Alteration of 1,2-Diacylglycerol Content in Myocardium From Diabetic Rats

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1,2-Diacylglycerol has been proposed to be a secondary messenger; therefore, in this study we evaluated the amount of 1,2-diacylglycerol in heart tissue from streptozocin-induced diabetic rats and examined the effect of insulin treatment on 1,2-diacylglycerol content. Diabetic rats had lower body and ventricular weights and higher ratios of ventricular to body weight, all of which shifted toward normal values after 4 wk of untreated diabetes followed by 4 wk of insulin treatment. The contents of major phospholipids were significantly depressed in the diabetic rat hearts. In contrast, the triglyceride and cholesterol contents in the myocardium were increased by streptozocin injection and completely normalized by insulin treatment, and glucose levels returned to normal. The 1,2-diacylglycerol content in the myocardium was also significantly elevated in the diabetic rats compared with age-matched controls. Moreover, the 1,2-diacylglycerol content was significantly higher in rats with 4 wk of diabetes than in those with 8 wk of diabetes. Insulin treatment in the diabetic rats, however, did not produce any decrease in 1,2-diacylglycerol content. The results of this study suggest that the development of cardiomyopathy induced by streptozocin injection is associated with a high 1,2-diacylglycerol level, which may result in the activation of protein kinase C. Insulin is one of the agonists that generates 1,2-diacylglycerol in myocytes; however, the relationship between the sustained 1,2-diacylglycerol level and the normalization of diabetes by insulin administration is unclear. *Diabetes* 37: 1168–72, 1988

Abnormalities of the myocardium independent of atherosclerotic coronary artery disease and hypertension have been suggested in experimental animals (1) and humans (2) with diabetes mellitus. Cardiomyopathy associated with diabetes is characterized by diminished left ventricular performance, e.g., diastolic compliance and shortening velocity (3), both of which can be improved by insulin treatment (4). In addition to physiological alterations, biochemical observations of altered myosin isoenzyme distribution (5) and decreased uptake of Ca^{2+} by sarcoplasmic reticulum have been demonstrated in diabetic animals (6).

It has recently been recognized that hormones eliciting intracellular mobilization of Ca^{2+} also cause phosphoinositide hydrolysis, resulting in an accumulation of 1,2-diacylglycerol in membranes (7,8). Changes in 1,2-diacylglycerol in the tissue are thought to have multiple metabolic roles. This lipid is a central intermediary metabolite in phospholipid and triglyceride synthesis and a potential source of arachidonate, the precursor of prostaglandins and eicosanoids. It also activates protein kinase C. Therefore, it has been proposed to be a secondary messenger in the intracellular signal transduction system (8), although 1,2-diacylglycerol may be generated from phospholipids other than phosphoinositides (9,10). Despite recent interest in the role of 1,2-diacylglycerol, difficulty in accurately estimating the amount of 1,2-diacylglycerol remains. We recently established a method using thin-layer chromatography and a flame ionization detector (TLC-FID) technique for the quantitation of this lipid (11).

The function and role of 1,2-diacylglycerol in the myocardium are still unknown. This study was undertaken in an attempt to evaluate the amount of 1,2-diacylglycerol in hearts from streptozocin (STZ)-induced diabetic rats and to examine the relationship between 1,2-diacylglycerol accumulation in the myocardium and insulin treatment in diabetic rats.

MATERIALS AND METHODS

Male Wistar rats weighing 200–230 g were used. Diabetes was induced by a single injection of STZ (65 mg/kg i.v.,

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dissolved in 0.05 M citrate buffer, pH 4.5) into the tail vein of animals lightly anesthetized with ether. Control animals from the initial group were injected with citrate buffer only. All rats had unrestricted access to food and water until they were killed. Diabetic animals were killed 4 and 8 wk after the STZ injection. Nondiabetic control animals were age-matched accordingly. The animals in the insulin-treated diabetic group received 3 U s.c. of protamine zinc insulin daily for 4 wk before being killed at 8 wk. Insulin was given at ~1700 each day. Plasma samples were collected at death and analyzed for glucose by the glucose oxidase method and for insulin by radioimmunoassay procedures.

Extraction and analysis of lipids. After the animals were anesthetized with ether, the hearts were rapidly excised and washed thoroughly with cold saline; two 60- to 80-mg tissue samples from the left ventricle near the apex were obtained, one piece for neutral lipid analysis and the other for phospholipid analysis. These pieces were frozen immediately in liquid N_2. After the two pieces from each ventricle and the remaining ventricle were weighed, the samples were placed in 5.5 ml of a chilled chloroform/methanol mixture (2:1 vol/vol) containing 0.01% butylated hydroxytoluene as an antioxidant and cholesteryl acetate (0.1 and 0.4 mg per tube for neutral lipid and phospholipid analysis, respectively) as an internal standard (12). The tissues were homogenized for 20 s with a motor-driven Potter-Elvehjem homogenizer maintained on ice, and the homogenate was then chilled on ice for 30 min and filtered through filter paper. The filtrate was evaporated to dryness under a stream of N_2 at 30°C.

To analyze the phospholipids, dried lipids were resuspended in 20 μl of chloroform/methanol (2:1) and applied to 75-μm silica gel–precoated thin-layer rods (Chromarods-SIII, Iatron, Tokyo) that were activated by being passed through the FID before use. A four-step development method, with some modifications as described by Innis and Clandinin (13), produced good separations of each of the phospholipids and cholesteryl acetate. The first and second developments of the chromatogram were carried out in a solvent system of chloroform/methanol/H_2O (50:25:2) until the front had migrated ~7 cm. The third and fourth developments were carried out with a dichloroethane/chloroform/acetic acid (46:6:0.05) solvent system until 9 cm and an *n*-hexane/diethyl ether/acetic acid (98:1:1) solvent system until 11 cm. After the rods were thoroughly dried in an oven at 50°C for 15 min, they were subjected to an Iatron TH-10 TLC analyzer under the following conditions: hydrogen flow rate 160 ml/min, scan speed 2.39 s/cm, and air flow rate 2000 ml/min. The peak areas were calculated with a potentiometric recorder (Chromatocorder 11, System Instrument, Tokyo). The phospholipids in the sample were determined by calculating the ratio of the area and the weight of the standard phospholipid. Each sample was analyzed with four Chromarods, and the results were averaged.

To separate the neutral lipids from phospholipids, we applied lipid extracts dissolved in 0.5 ml of chloroform to a 0.5-ml silicic acid column (minus 325 mesh from Bio-Rad, Richmond, CA) equilibrated with chloroform. Neutral lipids were eluted with 7 ml of chloroform, concentrated under a stream of N_2 at 30°C, and dissolved in 20 μl of chloroform. A three-step development method in the same direction was also carried out for neutral lipid quantitation with the TLC-FID technique (11). Chromarods were spotted with 1.5 μl of samples and developed twice in a solvent system containing 1,2-dichloroethane/chloroform/acetic acid (46:6:0.05) until the solvent front had migrated ~9 cm. The last development was stopped at ~11 cm in a solvent system of *n*-hexane/diethyl ether/acetic acid (98:1:1).

Cholesteryl acetate and butylated hydroxytoluene were purchased from Wako (Osaka, Japan). STZ and all other neutral lipid and phospholipid standards were purchased from Sigma (St. Louis, MO).

Statistical analysis. All data are presented as means ± SE. Lipid contents in the myocardium are expressed on a wet-tissue-weight basis. For data comparisons, a two-way analysis of variance (ANOVA) was carried out without the insulin-treated diabetic group. When the *F* value was significant, differences within the groups, including the insulin-treated diabetic group, were assessed with one-way ANOVA and then Duncan's multiple-range test or Student's *t* test for unpaired data. $P < .05$ was considered statistically significant.

RESULTS

General features of diabetic animals. Four and 8 wk after STZ injection, the diabetic animals exhibited significantly depressed body and ventricular weights. Elevated ratios of ventricular weight to body weight were observed in the 8-wk-diabetic rats but not in the 4-wk-diabetic rats (Table 1).

TABLE 1
Physical and biochemical variables in control, diabetic, and insulin-treated diabetic rats 4 and 8 wk after streptozocin injection

	4 wk		8 wk		
	Control	Diabetic	Control	Diabetic	Insulin-treated diabetic
n	9	7	10	8	7
Body weight (g)	431 ± 14	278 ± 15*	492 ± 2	309 ± 10*	390 ± 20*†
Ventricular weight (mg)	919 ± 21	624 ± 33*	1176 ± 60	882 ± 26*	1038 ± 36†‡
Ventricular weight/ body weight (mg/g)	2.14 ± 0.03	2.18 ± 0.07	2.36 ± 0.05	2.86 ± 0.03*	2.66 ± 0.07*§
Plasma glucose (mg/dl)	160 ± 7	629 ± 81*	183 ± 8	639 ± 48*	187 ± 22†
Plasma insulin (μU/ml)	17.1 ± 1.1	9.9 ± 2.2*	23.8 ± 2.5	10.1 ± 1.6‡	67.9 ± 7.7*†

Values are means ± SE.
*$P < .01$ and ‡$P < .05$ vs. control rats.
†$P < .01$ and §$P < .05$ vs. age-matched diabetic rats.

TABLE 2
Myocardial phospholipid contents in control, diabetic, and insulin-treated diabetic rats 4 and 8 wk after streptozocin injection

	4 wk		8 wk		
	Control	Diabetic	Control	Diabetic	Insulin-treated diabetic
Cardiolipin	3.90 ± 0.39	2.89 ± 0.16*	3.57 ± 0.13	2.97 ± 0.21	3.47 ± 0.34
Phosphatidylethanolamine	8.69 ± 0.25	7.78 ± 0.38	9.08 ± 0.14	7.42 ± 0.31*	8.78 ± 0.79†
Phosphatidylcholine	10.49 ± 0.21	10.38 ± 0.30	10.30 ± 0.22	9.24 ± 0.23*‡	9.58 ± 0.72
Sphingomyelin	0.45 ± 0.03	0.52 ± 0.04	0.43 ± 0.01	0.56 ± 0.03*	0.49 ± 0.06
Lysophosphatidylcholine	0.07 ± 0.01	0.09 ± 0.01	0.09 ± 0.01	0.11 ± 0.06	0.07 ± 0.02

Values are μg/mg wet wt and given as means ± SE.
*$P < .05$ vs. control values.
†$P < .05$ vs. age-matched diabetics.
‡$P < .05$ vs. diabetic values at 4 wk.

The presence of diabetes in the STZ-injected animals was confirmed by elevated plasma glucose and depressed plasma insulin concentrations compared with control rats (Table 1).

Daily injections for the last 4 wk with 3 U s.c. of insulin in the diabetic rats completely reversed the plasma glucose level and improved the growth and development of cardiac hypertrophy. However, body weight was significantly lower and the ratio of ventricular weight to body weight was significantly higher in the insulin-treated diabetic rats than in the control group. Insulin treatment maintained the plasma insulin at a high level.

Phospholipid contents in myocardium. Major phospholipids such as phosphatidylcholine, phosphatidylethanolamine, and cardiolipin in the 8-wk-diabetic rat hearts were lower than in the age-matched control rats, whereas the 4-wk-diabetic rats were found to possess only a significantly lower level of cardiolipin (Table 2). Insulin injection into the diabetic rats inhibited the decrease in these phospholipids, although the elevation of phosphatidylcholine by insulin treatment appeared to be only partial. On the other hand, the sphingomyelin content in the 8-wk-diabetic rats was higher than in the control rats. Lysophosphatidylcholine content appeared higher in the diabetic rats, although the difference was not significant. Phosphatidylserine and phosphatidylinositol could not be completely separated for determination of their contents with the method described above.

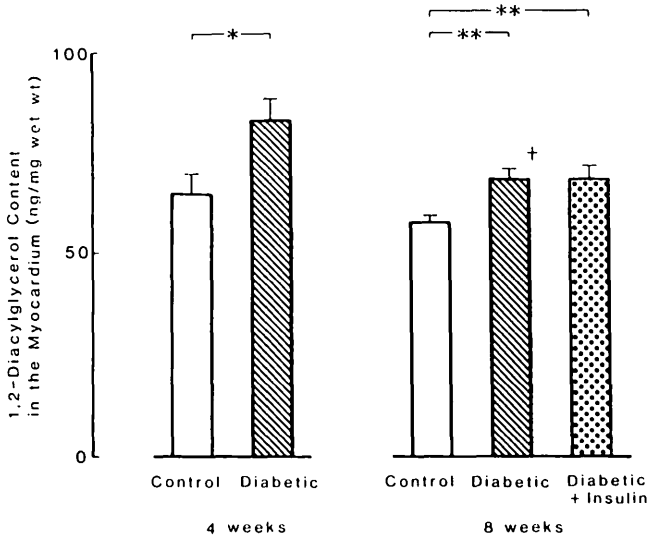

FIG. 1. 1,2-Diacylglycerol content in myocardium from control, diabetic, and insulin-treated diabetic animals 4 and 8 wk after streptozocin injection. *Bars* represent means ± SE. *$P < .05$; **$P < .01$; +$P < .05$ vs. diabetic animals at 4 wk.

Neutral lipid contents in myocardium. The 1,2-diacylglycerol contents determined in the myocardium of diabetic rats were 83.1 ± 5.1 ng/mg wet wt at 4 wk and 68.2 ± 2.1 ng/mg wet wt at 8 wk, values that were significantly higher than in the corresponding control rats (64.5 ± 4.7 ng/mg wet wt at 4 wk and 57.0 ± 1.7 ng/mg wet wt at 8 wk) (Fig. 1). The 1,2-diacylglycerol levels in control groups were about the same as those obtained after decapitation without anesthesia (11). Insulin treatment in the diabetic rats, however, did not decrease in the 1,2-diacylglycerol content of the myocardium (68.2 ± 3.1 ng/mg wet wt).

In contrast, the triglyceride contents in the myocardium of diabetic rats were 2.59 ± 0.36 μg/mg wet wt at 4 wk and 2.98 ± 0.42 μg/mg wet wt at 8 wk, values that were significantly higher than in the corresponding control rats (1.32 ± 0.15 and 1.18 ± 0.08 μg/mg wet wt, respectively), whereas insulin treatment completely returned the triglyceride content to the control level (1.12 ± 0.14 μg/mg wet wt) (Fig. 2).

With respect to cholesterol content (excluding cholesterol esters), there was no significant elevation in the 4-wk-diabetic rats, but the cholesterol content was significantly increased in 8-wk-diabetic compared with age-matched control rats (1.65 ± 0.04 vs. 1.46 ± 0.03 μg/mg wet wt; Fig. 3). The significant regression in cholesterol content, similar to that for triglyceride content in the myocardium, was observed with daily insulin treatment (3 U) in diabetic rats (1.48 ± 0.04 μg/mg wet wt).

DISCUSSION

In this study, STZ injected into rats led to the development of a diabetic state characterized by elevated plasma glucose and depressed plasma insulin levels. In addition to hyperglycemia, other biochemical abnormalities occur in diabetes. Diabetic animals have higher plasma free–fatty acid, triglyceride, and cholesterol concentrations (14–16) than human patients (17). Such severe diabetes mellitus in rats is associated with marked changes in myocardial function. Di-

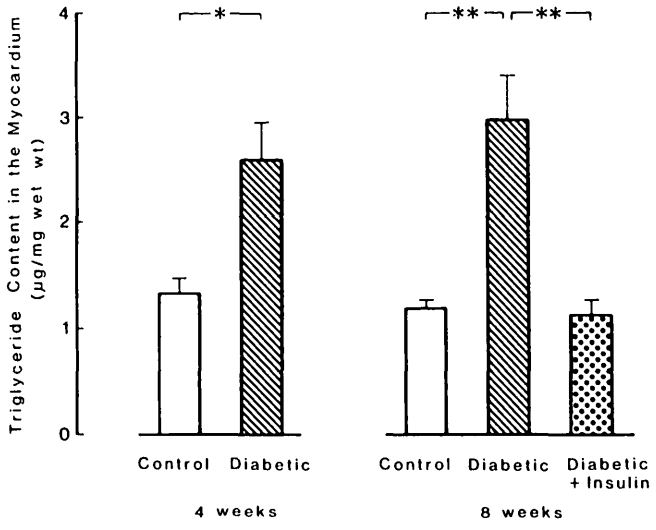

FIG. 2. Triglyceride content in myocardium from control, diabetic, and insulin-treated diabetic animals 4 and 8 wk after streptozocin injection. *Bars* represent means ± SE. *$P < 05$; **$P < 01$.

abetic cardiomyopathy is characterized by a depression of contractility and a marked slowing of the relaxation process (18). Such myocardial dysfunction in diabetic hearts is suggested to be due to enhanced sensitivity to high levels of elevated circulating lipids (14). The tissue lipid contents have also been evaluated in diabetic animals, and it was found that the triglyceride and cholesterol contents were high in the heart (16,19,20) and small intestine (21). Moreover, there is evidence that more triglyceride and cholesterol accumulate in the left ventricle and septum in diabetic patients compared to nondiabetic control subjects without heart disease (2). Our results are consistent with those results regarding to myocardial lipids. On a wet-weight basis, the hearts from 8-wk-diabetic animals revealed a decrease in major phospholipids, e.g., phosphatidylethanolamine and phosphatidylcholine, as reported earlier in diabetic rat sciatic nerves (22), whereas total phospholipid content from the isolated membranes of hearts in diabetic rats are elevated above those in control rats (6). A decrease in myocardial phospholipid content may be associated with the development of myocardial dysfunction if we consider the fact that abnormalities in ion transport systems of subcellular membranes develop with time (6,23), although such defects are not evident until 28 days after STZ injection. The contents of the minor phospholipids, sphingomyelin and lysophosphatidylcholine, showed a tendency to increase; however, the reason is still unclear, although lysophosphatidylcholine accumulation has been shown to be a consequence of ischemic myocardial damage (24). Unfortunately, the TLC-FID method used here could not totally reliably discriminate the phosphatidylserine and phosphatidylinositol fractions. These lipid alterations in the diabetic hearts, which occurred as a result of the disease process, were reversed after 4 wk of untreated diabetes followed by 4 wk of insulin treatment. A number of experimental diabetes studies have demonstrated that adequate insulin treatment completely normalized the myocardial function abnormalities and the lipid metabolism disorder (4,25).

It is generally accepted that the accumulation of 1,2-diacylglycerol in tissues results from the breakdown of phosphoinositides by phospholipase C. On receptor activation, phosphoinositide hydrolysis results in the generation of two secondary messengers, inositol triphosphate and 1,2-diacylglycerol, which are believed to be responsible for the intracellular mobilization of Ca^{2+} and the activation of protein kinase C, respectively (7,8). There is evidence that very small amounts of 1,2-diacylglycerol may play an important role as a central component of several pathways and by its activation of protein kinase C, suggesting that it is necessary to accurately estimate the 1,2-diacylglycerol content in tissues (26). Few direct measurements of 1,2-diacylglycerol have been conducted (27). We established a method using TLC-FID for quantitating the amount of 1,2-diacylglycerol in which cholesteryl acetate was chosen as an internal standard (11).

This study was carried out to determine the amount of 1,2-diacylglycerol present in diabetic cardiomyopathy. The elevation in 1,2-diacylglycerol content in the heart can probably be attributed to high levels of circulating and cardiac catecholamines in diabetic animals (28) because plasma catecholamines are elevated in diabetes (29,30), and 1,2-diacylglycerol in the myocardium is increased by exogenous norepinephrine in rats (31). In contrast to the time courses of the catecholamines in diabetic subjects (28), note that the 1,2-diacylglycerol content was lower at 8 wk after STZ injection than at 4 wk. Furthermore, another distinct aspect of 1,2-diacylglycerol is that insulin treatment failed to normalize this lipid content. The elevation in 1,2-diacylglycerol content in the heart may be associated with the development of diabetic cardiomyopathy, although the exact reason remains unclear. The classic pathway of diacylglycerol production is via hydrolysis of phosphoinositides, which can be stimulated by several agonists, e.g., acetylcholine, norepinephrine, histamine, and vasopressin. However, diacylglycerol may be generated from compounds other than phosphoinositides (9,10). It has also been reported that 1,2-diacylglycerol is partially derived from the degradation of phosphatidylcholine by phospholipase C in a preadipocytic cell line (26).

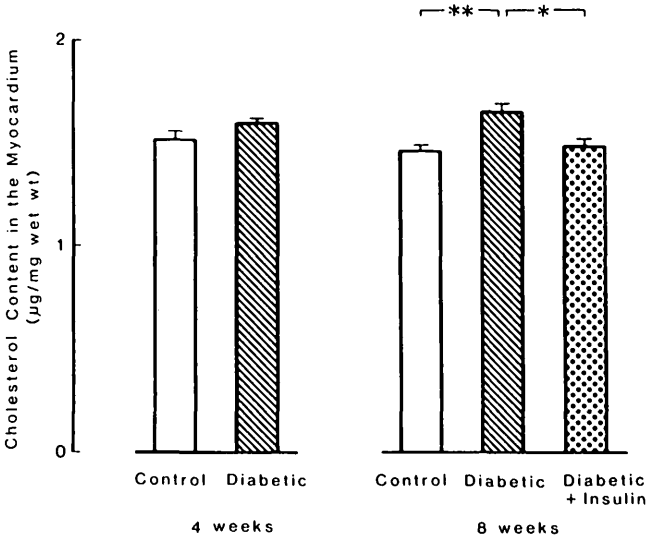

FIG. 3. Cholesterol content in myocardium from control, diabetic, and insulin-treated diabetic animals 4 and 8 wk after streptozocin injection. *Bars* represent means ± SE. *$P < .05$; **$P < .01$.

Insulin also stimulates the production of 1,2-diacylglycerol (32,33). However, the production of diacylglycerols by insulin stimulation was not always accompanied by phosphoinositide hydrolysis (34). Recently, Saltiel et al. (34) suggested that insulin caused the hydrolysis of a kind of glycolipid, resulting in the generation of diacylglycerol. Moreover, Farese et al. (35) suggested that the initial increase in diacylglycerol elicited by insulin is due to both phosphoinositide hydrolysis and de novo phosphatidic acid synthesis and that the sustained elevation is due to the latter, which is converted directly to diacylglycerols. Both events are considered to be important for generating 1,2-diacylglycerol as a secondary messenger for transduction mechanisms with respect to the action of hormones. The sustained 1,2-diacylglycerol level in the insulin-treated diabetic hearts herein may be explained by the direct effects of insulin on the generation of 1,2-diacylglycerol in the tissue. Species of diacylglycerol produced by insulin are different from those produced by α_1-adrenergic agents (34). Thus, these observed increases in 1,2-diacylglycerol levels in diabetic and insulin-treated diabetic rats may result from hydrolysis of different substrates and increased diacylglycerols may contain different species of fatty acids.

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