Animal Models of Diabetic Complications Consortium
(U01 DK61018)

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"Generating Mouse Mutants With Diabetic Nephropathy"
Vanderbilt University School
Principal Investigator

Raymond C. Harris, M.D.
Co-Investigator: Takamune Takahashi, M.D./Ph.D.
Co-Investigator: Chuan-ming Hao, M.D./Ph.D.

Address: C-3121 MCN Nashville, TN 37232
Phone: 615 322-2150
E-mail: ray.harris@vanderbilt.edu

Table of Contents

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Part A:
Principal Investigator's Summary

Given the increasing evidence implicating endothelial dysfunction in the pathogenesis of diabetic complications, particularly nephropathy, retinopathy, neuropathy, and macrovascular disease, the Vanderbilt component of the AMDCC is focusing on eNOS and prostacyclin synthase (PGIS), two endothelial genes encoding biochemically interrelated enzymes and in which polymorphisms associated with altered enzyme activity have been implicated in human diabetic nephropathy.

Both PGIS and eNOS activity are important for the maintenance of normal endothelial function. COX2 appears to be the major source of urinary prostacyclin excretion in man, and prolonged COX2 inhibition is associated not only with reduction of PGIS but also with excess cardiovascular mortality from thrombotic events. This is consistent with a cardioprotective action of prostacyclin. Functionally significant polymorphisms in eNOS and PGIS have been identified in humans. ENOS and PGIS activity are not only topographically linked but also biochemically linked through oxidative stress, which not only uncouples eNOS, but also results in increased peroxynitrite levels, which directly reacts with and inactivates prostacyclin synthase. Both eNOS uncoupling and peroxynitrite-induced inactivation of prostacyclin synthase have been demonstrated to be direct consequences of hyperglycemia. It has been hypothesized that as a result of this, diabetics exhibit impaired endothelial dependent acetylcholine induced vasodilation and glomerular barrier function, which is reflected as albuminuria. This may also be associated with the global cardiovascular disease associated with diabetic nephropathy.

Responsible Investigator: Raymond C. Harris, M.D.

1. Project Accomplishments:

Recent Progress and Major Accomplishments
I Proposed Goals of the Vanderbilt AMDCC

The goal of **Aim 1** is to determine the role of endothelial eNOS activity in the progression of diabetic nephropathy by generating floxed eNOS mice and studying them in the DN susceptible DBA2/J Akita mouse. Although our original attempts were not successful at producing a successfully floxed mouse that went germline, during the past year, we reinjected two additional ES clones. We have successfully generated the floxed eNOS mice using these two ES clones (1A6, 6A9) with the assistance of JAX lab. To confirm feasibility of Cre-loxP mediated eNOS gene targeting in these floxed eNOS lines, we conducted the following experiments. First, we crossed the floxed (flox/+) eNOS mice with Tie2-Cre mice and examined Cre-mediated deletion of floxed eNOS exons in lung (vascular rich organ) of the offspring, comparing with tail (vascular poor tissue), by genomic PCR. Second, we also crossed the floxed (flox/+) eNOS mice with Sox2-Cre mice to generate lox/+ eNOS mice (in which floxed eNOS allele is totally converted to lox allele). In both studies, floxed eNOS allele from both ES lines was converted to lox (floxed exons-removed) allele in the presence of Cre. No lox allele was detected in the floxed eNOS mice that do not carry Cre transgene. Thus, we confirmed that floxed eNOS exons can be deleted by Cre in our recombinant mice. Lastly, we have generated homozygous mice for lox allele (lox/lox) as well as floxed eNOS allele (flox/flox) using lox/+ or flox/+ mice.

Our plans for the upcoming year include:

1) *Phenotyping the homozygous (flox/flox or lox/lox) mice and comparing with wild-type (+/+) littermate mice.* We will characterize these mice by measuring systolic blood pressure, eNOS expression levels (Western blot analysis, Immunohitochemistry), and macro- and microscopic pathological examination. We anticipate that flox/flox mice will present with the same phenotype as wild-type mice, while lox/lox mice will have phenotypes more characteristic of eNOS knockout (eNOS-/-) mice.
2) *Backcrossing floxed eNOS mice to the DBA2 strain*. To perform conditional knockout studies on a diabetic nephropathy-sensitive strain, we are currently backcrossing the floxed eNOS mice as well as inducible endSCL-CreER mice to DBA2 strain. Backcrosses of floxed eNOS mice are being performed at JAX lab.
3) *Generating homozygous floxed (flox/flox) eNOS mice that carry VEcadherin-Cre (or endSCL-CreER) transgene.* Once we have generated this mouse, we will induce diabetes using STZ injections and examine their renal phenotype. We will ask whether deletion (or reduction) of endothelial eNOS gene may accelerate diabetic

nephropathy by this experiment. We are currently crossing VEcadherin-Cre flox/+ eNOS mice with flox/flox eNOS mice.

The goal of **Aim 2** is to determine the role of endothelial prostacyclin synthase in the progression of diabetic nephropathy nephropathy by generating floxed PGIS mice and studying them in the DN susceptible DBA2/J Akita. In this regard, we have completed the construct and have electroporated 129P3ES cells with the floxed PGIS targeting vector. The original screen of ES cells was negative. The construct was re-electroporated into ES cells and rescreened. As the result of additional blastocyst injections, we obtained 4 chimeras from1C11. We have recently been able to confirm that one of the males had both color coat transmission and germline transmission of transgene. We are in the process of generating mice to test whether the transgene can be successfully deleted.

Plans for the Upcoming Year: Now that we have successfully developed the floxed eNOS mice, we will ship them to Jackson Labs for speed congenic backcrossing to 129/sv and DBA2/J Akita backgrounds. We have also made the Sox2Cre mice available to Jackson Labs for backcrossing to DBA2/J and 129/sv backgrounds so that we will be able to selectively knock out eNOS in the endothelium. We are also hopeful that we will have PGIS synthase floxed mice available for backcrossing sometime in late 2010.

II Ongoing Studies of Murine Models of Diabetic Nephropathy

A) Characterization of the role of endothelial nitric oxide synthase deficiency in development of diabetic nephropathy.

1)eNOS Dysfunction and Compensatory COX-2 Upregulation in Diabetic Nephropathy Impaired eNOS activity may be involved in the pathogenesis of diabetic nephropathy (DN). We studied a model of type II diabetes, *db/db* (BKS) mice, at 17, 26, 36 and 52 weeks. Moderate mesangial expansion was found at 26 weeks, with progressive increases with aging. GFR increased from 276±38 ul/min/mouse at 17 wks to 354±29 at 26 wks and 408±35 at 36 wks, followed by a decline to 221±71 at 52 wks (BKS control: 289±19). Neither immunohistochemistry nor immunoblotting indicated any significant alteration of glomerular eNOS monomer expression, but eNOS dimerization progressively decreased beginning at 26 wks old (dimer/monomer ratio: 0.28±0.02 at 14 wks; 0.23±0.03 at 26 wks; 0.18±0.02 at 36 wks and 0.13±0.04 at 52 wks), indicating eNOS uncoupling. In addition, there was decreased phosphorylation of eNOS at Ser 1177, an essential step in eNOS activation, beginning at 26 wks, without significant change of phosphorylation of Thr 495, a marker of enzyme inhibition. Immunofluorescent localization confirmed increased COX-2 expression in glomerular mesangium, endothelium and Bowman's capsule by 26 wk. In endothelium, the increased COX-2 partially co-localized with eNOS, suggesting the possibility of a compensatory role for COX-2 in the face of defective eNOS activity. In *db/db* (BKS) mice with eNOS

deficiency (eNOS KO-*db/db*), glomerular COX-2 expression was further increased. Administration of a COX-2 specific inhibitor, SC 58236 (6mg/L in drinking water) to eNOS KO-*db/db* and age matched (26 wks) eNOS KO mice for 4 weeks accelerated renal injury in eNOS KO-*db/db*, indicated by more marked mesangial expansion and nodular sclerosis and further declines in GFR (from 188±31 to 132±7 ul/min/mouse in eNOS KO-*db/db* vs. 244±27 to 175±6 in eNOS KO). *T*hese studies indicate that eNOS uncoupling and impaired phosphorylation at Ser 1179 progressed after 26 wks of age in *db/db* (BKS) mice. The upregulated COX-2 in mesangium and endothelium and eNOS deficiency in aged *db/db* mice, along with the detrimental effect of COX-2 inhibition to eNOS KO-db/db mice, suggest that increased COX-2 may play a compensatory role in the face of eNOS dysfunction as diabetic nephropathy progresses.

2) ACE Inhibition Decreases Proteinuria and Retards Progression of Renal Injury in Type II Diabetic Mice with Endothelial Nitric Oxide Synthase Deficiency Diabetes causes microvascular dysfunction, which contributes to the development of ESRD. There is increasing evidence that alterations in expression and/or activity of endothelial nitric oxide synthase (eNOS) are associated with human diabetic nephropathy (DN). We previously reported that in a model of type II diabetes, *db/db* mice on the BKS background, crossing with eNOS knockout mice (*db/db* eNOS $^{-/-}$) induces an accelerated model of DN with distinct features of progressive nephropathy similar to human DN, including significant and early onset albuminuria, arteriolar hyalinosis, mesangiolysis and focal segmental and nodular glomerulosclerosis. Since progression of human DN is retarded with inhibition of the renin-angiotensin system, we tested whether this mouse model of DN is also sensitive to RAS inhibition by treating with the ACEI, captopril. *db/db* eNOS $^{-/-}$ mice, age 8-13 weeks (9.9±1 wks (con) n=9; 9.6±1 wks (cap) n=7) were treated for up to 14 weeks. One captopril-treated mouse died after 6 wks treatment and 2 control mice died during the period of treatment at 12 weeks and 1 at 14 wks. Although albuminuria was comparable at baseline (con vs. cap: 1895±163 vs. 1629±260 µg/mg Alb/Cr), it was significantly lower in captopril-treated mice at 5 wks of treatment (1443±211 vs. 482±126 µg/mg Alb/Cr), an effect that persisted to the last measurement at 11 wks of treatment (3398±1517 vs. 313±55 µg/mg Alb/Cr). Glomerulopathy in captopril-treated mice was markedly less than in control mice, with mild-moderate mesangial expansion but minimal mesangiolysis or glomerulosclerosis. These results further indicate that *db/db* eNOS $^{-/-}$ may be a useful model of human DN to test novel therapeutic approaches and suggest an important role for eNOS to counteract angiotensin-mediated glomerulopathy in DN. We are currently expanding these studies to determine the role of hypertension control vs. inhibition of the renin-angiotensin system. We are now completing a 12 week trial of treatment of an ACE inhibitor (captopril) vs. “triple therapy” (hydralazine, hydrochlorothiazide and reserpine).

B) The Role of Superoxide Dismutase-1 is Development of Diabetic Nephropathy

Renal phenotyping of B6-Akita-SOD1 -/-, B6-Akita-SOD3-/-, B6-Akita-SOD1-/-SOD3-/- mice We have generated B6-Akita mice that lack gene expression of SOD1 or SOD3 or both = using SOD1 or SOD3 knockout mice, and have examined their renal phenotype up to 30 weeks of age. B6-Akita SOD1-/- and B6-Akita SOD1-/-SOD3-/- mice showed significantly increased albuminuria, lower GFR, and increased mesangial expansion as compared with B6-Akita mice. Conversely, SOD3 deficiency did not exhibit significant renal effects in B6-Akita mice. A significant renal phenotype was also not observed in non diabetic SOD knockout mice. Interestingly, mesangial expansion in B6-Akita SOD1-/-SOD3-/- mice was more prominent than B6-Akita SOD1-/- mice at 30 weeks of age, whereas significant differences were not observed between these two groups at 20 weeks of age; this finding suggests the development of progressive diabetic nephropathy in B6-Akita SOD1-/-SOD3-/- mice. In aggregate, our data suggest an important role of SOD enzyme (in particular SOD1 isoenzyme) in the pathogenesis of diabetic nephropathy.

For the upcoming year we plan the following studies:

Phenotypic analysis of the SOD-deficient B6-Akita mice. We will further determine the renal phenotype of SOD-deficient diabetic mice (especially with 30 week-old mice), including GFR measures, assessment of glomerular nitric oxide production and eNOS activity, and morphological analysis.

Phenotypic analysis of db/db SOD1-/- mice. Since it is now evident that SOD1 deficiency accelerates murine diabetic nephropathy, we have also generated SOD1-deficient diabetic mice using the *db/db* strain. We will analyze the renal phenotype of this diabetic mouse. Because the *db/db* strain is more susceptible to diabetic kidney disease than B6-Akita, we anticipate that progressive diabetic nephropathy is developed in SOD1-deficient *db/db* mice.

Collaboration:

With Jax: The development of the floxed eNOS mouse was a collaboration with Jackson Labs. As indicated above, when our floxed mice are available, we have made arrangements with Jackson Labs to undertake the appropriate backcrosses onto the strains of interest.

With the MMPCs: We will continue to utilize the Phenotyping facilities at the Vanderbilt MMPC for functional characterization of the mice generated in this project.

Responses to Previous EAC comments

- *Endothelial dysfunction, indicated by abnormal albuminuria, correlates well with diabetic microvascular complications. Dr. Harris' focus on developing endothelial restricted deletions of two enzymes, critical to endothelial cell homeostasis, is justified. Slides indicate failure to generate germline transmission of targeted alleles. Alternative approaches were not included on slides and should be provided. The phenotypic characterization of 2 mouse lines with targeted deletion in eNOS, Sod1, are high quality and demonstrate nephropathy.*

As indicated in our current progress report, we have now been able to develop mice with the eNOS locus successfully floxed. We will backcross them to "nephropathic" backgrounds and they should be available for study in the near future.

- *The SOD 1 KO findings are interesting. As noted in the general NEPHROPATHY comments (above) the other diabetic nephropathy risk factors need to be assessed including blood pressure. Where in the kidney is the SOD1 normally expressed as compared to the other SODs?*

SOD1 is expressed everywhere including glomerulus and tubules. Its expression is ubiquitous. Stronger immunoreactivity is observed in vascular cells (endothelial cells) and tubular cells. There is one Diabetes paper, showing strong SOD1 immunoreactivity in podocyte in human kidney. However, we cannot see this finding in mouse kidney. SOD3 is expressed in artery and glomerulus (vascular wall) and SOD2 is highly expressed in tubules (especially in proximal tubules)

- *Dr. Harris has accomplished the proposed goals of the P&F. Results pending on phenotypic variability in out bred CD1 diabetic mice. The BC experiment between diabetic Akita and CD-1 mice with low and high ACRs has not been informative. We think the proposed plans to follow F1 progeny and increase F1 numbers make the most sense. If DN does develop in lines derived from CD1 high albumin "excretors" the experiment would be proof of principle for second order studies using this approach.*

We did follow the F1 progeny for 12 months and none of them developed significant proteinuria. However, as indicated in the previous project report, these studies were limited by the unexpectedly high mortality we saw in the CD1 mice. We had been able to generate only a small fraction of the F1 progeny that we had expected because of this unexpected early mortality. Therefore, we do not think that the F1 backcross was sufficiently powered to make any determination.

- *The P&F project applies interesting and potentially useful approach. The usual suspects need assessing e.g. weight gain/food intake, kidney weight and blood pressure.*

We completely agree with these comments. If we undertake a more extensive study of the outbred mice in the future, we will include these parameters in our study design.

Recent Publications

Fujita, H, Fujishima, H, Chida, S, Takahashi, K, Qi, Z, Kanetsuna, Y, Breyer, MD, Harris, RC, Yamada, Y and Takahashi, T. Reduction of renal superoxide dismutase in a murine model of progressive diabetic nephropathy. JASN. I20:1303-13, 2009

Cheng, H, Fan, X, Guan, Y, Moeckel, GW, Zent, R and Harris, RC. Distinct roles for basal and induced COX-2 in podocyte injury JASN 20:1953-62, 2009

Chen, J-K, Chen, J, Thomas, G., Kozma, SC and Harris, RC. S6 kinase 1 knockout inhibits uninephrectomy- or diabetes-induced renal hypertrophy. Am. J. Physiol (Renal)297:F585-93, 2009

Brosius, F, Alpers, C, Bottinger, E,, Breyer, M, Coffman, T, Harris, R, Kakoki, M, Kretzler, M, Leiter, E,Levi, M, McIndoe, R, Sharma, K, Smithies, O, Susztak, K, Takahashi, N and Takahashi, T. Mouse Models of Diabetic Nephropathy. JASN In Press

Harris, R.C. "Diabetic nephropathy" in Cecil's Textbook of Medicine. 35th ed. 2010

Manuscript submitted

Hiroki Fujita, Hiromi Fujishima, Keiko Takahashi, Kayoko Kagaya, Tsukasa Morii, Zhonghua Qi, Takahiko Shimizu, Takuji Shirasawa, Matthew D. Breyer, Raymond C. Harris, Yuichiro Yamada, and Takamune Takahashi Deficiency of cytosolic CuZn-superoxide dismutase (SOD1) accelerates renal injury in C57BL/6-*Ins2Akita* diabetic mice