Overexpression of lipoic acid synthase gene alleviates diabetic nephropathy of Lepr\textsuperscript{db/db} mice

Yingzheng Zhao, Tingting Yan, Cheng Xiong, Meiyu Chang, Qiyu Gao, Sanqiao Yao, Weidong Wu, Xianwen Yi, Guangcui Xu

ABSTRACT

Introduction Diabetic nephropathy (DN) develops in about 40% of patients with type 2 diabetes and remains the leading cause of end-stage renal disease. The mechanisms of DN remain to be elucidated. Oxidative stress is thought to be involved in the development of DN but antioxidant therapy has produced conflicting results. Therefore, we sought to define the role of antioxidant in retarding the development of DN in this study.

Research design and methods We generated a new antioxidant/diabetes mouse model, Lias\textsuperscript{H/H} Lepr\textsuperscript{db/db} mice, by crossing Lias\textsuperscript{H/H} db/db mice with Lias\textsuperscript{H/H} mice, which have overexpressed Lias gene (~160%) compared with wild type, and also correspondingly increased endogenous antioxidant capacity. The new model was used to investigate whether predisposed increased endogenous antioxidant capacity was able to retard the development of DN. We systemically and dynamically examined main pathological alterations of DN and antioxidant biomarkers in blood and kidney mitochondria.

Results Lias\textsuperscript{H/H} Lepr\textsuperscript{db/db} mice alleviated major pathological alterations in the early stage of DN, accompanied with increased antioxidant defense. The model targets the main pathogenic factors by exerting multiple effects such as hypoglycemic, anti-inflammation, and antioxidant, especially protection of mitochondria.

Conclusion The antioxidant animal model is not only very useful for elucidating the underlying mechanisms of DN but also brings insight into a new therapeutic strategy for clinical applications.

INTRODUCTION

Diabetes mellitus (DM) is the leading cause of cardiovascular and renal disease worldwide. In the last decades, the prevalence of type 2 diabetes mellitus (T2DM) has been increasing. Among microvascular complications of diabetes, DN develops in about 40% of patients with T2DM and T1DM and remains the leading cause of end-stage renal disease.\textsuperscript{1,3}

The prevalence of diabetes including T1DM and T2DM is 11.6% in Chinese adults; this may represent up to 113.9 million adults.\textsuperscript{4} Among those with diabetes, the prevalence of chronic kidney disease is as high as 63.9%.\textsuperscript{4}

The mechanisms of DN remain uncertain.\textsuperscript{5} Increased oxidative stress is proposed to be a major mechanism.\textsuperscript{1} Hyperglycemia is a major contributor to reactive oxygen species (ROS) production in mitochondria\textsuperscript{12} and consequently oxidative stress.\textsuperscript{8} Accumulative evidence suggests that oxidative stress derived from impaired antioxidant defenses has been implicated in the pathogenesis of DN.\textsuperscript{8} However, antioxidant therapy has produced conflicting results.\textsuperscript{10}

We postulate that impairment of antioxidant defense plays a critical role in the prevention and treatment of DN. The mouse models of antioxidant could be useful for a better understanding of the role of antioxidants in the onset and development of DN, although they usually exhibit modest kidney abnormalities and can only mimic the early stage of human DN.\textsuperscript{11}

Significance of this study

What is already known about this subject?

- Oxidative stress is thought to be involved in the development of diabetic nephropathy (DN) but antioxidant therapy has produced conflicting results.

What are the new findings?

- We generated a new antioxidant/diabetes mouse model by crossing Lias\textsuperscript{H/H} db/db mice, a common type 2 diabetes mouse model, with Lias\textsuperscript{H/H} mice, which have overexpressed Lias gene (~160%) compared with wild type, and also correspondingly increased endogenous antioxidant capacity.
- The strengthened endogenous antioxidant capacity animal model significantly attenuated pathological changes of early stage in DN including microalbuminuria, mesangial matrix expansion, podocyte foot process effacement, and chronic inflammatory response, accompanied with increased antioxidant content in the blood and kidney mitochondria.
- Strengthened antioxidant defense would be a valuable strategy for prevention and treatment of human DN.

How might these results change the focus of research or clinical practice?

- Strengthened antioxidant defense would be a valuable strategy for prevention and treatment of human DN.
α-lipoic acid (LA) is a potent antioxidant, catalyzed by lipoic acid synthase (Lias) in the mitochondria. To test our hypothesis, we generated two mouse lines by modifying 3′ untranslated regions of Lias gene (Lias) to influence mRNA stability. These models exhibit different levels of gene expression (LiasLow/Lias−10% and LiasHigh/Lias+160% of wild type) with corresponding LIAS expression, determined by real-time quantitative (qRT)-PCR and western blot. Our previous study showed that total plasma antioxidant capacity was significantly increased and lipid peroxidation induced by oxidative stress was exhibited in animal model with high expression of Lias gene.15 And the LiasH/H animals did not show any abnormal behaviors and performance in all generation in our long-term observations. By crossing LiasH/H with LeprH/H mice, the most widely used model of type 2 diabetes,13 we generated the LiasH/HLeprH/H mouse model. Our novel LiasH/HLeprH/H mice mimic gain-of-function of antioxidant by creating a strengthened endogenous antioxidant microenvironment. They may amplify subtle changes that may not be apparent under basal conditions and will be helpful to identify LA targeted diagnostic biomarkers in mitochondria, and decipher the mechanisms of immune regulations during the development of the disease.

In this study, we sought to determine whether overexpression of Lias gene could attenuate oxidative stress and chronic inflammation in mice with DN. Our data demonstrated that LiasH/HLeprH/H mice with increased endogenous antioxidant level showed alleviated major pathological alterations of T2DM at the early stage of DN.

RESEARCH DESIGN AND METHODS

Animal models
Eight-week-old C57BL/J db/+ and db/db mice were purchased from Jackson Laboratories (Bar Harbor, Maine, USA). Mice were fed ad libitum in a specific pathogen-free animal facility. LiasH/H mice on the same genetic background were mated with db/+ mice to finally obtain LiasH/HLeprH/H mice. Male LiasH/HLeprH/H mice will be used in our experiments since the lack of gender differences in the severity of renal injury in the LeprH/H db mouse model of DN.14 All animals were fed a normal chow produced according to NIH-07 standard and meet the hygienic standard of experimental animal compound feed (GB 14924.2) with 4% fat and 20.5% protein content (Puluteng, Shanghai, China). Water ad libitum. Animal experiments were carried out in accordance with the guidelines of the Institutional Animal Care and Use Committee of Xinxiang Medical University.

Measurement of blood metabolic variables
Blood glucose was monitored using OneTouch Verio Flex meter (Johnson-Johnson, New Brunswick, New Jersey, USA) after 5-hour fasting. Glycated hemoglobin (HbA1c) was measured from red cell lysates using high performance liquid chromatography (Bio-Rad, Richmond, California, USA). Serum levels of triglyceride (TG) and cholesterol (CHO) were determined using commercial assay kits (Jian-Cheng, Nan Jing, China). Levels of blood creatinine and blood urea nitrogen (BUN) were determined using commercial assay kits (Jian-Cheng, NanJing, China), and microalbuminuria was determined using ELISA kits (Ela science, Wuhan, China) following the manufacturer’s instruction.

Intraperitoneal glucose tolerance test (IPGTT)
All mice were fasted for 16 hours with free access to water before intraperitoneal injection of a glucose solution (0.5 mg/kg of body weight). Blood glucose was measured by the glucometer at designed timepoints after intraperitoneal injection of the glucose solution.

Measurement of urinary albumin excretion and creatinine concentration
Mice were singly placed in metabolic cages for 24 hours after acclimation and then food consumption, water intake, body weight, and urine output were recorded daily. Urinary albumin excretion per day was determined by mouse urinary albumin ELISA kit (Nanjing Bianzhen Biological Technology, China). Creatinine concentration in serum was measured by sarcosine oxidase method.

ELA to assess oxidative stress and inflammation markers
Blood levels of total autooxidizing capability (T-AOC), reduced glutathione (GSH) and malondialdehyde (MDA) were measured using commercial kits purchased from the Nanjing Jiancheng Bioengineering Institute (Nanjing, China) following the manufacturer’s instructions. Mitochondria were isolated from kidney cortex. GSH concentration was determined using a chromogenic assay (Thermo, EnSpire 2300, USA). MDA concentration was assayed using a chromogenic assay. This assay measures free and protein-bound MDA without undue interference from the other lipid peroxidation products.15 Superoxide dismutase 2 (SOD2) activity was measured using the Beauchamp and Fridovich method.16 Levels of T-AOC were measured using commercial kits (Cell Biolabs, USA). The net absorbance values (at 490 nm) of antioxidants are compared with a known uric acid standard curve. Results are expressed as ‘μM copper reducing equivalents’ (μM CRE). The levels of serum interleukin (IL)-1β, IL-6, tumor necrosis factor-α (TNF-α) and monocyte chemotactic protein-1 (MCP-1) in each group were analyzed by using ELISA kits (Dingguo Biotechnology, Beijing, China) according to the manufacturer’s instructions. The color generated was determined by measuring the optical density (OD) value at 450 nm with a spectrophotometric microtiter plate reader (Thermo, EnSpire 2500, USA). Levels of serum C-reactive protein (CRP) were determined using ELISA kits (Dingguo Biotechnology, Beijing, China).

Renal histopathology
The kidneys from mice were fixed in 4% paraformaldehyde at room temperature (RT) and then embedded in paraffin. Tissues were prepared as sections 3 μm in thickness and stained with periodic acid–Schiff (PAS) for examinations by light microscopy. The degree of
glomerular injury was evaluated according to the degree of mesangial matrix expansion (MME) examined in 30 glomeruli per kidney with a ×100 objective oil immersion lens from each group of mice on PAS-stained kidney tissue sections. Sixteen slides of mouse kidney covering the whole spectrum of renal cortical were evaluated by an experienced animal pathologist. MME was examined in a blinded fashion and scored from 0 to 4 according to the ratio of glomerular expansion area/normal area: score 0, a normal glomerulus; score 1, increased mesangial matrix, 25% of glomerular tuft; score 2, MME of 25%–50% of glomerular tuft; score 3, MME of 50%–75%; and score 4, MME of >75% of the tuft.

Kidney mitochondrial dysfunction
Cytosolic and mitochondrial fractions were separated using a Mitochondria Isolation Kit (Beyotime, Shanghai, China) following the manufacturer’s instructions. The mitochondrial SOD2 assay kit was used to determine the enzyme activities of SOD2 according to the manufacturer’s instructions (Beyotime Biotechnology, Shanghai, China). ATP Colorimetric Assay Kit was used to measure the ATP content in the kidney cortex (Shanghai, China). The right kidney cortical tissues were immediately fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer for 3 hours at RT. After, the specimens were postfixed with 1% osmium tetroxide. Ultrathin sections (70–80nm thick) were made on an RMC Power Tome XL ultramicrotome (Shanghai Leica Instrument, Shanghai, China), stained with both uranyl acetate and lead citrate, and examined using Hitachi H-7500 transmission electron microscopy (Hitachi, Japan). Glomerular basement membrane (GBM) thickness was measured at 20 different sites of the GBM.

Statistical analysis
The data were expressed as mean±SE. Student’s t-test was used for the comparison between $Lias^{+/+}Lepr^{db/db}$ and $Lias^{+/+}Lepr^{db/db}$ mice or one-way analysis of variance for the two strains plus $Lias^{+/+}Lepr^{db/db}$ mice. Results were considered to be statistically significant when p value was less than 0.05.

RESULTS
Alterations of metabolic parameters in $Lias^{H/H}Lepr^{db/db}$ mice
To assess the impact of enhanced endogenous antioxidant activity derived from overexpressing $Lias$ gene on metabolisms of the diabetic mice, we compared body weight and blood glucose levels between $Lias^{H/H}Lepr^{db/db}$ and $Lias^{+/+}Lepr^{db/db}$ mice. The body weights of male $Lias^{+/+}Lepr^{db/db}$ mice were markedly heavier than that of $Lias^{+/+}Lepr^{db/db}$ (table 1). The body weights of male $Lias^{H/H}Lepr^{db/db}$ mice at 7 months old was significantly heavier than their $Lias^{+/+}Lepr^{db/db}$ littermates (table 1), likely due to metabolic regulation of LA since LA is a cofactor

Table 1 Biological characteristics of three genotypes of mice at 7 months old

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>$Lias^{+/+}Lepr^{db/db}$</th>
<th>$Lias^{+/+}Lepr^{db/db}$</th>
<th>$Lias^{H/H}Lepr^{db/db}$</th>
<th>$P_1$</th>
<th>$P_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>31.54±1.05</td>
<td>65.56±2.03</td>
<td>74.27±1.71</td>
<td>0.001</td>
<td>0.001</td>
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<tr>
<td>Right kidney weight (mg)</td>
<td>244.26±8.04</td>
<td>242.58±7.61</td>
<td>248.85±13.90</td>
<td>0.909</td>
<td>0.669</td>
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<td>Chow intakes (g/day)</td>
<td>10.11±0.09</td>
<td>16.03±0.72</td>
<td>13.98±0.18</td>
<td>0.001</td>
<td>0.093</td>
</tr>
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<td>Water intakes (mL/day)</td>
<td>14.60±0.84</td>
<td>13.33±0.05</td>
<td>11.15±0.11</td>
<td>0.035</td>
<td>0.001</td>
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<tr>
<td>Urine output (mL/day)</td>
<td>2.21±0.12</td>
<td>16.94±0.51</td>
<td>13.44±0.65</td>
<td>0.001</td>
<td>0.001</td>
</tr>
<tr>
<td>TG (mM)</td>
<td>0.75±0.030</td>
<td>1.35±0.10</td>
<td>1.16±0.11</td>
<td>0.001</td>
<td>0.542</td>
</tr>
<tr>
<td>CHO (mM)</td>
<td>2.37±0.21</td>
<td>6.06±0.62</td>
<td>9.35±0.77</td>
<td>0.001</td>
<td>0.015</td>
</tr>
</tbody>
</table>

All mice were male at 7 months of age. Data are represented as mean±SEM (n=10). $P_1$ represents p value of $Lias^{+/+}Lepr^{db/db}$ vs $Lias^{+/+}Lepr^{db/db}$ mice and $P_2$ stands for p value of $Lias^{H/H}Lepr^{db/db}$ vs $Lias^{H/H}Lepr^{db/db}$ mice.

CHO, cholesterol; TG, triglyceride.
of several mitochondrial enzymes. We also found that the body weight in male Lias<sup>H/H</sup> mice was higher than that of male Lias<sup>+/+</sup> mice. The body may be regulated by metabolic homeostasis by storing body weight at the expense of excessive energy production in male Lias<sup>H/H</sup> mice.

We next determined blood glucose levels by measuring both glucose concentration and HbA1c levels at 7 months. Blood HbA1c concentrations in Lias<sup>H/H</sup>Lepr<sup>db/db</sup> mice were significantly lower than that of Lias<sup>+/+</sup>Lepr<sup>db/db</sup> mice (data not shown). HbA1c and fasting blood glucose levels at 7-month-old Lias<sup>H/H</sup>Lepr<sup>db/db</sup> mice were considerably lower than Lias<sup>+/+</sup>Lepr<sup>db/db</sup> mice (figure 1A and B), suggesting that increased endogenous antioxidant activity retarded the development of diabetes. To help diagnose type 2 diabetes, we also examine the mouse IPGTT at 7 months. The result showed that compared with Lias<sup>H/H</sup>Lepr<sup>db/db</sup> mice, Lias<sup>+/+</sup>Lepr<sup>db/db</sup> mice showed impaired glucose tolerance, as manifested by obviously increased glucose levels after glucose injection (p<0.05, all timepoints) (figure 1C).

**Reduction of systemic lipid content in Lias<sup>H/H</sup>Lepr<sup>db/db</sup> mice**

Lipid metabolism disorder is associated with development of DN. Thus, concentrations of TG and total CHO in circulation were measured in the all animals. TG level at 7 months was markedly lower in Lias<sup>H/H</sup>Lepr<sup>db/db</sup> mice than in Lias<sup>+/+</sup>Lepr<sup>db/db</sup> mice (table 1). However, total CHO concentration at 7 months was not significantly different between these two groups (table 1). The results indicated that increased antioxidant activity resulted in hypolipidemia status, which may contribute to the renal protective effect.

**Levels of plasma inflammatory mediators in Lias<sup>H/H</sup>Lepr<sup>db/db</sup> mice**

In patients with T2DM, serum levels of proinflammatory cytokines such as interleukin-1β (IL-1β) and interleukin-6 (IL-6) are elevated and have been widely used as a biomarker of T2DM. In addition, in patients with T2DM, elevated serum levels of CRP are closely associated with an increase in microalbuminuria excretion and renal dysfunction. To investigate potential anti-inflammatory role of enhanced endogenous antioxidant activity in the mouse model, we monitored serum concentrations of IL-1β, TNF-α, IL-6, CRP and MCP-1. The results showed that serum CRP, IL-1β and MCP-1 levels in Lias<sup>H/H</sup>Lepr<sup>db/db</sup> mice at 7 months of age were significantly lower than those of Lias<sup>+/+</sup>Lepr<sup>db/db</sup> mice (figure 1G–J), suggesting the inflammation was attenuated in Lias<sup>H/H</sup>Lepr<sup>db/db</sup> mice.

**Upregulation of antioxidant activity ameliorated renal dysfunction**

To investigate whether elevated endogenous antioxidant activity blocked the development of DN over time, urine albumin was examined before the mice were sacrificed. Our results showed that albuminuria was noticeably decreased in Lias<sup>+/+</sup>Lepr<sup>db/db</sup> mice, which was significantly alleviated in Lias<sup>H/H</sup>Lepr<sup>db/db</sup> mice (figure 1D). The concentrations of serum creatinine and BUN in Lepr<sup>db/db</sup> mice during the early stage of diabetes did not differ significantly from those in non-diabetic mice of the same age (data not shown). However, after persistent hyperglycemia, the serum concentrations of creatinine were significantly increased in Lias<sup>H/H</sup>Lepr<sup>db/db</sup> mice but this increase was partially offset in Lias<sup>+/+</sup>Lepr<sup>db/db</sup> mice (figure 1E). Serum BUN in Lias<sup>H/H</sup>Lepr<sup>db/db</sup> (for an unknown reason) and Lias<sup>+/+</sup>Lepr<sup>db/db</sup> mice significantly increased (figure 1F). The concomitant increases in BUN and creatinine suggested that the glomerular function was compromised. However, occurrence of hydration following persistent glycosuria likely contributed to the increased BUN in the diabetic animals.

**Overexpression of Lias gene reduced oxidative stress in plasma and kidney mitochondria**

To determine whether enhanced endogenous antioxidant activity systematically decreased oxidative stress, we measured plasma antioxidant content using a variety of biomarkers. We found that both T-AOC and reduced glutathione (GSH) in plasma significantly increased, while MDA in plasma significantly decreased in Lias<sup>H/H</sup>Lepr<sup>db/db</sup> mice (figure 2A–C). Mitochondrial dysfunction is a major source to produce ROS. Investigating mitochondrial redox status would gain a better understanding of DN mechanisms and prove our hypothesis that impairment of antioxidant defense plays a critical role in the prevention and treatment of DN. Thus, we examined entire antioxidant content T-AOC, antioxidant biomarker GSH and SOD2 activity and oxidative stress biomarker MDA in mitochondria isolated from kidney cortex. The results showed the highest T-AOC level in Lias<sup>H/H</sup>Lepr<sup>db/db</sup> mice (figure 2D). Our results also demonstrated that the level of GSH in Lias<sup>+/+</sup>Lepr<sup>db/db</sup> mice was suppressed by diabetes compared with non-diabetic Lias<sup>+/+</sup>Lepr<sup>db/db</sup> mice (figure 2E). Increased endogenous antioxidant capacity in Lias<sup>H/H</sup>Lepr<sup>db/db</sup> mice elevated GSH level to close statistical significance (p=0.053) (figure 2E). SOD2 activity did not show any significant difference between Lias<sup>H/H</sup>Lepr<sup>db/db</sup> and Lias<sup>+/+</sup>Lepr<sup>db/db</sup> mice (figure 2F). But we found that MDA in the mitochondria significantly decreased in Lias<sup>H/H</sup>Lepr<sup>db/db</sup> mice compared with Lias<sup>+/+</sup>Lepr<sup>db/db</sup> mice (figure 2G). Furthermore, ATP yield in the kidney of Lias<sup>H/H</sup>Lepr<sup>db/db</sup> mice was lower than that in Lias<sup>+/+</sup>Lepr<sup>db/db</sup> mice (figure 2H). We concluded that decreased ATP production in Lias<sup>H/H</sup>Lepr<sup>db/db</sup> mice may serve as an adaptive mechanism to decrease ROS levels at the expense of ATP yield in states of excess substrate supply.

Because the NF-κB signal pathway plays a key role in orchestrating inflammatory response, we examined the effect of overexpression of Lias gene on the activation of NF-κB. Nuclear p65 expression was evidently increased in Lias<sup>+/+</sup>Lepr<sup>db/db</sup> mice (figure 3A) but significantly inhibited in Lias<sup>H/H</sup>Lepr<sup>db/db</sup> mice, indicating that enhanced endogenous antioxidant activity alleviated inflammatory response in diabetes.
Nrf2 is a transcription factor that plays a pivotal role in the endogenous antioxidant system. Our results showed that the protein level of Nrf2 was largely diminished in Lias\(^{+/+}\)Lepr\(^{db/db}\) mice compared with non-diabetic Lias\(^{+/+}\)Lepr\(^{db/+}\) mice. However, the decline of Nrf2 level could be partially prevented in Lias\(^{H/H}\)Lepr\(^{db/db}\) mice.
Metabolism

Since plasma and tissue LA cannot directly be measured due to a technical obstacle, changes of LA levels in kidney mitochondria were estimated by evaluating LIAS protein expression using western blot (figure 3C) and quantitatively assessed by densitometry. It was shown that levels of LIAS protein were markedly decreased in \(L\)ias\(^+/+\)Lepr\(^db/db\) mice, which could be partially restored in \(L\)ias\(^H/H\)Lepr\(^db/db\) mice.

Kidney histological alterations

To investigate the potential role of increased \(L\)ias gene expression in diabetic glomerular injury, we examined MME, foot process effacement, proximal tubule mitochondria and GBM thickness. Notably, the extent of MME was significantly larger in \(L\)ias\(^+/+\)Lepr\(^db/db\) mice (figure 4B) than \(L\)ias\(^R/R\)Lepr\(^db/db\) mice (figure 4A) but the expansion was attenuated in \(L\)ias\(^H/H\)Lepr\(^db/db\) mice (figure 4C). Semiquantitative analysis revealed significantly higher MME scores in \(L\)ias\(^+/+\)Lepr\(^db/db\) mice compared with \(L\)ias\(^H/H\)Lepr\(^db/db\) mice (figure 4D). Podocyte foot process effacement, assessed using transmission electron microscopy, were severer in \(L\)ias\(^+/+\)Lepr\(^db/db\) mice (figure 4F), compared with \(L\)ias\(^R/R\)Lepr\(^db/db\) mice (figure 4E) but significantly improved in \(L\)ias\(^R/R\)Lepr\(^db/db\) mice (figure 4G). In addition, there were a few damaged mitochondria in proximal tubules of kidney of \(L\)ias\(^+/+\)Lepr\(^db/db\) mice (figure 4H). However, more damaged mitochondria were observed in \(L\)ias\(^+/+\)Lepr\(^db/db\) mice (figure 4I) than in \(L\)ias\(^R/R\)Lepr\(^db/db\) mice (figure 4J), which suggested the involvement of mitochondria in the development of DN.

Figure 2 Antioxidant content in serum and renal mitochondria in 7-month-old \(L\)ias\(^+/+\)Lepr\(^db/db\) and \(L\)ias\(^R/R\)Lepr\(^db/db\) mice. Serum T-AOC (A), GSH (B) and MDA (C). Renal mitochondrial T-AOC (copper reducing equivalents) (D), GSH (E), SOD2 activity (F) and MDA (G). ATP production in renal mitochondria (H). Results represent the mean±SEM. *p<0.05 and **p<0.01. GSH, reduced glutathione; MDA, malondialdehyde; SOD2, superoxide dismutase 2; T-AOC, total autoxidizing capability.
were not significantly different among the three strains (data not shown).

**DISCUSSION**

In this study, we found that enhanced endogenous antioxidant activity are associated: (1) significantly decreased blood glucose and HbA1c levels; (2) attenuated pathological changes of DN and improved renal function, as evidenced by improvements of serum creatinine, albuminuria, MME and podocyte foot process effacement; (3) decreased inflammatory response including the expression of proinflammatory cytokines/chemokines and NF-κB activity; and (4) reduced oxidative stress and strengthened antioxidant defense including increased NRF2 and Lias production. This change of endogenous antioxidant levels is within a physiological level. The concentration of the peak is higher than by 200% compared with the baseline when they gave LA (600 mg), which falls within pharmaceutical dose to healthy volunteers.24 Hence, we estimate that upregulation by ~60% of the Lias gene expression cannot reach that pharmaceutical level. Taken together, these results suggest that the new double mutant mouse model is a valuable tool to elucidate the antioxidant role in the development of DN derived from type 2 diabetes.

Mitochondria are a major source for oxidative stress and also a vulnerable target by oxidative stress. Thus, our research focused on potential mitochondrial protection by increased endogenous antioxidant capacity. Our results showed that total antioxidant content and GSH concentrations in cortex mitochondrial were higher in LiasH/HLeprdb/db mice. These findings are consistent with our previous observation that GSH content is significantly reduced if Lias gene expression level drops by 50%.25 GSH is the predominant intracellular non-protein thiol compound, and renal GSH levels are decreased during...
Other investigators also showed that the effect of LA on renoprotection was associated with reduced oxidative stress and restoration of renal cortical levels of GSH. MDA is a peroxide formed by oxygen free radicals attacking unsaturated fatty acids in biomembrane, which can reflect the degree of lipid peroxidation in organism and the degree of damage of cells attacked by free radicals. Our results showed that MDA in both serum and mitochondria significantly decreased in Lias\(^{+/-}\)Lepr\(db/db\) mice compared with Lias\(^{+/+}\)Lepr\(db/db\) mice. In addition, the results from this study also revealed that mitochondrial damage in proximal tubules of Lias\(^{+/-}\)Lepr\(db/db\) mice noticeably decreased compared with Lias\(^{+/+}\)Lepr\(db/db\) mice, which confirmed our previous observation using different Lias mouse model. These results strongly suggest that damage of mitochondria impairment in proximal tubules plays a determinant role in the progression of DN. In this study, we demonstrated that elevated Lias gene expression limited the rise of serum CRP and inflammatory cytokines/chemokines, IL-1\(\beta\) and MCP-1 in Lias\(^{+/-}\)Lepr\(db/db\) mice compared with Lias\(^{+/+}\)Lepr\(db/db\) mice. The present study confirmed our previous observation that endogenous LA may have anti-inflammatory effects in a model of lipopolysaccharide (LPS)-induced inflammation in Lias\(^{+/+}\) mice. Our new model also confirmed that other investigation that LA supplementation exerts anti-inflammatory action by decreasing the expression of inflammatory markers such as TNF-\(\alpha\) and MCP-1 in kidney tissue.

Figure 4  Representative images of mesangial matrix expansion (MME), ultrastructural alterations of the podocytes and proximal tubule mitochondrial damage in 7-month-old mouse kidneys. (A) Lias\(^{+/-}\)Lepr\(db/db\), (B) Lias\(^{+/-}\)Lepr\(db/db\) and (C) Lias\(^{+/-}\)Lepr\(db/db\) mice, the sections stained with periodic acid–Schiff (PAS). Original magnification x400. (D) Quantitative analysis of mesangial expansion in kidney glomerulus from the three strains. Data are expressed as mean±SEM. Podocyte foot process effacement was significantly increased in Lias\(^{+/-}\)Lepr\(db/db\) mice (F) compared to Lias\(^{+/+}\)Lepr\(db/db\) mice (E). The increase was significantly improved in Lias\(^{+/-}\)Lepr\(db/db\) mice (G). Original magnification, x30,000. A large numbers of damaged mitochondria (blue arrowheads) in kidney proximal tubules of Lias\(^{+/-}\)Lepr\(db/db\) mice (I), and the damaged mitochondria were seldom observed in proximal tubules of Lias\(^{+/-}\)Lepr\(db/db\) mice (J) and Lias\(^{+/-}\)Lepr\(db/db\) mice (H). Original magnification x30,000.
most well-defined transcription factors that are highly activated in renal tissues of patients with human DN.37 Under hyperglycemic environment, NF-κB is activated and induces multiple key proinflammatory genes.38 Ying and coworkers have reported that the pretreatment of human umbilical vein endothelial cells with LA, but not and coworkers have reported that the pretreatment of human DN.37 activated in renal tissues of patients with human DN.37

In conclusion, the pathogenesis of DN is complex involving numerous factors. Liao11/11 Lepd/db/db mouse model can resolve main factors by exerting multiple pharmacological effects such as hypoglycemic, anti-inflammation, and antioxidant, especially protection of mitochondria, all of which contributed to attenuation of DN. Thus, this antioxidant animal model is not only very useful for elucidating the underlying mechanisms of DN but also brings insight into a new therapeutic strategy for clinical applications.

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Contributors Conceptualization: GX and XY. Methodology: YZ, TY, and CX. Validation: GX, SY and WW. Data curation: YZ, TY, MC, OG and GX. Writing—original draft preparation: YZ and GX. Writing—review and editing: XY and WW. Supervision: GX and SY. Project administration: WW and SY. Manuscript submission: XY. All authors have read and agreed to the published version of the manuscript.

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Competing interests None declared.

Patient consent for publication Not required.

Ethics approval Animal experiments were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of Xinxiang Medical University (protocol number XFYLL-2017086, approved March 3, 2017).

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement Data are available in a public, open access repository. Data are available on reasonable request.

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