Glycation end-product cross-link breaker reduces collagen and improves cardiac function in aging diabetic heart

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Both diabetes mellitus (DM) and aging increase collagen in the heart and vessels and reduce their compliance (3, 5, 19, 28). The extent to which these changes occur in the aged diabetic heart is relatively unknown, particularly with respect to collagen types. In addition, glycation affects the interactions of collagen with cells and other matrix components, but the most damaging effects are caused by the formation of glucose-mediated intermolecular cross-links, i.e., advanced glycation end products (AGEs) (20). AGEs accumulate slowly on proteins with low turnover rates, such as collagen and elastin, covalently modifying their structure and function (5, 24). Associated with increased AGEs, there is an increase in total collagen (5). The increased accumulation of collagen in the extracellular compartment of the myocardium occurs in both the diabetic heart (3) and the aged heart (5, 28) and serves as a target protein for AGE accumulation, but less is known in the aged diabetic heart. This modification of the collagen protein, one of the major components of the matrix, has been implicated in the loss of ventricular compliance and diastolic dysfunction (5).

The first objective of the present investigation was to determine the extent to which collagen types I and III, which account for ~96% of the total collagen in heart (1), are increased in the aged diabetic heart. Second, we explored the effects of the AGE cross-link breaker phenyl-4,5-dimethylthiazolium chloride (ALT-711) to determine whether it improved function of the heart and also whether it reduced collagen in the heart. These goals are important because almost no information is available on the distribution of these collagen types either in the aged diabetic heart or, conversely, in their distribution following treatment with AGE cross-link breakers.

METHODS

Chronic Animal Model

Twelve healthy, male mongrel dogs, aged 9–12 yr and weighing 24–34 kg, were obtained from LBL Kennels (Reelsville, IN). The protocol was approved by the Institutional Animal Care Committee of New Jersey Medical School. The relative lack of coronary atherosclerosis in this species simplifies the consideration of variables affecting the myocardium (2). The animals were screened for diabetes before entering the study. Ages were obtained by documentation from the vendor, supplemented by eye, fur, and dental examination. Animals with evidence of pericardial or valvular disease by echocardiography were excluded. One animal died during the study.

†Deceased 20 October 2001.

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of an arrhythmia during biopsy during the second study, and one died of cardiac tamponade during surgery in the second study. For hemodynamics and other physiological parameters, the remaining 10 dogs were studied before diabetes and after the induction of diabetes, and then diabetics were studied after treatment with ALT-711, each serving as its own control with baseline echocardiographic studies of left ventricular (LV) function. After the initial hemodynamic measurement, the same mongrels were studied again 5 mo after the induction of diabetes with alloxan monohydrate (25–35 mg/kg in sterile saline, administered intravenously for 2–3 doses at weekly intervals). Fasting blood sugar was measured at 1- to 2-day intervals until a stable elevation was obtained. Thereafter, blood was taken weekly for glucose determination (3). Glycosylated hemoglobin A1c (Hb A1c) was measured by affinity chromatography (3). Five animals were fed with the cross-link breaker ALT-711 (1 mg/kg) daily for 1 mo before the terminal study. After hemodynamic studies, the hearts of control, diabetic, and drug-treated (ALT-711) diabetic groups of animals were harvested and stored at −80°C for collagen analysis.

**Hemodynamic Studies**

Aged dogs were anesthetized with ketamine (5 mg/kg iv) and fentanyl (20 µg/kg iv) after induction with pentobarbital sodium, followed by fentanyl and ketamine drip at 0.7 µg·kg⁻¹·min⁻¹ and 0.16 mg·kg⁻¹·min⁻¹, respectively. A cuffed endotracheal tube was inserted, and ventilation was controlled with a Harvard respiration pump to maintain the arterial pH and PO₂ within physiological range. Under sterile conditions, a size 7-Fr micromanometer catheter (Millar Instruments, Houston, TX) was introduced into the proximal aorta via the carotid artery and connected with a Honeywell physiological recording system. LV end-diastolic and end-systolic volumes were measured by two-dimensional echocardiography (ATL Ultramark VI) with 3.5-MHz transducers and calculated using an area-length method with a cylinder-ellipsoid model: 5/6 of the cross-sectional area at the papillary muscle length times the length from a LV long-axis view. LV ejection fraction (LVEF) was calculated as LV end-diastolic volume minus LV end-systolic volume divided by LV end-diastolic volume, expressed as a percentage (25). LV mass was calculated by subtracting the LV endocardial volume from the epicardial volume and multiplying the difference by a muscle density coefficient of 1.05. The endocardial and epicardial tracing was performed according to the convention of the American Society of Echocardiography. Aortic stiffness index (18) was calculated on the basis of the formula for a cylinder: $\beta = 127.32SP - DP/(d_{max}^2 - d_{min}^2)$, recorded from a Millar catheter 2 cm from the aortic valve (where SP is systolic pressure, DP is diastolic pressure, and d is diameter). Systolic and diastolic dimensions of the ascending aorta were assessed by M-mode echocardiography.

**Biochemical Studies**

Dogs were killed and their hearts excised. LV endo- and epicardiums were quickly frozen in liquid nitrogen and stored at −80°C until used for biochemical measurements.

**Collagen extraction.** Collagen types I and III were extracted as described (4), with the following modification. Briefly, ~500 mg of tissue were suspended in 8 ml of 50 mM Tris buffer (pH 7.4), 1 M NaCl, and 5 mM EDTA with protease inhibitor cocktail (Sigma) and homogenized using a Polytron homogenizer. The homogenate was centrifuged at 26,000 g, and the supernatant was discarded. The pellet was extracted with 0.5 M acetic acid for 24 h. This was followed by 0.5 M acetic acid containing pepsin (1 mg/ml) for 60 h at 4°C. The pepsin extraction was repeated for another 24 h, followed by a third pepsin extraction. Total collagen was precipitated from the pepsin extracts by adding NaCl to a 5% final concentration at 4°C and collected by centrifugation. Extracted collagen pellets were resolubilized in 0.5 M Tris-HCl (pH 7.4) and used for the immunoblot analyses of collagen type I and III.

**Immunoblot analysis of collagen types I and III.** Pepsin extract (6.4 mg equivalent wet wt tissue) was subjected to 8% SDS polyacrylamide gel electrophoresis, and proteins were then transferred to nitrocellulose membranes. Molecular weight markers and standard bovine collagen (Abcam, Cambridge, UK) were used as internal controls. The membranes were probed with rabbit polyclonal antibodies for collagen type I (1:5,000; Abcam) or collagen type III (1:5,000; Abcam) and with secondary anti-rabbit antibody (1:5,000; American). The blots were developed using an enhanced chemiluminescence reagent kit (Perkin-Elmer). Relative densities were scanned, quantified, and expressed as arbitrary units.

**Cross-linking by collagen solubility profile.** To determine myocardial solubility, LV tissue samples (300 mg) were homogenized in 50 mM Tris (pH 7.5), 5 mM EDTA, protease inhibitors, and 20 mM NaF. The pellet was digested with 2.5 ml of 200 µg/ml pepsin in 0.5 M acetic acid at 37°C (6, 16) for 2 and 24 h. Collagen content was determined by measuring hydroxyproline content after 2 and 24 h of pepsin digestion and acid hydrolysis with 6 N HCl for 20 h at 110°C (26). Collagen solubility was calculated as the hydroxyproline content after 2 h of digestion as a percentage of the total collagen recovered after 24 h of pepsin digestion.

**Statistical Analysis**

Data are expressed as means ± SE. Comparison between two values was performed using Student’s t-test. For multiple comparisons among different groups of data, significant differences were determined by the Bonferroni or Newman-Keuls methods. A P value of <0.05 was considered statistically significant. Because measurements presented were not obtained in all animals, n values for each parameter are listed in the text or figures.

**RESULTS**

**Physiological Data**

Blood glucose concentration of aging diabetic (Ag + DM) dogs was considerably higher than in Ag dogs but was not affected by the administration of ALT-711 in Ag + DM + ALT-711 dogs (Table 1). Hyperglycemia persisted throughout the study period and did not differ between the diabetic group and the diabetic group treated with ALT-711. Hb A1c levels were increased more than twofold at each of the monthly determinations in Ag + DM vs. Ag groups (P < 0.05). Body weight did not differ among the experimental groups. Five months after the induction of DM, LV weight in hearts from Ag + DM dogs increased by 14% above that in Ag controls (P < 0.05). The increase in LV weight in Ag + DM hearts was reversed by ALT-711 (P < 0.05). Taken together, the data indicate that hyperglycemia induces an increase in LV mass of the aging dog, which can be reversed by ALT-711 treatment.
Table 1. Baseline values in Ag, Ag + DM, and Ag + DM + ALT-711-treated mongrel dogs

<table>
<thead>
<tr>
<th></th>
<th>Ag (n = 10)</th>
<th>Ag + DM (n = 7)</th>
<th>Ag + DM + ALT-711 (n = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood glucose, mg/dl</td>
<td>81 ± 7</td>
<td>194 ± 8.7†</td>
<td>199 ± 17‡</td>
</tr>
<tr>
<td>Hb A1c, %</td>
<td>2.2 ± 0.1</td>
<td>5.2 ± 0.5†</td>
<td>5.5 ± 0.2‡</td>
</tr>
<tr>
<td>Body weight, kg</td>
<td>31 ± 1.1</td>
<td>32 ± 1.4</td>
<td>31 ± 1.7</td>
</tr>
<tr>
<td>LV mass, g/kg</td>
<td>4.3 ± 0.2</td>
<td>4.9 ± 0.2†</td>
<td>4.3 ± 0.2*</td>
</tr>
<tr>
<td>HR, beats/min</td>
<td>81 ± 3</td>
<td>80 ± 3</td>
<td>85 ± 4</td>
</tr>
<tr>
<td>LVEF, %</td>
<td>52 ± 2</td>
<td>39 ± 2†</td>
<td>55 ± 4*</td>
</tr>
<tr>
<td>SV, ml/kg</td>
<td>1.8 ± 0.1</td>
<td>1.3 ± 0.1†</td>
<td>2.1 ± 0.1*</td>
</tr>
<tr>
<td>Aortic stiffness, mmHg/ml</td>
<td>83 ± 9</td>
<td>143 ± 27†</td>
<td>83 ± 12*</td>
</tr>
<tr>
<td>LVEDV, ml/kg</td>
<td>3.6 ± 0.1</td>
<td>3.5 ± 0.2</td>
<td>3.8 ± 0.3</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SE; n, no. of animals studied. Ag, normal aging dogs; Ag + DM, Ag + diabetic mellitus; Ag + DM + ALT-711, Ag + DM + treatment with cross-link breaker ALT-711; LV, left ventricular; HR, heart rate; LVEF, LV ejection fraction; SV, stroke volume; LVEDV, LV end-diastolic volume. †P < 0.05 vs. Ag; ‡P < 0.05 vs. Ag + DM; §P < 0.05 vs. Ag.

Hemodynamic Parameters

Five months after the induction of DM, heart rate did not differ among the experimental groups, but, as shown in Table 1, the Ag + DM group exhibited a 25% reduction in LVEF (P < 0.05), which was accounted for by a decrease in stroke volume (SV). SV was lower in the Ag + DM group by 28% (1.3 vs. 1.8 ml/kg in Ag group, P < 0.05). LVEF and SV in the Ag + DM dogs were restored to values measured in Ag dogs following 4 wk of treatment with ALT-711 (Table 1). Treatment of Ag + DM dogs with ALT-711 restored LVEF and SV to values measured in Ag dogs (P < 0.05). As shown in Table 1, aortic stiffness increased ~1.7-fold (P < 0.05) after 5 mo of diabetes. The normalization of LVEF following ALT-711 treatment was accompanied by a 42% (P < 0.05) decrease in aortic stiffness (Table 1). Baseline LV end-diastolic volume did not differ among the experimental groups.

Collagen Types

To determine whether DM alters collagen types I and III in the aging heart, immunoblot analysis was performed on the pepsin-treated collagen extracts. As shown in Fig. 1A, collagen type I was increased (2-fold) in Ag + DM compared with Ag hearts (P < 0.05). ALT-711 reversed the upregulation in collagen I levels in Ag + DM hearts (P < 0.05). We next asked whether collagen III would exhibit a similar distribution pattern. Collagen III increased approximately threefold in the hearts of Ag + DM dogs compared with Ag hearts (P < 0.05). ALT-711 also reversed the upregulation of collagen III content in Ag + DM hearts (Fig. 1B). Taken together, DM upregulates collagen type I and III content in the Ag + DM myocardium. ALT-711 reverses DM-induced alterations in collagen type I and type III content in the Ag + DM heart.

Collagen Solubility

The percent solubility of myocardial LV collagen tended to fall with DM but increased significantly (P < 0.05) from 34 ± 4.9 to 51 ± 6.8% after treatment with ALT-711 (Fig. 1C).

DISCUSSION

DM is associated with an increased risk of cardiovascular disease (7, 12, 21). Increased intracellular AGE formation plays a critical role in the development of diabetic complications (19); conversely, inhibition of AGE formation can prevent the development of diabetic complications. AGE cross-links contribute to arterial stiffening in humans, supporting experimental studies performed in diabetic and nondiabetic animal models (2). Some investigations suggest that collagen cross-linking and turnover rate may be as important as the total amount of collagen found in the aged heart (17). Cross-linking among collagen molecules has the potential to increase the strength of the interstitium by covalently linking the fibrillar collagen molecules to-covalently linking the fibrillar collagen molecules to-

Fig. 1. Collagen type I (A) and collagen type III protein levels (B) determined by immunoblot analysis in aging (Ag, open bar), aging + diabetes (Ag + DM, hatched bar), and aging + diabetes + ALT-711 (Ag + DM + ALT, filled bar)-treated animals (n = 5–7). Type I collagen and type III collagen were significantly increased in Ag + DM compared with Ag and significantly decreased in Ag + DM + ALT compared with Ag + DM. C, left ventricular collagen solubility studies show an increase in percent solubility in Ag + DM dogs following treatment with ALT-711 (n = 5–6). Values are expressed as means ± SE. *P < 0.05, different from Ag or Ag + DM + ALT different from Ag + DM.
together (22, 24). ALT-711, an agent that reverses AGE cross-linking, improves arterial compliance and reduces arterial pulse pressure in older individuals with a stiffened vasculature (6, 14, 15, 29). AGEs covalently modify proteins by forming bonds with amino groups on other proteins, resulting in a matrix of cross-links. In the present study, we demonstrated that the AGE cross-link breaker ALT-711 reversed DM-induced increases in collagen of the aged diabetic heart. 

Hyperglycemia dominates the pathophysiology and clinical course of DM. The Framingham Study has provided compelling evidence for the existence of a primary cardiomyopathy in DM (8, 9, 13). The goal of the present study was to test the hypothesis that the cross-link breaker ALT-711 would preserve pump function in the aged DM dog heart and reduce the increased levels of collagen in the aged DM heart. Investigations were performed in aged dogs with alloxan-induced DM. Five months after the induction of DM in aged dogs, LVEF, and LVSV were significantly impaired, and aortic stiffness was increased. These indexes of depressed pump function and decreased vascular compliance were reversed after 1 mo of treatment with the AGE cross-link breaker (ALT-711). We also demonstrated that both DM and aging increased collagen in the heart and that this process was reversed with the AGE cross-link breaker ALT-711, consistent with the hypothesis that decreased LV compliance in the diabetic heart is the result of increased extracellular matrix deposition (5). In further support, we found that the solubility of collagen increases in the hearts of aged diabetic dogs treated with ALT-711, which was also observed in young DM rats (6). It has also been proposed that incubation of AGE cross-linked collagen with ALT-711 in vitro restores matrix metalloproteinase digestibility of collagen (14), and evidence has been presented that AGE formation associated with DM reduces extracellular matrix degradation and angiogenesis (27).

In the present investigation, LV mass increased in the aged DM heart, which has been observed in aged DM humans (11, 23); furthermore, LV mass fell with ALT-711 treatment. This could also have contributed to the improved LV function obtained in the aged DM dogs treated with ALT-711.

Relatively little is known about the changes in distribution of subtypes of collagen in aging or DM, and virtually nothing is known about this in aged diabetic hearts. In the aging human heart, with the use of transmission electron microscopy, type I collagen fibers were shown to be increased in thickness and number (10). A major finding of the present investigation was that DM superimposed on aging increases both types I and III collagen, which accounts for ~96% of total collagen in the heart, approximately two- to threefold. We found that ALT-711 decreased collagen type I and type III in aged DM hearts, indicating that cross-links may be important for increases in total amounts of types I and III collagen as well as for some of the hemodynamic alterations in aged dogs with DM. Taken together, the present study provides evidence for DM-induced alterations in collagen types I and III in the aged, large mammalian heart and for reversal of these alterations by the cross-link breaker ALT-711, which was associated with reversal of the adverse effects of DM on the function of the aged heart and aorta. It has also been proposed that incubation of AGE cross-linked collagen with ALT-711 in vitro restores matrix metalloproteinase digestibility of collagen (14), and evidence has been presented that AGE formation associated with DM reduces extracellular matrix degradation and angiogenesis (27).

In summary, the induction of DM in aging dogs induced LV systolic dysfunction, increased aortic stiffness, and increased collagen types I and III protein content. Here, we report that the AGE cross-link breaker ALT-711 reversed DM-induced myocardial collagen types I and III accumulation, aortic stiffness, LV dysfunction, and increased LV mass. These results support a causative role for collagen-linked glycosenylation in the biochemical and hemodynamic alterations in the aging diabetic myocardium and aorta and that a collagen cross-link breaker could possibly provide therapeutic potential in the treatment of aging diabetic patients.

DISCLOSURES

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