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Functional Defect and Its Possible Mechanism of Diabetic Cardiomyopathy*

Hae Won Kim[1]**, Hee Ran Lee[2], Yeon Jin Jang-Yang[3], Hyoungsup Park[1] and So Young Park[1]

Departments of Pharmacology[1], Physiology[3], University of Ulsan College of Medicine; Division of Pharmacology and Toxicology[2], Asan Institute for Life Sciences, Seoul 138-040, Korea

ABSTRACT

Oxidative modification of cellular proteins and lipids may play a role in the development of diabetic complications. Diabetic cardiomyopathy has been suggested to be caused by the intracellular Ca^{2+} overload in the myocardium, which is partly due to the defect of calcium transport of the cardiac sarcoplasmic reticulum (SR). In the present study, the possible mechanism of the functional defect of cardiac SR in diabetic rats was studied. Both of the maximal Ca^{2+} uptake and the affinity for Ca^{2+} were decreased in the diabetic rat SR in comparison with the control. To investigate whether the functional defect of the cardiac SR in streptozotocin-induced diabetic rat is associated with the oxidative changes of cardiac SR proteins, the carbonyl group content and glycohemoglobin levels were determined. The increase in carbonyl group content of cardiac SR (2.30 nmols/mg protein, DM; 1.78, control) and in glycohemoglobin level (13~17%, DM; 3~5%, control) were observed in the diabetics. The extent of increase in calcium transport by phospholamban phosphorylation was greater in the diabetic cardiac SR membranes than that in the control. The phosphorylation levels of phospholamban, as determined by SDS-PAGE and autoradiography with [γ^{32}P]ATP, were increased in diabetic cardiac SR. These results suggest that the impaired cardiac SR function in diabetic rat could be a consequence of the less-phosphorylation of phospholamban in the basal state, which is partly due to the depleted norepinephrine stores in the heart. Furthermore, the oxidative damages in cardiac SR membranes might be one of the additional factors leading to the diabetic cardiomyopathy.

Key Words: Heart, Sarcoplasmic reticulum, Phospholamban, Diabetic cardiomyopathy, Oxidative damages

Abbreviations: EDTA, ethylenediaminetetraacetic acid; EGTA. ehtylene glycol-bis (β-aminoethyl ether) N, N, N', N'-tetraacetic acid; SR, sarcoplasmic reticulum; TCA, trichloroacetic acid; TFA, trifluoroacetic acid

INTRODUCTION

Contraction and relaxation of cardiac muscle are regulated by physiological and pharmacological interventions involving second messengers, which transmit signals in the interior of the cell. One of these messengers is cyclic AMP (cAMP) and another is calcium. In cardiac muscle, the two major second messengers (cAMP and Ca^{2+}) are integrated at the level of sarcoplasmic reticulum (SR) protein phosphorylation via the activation of protein kinases, cAMP-dependent and

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**To whom correspondence should be addressed

Ca^{2+} • calmodulin-dependent, respectively (Kranias, 1985). In particular, calcium transport across the SR is regulated by the phosphorylation of phospholamban, a 27,000-dalton membrane-bound proteolipid. Dephosphorylated phospholamban is an inhibitor of cardiac SR Ca^{2+}-ATPase and upon phosphorylation the inhibitory effect of phospholamban is relieved and, thus, that stimulated the SR Ca^{2+} transport (Kim, 1992; Kim *et al.*, 1990). The stimulatory effects of the protein kinases on SR function can be reversed by a protein phosphatase which dephosphorylates phospholamban (Kranias and DiSalvo, 1986; Kranias *et al.*, 1988). Therefore, phspholamban appears to be an important regulator of SR function and, thus, a modulator of cardiac muscle function in general.

In diabetic patients, heart failure is the leading cause of death (Dhalla *et al.*, 1985). Several studies have revealed the presence of cardiac dysfunctions and ultrastructural abnormalities in chronic diabetes (Dhalla *et al.*, 1985; Garber *et al.*, 1983). Cardiomyopathy in chronic diabetes has been associated with the alterations in the sarcolemmal membrane and in myosin ATPase (Garber *et al.*, 1983; Makino *et al.*, 1987), and the defective SR Ca^{2+} uptake and Ca^{2+}-ATPase activity (Afzal *et al.*, 1989; Penpargkul *et al.*, 1981). The decreased activity of myofibrillar ATPase has been suggested to explain the impaired ability of diabetic heart to generate contractile force. On the other hand, impaired relaxation of diabetic hearts has been attributed to the defective SR fuction. There has been suggestions that oxidative stress may be a common pathway linking diverse mechanisms for the pathogenesis of complications in diabetes. Recently, several reports clearly showed underlying evidence that diabetes with complications is associated with increased chemical modification of proteins and lipids and that this damage appears to be largely oxidative in origin and is sufficient to explain the altered function of proteins (Baynes, 1991; Wolff *et al.*, 1991).

Although a defect in the capacity of the cardiac SR isolated from chronically diabetic rats to accumulate calcium has been observed, its direct relationship to the phospholamban regulation on the SR Ca^{2+}-ATPase by phosphorylation has not been established yet. The plasma catecholamine level in the diabetic subjects is known to be decreased in chronic status (Kahn *et al.*, 1986), it is possible that the defect in cardiac SR calcium uptake during chronic diabetes may be partly due to the decreased level of norepinephrine in myocardium in addition to the defect in SR Ca^{2+}-ATPase itself. Therefore, this study was done to determine whether the decrease of the cardiac SR function in streptozotocin-induced diabetic rat is associated with the oxidative damages of cardiac SR, and the phosphorylation level of phospholamban.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (8 wk) weighing about 250 g were randomly divided into two groups. One animal group was made diabetic with a single injection of streptozotocin (65 mg/kg i.p.) dissolved in citrate buffer (pH 4.5) whereas the other group of control animals was injected with citrate buffer. In a diabetic group, the development of diabetes mellitus (DM) was confirmed by urine test after one week of streptozotocin injection. All rats were maintained on normal animal chow and water *ad libitum* for 8~12 wk, after which the rats were fasted 5h prior to the blood sampling. Blood samples were taken and assayed for glycohemoglobin using affinity chromatography (Glyc-Affin GHb, Iso Lab Inc.; Akron, OH) and for glucose using glucose analyzer (Beckman). Routine urine analysis was done by Diastix and Ketostix (Miles Sankyo Co.; Tokyo, Japan). Since Thompson *et al.* (1991) reported that the severity of diabetes is a major determinant of myocardial damages in the rat, hearts with the glycohemoglobin level above 13% were excised and placed in ice-cold saline for 5~10 min prior to use.

Preparation of cardiac sarcoplasmic reticulum vesicles

Membrane fraction enriched with sarcoplasmic reticulum (SR) was isolated according to the slight modification of the method by Feher and LeBolt (1990). Briefly, the ventricular tissue removed large vessels and aorta, was homogenized in a Virtis hemogenizer at medium speed for 60 sec in a medium containing 10 mM imidazole, pH 7.0, 1 M KCl, and 10 mM sodium metabisulfite. The homogenate was centrifuged at 4,000 g for 20

min, and the supernatant was again centrifuged at 11,000 g for 25 min. The supernatant was centrifuged at 40,000 g for 25 min and this supernatant was again centrifuged at 100,000 g for 30 min. The resultant pellet was suspended in 10 mM imidazole, pH 7.0, 0.6 M KCl, and 0.3 M sucrose and then centrifuged at 100,000 g for 45 min. The final pellet was suspended in 10 mM imidazole, pH 7.0, 0.1 M KCl, and 0.3 M sucrose. Protein was measured by the method of Lowry *et al.* (1951) and Ca^{2+} transport assay was completed within 2 h.

Determination of calcium uptake

Calcium uptake was determined at 37℃ by using $^{45}CaCl_2$ and a modification of the Millipore filtration technique described by Martonosi and Feretos (1964). In the Millipore filtration assay, the rate of Ca^{2+} uptake was determined in a medium containing 50 μg of SR per ml, 0.1 M KCl, 5 mM $MgCl_2$, various amounts of $^{45}CaCl_2$, 0.5 mM EGTA, 5 mM ATP, 2.5 mM oxalate, 5 mM NaN_3, and 40 mM histidine-HCl, pH 7.0, using Millipore filters with a pore size of 0.45 μm (type GS). Calcium uptake was initiated by the addition of 5 mM ATP. The initial rates of Ca^{2+} uptake were calculated using least squares linear regression analysis of the 20-, 40-, and 60-sec values of Ca^{2+} uptake.

Determination of protein carbonyl content by reduction with $NaBH_4$

The carbonyl group content of cardiac SR was measured by the method of Oliver *et al.* (1987). Cardiac SR vesicles (0.3~0.5 mg protein in 200~400 μl) and 10 μl of 1% CHAPS were mixed with 25 μl of 1 M Tris-HCl, 10 mM EDTA, pH 8.5, and 40 μl of 100 mM NaOH in a 1.5 ml Sarstedt tube fitted with an O-ring and a cap. Then 30 μl of 0.1 M [^{3}H]$NaBH_4$ (specific activity, 50 mCi/mmol) in 0.1 N NaOH was added to the mixture and incubated at 37℃ for 30 min, without cap in the hood. After incubation the protein was precipitated with 1 ml of 20% TCA and left for 5 min in the hood without cap, and was centrifuged at 16,000 g for 15 min with cap. The precipitate was washed twice with 1 ml of 10% TCA. The precipitate was then dissolved in 1 ml of 6 M guanidine, 20 mM NaH_2PO_4, pH 2.3 (adjusted pH with TFA) and incubated at 37℃ for 15 min. The sample was sonicated in a bath-type sonicator (Virtis, Virsonic 300) for 10 min and the radioactivity of 0.5 ml aliquot was counted by a liquid scintillation counter (Packard, Tri-Carb 2500 TR). An aliquot was evaporated to dryness and then redissolved and retained for protein determination by the method of Lowry *et al.* (1951).

Phosphorylation of phospholamban

Phosphorylation of cardiac SR (0.5 mg/ml) was carried out, in 50 mM potassium phosphate, pH 7.0, 10 mM $MgCl_2$, 5 mM azide, 10 mM NaF, 0.5 mM EGTA, 50 units of catalytic subunit of the cAMP-dependent protein kinase, and 50 μM [γ^{32}P] ATP for 10 min at 30℃. Reactions were terminated by the addition of SDS-polyacrylamide gel electrophoresis sample buffer (62.5 mM Tris-HCl, pH 6.8, 5% glycerol, 1.5% SDS, 0.05% bromophenolblue, and 5% β-mercaptoethanol).

For investigation of the effect of phospholamban phosphorylation on Ca^{2+} uptake, control or diabetic cardiac SR vesicles were phosphorylated under the same conditions as the above using unlabeled ATP. The phosphorylation reaction was initiated by the addition of ATP. Non-phosphorylated vesicles were also incubated under identical conditions, but in the absence of ATP. After 2 min of incubation at 30℃, the reaction mixture was diluted 2-fold with ice-cold 40 mM imidazole, pH 7.0, 0.3 M sucrose (Buffer A) and centrifuged at 100,000 g for 30 min. The pellet was resuspended in Buffer A, and the protein was determined by the method of Lowry *et al.* (1951). The non-phosphorylated and phosphorylated vesicles were used for Ca^{2+} uptake to determine the SR function in control or diabetic hearts.

Polyacrylamide gel electrophoresis

Gel electrophoresis was performed on sodium dodecyl sulfate (SDS) polyacrylamide gels (10~18%) according to the method of Laemmli (1970). Autoradiograms were obtained from stained gels using Kodak film.

RESULTS

Animal conditions

In contrast to nondiabetic ones, rats treated with streptozotocin develop characteristics that

confirm diabetes. After 8~12 weeks of a single streptozotocin-injection, body weight of rats decreased significantly compared to the nondiabetic ones. Table 1 shows that the diabetic rats used in this study exhibited high glycohemoglobin levels in addition to the decreased heart weights.

Table 1. Conditions of diabetic animals. Values shown represent means±SE of 21 rats

Chracteristics	Nondiabetic	Diabetic*
Body weight, g	463.5±14.4	262.6±11.1
Heart weight, g	0.93±0.07	0.64±0.03
Glycohemoglobin, %	4.24±0.09	15.15±0.37

*Significant difference from nondiabetic, $p<0.05$.

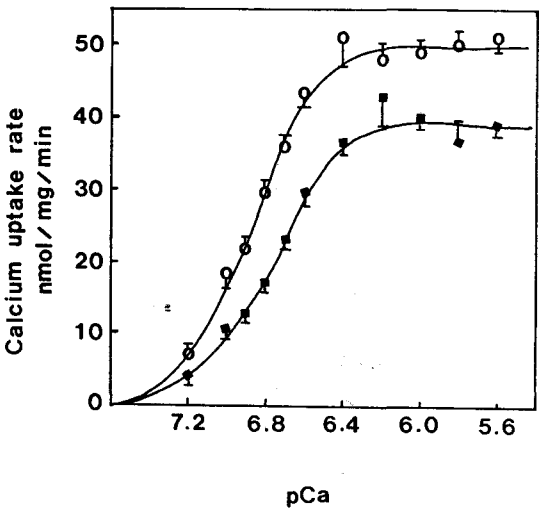

Fig. 1. Calcium dependence of calcium uptake by cardiac sarcoplasmic reticulum Ca^{2+}-ATPase. The initial rates of calcium uptake by sarcoplasmic reticulum vesicles (50 μg/ml) from control (○) and diabetic (■) rat heart were determined over various free calcium concentrations, as described in the Materials and Methods. Each value represents mean±SE of six different preparations.

Calcium transport of cardiac sarcoplasmic reticulum

Rates of Ca^{2+} uptake in cardiac sarcoplasmic reticulum (SR) fractions were examined in control and diabetic rats at various concentrations of Ca^{2+}. The data in Fig. 1 confirm those reported previously (Afzal *et al.*, 1989; Penpargkul *et al.*, 1981). Maximum Ca^{2+} uptake rates of SR (40 nmol/min/mg diabetic SR vesicles compared with 50 nmol/mg control SR vesicles) were depressed in hearts from chronically diabetic rats. The affinity of Ca^{2+}-ATPase for Ca^{2+} were also decreased in the diabetic rat heart in comparison with the control. The EC_{50} values for Ca^{2+} was 0.18 μM for Ca^{2+} uptake in diabetic SR vesicles compared with 0.13 μM Ca^{2+} in control SR vesicles.

Determination of SR protein carbonyl content

To investigate whether the functional defect of the cardiac SR in streptozotocin-induced diabetic rat is associated with the oxidative changes of cardiac SR proteins, the carbonyl group content was determined. The increase in carbonyl group content, as an index of oxidized protein, of cardiac SR was observed in the diabetic rats (Table 2).

Table 2. Protein carbonyl group content by borohydride method. The carbonyl group content of cardiac SR (0.3~0.5 mg protein in 200~400 μl) was measured by reduction with [^{3}H]$NaBH_4$ in 0.1 N NaOH. After reduction the protein was precipitated with 20% TCA and centrifuged at 16,000 g for 15 min. The precipitate was washed and dissoved in 6 M guanidine, 20 mM NaH_2PO_4, pH 2.3 (with TFA), and the radioactivity of the sample was counted. Each value represents the mean ±SE of triplicates of 6 different preparations

	Carbonyl content	
	n mol/mg prot	%
Control	1.78±0.08	100.0±4.5
DM	2.30±0.09	129.2±5.1

This data correlate well with the recent report (Jang-Yang *et al.*, 1993) that the hepatic intracellular proteins are oxidatively modified in the chronic diabetes and the damages could be prevented by the administration of vitamin E.

Effect of phosphorylation on Ca^{2+} uptake

To determine whether the decrease of the cardiac SR function in streptozotocin-induced diabetic rat is associated with the level of phospholamban phosphorylation, phosphorylation of phospholamban by the catalytic subunit of the cAMP-dependent protein kinase was studied. The phosphorylation of the cardiac SR membranes from both control and diabetic rats resulted in an increase of the initial rates of Ca^{2+} transport, compared to non-phosphorylated control and non-phosphorylated diabetic SR, respectively (Table 3). The extent of increase of the Ca^{2+} transport rates were greater between phosphorylated-diabetic and nonphosphorylated-diabetic cardiac SR membranes, than between phosphorylated-control and nonphosphorylated-control SR membranes. From this findings, it is tentatively suggested that the apparent phosphorylation levels of cardiac SR in basal state are quite different in control or in diabetic hearts.

Phosphorylation of phospholamban with [γ^{32}P] ATP

Phospholamban from control and diabetic cardiac SR was phosphorylated with [γ^{32}P]ATP and the catalytic subunit of cAMP-dependent protein kinase. After phosphorylation of phospholamban,

Table 3. Effect of cAMP-dependent phosphorylation on calcium uptake by sarcoplasmic reticulum from control and diabetic rat heart. Phosphorylation of cardiac SR (0.5 mg/ml) was carried out, in the presence of 50 mM potassium phosphate, pH 7.0, 10 mM $MgCl_2$, 5 mM azide, 0.5 mM EGTA, 50 units of catalytic subunit of the cAMP-dependent protein kinase, and 5 mM ATP. The phosphorylation reaction was initiated by the addition of ATP and was terminated by the dilution with ice-cold 40 mM imidazole, pH 7.0, 0.3 M sucrose and centrification. The nonphosphorylated and phosphorylated vesicles were used for Ca^{2+} uptake to determine the SR function in control and diabetic rat hearts. The results are expressed as percent stimulation over nonphosphorylated vesicles, shown in Fig. 1. Each value represents the mean±SE of five different preparations

pCa	Ca^{2+} uptake (% of nonphosphorylated)	
	Control	DM
7.0	165.7±9.4	186.9±9.8
6.8	143.9±10.2	173.1±11.2
6.0	117.2±6.6	129.7±9.4

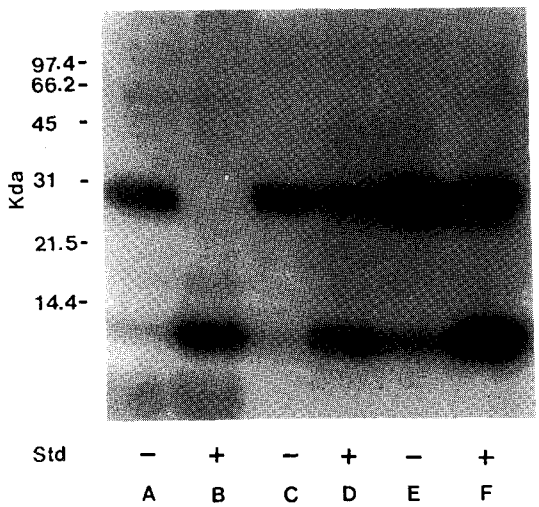

Fig. 2. Autoradiogram of SDS-polyacrylamide (10 ~18%) gels of cardiac sarcoplasmic reticulum vesicles from control (C, D) and diabetic (E, F) rat heart. Sarcoplasmic reticulum membranes (10 μg/lane) were phosphorylated at 30℃ for 2 min in the presence of catalytic subunit of the cAMP-dependent protein kinase and [γ^{32}P]ATP, as described in Materials and Methods. Some samples (+) were placed in a boiling water bath for 5 min prior to electrophoresis, and the others (−) were not. (A, B); canine cardiac sarcoplasmic reticulum membranes as a positive control.

SDS-polyacrylamide gel electrophoresis was done followed by autoradiography of phosphorylated phospholamban. Interestingly, the level of phospholamban phosphorylation was higher in phospholamban from diabetic hearts than in phospholamban from control hearts (Fig. 2).

DISCUSSION

The attenuation of the ability of diabetic heart to generate cardiac relaxation has been suggested to be due to the depression in sarcoplasmic reticulum (SR) Ca^{2+} uptake (Afzal *et al.*, 1989). Results of this study demonstrate that a decrease in calcium transport activity of the cardiac SR, in terms of the maximum Ca^{2+} uptake rate and affinity of Ca^{2+}-ATPase for Ca^{2+}.

Conflicting results have been presented regarding the relative roles of microvascular changes, interstitial fibrosis, and metabolic derangements in the pathogenesis of diabetic cardiomyopathy. Although the cardiac dysfunction is frequently associated with the enhanced coronary atherosclerosis in diabetic patients, evidence has been accumulated for the existence of a specific diabetic cardiomyopathy (Zarich and Nesto, 1989). There are several hypotheses regarding the pathogenesis of diabetic cardiomyopathy. One is the oxidative stresses and the other is the diabetic autonomic neuropathy.

There has been some suggestions that oxidative modification of cellular proteins and lipids might play a role in the development of diabetic complications. *In vitro,* free radicals, hydrogen peroxide, and alphaketoaldehydes produced by glucose autoxidation appear to be primary mediators of protein modification and peroxidation of protein-associated lipid under physiological conditions (Wolff *et al.*, 1991). However, *in vivo,* the involvement of the oxidative pathway in the development of diabetic complications is still uncertain. In this study, the increase of the carbonyl group content in cardiac SR protein from the diabetic rats was observed, which correlate well with the recent report of diabetic hepatic protein oxidation by Jang-Yang *et al.* (1993). However, the involvement of the peroxidation of protein-associated lipid in the diabetic cardiomyopathy needs to be determined.

Defective β-adrenergic responsiveness has been suggested for the characteristic feature of diabetes. Schaffer *et al.* (1991) reported that the basis for decreased β-adrenoceptor resposiveness in the diabetic rat heart is the reductions both in β-receptor number and in isoproterenol-induced activation of adenylate cyclase in the diabetic heart. Although the relationship of autonomic nervous system dysfunction to diabetic cardiomyopathy is suggested, the exact nature of the relationship is not completely clear.

In this study, we have shown that the phosphorylation of phospholamban with the catalytic subunit of cAMP-dependent protein kinase resulted in the improved SR Ca^{2+} pump activity in both the control and the diabetic hearts. The degree of stimulation of Ca^{2+} transport is greater in SR prepared from diabetic hearts than that in SR from the control, which is different from the previous report (Schaffer *et al.*, 1991). The conflict between results by other investigators (Schaffer *et al.*, 1991) and ours makes an absolute comparison virtually impossible. Part of the problem arises from the difference in animal models, such as other study using the rat model of non-insulin-dependent diabetes and our study using the chemically induced model of insulin-dependent diabetes. In accordance with our above result, we found that the phosphorylation band of phospholamban from diabetic hearts using [γ^{32}P]ATP was strong, compared with the phospholamban band of control hearts. This may suggest that in basal state, phospholamban is less-phosphorylated in diabetic hearts than in control hearts. Since phospholamban is an inhibitor of cardiac SR Ca^{2+}-ATPase and phosphorylation of phospholamban stimulates SR calcium transport (Kim, 1992; Kim *et al.*, 1990), the defect in SR function of chronic diabetic rats may partly due to the less-phosphorylation of phospholamban in the basal state.

It is interesting to determine the mechanism for this less-phosphorylation of phospholamban in the diabetic hearts. Phospholamban can be phosphorylated by three different protein kinases; cAMP-dependent, Ca^{2+} • calmodulin-dependent, and Ca^{2+} • phospholipid-dependent. As we mentioned in the Introduction, cAMP and Ca^{2+} is the two major second messengers to modulate SR function. In the physiological status, norepinephrine is the major candidate to modulate cAMP

and Ca^{2+} in myocardium. Kahn *et al.* (1986) reported that plasma catecholamine levels were significantly reduced in diabetic subjects. Furthermore, myocardial catecholamine depletion frequently occurs in patients with heart failure. Thus, the decreased NE content in heart homogenate from diabetic rats (data not shown) might be the possible cause of the less-phosphorylation of phospholamban. However, the direct involvement of the decreased myocardial norepinephrine level remains to be clarified.

From these above findings, we could conclude that cardiac dysfunction in chronically diabetic rats may partly due to the less-phosphorylation of phospholamban in the basal state possibly because of the depleted norepinephrine content in the myocardium. Furthermore, the oxidative damages in cardiac SR membranes might be one of additional factors leading to the diabetic cardiomyopathy. However, the direct evidence for cardiac muscle dysfunction due to oxidative damages in terms of lipid peroxidation and DNA damages still remains to be determined.

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=국문초록=

당뇨성 심근질환에서의 근장그물 기능이상과 그 작용기전

울산대학교 의과대학 약리학교실[1], 생리학교실[2]
아산생명과학연구소 약리 • 독물학연구실[3]

김혜원[1] · 이희란[3] · 장연진[2] · 박형섭[1] · 박소영[1]

스트렙토조토신으로 당뇨를 유발시킨 쥐의 심근 근장그물에서 칼슘이동이 저하됨을 볼 수 있었다. 칼슘이동의 저하는 최대칼슘 uptake의 감소와 칼슘에 대한 affinity의 감소로 나타났다. 이러한 심근 근장그물의 기능저하가 나타나는 작용기전이 심근 근장그물 단백의 산화성 손상과 관계가 있는지를 살펴보았다. 당뇨쥐에서는 glycohemoglobin과 carbonyl group의 양이 현저히 증가됨을 볼 수 있었다. 한편으로 cyclic AMP 의존성 protein kinase의 catalytic subunit에 의한 phospholamban 인산화에 의해 심근 근장그물 칼슘이동의 증가를 보였고, 이 증가는 대조군에 비하여 당뇨군에서 훨씬 현저하게 나타났다. SDS-polyacrylamide를 이용한 전기영동후 autoradiogram을 통하여 확인한 phospholamban 인산화는 당뇨군에서 진한 band로 나타남이 확인되었다. 이상의 결과로 미루어 당뇨군의 심근 근장그물 기능저하는, 기초상태에서 아마도 심근내 저하된 norephinephrine 양으로 인하여 phospholamban 인산화 정도가 적으므로 근장그물 Ca^{2+}-ATPase 억제가 나타남을 제시해 주며, 근장그물 단백의 산화성 손상도 당뇨성 심근질환을 일으킬 수 있는 또 다른 요인 중의 하나로 생각된다.