PROJECT TITLE: Using new mouse models to identify antioxidant roles in diabetic nephropathy

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BRIEF OVERVIEW OF PROJECT. Diabetic nephropathy (DN) is the single largest cause of end-stage renal disease. Although treatment has improved, renal failure remains a major health burden. Thus, additional strategies to protect the kidney against diabetic complications are required. Since oxidative stress is considered to be important in the development of DN, antioxidant therapy is a plausible strategy for treatment of these oxidative stress-related diseases. However, outcomes of most clinical trials for antioxidant therapy are disappointing. One possibility is that the antioxidants used in the clinic trials were not able to protect mitochondria, which is believed to be a major source for generation of reactive oxygen species (ROS). Another speculation is the initiation and severity of DN is dependent on the levels of endogenous antioxidants in patients. Thus, the purpose of this project is to develop a new antioxidant model to test these hypotheses and improve DN treatment.

Among all antioxidants, alpha-lipoic acid (1, 2-dithiolane-3-pentanoic acid, LA) has the following unique features and was thus selected for development of antioxidant animal model. 1). LA is produced by lipoic acid synthase (Lias) in mitochondria and thus potentially protects mitochondria (1). 2). LA plays a central role in the body's antioxidant network by effectively ROS, recycling other antioxidants like such as vitamin C, vitamin E, glutathione, coenzyme Q10, and ubiquinone, and self-regeneration through three cellular enzymes (2-5). LA also can ameliorate cellular antioxidative defense by transcriptional regulation (6). 3). Multipy clinical trials have shown that lipoic acid suppementation effectively attenuates diabetic polyneuropathy (7; 8). 4). LA is an essential cofactor in the several mitochondrial enzymes (9) and may participate in glucose metabolism. To test my hypotheses, we have created hyper- and hypomorphic mouse models with varying levels of *Lias* gene expression. These mice are then crossed with diabetic mice. As I anticipated, the diabetic mice with low antioxidant capacity are more susceptible to reactive oxygen species (ROS) and consequently show accelerated development of DN. On the other hand, diabetic mice with a strengthened antioxidant reservoir are more resistant to ROS and retard the development of DN. In summary, the hyper- and hypomorphic *Lias* mice provide us a platform for comparative analysis of endogenous lipoic acid in the development of DN.

Major Accomplishments

Systemic pathological changes. We have generated $Lias^{High/High}$ and $Lias^{Low/Low}$ mice as antioxidant models by modification of 3'-untranslated region (3'-UTR) of *Lias* gene. The most important phenotype of the hyper- and hypomorphic mice is that they have different levels of *Lias* gene expression (150% for $Lias^{High/High}$ and 25% for $Lias^{Low/Low}$ mice) with corresponding different redox status (due to technical difficulty, plasma and tissue lipoic acid cannot be measured). In addition, they do not show significantly different metabolic changes. Therefore, they can serve as antioxidant models to test the impact of endogenous antioxidant capacity on the development of the diseases implicated in oxidative stress. For this project,

Lias$^{High/High}$ and *Lias*$^{Low/Low}$ mice have been crossed with *Ins2*$^{Akita/+}$ mice, a well-studied non-obese hypo-insulinemic diabetic mouse. The *Ins2*$^{Akita/+}$ mice have a mutation changing cysteine 96 to tyrosine in the insulin 2 gene and exhibit marked hyperglycemia as early as 4 weeks of age (10). Male mice are characterized and used for the study and females, which only have a mild phenotype, are excluded in this project.

Table 1. Laboratory data in experimental animals

Parameters	*Lias*$^{H/H}$ n=8	*Lias*$^{L/L}$ n=8	*Lias*$^{+/+}$*Ins2*$^{Akita/+}$ n=6	*Lias*$^{H/H}$*Ins2*$^{Akita/+}$ n=5	*Lias*$^{L/L}$*Ins2*$^{Akita/+}$ n=4	*P* Value
Diet intake (g/day)	3.3±0.5	2.9±0.6	7.6±0.6	8.4±0.6	9.4±0.8	NS
Water intake (ml/day)	3.3±0.3	3.0±0.4	26.8±1.3	26.5±0.6	29.5±0.8	NS
Body weight (BW, g)	29.6±2.6	28±2.2	20.7±1.0	25.6±0.9[a]	19.6±1.9	<0.05
Plasma						
Glucose (mg/dl)	145±11	128±9	534±45	629±68	517±56	NS
Cholesterol (mg/dl)	79.2± 2.2	58.4±5.6	78±7	64±13	84±13	NS
Triglyceride (mg/dl)	62.1±3.2	50.3±5.4	55±9	52±5	63±13	NS
Kidney						
Urine volume (ml/day)	NA	NA	22.5±4.2	21.5±8.0	25.3±6.7	NS
Kidney weight (KW, g)	NA	NA	0.29±0.04	0.30±0.05	0.29±0.11	NS
KW/BW (mg/g)	NA	NA	14±1.3	12±0.9	15±1.4	NS

Data shown are mean values ±SEM for the male mice at 7 months. *P* Values are for comparisons among *Lias*$^{+/+}$*Ins2*$^{Akita/+}$, *Lias*$^{H/H}$*Ins2*$^{Akita/+}$ *and Lias*$^{L/L}$*Ins2*$^{Akita/+}$mice. n: mouse number. NA: not available

Lias$^{High/High}$*Ins2*$^{Akita/+}$ and *Lias*$^{Low/Low}$*Ins2*$^{Akita/+}$ mice exhibit the diabetic phenotype. Lean body weight is a feature of diabetic *Ins2*$^{Akita/+}$ mice. Over-expression of *Lias* gene result in significant retention of body weight compared with *Lias*$^{Low/Low}$*Ins2*$^{Akita/+}$ mice and *Ins2*$^{Akita/+}$ mice as shown in Table 1.

Lias$^{High/High}$*Ins2*$^{Akita/+}$ and *Lias*$^{Low/Low}$*Ins2*$^{Akita//+}$ mice reveal hyperglycemia at 4 weeks of age (data not shown) and hyperglycemia reaches the highest level as shown in Table 1 at 7 months of age when the mice are sacrificed. However, there is no significant difference in the severity of hyperglycemia among *Lias*$^{High/High}$*Ins2*$^{Akita/+}$ and *Lias*$^{Low/Low}$*Ins2*$^{Akita/+}$ and *Ins2*$^{Akita/+}$ mice although plasma glucose levels in *Lias*$^{High/High}$*Ins2*$^{Akita/+}$ are higher than *Lias*$^{Low/Low}$*Ins2*$^{Akita/+}$ mice. Likewise, plasma total cholesterol levels in *Lias*$^{High/High}$*Ins2*$^{Akita/+}$ are lower than *Lias*$^{Low/Low}$*Ins2*$^{Akita/+}$ despite being non-significant as shown in Table 1. We reported in the previous project, *Lias*$^{+/-}$ mice with approximately 50% *Lias* gene expression had significantly increased plasma cholesterol in diabetic *Lias*$^{+/-}$*apoE*$^{-/-}$ mice (11; 12). Based on this result, it is anticipated that an increased *Lias* gene expression may further reduce plasma cholesterol levels. However, current data do not support this speculation. A similar situation was observed in plasma triglyceride levels (Table 1). However, we have not detected significant changes in plasma triglyceride in *Lias*$^{+/-}$*apoE*$^{-/-}$ mice and diabetic *apoE*$^{-/-}$ mice feeding on dietary lipoic acid (13) although other investigators do (14).

Kidney pathologic changes. Individual mice are housed in metabolic cages designed for mouse urine albumin excretion determination. Body weight, food and water intake, and urine volume are monitored in a 24 h period for 2 consecutive days. Renal changes in *Lias*$^{High/High}$ *Ins2*$^{Akita/+}$ and *Lias*$^{Low/Low}$*Ins2*$^{Akita/+}$ mice are assessed by the measurements of urine albumin

excretion. Urine albumin is measured with a mouse Albuwell ELISA kit (Exocell Inc. Philadelphia, PA). Albumin excretion rate is almost 2-fold higher in *Lias*$^{Low/Low}$*Ins2*$^{Akita/+}$ mice than *Lias*$^{+/+}$*Ins2*$^{Akita/+}$ mice (Figure 1), and 1-fold higher than *Lias*$^{+/-}$ *Ins2*^{Akita} mice (11) at 7 months of age. Dietary intake and water consumption does not show any significant difference among the three groups of mice (Table 1).

Figure 1

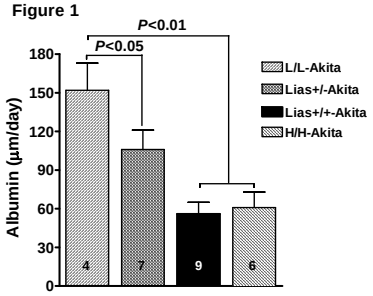
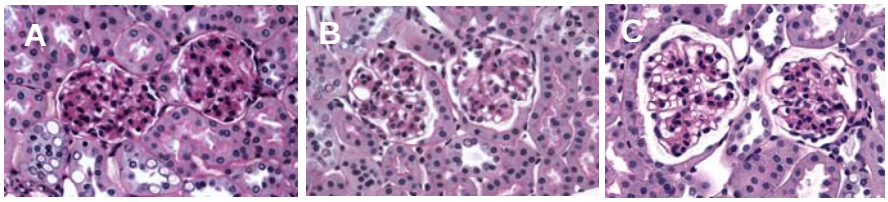


Table 1 shows kidney weight-to-body weight ratio (KW/BW) is higher in *Lias*$^{Low/Low}$*Ins2*$^{Akita/+}$ and lower in *Lias*$^{High/High}$*Ins2*$^{Akita/+}$ mice (*P*=0.09) than in *Lias*$^{+/-}$*Ins2*$^{Akita/+}$ mice, suggesting that increased *Lias* gene expression may attenuate potential diabetic renal hypertrophy. The possibility is supported by the data that transforming growth factor β 1 (TGFβ1) gene expression increases in *Lias*$^{Low/Low}$*Ins2*$^{Akita/+}$ mice (*P*=0.06) compared with *Lias*$^{+/+}$*Ins2*$^{Akita/+}$ mice (Table 2) whereas TGFβ1 gene expression is significantly reduced in *Lias*$^{High/High}$*Ins2*^{Akita} mice (Table 2). The results indicate that change of *Lias* gene expression could regulate TGFβ1 gene expression likely through quenching oxidative stress. It is well know that TGFβ1 plays an important role in kidney hypertrophy of DN (15). TGFβ1 expression is elevated in human and experimental diabetic nephropathy (16) and oxidative stress is involved in the process (17).

Renal pathological changes are assessed by light and electron microscopy. Figure 2 shows representative glomerular light micrographs from *Lias*$^{High/High}$*Ins2*$^{Akita/+}$ and *Lias*$^{Low/Low}$*Ins2*$^{Akita/+}$ mice. Moderate mesangial expansion, as evidenced by increased accumulation of periodic acid-Schiff (PAS)-positive material in the mesangial area, is observed in 7-month-old *Lias*$^{Low/Low}$*Ins2*$^{Akita/+}$ mice (Figure 2A), whereas mesangial expansion is mild in 7-month-old *Lias*$^{High/High}$*Ins2*$^{Akita/+}$ mice (Figure 2B). Semi-quantitative analysis of PAS-stained kidney sections reveals higher mesangial expansion score in *Lias*$^{Low/Low}$*Ins2*$^{Akita/+}$ mice as compared with *Lias*$^{High/High}$*Ins2*$^{Akita/+}$ and *Lias*$^{+/+}$*Ins2*$^{Akita/+}$ mice although this difference does not reach significance likely due to a relatively small sample number (data not shown). Transmission electron microscopy examination of *Lias*$^{Low/Low}$*Ins2*$^{Akita/+}$ mouse glomeruli shows structural irregularities of glomerular basement membranes (GBM) (data not shown). Podocyte effacement, although mild, is primarily detected in *Lias*$^{Low/Low}$*Ins2*$^{Akita/+}$ mice (data not shown). However, glomerulosclerosis, arteriolar hyalinosis and focal tubulointerstitial fibrosis is not observed in glomeruli at 7 month of age. In addition, no electron dense deposits are observed in the glomeruli of all these mice.

Figure 2

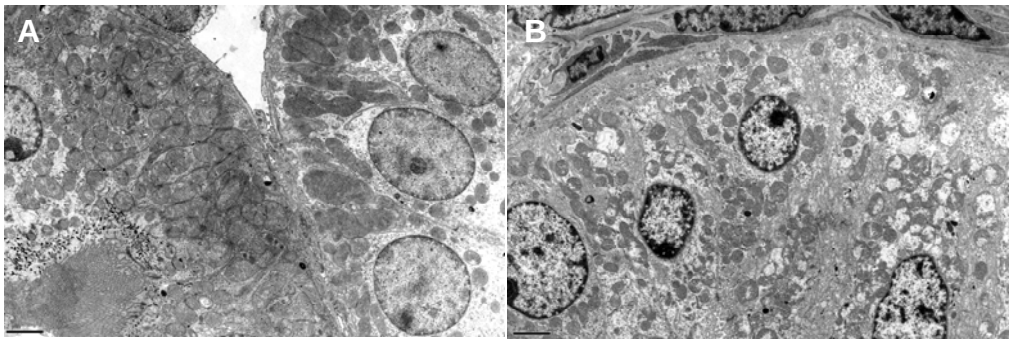


Lias$^{Low/Low}$*Ins2*^{Akita} *Lias*$^{+/+}$*Ins2*^{Akita} *Lias*$^{High/High}$*Ins2*^{Akita}

Renal proximal tubules represent most metabolically active structural and functional components in the nephron and account for reabsorption of 99% of glucose load filtered by glomeruli. It is shown that renal proximal tubular function is disturbed by high glucose-induced oxidative stress in the early stages of DN (18). Proximal tubule epithelial cells, possessing numerous mitochondria, provide a window to observe and test the effect of lipoic acid on protection of mitochondria. As I mentioned above, lipoic acid is produced in mitochondria and has the potential to protect mitochondria. In my current project data showed significantly more damaged mitochondria 7-

months-old *Lias*$^{Low/Low}$*Ins2*$^{Akita/+}$ mice (Figure 3B) than *Lias*$^{High/High}$*Ins2*$^{Akita/+}$ mice (Figure 3A). These results confirm our previous observation that many mitochondria got damaged in proximal tubule epithelial cells of *Ins2*$^{Akita/+}$ mice with 50% *Lias* gene expression (11). Moreover, a further decrease in *Lias* gene expression results in more mitochondrial damage. In contrast, the number of damaged mitochondria in *Lias*$^{High/High}$*Ins2*$^{Akita/+}$ mice is markedly rescued compared with *Lias*$^{+/-}$*Ins2*$^{Akita/+}$ mice. Taken together, the result strongly suggests that mitochondrial damage is a predominant pathological feature of DN and lipoic acid conducts a protective effect for mitochondria. We are isolating proximal tubule cells from mice with different *Lias* gene expression to examine superoxide production and its impact on mitochondrial functions.

Figure 3



Lias$^{High/High}$*Ins2*^{Akita} mice *Lias*$^{Low/Low}$*Ins2*^{Akita} mice

Mitochondria play a critical role in apoptosis (19; 20). ROS produced by the mitochondria can be involved in apoptosis (21). Since a large number of injured mitochondria occur in *Lias*$^{Low/Low}$*Ins2*$^{Akita/+}$ mice, we next examined apoptotic cells using TUNEL method following the manufacturer's protocol (Millipore). Significantly different numbers of apoptotic cells are detected in the sections from *Lias*$^{Low/Low}$*Ins2*$^{Akita/+}$ mice and *Lias*$^{High/High}$*Ins2*$^{Akita/+}$ mice (data not shown). Currently, we completed three mice in each group and will test more mice in the next 2 months when the mice reach 7 month of age (note: The grant period began last September but I did not receive the grant account number from the University of North Carolina until January).

Oxidative stress. The pathological changes in the diabetic mice with different *Lias* gene expression are accompanied with oxidative stress. Table 2 shows *Lias* gene expression decreased in all diabetic mice at 7 months of age. Among three genotypes of the diabetic mice, *Lias* gene expression is reduced by around 20% in *Lias*$^{High/High}$*Ins2*$^{Akita/+}$ mice compared with *Lias*$^{High/High}$ mice whereas *Lias*$^{Low/Low}$*Ins2*$^{Akita/+}$ and *Lias*$^{+/+}$*Ins2*$^{Akita/+}$ mice dropped more than 40% compared with corresponding non-diabetic mice, suggesting that DN consumes more endogenous *Lias* reservoir in the two groups. On the other hand, gene expression of *Sod2*, the mitochondrial superoxide dismutase, increases in order to compensate for loss of Lias in *Lias*$^{Low/Low}$*Ins2*$^{Akita/+}$ mice.

Table 2. Gene expression in kidney cortex

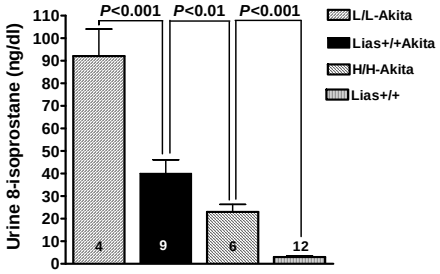
	Lias$^{+/+}$	*Lias*$^{H/H}$	*Lias*$^{L/-}$	*Lias*$^{+/+}$*Ins2*$^{Akita/+}$	*Lias*$^{H/H}$*Ins2*$^{Akita/+}$	*Lias*$^{L/-}$*Ins2*$^{Akita/+}$
Lias	1.00±0.09(4)	1.58±0.11(4)	0.25±0.15(4)	0.43±0.14(6)	1.26±0.16^{a,b}(5)	0.14±0.25^{a,b}(4)
Sod2	NA	NA	NA	1.00±0.16(6)	1.05±0.25(5)	1.65±0.21^{a}(4)
TGFβ1	NA	NA	NA	1.00±0.11(6)	0.67±0.16(5)	1.32±0.19(4)
GLUT1	NA	NA	NA	1.00±0.18(6)	0.90±0.31(5)	0.98±0.27(4)

Samples were taken at 7 months of age of the mice. Data shown are mean values ±SEM. a: P<0.05 vs *Lias*$^{+/+}$*Ins2*$^{Akita/+}$ mice; b: P<0.05 vs non-diabetic corresponding *Lias*$^{H/H}$ mice or *Lias*$^{L/-}$ mice. mRNA levels

of *Lias*$^{+/+}$*Ins2*$^{Akita/+}$ mice were used as references and set to 1.00 for Sod2, *TGFβ*1 and *GLUT1* gene expression. mRNA of *Lias*$^{+/+}$mice were used as reference for *Lias* gene expression.

I also examined a reliable lipid peroxidation marker, 8-isoprostane, in urine. 7-month-old *Lias*$^{Low/Low}$*Ins2*$^{Akita/+}$ mice show considerably higher levels than *Lias*$^{+/+}$*Ins2*$^{Akita/+}$ mice (Figure 4). On the contrary, the same aged *Lias*$^{High/High}$*Ins2*$^{Akita/+}$ mice reveal significantly lower urinary 8-isoprostane level than *Lias*$^{+/+}$*Ins2*$^{Akita/+}$ mice (Figure 4). The data suggest that the diabetic mice with low antioxidant capacity lead to increased oxidative stress. On the other hand, the diabetic mice with a strengthened antioxidant reservoir are more resistant to ROS and retard the development of DN.

Figure 4



In summary, *Lias*$^{High/High}$*Ins2*$^{Akita/+}$ and *Lias*$^{Low/Low}$*Ins2*$^{Akita/+}$ mice show different degrees of diabetic pathologic changes and kidney oxidative stress. The models further clarify the role of antioxidants in the development of diabetic nephropathy and will help identify potential therapeutic targets for effective antioxidant therapy.

References:

1. Shay KP, Hagen TM: Age-associated impairment of Akt phosphorylation in primary rat hepatocytes is remediated by alpha-lipoic acid through PI3 kinase, PTEN, and PP2A. *Biogerontology* 10:443-456, 2009
2. Biewenga GP, Haenen GR, Bast A: The pharmacology of the antioxidant lipoic acid. *Gen Pharmacol* 29:315-331, 1997
3. Packer L, Kraemer K, Rimbach G: Molecular aspects of lipoic acid in the prevention of diabetes complications. *Nutrition* 17:888-895, 2001
4. Liu J: The effects and mechanisms of mitochondrial nutrient alpha-lipoic acid on improving age-associated mitochondrial and cognitive dysfunction: an overview. *Neurochem Res* 33:194-203, 2008
5. Packer L, Witt EH, Tritschler HJ: alpha-Lipoic acid as a biological antioxidant. *Free Radic Biol Med* 19:227-250, 1995
6. Suh JH, Shenvi SV, Dixon BM, Liu H, Jaiswal AK, Liu RM, Hagen TM: Decline in transcriptional activity of Nrf2 causes age-related loss of glutathione synthesis, which is reversible with lipoic acid. *Proc Natl Acad Sci U S A* 101:3381-3386, 2004
7. Ziegler D: Treatment of diabetic neuropathy and neuropathic pain: how far have we come? *Diabetes Care* 31 Suppl 2:S255-261, 2008
8. Cowell RM, Russell JW: Nitrosative injury and antioxidant therapy in the management of diabetic neuropathy. *J Investig Med* 52:33-44, 2004
9. Reed LJ: A trail of research from lipoic acid to alpha-keto acid dehydrogenase complexes. *J Biol Chem* 276:38329-38336, 2001
10. Yoshioka M, Kayo T, Ikeda T, Koizumi A: A novel locus, Mody4, distal to D7Mit189 on chromosome 7 determines early-onset NIDDM in nonobese C57BL/6 (Akita) mutant mice. *Diabetes* 46:887-894, 1997
11. Yi X, Xu L, Hiller S, Kim HS, Nickeleit V, James LR, Maeda N: Reduced expression of lipoic acid synthase accelerates diabetic nephropathy. *J Am Soc Nephrol* 23:103-111, 2012
12. Yi X, Xu L, Hiller S, Kim HS, Maeda N: Reduced alpha-lipoic acid synthase gene expression exacerbates atherosclerosis in diabetic apolipoprotein E-deficient mice. *Atherosclerosis* 223:137-143, 2012

13. Yi X, Maeda N: alpha-Lipoic acid prevents the increase in atherosclerosis induced by diabetes in apolipoprotein E-deficient mice fed high-fat/low-cholesterol diet. *Diabetes* 55:2238-2244, 2006
14. Zhang WJ, Bird KE, McMillen TS, LeBoeuf RC, Hagen TM, Frei B: Dietary alpha-lipoic acid supplementation inhibits atherosclerotic lesion development in apolipoprotein E-deficient and apolipoprotein E/low-density lipoprotein receptor-deficient mice. *Circulation* 117:421-428, 2008
15. Sharma K, Ziyadeh FN: Hyperglycemia and diabetic kidney disease. The case for transforming growth factor-beta as a key mediator. *Diabetes* 44:1139-1146, 1995
16. Yamamoto T, Nakamura T, Noble NA, Ruoslahti E, Border WA: Expression of transforming growth factor beta is elevated in human and experimental diabetic nephropathy. *Proc Natl Acad Sci U S A* 90:1814-1818, 1993
17. Ziyadeh FN, Han DC, Cohen JA, Guo J, Cohen MP: Glycated albumin stimulates fibronectin gene expression in glomerular mesangial cells: involvement of the transforming growth factor-beta system. *Kidney Int* 53:631-638, 1998
18. Han HJ, Lee YJ, Park SH, Lee JH, Taub M: High glucose-induced oxidative stress inhibits Na+/glucose cotransporter activity in renal proximal tubule cells. *Am J Physiol Renal Physiol* 288:F988-996, 2005
19. Hoppins S, Nunnari J: Cell Biology. Mitochondrial dynamics and apoptosis--the ER connection. *Science* 337:1052-1054, 2012
20. Orrenius S: Mitochondrial regulation of apoptotic cell death. *Toxicol Lett* 149:19-23, 2004
21. Fleury C, Mignotte B, Vayssiere JL: Mitochondrial reactive oxygen species in cell death signaling. *Biochimie* 84:131-141, 2002