CARDIOPULMONARY SUPPORT AND PHYSIOLOGY

INSULIN STIMULATES PYRUVATE DEHYDROGENASE AND PROTECTS HUMAN VENTRICULAR CARDIOMYOCYTES FROM SIMULATED ISCHEMIA

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Impaired myocardial metabolism after cardioplegic arrest results in persistent anaerobic lactate production. Insulin may protect the heart from ischemia and reperfusion by enhancing myocardial metabolic recovery. However, the stimulation of glycolysis during ischemia may be detrimental because of an accumulation of metabolic end-products. We examined the effect of insulin on quiescent human ventricular cardiomyocytes subjected to simulated cardioplegic ischemia and reperfusion. *Methods:* **Primary cardiomyocyte cultures were established from patients undergoing corrective repair of tetralogy of Fallot. Cells were exposed to varying concentrations of glucose and insulin during 30 minutes of stabilization in 10 mL of phosphate-buffered saline solution. Ischemia was simulated by exposing the cells to a low volume (1.5 mL) of deoxygenated phosphate-buffered saline solution for 90 minutes followed by 30 minutes of simulated reperfusion in 10 mL of normoxic phosphate-buffered saline solution. Cell viability was assessed by trypan blue exclusion. The activity of mitochondrial pyruvate dehydrogenase was measured in 3 states: stabilization, ischemia, and reperfusion. In addition intracellular lactate, adenine nucleotides, extracellular lactate, pyruvate, and acid release were measured.** *Results:* **Higher ambient glucose concentrations resulted in greater cellular injury although insulin-treated cells displayed less injury after ischemia and reperfusion. Insulin increased the pyruvate dehydrogenase activity by 31% in cardiomyocytes and reduced extracellular lactate production by 40%. Intracellular adenosine triphosphate was improved by 75% in cells exposed to high glucose concentrations in the presence of insulin.** *Conclusions:* **Insulin protected human ventricular cardiomyocytes from ischemia and reperfusion. This protection may be due to a stimulation of pyruvate dehydrogenase activity which resulted in improved aerobic metabolism. (J Thorac Cardiovasc Surg 1998;116:485-94)**

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Cardioplegic arrest and aortic crossclamping during surgery induces anaerobic myocardial metabolism with a net production of lactate. In patients undergoing coronary bypass surgery, we observed persistent lactate release during reperfusion, suggesting a delayed recovery of normal aerobic metabolism.[1] In a previous canine model of cardioplegic arrest and reperfusion, cardiac metabolism was evaluated with lactate infusions labeled with radioactive carbon (^{14}C).[2] After cardioplegic arrest, there was ^{14}C-lactate extraction by the heart despite a net myocardial lactate release. This suggests that exogenous administration of lactate can alter myocardial substrate preference during cardioplegic arrest. However, the balance between lactate production

and lactate oxidation appears to favor anaerobic metabolism. In addition, the detection of myocardial lactate release grossly underestimates the true amount of anaerobic glycolysis because lactate produced by ischemic regions of the heart may be extracted by normally functioning myocardium. The extent of anaerobic lactate production correlated with depressed postoperative left ventricular function. Improving the transition from anaerobic lactate release to aerobic lactate oxidation during reperfusion may improve the tolerance to ischemia and enhance left ventricular function.

In 1965, Sodi-Pollaris and colleagues[3] demonstrated a beneficial effect of an insulin solution on the electrocardiographic abnormalities in patients with acute myocardial infarction. Since that initial report, several investigators[4-6] have attempted to use glucose and insulin solutions to improve myocardial tolerance to ischemia. Unlike acute myocardial infarction, cardioplegic arrest during a cardiac operation provides a unique clinical situation in which myocardial ischemia can be anticipated and metabolic interventions introduced to improve cellular susceptibility to ischemia. An early study by Hearse and colleagues[6] demonstrated a detrimental effect of exogenous glucose infusions during ischemic cardiac arrest. However, only a single cardioplegic infusion was administered, and the deleterious effects were attributed to an accumulation of metabolic end-products such as lactate and acid. Using a similar isolated, rat heart model, Doherty and colleagues[4] demonstrated a benefit of high doses of glucose and insulin by providing intermittent perfusion with hypothermic crystalloid cardioplegia. This model effectively "washed out" the end products of glycolysis and prevented the cellular acidosis. The controversy regarding the effects of glycolytic stimulation during ischemia continues.[7, 8] We hypothesized that glycolytic stimulation during a "low flow" cardioplegic arrest condition may have a beneficial effect on cardiomyocyte survival. Specifically, we believe that stimulation of mitochondrial pyruvate dehydrogenase (PDH) will allow an earlier recovery of aerobic metabolism after ischemia and improve cellular survival.

Kobayashi and Neely[9] demonstrated that the activity of mitochondrial PDH was inhibited during the first 2 minutes of reperfusion after ischemia and remained depressed for as long 45 minutes. Stimulation of PDH after ischemia may lead to an improved transition from anaerobic to aerobic metabolism. Weiss and Hiltbrand[10] demonstrated that ATP produced from oxidative phosphorylation was preferentially used for contractile function. Thus, enhanced myocardial metabolic recovery after ischemia may increase the ratio of aerobic to glycolytic ATP production and result in improved functional recovery. Insulin has been shown to stimulate mitochondrial PDH in adipocytes and hepatocytes.[11-13] We hypothesized that insulin would stimulate PDH in cardiomyocytes before ischemia and prevent the inhibition of PDH activity after ischemia.

This study used quiescent human ventricular cardiomyocytes to evaluate the effect of insulin on the cellular response to simulated ischemia and reperfusion. The quiescent nature of these cardiomyocytes simulates cardioplegic arrest. In addition, the isolated cell culture model removes the possible confounding effects of other cell types and organ systems. The effects of circulating free fatty acids[14] on myocardial substrate use are also absent in our model. Thus, this model permits an extensive metabolic evaluation of isolated human cardiomyocytes subjected to simulated low-flow cardioplegic arrest and reperfusion.

Methods

Human ventricular cardiomyocyte culture. Cultures of human ventricular cardiomyocytes were established as previously described.[15, 16] In brief, 20 mg biopsies were obtained from the right ventricle of patients undergoing corrective repair of tetralogy of Fallot. The myocardial biopsies were washed in phosphate-buffered saline solution without calcium (phosphate-buffered saline solution [PBS]: NaCl 136.9 mmol/L, KCl 2.7 mmol/L, Na_2HPO_4 8.1 mmol/L, KH_2PO_4 1.5 mmol/L). After removing connective tissue, the myocytes were separated with enzymatic digestion with a mixture of 0.2% trypsin (Difco Laboratories, Detroit, Mich.) and 0.1% collagenase (Worthington Biochemical Corp., Freehold, N.J.). The isolated cells were cultured at 37° C in 5% carbon dioxide and 95% air in Iscove's modified Dulbecco's medium (GIBCO laboratories, Grand Island, N.Y.) containing 10% fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.1 mmol/L β-mercaptoethanol. Purification was achieved with the dilution cloning technique. Enzymatically isolated cells were seeded at a low density (50 to 100 cells per 9-cm diameter culture dish). At this low density, individual cardiomyocytes can be distinguished morphologically by their rectangular shape and large size (40 × 80 μm) from contaminating cells such as fibroblasts and endothelial cells. Culture purity greater than 95% was demonstrated in passage 3 with a fluorescent monoclonal antibody staining for human ventricular myosin heavy chain (Rougier Bio-Tech Ltd., Montreal, Quebec, Canada). Cells passaged 2 to 7 times with a time from primary culture of less than 60 days were used for this study.

Experimental protocols. The cardiomyocytes were studied in PBS with magnesium and calcium ($MgCl_2$ 0.49 mmol/L, $CaCl_2$ 0.69 mmol/L). Our in vitro technique to simulate ischemia and reperfusion has been previously described in detail.[17, 18] Cells were stabilized for 30 minutes in 10 mL of normoxic (Po_2 = 150 mm Hg) PBS with varying concentrations of insulin and glucose. Cardioplegic ischemia was simulated by placing the cells into a sealed Plexiglas chamber flushed with 100% nitrogen and exposing the cells to a low volume (1.5 mL) of deoxygenated PBS. Deoxygenated PBS was prepared by degassing normoxic PBS with 95% nitrogen and 5% carbon dioxide until the measured Po_2 reached 0 mm Hg. Reperfusion was accomplished by exposure to 10 mL of normoxic PBS for 30 minutes. A small sample of deoxygenated PBS (2 mL) was placed into a center dish in the chamber to monitor temperature and to confirm the absence of oxygen at the end of the ischemic period. The temperature was maintained at 37° C throughout the experiment. The osmolality of the degassed PBS solutions was maintained between 280 and 320 mOsm/L with sodium chloride or water as required.

Assessment of cellular injury. Cellular injury was assessed with non-confluent plates of cardiomyocytes (approximately 337,000 cells per 9 cm diameter culture dish) cultured for 4 to 5 days after the last passage. After the intervention of interest, cell plates were incubated with 0.4% trypan blue dye (Sigma Chemical Company, St. Louis, Mo.) dissolved in normal saline solution and assessed for injury under an inverted light microscope (Nikon Canada Instrument Inc., Mississauga, Ontario) at ×200 magnification. Injured cells were unable to exclude the large molecular weight dye and stained blue. The number of blue-stained cells was counted from 5 standard locations on each plate and expressed as a percentage of the total number of cells. All counts were performed by a single observer who was blinded to the intervention.

After a dose-response analysis for both glucose and insulin, we chose to compare 4 groups. A physiologic glucose concentration of 5 mmol/L was compared with a glucose concentration of 100 mmol/L. The latter concentration approximates the glucose concentration in the cardioplegic solution currently used at our institution. The effects of 10 IU/L of insulin was then evaluated at both levels of glucose: group 1, 5 mmol/L glucose; group 2, 5 mmol/L glucose and 10 IU/L insulin; group 3, 100 mmol/L glucose; group 4, 100 mmol/L glucose and 10 IU/L insulin.

Biochemical measurements. The activity of PDH was measured after each intervention of interest with the modifications described by Robinson and colleagues[19] of the method of Sheu and colleagues.[20] Cell extracts were aliquoted into separate Eppendorf tubes containing PBS. After incubation for 10 minutes, cells were treated with a buffer containing 25 mmol/L sodium fluoride and snap frozen in liquid nitrogen. Cell extracts were then reacted with a ^{14}C-pyruvate containing buffer in an open Eppendorf insert and placed in sealed containers containing benzothonium hydroxide to trap $^{14}CO_2$. The reaction was stopped with 10% trichloroacetic acid, and $^{14}CO_2$ collected for 1 hour. After the $^{14}CO_2$ collection, the cell inserts were removed and scintillation fluid was added to the exposed benzothonium hydroxide. The collected

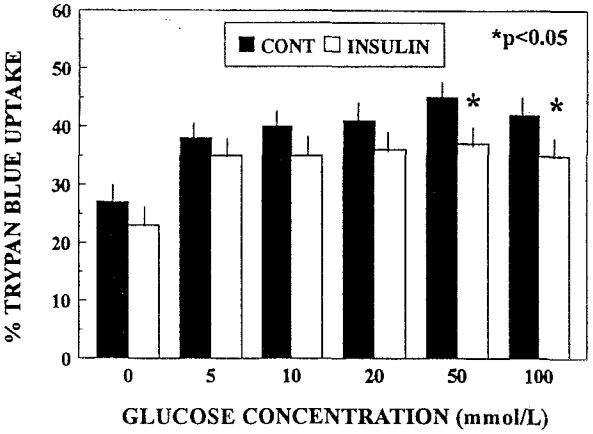

Fig. 1. The effect of glucose and insulin on cellular injury after simulated ischemia and reperfusion. (glucose effect: $F = 6.48$, $P < .0001$, by 2-way ANOVA). The addition of 10 IU/L of insulin at each glucose concentration reduced cellular injury (insulin effect: $F = 24.26$, $P < .0001$). Significant differences between insulin- and non-insulin–treated groups were specified at 50 and 100 mmol/L glucose.

$^{14}CO_2$ was then counted in a β-counter. PDH activity was calculated after correction for protein content and expressed as nanomoles of pyruvate oxidized per milligram of protein per minute.

Confluent cultures of cardiomyocytes (approximately 600,000 cells per culture dish) cultured for 5 to 10 days from the last passage were used for biochemical analysis. After being removed from the culture dish, the cardiomyocytes and the extracellular fluid recovered from each intervention were analyzed for lactate by an enzymatic method (Stat-Pack rapid lactate test kit; Behring Diagnostics, La Jolla, Calif.).

In a separate series of experiments, extracellular lactate and pyruvate were measured in the supernatant of each plate. Supernatants were collected and treated with a measured volume of 6% perchloric acid to inhibit enzymatic degradation. Lactate concentrations were determined as described earlier. Pyruvate concentrations were determined using a lactate dehydrogenase coupled reaction.[19] The concentration of hydrogen ion in the extracellular fluid was determined by measuring the pH value with a blood gas analyzer (1312 Blood Gas Manager; Instrumentation Laboratory, Milano, Italy).

Confluent plates of cardiomyocytes were used to determine cellular adenine nucleotide contents after the intervention of interest. The specimens were flash frozen in liquid nitrogen and then freeze-dried. Specimens were analyzed by high-performance liquid chromatography to determine the myocardial concentrations of adenosine triphosphate (ATP), adenosine diphosphate (ADP) and adenosine monophosphate (AMP). We also measured the metabolites adenosine, inosine, hypoxanthine and xanthine. Total adenine nucleotides were determined as the sum of ATP, ADP and AMP. Total degradation products

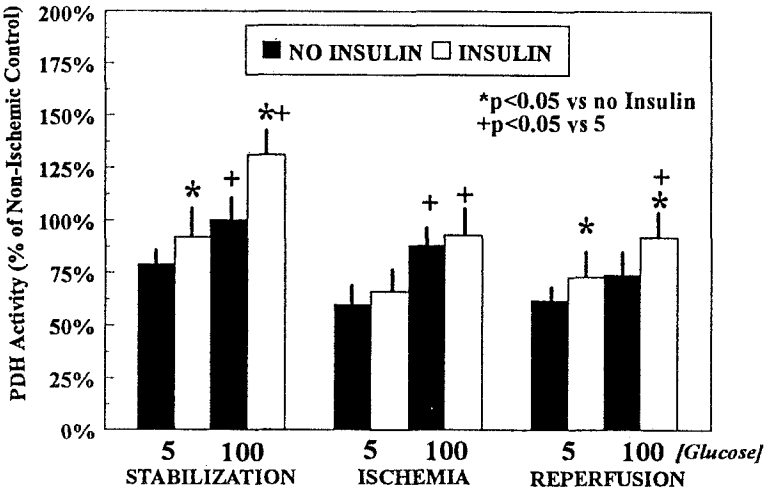

Fig. 2. PDH activity after 30 minutes of exposure to glucose and insulin *(stabilization),* 90 minutes of ischemia, and 30 minutes of reperfusion. Insulin exposure increased PDH activity at both levels of glucose before ischemia and prevented PDH inactivation after reperfusion. Results are expressed as a percentage of control values obtained at baseline or from cells exposed to equivalent volumes of normoxic PBS for equivalent time periods.

were calculated as the sum of adenosine, inosine, hypoxanthine, and xanthine. Energy charge represents the usable high-energy phosphate pool and was calculated as: energy charge = (ATP + 0.5 ADP)/(ATP + ADP + AMP). The DNA in the cell extracts was recovered in 5% perchloric acid and quantitated with a spectrophotometric, diphenylamine color reaction with calf thymus DNA as the standard.[1] Intracellular and extracellular lactate and adenine nucleotide values were then corrected for DNA content from each plate.[18]

Non-ischemic control cardiomyocytes were subjected to similar protocols with equivalent volumes of normoxic PBS (Po_2 = 150 mm Hg) for equal time periods as their ischemic counterparts. Baseline biochemical measurements were made after removing the culture media and washing the cells with normoxic PBS. In a separate series of experiments designed to eliminate the effects of glucose, cells were exposed to 5 or 100 mmol/L of mannitol with or without insulin.

Statistical analysis. The SAS Statistical Package (SAS Institute, Cary, N.C.) was used for analysis of all data. Data are expressed as the mean ± standard error with 8 plates per group, unless otherwise specified. Biochemical end points are expressed as a percentage of the value obtained from non-ischemic control plates exposed to an equivalent volume of normoxic PBS for the equivalent period of time. Analysis of variance (ANOVA) was used to simultaneously compare groups at different time periods. When statistically significant differences were found, they were specified by the Duncan's multiple range test.

Results

Assessment of cellular injury. An insulin dose response curve was performed with a glucose concentration of 5 mmol/L (physiologic) and 100 mmol/L (experimental). Increasing the insulin concentration from 0 to 100 IU/L did not reduce cellular injury at a glucose concentration of 5 mmol/L (0, 39% ± 3%; 10, 35% ± 2%; 50, 35% ± 3%; 100, 37% ± 2%; F = 0.27, P = .84 by ANOVA). However, when cells were exposed to a glucose concentration of 100 mmol/L, the addition of insulin reduced cellular injury (no insulin 48% ± 3% vs 10 IU/L insulin 37% ± 2%; F = 4.48, P = .01 by ANOVA). No further protection could be demonstrated by increasing the insulin concentration from 10 IU/L to 100 IU/L (50, 38% ± 1%; 100, 39% ± 2%; $P > .05$ compared with 10 IU/L insulin by Duncan's multiple range test).

A glucose dose-response curve (Fig. 1) demonstrated higher cell injury with increasing glucose concentration (by 2-way ANOVA, glucose effect F = 6.48, $P < .0001$). The addition of 10 IU/L of insulin at each glucose concentration reduced cellular injury (insulin effect F = 24.26, $P < .0001$). There was no interactive effect between glucose concentration and insulin treatment (glucose-insulin effect F = 2.05, P = .08). Significant differences between insulin- and non-insulin–treated groups were found at 50 and 100 mmol/L glucose.

Effect on PDH activity. Fig. 2 demonstrates PDH activity after stabilization, ischemia, and reperfusion. Insulin exposure increased PDH activity at

both levels of glucose. Cells exposed to 100 mmol/L glucose also increased PDH activity compared with 5 mmol/L glucose with or without insulin. PDH activity was inhibited after ischemia in the groups with 5 mmol/L of glucose, although those cells exposed to 100 mmol/L of glucose with or without insulin retained PDH activities near control levels. After reperfusion, only those cells exposed to 100 mmol/L of glucose ***and*** insulin retained normal PDH activities; all other groups had inhibited activity.

To eliminate a glucose effect, cells were subjected to 5 or 100 mmol/L of mannitol with or without 10 IU/L of insulin. Fig. 3 illustrates the ability of insulin to stimulate PDH independent of glucose.

Effect on intermediary metabolites. Fig. 4 *(top panel)* displays the intracellular lactate accumulation that was highest in cells exposed to 100 mmol/L of glucose and insulin during stabilization and reperfusion. However, after 90 minutes of ischemia, intracellular lactate accumulation in cells exposed to 100 mmol/L of glucose was lower when insulin was added. Ischemia and reperfusion increased intracellular lactate in all groups.

Fig. 4 *(middle panel)* illustrates extracellular lactate release measured in the supernatant over each plate. Ischemia resulted in a large increase in lactate release in all groups. Extracellular lactate release remained elevated after reperfusion in all groups, suggesting persistent anaerobic metabolism. Lactate release was lower in the insulin-treated cells.

Fig. 4 *(bottom panel)* displays total cellular lactate. Insulin treatment lowered total cellular lactate during stabilization, ischemia, and reperfusion. Similarly, extracellular pyruvate concentrations were lower in the insulin-treated cells. The lactate/pyruvate ratios were higher in the cells exposed to high concentrations of glucose. Table I displays the lactate, pyruvate, PDH, and pH measurements after each intervention.

Adenine nucleotides. The intracellular levels of adenine nucleotides and their metabolites are given in Table II. Fig. 5 illustrates the fall in ATP in all groups after ischemia. Intracellular ATP was best preserved in the group with 100 mmol/L glucose and insulin compared with the groups with 5 mmol/L glucose. Reperfusion resulted in only a small increase in intracellular ATP, with all 4 groups remaining well below non-ischemic control values. Similarly, ischemia resulted in a significant fall in total adenine nucleotides in all groups; however, they were best preserved in the group with 100 mmol/L glucose and insulin. After reperfusion, total adenine nucleotides were higher in the high-glucose groups but remained below 50% of control values.

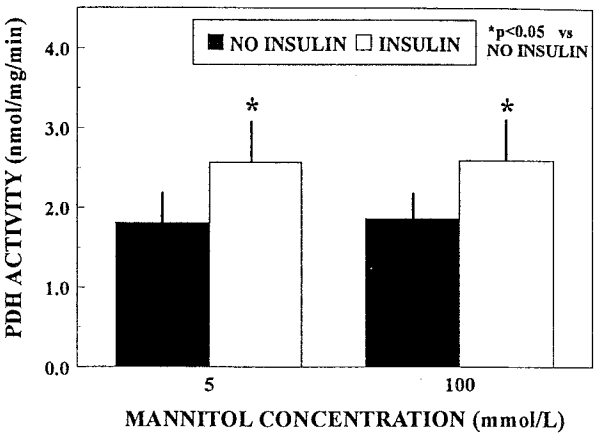

Fig. 3. PDH activity after 30 minutes of exposure to mannitol (5 or 100 mmol/L) and insulin (0 or 10 IU/L). Insulin resulted in similar PDH stimulation at both levels of mannitol, indicating that the stimulatory effect is independent of a glucose transport effect.

Discussion

During ischemia, fatty acid oxidation is inhibited, and glycolytic ATP production predominates. In a clinical study examining patients who were undergoing coronary bypass operation, we found that ^{14}C-labeled fatty acid oxidation was inhibited for as long as 60 minutes after cardioplegic arrest.[22] In addition, during reperfusion there is a delay in the recovery of normal aerobic glucose metabolism. Several clinical trials at our institution demonstrate persistent lactate release during reperfusion, which suggests continued anaerobic glycolysis.[1, 23, 24] Because net myocardial lactate release is an underestimate of total lactate production, due to a low level of continuous myocardial lactate extraction, we believe that cardioplegic arrest results in a significant impairment of normal aerobic metabolism. Improving the transition from anaerobic to aerobic metabolism after cardioplegic arrest should improve tolerance to ischemia and enhance the recovery of ventricular function.

Several investigators have attempted to stimulate glucose oxidation during reperfusion to improve myocardial functional recovery.[25-27] These authors used dichloroacetate to stimulate PDH activity that resulted in an improvement in glucose oxidation and myocardial functional recovery after ischemia. However, Mazer and colleagues[28] found that dichloroac-

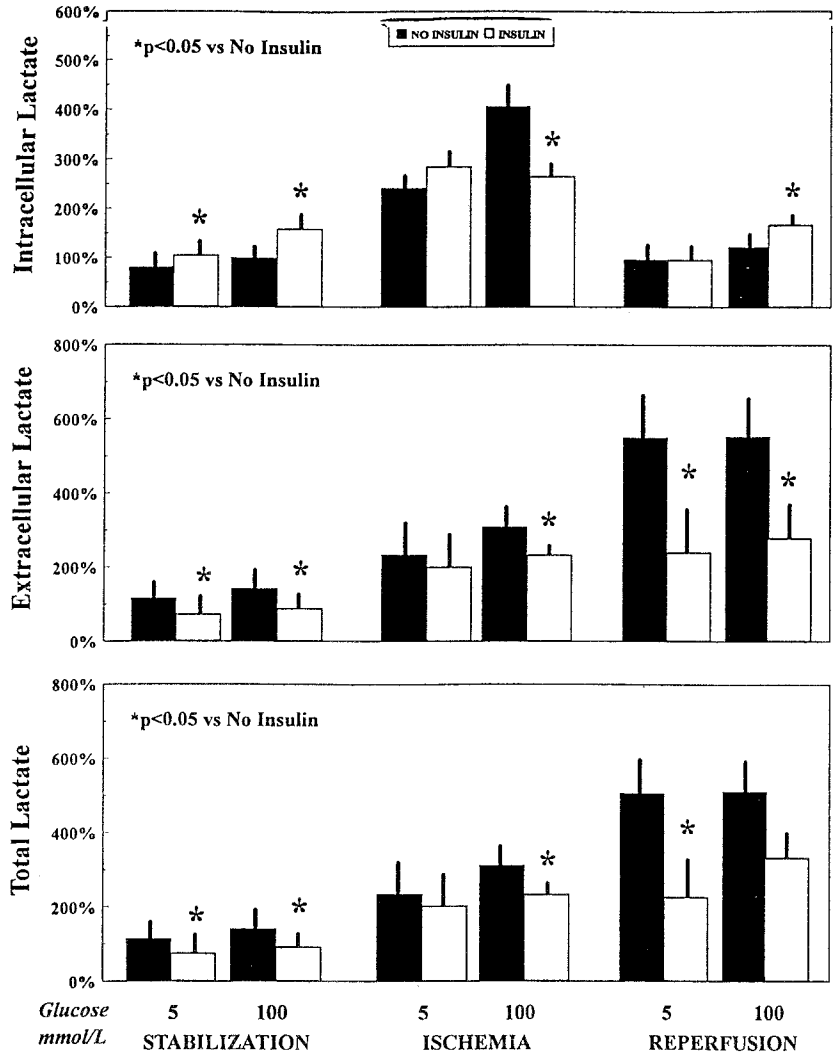

Fig. 4. Top panel, Intracellular lactate accumulation after 30 minutes of stabilization, 90 minutes of ischemia, and 30 minutes of reperfusion. Lactate extraction during stabilization and reperfusion was higher in group 4. However, lactate accumulation was lower in group 4 after ischemia. **Middle panel,** Extracellular lactate release into the supernatant over each plate. Lactate release increased significantly with ischemia and remained elevated during reperfusion, suggesting persistent anaerobic metabolism. Insulin treatment reduced lactate release at both glucose concentrations. **Bottom panel,** Total cellular lactate increases during ischemia and remains elevated during reperfusion. Insulin treatment reduced total cellular lactate during stabilization, ischemia, and reperfusion. Results are expressed as a percentage of control values obtained at baseline or from cells exposed to equivalent volumes of normoxic PBS for equivalent time periods.

etate did not improve systolic function in the in vivo porcine heart, despite an increase in glucose oxidation. Therefore alternative methods of PDH stimulation may prove to be superior to dichloroacetate in enhancing myocardial functional recovery after ischemia.

Insulin has been shown to stimulate PDH activity in adipocytes,[12] hepatocytes,[11] and cardiomyocytes.[13] The mechanism by which insulin stimulates PDH remains controversial. The PDH complex is regulated by a PDH phosphatase and a PDH kinase. Phosphorylation of the complex by PDH kinase renders it inactive, although dephosphorylation by the phosphatase returns the complex to its active form. Lilley and colleagues[13] found that insulin in bovine heart mitochondria preferentially stimulates

Table I. *Extracellular lactate and pyruvate production*

	Time period (min)				
	5	*5 + I*	*100*	*100 + I*	*Control*
Stabilization					
Lactate (mol/min/g)	1.6 (0.3)	1.2 (0.1)*	1.8 (0.2)	1.2 (0.2)*	1.5 (0.6)
Pyruvate (mol/min/g)	33 (5)	21 (3)*	30 (6)	19 (2)*†‡	36 (5)
Lactate/pyruvate ratio	52 (8)	60 (9)	63 (6)	65 (9)†	47 (5)
Extracellular pH	7.27 (0.04)	7.21 (0.03)*	7.19 (0.05)	7.21 (0.04)	7.25 (0.05)
PDH activity (nmol/mg)	0.76 (0.04)†	0.88 (0.09)*†	0.96 (0.13)‡	1.26 (0.12)*†‡	0.96 (0.09)
Ischemia					
Lactate (mol/min/g)	3.0 (0.6)†	2.7 (0.5)†	4.6 (0.5)†‡	2.9 (0.6)*†	1.1 (0.3)
Pyruvate (mol/min/g)	73 (11)	43 (8)*†	72 (10)	46 (8)*†‡	76 (10)
Lactate/pyruvate ratio	40 (3)†	64 (3)*†	65 (3)†	62 (20)†	20 (4)
Extracellular pH	7.10 (0.03)†	7.10 (0.04)†	7.03 (0.03)†	7.08 (0.04)†	7.24 (0.03)
PDH activity (nmol/mg)	0.62 (.04)	0.68 (0.04)†	0.91 (0.09)‡	0.96 (0.10)‡	1.03 (0.08)
Reperfusion					
Lactate (mol/min/g)	1.3 (0.4)†	1.0 (0.1)†	2.0 (0.3)†‡	1.2 (0.2)*†	0.4 (0.1)
Pyruvate (mol/min/g)	41 (6)†	27 (5)*†	42 (6)†	27 (6)*†‡	61 (5)
Lactate/pyruvate ratio	32 (5)†	40 (3)†	48 (4)†	47 (6)†	9 (1)
Extracellular pH	7.24 (0.04)	7.26 (0.03)	7.22 (0.04)	7.26 (0.03)	7.27 (0.03)
PDH activity (nmol/mg)	1.01 (0.11)†	1.20 (0.10)*†	1.22 (0.09)†	1.51 (0.08)*‡	1.64 (0.09)

All data are mean (SEM). Control plates assessed at baseline (stabilization) or after 120 minutes (ischemia) or 150 minutes (reperfusion) of incubation in normoxic phosphate-buffered saline solution.

*$P < .05$ vs no insulin.

†$P < .05$ vs control.

‡$P < .05$ vs 5.

the PDH phosphatase. In contrast, dichloroacetate causes PDH stimulation by inhibiting the PDH kinase.

The ability of pre-ischemic insulin exposure to stimulate post-ischemic PDH activity in human myocardial tissue has not previously been demonstrated. This study examined the clinically relevant effects of pre-ischemic exposure to insulin in the presence of physiologic or hyperglycemic concentrations of glucose. Insulin was found to stimulate PDH activity before ischemia and partially prevented the inhibition of PDH activity during reperfusion. In addition, insulin treatment reduced extracellular lactate release and improved the preservation of ATP. The beneficial metabolic effects of insulin were found to protect against cellular injury after simulated ischemia and reperfusion.

Cell culture model. The cardiomyocytes used in these studies have been extensively evaluated in previous reports.[15-18] These cardiomyocytes retain many characteristics of freshly isolated cells but have distinct differences. These cells become quiescent after enzymatic digestion and passaging. Other investigators [29] who have successfully cultured adult cardiomyocytes have reported that the cells lose their ability to contract. Despite an abundant supply of mitochondria and contractile proteins, the sarcomeres become disrupted during division and do not reestablish their characteristic functional format. However, the metabolic response of these cells after ischemia is similar to our intraoperative findings during cardiac operations.[1, 22-24] Therefore we believe that these cells provide a unique opportunity to evaluate the cellular response to ischemia and reperfusion and the effects of metabolic interventions such as insulin.

Simulated ischemia and reperfusion. We are able to produce a deoxygenated PBS solution with a measured Po_2 of 0 mm Hg.[17] Exposing cells to 90 minutes of ischemia resulted in significant cellular injury as assessed by trypan blue exclusion. Reducing the volume of the solution over the cells from 10 mL to 1.5 mL and exposure to an anoxic atmosphere resulted in an accumulation of the products of ischemic metabolism and a marked reduction in the extracellular pH. Therefore this model is similar to the effects of global ischemia on the heart. Unfortunately, the volume overlying the cells remains greater than the solution to which the cells are exposed during global ischemia and may actually represent a form of low-flow ischemia, analagous to limited cardioplegic perfusion.

We believe that our model represents low-flow ischemia rather than hypoxia because of the volume

Table II. *Intracellular adenine nucleotide concentrations*

	Time period (min)				
Nucleotide	*5*	*5 + I*	*100*	*100 + I*	*Control*
Stabilization					
ATP	1.9 (1.0)	2.7 (1.0)*	3.1 (1.5)*†	2.5 (0.9)	2.0 (1.0)
ADP	0.8 (0.2)*	1.0 (0.4)	0.9 (0.2)*	1.3 (0.3)	1.6 (0.3)
AMP	0.1 (0.1)*	0.3 (0.2)*	0.7 (0.4)	0.9 (0.3)	1.1 (0.2)
Inosine	0.4 (0.2)*	0.4 (0.2)*	0.6 (0.2)*	0.9 (0.3)	1.1 (0.2)
Adenosine	0.3 (0.2)*	0.4 (0.2)*	1.0 (0.3)	1.4 (0.5)	1.1 (0.3)
Energy charge	0.81 (0.08)	0.80 (0.12)	0.77 (0.15)	0.75 (0.13)	0.71 (0.10)
Ischemia					
ATP	0.3 (0.1))*	0.3 (0.1)*	0.2 (0.1)*	0.5 (0.2)*‡	1.8 (0.2)
ADP	0.2 (0.1)*	0.3 (0.1)*	0.2 (0.2)*	0.3 (0.1)*	0.8 (0.1)
AMP	0.1 (0.1)*	0.1 (0.1)*	0.1 (0.1)*	0.1 (0.1)*	0.8 (0.1)
Inosine	0.8 (0.2)	0.9 (0.4)	0.9 (0.6)	1.3 (0.3)*	0.5 (0.2)
Adenosine	0.2 (0.1)	0.4 (0.2)	0.4 (0.2)	0.6 (0.2)	0.3 (0.2)
Energy charge	0.72 (0.04)	0.64 (0.07)	0.71 (0.08)	0.70 (0.09)	0.65 (0.02)
Reperfusion					
ATP	0.4 (0.1)*	0.4 (0.1)*	0.6 (0.2)*	0.7 (0.2)*†	1.9 (0.4)
ADP	0.2 (0.1)*	0.2 (0.1)*	0.4 (0.1)*	0.4 (0.1)*	0.9 (0.2)
AMP	0.2 (0.1)*	0.2 (0.1)*	0.6 (0.2)†	0.3 (0.2)	0.5 (0.2)
Inosine	0.3 (0.1)*	0.5 (0.2)	0.7 (0.3)†	0.6 (0.1)	0.6 (0.3)
Adenosine	0.2 (0.1)*	0.2 (0.1)	0.3 (0.1)	0.3 (0.1)	0.4 (0.1)
Energy charge	0.61 (0.03)*	0.61 (0.06)*	0.57 (0.10)*	0.66 (0.07)‡	0.71 (0.07)

All values are reported as millimoles per gram DNA; mean (STD). Control plates assessed at baseline (stabilization) or after 120 minutes (ischemia) or 150 minutes (reperfusion) of incubation in normoxic phosphate-buffered saline solution.

*$P < .05$ vs control.

†$P < .05$ vs 5 mmol/L glucose.

‡$P < .05$ vs no insulin.

differences. When the cells are exposed to 90 minutes of hypoxia with 10 mL of deoxygenated PBS, the biochemical abnormalities are much less severe and cellular injury is only 25% to 30%, compared with approximately 45% with 1.5 mL of anoxic PBS.[30] Therefore the volume changes over the culture dishes result in a greater ischemic insult than occurs with simple hypoxia and reoxygenation. There was an increase in PDH activity after an increase in PBS volume to 10 mL in the control plates. This increase in PDH activity in the control plates was due to a decrease in the extracellular lactate concentration and a sharp fall in the lactate/pyruvate ratio causing inhibition of the PDH kinase. Similarly, stabilization in PBS with 5 mmol/L of glucose resulted in an inhibition of PDH activity, compared with cells recovered directly from culture media. This inhibition was associated by a rise in the lactate/pyruvate ratio. However, PDH activity was higher in cells exposed to high glucose or insulin.

Insulin's effect on PDH activity. We found that insulin treatment resulted in a stimulation of PDH activity. Similar findings have been reported by several authors in various organ systems.[11-13] Kobayashi and Neely[9] found that PDH activity was inhibited after ischemia within 2 minutes of reperfusion. We found that PDH activity was inhibited at the end of ischemia. Our technique of measuring PDH involves approximately 10 minutes of incubation in PBS. Thus, our measurement of PDH activity after ischemia may correlate with 10 minutes of reperfusion in Kobayashi and Neely's model. In addition, Kobayashi and Neely demonstrated a 55% inhibition of PDH activity during reperfusion. The magnitude of PDH inhibition in our model was much less severe (20% to 45% at end of ischemia). Cells treated with high glucose displayed better preservation of PDH activity after ischemia. High extracellular glucose concentrations may increase intracellular diacylglycerol levels with a resultant stimulation of protein kinase C. Benelli and colleagues[11] found that protein kinase C mediated insulin's ability to stimulate PDH activity in cultured hepatocytes. After 30 minutes of reperfusion, PDH activities were still depressed compared with control, non-ischemic values; however, the high-glucose– and insulin-exposed cells displayed a better recovery of PDH activity. Because our experimental protocol involved only a 30-minute exposure to insulin, we do not feel that insulin's stimulatory

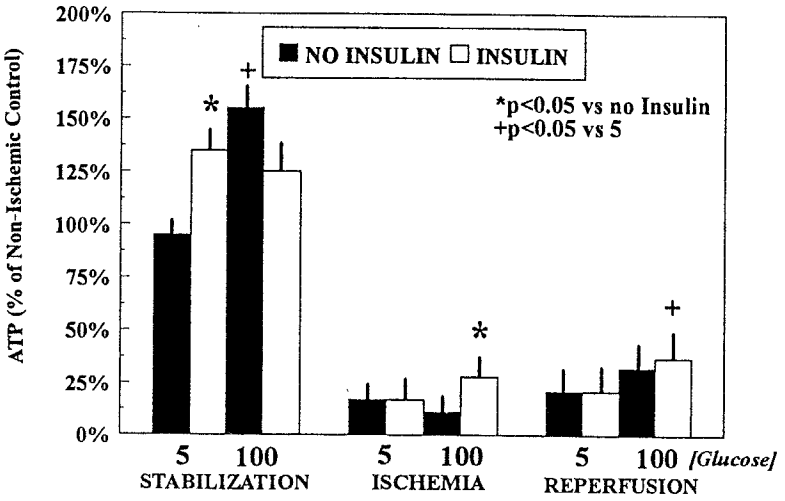

Fig. 5. Intracellular ATP levels after stabilization, after 90 minutes of ischemia, and after 30 minutes of reperfusion. ATP fell significantly in all groups but was better preserved in group 4 (100 mmol/L glucose and 10 IU/L insulin). Results are expressed as a percentage of control values obtained at baseline or from cells exposed to equivalent volumes of normoxic PBS for equivalent time periods.

effect involves an increase in protein synthesis. Rather, insulin exposure results in an increased activity of native PDH.

Insulin's effect on intermediate metabolites. Insulin-treated cells produced less lactate and pyruvate than non-insulin–treated cells. After ischemia, intracellular lactate accumulation was significantly higher in the 100 mmol/L glucose group. Insulin reduced intracellular lactate to levels similar to that in groups with 5 mmol/L of glucose. However, lactate extraction was higher after reperfusion in cells exposed to 100 mmol/L glucose and insulin. Both intracellular and extracellular lactate concentrations were significantly higher in all groups during reperfusion, compared with the non-ischemic controls. This persistent anaerobic glycolysis has been observed in patients who are undergoing cardiac operation.[1, 22-24]

Insulin's effect on adenine nucleotides. Insulin treatment resulted in a better preservation of intracellular ATP and total adenine nucleotides after ischemia and reperfusion. This may be due to the higher activity of PDH during reperfusion. Higher PDH activity improves the efficiency of ATP production by stimulating oxidative metabolism. However, both ATP and total adenine nucleotide levels remained well below control values. The improvement in high-energy phosphates may simply be due to higher preischemic values. However, preischemic ATP levels were higher in the 100 mmol/L glucose group than in the 100 mmol/L glucose and insulin group, yet ATP levels were better preserved in the latter group.

The ratio of oxidatively derived ATP to ATP derived from glycolysis may be higher in the insulin-treated group. We did not directly measure oxidative phosphorylation in these studies. Weiss and Hiltbrand[10] described a functional compartmentation of energy production in isolated rabbit hearts. They reported that aerobically derived ATP was preferentially used for contractile function whereas ATP derived from anaerobic glycolysis was used preferentially to support sarcolemmal function. We are unable to measure contractile function in this model. Demonstration of an improved transition from anaerobic to aerobic metabolism with an evaluation of the effect on contractile function will require further investigation.

Significance. We have demonstrated that preischemic exposure to insulin improves cellular tolerance to simulated ischemia and reperfusion in isolated human ventricular cardiomyocytes. We believe that this is the first report to demonstrate that in isolated human myocardial tissue:

1. The activity of PDH is inhibited by 40% after ischemia and remained depressed after 30 minutes of reperfusion.

2. Insulin stimulated PDH activity by 31% before ischemia and partially prevented the inhibition of PDH activity after reperfusion.

3. Insulin treatment reduced extracellular lactate release by 37% after ischemia and by 40% after reperfusion. Intracellular high-energy phosphate levels were improved by 75% in cells exposed to high glucose and insulin, and total pool size of purine nucleotides was increased.

The results of these investigations suggest that insulin treatment can enhance human myocardial PDH activity, improve the transition from anaerobic to aerobic metabolism, and result in improved cellular survival after ischemia and reperfusion. These findings may prove important in clinical situations of controlled ischemia, such as cardioplegic arrest during cardiac operations. Although we have performed an extensive evaluation of the metabolic response to ischemia, the results require confirmation in a whole organ model.

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