ANIMAL MODELS OF DIABETIC COMPLICATIONS CONSORTIUM

(U01 DK61018)

UPDATE REPORT
(September 2001 –January 2004)

Matthew D. Breyer, MD
Program Director

Matthew D. Breyer, MD
Vanderbilt University
Department of Medicine and Molecular Physiology and Biophysics
Division of Nephrology
New York, NY 10032
Phone: (615) 343-9867 Fax: (615)343-4704
Email: matthew.Breyer@vanderbilt.edu

Table of Contents

ANIMAL MODELS OF DIABETIC COMPLICATIONS CONSORTIUM

(U01 DK61018)

PART A:

PRINCIPAL INVESTIGATOR'S SUMMARY

Program Accomplishments:

The major theme of the AMDCC project at Vanderbilt is the identification and characterization of genetic modifiers causing diabetic nephropathy. Within this theme three related topics of research are: 1) characterization of candidate gene mutations exacerbating nephropathy in mice; 2) characterization of ENU mutants exhibiting diabetic nephropathy, and 3) Establishment of Phenotyping capability for renal function in diverse strains of mice.

Major accomplishments include:

1) Determination that diabetic nephropathy is more severe in mice with genetic disruption of genes participating in lipid metabolism. These include: ApoE, LDLR and Cyp4a14 knockout mice.
2) The identification of five novel ENU mutants that exhibit excess albuminuria in the setting of diabetes as compared with other mice of the same strain.
3 The establishment of techniques to measure GFR in mice using FITC-inulin clearance
4) Demonstration of dramatic differences in the severity of albuminuria and histopathological changes in different strains of inbred mice including KK, DBA, A/J, and C57BL/6J

Drs. Fogo and Breyer (Project 3) have carried out extensive studies defining the pathological characteristics of streptozotocin induced diabetic nephropathy in several different strains of mice. Further studies using albuminuria, and GFR to define the functional consequences of diabetic nephropathy according to mouse strain are ongoing.

Interrelationship of projects:

Project 1: Project 1 – " Characterization of Candidate genes predisposing to diabetic nephropathy.

This project is focused primarily on type II diabetes and defining the role of dyslipidemia and hypertension in exacerbating diabetic nephropathy. Project 2 is defining characterizing type I diabetes. Both are utilizing techniques to measure GFR and albuminuria in specific strains that are being developed by project 3.

Project 2

In collaboration with Dr. Gene Rinchik a member of our consortium at University of Tennessee, in a project directed by Dr. Breyer, we have generated over 300 diabetic ENU mutants and identified five mutants with increased albuminuria and . Drs. Breyer and Rinchik continue to collaborate on the mouse studies related to XXXXX (see Project #). Drs. Breyer, and Fogo have collaborated in characterization of renal pathological changes occurring in diabetic strains.

Project 3
Project 3 involves close work with projects 1 and 2. Within the AMDCC, Dr. Breyer has collaborated with Drs. Fogo and Harris in the development of techniques to measure GFR in mice. Project 3 is critical for establishing criteria for measuring renal function and establishing normal ranges for glucose, HbA1C, GFR, albuminuria and renal histopathologic criteria. Research design continues to involve Dr. Breyer, the Project leader of Project 2 who is a co-investigator in most of the studies in this report. In addition, there continues to be a shared theme with Project # 1, which has led to an improved understanding of the role of dyslipidemia and the genesis of diabetic nephropathy. Based on the work of these two Projects, we believe that there are shared overall processes between the component projects of the Vanderbilt AMDCC site.

CORES:
The Vanderbilt AMDCC does not have formal COREs as part of its structure. Rather the VU-AMDCC builds on existing infrastructure at Vanderbilt, including the Mouse Metabolic Phenotyping center (MMPC) and the Vanderbilt Ingram Cancer Center which provide mouse histopathological characterization. Each project has interacted with these cores including the Vanderbilt MMPC and the Vanderbilt small animal imaging core. In addition project 2 closely interacts with the Tennessee Mouse Genome Consortium (TGMC) in order to develop technology to map novel ENU induced mutations identified to confer risk for diabetic nephropathy.

Collaborations with other Groups (Including Core Facilities):

Dr. Breyer has carried out a major collaboration with Dr. Kumar Sharma and Erwin Bottinger in the AMDCC program at Jefferson and Einstein to evaluate the adequacy of HPLC determined serum creatinine as an endogenous marker for determining GFR in mice. These studies should provide a rapid and validated measure of GFR in mice.

Pertinent non-AMDCC Collaborations

The GFR studies represent a very close collaboration with Dr. Streamson Chua (Columbia UNIVERSITY) who has provided mice with leptin receptor mutations (i.e. db/db) on DBA and FVB strains. In addition there have been important contributions from Dr. Youfei Guan, Dr. Alyssa Hasty and Dr. Jorge Capdevila (Vanderbilt School of Medicine) who have provided us with access to the 129svJ db/db, LDLR-/- and CYP4a14-/- mice respectively.

ANIMAL MODELS OF DIABETIC COMPLICATIONS CONSORTIUM

(U01 DK61018)

PART B:

UPDATE BY PROJECT LEADERS

Responsible Investigators: **Raymond C. Harris, M.D.**

Project Number and Title: **Project 1 – " Characterization of Candidate genes predisposing to diabetic nephropathy"**

A. Rationale and Relevance:

Modifier genes have been proposed to be important for the development of diabetic nephropathy, the rate of progression of the injury (from microalbuminuria to ESRD) or both. Population studies have identified a number of potential candidate genes that may predispose to progressive nephropathy in either type I or type II diabetes. In this regard, we are concentrating on the possible interaction of altered endothelial function and altered regulation of lipoproteins as exacerbating factors in type II diabetes.

The *db/db,* or *Lepr*^{db} strain, expresses a spontaneous mutation of the leptin receptor. Homozygous *db/db* mice become obese by 3 to 4 weeks of age. Plasma insulin is elevated by 10 to 14 days and blood sugar is elevated by 4 to 8 weeks. It is well known that the course and severity of the disease is influenced by genetic background of the mice. On the C57Bl/6J background, islet ß-cells undergo compensatory hyperplasia, and the mice display continued hyperinsulinemia throughout an 18-to 20-month life span. These animals manifest delayed wound healing, but published reports indicate that *db/db* mice on the C57Bl6 background develop minimal nephropathy. In contrast, when *db/db* is expressed on the C57BLKs background, the diabetes is more severe due to an insulitis that results in progressive depletion of the insulin-producing ß-cells of the pancreatic islets, and death by 10 months of age. Therefore, for the db/db studies, modifier genes are being tested on both C57Bl/6J and C57BLKS backgrounds.

There is increasing evidence also indicates that a genetic susceptibility to development of DN is related to abnormalities in lipid metabolism [Quinn, Angelico, Warram, Krolewski 1996]. Apoliprotein (apo) E is an efficient ligand for the receptor-mediated clearance of remnant lipoproteins (chylomicron remnants and IDL) and mediates the clearance of remnant lipoproteins through its interaction with at least two different receptors: the LDL receptor. ApoE deficiency causes increased susceptibility to atherosclerosis, and there is an association between ApoE2 polymorphisms and development of nephropathy in Caucasians with type I diabetes [Araki et al 2000]. In addition, we are examining the potential role of inducing hyperlipidemia by deletion of the LDL receptor.

The CYP4a family represents another pathway increasingly appreciated as impacting on lipid metabolism. The CYP4a's mediate Ω-hydroxylation of fatty acids (including linoleate and arachidonate) (1-4). Knockout of Cyp4a14 activity causes hypertension and an insulin resistance syndrome in mice (3). In man, increased risk of diabetic nephropathy has been observed with both dyslipidemia and hypertension, both of which are features present in the Cyp4a14 null mouse.

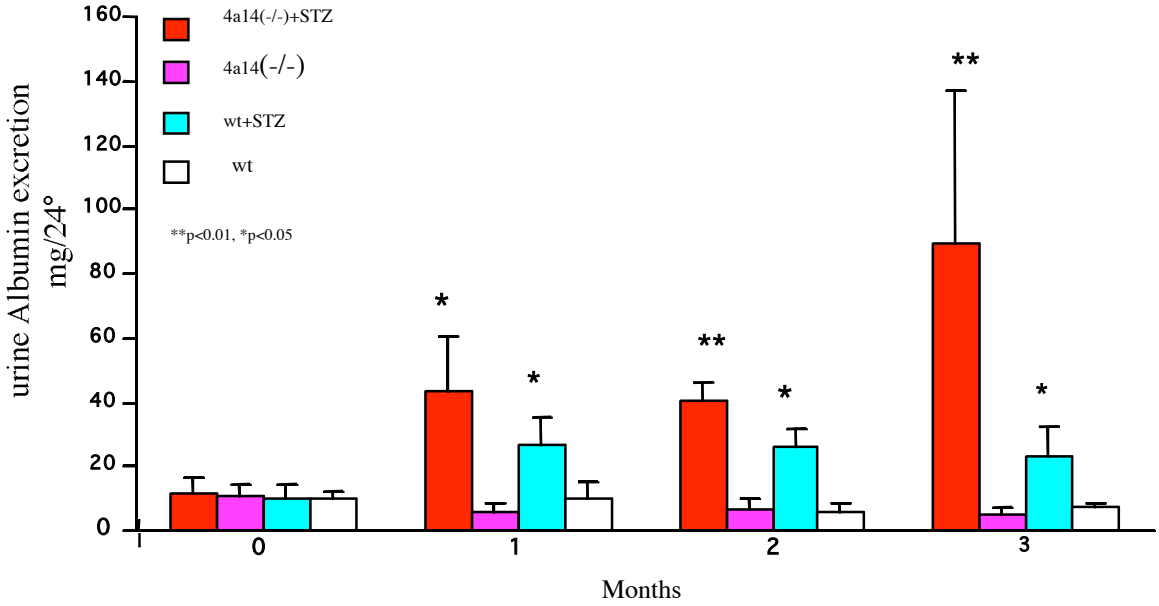
B. Summary of accomplishments

We have completed the backcrosses (10 generation backcross) of LDLR, apoE and eNOS knock-out mice to C57BLKS/J and have begun to examine assess development of diabetic nephropathy. In 26 week old non-obese C57BLKS mice (control), albumin to creatinine ratio (ACR) was 94 ±10 mg/g (n=3), higher than in C57BL6/J mice. GFR was 199±3 µl/min/mouse (n=3). Albumin/creatinine ratio at 26 weeks was higher in diabetic C57BLKS *db/db* mice than their non-diabetic littermates, being 143±28 (n=7) GFR was not different 175 (n=10).

C57BL/6J *db/db* mice with LDL receptor (LDLR-/-) deletion, *db/db* mice had an albumin/creatinine ratio of 152±13 (n=6) and GFR of 279±95 (n=4), however these mice had not been backcrossed onto the BLKS strain. In preliminary studies, we have examined the 6th backcross (expected to be >90% pure) of LepR$^{db/db}$ mice on the C57BLKS background. Of note **16** week db/db mice with concomitant LDL receptor deletion, had an albumin/creatinine ratio of 224 mg/g while in the **16** week mice with ApoE gene deletion, *db/db* mice had a dramatically elevated ACR of 659µg/mg. These numbers are still small in each group, but these preliminary results suggest the possibility that abnormalities in lipid metabolism, and especially abnormalities in ApoE function, may greatly exacerbate nephropathy in these mice.

Another approach to examining dyslipidemia is and potential for lipotoxicity is provided by studies in Cyp4a14 knockouts. Cyp4a14-/- 129svJ mice exhibit markedly increased albuminuria as compared to their wild-type controls following 16 weeks of STZ induced hyperglycemia (figure 1).

Figure 1: Development of progressively increasing albuminuria in diabetic 4a14-/- mice. Bars indicate 24° albumin excretion in both diabetic, non-diabetic, wt and 4a14-/- mice.



C. Plans for the coming year

As described C57BLKS is known to be more susceptible to the development severe diabetic nephropathy. We are currently completing the backcross at the 10th generation of LDLR, ApoE and eNOS to C57BLKS. Therefore, we should be able to provide definitive assessment of the effect of these modifier genes on *db/db* mice on this strain within the coming 18 months.

Additional studies will complete the characterization of diabetic nephropathy in STZ treated Cyp4a14 wt and knockout mice on the 129svJ background. We have recently received the 129svJ*LepR*$^{db/db}$ mice and which will facilitate the intercross of the Cyp4a14 knockout onto the leptin receptor mutant model of type II diabetes. We anticipate this combined mutant will exhibit a dramatic form of diabetic nephropathy.

D. Significant achievement and its importance

Preliminary studies of models of type II diabetes combined with mutations of genes involved in lipid metabolism (LDLR-/-, ApoE-/-, and Cyp4a14-/-) are all suggestive of enhanced progression of diabetic nephropathy. Since ApoE polymorphisms have been associated with diabetic nephropathy in humans, it may well be that these mutations confer the necessary milieu for mice to develop nephropathy similar to that seen in people.

Publications

Manuscript on Cyp4a14-/- mice in preparation

Responsible Investigators: **Matthew D. Breyer, M.D.**

Project Number and Title: **Project 2 – " A screen for dominant ENU mutants developing diabetic nephropathy"**

A. Rationale and Relevance:

Accumulating evidence suggests that, as in man, genetic modifiers are a critical determinant of the extent of renal injury in mice(5, 6). The extent of renal injury developing in insulin dependent diabetic mice (following streptozotocin treatment) is dramatically influenced by the genetic background. Even in "susceptible" strains, it remains unclear whether predisposition to histopathologically defined glomerulosclerosis is accompanied by changes in renal function including proteinuria or diminished glomerular filtration rate (GFR) as observed in human DN (7).

Similarly, modifier loci appear to play a crucial role in the development of DN observed in mice carrying mutation of the leptin receptor (i.e. db/db mice). On the C57BL/6 background these mice do not develop nephropathy, whereas they develop significant glomerulosclerosis and proteinuria on the C57BLKS strain (8). C57/BLKS comprises a strain distinct from C57BL/6 and apparently was contaminated by DBA when the colony was pen-bred in the 1940's. C57BLKS differs from C57BL/6 at multiple loci including the major histocompatibility H2 locus (www.jax.org/ listed under C57BLKS). It is apparent that some combination of the differences at these loci is critical for the development of nephropathy, however exactly how this occurs and where these quantitative trait loci (QTLs) map may be difficult to determine since epistatic interaction between multiple alleles may be necessary to develop nephropathy. Mapping a QTL involves segregation of alleles. In order for an isolated QTL to be successfully mapped, it must have a measurable phenotypic effect when separated from the other genes contributing to the trait. Separation of an individual allele from the genes in a particular strain may progressively diminish the severity of the phenotype, often to the point where the phenotype is lost.

Mutagenesis offers distinct advantages over QTL mapping in the analysis of complex traits (9). In contrast to QTL mapping, mutagenesis introduces individual mutations that result in the phenotype for which the particular screen is designed. Chemical mutagenesis induces a high frequency of mutations affecting potentially every gene that might contribute to a given trait. Genome-wide random mutagenesis has recently been applied to identify functions of novel genes important for a neurologic function, allergy and immunology, and embryonic development and dysmorphology (10-12). These screens rely on casting a wide net, in terms of the types of phenotypes sought (11, 13).

In this project we are utilizing *N*-ethyl-*N*-nitrosourea (ENU) to perform a "sensitized screen" for mutants that induce renal dysfunction with diabetes mellitus being the "sensitizing" condition. ENU is a potent point mutagen that acts by transferring its ethyl group to oxygen or nitrogen radicals in DNA, which, if not corrected, results in mis-pairing with resulting A/T to T/A transversions and A/T to G/C transitions (14). ENU mutagenesis provides an unbiased approach for identifying novel and

unpredicted genes that may contribute to the development of DN throughout the genome. In characterizing the resultant mutants it will be important to differentiate pedigrees with non-diabetic forms of renal injury (e.g. hydronephrosis, polycystic kidneys, spontaneous focal segmental glomerular sclerosis) from those with diabetic renal disease. Strategies for excluding these other causes for renal failure include characterization of anatomy by renal ultrasound (see below), examination of pathologic material and breeding them to in the absence of the diabetic sensitizing condition. These and other approaches for characterizing the both the gene and phenotypes are outlined below.

B. Summary of Accomplishments

GENERATING novel Mouse mutants with diabetic nephropathy:

During the prior year of support, we identified five type I diabetic Akita C57BL/6J ENU mutants exhibiting increased albuminuria. These mutants exhibiting increased albuminuria with a Ualb:Creat ratio averaging 340.2±50 µg/mg. This value is more than 3 standard deviations more than the typical levels of albuminuria in C57BL/6J^{ins2akita} mice, defining them as prime candidates for founders carrying novel mutations conferring susceptibility to diabetic nephropathy.

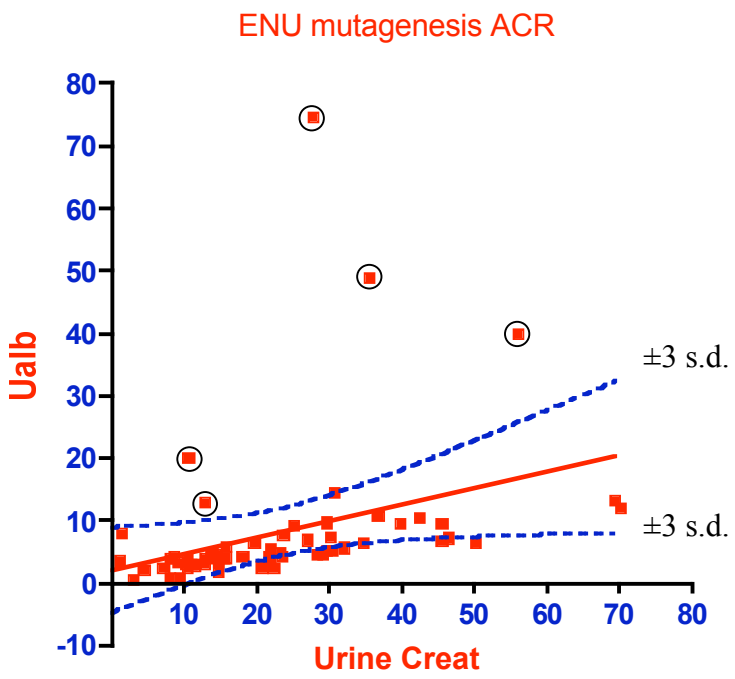


Figure 1: Urine albumin concentration as a function of Urine Creatinine concentration in ENU mutagenized diabetic C57BL/6J^{Ins2Akita} mice. Dashed lines represent ±3 SD limits of the regression line for this relationship. Data points encircled represent those animals that are statistical outliers.

Importantly diabetic progeny from three of these mice crossed with wild-type C57BL6/ females have now also been identified as exhibiting increased albuminuria, consistent with the existence of a dominant mutation associated with albuminuria. Equally relevant to the goals of this project, is the observation that the non-diabetic progeny do not exhibit albuminuria, consistent with a requirement for hyperglycemia to induce albuminuria.

C. Plans for the coming year

The top priority for the upcoming year will be to more fully phenotype the ENU mutants so as to determine how closely the phenotype exhibited by the founders of each

albuminuric ENU mutant line, matches that of diabetic nephropathy. This will include performing a full metabolic panel on plasma including the determination of plasma lipids, electrolytes, serum creatinine/BUN GFR, and imaging of the kidneys by micro-CT scan or ultrasound.

Further phenotyping of the progeny of each albuminuric founder mouse will also be undertaken. This includes those progeny that are presently non-diabetic – they did not inherit the $Ins2^{akita}$ transgene. The non-diabetic mice do not exhibit albuminuria but may still possess the ENU induced mutation. Induction of diabetes in these mice using streptozotocin should reveal a subset of 50% of the non-diabetic progeny that develop excessive albuminuria following induction of STZ.

We hope to complete a manuscript describing the severity of nephropathy in the C57BL/6J akita mouse as well as continue back-crossing the Akita mutation to Balb/C and DBA2J strains.

In the coming year we hope to confirm the full phenotype albuminuria develops in an autosomal dominant fashion in the ENU mutant mice. F2 intercrosses will be performed to determine whether the homozyotes develop more severe disease or are lethal. Lines expressing the mutant allele will be outcrossed to DBA/2J, A/J, and 129 P3/J to determine whether the dominant mutation leading to albuminuria and diabetes can be detected in F1 outcrosses and F2 intercrosses of these two strains. These particular strains have been picked by the availability of complete genomic sequence, availability of markers and recombinant inbred lines. These studies should ultimately allow mapping of the mutation to a specific chromosomes and identification of markers to flanking its location.

D. Most significant achievement.

The identification of five diabetic ENU induced mutants that exhibit progressively worsening albuminuria, significantly exceeding that detected in other diabetic siblings.

Publications

Manuscript on diabetic nephropathy in Akita mouse in preparation

Responsible Investigators: **Matthew Breyer M.D. and Agnes Fogo M.D.,**

Project Number and Title: **Project 3 – " Phenotypic Screens for diabetic nephropathy in inbred strains of mice"**

A. Rationale and relevance

1. Phenotyping renal function in mice:

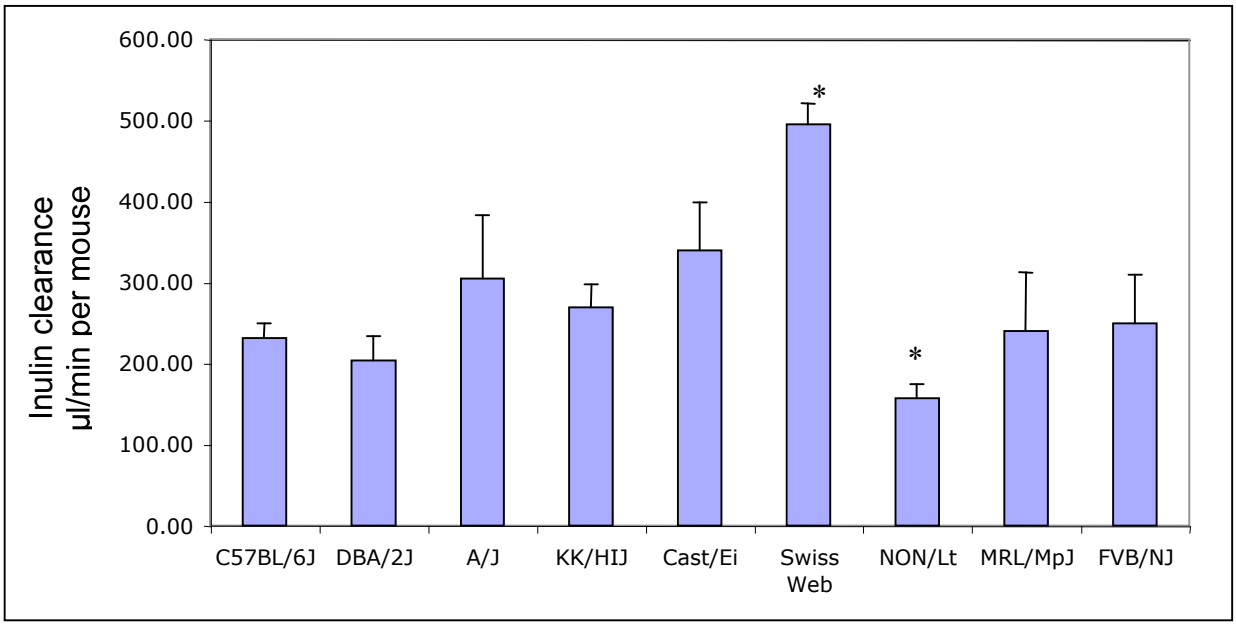
The ability to identify renal disease in mice has been significantly hampered by the lack of the routine and easy methods to determine GFR and established normal ranges of albuminuria. Measurement of GFR has been particularly problematic. Physiological characteristics of mice including limited blood volume and the presence of high levels of non-creatinine chromagens in the blood, complicate the use of endogenous creatinine clearance as an index of GFR. For example, using high performance liquid chromatography (HPLC) creatinine levels in mice are approximately 9 μM (<0.2mg/dl) or less than 20% of typical values obtained with the widely used alkaline picrate method (~45μM). It is likely cross-reacting non-creatinine chromagens account for the discrepancy between the two methods. Although similar chromagens exist in man, these generally contribute to less than 10% of measured circulating creatinine levels, using similar technique. This circumstance requires the use of more rigorous methods to determine GFR in conscious mice. The present studies established the feasibility of serially measuring GFR in conscious mice using FITC-inulin clearance.

B. Summary of Accomplishments

Two non-radioactive methods for determining glomerular filtration rate (GFR) in conscious mice using fluorescein isothiocyanate labeled inulin (FITC-inulin) were evaluated. The first method measured GFR using clearance kinetics of plasma FITC-inulin following a single bolus injection. Based on a two-compartment model, estimated GFR was 236.69 ± 16.55 and 140.20 ± 22.27 μl/min in male and female C57BL/6J mice, respectively. Total or 5/6 nephrectomy reduced inulin clearance to zero or 32.80 ± 9.32 μl /min, respectively. Conversely, diabetes mellitus induced by streptozotocin (STZ) was associated with increased GFR. The other approach measured urinary Inulin clearance using intra-peritoneal micro-osmotic pumps to delivery FITC-inulin and metabolic cages to collect timed urine samples. This approach yielded similar GFR values of 211.11 ± 26.56 and 157.36 ± 20.02μl/min in male and female mice, respectively. These studies demonstrate the feasibility of repeated non-isotopic measurement of inulin clearance in conscious mice.

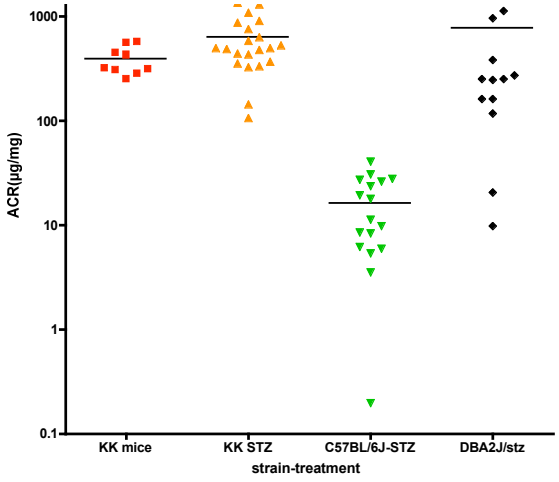
In the past year the glomerular filtration rate (GFR) has been determined for several different strains of mice. Significant differences in GFR of non-diabetic mice can be seen (figure 2), and most of these differences persist when corrected for mouse body weight. In particular the GFR for the out-bred Swiss white mice are higher than C57BL/6J while the NON/LtJ GFR is lower than other strains.

FIG 2: GFR measured in normal (non-diabetic mice) is influenced by genetic strain.



Studies examining the levels of albuminuria in several strains of inbred diabetic mice are also being characterized. In these studies we've determine that DBA2/J and KK/HiJ develop significantly more albuminuria than in diabetic C57BL6/J mice of comparable age, gender, duration and severity of diabetes (induced by low dose STZ). Interestingly non-STZ treated KK mice also exhibit substantial albuminuria as compared to diabetic C57BL6/J mice (Figure 3).

Figure 3: Log10 plot of Alb:Cre ratio in diabetic KK, C57BL/6J and DBA2/J mice. Measurements were made following 15 weeks of hyperglycemia in male mice. Symbols represent ACR in individual mice.



C. Plans for the coming year

Further studies will determine the GFR in ***diabetic*** mice of several strains and determine whether GFR falls in these diabetic mice to levels below that determined to be normal for non-diabetic mice of the same strain.

In the coming year we plan to establish additional normal ranges for renal function in several inbred stains of mice including KK/HIJ, C57BL/6J, 129p3J, DBA/2J, A/J, Cast/EiJ, MRL.

Parameters for histopathology will be evaluated including mesangial volume/glomerular volume, and GBM thickness. Finally albuminuria will be determined in several strains of diabetic mice focusing on C57BL/6J, NON/LtJ, KK/HiJ and DBA/2J.

Collaborations between the Einstein/Jefferson Group and the Vanderbilt Group will be undertaken to utilize microarray to identify specific genes that are up-regulated in kidneys of diabetic strains exhibiting greater susceptibility to nephropathy than those resistant to nephropathy.

D. Significant Achievement

Establishing strain dependence of diabetic nephropathy as determined by albuminuria and histopathological changes. The studies suggest KK/HiJ and DBA2/J mice provide good candidate strains for further exploration as to whether they may develop diabetic nephropathy including renal failure.

Publications:

Qi Z, Whitt I, Mehta A, Jin J, Zhao M, Harris RC, Fogo AB, Breyer MD Serial determination of glomerular filtration rate in conscious mice using FITC-inulin clearance. Am J Physiol Renal Physiol. 2004 Mar;286(3):F590-6. Epub 2003 Nov 04.

Stephen Dunn, Zhonghua Qi, Erwin Bottinger, Matthew Breyer, Kumar Sharma. Utility of endogenous creatinine clearance as a measure of renal function in mice **Kidney international** (in revision).

REFERENCES

1. Pass, G.J., Becker, W., Kluge, R., Linnartz, K., Plum, L., Giesen, K., and Joost, H.-G. 2002. Effect of Hyperinsulinemia and Type 2 Diabetes-Like Hyperglycemia on Expression of Hepatic Cytochrome P450 and Glutathione S-Transferase Isoforms in a New Zealand Obese-Derived Mouse Backcross Population. *J Pharmacol Exp Ther* 302:442-450.
2. Barclay, T.B., Peters, J.M., Sewer, M.B., Ferrari, L., Gonzalez, F.J., and Morgan, E.T. 1999. Modulation of Cytochrome P-450 Gene Expression in Endotoxemic Mice Is Tissue Specific and Peroxisome Proliferator-Activated Receptor-alpha Dependent. *J Pharmacol Exp Ther* 290:1250-1257.
3. Holla, V.R., Adas, F., Imig, J.D., Zhao, X., Price, E., Jr., Olsen, N., Kovacs, W.J., Magnuson, M.A., Keeney, D.S., Breyer, M.D., et al. 2001. Alterations in the regulation of androgen-sensitive Cyp 4a monooxygenases cause hypertension. *Proc Natl Acad Sci U S A* 98:5211-5216.
4. Muerhoff, A.S., Griffin, K.J., and Johnson, E.F. 1992. The peroxisome proliferator-activated receptor mediates the induction of CYP4A6, a cytochrome P450 fatty acid omega-hydroxylase, by clofibric acid. *J Biol Chem* 267:19051-19053.
5. Adler, S.G., Pahl, M., and Seldin, M.F. 2000. Deciphering diabetic nephropathy: progress using genetic strategies [editorial]. *Curr Opin Nephrol Hypertens* 9:99-106.
6. Zheng, F., Striker, G.E., Esposito, C., Lupia, E., and Striker, L.J. 1998. Strain differences rather than hyperglycemia determine the severity of glomerulosclerosis in mice. *Kidney Int* 54:1999-2007.
7. Ruggenenti, P., Perna, A., Gherardi, G., Benini, R., and Remuzzi, G. 2000. Chronic proteinuric nephropathies: outcomes and response to treatment in a prospective cohort of 352 patients with different patterns of renal injury. *Am J Kidney Dis* 35:1155-1165.
8. Gartner, K. 1978. Glomerular hyperfiltration during the onset of diabetes mellitus in two strains of diabetic mice (c57bl/6j db/db and c57bl/ksj db/db). *Diabetologia* 15:59-63.
9. Nadeau, J.H., and Frankel, W.N. 2000. The roads from phenotypic variation to gene discovery: mutagenesis versus QTLs. *Nat Genet* 25:381-384.
10. Justice, M.J. 2000. Capitalizing on Large-scale mouse mutagenesis screens. *Nature Reviews| Genetics* 1:109-115.
11. Hrabe de Angelis, M.H., Flaswinkel, H., Fuchs, H., Rathkolb, B., Soewarto, D., Marschall, S., Heffner, S., Pargent, W., Wuensch, K., Jung, M., et al. 2000. Genome-wide, large-scale production of mutant mice by ENU mutagenesis. *Nat Genet* 25:444-447.
12. Nolan, P.M., Peters, J., Strivens, M., Rogers, D., Hagan, J., Spurr, N., Gray, I.C., Vizor, L., Brooker, D., Whitehill, E., et al. 2000. A systematic, genome-wide, phenotype-driven mutagenesis programme for gene function studies in the mouse. *Nat Genet* 25:440-443.
13. Nolan, P.M., Peters, J., Vizor, L., Strivens, M., Washbourne, R., Hough, T., Wells, C., Glenister, P., Thornton, C., Martin, J., et al. 2000. Implementation of a large-scale ENU mutagenesis program: towards increasing the mouse mutant resource. *Mamm Genome* 11:500-506.
14. Justice, M.J., Noveroske, J.K., Weber, J.S., Zheng, B., and Bradley, A. 1999. Mouse ENU mutagenesis. *Hum Mol Genet* 8:1955-1963.