Correlation between Metabolic and Histopathological Changes in the Myocardium of the KK Mouse
Effect of Diltiazem on the Diabetic Heart

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SUMMARY:
Serial changes in myocardial parameters related to non-insulin-dependent diabetes mellitus were investigated in DDY mice (control), untreated KK mice, and KK mice treated with diltiazem (150 µg/kg body weight, KKd1; or 300 µg/kg body weight diltiazem, KKd2). The isozyme ratio of lactate dehydrogenase (LDH) $(LDH_1+LDH_2)/(LDH_4+LDH_5)$ was used as an index of aerobic metabolism of myocardial tissue. Mean blood sugar levels did not vary between 5 weeks and 30 weeks of age, ranging from 108 (range 60-198) mg/dl in DDY mice to 170 (range 110-282) mg/dl in KK mice. The ratio of heart weight to body weight was larger in KK mice than in DDY mice at 20 weeks of age, but was unaffected by diltiazem treatment. The LDH isozyme ratio showed that DDY mice were in an aerobic state at 15 and 20 weeks of age, while KK mice were in an anaerobic state at 10 and 15 weeks of age. The KKd1 and KKd2 groups exhibited the same LDH isozyme ratios as untreated KK mice; diltiazem had no effect on the LDH isozyme ratio at 20 and 30 weeks of age. The mean diameter of myocytes was increased in KK mice but diltiazem had no effect on this parameter. Interstitial fibrotic changes appeared at 15 weeks in untreated KK mice and progressed with age. These changes were completely suppressed in KK mice treated with diltiazem. These results suggest that hyperglycemia induces an anaerobic state in heart muscle, leading to muscle hypertrophy, degeneration, and fibrosis and that calcium antagonists may suppress these pathological changes. (Jpn Heart J 34: 617-626, 1993)

Key Words:
Lactate dehydrogenase composition Interstitial fibrosis Calcium channel blocker Diabetic cardiomyopathy

Macroangiopathy such as coronary arteriosclerosis and myocardial injury not attributable to hypertension are often associated with diabetes mellitus. Congestive heart failure in diabetes1) is caused by a reduction in myocardial contractility and an increase in chamber stiffness.2,3) The metabolic changes
that occur in myocardial cells typically involve altered energy production related to reduced insulin activity, such as a reduction in ATPase activity, which can affect myocardial contractility. Histological changes include a narrowing of the small vessels of the myocardium and a thickening of vessel walls caused by endothelial cell proliferation, an accumulation of mucopolysaccharides, hypertrophy of myocytes, and fibrosis of the myocardial interstitium. These changes are classified as diabetic cardiomyopathy. Although metabolic changes and tissue damage in diabetic cardiomyopathy have been examined, few studies have quantitatively examined the natural course of such myocardial lesions in insulin-dependent and non-insulin-dependent diabetes mellitus (NIDDM, hyperglycemic hyperinsulinemic diabetes mellitus).

KK mice, developed from Japanese native mice in 1957, offer an appropriate model of NIDDM, because, in addition to obesity, polyphagia, polyposia, glucosuria, abnormal glucose tolerance, and hyperinsulinemia, they also develop such histological changes as hypertrophy and hyperplasia of Langerhans’ islands, hypertrophy and degranulation of B cells, increases in ribosomes and endoplasmic reticulum in B cells, and enlargement of the Golgi zone. Myocardial lesions, such as calcification of the myocardium, degeneration of the myocardium and fibrosis of the myocardial interstitium, have also been observed in KK mice. Since these lesions may reflect excess intracellular calcium, the purpose of this study was to evaluate both the natural course of metabolic and histopathological changes in KK mice and the ability of diltiazem, a calcium antagonist, to arrest these changes.

**Materials and Methods**

**Materials**

Male DDY mice (Shizuoka Experimental Animal Center, Hamamatsu, Japan), received daily ip injections of physiologic saline and served as the control group (DDY group). Male KK mice (Clea Japan, Tokyo, Japan), which served as a model of NIDDM, were divided into 3 groups. The KKS group received a daily ip injection of physiologic saline (10 ml/kg body weight/day). The KKd1 group received a daily ip injection of 150 μg/kg body weight of diltiazem, while the KKd2 group received a daily ip injection of 300 μg/kg body weight of diltiazem.

**Methods**

Myocardial parameters (see below) were assessed at 5, 10, 15, 20, and 30 weeks. Necropsy examinations were also performed at the termination of the experiment.

**Age and body weight:** The animals were fed a conventional solid stock diet ad
libitum. Body weight was measured weekly from the age of 5 weeks.

**Blood sugar level:** Weekly blood samples were collected after cutting the tail. Blood glucose level was determined using both test paper (Glucosticks®) and a Glucostar M® measuring instrument (Miles-Sankyo Co. Ltd., Tokyo, Japan).

**Heart weight:** Animals were sacrificed by cervical dislocation at 5, 10, 15, 20, and 30 weeks. A thoracotomy was performed, and the heart was excised. The blood in the cardiac cavities was washed out with phosphate buffer (0.2M, pH 7.5, 0.2M KH₂PO₄, 0.2M K₂HPO₄, [P]), the buffer on the surface was wiped off with filter paper, and the total weight of the heart was determined.

**Myocardial protein content, LDH content, and LDH composition:** The procedures for the microdetermination of myocardial protein content, LDH content and LDH composition are described in a previous publication¹⁰. After the mice were sacrificed by cervical dislocation, part of the left ventricular free wall was removed, suspended in [P] 50 times the wet weight of the myocardial tissue, and homogenized in an ultradisperser (Yamato, Tokyo, Japan). The homogenate was centrifuged, and the supernatant [S] was used for measurements. The protein content was determined by adding 1 to 5 μl of [S] to 0.8 ml of distilled water. The absorbance at a wave length of 595 μm was measured with a Hitachi Model 200-20 Spectrophotometer (Hitachi, Ltd., Tokyo, Japan) after the addition of 0.2 ml Coomassie Brilliant Blue G250 (Bio-Rad, Richmond, USA). To determine LDH activity, 5 μl of [S] was applied to an LDH Monotest (Boehringer-Mannheim, Mannheim, Germany). The LDH composition was examined by cold agar gel plate electrophoresis (LDH isozyme kit, Wako Pure Chemicals, Osaka, Japan). Because aerobic metabolism is reflected by LDH₁ and LDH₂ levels, and anaerobic metabolism by LDH₃ and LDH₅ levels, the ratio of (LDH₁+LDH₂)/(LDH₃+LDH₅) was used as an index of the metabolic state.

**Myocardial tissue:** Part of the left ventricular free wall was fixed with formalin for histopathological examination and stained with hematoxylin-eosin. The diameter of 100 myocytes from each heart was measured at the level of the nucleus in vertical cross-sections of the cells. Fibrosis of the myocardial interstitium was determined quantitatively by the point counting method¹¹,¹² using a 10 μm square grid at a magnification of 400. At least 2,000 points were measured from each cross-section. The extent of fibrosis was expressed as the percent of the entire myocardial matrix.

**Statistical analysis**

The measured values are expressed as the mean ±SD and as the mean ±SEM. The difference between two values was assessed using analysis of varia-
tion (ANOVA) with a one-way layout, followed by Scheffe's multiple comparison. The difference was regarded as significant when $p$ was less than 0.05.

**RESULTS**

**Blood sugar level**

No differences (ANOVA) were observed among the DDY, KKS, KKD1 and KKD2 groups. However, the blood sugar levels were elevated from DDY control values in the KKS, KKD1, and KKD2 groups after 10 weeks of age (Table I).

<table>
<thead>
<tr>
<th>Age (weeks)</th>
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<th>10</th>
<th>15</th>
<th>20</th>
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<td>165±56</td>
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<td>165±37</td>
<td>145±14</td>
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Hwt/Bwt (%)

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<td>n=7</td>
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<td>KKS</td>
<td>4.01±0.46</td>
<td>4.22±0.50</td>
<td>4.10±0.24</td>
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<td>4.94±0.17</td>
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$t$-LDH/t-Prot (IU/mg)

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<th>10</th>
<th>15</th>
<th>20</th>
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<td>DDY</td>
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<td>KKS</td>
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<td>2.41±0.57</td>
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<td>2.23±0.31</td>
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<td>n=7</td>
<td>n=10</td>
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<tr>
<td>KKD1</td>
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<td>KKD2</td>
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<td>2.37±0.53</td>
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BS=blood sugar; Hwt/Bwt=mouse heart weight per mouse body weight; $t$-LDH/t-Prot=total lactate dehydrogenase activity in the whole heart per total weight of the protein in the whole heart; DDY=DDY group (10 ml/kg body weight saline daily i.p.); KKS=KKS group (10 ml/kg body weight saline daily i.p.); KKD1=KKD1 group (150 μg/kg body weight diltiazem daily i.p.); KKD2=KKD2 group (300 μg/kg body weight diltiazem daily i.p.). The values are mean ±SD. * $p<0.05$, ** $p<0.01$ and *** $p<0.001$. 
Heart weight/body weight ratio

The heart weight/body weight ratio was increased in the KKSs group at 20 weeks compared with DDY group (p<0.05). However, the ratios from the KKSs, KKD1 and KKD2 groups did not differ significantly (Table I). Thus, diltiazem treatment did not alter the heart weight/body weight ratio.

Total LDH/total protein ratio in the myocardium

In the DDY group, the ratio of the total LDH content to the total protein content increased gradually until 20 weeks of age but decreased slightly at 30 weeks of age. However, the ratios did not change significantly after 15 weeks of age. The total LDH/total protein ratio was slightly higher in the KKD2 group as compared with the KKSs group at all ages: the difference was significant between 15 and 30 weeks (Table I).

LDH composition

In the DDY group, the (LDH1+LDH2)/(LDH4+LDH5) ratio was increased significantly from 5 weeks to 20 weeks of age (p<0.001), indicating increased aerobic metabolism (Fig. 1). In the KKSs group, this ratio was significantly lower than the DDY group at 10 weeks (p<0.05), 15 and 20 weeks (p<0.001), and 30 weeks (p<0.001).
weeks (p<0.01) compared with the DDY group, suggesting a shift to anaerobic metabolism in KK mice. The diltiazem-treated group (KKd2) differed significantly from the KKs control group at 15 weeks (n<0.001), but did not differ from the KKs group between 20 and 30 weeks. Thus, diltiazem may affect the metabolic status of the myocardium in KK mice at the early stage of treatment.

**Myocyte size and interstitial fibrosis**

The mean diameter of the myocytes increased with age in all groups (Fig. 2). In the DDY group, the diameter increased gradually between 5 and 30 weeks, with the diameter at 30 weeks significantly larger than the value at 5 weeks. However, the increase was sharper in KKs and KKd2 groups; the myocyte diameter was significantly larger at 10, 15, 20 and 30 weeks compared with 5 weeks (p<0.05). There was no significant difference in the diameters among the
DDY group, KKS group and KKD2 group at each age, suggesting that diltiazem did not prevent myocyte hypertrophy.

The percent fibrosis of the myocardial interstitium did not change in the DDY group throughout the observation period. By contrast, fibrosis increased significantly in the KKS group at 15 weeks (p<0.05), and continued to increase thereafter with age (p<0.05 at 20 weeks, p<0.001 at 30 weeks) (Fig. 2 and 3). This effect was blocked completely by diltiazem treatment. As shown in Figure 3, the percent fibrosis in the KKD2 group did not differ from the DDY group throughout the observation period. The fibrosis in the DDY group and the KKD2 group was significantly less than that in the KKS group at 20 weeks (p<0.01 in the DDY group, p<0.05 in the KKS group) and 30 weeks (p<0.05 in both groups).

**DISCUSSION**

This study quantitatively assessed the effects of diltiazem and histology of the myocardium in spontaneously hyperglycemic and hyperinsulinemic KK mice.
Abnormal myocardial anaerobic metabolism in diabetic cardiomyopathy is a consequence of deficient insulin activity. The expression and activity of LDH isozymes reflect these changes in myocardial metabolism. It is well known that decreased LDH1 levels and increased LDH5 levels are characteristic of ischemic cardiac muscle. LDH5 has been found to increase during anoxic metabolism in experimental dogs, and LDH4 and LDH5 have been found to increase in the hypertrophic heart in humans and guinea pigs. It is interesting to note the findings reported by Revis et al. that the (LDH1+LDH2)/(LDH4+LDH5) ratio is correlated with the degree of fibrosis in experimental cardiac hypertrophy. Moreover, the LDH1/LDH5 ratio in platelets decreases in diabetic patients. In the present paper, the LDH ratio ((LDH1+LDH2)/(LDH4+LDH5)) was similar in KK mice and DDY mice at 5 weeks of age. This ratio increased in the DDY controls with age, suggesting a shift to aerobic metabolism, while myocardial metabolism remained in an anaerobic state in KK mice. However, the LDH ratio did not predict fibrosis in my studies, since similar LDH ratios were found in both KKS and KKd2 groups.

Degeneration and necrosis of the myocytes, and fibrosis and calcification of the myocardial matrix have been observed in KK mice. The diameter of myocytes increased with age in all groups, and the increase was greater in KK mice than in DDY mice; the diameter of myocytes at 30 weeks was significantly greater in KK mice compared with DDY mice.

The pathogenesis of fibrosis in the heart of diabetic patients is unknown. Van Hoeven et al. examined the hearts obtained at autopsy with the microscopic grading of fibrosis and the histochemical determination of the amount of collagen per total collagenous protein in the hypertensive or diabetic hearts. In their study the amount of microscopic fibrosis was lowest in hypertensive hearts, midrange in diabetic hearts, and highest in hypertensive-diabetic hearts. Replacement fibrosis (an area of scar formation, scar that replaced viable muscle cells), perivascular fibrosis or interstitial fibrosis of diabetic hearts had also increased in the same order. Electron microscopic study of myocardial samples taken from alloxan-diabetic dogs also revealed a specific increase in the number of both collagen fibers and mitochondria, in addition to a widening of Z-bands. The present study also demonstrated long-term interstitial fibrosis of the diabetic heart. In DDY mice, there was no change in percent interstitial fibrosis until 30 weeks. In KK mice, the percent fibrosis was elevated significantly after 20 weeks. These findings suggest that the myocytes develop mild hypertrophy starting at 10 weeks of age, and that the metabolic cycle in myocytes becomes markedly anaerobic at 15 to 20 weeks. Marked fibrosis progresses after 15 weeks.

Although diltiazem has been reported to inhibit insulin secretion, it does not affect blood sugar control in humans. Diltiazem promotes aerobic myo-
cardial metabolism, presumably by increasing the oxygen supply and reducing oxygen demand through its actions as a calcium antagonist. Because the myocardial calcium content is markedly higher in the KK mouse than in the DDY mouse, calcium antagonists were expected to be effective in KK mice. Diltiazem had little effect on the blood sugar level, heart weight, or the total protein ratio in KK mice, and it produced only a modest, transient increase in the ratio of \((\text{LDH}_1+\text{LDH}_2)/(\text{LDH}_4+\text{LDH}_5)\) at 15 and 20 weeks of age. Diltiazem markedly suppressed the progression of fibrosis in KK mice after 15 weeks of age. These findings suggest that diltiazem has a strong preventive effect on myocardial degeneration and fibrosis. In this regard, it is important to note the study of Villee et al reports that the production of type III collagen increased in association with an increase in glucose concentration in cultured fibroblasts. Thus, it is possible that the beneficial effect of calcium antagonists on myocardium in KK mice may be due to direct inhibition of collagen production by fibroblasts, rather than prevention of myocardial cell degeneration. The results shown in this paper strongly suggest that calcium antagonists, such as diltiazem, are effective against cardiac lesions caused by NIDDM.

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**REFERENCES**


