Effects of Glucose Intolerance on Myocardial Function and Collagen-Linked Glycation

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In experimental diabetes, diastolic dysfunction of the left ventricle has been associated with collagen-linked glycation. To determine whether less severe hyperglycemia may have similar effects, we gave alloxan to mongrel dogs (group 2) to induce impaired glucose tolerance (IGT) for comparison with normal subjects (group 1). After 6 months, hemodynamic studies were performed in the anesthetized animals. Basal heart rate, aortic pressure, and ejection fraction were comparable in the two groups, but calculated chamber stiffness was increased in group 2, associated with a reduced end diastolic volume and increased pressure. During infusion of dextran, the volume and pressure responses were similarly abnormal in group 2. In the myocardium, the collagen concentration rose with an increased interstitial distribution histologically. To assess glycation, collagen was extracted, digested with collagenase, and measured for fluorescence. Advanced glycation end products were increased in group 2 to 10.6 ± 1.6 vs. 6.9 ± 0.7 fluorescent units (FU)/mg collagen in group 1 ($P < 0.01$). To assess whether this could be pharmacologically prevented, we administered enalapril to inhibit ACE during the 6 months of glucose intolerance to group 3. This resulted in normal glycation and significant reduction in chamber stiffness increment. We gave group 4 animals aminoguanidine daily for 6 months, which prevented abnormal collagen glycation and chamber stiffness. Thus, in animals with IGT, collagen-linked glycosylation appeared to be a major factor affecting diastolic function and was shown to be amenable to pharmacological intervention. *Diabetes* 48:1443–1447, 1999

Although myocardial abnormalities have been described in diabetes, the glycemic threshold required for these alterations has not been defined. Impaired glucose tolerance (IGT) in humans has been associated with enhanced morbidity and mortality (1), but comorbid conditions may confound this relationship.

To assess the cardiac effects of isolated glucose intolerance, this issue has been examined in the healthy mongrel dog. Cardiac dysfunction in diabetes is often characterized by an early increase of diastolic stiffness (2), which can progress to systolic dysfunction (3). Relatively short-term diabetes in the dog demonstrates diastolic dysfunction (4). The issues addressed include whether the increased collagen-linked glycation (5) and concentration (4,6,7) observed in diabetic myocardium can be induced by glucose intolerance. These findings are also related to the measured left ventricular diastolic stiffness both in the basal state and after a volume infusion challenge.

To elucidate these interactions, two pharmacological interventions that have been reported to modify myocardial collagen have been examined. First, inhibition of ACE has been shown to prevent abnormal increases of the collagen volume fraction and diminished diastolic compliance of the left ventricle in the spontaneous hypertensive rat (8). Further, in Syrian hamster cardiomyopathy, ACE inhibition improved the elasticity of myocardial collagen despite unchanged concentrations (9). These findings suggest a potential effect of this treatment in the canine model of IGT.

As an alternative approach, aminoguanidine has been shown to be efficacious in preventing collagen-linked glycation in myocardium of the diabetic rat (2). Increased diastolic stiffness demonstrated in the isolated papillary muscle was found to be prevented by treatment with the guanidine. We used aminoguanidine over the course of 6 months of IGT to test the view that the collagen alterations in mild hyperglycemia are preventable.

RESEARCH DESIGN AND METHODS

Chronic animal model. Healthy, male mongrel conditioned dogs, ages 2–3 years, were obtained from LBL Kennels (Reelsville, IN). The protocol was approved by the Institutional Animal Care Committee according to the "Principles of Laboratory Animal Care" (National Institutes of Health publ. no. 85–23, revised 1985). The relative lack of coronary atherosclerosis in this species simplifies the consideration of variables affecting the myocardium. The mixed dog food diet consisted of Purina Pedigree diet (Vernon, CA) and Hill Science Diet (Topeka, KS), of which ~22% of calories were protein, ~16% were lipid, and ~62% were carbohydrate.

Glucose intolerance was produced with alloxan monohydrate in sterile saline administered intravenously in two 20 mg/kg doses separated by a 1-week interval. Fasting plasma glucose, determined by the glucose oxidase method (10), was measured two to three times weekly for the initial months and once a week thereafter. In addition, glycosylated hemoglobin A 1c (HbA_{1c}) was measured by affinity chromatography (11) and body weight was determined, both at monthly intervals. The normal range for HbA_{1c} in the dog is 1.2–2.0% and was reproducible in the normal controls (group 1). IGT was considered to be present on the basis of increased levels of HbA_{1c} and fasting blood glucose. In a previous comparison of values before and after administration of alloxan, we observed that increments of these parameters after alloxan correlated with a reduced clearance of glucose during an intravenous tolerance test (12). After larger dosages of alloxan in the dog, the fasting blood sugar rose to 172 ± 17 mg/dl and the HbA_{1c} increased to 3.9 ± 3% (4). Using the same assay in stable human diabetic patients at a fasting blood glucose of 175 ± 36 mg/dl, the HbA_{1c} was 10.9 ± 1.4% (13). Erythrocyte hemoglobin in the dog appears to be less responsive to hyperglycemia than in

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AGE, advanced glycosylation end product; IGT, impaired glucose tolerance.

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TABLE 1
Blood composition in the glucose-intolerant state

Group	Glucose (mmol/l)		HbA_{1c} (%)		Body wt (kg)	
	Baseline	Mean for 6 months	Baseline	Mean for 6 months	Baseline	At 6 months
1	4.6 ± 0.2	4.5 ± 0.2	1.8 ± 0.1	1.7 ± 0.2	25.1 ± 0.3	26.0 ± 0.6
2	4.8 ± 0.3	5.9 ± 0.3*	1.7 ± 0.1	2.9 ± 0.3*	24.5 ± 0.7	25.3 ± 0.2
3	4.5 ± 0.3	5.5 ± 0.3*	1.8 ± 0.2	3.2 ± 0.5*	23.6 ± 0.4	24.9 ± 0.5
4	4.7 ± 0.4	5.8 ± 0.3*	1.9 ± 0.3	3.3 ± 0.4*	24.7 ± 0.5	25.0 ± 0.4

Data are means ± SE. In group 1, values are at baseline and 6 months. In diabetic groups (groups 2–4), values represent baseline and the mean for the monthly values during the 6-month period. *Values for HbA_{1c} and fasting glucose after administration of alloxan were significantly elevated above their baseline values as well as those of the controls in group 1 ($P < 0.05$). After 6 months, body weight did not differ from baseline.

humans. The quantitative relation of this phenomenon to glycation of collagen in this species has not been ascertained, but these are clearly dissociated by aminoguanidine, as indicated in group 4 of this study. As a marker of a hyperglycemic effect in tissue, it is noteworthy that the accumulation of type VI collagen in the diabetic mouse heart (12) was also observed in the glucose-intolerant dog (14).

All animals survived the 6-month observation period. Concerning the question of a direct toxic effect of alloxan on myocardium, previous observations in this model have indicated that diminished myocardial compliance and increases of collagen concentration in diabetes were not present when the pancreatic effects of alloxan and resultant diabetes were prevented (15). Four groups of animals were formed for a 6-month study. Group 1 consisted of normal controls ($n = 8$). Groups 2 and 3 received alloxan (n = 8 in each group) to induce IGT. Two of eight in each group were resistant to alloxan and were excluded from the study. The glucose-intolerant animals of group 2 remained untreated ($n = 6$); group 3 animals were glucose intolerant and were treated orally with the ACE inhibitor enalapril 0.1 mg/kg b.i.d. ($n = 6$) (Merck, West Point, PA). This dosage inhibited the acute hypertensive response to intravenous angiotensin I. To compare the responses to enalapril with the classic inhibitor of advanced glycosylation, aminoguanidine, group 4 animals with glucose intolerance were treated daily throughout the 6-month course with aminoguanidine 20 mg/kg p.o. ($n = 4$) (Alteon, Ramsey, NJ). Additional animals in group 4 were not used, since none of the animals in this group showed an increase in left ventricular diastolic stiffness or collagen glycation, as expected from previous findings (5). Both enalapril and aminoguanidine were given daily over a 6-month period and were administered orally in a capsule covered with a small amount of meat before mealtime to avoid the potential complications of gastric instillation. These agents were omitted on the day of study.

Hemodynamic study. After 6 months of IGT, the animals were anesthetized with chloralose 75 mg/kg i.v. A cuffed endotracheal tube was inserted and respiration was controlled by a Harvard respiration pump to maintain the arterial pH and PO_2 within physiological ranges. In the intact animals of the four groups, simultaneous measures of left ventricular pressure and volume were made in the basal state before volume loading; 10% dextran 40 (3 ml · min^{-1} · kg^{-1} over 3 min) was infused to observe diastolic pressure and volume responses. This volume is less than the amount that increases intrapericardial pressure as ventricular volume is expanded (16). Thus, an artifactual elevation of ventricular filling pressure due to the extrinsic compression of the myocardium was avoided.

The methods used for left ventricular pressures—echocardiography-derived volume measurements (17) and the calculation of chamber stiffness (18,19)—as used in our laboratory have been previously described (4).

Myocardial composition. After the hemodynamic study and thoracotomy, the subjects were killed when their heart was cold arrested with iced-Ringer's solution. To assess left ventricular weight, the left ventricle and septum were used after removing the atria, remnants of the aorta, and the pulmonary artery as well as right ventricle. Transmural sections from the mid-left ventricle were trimmed of epicardial adipose tissue and vasculature and stored at –70° C for subsequent assays. Sections were also placed in formalin and embedded in paraffin for treatment with 0.1% Picro-Sirius red F_3Ba (20). Photographs obtained during microscopy were used to calculate the collagen volume fraction of the myocardial interstitium, as previously described (4).

Advanced glycosylation. To determine collagen-linked fluorescence as a measure of advanced glycosylation products, samples of left ventricular myocardium were minced, delipidated, and washed three times in phosphate-buffered saline (21). The tissue was subjected to sequential extraction steps. The initial extraction used 2 ml of 0.5 mol/l acetic acid overnight at 4° C. The precipitate was extracted in 1% pepsin in 0.5 N acetic acid for 24 h at 4° C. After two repetitions, the samples were centrifuged and the supernatant saved for subsequent analysis. The undigested material was treated with a 1% proteinase K solution in 0.1% sodium dodecyl sulfate at 37° C overnight with shaking. Next, 500 µl of 5% collagenase VII (Sigma, St. Louis, MO) in phosphate buffer solution was added to this precipitate; 1 µl each of chloroform and toluene were added to this and a blank tube containing collagenase. After incubation at 37° C for 24 h, samples were centrifuged for 3 min in a microfuge. A portion was acid hydrolyzed with 6 N HCl at 110° C for 24 h to quantitate hydroxyproline content (22). Fluorescence measurements were taken at an excitation wavelength of 370 nm and an emission wavelength of 430 nm with a Perkin-Elmer spectrophotofluorometer (Norwalk, CT).

Statistics. Data are expressed as means ± SE. When only one statistical comparison was used between a control and intervention, Student's t test for paired data was used; the unpaired t test was determined when comparing two groups. Analysis of variance was performed using Duncan's multiple range test when P values were significant at an alpha level of <0.05.

RESULTS

A significant rise in HbA1c and fasting plasma glucose compared with baseline values was observed in the three glucose-intolerant groups (groups 2–4), whereas group 1 controls exhibited no changes over time. These parameters in the two intervention groups (groups 3 and 4) did not significantly differ from the untreated diabetics, and there was no change of body weight in any group (Table 1). A hemodynamic study in the basal state revealed that heart rate, arterial pressure, and ejection fraction for groups 2–4 were comparable with that of group 1 controls (Table 2). Left ventricular end diastolic pressure was increased only in the untreated diabetic animals of group 2, associated with a reduced end-diastolic volume. Calculation of left ventricular chamber stiffness in the basal state indicated a higher level for group 2 animals compared with normals and the treatment groups (Table 2).

During the systemic infusion of dextran, heart rate and arterial pressure responses in the experimental groups were similar to group 1 and an ejection fraction increase was observed in all but the untreated diabetic animals (group 2) (Table 2). When compared with baseline, the end-diastolic pressure increased to a greater extent in group 2 than in the treated diabetic animals of groups 3 and 4. The end-diastolic volume increase was largest in the control (group 1) and aminoguanidine (group 4) groups. Although groups 2 and 3 had similar end-diastolic volumes during infusion, the former had a significantly higher distending pressure in diastole.

During dextran infusion, diastolic stiffness increased most in group 2 and was significantly less in group 3. Each of the IGT dogs treated with aminoguanidine exhibited a basal and post-dextran chamber stiffness approximating that of normal controls. There was no difference in left ventricular weight between the groups (Table 3).

Collagen-linked advanced glycosylation end products (AGEs) isolated from left ventricle were significantly greater in group 2 than in group 1 (Table 3). We found that <10% of

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TABLE 2
Left ventricular hemodynamics

Group	Heart rate per minute	Systolic arterial pressure (mmHg)	Ejection fraction (%)	Left ventricular end diastolic Pressure (mmHg)	Left ventricular end diastolic Volume (ml/m^2)	Chamber stiffness (mmHg · M^{-2} · ml^{-1})
Basal state						
1	106 ± 6.1	150 ± 3.9	48.8 ± 3.6	6.2 ± 0.6	67.4 ± 4.1	20.9 ± 4.8
2	109 ± 8.6	149 ± 8.8	51.5 ± 5.6	10.1 ± 0.9*	55.6 ± 4.6*	46.9 ± 6.4*
3	118 ± 9.9	143 ± 4.9	44.7 ± 7.4	6.8 ± 0.6	50.3 ± 4.9*	35.7 ± 5.1*
4	113 ± 7.6	146 ± 9.1	47.0 ± 8.3	5.9 ± 1.1	62.5 ± 6.2	24.6 ± 5.9
Response to dextran infusion						
1	110 ± 6.9	158 ± 9.2	61.8 ± 2.1	10.1 ± 0.9	87.7 ± 5.2	36.0 ± 3.5
2	111 ± 8.0	151 ± 9.3	53.8 ± 11.1	21.3 ± 1.2†	61.8 ± 5.9†	132.9 ± 15.8†
3	122 ± 11.5	137 ± 6.9	62.3 ± 19.1	14.0 ± 0.7‡	65.0 ± 3.8	72.3 ± 10.1‡
4	117 ± 10.7	148 ± 11.4	58 ± 14	8.8 ± 1.7‡	78.4 ± 6.9‡	43.5 ± 6.1‡

Data are means ± SE. *Basal end-diastolic pressure was increased in group 2 vs. group 1 ($P < 0.05$); there was no difference in groups 4 vs. 1; in groups 2 and 3, end-diastolic volume was reduced ($P < 0.05$) and chamber stiffness was enhanced ($P < 0.05$). After dextran infusion, †abnormalities in group 2 were significantly greater vs. group 1 ($P < 0.05$); ‡in group 3, diastolic pressure and chamber stiffness were less than in group 2 ($P < 0.05$); group 4 did not differ from group 1, with each parameter significantly improved vs. group 2 ($P < 0.05$).

the fluorescent material was present in the precollagenase fractions and did not differ between normal controls and diabetic animals. Treatment with aminoguanidine or enalapril prevented the increment observed in the untreated animals of group 2.

Total collagen concentrations were increased in groups 2–4 above those of group 1, with no intergroup differences among the IGT groups (Table 3). Histochemical staining of myocardium from a control animal (Fig. 1*A*) and a group 2 animal (Fig. 1*B*) demonstrates an interstitial distribution of the collagen increment. The collagen volume fraction determined with an automatic image analyzer was calculated as 2.7 ± 0.3% in controls and 5.4 ± 0.4% in group 2.

DISCUSSION

Hyperglycemia associated with diabetes is known to be associated with an increased risk of cardiovascular disease, including heart failure, without significant coronary disease (23–25). The concept of a graded relationship between glucose and cardiovascular events in the nondiabetic range has been advanced (1), but the individual tissue responses, in contrast to frank diabetes, remain relatively undefined.

An increase of left ventricular diastolic stiffness without systolic dysfunction characterized this canine model of IGT compared with normal controls. Diastolic dysfunction was associated with an increased concentration of collagen in myocardium in the absence of left ventricular hypertrophy. On morphological study, interstitial fibrosis was observed in diabetic animals without evidence of replacement fibrosis. Although a change in collagen phenotypes may occur during cardiac remodeling, a disproportionate increase of type I collagen, which may enhance stiffness, compared with types III and V has not been found in the myocardium of the glucose-intolerant model (26). Prevention of the cross-link abnormality as well as the stiffness increment by aminoguanidine, despite unchanged collagen concentrations, suggests that this property of collagen is a basis for altered cardiac function in the presence of glucose intolerance.

TABLE 3
Influence of glucose intolerance on myocardial collagen

Group	Collagen Concentration (μg/mg dry wt)	Collagen AGE (FU/mg collagen)	Left ventricular wt (g/kg)
1	2.4 ± 0.4	6.9 ± 0.7	4.5 ± 0.3
2	3.9 ± 0.5*	10.6 ± 1.6*	4.4 ± 0.4
3	4.3 ± 0.7*	5.6 ± 0.9	4.8 ± 0.6
4	4.1 ± 0.6*	5.4 ± 0.8	4.7 ± 0.9

Data are means ± SE. *Collagen-linked AGEs in fluorescent units (FU) were significantly increased in group 2 vs. group 1 ($P < 0.05$). Levels were normal in groups 3 and 4. Collagen concentrations were increased above those of group 1 in all three post-alloxan groups ($P < 0.05$).

Advanced glycosylation of collagen observed during IGT was presumed to provide cross-links that conferred enhanced stiffness to protein in group 2 animals. Although characterized by fluorescence of collagen, an increase of nonfluorescent glycosylated products may be elicited (27). Aminoguanidine has been used to modify the glucose-derived cross-link formation in diabetes without affecting enzymatically derived collagen cross-links (28). The equivalent effects of the ACE inhibitor intervention in terms of normalizing collagen-linked fluorescence in group 3, whereas only aminoguanidine prevented the increased diastolic stiffness of myocardium in group 4, suggests that the latter also acted on the nonfluorescent cross-links that appear to be present in the tissue of untreated diabetic subjects (27). The associated normal levels of chamber stiffness in the aminoguanidine group supports the view that collagen cross-links in the untreated diabetic animals were sufficient to reduce diastolic compliance (2).

That AGEs can directly alter the physical and structural properties of extracellular matrix by inducing collagen cross-linking has been well established (29). Whether their production and disposition is affected by the receptors in the cell membranes of neighboring cells in myocardium has not been ascertained.

Although arterial pressure was not measured in the awake state, the levels in the experimental groups were not significantly different from those of the control group. The small ele-

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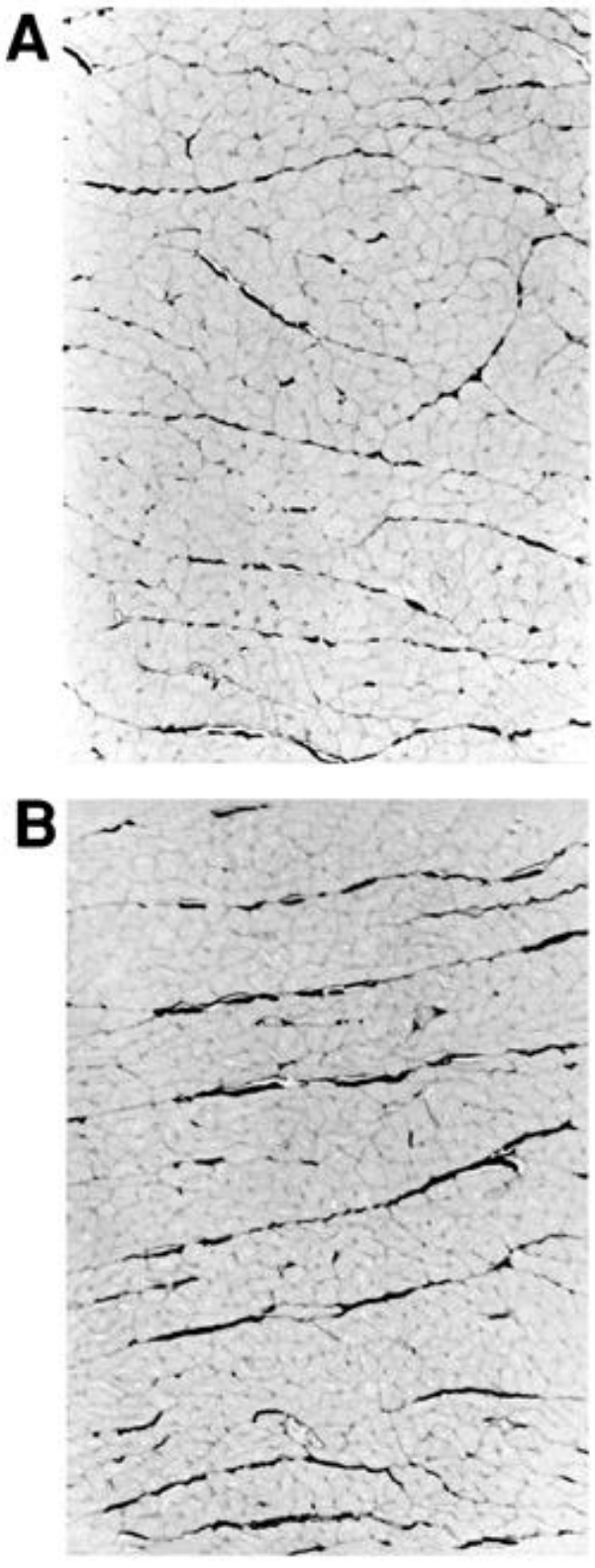

FIG. 1. *A*: Sirius red stain of left ventricular myocardium for interstitial collagen is depicted in a normal control animal representative of group 1. Original magnification ×40. *B*: Sirius red stain in a glucose-intolerant dog of group 2, 6 months after alloxan administration, indicates greater accumulation of interstitial collagen with no evidence of replacement fibrosis. Original magnification ×40. The average collagen volume fraction for the group was increased approximately twofold above the level in control animals. Some heterogeneity of myocyte size is present compared with *A*.

vation of arterial pressure in the normal animals is consistent with an effect of anesthesia. Pressure was not higher in the untreated diabetic animals, suggesting that chronic hypertension was not present at this stage of mild diabetes of limited duration. The absence of left ventricular hypertrophy in the experimental groups supports this view. Levels in group 3 were slightly but not significantly lower than for untreated diabetic animals, consistent with the response to ACE inhibitors in normotensive subjects. A previous study of enalapril in normotensive conscious dogs after myocardial infarction showed no reduction of arterial pressure at dosages that improved left ventricle function (30).

The minimal effect of ACE inhibitors in normalizing chamber stiffness in the basal state is analogous to observations after chronic treatment with β-aminoproprionitrile to reduce collagen cross-links (31). Diminished myocardial stiffness was detected only after a volume challenge. Similarly, after administration of ACE inhibitors in group 3, the infusion of dextran increased end-diastolic volume at a lower distending pressure and the calculated chamber stiffness was significantly less than in the untreated diabetic animals. The mechanism of ACE inhibitor action may be related to the nitric oxide (NO) system, which has been found to modulate collagen levels (32). Production of NO by the heart has been found to be reduced in diabetes (33). Because ACE inhibition promotes NO accumulation in myocardium (34), it is noteworthy that increased NO production in diabetic renal tissue (35), which has been postulated to diminish accumulation of AGEs may similarly affect heart muscle. In contrast, aminoguanidine is known to quench the response to endogenous NO (36). If this occurs in the background of reduced NO formation in the diabetic heart (33), the therapeutic mechanism would appear to differ from that of ACE inhibition.

The persistence of collagen accumulation after pharmacological intervention in the two treatment groups suggests that concentration increases were unrelated to advanced glycosylation. Improved diastolic function occurred despite increased myocardial collagen concentrations. Of interest is the observation that diminished collagenase gene expression in the diabetic kidney may be a basis for collagen accumulation (37); this has recently been observed in myocardium (38).

This study, if applicable clinically, suggests that patients who have chronic IGT may have myocardial dysfunction, which may contribute to the enhanced mortality reported by Gerstein (1). Moreover, when IGT coexists with hypertension, a greater degree of left ventricular diastolic stiffness may be observed compared with hypertensive patients with normal tolerance (39,13).

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