Negative Inotropic Effect of Insulin in Papillary Muscles From Control and Diabetic Rats

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Summary
The inotropic effects of insulin in the rat heart are still incompletely understood. In this study, the effects of insulin on cardiac contraction were studied in right ventricular papillary muscles from both control rats and rats with chronic diabetes (lasting 16 weeks). Diabetes was induced by the application of streptozotocin (STZ) and the development of diabetes was documented by increased levels of blood glucose, by reduction in body weight and by decreased plasma concentrations of insulin. The contraction was significantly smaller in diabetic rats. Insulin (80 IU/l) reduced the contraction force in both control and diabetic groups. The post-rest potentiation of contraction was not influenced by insulin in control rats, but insulin increased it in diabetic rats. The negative inotropic effect of insulin was preserved in the presence of cyclopiazonic acid (3 µmol/l), a blocker of sarcoplasmic reticulum (SR) Ca²⁺ pump, in both control and diabetic groups. In contrast, the negative inotropic effect of insulin was completely prevented in the presence of nifedipine (3 µmol/l), a blocker of L-type Ca²⁺ current. We conclude that insulin exerts a significant negative inotropic effect in rat myocardium, both control and diabetic. This effect is probably related to processes of SR Ca²⁺ release triggering, whereas SR Ca²⁺ loading is not involved.

Key words
Insulin • Rat • Heart • Contraction • Diabetes

Introduction
Prevalence and mortality from cardiovascular diseases is several fold higher in patients with diabetes mellitus than in the general population. One of the most important cardiovascular complications of insulin-dependent diabetes mellitus is a diabetic cardiomyopathy characterized by an early diastolic and later systolic dysfunctions (Maghoub and Abd-Elfattah 1998), microangiopathy, hypertrophy of cardiac myocytes (Gargiulo et al. 1998) and finally by heart failure (Jarret 1989). The mechanisms of diabetic cardiomyopathy are still not completely understood. Nevertheless the most important mechanisms are metabolic disturbances (depletion of glucose transporter 4, increased free fatty acids, carnitine deficiency, changes in calcium homeostasis), myocardial fibrosis (association with increases in angiotensin II, IGF-I, and inflammatory cytokines), small vessel disease (microangiopathy, impaired coronary flow reserve, and endothelial...
dysfunction), cardiac autonomic neuropathy (denervation and alterations in myocardial catecholamine levels), and insulin resistance (for review see Fang et al. 2004).

In experimental animal studies, diabetes is most commonly induced by the application of streptozotocin (STZ). In STZ-treated rats, diminished cardiac contractility was found (Penpargkul et al. 1980, Brown et al. 2001) and this was related to significant alterations of myocardial calcium metabolism: reduction of sarcoplasmic reticulum (SR) Ca\(^{2+}\)-ATPase (Ganguly et al. 1983), of sarcolemmal Ca\(^{2+}\)-ATPase (Heyliger et al. 1987), of Na\(^+-\)Ca\(^{2+}\) exchanger (Makino et al. 1987), of L-type calcium current (Chattou et al. 1999), of ryanodine-sensitive Ca\(^{2+}\) channels (Teshima et al. 2000), and decreased cross-bridge cycle rate (Ishikawa et al. 1999). Action potential durations measured in both atrial and ventricular tissue were found to be prolonged in STZ-treated rats, probably due to decreased potassium conductance (Nobe et al. 1990, Jourdon and Feuvray 1993).

Insulin, the hormone produced by beta cells of the islets of Langerhans, is one of the most important regulators of carbohydrate, lipid and protein metabolism. Insulin is known to regulate gene expression, ion currents, cell growth and apoptosis (Mayers and White 1996). In cardiac myocytes, insulin profoundly affects calcium handling, e.g. it stimulates L-type calcium current (Aulbach et al. 1999), Na\(^+-\)Ca\(^{2+}\) exchanger (Ballard et al. 1994), interaction between insulin-receptor substrate proteins and SR Ca\(^{2+}\)-ATPase (Algenstaedt et al. 1997). The effects of insulin on overall cardiac contraction force vary among species. Positive inotropic effect of insulin was described in the guinea pig (von Arnim and Bolte 1980) and rabbit (Snow 1976). In piglets, the inotropic effect of insulin was biphasic, initial negative action was followed by an increase in the contraction force (Lee and Downing 1976). In the rat, the effects of insulin on cardiac contraction are still unclear. The results reported so far are conflicting because positive inotropic effect (Sethi et al. 1991), negative inotropic effect (Farah and Alousi 1981) and even no effect (Ren et al. 1999, Schmidt and Koch 2002) of insulin on cardiac contraction force were reported.

In this study, the effects of insulin on cardiac contraction in healthy control rats as well as in rats with STZ-induced diabetes were investigated in detail and possible mechanisms of the effects were further addressed.

**Material and Methods**

All experiments were conducted in accordance with European Directive for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (86/609/EU) and were approved by the University Committee for Experiments on Laboratory Animals (Charles University, Czech Republic).

**Rat model of diabetes**

The experiments were performed in 45 adult male rats (Velaz, Prague, Czech Republic). Diabetes was induced in 25 rats with an intravenous injection (tail vein) of streptozotocin (Sigma-Aldrich, USA; 65 mg/kg body weight) dissolved in citrate buffer (pH 4.5). Control rats (n=20) received an injection of citrate buffer only. The action of streptozotocin was confirmed by the occurrence of severe hyperglycemia. The plasma glucose levels were measured before the administration of STZ and then weekly by an enzymatic method (Bio-La-Test, Lachema, Czech Republic). Only animals that had fasting blood glucose level higher than 18 mmol/l (n=22) were considered as diabetic. Insulin concentrations in the plasma were measured using a commercial radioimmunoassay kit (LINCO Research, MO, USA) with rat insulin as the standard.

**Contraction experiments**

Animals were anesthetized with an intraperitoneal injection of urethane (1.5 g/kg b.w.) 10 min after having received heparin (500 U, i.p.) and their hearts were quickly excised. The papillary muscles were dissected from the right ventricle and placed in an experimental chamber, where they were attached to an isometric force transducer F30 (Hugo Sachs, Germany). After a stabilization period (>30 min) we recorded: 1) steady-state contractions at various stimulation frequencies (1 Hz. Double-chamber stimulation (stimulator Pulemaster A300, WPI, USA) was used. Square-wave voltage pulses had a duration of 1 ms and amplitude ≥ 50 % above threshold. Data were recorded using the data acquisition system DiSys (Merlin, Czech Republic). The preparation was perfused with a 36 °C warm, oxygenated solution at a constant flow rate (6-10 ml/min). After a stabilization period (>30 min) we recorded: 1) steady-state contractions at various stimulation frequencies (3, 2, 1, 0.5, 0.3, 0.2 and 0.1 Hz); 2) post-rest potentiation contractions – steady-state (1 Hz) stimulation interrupted by a period of rest (10, 30, 60,
120 and 300 s). Contractions were measured in arbitrary units (a.u.). Time course of contractions was characterized using time-to-peak (time from resting tension to the peak of contraction, TTP) and half-maximal relaxation time (R/2). The resting tension was taken as zero.

**Solutions and chemicals**

The composition of the Tyrode solution was as follows (in mmol/l): NaCl 137, KCl 4.5, MgCl₂ 1, CaCl₂ 2, glucose 10, Hepes 5; pH was adjusted to 7.4 with NaOH. Insulin (Sigma-Aldrich, USA) was used at concentration 2.8 mg/l (80 IU/l), cyclopiazonic acid (Sigma-Aldrich) was used in the concentration of 3 µmol/l and nifedipine (Sigma-Aldrich) was dissolved in dimethyl sulfoxide and used the concentration of 3 µmol/l. Solutions containing nifedipine were protected from the light. All other chemicals were from Sigma or Lachema (Czech Republic).

**Statistics**

Data are presented as mean ± S.E.M. Statistical comparisons were performed by Student’s t-test for unpaired (control vs. diabetic rats) and paired (various interventions in the same animals) data groups, after testing for normality of distribution. Differences at p ≤ 0.05 were considered significant.

**Results**

**Basic characteristics of diabetic rats**

To verify the development of insulin-dependent diabetes, we monitored the blood glucose level, body weight (BW) and plasma insulin concentrations in both control and diabetic groups. Before administration of streptozotocin (STZ) there were no significant differences between control and diabetic animals (Fig. 1). In control rats the blood glucose level did not exceed 9 mmol/l during the whole experiment (16 weeks). In diabetic animals, glycemia was significantly increased from the first week after STZ injection to the end of the experiment (Fig. 1). Body weight of control rats increased gradually throughout the whole experiment. In contrast, BW of diabetic animals increased only slightly after STZ administration, and these values were significantly lower than BW of control rats (Fig. 1). Plasma insulin concentrations in the control group did not change significantly throughout the whole experiment, but in diabetic rats insulin concentrations dropped significantly after injection of STZ (Fig. 1).

![Fig. 1](image)

**Fig. 1.** Blood glucose, body weight and plasma insulin concentration in control and diabetic rats. Empty bars: control group (n=20), filled bars: diabetic group (n=22). * significantly different from value in the control group (p<0.05), # significantly different from the value before STZ or vehicle injection (p<0.05).
The effect of experimental diabetes and insulin on papillary muscle contraction.

Chronic diabetes decreased the contraction force (CF) of papillary muscles considerably (Fig. 2A). Furthermore, it decreased the dependence of CF on stimulation frequency, especially at slower rates (<1 Hz) (Fig. 2B, C). Insulin (80 IU/l), surprisingly, reduced CF in both experimental groups, i.e. at stimulation frequency of 1 Hz from 47±2.8 a.u. to 35±2.8 a.u. (n=5, p<0.05) in control rats and from 23±2.9 a.u. to 14±1.7 a.u. (n=6, p<0.05) in diabetic rats (Fig. 2A). The negative inotropic effect of insulin occurred at all stimulation frequencies tested (Fig. 2B, C). The frequency-dependence of the effect, however, was different in control and diabetic groups: in control rats; the effect was more pronounced at slow rates, whereas in diabetic animals larger effect occurred at fast rates (Fig. 2B, C).

Time-to-peak (TTP) was significantly longer in diabetic animals than in controls at all stimulation frequencies tested. In both experimental groups TTP shortened with increasing frequency of stimulation. In control rats, insulin application led to a decrease in contraction duration at all stimulation frequencies, whereas insulin had no effect on TTP in diabetic rats. For example, at stimulation frequency of 0.5 Hz in the control group TTP was 74±2.4 ms in insulin-free solution and 58.9±1.3 ms with insulin, in STZ rats TTP was 78.2±2.1 ms and 73.4±4.3 ms, respectively. Similarly, R/2 was prolonged by chronic diabetes at all stimulation frequencies tested. In the control group, insulin shortened R/2 at all stimulation frequencies, in diabetic rats no effect of insulin on relaxation kinetics was found (e.g. at stimulation frequency of 1 Hz in the control group R/2...
was 43±1.8 ms without insulin and 36.5±0.8 ms with insulin, while in the diabetic group R/2 was 49.2±1.8 ms and 51.2±1.3 ms, respectively).

**Negative inotropic effect of insulin and sarcoplasmic reticulum**

In rats, the first contraction after a period of rest is increased. This phenomenon is called post-rest potentiation of contraction and it mainly reflects function of the Na+/Ca2+ exchange and of the sarcoplasmic reticulum Ca2+ pump (Shattock and Bers 1989, Bers and Christensen 1990). To obtain a better insight into the mechanisms by which diabetes and/or insulin influence the contractility, we further investigated how diabetes and/or insulin affect the post-rest potentiation of contraction. Steady-state stimulation (1 Hz) was interrupted by a rest period (RT) of variable duration (from 10 to 300 s). The first post-rest contraction was always increased and the maximal potentiation of contraction in control rats occurred after a period of 60 s (Fig. 3B). Insulin did not influence the post-rest potentiation in control rats.

![Fig. 3. The effect of insulin on the post-rest potentiation of contraction in control and diabetic rats. A. Contraction force (CF) before rest period (RP) at stimulation frequency of 1 Hz. Left part: control rats (n=5), right part: diabetic rats (n=6). Empty bars: baseline, filled bars: insulin containing solution (80 IU/l). * significantly different from baseline (p<0.05), # significantly different from control rats (p<0.05). B. The effect of insulin on post-rest contraction in control rats (n=5). Left inset: CF before and after RP in insulin-free solution. Right inset: CF before and after RP in insulin-containing solution. C. The effect of insulin on post-rest contraction in diabetic rats (n=6). Left inset: CF before and after RP in insulin-free solution. Right inset: CF before and after RP in insulin-containing solution. Open squares: baseline, filled circles: insulin (80 IU/l). * significantly different from baseline (p<0.05), # significantly different from control rats (p<0.05).](image)

In diabetic myocardium the post-rest potentiation of contraction was preserved, however, the increase in post-rest contraction was less pronounced (Fig. 3C). Furthermore, the dependence of potentiation on the duration of RP was flatter in diabetic rats than in control rats. Application of insulin in diabetic rats resulted in a significant increase in the post-rest potentiation of contraction (Fig. 3C).

To address the role of sarcoplasmic reticulum in more detail, we used cyclopiazonic acid (CPA), a selective blocker of sarcoplasmic reticulum Ca2+ pump (SERCA2). CPA (3 µmol/l) showed a negative inotropic effect and this effect was similar in both control and diabetic animals, e.g. at stimulation frequency of 1 Hz from 47±2.8 a.u. to 26±2.7 a.u. (n=5, p<0.05) in control rats and from 23±2.9 a.u. to 14±1.5 a.u. (n=6, p<0.05) in diabetic rats (Fig. 4). Furthermore, CPA slowed down the relaxation, especially the second half, e.g. at stimulation...
frequency of 2 Hz the time to 90 % relaxation prolonged from 57.2±2.4 ms at baseline to 70.4±1.9 ms (p<0.05, n=5) in the presence of CPA in control rats, from 68.8±3.4 ms at baseline to 98.7±4.5 ms (p<0.05, n=6) in CPA in diabetic animals.

Administration of insulin on top of CPA further decreased the contraction, again in both control and diabetic rats (Fig. 4). The insulin-dependent decrease of contraction in the presence of CPA (Fig. 4) was similar to that in the absence of CPA (Fig. 2). This suggests that the Ca\(^{2+}\) uptake to sarcoplasmic reticulum by SERCA2 is not a major determinant of the negative inotropic effect of insulin and that different mechanisms must be involved.

**Fig. 4.** Negative inotropic effect of insulin in the presence of CPA. A. Contraction force (CF) at stimulation frequency of 1 Hz. Left part: control rats (n=5), right part: diabetic rats (n=6). Empty bars: baseline, shaded bars: CPA (3 µmol/l), filled bars: CPA (3 µmol/l) + insulin (80 IU/l). * significantly different from baseline (p<0.05), † significantly different from CPA (p<0.05). B. CF at various stimulation frequencies (0.1 - 3 Hz) in control animals (n=5). C. CF at various stimulation frequencies (0.1 - 3 Hz) in diabetic animals (n=6). Values were normalized to CF at 1 Hz at baseline. Open squares: control solution, filled circles: CPA (3 µmol/l), filled triangles: CPA (3 µmol/l) + insulin (80 IU/l). * significantly different from baseline (p<0.05), † significantly different from CPA (p<0.05). Left inset: Effect of CPA and CPA+insulin on CF at stimulation frequency of 1 Hz in controls. Right inset: Effect of CPA and CPA+insulin on CF at stimulation frequency of 1 Hz in diabetic rats.

**Negative inotropic effect of insulin and L-type Ca\(^{2+}\) current**

Sarcolemmal Ca\(^{2+}\) transport through L-type Ca\(^{2+}\) channels (L-type Ca\(^{2+}\) current, I\(_{CaL}\)) is another factor possibly involved in the negative inotropic effect of insulin. Therefore, we investigated the inotropic effects of insulin in the presence of nifedipine, a blocker of I\(_{CaL}\). Nifedipine (3 µmol/l) diminished the contraction force in both experimental groups (Fig. 5) and this effect was strongly dependent on stimulation frequency as reported previously (Schouten and ter Keurs 1991). The negative inotropic effect of nifedipine was more pronounced at high frequencies, e.g. contraction in the presence of nifedipine was reduced to 91±4.9 % of control contraction at stimulation frequency of 0.1 Hz compared to 9.4±0.7 % (n=5, p<0.05) of control contraction at stimulation frequency of 3 Hz in control rats; 89.3±5.2 % vs. 27.1±1.8 % (n=6, p=0.05) in diabetic rats. Subsequent administration of insulin on top of nifedipine did not affect the contraction further (Fig. 5). This lack of effect of insulin in the presence of nifedipine was also observed at low frequencies of stimulation, in which the reduction of contraction by nifedipine was only minor (Fig. 5B, C).
Discussion

The main finding of the present study is that insulin exerts a significant negative inotropic effect in papillary muscles of both healthy control rats and rats with chronic diabetes. The data indicate that the negative inotropic effect of insulin is related to $I_{CaL}$ and processes of triggering SR Ca$^{2+}$ release. On the other hand, processes of SR Ca$^{2+}$ loading, namely the SR Ca$^{2+}$ pump, are unlikely to contribute to this effect. Furthermore, the data confirm a negative inotropic effect of chronic diabetes described in previous studies (Penpargkul et al. 1980, Brown et al. 2001).

Inotropic effect of insulin in the rat heart

The effects of insulin on cardiac contractility in rat were so far poorly investigated and conflicting data reported. Positive inotropic effect (Sethi et al. 1991), negative inotropic effect (Farah and Alousi 1981) and even no effect (Schmidt and Koch 2002, Ren et al. 1999) of insulin on cardiac contraction force were found. In our experiments, insulin (80 IU/l) decreased the contraction force by ~25 % in papillary muscles of healthy rats and by ~40 % in papillary muscles of diabetic rats. Reasons of the discrepancies between the studies are not clear, but the differences in experimental design could contribute to this. The concentration of extracellular Ca$^{2+}$, a crucial factor for cardiac contractility, was different in these studies and it was previously shown that the inotropic effect of insulin is dependent on extracellular Ca$^{2+}$ (Schmidt and Koch 2002). At Ca$^{2+}$ concentration of 2.5 mmol/l insulin did not influence the contraction force, at lower Ca$^{2+}$ concentrations a positive inotropic effect of insulin was found and at higher Ca$^{2+}$ concentrations a negative inotropic effect of insulin occurred (Schmidt and Koch 2002). These experiments, however, were performed in whole rat hearts. In papillary muscles the relationship between insulin and Ca$^{2+}$ concentration seems to be different. The site of origin of papillary muscles can also be of importance. No inotropic effect of insulin was found in left ventricle papillary muscles (Ren et al. 1999), whereas we found a negative inotropic effect of insulin in right ventricle papillary muscles. Previously, both electrophysiological and contractile differences were
reported between right and left rat ventricles (Nand et al. 1997, Casis et al. 1998). Furthermore, in diabetic rats the duration of the disease can be an important factor. A positive inotropic effect of insulin was shown in papillary muscles of rats with diabetes lasting 5-7 days (Ren et al. 1999). In contrast, a negative inotropic effect of insulin was found in our experiments, in which the duration of diabetes was much longer (16 weeks). The inotropic effect of insulin can be also dependent on the applied concentration (Schmidt and Koch 2002, Švíglerová et al. 2003).

In control rats, insulin accelerated both contraction and relaxation. This acceleration corresponds well with the reported cellular effects of insulin: stimulation of L-type calcium current (Aulbach et al. 1999), of Na⁺-Ca²⁺ exchanger (Ballard et al. 1994), and of interaction between insulin-receptor substrate proteins and SR Ca²⁺-ATPase (Algenstaedt et al. 1997). In diabetic rats, however, insulin had no effects on kinetics of either contraction or relaxation. This lack of kinetic effects of insulin in the diabetic group is possibly related to detrimental effects of chronic diabetes on components of the Ca²⁺ handling system (reduction of SR Ca²⁺-ATPase (Ganguly et al. 1983, Švíglerová et al. 2004), of sarcolemmal Ca²⁺-ATPase (Heyliger et al. 1987), of Na⁺-Ca²⁺ exchanger (Makino et al. 1987), of L-type calcium current (Chattou et al. 1999), of ryanodine-sensitive Ca²⁺ channels (Teshima et al. 2000) as well as decreased cross-bridge cycle rate (Ishikawa et al. 1999), thus preventing and counteracting the stimulating actions of insulin.

Mechanisms of the negative inotropic effect of insulin

The post-rest potentiation of contraction, i.e. increase in contraction after a period of rest, occurs in the rat heart, and it mainly reflects function of Na⁺-Ca²⁺ exchange and of SERCA2 (Shattock and Bers 1989, Bers and Christensen 1990). In our experiments, insulin did not influence the post-rest potentiation of contraction in control rats and in diabetic rats the post-rest potentiation was even more pronounced in the presence of insulin. This strongly suggests that the negative inotropic effect of insulin is not mediated by influencing processes of SR Ca²⁺ loading. To verify this further we also tested the effect of insulin in the presence of cyclopiazonic acid, a selective blocker of SERCA2. Administration of insulin on top of CPA decreased the contraction to the same relative extent as in its absence, in both control and diabetic rats. Such additive effects of insulin and CPA indicate again that Ca²⁺ uptake into the sarcoplasmic reticulum by SERCA2 is not a major determinant of the negative inotropic effect of insulin so that different mechanisms has to be involved.

Sarcolemmal Ca²⁺ transport through L-type Ca²⁺ channels (L-type Ca²⁺ current, I_{Ca,L}) and/or triggering of SR Ca²⁺ release are then the obvious candidates. Nifedipine, blocker of I_{Ca,L}, diminished the contraction force with a clear dependence on stimulation frequency as reported previously (Schouten and ter Keurs 1991). Administration of insulin in the presence of nifedipine did not affect the contraction further. This lack of the effect of insulin was also observed at low stimulation frequencies, in which the reduction of contraction by nifedipine was only minor. Based on these experiments, one could hypothesize that insulin inhibits I_{Ca,L} and the reduction of I_{Ca,L} leads to the negative inotropic effect. In the presence of nifedipine, which binds to I_{Ca,L} channels, this inhibitory action of insulin would be prevented. However, the effect of insulin on I_{Ca,L} was investigated directly using the patch-clamp method in rat isolated ventricular myocytes (Aulbach et al. 1999). In contrast to the above hypothesis, no inhibition but stimulation of I_{Ca,L} by insulin was found. If this is also the case in our experimental conditions of multicellular preparation, the impairment of triggering SR Ca²⁺ release by I_{Ca,L} (decreased efficiency of this process) remains as the most likely mechanism.

**Negative inotropic effect of diabetes**

STZ-treated rats developed diabetes with all characteristic clinical symptoms: increased blood glucose, decreased insulin plasma concentration, stagnant body weight. Chronic diabetes (lasting 16 weeks) led in our conditions to a considerable reduction of cardiac contraction force. In addition to this, both contraction and relaxation were slowed down in chronic diabetes. These findings are in good agreement with earlier studies (Brown et al. 1996, Ren et al. 1999). With regard to basic mechanisms of the negative inotropic effect of diabetes a considerable amount of work was done on cellular and molecular level. A number of diabetes-induced changes was found on the level of Ca²⁺ handling: reduced I_{Ca,L} (Lee et al. 1992), decreased number of SR ryanodine receptors (Yu et al. 1994), decreased mRNA and protein levels of SERCA2 (Kim et al. 2001), further inhibition of SERCA2 by increased level of unphosphorylated phospholamban (Kim et al. 2001), decreased mRNA and protein levels of Na⁺-Ca²⁺ exchanger (Hattori et al. 2000). Significant alterations were also described for contractile proteins: diminished Ca²⁺ sensitivity, shifts in
myosin isoenzymes (from V1 myosin with high ATPase activity to V3 myosin with low ATPase activity; for review see Malhotra and Sanghi 1997).

In this study the possible mechanisms underlying the negative inotropic effect of insulin were investigated only indirectly. To understand this effect in detail, cellular experiments employing measurement of ionic currents (I_{Ca}) and intracellular Ca^{2+} transients will be necessary.

Translation of our findings to the clinical situation is by far not straightforward. Profound interspecies differences in the effect of insulin exist (Snow 1976, Lee and Downing 1976, von Armin and Bolte 1980, Sethi et al. 1991, Farah and Alousi 1981, Ren et al. 1999, Schmidt and Koch 2002) and therefore it must be first verified whether the negative inotropic effect of insulin also occurs in the human heart. Second, in this study only an acute single administration of insulin was tested. Therefore, an obvious question is whether chronic repeated application of insulin (as in the case of diabetic patient) has similar or different effects. Anyway, if insulin exerts some direct effects on contractility in the human heart, the consequences of insulin application for (diabetic) cardiomyopathy, its development and treatment must be very carefully considered.

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References


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