 exon 3 in proximal tubuli in mice. For example, Ksp1-Cre (proximal and distal tubular promoter) and/or Pepck-Cre transgenic (proximal tubular promoter)

- Subject these mice to multiple low dose STZ protocol and other diabetes models

- Send Mpv17^{flex3} mice to JAX lab

Aim 2: whether Mpv17-deficiency accelerates and increases podocyte apoptosis and depletion leading to progressive glomerulosclerosis and/or nodules in diabetic mouse models

- Complete characterization of diabetes in Mpv17-/- knockout mice

We completed this milestone and our observations demonstrate that the Mpv17-/- mice have significantly improved glucose control compared with Mpv17+/+ mice when exposed to diabetes induced by STZ or Akita models (see figure below).

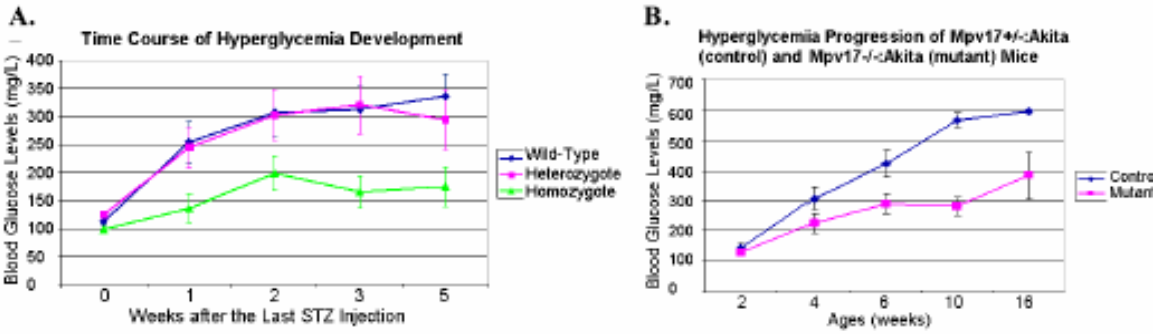

Figure 4. Hyperglycemia progression of Mpv17-/- and control mice subjected to MLDS and $Ins2^{Akita}$-induced diabetes. **A.** Time course of hyperglycemia development of Mpv17-/- mice and control mice (Mpv17+/+ and Mpv17+/-) in MLDS; **B.** Time course of hyperglycemia development of Mpv17-/-:$Ins2^{Akita}$ and control mice (Mpv17+/-: $Ins2^{Akita}$).

- Discuss approach to explore mechanism by which Mpv17 deficiency protects against development of type I diabetes.
- Explore strategies to evaluate whether Mpv17-/- alters metabolic phenotype in type II db/db mice.
-

Based on concerns expressed by the EAC, we are currently not pursuing these two milestones.

<u>B. Studies of role of Cd36 scavenger receptor expression in tubular epithelia in DN (Aims 3 and 4; Co-PI: Susztak)</u>

Based on results previously reported, we conclude that increased proximal tubular CD36 expression plays an important role in diabetic tubular degeneration and renal disease progression, supporting our hypothesis. Ongoing studies are focused on determining the mechanism of CD36 mediated tubular degeneration. CD36 is a fatty acid transported and scavenger receptor, we were interested to analyze whether increased fatty acid uptake via CD36 would be responsible for the observed phenotype. We performed Oil Red-O staining (to detect intracellular lipid accumulation) on control diabetic and transgenic diabetic animals. Our analysis showed increased lipid accumulation in renal tubular epithelial cells. We are currently performing quantitative analysis of total triglyceride contents in kidney homogenates of wild type and transgenic diabetic mice. In addition we have analyzed human kidney biopsy samples obtained from control and diabetic patients (n=5 per groups). We found increased Oil-red-O staining in human diabetic samples as well, indicating that similar mechanism is present in patients with diabetes. Additional studies are being performed to determine the mechanism of the increased lipid uptake mediated tubular damage, examining enzymes on the lipid uptake, oxidation and storage pathways.

Plans for the Upcoming Year

A. Studies of Mpv17-family proteins in DN (Aims 1 and 2; PI: Bottinger)

Priorities are:

1. Generation and characterization of mice carrying Mpv17l^{flex3} alleles for conditional deletion of Mpv17l.

1.1. Use these mice to study the role of Mpv17l in diabetic nephropathy and experimental models of diabetes.

2. Continue characterization of the roles of human Mpv17l type 1 and type 2 splice isoforms in diabetic nephropathy

3. Identify the mitochondrial substrates for Htra2 protease.

4. Continue studies to identify the mechanism of downregulation of Mpv17 family members by glucose and tgfb.

B. Studies of role of Cd36 scavenger receptor expression in tubular epithelia in DN (Aims 3 and 4; Co-PI: Susztak)

1, First priority for the coming year is the completion of the characterization of the CD36 transgenic animals.

A, In addition to the STZ induced diabetic mice we also generated mice with combined CD36 and Akita genotype and with combined CD36/dbdb genotype. Preliminary results indicate that these animals also suffer from premature mortality, renal phenotype analysis is ongoing.

B, Further studies will be aimed to determine the mechanism of CD36 mediated tubular damage and its relationship to lipid uptake an accumulation. We are working on quantitative characterization of lipid accumulation in the kidney and gene expression analysis of lipid uptake, oxidation and storage enzymes. We also would like to set-up a new in vivo imaging assay to monitor lipid uptake in the kidneys (as such studies yielded important information in the heart and cardiovascular complication field). Furthermore the effect of lipid accumulation on mitochondrial biology will also be studied.

C, The in vivo studies will be complemented with in vitro experiments determining the effect of CD36 and lipid uptake on tubular epithelial cells including potential lipotoxicity, apoptosis etc. These studies will be driven primary by our in vivo findings of the diabetic sglt2CD36 transgenic animals.

2. Collaboration:

With other AMDCC PIs

Kumar Sharma, UCSD, is analyzing serum creatinine levels in our various mouse models

Jim Kern is analyzing eyeballs from RAGE-akita and Akita/Cd2ap+/- mice for lesions of diabetic retinopathy

We are collaborating with Tom Coffman at Duke on identification of genetic loci that underlie differential DN phenotype manifestations in DBA/2J (susceptible) and C57BL/6J (resistant) strains for albuminuria (Duke) and podocyte depletion (Bottinger).

With Jax

Dr. Susztak is working with JAX to deposit sgtl2-Cd36 transgenic mice. For update also 3. Response to EAC, paragraph d.

With the MMPCs

None

With other non-AMDCC PIs

Robert Williams, University of Tennessee

3. **Address previous EAC comments:**

a. No representative was present from Dr. Bottinger's group.

Dr. Bottinger was registered and prepared to attend the fall 2007 meeting, but had to cancel on the evening prior to the meeting because of a family emergency. This was communicated and cleared at the time with AMDCC Program Staff.

b. Mpv17 null mice have improved glucose metabolism. Given Dr. Bottinger's written summary, this particular line may be problematic. The *Mpv17* and *Mpv17l* story is interesting kidney cell biology. Mpv17 interacts with Htra2, a mammalian orthologue of a bacterial stress sensor.

The improved glucose metabolism in both, Akita and STZ models that are null for Mpv17, was an accidental observation that we made in the course of studies to examine the effect of Mpv17-deficiency on diabetes-induced glomerular lesions. Because Mpv17-null mice don't develop significant hyperglycemia, the studies proposed in Specific Aim 2 of our original proposal could not be completed. Following the EACs concerns we have placed work on Specific Aim 2 on hold.

c. What is the final verdict on the RAGE-akita mice? Other complications? Positive or negative, this data needs to be made available to the community.

We have completed 24-wk and 40-wk age analysis of diabetic nephropathy phenotypes in RAGE-akita mice. There is no significant difference between RAGE-akita and akita groups with respect to hyperglycemia, albuminuria, glomerular histopathology, or survival. We have cleaned and formatted the data and are in the process of depositing the data with Rick McIndoe's group to make it available to AMDCC and general public.

d. Dr. Susztak's studies on the CD36 scavenger receptor are progressing; overexpressing CD36 exacerbates albuminuria and tubulointerstitial disease in the context of hyperglycemia. CD36 mice should be reposited with JAX by Spring 2008.

Dr. Susztak is working together with the Jackson Laboratory to reposit the sglt2CD36Tg animals at Jax. She has already uploaded information to their website and contacted Ed Leiter regarding the animals. Dr. Susztak is currently working with 2 different transgenic lines, with somewhat different level of expression of CD36. She has been asked to further characterize these two founder lines and reposit only one transgenic line. She is in the process of doing these experiments. Dr. Susztak anticipates to finish them by September 2008 and send animals to Jax during the fall of 2008.

e. We look forward to seeing Dr. Susztak's comparison of the SGLT and PEPCK PT promoters.

Dr. Susztak has imported the sglt2cre and PEPCK cre animals into her laboratory and is performing side by side comparisons on their cre expression. Preliminary results indicate that cre expression appears to be higher in the PEPCK cre animals, but these animals also express the cre recombinase in the liver. In addition, in most of these animals the cre expression shows significant variation based on their diet (salt and acid load). Dr. Susztak wants to propose to generate a new proximal tubular specific cre line using the NaPi promoter.

4. Publications:

A. Studies of Mpv17-family proteins in DN (Aims 1 and 2; PI: Bottinger)

Presented at Keystone Meeting J6: Molecular, Cellular, Physiological and Pathogenic Responses to Hypoxia January, 15-20, Vancouver, British Columbia)

> *Mpv17-like is a novel mitochondrial membrane protein that interacts with Omi/HtrA2 and protects against ROS-induced mitochondrial dysfunction and apoptosis*
> Stefanie Krick, Shaolin Shi, Wenjun Ju, Peter Mundel and Erwin P Bottinger.
> Department of Medicine, Mount Sinai School of Medicine, New York, NY 10029, USA.

Abstracts submitted for the Annual Scientific Meeting of the American Society of Nephrology November 2008, Philadelphia

> *An emerging role of Mpv17l as a mitochondrial protector against oxidative stress-induced renal tubular injury in humans*
> Stefanie Krick, Bernd Krüger, Bernd Schröppel and Erwin P Bottinger
>
> The mitochondrial Mpv17 protein protects podocytes against mitochondrial oxidative stress, dysfunction, and apoptosis
> *Stefanie Krick, Liping Yu, Shaolin Shi, Wenjun Ju, Bernd Schroppel, Steven Dikman and Erwin Bottinger*

Manuscript in Revision for publication in Proc Natl Acad Sci USA:

> *Mpv17l Protects against Mitochondrial Oxidative Stress and Apoptosis by Activation of Omi/HtrA2 protease*
> Stefanie Krick, Shaolin Shi, Su-yi Tsai, Wenjun Ju, Christian Faul, Peter Mundel and Erwin P Böttinger.

B. Studies of role of Cd36 scavenger receptor expression in tubular epithelia in DN (Aims 3 and 4; Co-PI: Susztak)

(presented at the Annual Meeting of the American Society of Nephrology San Francisco 2007)

> *Increased tubular epithelial degeneration in sglt2CD36 transgenic diabetic mice*
> Ana M Garcia, Antje Gruenwald, Velasco Cimica, Erwin Bottinger and Katalin Susztak

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Part B:

Update by Individual Project Leaders

Project 1: "Role Mpv17-family members in DN"

Responsible Investigator: Bottinger, Erwin

1. **Project Accomplishments:**

Studies to characterize Mpv17l in proximal tubular epithelial biology and response to diabetes in experimental models and in human

Mpv17l is a novel member of the Mpv17/PMP22 protein family which exists in two isoforms and shares high sequence homology with the inner mitochondrial membrane protein Mpv17, of which absence or malfunction causes oxidative phosphorylation failure and mtDNA depletion. In contrast to the previously reported peroxisomal and cytosolic localization of the Mpv17l isoforms, we show that both Mpv17l isoforms are localized in mitochondria. In diabetic and non-diabetic kidney disease, Mpv17l is downregulated at an early disease stage, indicating that Mpv17l is a common target of diverse ROS-generating extracellular signals. Silencing of Mpv17l expression causes increased mitochondrial ROS generation, depolarization of the inner mitochondrial membrane potential and increased apoptosis whereas Mpv17l overexpression has the opposite effect which is associated with retention of Omi /HtrA2, Smac/DIABLO, and cytochrome c in the mitochondrial intermembrane space. These antioxidant and anti-apoptotic effects of Mpv17l are linked to an interaction-dependent regulation of the mitochondrial serine protease Omi/HtrA2. Our findings identify the mitochondrial Mpv17l-Omi/HtrA2 complex as a novel ROS sensor and effector to reduce mitochondrial oxidative stress thereby protecting cells against ROS-induced mitochondrial dysfunction and apoptosis.

Mpv17l is a close family member of the Mpv17 protein, whose absence causes proteinuria and glomerulosclerosis in mice. We have observed previously that the downregulation of Mpv17l in proximal tubular cells correlates with the severity of diabetic and non-diabetic kidney disease in mice. We show that in human proximal tubular epithelial cells (PTEC), both Mpv17l splice variants (type 1, 2) are downregulated by TGF 1, which was associated by mitochondrial membrane depolarization (ΔΨm). In contrast to previous studies, we detected Mpv17l type 1-localization in mitochondria and Mpv17l type 2 colocalizes with mitochondrial and nuclear markers. Isoform-specific overexpression causes protection against ROS-induction, loss of ΔΨm and apoptosis. In addition, Mpv17l type 2 overexpression increases cell proliferation. Furthermore, both isoforms are binding partners of the mitochondrial serine protease Omi/HtrA2. The protective and proliferative effects of Mpv17l type 2 are functionally linked to the Omi/HtrA2 protease activity. These results are underscored by in vivo studies, demonstrating that the Mpv17l isoforms are differentially regulated in renal ischemia-reperfusion injury.
In summary, the human Mpv17l isoforms exert different roles in the protection against oxidative stress. This is partially mediated by mitochondrial Omi/HtrA2, which is an interaction partner of both isoforms and capable of regulating Mpv17l levels via a negative feedback loop. These findings show that our previous studies in mouse models also apply to the human system which could contribute to the development of new therapeutic strategies targeting oxidative stress in

acute tubular injury.

Generation of mice carrying Mpv17l^{flex3} alleles for conditional deletion of Mpv17l

We generated Mpv17^{flex3} allele carrying mice through targeted allele replacement. However, we noticed difficulties in PCR based genotyping strategies and confirmed by Southern analysis that the 5' arm of the targeting vector was apparently rearranged. Thus, although we achieved germline transmission, we decided to terminate this line and start the process of generating mice with conditional Mpv17^{flex3} allele.

Studies to characterize Mpv17 in podocyte biology and response to diabetes

Mutations in the gene encoding the inner mitochondrial membrane protein Mpv17 cause mitochondrial DNA depletion syndromes characterized by mitochondrial oxidative phosphorylation failure and early death [Nat Genet, 38:576]. Mpv17-deficient mice develop proteinuria and glomerulosclerosis associated with glomerular overproduction of reactive oxygen species [Cell, 62:425; Am J Pathol, 154:1067]. We observed significant loss of Mpv17 protein expression in podocytes in various murine models of glomerular diseases, including Tgfb1 transgenic mice and diabetic db/db mice, and hypothesized that Mpv17 may negatively regulate mitochondrial ROS production and apoptosis. To test this hypothesis, we inactivated or overexpressed Mpv17 using siRNA or transgenesis in cultured murine podocytes. Together these studies demonstrate that Mpv17 protects podocytes against oxidative stress-induced mitochondrial dysfunction and apoptosis. Coimmunoprecipitations and pull-down assays showed that Mpv17 interacted with and activated the mitochondrial serine protease HtrA2/Omi, which is mutated in humans with Parkinson's disease. Mpv17 protein expression is also downregulated in various human glomerular diseases, including FSGS, membraneous GN and diabetic nephropathy. In conclusion, Mpv17 protects podocytes against mitochondrial oxidative stress, mitochondrial dysfunction and apoptosis through activation of mitochondrial HtrA2/Omi protease. These finding provide novel insights into mechanisms of mitochondrial dysfunction and apoptosis, and podocyte injury in experimental and human glomerular diseases.

2. **Collaboration:**

   See also Part A

3. **Publications:**

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Project 2: "Role of Cd36 in tubulo-interstitial of DN"

Responsible Investigator: Susztak, Katalin

1. Detailed Project Accomplishments

1, Human CD36 cDNA was cloned under the murine sglt2 (sodium/glucose co-transporter) promoter. The targeting vector was injected to FvB oocytes.

2, We identified the founders carrying the transgene by genomic PCR.

3, We characterized the transgene expression. Transgenic animals had ~100 fold increase in the transgene (human CD36) mRNA levels expression compared to wild type mice. Western blots failed to show an increase of the transgene expression at baseline. Animals challenged with a high sodium and high glucose diet or were made diabetic to enhance the transgene expression (as the sglt2 promoter is known to be sensitive to these measures). Following enhanced salt and glucose load transgenic animals had approximately 3-4 fold higher level of CD36 levels at the whole kidney level, however the results of this treatment was variable. Diabetes induced a similar (3-4 fold) increase in protein expression with a more consistent results. Immunohistochemistry analysis also identified the Cd36 transgene in the proximal tubules, as it co-localizes with proximal tubular markers. Thereby we concluded that we successfully generated transgenic animals with CD36 overexpression in the proximal tubules.

4, Phenotype analysis of transgenic animals are in progress

A, No obvious phenotype was observed in control transgenic animals. Animals exhibit normal life-span and renal histology did not show obvious abnormalities.

B, In order to analyze the role of CD36 in diabetic nephropathy, animals were made diabetic by multiple low dose streptozotocin injection. Wild type (FvB) animals showed hyperglycemia and polyuria and polydipsia following STZ injection, but they exhibited normal life span. Transgenic diabetic animals exhibited weight loss and increased mortality starting at 9-10 weeks of age mortality reaching 100% by 13 weeks of age. Histological analysis (by PAS staining) showed dilated tubules with tubular epithelial degeneration and mildly increased interstitial infiltration. Tubular apoptosis rate (quantified by TUNEL stain) in the kidney cortex was significantly increased (0.373% vs. 1.5%) in transgenic diabetic animals compared to wild type diabetic animals. Interestingly, while tubular apoptosis and degeneration was marked in these animals the degree of tubulointerstitial fibrosis was less pronounced. Serum samples were taken from animals and sent to the laboratory of Dr Kumar Sharma (UCSD) for serum creatinine analysis by HPLC analysis.

C, Studies determining the mechanism of CD36 mediated tubular degeneration are ongoing. CD36 is a fatty acid transported and scavenger receptor, we were interested

to analyze whether increased fatty acid uptake via CD36 would be responsible for the observed phenotype. We performed Oil Red-O staining (to detect intracellular lipid accumulation) on control diabetic and transgenic diabetic animals. Our analysis showed increased lipid accumulation in renal tubular epithelial cells. We are currently performing quantitative analysis of total triglyceride contents in kidney homogenates of wild type and transgenic diabetic mice. In addition we have analyzed human kidney biopsy samples obtained from control and diabetic patients (n=5 per groups). We found increased Oil-red-O staining in human diabetic samples as well, indicating that similar mechanism is present in patients with diabetes. Additional studies are being performed to determine the mechanism of the increased lipid uptake mediated tubular damage, examining enzymes on the lipid uptake, oxidation and storage pathways.
Our studies suggest that increased proximal tubular CD36 expression plays an important role in diabetic tubular degeneration and possibly renal disease progression.

2. Collaboration:

We are working together with the Jackson Laboratory to reposit the sglt2CD36Tg animals at Jax. We have already uploaded information to their website and contacted Ed Leiter regarding the animals. We are currently working with 2 different transgenic lines, with somewhat different level of expression of CD36. We have been asked to further characterize these two founder lines and reposit only one transgenic line. We are in the process of doing these experiments. We hope to finish them by September 2008 and send animals to Jax during the fall of 2008.

3. Publications:

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