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Prevention and Reversal of Diabetic Nephropathy in db/db Mice Treated with Alagebrium (ALT-711)

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Key Words
Advanced glycation end products (AGE) · Diabetic nephropathy · Proteinuria · Renal function, diabetes · Anti-AGE therapy · ALT-711 (alagebrium)

Abstract
Background: Alagebrium (ALT-711) has been shown to improve renal dysfunction in animal models of diabetes. ***Methods:*** To test its effects in diabetic nephropathy (DN), ALT-711 was administered (1 mg/kg daily i.p.) to 9-week-old female db/db mice (n = 15, group A1) for 3 weeks and to 3-month-old (n = 15, group A2), 7-month-old (n = 7, group A3), and 12-month-old (n = 5, group A4) female db/db mice for 12 weeks, while a similar number of diabetic and nondiabetic mice were used as controls. The εN-carboxymethyllysine (CML) levels in serum, urine, skin, and kidney tissue were measured by enzyme-linked immunosorbent assay. The renal morphometric parameters were assessed by electron and light microscopy. ***Results:*** By the 3rd week of treatment, the serum CML level decreased by 41%, and the urinary CML concentration increased by 138% from baseline, while the urinary albumin/creatinine ratio was lower ($p < 0.05$) in diabetic and nondiabetic group A1 mice. After 3 months of treatment, serum, skin, and kidney CML levels and urinary albumin/creatinine ratio were lower ($p < 0.05$) and the urinary CML levels higher ($p < 0.05$) in treated group A2, A3, and A4 animals compared with groups which received phosphate-buffered saline, with a similar pattern observed in nondiabetic mice. The renal morphological parameters characteristic of DN decreased in treated compared with untreated mice. ***Conclusion:*** Alagebrium may prevent, delay, and/or reverse established DN in db/db mice by reducing the systemic advanced glycation end product pools and facilitating the urinary excretion of advanced glycation end products.
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Introduction

Diabetic nephropathy (DN) currently represents the most common cause of end-stage renal disease requiring renal replacement therapy [1]. Although the genetic susceptibility is an important factor in DN, exposure of renal tissues to chronic hyperglycemia seems to be the initiating factor [2], acting through several pathogenic pathways, including the formation of advanced glycation end products (AGE) [3–5].

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The importance of AGE in the pathogenesis of DN has been demonstrated in many in vitro and animal experiments and is further supported by results of interventional studies, demonstrating the beneficial effects of several AGE inhibitors and breakers [3–6].

Alagebrium (3-phenacyl-4,5-dimethylthiazolium chloride, ALT-711), a representative of a novel class of thiazolium derivatives, is a recently introduced compound that is thought to act via cleavage of preformed AGE protein cross-links in tissues [6]. The administration of alagebrium to various animal models of diabetes, aging, and spontaneous hypertension has resulted in an improvement in large-vessel elasticity, a decrease in stiffness and peripheral resistance, and an attenuation in several functional and structural manifestations of diabetes- and non-diabetes-related nephropathy [7–13]. In addition, clinical studies demonstrated either a reversal or an improvement of established cardiovascular complications of aging, such as arterial and myocardial stiffening and diastolic heart failure [6].

The aim of the current study was to examine the effects of alagebrium on DN in db/db mice. First, we studied relatively young db/db mice with almost no signs of DN treated with alagebrium for 3 weeks. Then, we studied db/db mice of various ages and variable degrees of DN treated with alagebrium for 3 months.

Study Design and Methods

Protocol A: Short-Term Study (3 Weeks)

Fifteen female 9-week-old diabetic BKS.Cg-m+/+ Lepr^{db} mice (*db/db+/+;* 36–43 g; Jackson Laboratories, Bar Harbor, Me., USA) and 15 age-matched, female nondiabetic mice, from the same colony (*db/db+/–;* 15–20 g), were treated with alagebrium (1 mg/kg/day i.p.; Alteon, Parsippany, N.J., USA) for 3 weeks, and a similar number of mice from each group were treated with the same volume of phosphate-buffered saline as controls.

Protocol B: Long-Term Study (3 Months)

Diabetic BKS.Cg-m+/+ Lepr^{db} mice (*db/db+/+;* Jackson Laboratories) aged 3 months (n = 29), 7 months (n = 11), and 12 months (n = 9) and a group of nondiabetic mice (db/db+/–) aged 3 months (n = 20) and 7 months (n = 9) were followed during treatment with alagebrium (1 mg/kg/day i.p.) or phosphate-buffered saline (same volume) for 3 months. Baseline and biweekly body weights and fasting blood glucose levels were evaluated throughout.

Blood for serum εN-carboxymethyllysine (CML) and 24-hour urine for CML, creatinine, and albumin measurements were collected at baseline and 1, 2, and 3 weeks (short-term study) and 3 months (long-term study) after initiation of treatment. At the end of both studies, the mice were sacrificed, and skin and kidneys were removed to obtain samples for CML measurement and for histopathological studies.

Throughout both studies, baseline and biweekly food and water intakes, body weight, and fasting blood glucose were evaluated. All mice were housed in a temperature-controlled animal facility (23°C), with a 12-hour light/dark cycle and provided with food and water ad libitum. All procedures were approved by the Institutional Animal Care and Use Committee.

Assays

Fasting blood was obtained from the tail vein, and the glucose levels were measured by means of a Glucometer Elite XL 3901E (Bayer, Tarrytown, N.Y., USA). Serum, 24-hour urine, skin tissue (from the back of the mice), and renal tissue samples were analyzed for CML immunoreactivity by enzyme-linked immunosorbent assay, as previously described [14], as was the urinary albumin concentration [14]; the urinary creatinine level was measured by a standard colorimetric method [14].

Histopathological Studies

Light Microscopy. Kidneys removed from mice in both protocols were fixed in 10% buffered formalin and were paraffin embedded. Sections (5 μm thick) were stained with periodic acid-Schiff and examined under a light microscope for glomerular volume estimation [15–17].

Electron Microscopy. One-millimeter cubes of kidney cortices were fixed in 2.5% glutaraldehyde in Millonig's buffer, postfixed in 1% osmium tetroxide, dehydrated through a series of ethanol washes, and embedded in Poly/Bed 812 (Polysciences, Warrington, Pa., USA). Sections (1 μm thick) were cut, stained with toluidine blue, and examined [15–17]. The following morphometric parameters were chosen for analysis: total mesangium, total mesangial matrix, mesangial/glomerular fraction, and glomerular basement membrane width.

Statistics

All data are given as mean ± SEM. Differences of variables between the treated and untreated groups were analyzed by the Mann-Whitney unpaired U test. Differences of variables at different time points in either group were analyzed by a two-sided Wilcoxon matched-pair signed-rank test. Statistically significant differences were defined as $p \leq 0.05$. Data analysis was performed using the SPSS statistical program (SPSS version 10.0 for Windows).

Results

Short-Term Study: Effects of 3-Week Alagebrium Treatment on Young Diabetic and Nondiabetic Mice

Body weight and fasting blood sugar levels did not change significantly during the study period in any group.

Initially, alagebrium treatment produced a significant increase of serum CML levels which returned to baseline by the 2nd week and decreased by 41% by the 3rd week compared with baseline in db/db mice (p = 0.043; fig. 1a). The urinary CML excretion increased by 138% compared with baseline in treated db/db mice (p = 0.043; fig. 1b). A

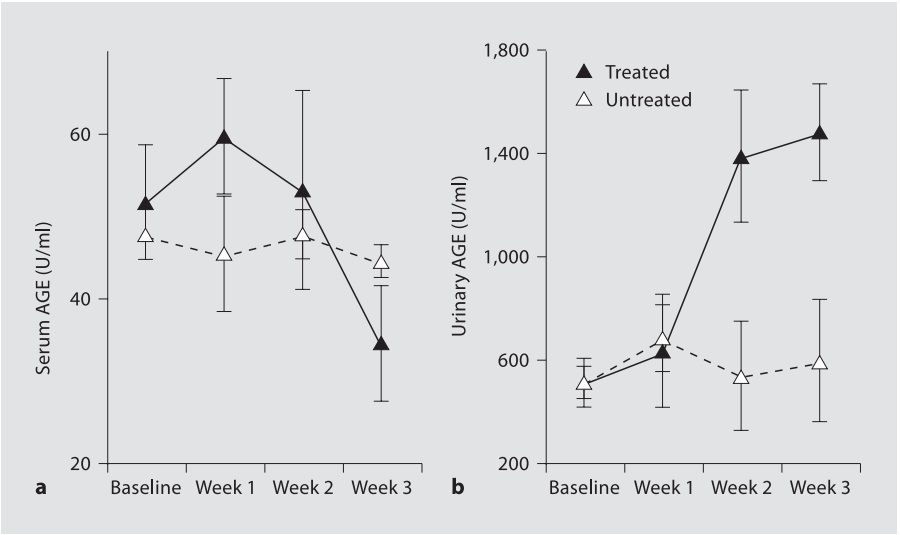

Fig. 1. Acute changes in serum (**a**) and urinary (**b**) CML levels in treated and untreated db/db mice (short-term study). Data are expressed as mean ± SEM. Fasting serum and 24-hour urine samples were obtained weekly in treated and untreated db/db mice and tested for CML, as previously described [14].

similar, although not statistically significant, trend was observed in serum and urinary CML levels in the treated nondiabetic mice.

The creatinine clearance did not change during the study period in any of the groups. The urinary albumin/creatinine ratio increased by 26% in the untreated db/db mice compared with the treated diabetic group, in which it remained unchanged (p = 0.033).

There was no difference either in the CML deposition in skin and kidney tissue or in the renal histological parameters between the different groups at the end of the study.

Long-Term Study: Effects of 3-Month Alagebrium Treatment on Diabetic and Nondiabetic Mice of Different Ages

In diabetic and nondiabetic mice of different ages, body weight and fasting blood sugar did not differ between treated and untreated groups at baseline or at the end of the treatment.

The serum CML levels at the end of the study were lower in each of the treated diabetic groups (3 months, 7 months, and 12 months of age) than in the corresponding untreated groups (p = 0.001, p = 0.034, and p = 0.025, respectively; table 1). At the end of the study, the urinary CML excretion rates were higher in the treated (3 months, 7 months, and 12 months of age) than in the untreated db/db mice (p = 0.013, p = 0.05, and p = 0.021, respectively; table 1). The same pattern was observed in treated compared with untreated nondiabetic mice regarding the serum CML levels (p = 0.04 and p = 0.05, respectively) or the urinary CML levels (p = 0.025 and p = NS, respectively; table 1).

At the end of the study, the creatinine clearance was lower in the untreated than in the treated 3-month-old, 7-month-old, and 12-month-old db/db mice (p = NS, p = 0.034, and p = 0.029, respectively). The urinary albumin/creatinine ratio was higher in the untreated than in the treated 3-month-old, 7-month-old, and 12-month-old db/db mice (p = 0.006, p = 0.031, and p = 0.014, respectively; fig. 2).

In db/db mice, the skin and kidney CML levels were lower in the 3-month-old, 7-month-old, and 12-month-old treated than in the untreated mice (skin CML: p = 0.05, p = 0.04, and p = 0.05, respectively; kidney CML: p = NS, p = 0.034, and p = 0.014, respectively; fig. 3a–d).

Most of the measured glomerular morphological parameters were less in treated than in untreated db/db mice, irrespective of age (fig. 4). No statistically significant differences were observed between treated and untreated nondiabetic mice.

Discussion

The current work demonstrates that both short- and long-term administration of alagebrium leads to a significant decrease in circulating CML levels and to a significant increase of urinary CML excretion in diabetic and nondiabetic mice. This effect was apparent in all groups studied. More importantly, the long-term treatment has a profound effect on diminishing the CML de-

Table 1. Long-term study: characteristics of diabetic (db+/db+) and non-diabetic (db+/db–) mice treated with alagebrium for 3 months

Parameters	3 months old		7 months old		12 months old	
	treated	untreated	treated	untreated	treated	untreated
Diabetic mice						
Number	15	14	6	5	5	4
Serum AGE (U/ml)						
Baseline	38.4 ± 2.3	39.2 ± 2	44.6 ± 5	42.7 ± 4.6	49 ± 3.8	46 ± 5.9
End	19.6 ± 1.7*	42.3 ± 3.2	24.9 ± 5.4*	44.7 ± 5.2	20 ± 4.5*	47.4 ± 3.7
Urinary AGE excretion (U/mg)						
Baseline	3,560 ± 890	3,478 ± 922	3,367 ± 240	3,206 ± 160	2,825 ± 144	3,188 ± 116
End	7,728 ± 140*	3,063 ± 771	10,067 ± 11,097*	2,900 ± 208	8,523 ± 370*	1,108 ± 470
Nondiabetic Mice						
Number	10	10	5	4		
Serum AGE (U/ml)						
Baseline	29.6 ± 2.9	28.9 ± 2	26.7 ± 2.7	31 ± 2.3		
End	17.4 ± 3.2*	30.1 ± 3.8	15 ± 2.7*	32.5 ± 2.5		
Urinary AGE excretion (U/mg)						
Baseline	4,600 ± 399	3,563 ± 213	3,485 ± 188	3,538 ± 123		
End	5,528 ± 323*	3,800 ± 339	4,618 ± 702*	3,625 ± 113		

Data are presented as mean ± SEM. * $p < 0.05$: significant differences between treated and untreated mice at the end of the study.

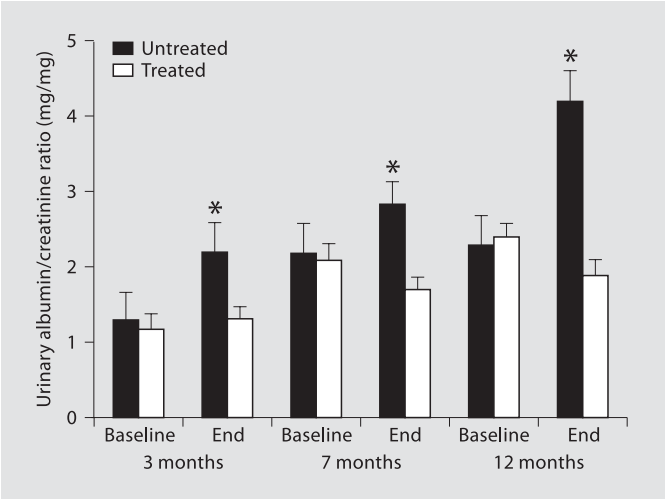

Fig. 2. The urinary albumin/creatinine ratio is much lower in db/db mice treated for 3 months with alagebrium (long-term study). Data are expressed as mean ± SEM. Urinary albumin/creatinine ratios were determined at the end of the study in 3-month-old, 7-month-old, and 12-month-old treated and untreated db/db mice. * $p < 0.05$: significantly different values between treated and untreated db/db mice.

position in tissues such as kidneys and skin, preventing, diminishing, or even reversing albuminuria and glomerular structural changes characteristic of diabetic animals, unrelated to age at the beginning of treatment.

Long-term treatment with alagebrium produced a sustained decrease of serum AGE levels in our db/db mice. Treatment of streptozotocin (STZ)-diabetic rats with alagebrium only prevented the increase in serum AGE levels observed in untreated rats [13]. Furthermore, treatment with alagebrium produced a marked and sustained increase of urinary AGE excretion in treated versus untreated db/db mice, while STZ-diabetic rats treated with alagebrium had less of an increase of urinary AGE excretion than untreated rats [13]. The reasons for these differences remain undetermined, but most likely reflect different species responses to the drug.

During the short treatment, the serum AGE levels actually increased initially during the 1st and 2nd weeks, followed by a reduction by the 3rd week, while an increase in urinary AGE excretion started to be evident by the 2nd week. These changes favor the postulation that alagebrium acts by cleaving tissue AGE, leading to release of AGE fragments to the circulation which end up being excreted in the urine. Increased urinary excretion in parallel with

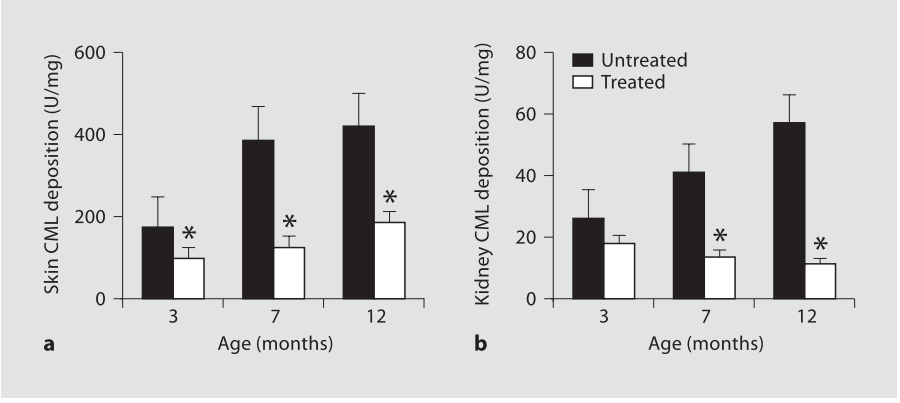

Fig. 3. The skin and renal levels of AGE are markedly diminished in db/db mice treated for 3 months with alagebrium (long-term study). Data are expressed as mean ± SEM. CML and protein concentrations were measured in homogenized samples from skin tissue from the back of the mice (**a**) and kidneys (**b**) after 3 months on treatment with alagebrium. * $p < 0.05$: significantly different values between untreated and treated db/db mice.

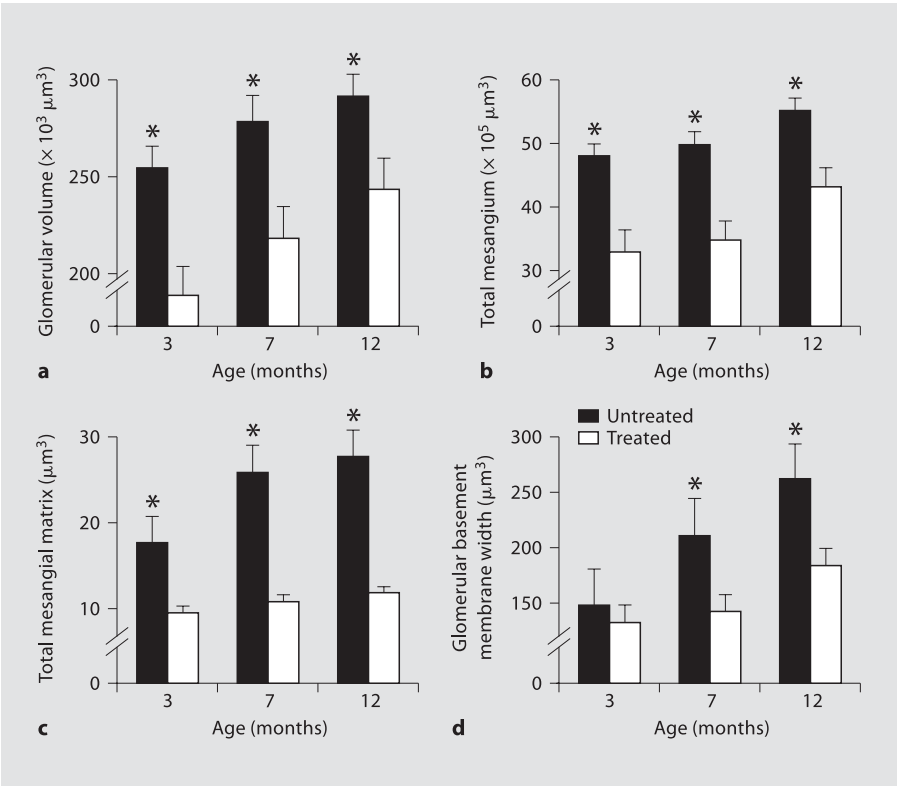

Fig. 4. Improvement and/or reversal of glomerular volume (**a**), total mesangial volume (**b**), total mesangial matrix (**c**), and glomerular basement membrane width (**d**) in db/db mice treated for 3 months with alagebrium (long-term study). Data are expressed as mean ± SEM. Kidney samples obtained at the end of 3 months of treatment were processed for electron and light microscopy, as described in the Methods section. * $p < 0.05$: statistically significant differences between treated and untreated db/db mice.

decreased circulating CML levels was also found in the long-term protocol in all age groups studied. It has been shown that AGE peptides are filtered across the glomerulus and are partially reabsorbed by the proximal convoluted tubule, while AGE-free adducts, which filter freely, are not well reabsorbed [18, 19]. Whether alagebrium itself has any direct effect on the AGE tubular transport system as well remains undetermined at present. Additionally, some data suggest that alagebrium could also reduce the formation of AGE through its chelating effect on copper [20], an effect that was not explored in the present study. Of interest, similar changes were observed in nondiabetic mice in the absence of hyperglycemia which demonstrates that AGE-induced protein cross-linking takes place in the body continuously, influenced by the diet and facilitated by the presence of diabetes and/or renal insufficiency [3, 4].

Alagebrium decreased the AGE accumulation in kidney tissue in all age groups studied, in contrast to the findings in the STZ-diabetic rats in which AGE accumulation was much more pronounced in the early- as compared with the late-intervention group [13]. Many in vi-

tro and in vivo studies have demonstrated that AGE can produce renal disease by a variety of mechanisms, including direct cross-linking of extracellular matrix proteins and disrupted receptor-mediated metabolism [3–5]. Recently it has been shown that AGE/RAGE interaction results in oxidative stress generation, local renin-angiotensin system activation, and transforming growth factor β-Smad signaling induction, contributing to the pathogenesis of DN [21]. Alagebrium treatment started either early or late has been shown to decrease the receptor for AGE expression, renal cortical nitrotyrosine levels (a marker of protein oxidative damage by peroxynitrite radicals), transforming growth factor –β1 gene and protein expression, connective tissue growth factor gene expression, and collagen IV gene and protein expression in STZ-diabetic rats [13]. Furthermore, alagebrium treatment of STZ-diabetic rats also inhibited protein kinase C α activation which helps to decrease AGE accumulation and ameliorate the expression of vascular endothelial growth factor and various extracellular matrix proteins, including fibronectin and laminin, which should provide renoprotection [13, 22]. Our data using a different animal model further support previous findings [3, 4, 13, 22]. We demonstrated attenuation of the diabetes-related increase in the albumin/creatinine ratio in all treatment groups, as it has been previously demonstrated in STZ-diabetic rats [13]. The effect of attenuating albuminuria paralleled the significant improvement noticed in several morphological features of DN in treated as compared with untreated mice.

Our results suggest that even if alagebrium therapy is started relatively late in the course of nephropathy, structural and functional manifestations of this disease can be delayed or reversed. This finding is consistent with results of other studies using AGE inhibitors, where even delayed administration was associated with similar functional effects [6, 18]. It is likely that the renoprotective action of alagebrium is via decreasing the AGE accumulation in renal tissues, but an additional beneficial effect derived from its antihypertensive action cannot be excluded [8].

Our data show that alagebrium decreased skin tissue CML accumulation in all age groups studied. Forbes et al. [13] demonstrated an improvement of tail tendon collagen solubility which was more remarkable in the early compared with the late interventional groups, an effect which could be attributed to the different time-treatment protocol used. Alagebrium in the form of a cream preparation has been shown to improve water content and elasticity of the aged rat skin in association with a reduction of skin-lipid- and glucose-derived AGE levels [6]. In addition, incubation of tail collagen AGE isolated from diabetic rats with alagebrium resulted in a significant decrease in cross-linking [6]. A decrease in skin tissue AGE accumulation has been previously shown to be associated with improved wound healing in diabetic mice [14] and with better glycemic control and cardiovascular disease outcome in diabetic subjects [23].

In summary, the above data further support a causative role of AGE in the initiation and progression of DN and a renoprotective effect of alagebrium, either in the form of the primary or secondary prevention. Further studies are needed to determine the exact nature and the mechanisms implicated in the effect of this compound in the course of DN.

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