

Combined AGE inhibition and ACEi decreases the progression of established diabetic nephropathy in B6 db/db mice

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The accumulation of advanced glycation end products (AGE) is a key factor in diabetic nephropathy (DN). Pyridoxamine inhibits AGE formation and protects against type I DN. Herein we tested: (1) whether C57BL6 db/db mice as a model of established type II DN resembled patients treated with drugs which inhibit angiotensin II action; (2) whether pyridoxamine was effective as a single therapy; and (3) whether pyridoxamine would add to the benefit of angiotensin-converting enzyme inhibition (ACEi) by enalapril. In first set of experiments mice were treated with ACEi (benazepril) and an angiotensin II receptor blocker (valsartan) combination for 16 weeks after the onset of diabetes. In second group, mice with established DN were treated with pyridoxamine for 8 weeks. In a third set, mice with established DN were treated with pyridoxamine and enalapril combination for 16 weeks. Benazepril and valsartan combination partially prevented the development and progression of DN. Pyridoxamine treatment, as single therapy, decreased the progression of albuminuria and glomerular lesions. The combination of pyridoxamine with enalapril reduced both mortality and the progression of DN. In conclusion, (1) C57 BL6 db/db mice are a model of progressive type II DN; (2) The combination of pyridoxamine with enalapril decreased progression of type 2 DN and overall mortality. Thus, pyridoxamine could be a valuable adjunct to the current treatment of established type II DN.

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KEYWORDS: diabetic nephropathy; type II diabetes mellitus; glomerular hypertrophy; advanced glycation end products; angiotensin-converting enzyme inhibitor; AGE inhibition

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Type II diabetic nephropathy (DN) is a major cause of end-stage renal disease and its prevalence is rising.^{1,2} While not the only factor, hyperglycemia has been shown to play a central role in the development and progression of DN. The means by which hyperglycemia induces renal changes involves multiple pathways, including activation of protein kinase C, increased reactive oxygen species, and accumulation of advanced glycation end products (AGE).^{3–8} AGE are derived from a non-enzymatic chemical reaction between carbohydrates and proteins, lipids or DNA.⁹ The rate of AGE formation in serum and tissues is significantly increased in patients with diabetes, due to the availability of excess free sugar. We found that the infusion of bovine serum albumin-derived AGE, formed *in vitro*, induced glomerular lesions in normal animals.¹⁰ The lesions resembled those found in DN. The suppression of AGE formation by aminoguanidine decreased albuminuria and the severity of glomerular lesions in diabetic rats.^{11,12} Similar effects were found in diabetic animals treated with other AGE inhibitors.^{13,14} Recently, Hudson *et al.* found that pyridoxamine, a non-toxic, safe, and well-tolerated vitamer from the B6 family, inhibited the glycoxidative breakdown of Amadori products to AGE, a key step in final AGE formation.^{5,15} Pyridoxamine treatment prevented the rise of plasma creatinine levels, albuminuria, and glomerular hypertrophy in rats with streptozotocin-induced type I diabetes mellitus.^{16,17} We postulated that pyridoxamine would reduce the rate of progression in type II diabetics with nephropathy. We selected C56BL6 (B6) db/db mice as the experimental model because 75% of them develop persistent hyperglycemia and hyperinsulinemia.¹⁸ In addition, all of those with persistent hyperglycemia develop progressive DN. Since the inhibition of angiotensin II action with either angiotensin-converting enzyme inhibitors (ACEi) or angiotensin II receptor blockers (ARB) has been shown to reduce the progression of nephropathy in patients with type II diabetics,^{19–22} we first asked whether the response to these drugs in diabetic db/db B6 mice resembled that in patients. We then asked whether pyridoxamine was effective as a single agent in these mice and finally, whether the addition of pyridoxamine would add to the benefits of ACEi treatment. Since many type II diabetics have already developed DN before they seek medical advice, we started

treatment with pyridoxamine or the combination of pyridoxamine and ACEi (enalapril) in B6 db/db mice 6–7 weeks after they had evidence of established DN.

RESULTS

General characteristics of B6 db/db mice

We examined the natural history of diabetes and nephropathy in 43 female B6 db/db mice. Seventy percent of them developed diabetes at 7–9 weeks of age, 95% had diabetes at 10–14 weeks of age, and all had developed diabetes at 15–24 weeks of age. Body weight increased for a period of 8 weeks after diabetes became established, and remained stable or increased only slightly thereafter. Serum insulin and leptin levels were increased in diabetic mice.¹⁸ Islet hypertrophy was evident but there was no insulinitis in mice up to 8 months of age (data not shown). Serum cholesterol and triglyceride levels were increased in diabetic db/db mice (data not shown). The mice remained normotensive. Urine albumin excretion increased in B6 db/db mice after 6 weeks of hyperglycemia ($n = 31$, albumin/creatinine, 0.11 ± 0.03 vs 6–8 weeks old, non-diabetic B6 db/db mice, 0.05 ± 0.01 , $P < 0.05$, Figure 1a). A further increase in urine albumin excretion was found in mice after 22 weeks of diabetes (0.25 ± 0.06 vs 0.11 ± 0.03 at 6 weeks, $P < 0.05$). In contrast, mice that returned to normoglycemic did not develop progressive albuminuria.¹⁸ Diabetic glomerulopathy in db/db mice is characterized by mesangial expansion, hypercellularity, and thickened glomerular basement membranes by light microscopy.¹⁸ Very few nodules were identified. Glomerular hypertrophy, increased cell number, and mesangial matrix

expansion (increased mesangial/glomerular fractional area) were present as early as 6 weeks after the onset of hyperglycemia (Figure 1b–d). The lesions were more severe after 22 weeks of diabetes (Figure 7a).

Combination therapy with benazepril and valsartan prevented the progression of DN

To test the effectiveness of ACEi and ARB combination on the development and progression of DN, female B6 db/db mice with stable hyperglycemia (≥ 200 mg/dl) between 8 and 10 weeks of age were randomized to receive 16 weeks of treatment with either benazepril plus valsartan ($n = 10$, 15 mg/kg/day of each drug in drinking water) or vehicle ($n = 9$).

General: Body weight was similar between vehicle (48.19 ± 0.8 g) and the combination therapy groups (49.07 ± 1.3 g) after 16 weeks of treatment (~ 25 weeks of age). There were no differences in glycemic and glycosylated hemoglobin levels between the two groups. Blood pressure was normal in the vehicle group. Benazepril plus valsartan treatment did not reduce blood pressure.

Albuminuria: Urine albumin excretion was significantly increased in the vehicle group after 16 weeks of hyperglycemia (0.22 ± 0.05 vs mice before the treatment, 0.05 ± 0.01 , $P < 0.01$). Combination therapy with benazepril and valsartan partially prevented the increase in urine albumin excretion (Figure 2a).

Histology and morphometry: Glomeruli from the vehicle group showed changes characteristic of DN, including increased glomerular size and mesangial expansion and hypercellularity (Figure 2b). The glomeruli were smaller and extracellular matrix expansion and hypercellularity were less accentuated after 16 weeks of treatment. Morphometric analysis showed that the glomerular volume and mean mesangial area were significantly decreased in the therapy group compared with the vehicle group (Figure 2b–e).

Pyridoxamine treatment decreased the progression of DN

As described in Materials and Methods, renal biopsies were performed in this group of animals 6 weeks after the onset of diabetes. All diabetic mice developed nephropathy at this time point, as evidenced by increased urine albumin excretion and glomerular hypertrophy, mesangial matrix expansion and hypercellularity (Figure 1a–d). Thus, we started pyridoxamine treatment 1 week after renal biopsy.

General: Food and water intake were similar between vehicle- and pyridoxamine-treated diabetic db/db mice during 8 weeks of follow-up. There were no differences in body weight, glycemic, and glycosylated hemoglobin levels between mice treated with vehicle and pyridoxamine at the end of study (23–25 weeks of age). The mice remained normotensive. Serum cholesterol and triglyceride levels were not significantly decreased by pyridoxamine treatment (data not shown). The mean serum pyridoxamine concentration was increased about fivefold in pyridoxamine-treated mice. Fifty percent of the mice died in the groups treated with

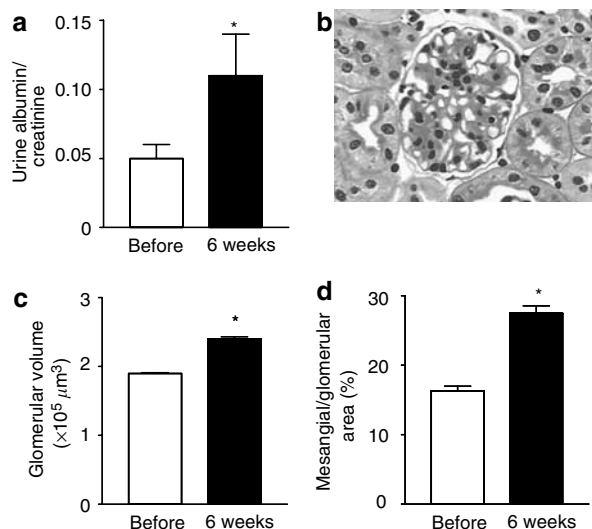


Figure 1 | Diabetic nephropathy was evident 6 weeks after the onset of hyperglycemia. (a) Increased urine albumin excretion.

* $P < 0.05$ vs mice before the onset of diabetes. **(b)** Renal biopsy at this time revealed increased glomerular size, moderate matrix expansion and an increased number of cells in the mesangial areas (periodic acid Schiff $\times 400$). **(c)** Glomerular volume was increased. * $P < 0.05$ vs prior to the onset of diabetes. **(d)** Mesangial area was also increased. The fractional mesangial area was expressed as the ratio of mesangial and glomerular surface area (%). * $P < 0.05$ vs mice before the onset of diabetes.

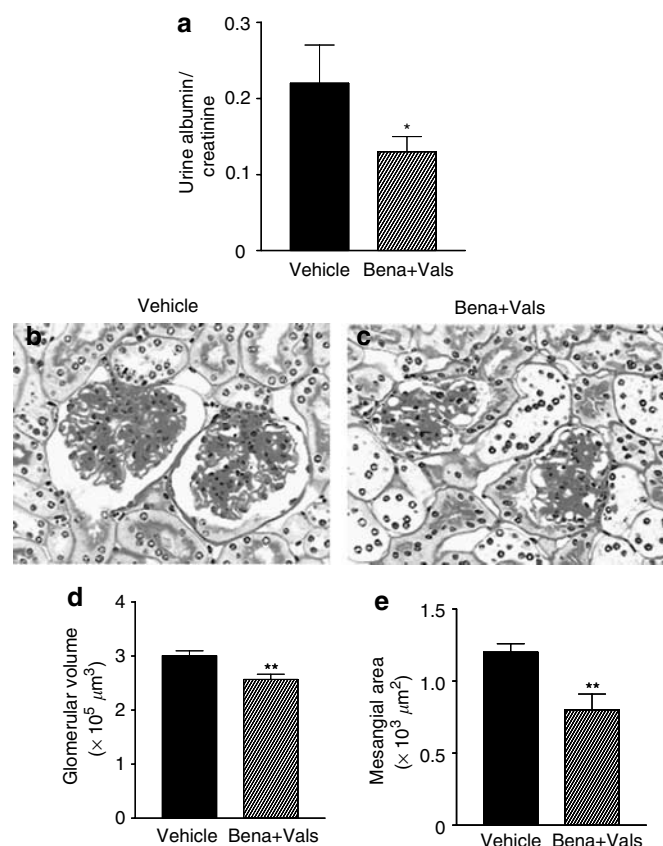


Figure 2 | Benazepril and valsartan combined therapy prevented the progression of diabetic nephropathy. Mice were treated with combination or vehicle immediately after the onset of diabetes. (a) Urine albumin excretion was decreased by the therapy (benazepril = Bena, Vals = valsartan). * $P < 0.05$ vs vehicle group. (b) and (c) Glomerular lesions were less severe in the treated group. Glomeruli from the vehicle group were large and the mesangial regions were expanded. Glomeruli in the therapy group were smaller in size and had reduced mesangial hypercellularity and sclerosis (periodic acid Schiff $\times 200$). (d) Glomerular volume was decreased in therapy group. ** $P < 0.01$ vs vehicle-treated mice. (e) Mesangial area expansion was decreased in therapy group. ** $P < 0.01$ vs vehicle-treated mice.

pyridoxamine and vehicle. Autopsy showed that pulmonary infection was the most frequent cause of animal death although the pathogen(s) were not defined. Complications following the open renal biopsy might have increased the number of infections observed in this group since most of the deaths occurred within 2–3 weeks after biopsy. Weight loss (≥ 5 g) was also a prominent feature of mice with infections. Mice whose death was due to obvious infections were excluded from the data analysis, except for overall mortality.

Albuminuria: At killing, urine albumin excretion was increased 2.3-fold from the onset of treatment in the vehicle group. Albuminuria was not increased in pyridoxamine-treated mice (Figure 3).

Histology and morphometry: Vehicle-treated diabetic db/db mice developed severe, diffuse glomerular lesions consisting of an increase in glomerular size and mesangial matrix (Figure 4a). These lesions were much less evident in pyridoxamine-treated mice (Figure 4b). Marked vascular

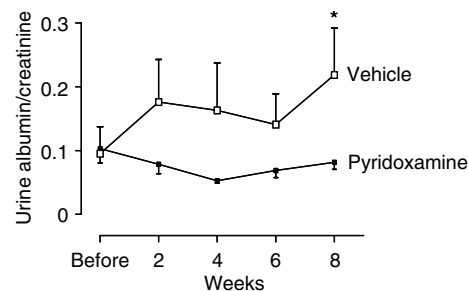


Figure 3 | Albuminuria did not increase after pyridoxamine treatment. Urine albumin excretion was increased from 6 to 15 weeks after the onset of hyperglycemia in vehicle-treated mice, but not in pyridoxamine-treated mice. * $P < 0.05$ vs mice before treatment or in pyridoxamine-treated mice at study end.

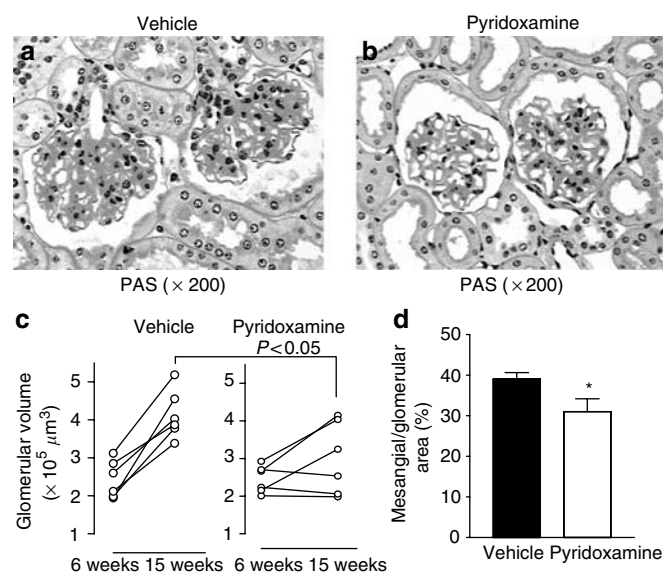


Figure 4 | Glomerular lesions were less prominent following pyridoxamine treatment. (a) After 15 weeks of persistent hyperglycemia, there was marked glomerular hypertrophy and matrix expansion in vehicle-treated mice (~ 24 weeks of age) (periodic acid Schiff $\times 200$). (b) Glomerular lesions were less severe in pyridoxamine-treated mice (periodic acid Schiff $\times 200$). (c) Glomerular volume was increased from 6 to 15 weeks after diabetes in vehicle-treated mice. This increase was blocked by pyridoxamine treatment ($P < 0.05$ vs vehicle-treated mice at 15 weeks). (d) Mesangial area expansion was less in pyridoxamine-treated mice. * $P < 0.05$ vs vehicle-treated mice.

pole scarring was frequently seen. Glomerular volume was increased 6 weeks after the onset of hyperglycemia, and was further increased after 15 weeks of hyperglycemia in all vehicle-treated mice ($4.1 \pm 0.3 \times 10^5 \mu\text{m}^3$ vs 6 weeks, $2.4 \pm 0.2 \times 10^5 \mu\text{m}^3$, $P < 0.01$, Figure 4c). In contrast, no significant increase in glomerular volume from 6 to 15 weeks after hyperglycemia developed in pyridoxamine-treated mice (6 weeks, $2.5 \pm 0.4 \times 10^5 \mu\text{m}^3$, 15 weeks, $3 \pm 0.5 \times 10^5 \mu\text{m}^3$, $P > 0.05$). There was a progressive increase in mesangial area after the onset of diabetes in vehicle-treated mice (15 weeks, $39.04 \pm 1.6\%$ vs 6 weeks, $27.6 \pm 1.1\%$, $P < 0.05$). A further increase, over a period of 8 weeks, was largely prevented by

pyridoxamine treatment (30.8 ± 0.9 vs $27.6 \pm 1.1\%$ at biopsy, $P > 0.05$, Figure 4d).

Combination therapy (16 weeks duration) with enalapril and pyridoxamine reduced both mortality and the progression of DN, whereas enalapril therapy did not

After DN was established, B6 db/db mice were randomized to receive (1) enalapril (10 mg/kg/day) plus pyridoxamine (250 mg/kg/day); (2) enalapril (10 mg/kg/day) plus pyridoxamine (100 mg/kg/day); (3) enalapril (10 mg/kg/day); or (4) vehicle. Mice were followed for 16 weeks. The outcomes were very similar in mice those received 250 mg/kg/day or 100 mg/kg/day of pyridoxamine, in combination with ACEi, treatment groups. Since four mice in the group of enalapril plus 250 mg/kg/day of pyridoxamine became normoglycemic shortly after treatment was begun, we present results here only from mice treated with enalapril plus 100 mg/kg/day of pyridoxamine.

General: Food and water intake were similar among all groups. There were no significant differences in body weight in mice at study end (data not shown). All diabetic db/db mice, including those treated with enalapril, remained normotensive. Blood glucose and total glycated hemoglobin levels were not affected by pyridoxamine or enalapril treatment. One major difference between groups was that vehicle-treated mice had a high mortality (66.7%, Figure 5). The combination of pyridoxamine with enalapril significantly reduced the death rate (22% vs vehicle, $P < 0.05$). Treatment with enalapril alone did not improve survival. Infection was the most prominent cause of animal death.

Albuminuria: A progressive increase in urine albumin excretion was found in vehicle-treated diabetic db/db mice. At 30–32 weeks of age, the urine albumin excretion rate in diabetic db/db mice was 0.27 ± 0.13 , a value significantly higher than in diabetic mice at 24 weeks of age (0.1 ± 0.04 , $P < 0.01$). Combination therapy with pyridoxamine and enalapril significantly reduced the progression of albuminuria (Figure 6). Enalapril as single agent did not decrease albuminuria in mice with established DN.

Histology and morphometry: There was a progressive increase in glomerular size in vehicle-treated diabetic db/db

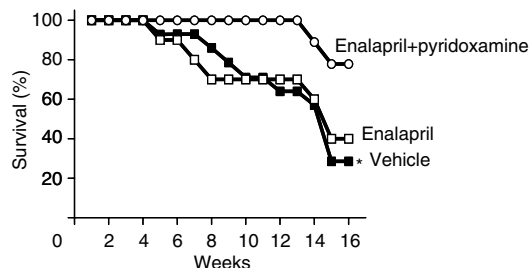


Figure 5 | Survival was increased in the combination enalapril and pyridoxamine group compared to either the vehicle or enalapril groups. * $P < 0.05$ vs those in the combination therapy group. Mice were followed for 22 weeks after the onset of diabetes (~31 weeks of age). In all, 66.7% of the vehicle-treated died during the follow-up.

mice (Figure 7a and d). This was associated with diffuse, conspicuous expansion of the mesangial matrix. Glomerular volume in diabetic db/db mice progressively increased from $2.4 \pm 0.03 \times 10^5 \mu\text{m}^3$, after 6 weeks of the diabetes, to $4.27 \pm 0.4 \times 10^5 \mu\text{m}^3$ after 15 weeks of the diabetes and was further increased to $6 \pm 0.5 \times 10^5 \mu\text{m}^3$ after 22 weeks of the diabetes. Combination therapy with enalapril and pyridoxamine reduced both glomerular hypertrophy and mesangial matrix expansion in diabetic db/db mice (Figure 7). Enalapril

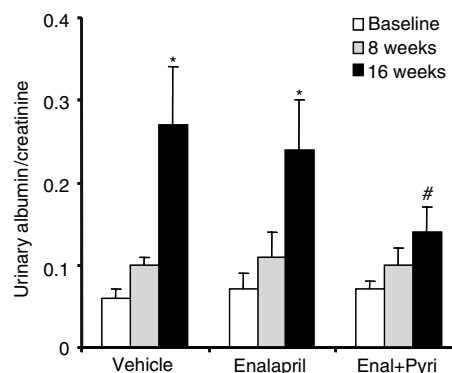


Figure 6 | The increase in albuminuria was blunted in the combination enalapril plus pyridoxamine group compared to the vehicle or enalapril treated mice after 22 weeks of hyperglycemia. * $P < 0.05$ vs pretreatment (6 weeks after the onset of hyperglycemia). The increase in albuminuria was prevented by combination enalapril and pyridoxamine therapy. # $P < 0.05$ vs vehicle-treated mice at study end.

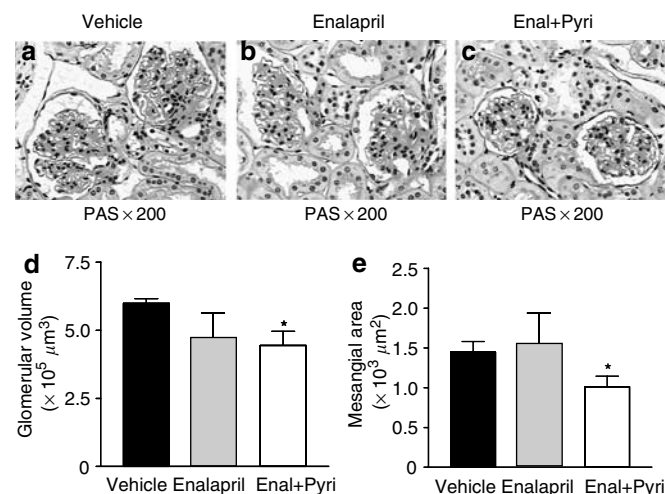


Figure 7 | Glomerular lesions in diabetic db/db mice were decreased after combination enalapril and pyridoxamine therapy. (a) Severe glomerular lesions characterized by increased glomerular size, mesangial sclerosis and cell proliferation were presented in vehicle-treated mice after 22 weeks of hyperglycemia (~31 weeks of age). (b) Glomerular hypertrophy was less pronounced in the enalapril treatment group compared to the vehicle-treatment group. (c) Glomerular histologic lesions were less severe in the combination enalapril and pyridoxamine (Enal + Pyri) group. (d) Decreased glomerular volume in mice treated with combination therapy. * $P < 0.05$ vs vehicle-treated mice. (e) Decreased mesangial area expansion in mice treated with combination therapy. * $P < 0.05$ vs vehicle-treated mice.

treatment did not affect glomerular size or mesangial area (Figure 7b, d, and e).

Decreased serum AGE levels in mice treated with pyridoxamine plus enalapril

Serum AGE levels were significantly increased in vehicle-treated diabetic db/db mice compared to age-matched db/+ controls (Figure 8). Pyridoxamine and enalapril combination therapy decreased, while enalapril alone did not affect AGE levels.

Pyridoxamine decreased type IV collagen expression

The expansion of mesangial area in vehicle-treated B6 db/db mice was associated with increased $\alpha 1(\text{IV})$ collagen mRNA levels and type IV collagen accumulation in glomeruli (Figure 9a). Mice in the pyridoxamine-treatment group had reduced mesangial area expansion, $\alpha 1(\text{IV})$ collagen mRNA levels and type IV collagen deposits (Figure 9b and c). In addition, pyridoxamine significantly reduced type IV collagen production in both normal and high glucose conditions in cultured mesangial cells isolated from diabetic db/db mice (Figure 10).

DISCUSSION

Both ACEi and angiotensin receptor blockade slowed, but do not stop the progression of established DN in patients.^{19–22} The accumulation of AGE is a key factor in the pathogenesis of DN.^{6,7} This led to the development of drugs that inhibit the formation of AGE.²³ Pyridoxamine is a newly discovered, potent inhibitor of AGE and, has an excellent safety profile. We undertook a study of its utility in the treatment of established DN in db/db mice, a model of type II diabetes. These mice have a leptin receptor mutation that causes polyphagia, obesity, and insulin resistance followed by diabetes mellitus.^{24,25} The phenotype of diabetes in db/db mice depends on the genetic background. KsJ db/db mice develop insulinitis and hypoinsulinemia.²⁶ On the other hand, B6 db/db mice develop hyperglycemia with persistent hyperinsulinemia and islet hypertrophy. Since the pattern in B6 db/db mice resembles the diabetic phenotype commonly seen in human type II diabetics, we selected

them as a model for pyridoxamine treatment. Since it has been reported that the hyperglycemia in B6 db/db mice could be transient and that glycemic levels were lower than that in KsJ db/db mice, we studied a large cohort of B6 db/db mice. We found that 75% of B6 db/db mice remained hyperglycemic, and had impaired glucose and insulin tolerance up to the termination of this study at 8 months of age.¹⁸ Importantly, we found that B6 db/db mice with persistent hyperglycemia developed progressive DN, and that urine albumin excretion progressively increased only in that cohort of B6 db/db mice with persistent hyperglycemia. Glomerular hypertrophy and mesangial matrix expansion also progressively worsened in the cohort with persistent hyperglycemia. As seen in patients, we found that the inhibition of angiotensin II with the combination of ACEi (benazepril) and ARB (valsartan), before DN became established,

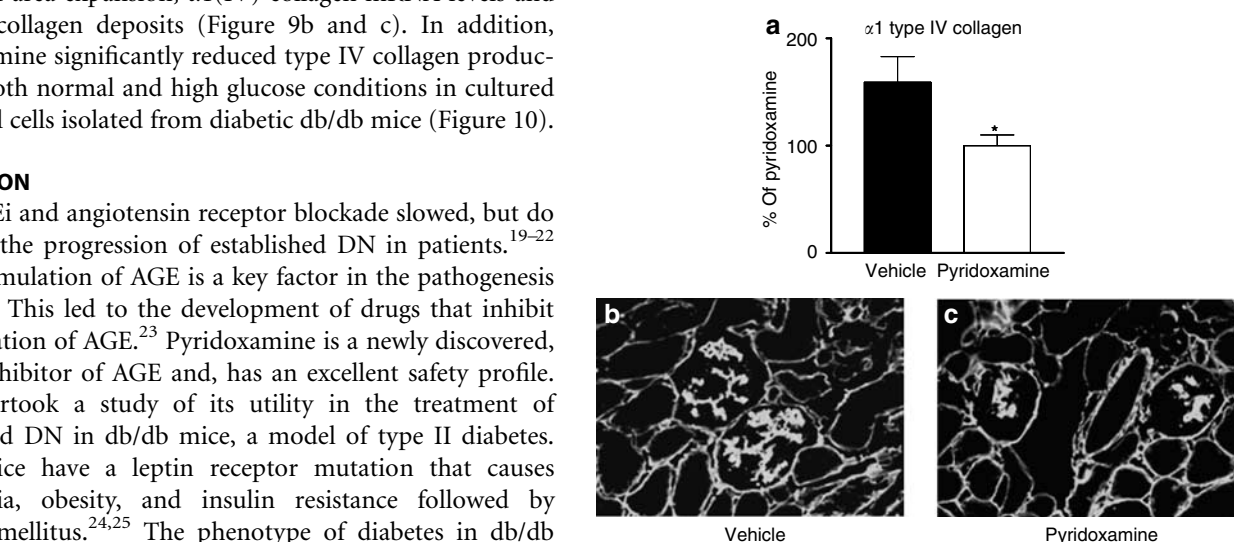


Figure 9 | Decreased type IV collagen expression in the pyridoxamine-treated group. (a) Decreased $\alpha 1$ type IV collagen mRNA expression in renal cortex of pyridoxamine-treated mice. * $P < 0.05$ vs vehicle-treated mice. (b and c) Glomerular type IV collagen accumulation was significantly reduced in pyridoxamine-treated mice (immunofluorescence microscopy $\times 200$).

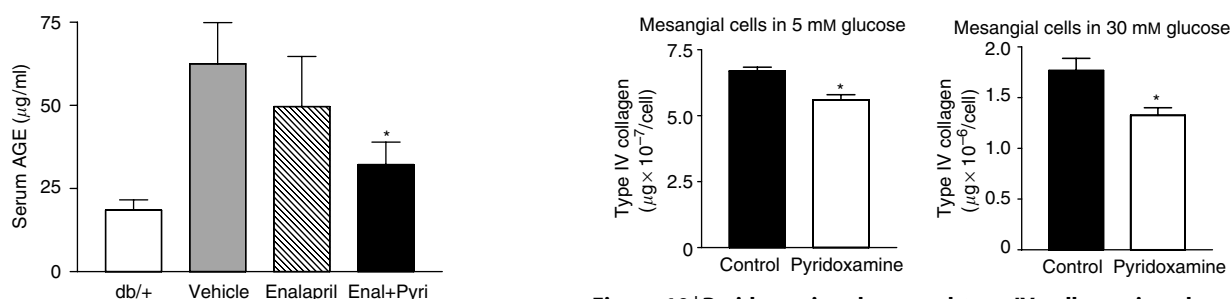


Figure 10 | Pyridoxamine decreased type IV collagen in cultured mesangial cells. Mesangial cells were cultured under 5 mM (normal) or 30 mM (high) glucose condition in the presence or absence of pyridoxamine. Type IV collagen produced by the cells were measured by enzyme-linked immunosorbent assay. Pyridoxamine significantly decreased type IV collagen production by mesangial cells. * $P < 0.05$ vs control.

Figure 8 | Decreased serum AGE levels in mice treated with pyridoxamine plus enalapril. Serum AGE levels were measured at killing (22 weeks after hyperglycemia) in mice treated with vehicle, enalapril, and pyridoxamine plus enalapril. Levels from age-matched db/+ mice (non-diabetic heterozygote control for db/db mice) were also determined. * $P < 0.05$ vs vehicle-treated mice.

prevented the progression of DN in B6 db/db mice. Thus, these mice provide a model for studying the pathogenesis of type II DN and for evaluating the effect of new antinephropathy drug(s).

Treatment with an ACEi and ARB combination did not decrease blood pressure and the mice remained normotensive. Thus, one limitation of this model is that hypertension, one of the important factors contributing to nephropathy in patients with diabetes, is not present in diabetic B6 db/db mice. This is similar to findings in some patients with type II diabetes.²⁷

We found that B6 db/db mice developed DN after 6 weeks of hyperglycemia, as evidenced by increased urine albumin excretion and biopsy proven glomerular lesions. Albuminuria and glomerular lesions were worsened with time in vehicle-treated diabetic db/db mice. However, neither albuminuria nor glomerular lesions progression was present in the pyridoxamine-treated group after 15 weeks of diabetes. Thus, we concluded that pyridoxamine treatment reduced the progression of DN in B6 db/db mice.

Since ACEi's and/or ANG II receptor blockers are currently the standard treatment for DN, we expected that the combination of pyridoxamine and enalapril would be more effective, than either drug alone. However, enalapril as a single treatment did not result in decreased progression of DN in db/db mice. The dose of (10 mg/kg/day) enalapril used in this study has been shown to reduce albuminuria in diabetic rats.^{28–30} Thus, it may be that other factors may contribute to the lack of effect of enalapril. Interestingly, we have preliminary data showing that captopril alone was not effective. However, since a combination of benazepril and valsartan successfully prevented the progression of DN in B6 db/db mice, these data suggest that either there are differences between ACEi's or that a more complete inhibition of ANG II may be required for the treatment of DN in db/db mice. In support of this postulate are the findings that a combination of ACEi and ANG II receptor blocker was more effective than ACEi alone in type II diabetic patients with nephropathy.³¹

We found that the combination of pyridoxamine and enalapril significantly reduced the progression of albuminuria and glomerular lesions in diabetic db/db mice. Furthermore, the combination treatment significantly reduced mortality. Infection was the principal cause of death in all groups of mice. The mechanism for the decreased mortality in the group treated with the combination of pyridoxamine and enalapril remains unclear.

The effects of pyridoxamine on DN were thought to be related to inhibition of the formation of tissue AGEs and advanced lipoxidation end products, to improvement of the altered redox status in diabetes, and to reduction of serum lipid levels.^{16,17,23} We found that combination treatment of pyridoxamine and enalapril decreased serum AGE levels in diabetic db/db mice while enalapril treatment alone did not have this effect. Interestingly, we found that type IV collagen mRNA expression in renal cortex and type IV collagen

accumulation in glomeruli was significantly reduced in pyridoxamine-treated B6 db/db mice. Using mesangial cells isolated from diabetic db/db mice, we found that high glucose in the culture medium led to significantly increased type IV collagen production. Pyridoxamine suppressed type IV collagen production by diabetic db/db mice mesangial cells in the presence of both elevated and normal glucose concentrations. Since mesangial cells isolated from diabetic db/db mice have been shown to exhibit phenotypic changes favoring the development and progression of diabetic glomerular lesions, this may partially explain the effect of pyridoxamine on cells under normal glucose condition.³² Thus, pyridoxamine may have a direct effect on type IV collagen production by mesangial cells.

In summary, we found that pyridoxamine prevented the progression of established DN and decreased mortality in B6 db/db mice, a model of type II diabetes.

MATERIALS AND METHODS

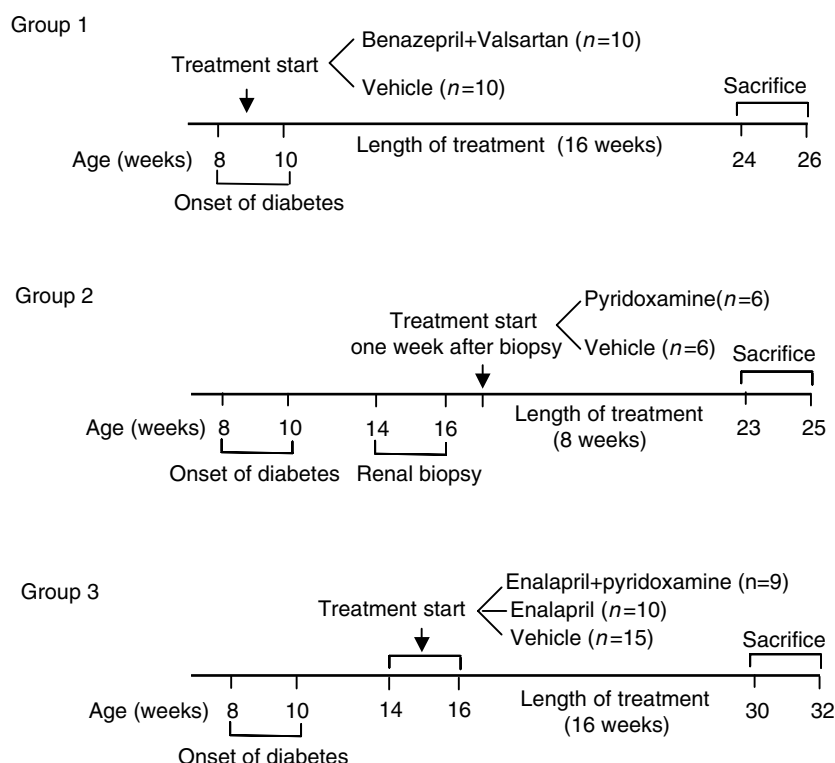
Experimental design

The purpose of these experiments was three-fold: (1) determine whether B6 db/db mice as a model of type II diabetes mellitus resembled that of patients, clinically, histologically and response to ACEi and/or ARB therapy, now considered to be standard-of-care; (2) determine whether pyridoxamine, an inhibitor of AGE formation was a safe and effective adjunct to ACEi treatment; and (3) determine whether pyridoxamine would add to the benefit of ACEi treatment.

Female B6 db/db mice were obtained from Jackson Laboratories (Bar Harbor, ME, USA). Because of the limited supply of these mice, the experiments were performed on mice obtained at three different times (Table 1, Time lines). In the first set of experiments (group 1), we tested the effectiveness of a combination of ACEi and ARB on the development and progression of DN. Mice with stable hyperglycemia (≥ 200 mg/dl) between 8 and 10 weeks of age were randomized to receive 16 weeks of either benazepril + valsartan ($n = 10$, 15 mg/kg/day of each drug in drinking water) or vehicle ($n = 9$).

In the second set of experiments (group 2), we tested the effectiveness of pyridoxamine on established DN. An open renal biopsy, from the lower pole of the left kidney, was performed on mice 6 weeks after the onset of hyperglycemia. Anesthesia was provided by ketamine and xylazine. We obtained ≥ 20 glomeruli per biopsy. One week after biopsy, mice with glomerular lesions, sustained hyperglycemia and an elevated albumin/creatinine ratio were randomized to receive either pyridoxamine ($n = 6$, 250 mg/kg/day in drinking water, provided by Biostratum Inc., Durham, NC, USA) or vehicle ($n = 6$). Mice were housed individually and the concentration of pyridoxamine in drinking water was adjusted daily based on the water intake. Mice were followed for 8 weeks. Blood pyridoxamine and lipid levels were determined at killing. Fifty percent of the mice died during the early follow-up period, due mostly to infection, and were not included in analyses. Plasma pyridoxamine concentrations were measured by reverse phase-high performance liquid chromatography with excitation and emission wavelengths of 293 and 393 nm as previously described.¹⁶

Since the second set of the experiments revealed that all female B6 db/db mice developed nephropathy after 6 weeks of hyperglycemia and the rate of infection was high after renal biopsy, we did not perform this procedure in the third set of animals (group 3).

Table 1 | Timeline of the experiments

The mice were followed for 6 weeks after the onset of diabetes and were then randomized to receive: (1) enalapril (10 mg/kg/day) plus pyridoxamine (250 mg/kg/day), $n = 11$; (2) enalapril (10 mg/kg/day) plus pyridoxamine (100 mg/kg/day), $n = 9$; (3) enalapril (10 mg/kg/day), $n = 10$; 4. Vehicle, $n = 15$. Mice were treated for 16 weeks.

Glycemia and glycosuria were monitored in animals twice per week in all experiments. Mice in which glycemic levels fell to < 200 mg/dl over a period of 3 consecutive weeks, an event which occurred most often within 3 weeks after the onset of hyperglycemia, were excluded from the studies and analyses. Total glycosylated hemoglobin was examined at the end of study on whole blood by boronate affinity chromatography (Sigma, St Louis, MO, USA). Urine samples were collected weekly. Blood pressure was recorded 1 week before killing using a sensor cuff device.³³ Serum AGE levels were measured by a competitive enzyme-linked immunosorbent assay based on an anti-CML monoclonal antibody (4G9), as previously described.³⁴

Urine albumin was measured by enzyme-linked immunosorbent assay using a kit from Bethyl Laboratory Inc. (Houston, TX, USA), as previously described.³³ Urine creatinine levels were measured in the same samples and the urine albumin excretion rate was expressed as the ratio of albumin to creatinine.

Renal histology and morphometry

At killing, the kidneys were perfused with a saline solution and subsequently perfuse fixed *in situ* with 4% paraformaldehyde. The tissues were embedded in glycol methacrylate, cut at a thickness of $2\ \mu\text{m}$ and stained with periodic acid Schiff.³³ The glomerular volume and mesangial area were determined by examining plastic-embedded sections using a digitizing tablet and video camera as

previously described.³³ The relative mesangial area was expressed as mesangial/glomerular surface area (%).

mRNA levels

Total RNA was isolated from renal cortex of the second set of animals, as described.³³ $\alpha 1$ type IV collagen mRNA levels were determined by real-time polymerase chain reaction (PCR).³³ Briefly, equal amounts of RNA from each sample were reverse transcribed with specific primers and amplified using the TaqMan one step reverse transcriptase-polymerase chain reaction master mix reagents kit and ABI Prism 7700 sequence detection system (Perkin Elmer Applied Biosystems, Wellesley, MA, USA). Taqman ribosomal RNA control reagents kits were used to measure the expression of 18S ribosomal RNA genes. The levels of $\alpha 1$ type IV collagen mRNA expression were normalized to 18S mRNA levels.

Type IV collagen immunofluorescence staining

Frozen kidney sections from the second set of animals were cut at a thickness of $5\ \mu\text{m}$. Sections were incubated with cold acetone for 10 min and coated with a rabbit anti-type IV collagen (Bioscience Resource Project, Kennebunkport, ME, USA) followed by a biotin-conjugated goat anti-rabbit IgG (Sigma, Saint Louis, MO, USA) and streptavidin-conjugated fluorescein isothiocyanate (Zymed Laboratories Inc., San Francisco, CA, USA).³³ Sections were examined by an investigator who was blinded to the experimental groups.

Mesangial cell type IV collagen

Mesangial cells were isolated from microdissected glomeruli and characterized as previously described.³⁴ In all, 30 000 cells were seeded in each well of a 24-well plate in medium containing 20%

serum to examine the effect of pyridoxamine on the production of type IV collagen by mesangial cells. After 24 h, cells were switched to normal glucose (5 mM) or high glucose (30 mM) medium containing 0.1% bovine serum albumin in the presence or absence of 30 ng/ml of pyridoxamine. Supernatant and cell lysate were collected 48 h after treatment. Type IV collagen in the supernatant and cell lysate were assayed by enzyme-linked immunosorbent assay, as previously described.³⁵

Statistical analysis

Values were expressed as mean \pm s.e. The two-tailed unpaired *t*-test was used to evaluate the differences of means for data obtained from mice treated with pyridoxamine or vehicle. The levels of significance was tested at $P < 0.05$. One way analysis of variance and post *t*-test were used for the comparisons of differences among groups of the third set of experiments. Survival curve was analyzed by the Prism software.

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