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Breakers of advanced glycation end products restore large artery properties in experimental diabetes

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ABSTRACT **Glucose and other reducing sugars react with proteins by a nonenzymatic, posttranslational modification process called nonenzymatic glycation. The formation of advanced glycation end products (AGEs) on connective tissue and matrix components accounts largely for the increase in collagen crosslinking that accompanies normal aging and which occurs at an accelerated rate in diabetes, leading to an increase in arterial stiffness. A new class of AGE crosslink "breakers" reacts with and cleaves these covalent, AGE-derived protein crosslinks. Treatment of rats with streptozotocin-induced diabetes with the AGE-breaker ALT-711 for 1–3 weeks reversed the diabetes-induced increase of large artery stiffness as measured by systemic arterial compliance, aortic impedance, and carotid artery compliance and distensibility. These findings will have considerable implications for the treatment of patients with diabetes-related complications and aging.**

Altered arterial wall properties that develop in diabetes may influence important determinants of circulatory function, such as peripheral resistance, neural control mechanisms, and cardiac work (1). In comparison to the small quantitative alterations (2–5), qualitative collagen changes may be of more importance: formation of advanced glycation end products (AGEs) plays a crucial role in the etiology of diabetic complications (6). Glucose-derived crosslinks on collagen are formed under hyperglycemic conditions, and these crosslinks drastically alter the structure and function of this protein (7–10). Increased arterial stiffness was observed in patients with diabetes and showed a strong correlation with blood glucose levels (11) and increased aorta and myocardial collagen advanced glycation (12).

Previously, our group has shown that arterial wall stiffness was increased in diabetic rats: characteristic input impedance of the ascending aorta, determined from phasic recordings of pressure and flow, was higher and carotid artery compliance lower in diabetic rats than in controls (13). Treatment of these diabetic rats with aminoguanidine, an inhibitor of AGE formation, significantly increased carotid artery compliance and decreased aortic impedance (14). Normalization of blood glucose levels reduced the amount of glycation products on collagen (15). These results suggested that the disturbances of vascular mechanical properties in experimental diabetes are caused by AGE accumulation (14).

To further substantiate this concept, we evaluated the effects of a newly developed AGE breaker compound ALT-711 on functional arterial wall properties in diabetic rats. ALT-711 is the stable 4,5-dimethylthiazolium derivative of the prototype compound *N*-phenacylthiazolium bromide (16), which has been shown to break AGEs *in vitro* and *in vivo*. Treatment of rats with streptozotocin-induced diabetes with ALT-711 for 1–3 weeks reversed the diabetes-induced increase of large artery stiffness as measured by systemic arterial compliance and characteristic aortic input impedance, and by carotid artery compliance and distensibility assessed *in vivo* and *in vitro*. These findings will have significant impact on the future treatment of patients with diabetes-related complications and aging.

MATERIALS AND METHODS

Animals. Male Wistar rats were made diabetic at the age of 9–10 weeks by i.p. injection of 70 mg/kg of streptozotocin. Only animals that developed blood glucose levels >15 mmol/liter were used. After 9 weeks of diabetes, the animals were divided into three groups: one group was studied to assess the exact hemodynamic changes that were caused by the diabetic state ($n = 13$), and two groups received ALT-711 (1.0 mg/kg per day i.p.) for either 1 or 3 weeks ($n = 8$–10 animals in each group) to assess possible reversal of the diabetes-induced cardiovascular abnormalities by treatment with this compound. Studies were carried out in a fixed scheme, allowing comparable exposure to hyperglycemia in all animals before therapy. Diabetes duration was 62 ± 5 days in untreated animals, 65 ± 2 days in the animals that subsequently were treated for 1 week (total diabetes duration 72 ± 2 days), and 60 ± 3 days in the animals treated for 3 weeks (total diabetes duration 80 ± 3 days).

Hemodynamic Measurements. Details regarding the surgical procedure and hemodynamic measurements have been described elsewhere (17). In summary, animals were anesthetized with 50 mg/kg of pentobarbital i.p., placed on a heating pad, intubated, and ventilated with a rodent respirator (Harvard Bioscience model 683, South Natick, MA). After midsternal thoracotomy, an adapted Doppler probe was placed around the ascending aorta to measure mean (cardiac output minus coronary blood flow) and phasic aortic blood flow (17). A 2F Millar Instruments (Houston, TX) catheter tip pressure transducer was advanced into the ascending aorta. After stabilization for 10 min, aortic blood flow and pressure were recorded on a beat-to-beat basis for 30 sec, averaged, and processed by a microcomputer system with analog-digital converter. In this way, systolic, diastolic, and mean arterial

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Abbreviations: AGE, advanced glycation end product; TPR, total peripheral resistance; SAC, systemic acterial compliance; Zc, characteristic input impedance of the aorta; BP, blood pressure; RBC, red blood cell.

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blood pressure (BP), mean cardiac output, and heart rate were determined.

The elasticity assessment of the arterial system is based on the concept that mechanically the arterial system can be regarded as a simple elastic system, which discharges during diastole into a single resistance representing the total peripheral resistance. Systemic arterial compliance (SAC) can be regarded as a global parameter, representing the mechanical properties of the whole aorta (18). The stiffer the arterial wall is, the lower the compliance. Liu *et al.* (19) have proposed a modification of this method, which is based on the measurement of the surfaces under the systolic and diastolic parts of the aortic pressure curve; this method is less dependent on actual BP. Pulsatile aortic pressure and flow signals were subjected to Fourier analysis and impedance modulus and phase-determined from the harmonic components. Corrections were made for the delay of the pressure transducer and flowmeter, which was linear (6.6/Hz) in the frequency range 0–50 Hz. Characteristic aortic input impedance (Zc) mainly represents the elastic modulus of the ascending thoracic aorta (20) and was taken as the average value of the modulus of impedance for high frequencies (fourth to 10th harmonic) (20). This parameter is independent of the reflection waves and is higher when the aorta is stiffer. The impedance modulus at 0 frequency represents total peripheral resistance (TPR). Cardiac output and TPR have been corrected for body surface area.

Carotid Artery Compliance. Both common carotid arteries were dissected free. Pressure was measured in the right carotid artery with a 2F Millar catheter tip pressure transducer. The diameter and change in diameter of the left carotid artery during the cardiac cycle were assessed by using a vessel wall echotracking system (Asulab S.A., Neuchâtel, Switzerland) using a 10-MHz probe (21), and converted to lumen areas and changes thereof (A and ΔA), assuming a circular cross-section of the vessel. Carotid artery cross-sectional compliance (CSC) was determined by computing the ratio of lumen area (ΔA) and phasic pressure oscillations (ΔP) as CSC = $\Delta A/\Delta P$.

***In Vitro* Carotid Artery Compliance and Distensibility.** The distal end of the left carotid artery first was ligated and then catheterized with a 9-gauge catheter and connected via a three-way stopcock to a reservoir filled with Tyrode's solution containing albumin (4%) at 130 mm H_2O, as described previously (22). The presence of albumin in incubating and flushing solutions maintained a physiological osmotic pressure gradient across the arterial wall and preserved the endothelial integrity and functions. A second catheter closed with a three-way stopcock pointing distally then was inserted into the proximal ligated end of the carotid artery. By this procedure, a pressure of 130 cm H_2O (approximately 100 mm Hg) was continuously maintained within an isolated segment of carotid artery (20–23 mm long). The two catheters then were clamped into a crossbar that maintained the artery at its *in vivo* length and prevented shortening on excision. Removed carotid artery segments were immersed in a bath containing a modified Krebs–Ringer bicarbonate solution (pH 7.4, 95% O_2, 5% CO_2, 38°C, 10 mM Hepes, 4% albumin, 25 mM glucose). A glass tube 30 cm long and 0.5 cm in diameter was filled with albumin-Tyrode's solution and connected to a manometer pressurized to 100 mm Hg and to the still-pressurized artery via a three-way stopcock. The artery then was perfused discontinuously for 30 sec every 20 min. Changes in the carotid artery diameter were determined with an ultrasonic microdimensiometer (Application Electronique, Montreuil, France), which allowed continuous measurement of the arterial diameter (with an accuracy of measurement better than 10 μm). During step-wise increase in pressure (75–150 mm Hg), diameter and lumen area changes were assessed in response to pressure (elasticity). The protocol was repeated after incubating the vessel with potassium cyanide to abolish smooth muscle cell function (passive elasticity). Carotid artery cross-sectional compliance (CSC) was calculated by the ratio of lumen area (ΔA) and pressure (ΔP) as CSC = $\Delta A/\Delta P$; carotid artery distensibility was calculated as compliance divided by baseline cross-sectional lumen area: $(\Delta A/A)/\Delta P$.

Collagen Crosslinking. Because it is likely that the observed improvements result from a reduced crosslinking of arterial wall extracellular matrix proteins, we assessed the effects of ALT-711 on collagen crosslinking. For this purpose, tails were removed and the tail collagen was dissected from the tendon sheet by gentle pulling. The tendons were cleaned free of debris and fat in 0.9% NaCl over ice, rolled into a ball, patted dry on paper towels, lyophylized, and transferred to capped polypropylene tubes. Samples were stored at −70°C until use. Collagen solubility was assessed by treating tail tendon collagen with pepsin (5.0 μg/ml) for 45 min, according to previously described methods (23, 24).

Red Blood Cell (RBC)-IgG Assay. Another biochemical marker of the effects of ALT-711 is its capacity to break crosslinks of IgG to RBCs (16). In a separate series of experiments, the biochemical effects of ALT-711 treatment were studied in STZ-diabetic male Lewis rats. After 3 months of diabetes, treatment with vehicle or ALT-711 administered orally by gavage in a dose of 10 mg/kg was initiated, and blood samples were collected after 1, 2, 5, and 10 weeks of treatment for erythrocyte-cell-surface IgG determinations by using an anti-IgG ELISA adapted for use with cellulose ester membrane-sealed 96-well microtiter plates (Multiscreen-HA, Millipore). Also, a separate series of diabetic rats were treated for 8 days with ALT-711 in doses varying from 0.01 to 10 mg/kg. Heparinized blood was washed three times with PBS; the packed RBCs were diluted 1:250–1:500 in PBS. Membrane-containing wells first were blocked with 0.3 ml of Superblock (Pierce), then washed with 0.3 ml of PBS/0.05% Tween, followed by 0.1 ml of PBS. RBCs were gently vortexed and 50-μl aliquots pipetted into wells. Cells then were washed, and 50 μl of a polyclonal rabbit anti-rat IgG (Sigma, diluted 1:25,000) was added. After incubation at room temperature for 2 hr, the cells were washed three times with PBS, once with Tris-buffered saline, and 0.1 ml of *p*-nitrophenyl phosphate substrate was added (1 mg/ml in 0.1 M diethanolamine buffer, pH 9.5). By this technique, the A410 of nondiabetic red cells was 0.10 ± 0.02 and the A410 of diabetic red cells was 0.57 ± 0.06 ($n = 4$; $P < 0.001$).

Statistical Analysis. All results are reported as mean ± SD, unless stated otherwise. Differences between groups were evaluated by using a one-way ANOVA with Student–Newman–Keuls correction for multiple comparisons, and linear trend analysis for treatment effects. A two-tail P value of <0.05 was considered statistically significant.

RESULTS

Hemodynamic Measurements. Body weight of the animals at randomization was 255 ± 15 g and comparable between the groups; ALT-711 treatment did not result in significant weight changes. Also, blood glucose levels were not different. Table 1 provides details on the metabolic and hemodynamic data, as well as results of hemodynamic measurements obtained in nondiabetic animals of comparable age. Between the three groups of diabetic animals, systolic, diastolic and mean arterial BP were not different. In comparison with nondiabetic age-matched control animals, body weight of the diabetic animals was lower, whereas BP and cardiac output were slightly higher. However, SAC was lower, and Zc was considerably higher, indicating significant alterations of vascular wall properties. Treatment with ALT-711 resulted in an increase in cardiac output, a reduction of total and indexed peripheral resistance, an increase of SAC, and a reduction of Zc, with increasing duration of treatment (Table 1).

Table 1. Glycemic and hemodynamic measurements performed in diabetic animals and diabetic animals treated with ALT-711 for 1 or 3 weeks

	Nondiabetic controls	9 weeks diabetes	Diabetes + ALT-711 for:	
			1 week	3 weeks
Blood glucose (mmol/liter)	6.0 ± 2.0	24.5 ± 3.2*	23.3 ± 2.9	24.0 ± 3.5
HbA1c (%)	1.3 ± 0.3	4.7 ± 0.4*	4.6 ± 0.5	4.6 ± 0.5
Body weight (g)	354 ± 29	258 ± 14*	255 ± 16	251 ± 15
Systolic BP (mm Hg)	126 ± 11	111 ± 12*	108 ± 11	109 ± 13
Diastolic BP (mm Hg)	98 ± 8	87 ± 15	83 ± 10	85 ± 16
Mean BP (mm Hg)	111 ± 10	98 ± 15	95 ± 10	96 ± 16
Heart rate (beats/min)	412 ± 66	321 ± 32*	296 ± 25	320 ± 34
Cardiac output (ml/min)	69 ± 6	59 ± 15	62 ± 10	75 ± 18†
Cardiac index (ml/min per cm^2)	0.19 ± 0.02	0.19 ± 0.05	0.21 ± 0.03	0.25 ± 0.07†
TPR (10^3.dyne.sec/cm^5)	128 ± 17	141 ± 46	126 ± 31	107 ± 34‡
TPR index (dyne.sec/cm^3)	359 ± 35	463 ± 170	429 ± 106	357 ± 123‡
SAC (10^{-3} ml/mm Hg)	6.9 ± 0.6	5.6 ± 1.8*	6.4 ± 1.6	7.3 ± 3.3†
Zc (10^3.dyne.sec/m^5)	8.7 ± 3.1	13.3 ± 4.6*	11.7 ± 4.2	8.6 ± 3.8†

Values are mean ± SD. HbA1c, glycated hemoglobin; cardiac index, cardiac output corrected for body surface area; TPR index, TPR corrected for body surface area.
*$P < 0.05$ vs. nondiabetic control animals.
†$P < 0.05$; ‡$P = 0.075$ by ANOVA vs. 9-week diabetic animals.

Carotid Artery Compliance and Distensibility *in Vivo*. Both systolic and diastolic carotid artery lumen diameter were significantly larger in animals treated 1 and 3 weeks. The change of vessel cross-sectional lumen area during the cardiac cycle was larger for both groups of ALT-711-treated animals, whereas the pressure changes were not different. Hence, the cross-sectional compliance of the carotid artery, which reflects the buffering capacities of the vessel, was significantly increased by 40% as a cause of the AGE breaker treatment (Fig. 1).

***In Vitro* Carotid Artery Distensibility.** Basal carotid artery diameter *in vitro* was 1.09 ± 0.17 mm in the untreated animals. In contrast, carotid artery diameter was 14–15% higher in the animals that were treated with ALT-711 for 1 and 3 weeks, respectively. Under no flow conditions, the intraluminal pressure was increased from 75 to 150 mmHg by 25-mmHg increments; in the untreated animals, diameter increased to a limited extent, from 1.09 ± 0.12 mm at 75 mmHg to 1.21 ± 0.16 mm at 150 mmHg. In the animals that were treated with ALT-711, this increase in diameter during increasing pressure levels was significantly higher (from 1.22 ± 0.13 to 1.38 ± 0.09 mm in those treated for 1 week, and from 1.24 ± 0.15 to 1.49 ± 0.08 mm in those treated for 3 weeks, both $P < 0.05$ vs. untreated). This finding implies that both carotid artery compliance and distensibility were markedly improved by treatment with ALT-711 (Fig. 2). After incubation of the vessels with potassium cyanide to abolish smooth muscle tone, a significant difference between the diameters of arteries obtained from untreated diabetic animals and those from 1- and 3-week-treated animals remained, indicating that the diabetes-induced increase of passive stiffness was reverted in the treated animals.

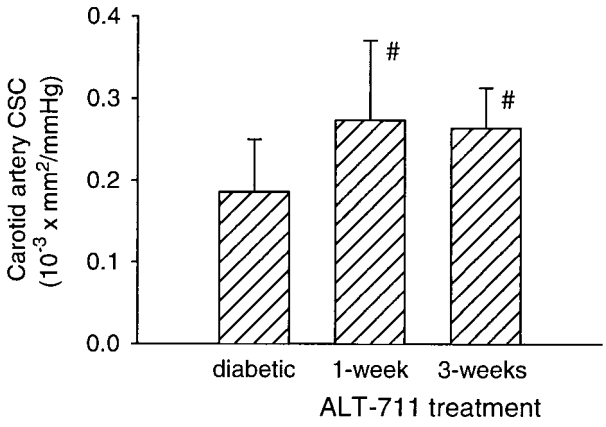

FIG. 1. ALT-711 significantly increases *in vivo* carotid artery cross-sectional compliance as assessed by ultrasound in diabetic animals, and animals treated with ALT-711 for 1 or 3 weeks. # reflects $P = 0.017$ (by ANOVA) for animals treated for 1 week, and $P = 0.012$ for those treated for 3 weeks.

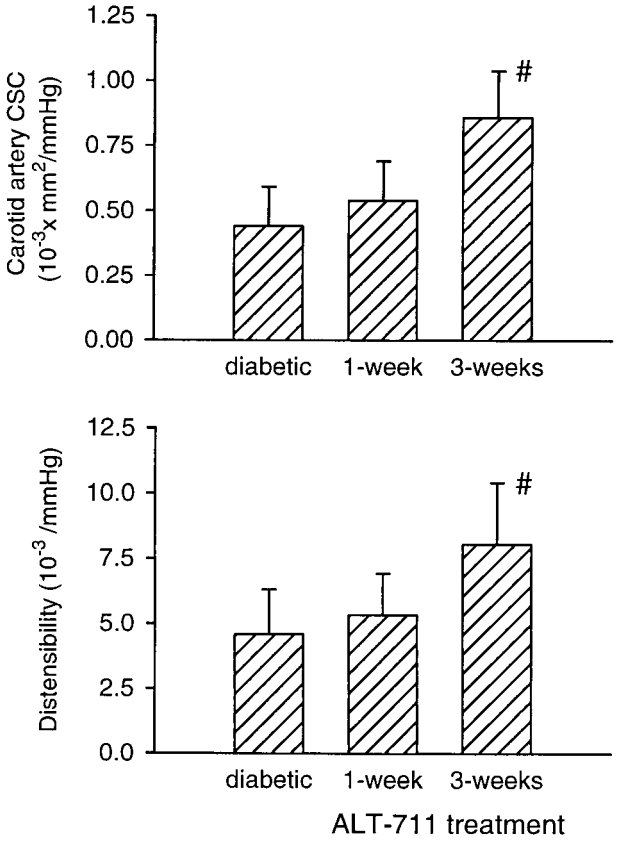

FIG. 2. ALT-711 improves *in vitro* carotid artery cross-sectional compliance and distensibility with increasing duration of treatment. Compliance and distensibility were calculated based on the increase of diameter and subsequent lumen area when pressure was increased from 75 to 125 mmHg under no flow conditions. #, $P < 0.05$ by ANOVA compared with diabetic animals.

Biochemical Markers of Protein Crosslinking. The increase in collagen crosslinking as a consequence of diabetes-induced AGE accumulation resulted in a marked decrease in the susceptibility of tail tendon collagen to pepsin digestion, as found in earlier studies (5, 23). By contrast, pepsin solubility of collagen, which was obtained from the animals that had been treated with ALT-711, was not different from that observed in nondiabetic controls (Fig. 3).

When compared with the control group, ALT-711 treatment decreased cell-surface IgG content by 90% after 1 week, which remained at this level during the subsequent weeks, whereas this parameter increased slightly further in the vehicle-treated animals (Fig. 4, *Upper*). The reduction of cell-surface IgG content proved to be dose-dependent (Fig. 4 *Lower*), and the EC_{50} for breaking RBC-IgG crosslinks, calculated by the INPLOT program, was 0.06 mg/kg.

DISCUSSION

The different approaches used in these studies consistently show the beneficial effects of treatment with the AGE breaker ALT-711 on arterial elasticity. *In vivo*, treatment increased SAC and reduced Zc. There was a decrease of carotid artery stiffness, by assessment with ultrasound *in vivo* and *in vitro*. These effects seemed to be related to the duration of the treatment, with stronger effects after 3 weeks than after 1 week. The finding of the marked increase of compliance and decrease of impedance cannot be attributed to differences in BP, which did not change during treatment; therefore it reflects intrinsic modifications of the mechanical properties of the arterial wall. The increase of SAC and decrease of Zc indicate that because of treatment with ALT-711 the stiffness of the aorta was reduced by 25% (SAC) to 35% (Zc) to levels comparable to those observed in nondiabetic animals. This improvement was comparable to our results achieved with preventive treatment with aminoguanidine in diabetic rats (14).

The augmented stiffness of the arterial wall in diabetes could be attributed to changes in intrinsic mechanical wall properties caused by histological differences, especially in the ratio of elastin to collagen content. However, it has been demonstrated that the absolute and relative concentrations of elastin and collagen in the vessel wall are unaltered in rats with streptozotocin-induced diabetes and in diabetic patients (5). Therefore, it is more likely that glucose-derived crosslinking of extracellular matrix proteins causes the increase in stiffness, and that the observed improvements after ALT-711 treatment result from a reduced crosslinking of these matrix proteins. Collagen obtained from tail tendons is the best accessible to evaluate the magnitude of this effect, because the hyperglycemia-induced changes in collagen will not differ between the locations of this protein. We found that there indeed was an increase in collagen crosslinking in diabetic rats as evidenced by a marked decrease in the susceptibility of tail tendon collagen to pepsin digestion; this finding also has been shown in earlier studies (5, 23). By contrast, pepsin solubility of collagen that was obtained from the animals that had been treated with ALT-711 was considerably improved, and not different from that observed in nondiabetic control rats. A related biochemical marker of diabetes-induced crosslinking is the increase of erythrocyte cell surface-bound IgG (16). It was shown that treatment with ALT-711 resulted in a reduction of red cell surface IgG content in diabetic rats, which proved to be dose-dependent.

Functional abnormalities and disturbed elastic properties of the aorta may precede structural changes in the aortic wall (25). The process of AGE formation on arterial wall matrix proteins may be related to the development of atherosclerosis in many different ways. It is supposed that reactive free radicals are formed during the glycation process, and these free radicals

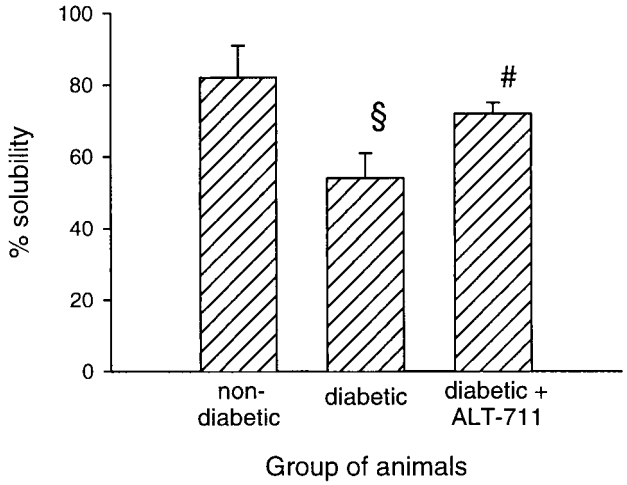

FIG. 3. Chronic treatment with ALT-711 increases pepsin-induced tail tendon collagen solubility in diabetic rats. §, $P < 0.05$ vs. age-matched nondiabetic control animals; #, $P < 0.05$ vs. diabetic animals. The ALT-711 group reflect animals that were treated for 3 weeks (1 mg/kg daily by i.p. injection).

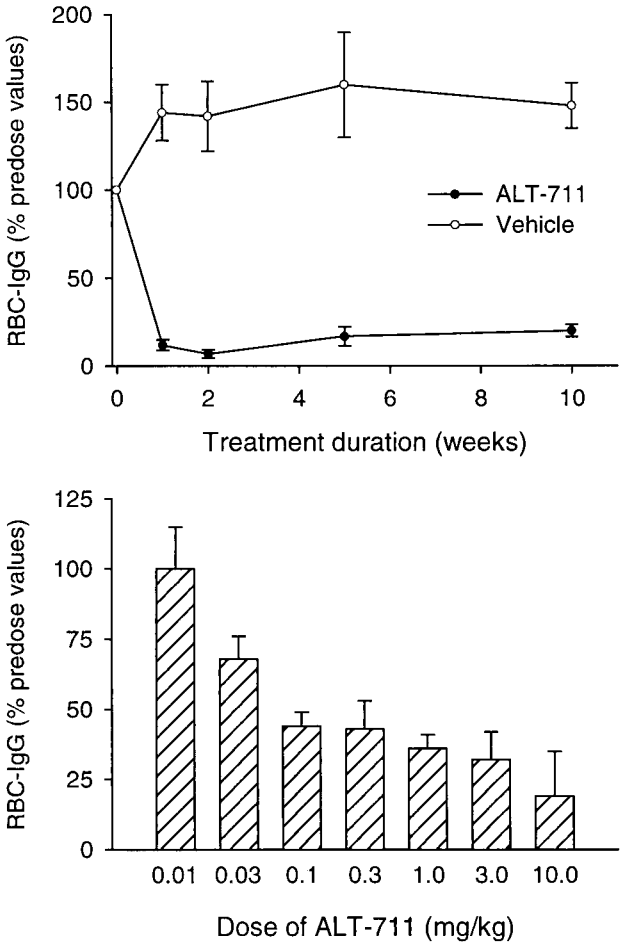

FIG. 4. ALT-711 decreases IgG crosslinked to the RBC surface. (*Upper*) Chronic treatment decreases RBC-IgG already after 1 week of chronic dosing. Values are means ± SD of the percent change with respect to day 0. All time points $P < 0.01$ vs. predose and vs. vehicle. (*Lower*) RBC-IgG levels decrease dose-dependently after 8 days of oral dosing with ALT-711. Diabetic rats were treated for 8 days with ALT-711 in doses varying from 0.01 to 10 mg/kg. All doses in the range 0.03–10.0 are $P < 0.01$ vs. predose values.

may interact with various arterial wall constituents. It has been demonstrated that AGEs inhibit a normal network formation by collagen (26). Also, glycation of collagen abolished the inhibitory effect of this matrix protein on human smooth muscle cell proliferation *in vitro* (27) and increased the adhesion of monocytes (28). Furthermore, the resulting abnormal elastic properties of the aorta constitute a greater work load for the heart, which can result in the development of hypertension and left ventricular hypertrophy.

The hallmark Diabetes Control and Complications Trial (DCCT) showed that near-normalization of blood glucose control by intensive insulin therapy reduced the risk of development of diabetic complications (29). However, intensive insulin therapy does not prevent nor cure complications, and this intensive therapy carries a high risk of side effects, especially occurrence of severe hypoglycemia. Thus, a large number of people still are prone to develop vascular complications, and additional pharmacological approaches to prevent these complications are warranted. Both inhibitors of AGE formation, like aminoguanidine, and AGE breakers, like ALT-711, may have a beneficial effect in this respect. Clinical studies with aminoguanidine are now ongoing. In our earlier studies in diabetic rats, a 30–35% lower arterial stiffness was observed in diabetic rats treated long-term with aminoguanidine compared with vehicle-treated animals, whereas cardiac output corrected for body surface area and total peripheral resistance did not differ (14). The latter effect may have been because of the (weak) effects of aminoguanidine as an inhibitor of the endothelial constitutive nitric oxide synthase. It must be reminded that aminoguanidine was given in our earlier studies as a preventive treatment, i.e., treatment started directly after the induction of diabetes. In contrast, in the current studies ALT-711 was given as a curative treatment, i.e., treatment started after diabetes already had existed for more than 2 months. Thus, both aminoguanidine and ALT-711 beneficially influenced arterial stiffness as assessed by aortic impedance, carotid artery compliance, and distensibility. The increase of cardiac output corrected for body surface area and reduction of peripheral resistance observed with ALT-711 in the absence of any effects on BP suggests that these breaker compounds by directly reducing vascular AGE-accumulation have an additional beneficial influence on the hemodynamic system.

As pointed out earlier, glucose-induced damage is not unique to diabetic patients. Even at normal levels of blood glucose there will be some degree of glycation and accumulation of AGE-related products over time (30). Increased accumulation of AGEs indeed has been demonstrated in the mesenteric artery tissue specimens obtained from humans, and was clearly associated with aging (31); the changes in the aorta that occur with aging are characterized by a progressive increase in aortic stiffness. Our findings therefore may have significant therapeutic potential in the future treatment of conditions characterized by increased AGE accumulation and protein crosslinking, such as diabetes-related complications, systolic hypertension, and aging.

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