Improvement of Hypoperfusion with Norepinephrine Injury by Ex Vivo Insulin in Isolated Diabetic Rat Hearts

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Abstract—Effects of insulin on contractile and energy metabolic dysfunctions during hypoperfusion (2 ml/min/g heart wt., 60 min) with 10-6 M norepinephrine were studied in paced hearts isolated from streptozotocin-diabetic rats. Insulin (2 mU/min/g heart wt.) was infused 20 min before and during hypoperfusion (pre-treated group) or 30 min after the onset of hypoperfusion (post-treated group). Hearts in the non-treated group were hypoperfused without insulin and other hearts in the control group were not hypoperfused. In the non-treated group, resting contractile force (CF) and resting left ventricular pressure (LVP) were significantly elevated to maximum levels within 30 min after hypoperfusion and these elevations were restored in the pre-treated group but not in the post-treated group. Developed CF was depressed in the non-treated group and improved significantly in the pre-treated group but not in the post-treated group. Developed LVP was depressed in the non-treated group, and depression was slightly larger in the pre-treated group. In the non-treated group, ATP and creatine phosphate contents in the left ventricle significantly decreased. Decreases in ATP and creatine phosphate contents in the inner layer were partially restored in the pre-treated group but not in the post-treated group. Lactate significantly increased in the non-treated group and increased even further in the insulin treated groups. These results indicate that contractile dysfunction during hypoperfusion with norepinephrine is improved by pre-treated insulin, as is partial recovery of energy metabolism.

The incidence of heart failure (1) and the mortality rate (2) following myocardial ischemia are significantly greater in diabetic than in non-diabetic patients. Insulin with or without glucose and potassium has been used in the treatment of myocardial ischemia, but the effects of insulin are still controversial. In non-diabetic patients with ischemic heart disease, insulin showed beneficial effects such as diminution of the frequency of ventricular arrhythmias (3) and improvement of myocardial contractility (4) or in some patients, did not (5). In diabetic patients with ischemic heart disease, insulin contributed to a decrease in the mortality rate (6) or in other cases, did not (7). Thus, there are diverse reports on the effects of insulin in clinical studies. In an experimental study using hearts isolated from diabetic rats, a beneficial effect of insulin infusion on mechanical dysfunction during anoxia was observed (8).

In our previous studies (9, 10), the hearts isolated from diabetic rats were more vulnerable to hypoperfusion and more susceptible to norepinephrine (NE) under hypoperfusion in comparison with the hearts isolated from non-diabetic rats. These dysfunctions observed in diabetic hearts were improved to non-diabetic heart levels by daily administration of insulin. The diverse results obtained in clinical trials suggested that it would be worth investigating whether insulin infusion is effective on diabetic hearts hypoperfused with NE. In the present study, therefore, the effects
of ex vivo insulin on mechanical and metabolic dysfunctions during hypoperfusion with NE in hearts isolated from diabetic rats were examined, and the effectiveness of insulin infused before and after the onset of the dysfunctions was also evaluated.

Materials and Methods

Animals and treatment: Male Sprague-Dawley rats weighing 200–250 g (234±4 g, n=25) were used. After overnight fasting, a diabetic state was induced by a single intravenous injection of 60 mg/kg streptozotocin (STZ, Sigma) dissolved in physiological saline. The diabetic state was assessed when the nonfasting blood glucose level was more than 300 mg/dl 3 days after the STZ administration. The level was checked by a simplified enzymatic method (Glucoboy set, Eiken Chemicals Co., Ltd.). The animals were fed ad libitum on tap water and rat chow and sacrificed 11–12 days after STZ treatment. The body weight of the diabetic rats at this time was 246±3 g.

Heart perfusion: The animals were anesthetized with ether, and after laparotomy, the blood samples were obtained from the inferior vena cava. The plasma glucose level measured by the enzymatic method (Gluneo, Shinotest Co., Ltd.) was 485±13 mg/dl. Then after thoracotomy, the hearts were quickly removed and perfused with the Langendorff apparatus as described in the previous report (9). The perfusate was Krebs-Henseleit solution (pH 7.4, 36°C) containing 120 mM NaCl, 4.8 mM KCl, 1.25 mM CaCl2, 1.2 mM MgSO4, 1.2 mM KH2PO4, 11 mM glucose and 25 mM NaHCO3, equilibrated with 95% O2-5% CO2.

To measure the left ventricular pressure (LVP), a balloon was placed in the left ventricle, filled with water and connected to a pressure transducer (Nihon Kohden, TP-101T and AP-620G). The isometric contractile force (CF) was measured by a force-displacement transducer (Nihon Kohden, SB-1T) through a thread connected to the cardiac apex. Resting LVP and resting CF were adjusted to 0 mmHg and 1 g, and changes in resting LVP and resting CF from the levels just before hypoperfusion were represented as ΔmmHg and Δg, respectively. The first derivatives of developed LVP (±dP/dt) and developed CF (±dF/dt) were monitored by differentiators (Nihon Kohden, ED-601G). Coronary perfusion flow (CPF) was measured by collecting the perfusate drops from the hearts. The hearts were perfused at the coronary flow rate for maintaining the coronary perfusion pressure (CPP) at 55 mmHg using a rotary pump (Tokyo Rikakikai, MP-3B). CPP was measured using an electric manometer (Nihon Kohden, TP-200T and AP-621G). Heart rate (HR) was measured by a cardiotachometer (Nihon Kohden, AT-601G). The hearts were paced at 300 beats/min using an electronic stimulator (Nihon Kohden, SEN-3301).

Experimental protocol: As shown in Fig. 1, the diabetic hearts were divided into four groups. In the control group (n=6), the hearts were perfused at 55 mmHg of CPP for 90 min. Thirty minutes after normoperfusion, the hearts in the other three groups were exposed to hypoperfusion by reducing CPF to 2 ml/min/g heart wt. for 60 min. Six minutes after the onset of hypoperfusion, the perfusate was changed to that containing 10−6 M norepinephrine (NE, Sankyo Co., Ltd.). Insulin (2 mU/min/g heart wt., Actrapid, Novo) was infused using an infusion pump (Nipro, SP-60) 20 min before and during hypoperfusion (pre-treated group, n=7) or 30 min after the onset of hypoperfusion (post-treated group, n=6). Six hearts were hypoperfused without insulin (non-treated group, n=6). At the end of hypoperfusion, the hearts were quickly frozen in liquid nitrogen for measurement of tissue substrates as described previously (9).

In the preliminary experiments, resting LVP and resting CF in the hearts isolated from non-diabetic rats did not change during hypoperfusion with NE.

Determination of myocardial energy metabolites: The frozen left ventricular wall was divided into inner and outer layers, corresponding to endocardium and epicardium, respectively, and the dried tissue was extracted by 0.6 M perchloric acid. The mixture was centrifuged at 10,000 rpm for 15 min at 2°C, and the supernatant was used for determination of tissue energy metabolites. The method of Fiske-Subbarow modified by
Fig. 1. Experimental protocol. All hearts (n=25) were isolated from diabetic rats 11–12 days after an intravenous injection of streptozotocin (60 mg/kg). The hearts were paced at 300 beats/min and initially perfused at 55 mmHg of coronary perfusion pressure (CPP). In the control group (n=6), the hearts were perfused at 55 mmHg of CPP for 90 min. Thirty minutes after normoperfusion, the other three groups were exposed to hypoperfusion (2 ml/min/g heart wt.) by reducing coronary perfusion flow and then perfused with the perfusate containing 10^{-6} M norepinephrine (NE) from 6 min after the onset of hypoperfusion. Six hearts were hypoperfused without insulin (non-treated group). Insulin (2 mU/min/g heart wt.) was infused 20 min before and during hypoperfusion (pre-treated group, n=7) or 30 min after the onset of hypoperfusion (post-treated group, n=6). At the end of perfusion, the hearts were quickly frozen in liquid nitrogen (arrows) for determination of tissue energy metabolites.

Furchgott and De Gubareff (11) was employed for measuring creatine phosphate (CP) and inorganic phosphate (Pi). ATP was determined by the firefly luminescence method (12) using a Lumiphotometer (Laboscience, TD-4000). Lactate was measured by the enzymatic method (Lactate UV test, Boehringer-Mannheim Co.).

Statistics: Data were analyzed statistically with Student’s t-test and analysis of variance (ANOVA) for multiple comparison.

Results

Mechanical performance: In the control group, 30 min after normoperfusion at CPP of 55.4±0.7 mmHg, CPF was 7.1±0.3 ml/min/g heart wt., and the following 60-min perfusion did not cause significant change in CPF. Resting CF and resting LVP did not change throughout perfusion period. Developed CF was 2.9±0.2 g and developed LVP was 54.4±4.6 mmHg 30 min after normoperfusion, and both of them decreased slightly but significantly for 60 min (81% and 85% of the levels 30 min after normoperfusion, respectively). The peak ±dF/dt (+98±9 and -76±8 g/sec 30 min after normoperfusion) and the peak ±dP/dt (+2040±120 and -880±90 mmHg/sec 30 min after normoperfusion) also decreased slightly but significantly for 60 min (90% of the level 30 min after normoperfusion in +dF/dt and 66% in −dF/dt, 91% in +dP/dt and 82% in −dP/dt, respectively).

CPF and CPP just before hypoperfusion were not significantly different among the three hypoperfused groups and were similar to those in the control group. CPP decreased rapidly within 5 min of hypoperfusion to about one third of the level just before hypoperfusion, and after a temporal increase by NE, it was maintained at almost the pre-NE level throughout the hypoperfusion period in these three groups.

Changes in resting CF and resting LVP are shown in Fig. 2. In the non-treated group,
Fig. 2. Changes in resting left ventricular pressure (LVP) and resting contractile force (CF) in isolated diabetic rat hearts in the non-treated group (closed circle), pre-treated group (open circle) and post-treated group (open triangle). Resting LVP was adjusted to 0 mmHg and resting CF was adjusted to 1 g before hypoperfusion. The values just before hypoperfusion are expressed as 0. Vertical lines are S.E.M. *P<0.05 and **P<0.01: Significantly different from the non-treated group. Other explanations are the same as described in Fig. 1.

Fig. 3. Changes in developed CF and the peak ±dF/dt in isolated diabetic hearts. All explanations and symbols are the same as described in Figs. 1 and 2.

they began to increase within 15 min of hypoperfusion with NE, reached the maximum levels around 30 min after and maintained these levels throughout hypoperfusion (3.9±0.3 g and 9.9±1.4 mmHg at the end of hypoperfusion, respectively). In the pre-treated group, no elevations in resting CF and resting LVP were observed during hypoperfusion (0.3±0.1 g and −1.0±0.3 mmHg at the end of hypoperfusion, respectively). In the post-treated group, these elevations were similar to those in the non-treated group (3.1±0.4 g and 10.1±4.4 mmHg at the end of hypoperfusion, respectively).

Changes in developed CF and the peak ±dF/dt are shown in Fig. 3. There were no significant differences in these parameters just before hypoperfusion among the three groups except for the developed CF in the post-treated group. With induction of hypoperfusion, developed CF decreased rapidly; and after a temporal increase by NE, it decreased gradually and was maintained at a steady level 30 min after hypoperfusion in the non-treated group (17% of the level just before hypoperfusion at the end of hypoperfusion). The developed CF in the post-treated group decreased (22%) similarly to that in the non-treated group. In the pre-treated group, the decrease in developed CF during hypoperfusion was significantly attenuated. Changes in the peak ±dF/dt during hypoperfusion were similar to those in developed CF.

Changes in developed LVP and the peak ±dP/dt are shown in Fig. 4. There were no significant differences in these parameters
just before hypoperfusion among the three groups. With induction of hypoperfusion, developed LVP decreased rapidly; and after a temporal increase by NE, it decreased gradually and was maintained a steady level 30 min after hypoperfusion in the non-treated group (67% of the level just before hypoperfusion at the end of hypoperfusion) and in the post-treated group (70%). The developed LVP was significantly attenuated in the pre-treated group. There were no significant differences in the peak $-dP/dt$ among the three groups throughout hypoperfusion. The peak $+dP/dt$ in the pre-treated group was slightly smaller than that in the non-treated group at the end of hypoperfusion.

Energy metabolism: Tissue contents of ATP, CP, Pi and lactate are shown in Table 1. In the non-treated group, ATP and CP contents decreased significantly to 30% and 45% of the control levels in the LV inner layer and to 47% and 57% in the outer layer, respectively. Pi and lactate contents increased significantly in comparison with the control levels, to 2.4- and 3.2-fold in the inner layer and to 2.1- and 3.1-fold in the outer layer, respectively. ATP, CP and Pi contents in the inner layer were significantly different from those in the outer layer, respectively, in the non-treated and post-treated groups. In the pre-treated group, ATP and CP contents in the inner layer increased significantly to 53% and 56%, respectively; and Pi content in the inner layer decreased significantly to 1.9-fold, but did not in the post-treated group (to 36%, 49% and 2.3-fold, respectively) in comparison with those in the non-treated group. Lactate content in pre-treated and post-treated groups increased (to 4.5- and 4.3-fold in the inner layer and to 4.4- and 4.3-fold in the outer layer, respectively) more than that in the non-treated group.

Discussion

In non-diabetic hearts, it has been reported that myocardial ischemia increases the release of NE and that catecholamines at high concentrations probably further aggravate the cardiac function impaired at low coronary flow rate (13). At 2 ml/min/g heart wt. of coronary flow rate with $10^{-6}$ M NE in the present study, significant elevations in resting CF and resting LVP were observed in diabetic hearts but not in non-diabetic hearts. These results are identical with those obtained in our previous study (10). In the present study, ex vivo treatment of insulin prior to hypoperfusion improved cardiac dysfunction during hypoperfusion with NE: it prevented the elevations of resting CF and resting LVP, and it attenuated the depression of developed CF and the decreases in tissue contents of ATP and creatine phosphate in the LV inner layer, and increased the content of lactate in both the layers. Thus, ex vivo insulin showed beneficial effects on mechanical dysfunction and energy metabolic disturbance in the hearts isolated from diabetic rats.

Diabetic hearts have a suppressed glucose uptake (14) and a depressed glucose utilization due to inhibition of the activities of phosphofructokinase (15) and pyruvate dehydrogenase (16). It has been reported that insulin increases myocardial glucose uptake
Table 1. Effects of ex vivo insulin on tissue ATP, CP, Pi and lactate contents during hypoperfusion with norepinephrine in isolated diabetic hearts

| Group        | n  | ATP (μmol/g dry wt.) | CP (μmol/g dry wt.) | Pi (μmol/g dry wt.) | Lactate (μmol/g dry wt.) |
|--------------|----|----------------------|---------------------|--------------------|--------------------------|
|              |    | ENDO                 | EPI                 | ENDO               | EPI                      | ENDO     | EPI     | ENDO | EPI |
| Control      | 6  | 21.22±1.02           | 20.09±0.64          | 24.64±1.21         | 24.76±1.30              | 25.84±0.95| 26.05±1.03| 5.15±0.44| 4.98±0.38 |
| Non-treated  | 6  | 6.27±0.60            | 9.36±0.74**         | 11.19±0.56         | 14.07±0.61**            | 62.68±1.89| 53.96±1.19**| 16.40±1.20| 15.32±1.12 |
| Pre-treated  | 7  | 11.16±0.60**         | 11.31±0.62          | 13.71±0.86*        | 14.96±0.48              | 49.67±1.83**| 49.27±2.35| 23.36±1.46**| 22.15±1.96** |
| Post-treated | 6  | 7.66±0.75            | 9.44±0.42**         | 11.96±0.96         | 16.06±0.67**            | 58.72±3.08| 50.61±1.54*| 22.20±1.19*| 21.18±0.83* |

Each value shows the mean±S.E.M. Abbreviation: CP, creatine phosphate; Pi, inorganic phosphate; ENDO, subendocardium; EPI, subepicardium. All values in non-treated, pre-treated and post-treated groups are significantly different from the control values. *P<0.05 and **P<0.01: Significantly different from the non-treated group. *P<0.05 and **P<0.01: Significantly different from ENDO.
(14) and glucose utilization due to the stimulation of pyruvate dehydrogenase activity (16) in the diabetic hearts. In the present study, the further increase in lactate content by insulin might result from stimulation of glycolysis. The restoration of cardiac glucose metabolism might result in the increases in ATP and creatine phosphate contents in the LV inner layer. Thus, improvement of energy metabolism might contribute to improvement of mechanical dysfunction.

It is likely that insulin influences myocardial ion homeostasis through the restoration of energy metabolism and/or through the activation of the enzymes that are associated with intracellular ion homeostasis. In diabetic hearts, decreases in the activities of sarcoplasmic Na⁺-K⁺-ATPase (17) and sarcolemmal Ca²⁺-ATPase (18) have been observed, and the restoration of Na⁺-K⁺-ATPase activity to a normal level by ex vivo insulin (19) has been reported. In normal hearts, increases in sarcoplasmic reticular Ca²⁺-ATPase (20) activity by in vitro insulin have been also observed. The restoration of the activities of these enzymes in diabetic hearts by insulin may contribute to restoration of intracellular ion homeostasis, resulting in improvement of mechanical dysfunction during hypoperfusion with NE in the present study.

In addition, it has been reported that insulin pretreatment restored NE-induced myocardial damage in a pathological study using normal rats (22). As described in our previous study (9), NE aggravated the cardiac dysfunction during hypoperfusion in hearts isolated from diabetic rats. Therefore, it is also conceivable that such a protective effect of insulin on NE injury can be attributable to the beneficial effects in the present study.

In contrast to the beneficial effects of insulin prior to hypoperfusion, treatment with insulin after the maximum elevations of resting CF and resting LVP did not show any significant ameliorative effects on mechanical and metabolic dysfunctions during hypoperfusion with NE. This may be due to insufficient quantity of insulin given to diabetic hearts. It might be effective if the dosage of insulin was increased or insulin was given for a longer period. Another possible reason is that during the 30-min hypoperfusion with NE, severe changes in the myocardium might occur in our preparations as shown in non-diabetic hearts (23) in which the significant decrease in high energy phosphate and the elevation in intracellular pH were observed during 15-min no-flow. Thus, these possibilities might injure the activities of enzymes that are associated with energy metabolism and/or myocardial ion homeostasis, resulting in insufficient restoration by post-treated insulin.

Developed LVP in the non-treated and post-treated groups was higher than that in the pre-treated group in which the elevation of resting LVP was markedly lower than that in the former two groups. The depressed developed LVP in the pre-treated group would not be caused by the increase in lactate content because lactate also increased in the post-treated group in which developed LVP was the same as that in the non-treated group. These results are consistent with the results in our previous studies (9, 10), in which developed LVP was lower along with lower myocardial stiffness and higher lactate content in non-diabetic hearts than in diabetic hearts. Developed LVP may be associated with not only contractile function but also the resting condition of the hearts.

In conclusion, when ex vivo treatment with insulin is given prior to hypoperfusion, mechanical dysfunction during hypoperfusion with NE is significantly restored, while energy metabolism is partially improved in the hearts isolated from diabetic rats.

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