THE SEVERITY OF DIABETES IS A MAJOR DETERMINANT OF MYOCARDIAL DAMAGE IN THE RAT

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Abstract: The severity of insulin-dependent diabetes mellitus had a marked effect upon the development of myocardial sequelae in the rat. Even with the same degree of hyperglycemia, glycosuria, polydipsia, and polyuria, moderately diabetic animals did not develop the degenerative ultrastructural changes seen in myocardium from more severely diabetic rats. These included decreased cardiocyte size, loss and disorganization of myofibrils, and loss of sarcoplasmic reticulum and transverse tubules. Since hyperglycemia and glycosuria are frequently used as the primary, and often sole, criteria for identifying diabetes in experimental animals, this study demonstrates the need to more specifically define the severity of the disease in studies of the heart.

Introduction

Heart disease remains one of the most serious complications of diabetes mellitus, even with good glycemic control by insulin replacement. Although coronary atherosclerosis plays a major role, a cardiomyopathy which does not have a macrovascular etiology is also a significant contributor (1-3).

We have recently observed that severe insulin dependent diabetes produces a progressive pattern of ultrastructural changes in rat myocardium, with loss and disorganization of cardiocyte contractile elements, deposition of extracellular matrix, and thickening of both the capillary endothelium and its basement membrane (4). These changes occurred in the absence of coronary artery disease and were almost entirely reversible with insulin treatment. Similar nonvascular effects of diabetes on myocardial ultrastructure and composition have been well described in many animal species (5-8) and linked with contractile (5,6,8-10) and biochemical (11-13) pathology.

Unfortunately, cardiomyopathy studies are often difficult to interpret because of differences in diabetic models. Reports of diabetic effects on myocyte ultrastructure, for example, range from no effect (14) to myocytolysis and contraction bands (5). The length of diabetes before changes are observed varies widely, as do the types of changes seen. Severity of diabetes is often not defined, typically being limited to measurements of blood glucose and glycosuria which are subject to daily fluctuations.

Our hypothesis for this diversity in reported observations is that the severity of diabetes has a more specific effect than has been previously recognized on the type, sequence, and severity of the complications which subsequently develop. The observations reported here support this hypothesis for structural alterations and agree with reports that contractile dysfunction can be corrected by injection of insulin at doses which do not correct the hyperglycemia (15,16).

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Materials and Methods

Experimental Animals. Diabetes was induced in male Sprague-Dawley rats at 150-180 g by a single injection of 55 mg/kg alloxan (Sigma, St. Louis MO) into a tail vein (4,17); control rats of the same weight and age were given equivalent volumes of sterile saline. Nonfasting plasma glucose was measured 3 days later by spectrophotometric assay. Alloxan-injected rats with values above 17 mM (300 mg/dl) were classified as hyperglycemic (4,17); any animals with values below this were excluded from the study. Saline-injected rats had values below 7.8 mM (140 mg/dl). Glucose concentrations were measured monthly to ensure that hyperglycemia in the alloxan-injected animals and euglycemia in the saline-injected animals were maintained. Rats were weighed weekly and urine glucose and ketones were estimated by dipstick.

Although most of the alloxan-injected animals developed a severe, stable diabetes as discussed in Results, a limited number instead exhibited the same degree of hyperglycemia and glycosuria but developed few of the other symptoms characteristic of this diabetic model. In contrast to the more severely diabetic rats, these moderately diabetic animals initially gained weight at the same pace as nondiabetic rats, retained more subcutaneous fat, and exhibited only sporadic ketonuria. They were identified 2 to 4 weeks after injection and assigned to the appropriate experimental groups. Moderately diabetic (MD), severely diabetic (SD), and nondiabetic control (NC) rats were exposed to identical experimental protocols thereafter.

Sacrifice and Tissue Collection. MD, SD, and NC rats were sacrificed 6, 12, or 26 weeks after induction of diabetes. Under full pentobarbital anesthesia (50-65 mg/kg IP) the heart was rapidly excised, cleared of blood, and fixed by vascular perfusion as previously detailed (4). Coronary perfusion in all cases began within one minute of thoracotomy.

Microscopy. The anterior papillary muscle of the left ventricle, widely used in studies of myocardial structure, function and biochemistry, was excised from each heart and prepared for light and electron microscopic study. Proximal, distal, and

intermediate regions of each papillary muscle were examined in both longitudinal and transverse section to ensure that results were representative of the entire muscle (4,18). After routine osmium postfixation, dehydration, and embedding in Spurr (4), thick sections (1 μm) were cut, stained with toluidine blue, and examined by light microscopy. Thin sections (70 nm) were cut, stained with lead citrate and uranyl acetate, and examined with a JEOL 100s electron microscope.

Morphometry. Cross-sectional areas of myocytes were measured at a magnification of 1,000x through a microscope equipped with a sidearm lens which superimposed the images of the myocardium and the planimeter cursor. To ensure that measurements were made near the midregion of each cylindrical cell, only profiles which contained a nucleus were included (18). For each animal, at least 50 cells from each of two transversely sectioned blocks of tissue were measured (18,19).

Statistics. Plasma glucose levels, body and heart weights, and myocyte cross-sectional areas at each diabetic interval were compared by one-way analysis of variance, followed by a Newman-Keuls test when a significant difference was indicated.

Results

More than 90% of the alloxan-injected animals developed marked hyperglycemia, glycosuria, polydipsia, and polyuria within 3 days. None of them later reverted to euglycemia, normal drinking, or absence of glycosuria. Approximately 14% entered diabetic coma and were euthanized, 75% were SD by the criteria discussed above, 8% were MD, and 3% could not be assigned to either group.

As illustrated in Table I, SD animals gained little weight while MD rats grew at a rate similar to that of the NC animals, even though the degree of hyperglycemia was the same. Differences in the body weights of NC and MD rats did not become evident until 26 weeks. Starvation was not a factor in the failure of SD rats to gain weight, as these animals in fact increase their food intake (17).

Both groups of diabetic rats consistently had urine glucose values above the dipstick maximum of 1 g/dl. SD animals also consistently had ketone levels above the dipstick maximum of 250 mg/dl, but this was more variable in MD rats which often showed little or no ketonuria. Neither glucose nor ketones were present in urine of NC animals. Subcutaneous fat was strikingly decreased in SD but retained in MD rats. All rats in both groups developed cataracts, and polydipsia and polyuria were equally prominent.

Heart weights of SD animals were significantly lower than those in the NC group at all intervals, while those of the MD rats kept pace through 12 weeks of diabetes but lagged thereafter (Table I). After 26 weeks, heart weights for this group were intermediate between those of NC and SD animals, indicating that the effect of diabetes was finally becoming evident. The ratio of heart weight to body weight increased in SD rats, reflecting the greater loss of body weight.

Myocytes of the SD animals were significantly smaller than those of the other two groups after 6 weeks (Fig. 1), and the difference increased as the diabetes progressed. After 26 weeks, the mean cross-sectional area of cells from these rats was approximately 75% that of the NC rats. Through 26 weeks, the mean myocyte cross-sectional area of MD animals did not differ from that of the NC group. Since altered cross-sectional area but not length occurs in hypertrophy from both pressure (20,21) and volume (20) overloading of the heart, this is a reliable index of cardiocyte size.

The SD rats in this study exhibited extensively altered myocardial ultrastructure, which we have previously described in detail (4) and will not repeat here. Briefly, loss and disorganization of myofibrils, transverse tubules, and sarcoplasmic reticulum occurred within 6 weeks, as did deposition of extracellular matrix. These occurred in a focal and progressive pattern, with both larger regions of myocardium affected and more damage per cell as diabetes continued. More than 60% of the myocytes showed structural damage after 26 weeks of diabetes, grouped in areas which also showed deposition of matrix and capillary changes. After 12 and 26 weeks, the additional matrix was quite prominent. Capillary ultrastructure was unchanged

Table I. Experimental Animals

Experimental group	(*n*)	Duration (days)	Sacrifice weight (g)	Plasma glucose (m*M*)	Heart weight (g)	Heart weight (g)/ body weight (100 g)
6-week Severely diabetic	5	42.0 ± 0.7	201.4 ± 12.8[a,b]	25.82 ± 3.70[b]	0.59 ± 0.03[a,b]	0.29 ± 0.01[a]
6-week Moderately diabetic	4	42.0 ± 0.8	353.8 ± 20.0	25.27 ± 4.45[c]	1.00 ± 0.06	0.28 ± 0.03
6-week Normal control	4	40.5 ± 0.6	412.5 ± 47.0	5.88 ± 0.52	1.02 ± 0.08	0.25 ± 0.01
12-week Severely diabetic	4	85.5 ± 2.5	208.3 ± 27.8[a,b]	23.30 ± 4.62[b]	0.63 ± 0.10[a,b]	0.30 ± 0.02[a,b]
12-week Moderately diabetic	4	89.3 ± 3.9	458.5 ± 12.8	25.53 ± 1.93[c]	1.13 ± 0.04	0.25 ± 0.01
12-week Normal control	4	83.0 ± 0.0	494.8 ± 51.9	5.56 ± 0.32	1.14 ± 0.10	0.23 ± 0.01
26-week Severely diabetic	3	181.0 ± 0.0	186.7 ± 15.0[a,b]	28.83 ± 1.55[b]	0.83 ± 0.16[b]	0.43 ± 0.07[a,b]
26-week Moderately diabetic	5	183.4 ± 0.9	382.2 ± 20.1[c]	25.38 ± 3.74[c]	0.98 ± 0.06[c]	0.26 ± 0.02
26-week Normal control	5	187.8 ± 6.6	575.0 ± 154.1	5.54 ± 0.30	1.15 ± 0.05	0.23 ± 0.03

Note. All values are presented as $\bar{X}$ ± SD.

[a] Significant difference between severely diabetic and moderately diabetic groups, $P \leq 0.05$ by Newman-Keuls test following one-way analysis of variance.

[b] Significant difference between severely diabetic and normal control groups, $P \leq 0.05$ by Newman-Keuls test following one-way analysis of variance.

[c] Significant difference between moderately diabetic and normal control groups, $P \leq 0.05$ by Newman-Keuls test following one-way analysis of variance.

during the first 6 weeks, but after 12 weeks endothelial hypertrophy with an increased number of vesicles was noted in affected areas. Thickening or partial reduplication of the basement membranes of these vessels was also noted. The effects of diabetes on myocardial structure were reversed by insulin replacement, confirming that they were the result of insulin deficiency rather than a cardiotoxic effect of the alloxan.

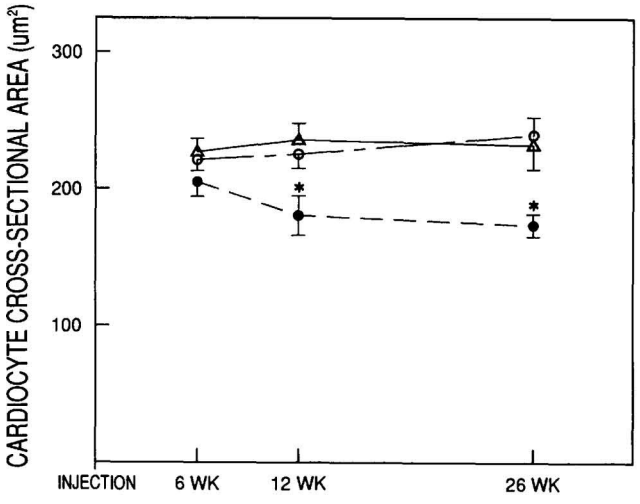

Figure 1. Cardiocyte cross-sectional areas ($\bar{X} \pm SD$) during the progression of moderate (○) or severe (●) forms of diabetes mellitus and in nondiabetic control animals (△). At least 50 cells from each of two regions of the left ventricular papillary muscle were measured by direct planimetry for each animal; only profiles containing a nucleus were included to ensure that cells were measured near their centers. Values from severely diabetic rats were significantly ($* = P \leq 0.01$) smaller than those from either moderately diabetic or nondiabetic animals after 12 and 26 weeks, but no differences were observed between the latter two groups.

Hearts of the MD animals, in contrast, showed comparatively minor structural damage (Fig. 2). No changes occurred during the first 12 weeks of diabetes. After 26 weeks, areas of myofibrillar misalignment were evident in the hearts of most rats, but the loss of myofibrils and other organelles observed in SD animals was seen in only two of the MD rats. One of these animals also showed endothelial hypertrophy and extracellular matrix deposition similar to that observed earlier in the severely diabetic group.

Discussion

Diabetic rats in both the SD and the MD groups exhibited the classic symptoms of hyperglycemia, glycosuria, polydipsia, and polyuria to the same degree. However, differences in weight gain, adipose tissue, and ketonuria confirmed that these two groups represented different regions on the spectrum of diabetic severities, and the effects on myocardial structure were markedly dissimilar. In contrast to more severe diabetes, the moderate expression of the disease had little or no effect on heart weight, myocyte size and ultrastructure, extracellular matrix, or capillary structure.

This is supported by the observations of Regan and coworkers (8,14) that only minor histologic changes and no ultrastructural damage occurred in the hearts of dogs which were intentionally made mildly diabetic by injecting low doses of alloxan. Rubenstein et al. (15) found in the streptozotocin diabetic rat that end-diastolic pressure, rate of pressure development, and peak systolic pressure were normalized by doses of insulin insufficient to correct the hyperglycemia, while other indices

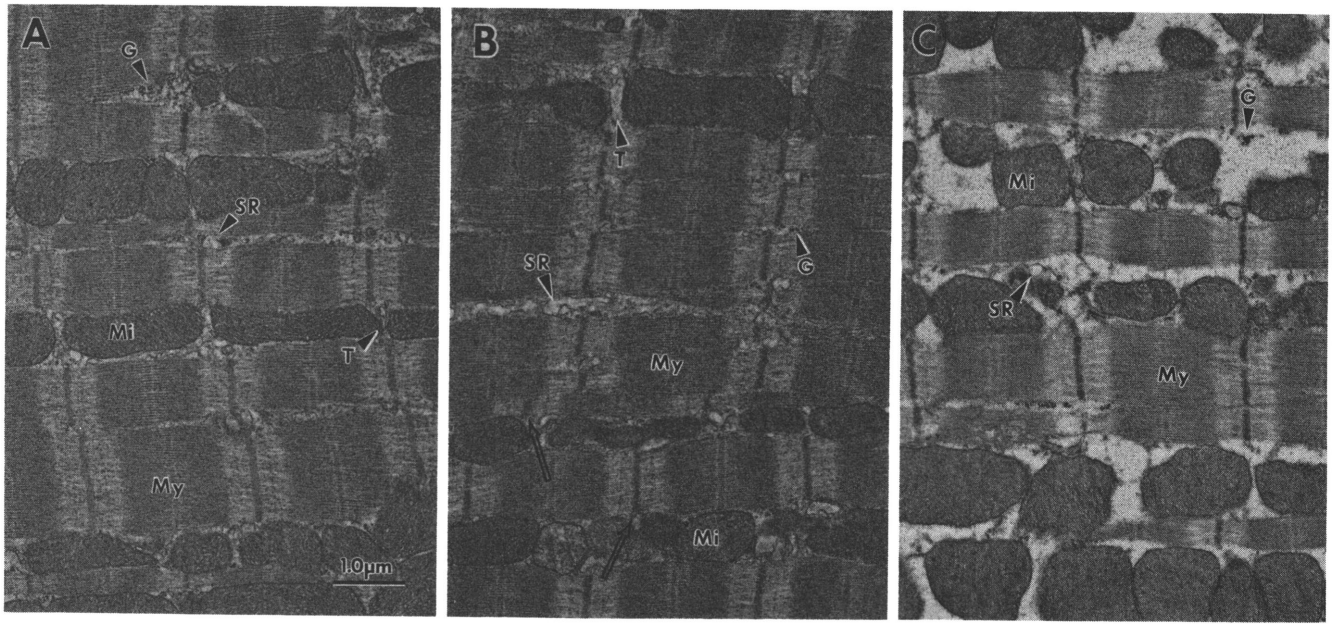

Figure 2. Electron micrographs of cardiac myocytes from nondiabetic (A), moderately diabetic (B), and severely diabetic (C) rats after 26 weeks of untreated diabetes. Normal cytoarchitecture has been maintained in cells from moderately diabetic animals except for a modest degree of myofibrillar misalignment (Z-lines indicated by open arrows), in contrast to the loss and disorganization of myofibrils (My), sarcoplasmic reticulum (SR), and transverse tubules (T) in cells from severely diabetic rats. Mitochondria (Mi) and glycogen (G) were not affected by diabetes. Myocardial structural damage in severe diabetes is presented in detail in reference 4.

of contractile function exhibited dose-dependent responses.

Rodrigues et al. (16) similarly observed that the hearts of spontaneously diabetic BB rats given small doses of insulin, which are necessary for survival but do not normalize blood glucose, had the same rate and degree of force development and relaxation as both nondiabetic rats and diabetic rats given enough insulin to maintain euglycemia. They concluded that persistent hyperglycemia alone is not sufficient to cause myocardial dysfunction and that other factors must be contributing to the depression of heart function during diabetes.

Using the same model, Malhotra et al. observed that moderating the severity of diabetes by daily injection of a small amount of insulin markedly delayed the appearance of myosin isoform changes and decreased activity of calcium-stimulated, but not actin-activated, myosin ATPase. Of particular interest, the diabetic animals in their study were very similar to the MD rats in ours.

We conclude that the severity of diabetes has an important effect on myocardial structure and must be considered when designing or interpreting studies of diabetic heart disease. Hyperglycemia and glycosuria are freqently cited as the primary, or even sole, criteria of diabetes in experimental or clinical investigations. While it undoubtedly plays a central role in many diabetic complications, this study demonstrates that hyperglycemia per se is not always sufficient to produce them.

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