Role of glucose metabolism in the recovery of postischemic LV mechanical function: effects of insulin and other metabolic modulators

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Gandhi M, Finegan BA, Clanachan AS. Role of glucose metabolism in the recovery of postischemic LV mechanical function: effects of insulin and other metabolic modulators. Am J Physiol Heart Circ Physiol 294: H2576–H2586, 2008. First published April 11, 2008; doi:10.1152/ajpheart.00942.2007.—The role of proton (H+) production from glucose metabolism in the recovery of myocardial function during postischemic reperfusion and its alteration by insulin and other metabolic modulators were examined. Rat hearts were perfused in vitro with Krebs-Henseleit solution containing palmitate (1.2 mmol/l) and glucose (11 mmol/l) under nonischemic conditions or during reperfusion following no-flow ischemia. Perfusate contained normal insulin (n-Ins, 50 mU/l), zero insulin (0-Ins), or supplemental insulin (s-Ins, 1,000 mU/l) or other metabolic modulators [dichloroacetate (DCA) at 3 mmol/l, oxenfinecine at 1 mmol/l, and N6-cyclohexyladenosine (CHA) at 0.5 μmol/l]. Relative to n-Ins, 0-Ins depressed rates of glycolysis and glucose oxidation in nonischemic hearts and impaired recovery of postischemic function. Relative to n-Ins, s-Ins did not affect aerobic glucose metabolism and did not improve recovery when present during reperfusion. When present during ischemia and reperfusion, s-Ins impaired recovery. Combinations of metabolic modulators with s-Ins stimulated glucose oxidation ∼2.5-fold in nonischemic hearts and reduced H+ production. DCA and CHA, in combination with s-Ins, improved recovery of function, but addition of oxenfinecine to this combination provided no further benefit. Although DCA and CHA were each partially protective in nonischemic hearts perfused with n-Ins, optimal protection was achieved with DCA + CHA; recovery of function was inversely proportional to H+ production during reperfusion. Although supplemental insulin is not beneficial, elimination of H+ production from glucose metabolism by simultaneous inhibition of glycolysis and stimulation of glucose oxidation optimizes recovery of postischemic mechanical function.

proton production; ischemia-reperfusion; contractile function; rat hearts

MYOCARDIAL ISCHEMIA IS A COMMON cause of morbidity and mortality, and there continues to be a great opportunity for the development of new cardioprotective interventions to reduce ischemic injury and enhance recovery of left ventricular (LV) mechanical function during postischemic reperfusion. Inasmuch as ischemia is associated with marked metabolic alterations, acute drug-induced modification of myocardial metabolism may provide useful therapeutic approaches to lessen ischemia and reperfusion injury (28, 41).

Insulin has been extensively investigated as a potential cardioprotective agent (1, 3, 8, 11, 32, 37, 40), but its effectiveness remains unclear. An initial meta-analysis of insulin trials suggested that insulin, in combination with glucose and K+ (GIK solution), reduces mortality due to myocardial infarction (11), but additional meta-analyses concluded that GIK provides either benefit (5) or no benefit (21). A large randomized controlled trial (CREATE-ECLA) to evaluate GIK (2) found no beneficial effects (32). The balance of beneficial and detrimental effects of insulin supplementation and GIK may be affected by several factors, including changes in plasma glucose (10, 42) and fatty acid concentrations (30), as well as direct or indirect effects on myocardial energy metabolism (6, 28, 41). Thus there is no clear consensus on the cardioprotective mechanisms or the effectiveness of insulin supplementation.

Investigations of the direct effects of insulin on recovery of postischemic LV function show that supplemental insulin (1,000 mU/l, 6.7 nmol/l at reperfusion) elicits modest benefits (9, 15). Similarly, insulin (300–5,000 mU/l) administered during reperfusion reduces infarct size (22). However, these studies compared hearts perfused in the absence of insulin with hearts perfused in the presence of insulin, and although they indicate that zero insulin is deleterious, they do not provide evidence that addition of insulin above normal plasma concentrations is beneficial. Other studies comparing very high levels of insulin (100 nmol/l) with no insulin also provide evidence of protection (49). The first objective of the present study was to determine whether improvement of LV function can be obtained with insulin supplementation compared with physiological concentrations of insulin (0.3–0.8 nmol/l).

Although reperfused hearts are not energy deficient (25), energy substrate preference is an important determinant of ischemia-reperfusion injury (7, 28, 41). This is particularly important when hearts are exposed to high levels of fatty acid, which preferentially inhibit glucose oxidation and, thereby, further impair the mismatch in “glycolysis-glucose oxidation coupling.” This prolongs intracellular acidosis during reperfusion, increases the potential for Na+ accumulation and Ca2+ overload, and impairs recovery of postischemic LV function (7, 26). An important action of some metabolic modulators is to improve the relative rates of glycolysis (conversion of glucose to pyruvate) and the subsequent oxidation of pyruvate by pyruvate dehydrogenase and enzymes of the tricarboxylic acid cycle. This prevents postischemic impairment of glycolysis-glucose oxidation coupling and, therefore, limits intracellular acidosis and reduces the potential for Ca2+ overload and LV dysfunction (4, 7). Insulin also elicits numerous metabolic alterations (stimulation of glucose uptake, promotion of glycogen storage, and acceleration of glycolysis and glucose...
oxidation) (6), which may influence recovery of LV function.

A second objective of the present study was to compare the effects of supplemental insulin on the pathways of myocardial glucose metabolism when administered alone with the effects of supplemental insulin administered in combination with other metabolic modulators with different mechanisms of action.

Drug-induced reductions in proton (H⁺) production from glucose metabolism have been achieved by partial inhibition of glycolysis with adenosine (13) or adenosine A₁ receptor agonists [N⁶-cyclohexyladenosine (CHA)] (14, 2) by limitation of glycogenolysis (and glycolysis) with ingliflozin (46), 3 by stimulation of glucose oxidation directly with dichloroacetate (DCA) (25, 31), or 4 by stimulation of glucose oxidation indirectly with oxifenicine (OXF) (23). Each of these agents, when studied alone, causes a partial reduction in H⁺ production or a partial improvement in the recovery of LV function. The use of insulin in combination with other metabolic modulators provides an approach to examine further the hypothesis that H⁺ production from glucose metabolism impairs recovery of posts ischemic LV function and that drug-induced inhibition of H⁺ production is beneficial. This study also determined whether drug combinations might elicit greater improvements in recovery of posts ischemic LV function.

MATERIALS AND METHODS

Animal Care

The investigation conforms to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, Revised 1985) and the guidelines of the Canadian Council on Animal Care. In addition, the protocol was approved by the Animal Care and Use Committee of the University of Alberta.

Heart Isolation and Measurement of LV Work

Hearts from pentobarbital sodium-anesthetized Sprague-Dawley rats (300–350 g body wt) that had been fed ad libitum were excised, their aortae were cannulated, and perfusion with Krebs-Henseleit solution (37°C, pH 7.4) was promptly initiated. Hearts were perfused initially in Langendorff mode for 10 min. Thereafter, hearts were switched to ejecting mode (13, 14, 17) and perfused aerobically at a constant workload (11.5 mmHg preload, 80 mmHg afterload) and rate (paced at 300 beats/min). Systolic and diastolic aortic pressures (mmHg) were measured using a pressure transducer (model TSD104A, Harvard Apparatus) attached to the aortic outflow line. Ultrasound flow probes (model T206, Transonic Systems) placed in the left atrial inflow line and aortic outflow line measured cardiac output (ml/min) and aortic flow (ml/min), respectively. Coronary flow was calculated as cardiac output – aortic flow, and LV work was calculated as cardiac output x LV developed pressure (systolic pressure – preload pressure)/1,000 and served as a continuous index of LV function.

Composition of Heart Perfusates

The standard perfusate (100 ml recirculating volume) used for all control groups consisted of a modified Krebs-Henseleit solution [4.7 mmol/l KCl, 118 mmol/l NaCl, 1.2 mmol/l KH₂PO₄, 1.2 mmol/l MgSO₄, 2.5 mmol/l CaCl₂, 25 mmol/l NaHCO₃, 1.2 mmol/l palmitate (prebound to 3% BSA), 11 mmol/l glucose, and 50 mU/l insulin]. Insulin concentrations in randomly selected perfusate samples were analyzed using an INS-EASIA kit (Bio-source, Medicorp, Montreal, PQ, Canada). Insulin concentration in normal perfusate was 48 ± 4 mU/l (0.3 mmol/l, n = 12), which is comparable to the plasma concentration of healthy male Sprague-Dawley rats (42–48 mU/l) (43).

Initial studies examined the effects of two concentrations of glucose: 11 mmol/l (as in standard Krebs-Henseleit solution) and 5.5 mmol/l (a commonly used concentration in experimental heart perfusate that approximates the human plasma concentration in the fasted state). To examine the effects of insulin, groups were perfused with normal insulin (n-Ins, 50 mU/l, 0.3 mmol/l), zero insulin (0-Ins), supplemental insulin (s-Ins, 1,000 mU/l, 6.7 mmol/l), and a positive control (CHA, 0.5 μmol/l). Additional groups were perfused with combinations of s-Ins and other metabolic modulators, DCA (3 mmol/l) to inhibit pyruvate dehydrogenase kinase and stimulate glucose oxidation, oxifenicine (OXF, 1 mmol/l) to inhibit fatty acid β-oxidation and indirectly stimulate glucose oxidation, and CHA (0.5 μmol/l) to inhibit glycolysis, which were selected on the basis of previous reports that these agents individually produce submaximal improvements in recovery of LV function (14, 23, 25, 31). Finally, the potential benefits of optimal treatments with DCA (3 mmol/l, during reperfusion), CHA (0.5 μmol/l, during ischemia and reperfusion), or DCA + CHA were also investigated.

Perfusion Protocol

Aerobic (nonischemic) group. Unless indicated otherwise, all groups were initially perfused for 45 min (baseline) with standard Krebs-Henseleit solution containing 11 mmol/l glucose, 1.2 mmol/l palmitate, and n-Ins. Hearts were then frozen for biochemical analysis or perfused in aerobic (nonischemic) mode for a further 35 min (treatment) with standard perfusate (untreated control) or a modified perfusate.

Ischemia-reperfusion group. Unless indicated otherwise, all groups were perfused aerobically for 45 min (baseline) with standard Krebs-Henseleit solution containing 11 mmol/l glucose, 1.2 mmol/l palmitate, and n-Ins. Hearts were then subjected to global, no-flow ischemia followed by reperfusion (30 min). Duration of ischemia was chosen to produce mild ischemia (15 min), where recovery of LV function in untreated hearts was ~50% of preischemic values (so that increases or decreases in recovery could be easily detected), or severe ischemia (25 or 30 min), where recovery of LV function in untreated hearts was <30% of preischemic values. Except as indicated, hearts were exposed to drugs or drug combinations immediately at the onset of reperfusion.

Measurement of Steady-State Rates of Glucose Metabolism

Glycolysis and glucose oxidation were measured from the rate of production of ³H₂O and ¹⁴CO₂, respectively, from [⁵-3H]glucose and [U-¹⁴C]glucose (13, 14, 17). Samples (3 ml) of perfusate were withdrawn at 10-min intervals during baseline, nonischemic treatment and reperfusion periods. Rates (μmol [⁵-3H]glucose or [U-¹⁴C]glucose metabolized·g dry wt⁻¹·min⁻¹) were calculated for each perfusion phase from linear time courses of ³H₂O and ¹⁴CO₂ accumulation (indicative of steady-state conditions).

Calculation of H⁺ Production From Exogenous Glucose Metabolism

The hydrolysis of glycolytically manufactured ATP releases two H⁺ per molecule of glucose, whereas the hydrolysis of ATP produced by the oxidation of pyruvate consumes one H⁺ per molecule of pyruvate (2 H⁺ per molecule of glucose). Thus, if the rate of glycolysis exceeds the rate of pyruvate oxidation, the net production is two H⁺ per molecule of exogenous glucose that passes through glycolysis but is not subsequently oxidized. Therefore, the rate of H⁺ production attributable to the hydrolysis of ATP from glucose metabolism was calculated as 2 x (rate of glycolysis – rate of glucose oxidation).
GLUCOSE USE AND RECOVERY OF POSTISCHEMIC LV FUNCTION

Assay of Glycogen Content, Glycogen Synthesis, and Glucose Uptake

Glycogen content (μmol glucosyl units/g dry wt), glycogen synthesis (μmol glucose·g dry wt⁻¹·min⁻¹), and glucose uptake (μmol·g dry wt⁻¹·min⁻¹) were calculated as described previously (17). Briefly, glycogen in ~150–200 mg of frozen heart was separated from exogenous glucose by alkaline extraction with 30% KOH and then subjected to acid hydrolysis to release radiolabeled and unlabeled glucose; total (radiolabeled and unlabeled) glucose provided a measure of glycogen content, whereas radiolabeled glucose was used to calculate the rate of incorporation of exogenous glucose into glycogen (net glycogen synthesis). Glucose uptake was measured as the sum of the rates of glycolysis and glycogen synthesis.

Materials

d-[5-3H]glucose and d-[U-14C]glucose were purchased from PerkinElmer Life and Analytical Science (Boston, MA); BSA (fatty acid free) from Equitech-Bio (Kerrville, TX); insulin (Human Bio-synthetic Regular) from Novo Nordisk (Mississauga, ON, Canada); DCA from BDH (VWR Canlab, Mississauga, ON, Canada); OXF from Fluka Chemie; and CHA from Sigma-Aldrich (Oakville, ON, Canada). All other chemicals were reagent grade.

Statistical Analysis

Values are means ± SE of n hearts. Student’s t-test was used to determine the significance of differences between two groups, and one-way ANOVA was used to detect differences among three or more groups. When ANOVA revealed significant differences, Bonferroni’s multiple comparison test was used to correct for multiple comparisons. Differences were considered significant when P < 0.05.

RESULTS

Aerobic Perfusion

Effect of glucose concentration. Levels of LV work (8.2 ± 0.31·min⁻¹·mmHg⁻¹, n = 14) and coronary flow (24.0 ± 1.5 ml/min, n = 14) were stable in hearts perfused with Krebs-Henseleit solution containing palmitate, n-Ins, and 11 mmol/l glucose during baseline conditions. In hearts perfused with 5.5 mmol/l glucose, LV work was lower (6.6 ± 0.51·min⁻¹·mmHg⁻¹, n = 12, P < 0.01), but coronary flow was not significantly different (20.1 ± 2.0 ml/min, n = 12). After 45 min, normal in vivo levels of glycogen (~150 μmol/g dry wt) (48) were reestablished in hearts perfused with 11 mmol/l glucose, but not in hearts perfused with 5.5 mmol/l glucose (Table 1). Although glucose uptake, glycogen synthesis, and glucose oxidation were also lower in the hearts perfused with 5.5 mmol/l glucose, there were no significant differences in rates of glycolysis or H⁺ production (Table 1).

Effect of insulin. The effects of insulin on LV function and glucose metabolism were first determined during 45 min of aerobic perfusion (baseline). When heart perfusate contained n-Ins, baseline LV work was stable and similar to values reported previously (13, 14, 17). LV function was not affected by insulin (Fig. 1A); therefore, changes in glucose metabolism could be measured independently of changes in contractility and energy demand. Rates of glycolysis, glucose oxidation, and H⁺ production from glucose metabolism were 43% (P < 0.01), 52% (P < 0.0001), and 40% (P < 0.01) lower, respectively, in hearts perfused with n-Ins (n = 6) than in hearts perfused with n-Ins (n = 5; Fig. 1, B–D). Rates of glucose uptake and glycogen synthesis were also 45% (P < 0.001) and 50% (P < 0.0001) lower, respectively, in the 0-Ins group (Fig. 1, E and F), which limited time-dependent glycogen accumulation (Table 2) by 17% (P < 0.05). In additional groups that were perfused for a further 35-min aerobic period (treatment) with n-Ins (n = 7), 0-Ins (n = 6), or s-Ins (n = 6), LV work remained stable and similar (Fig. 1A). Hearts perfused with 0-Ins during baseline and treatment periods continued to exhibit lower rates of glycolysis (by 41%, P < 0.01), H⁺ production (by 46%, P < 0.01), and glucose uptake (by 39%, P < 0.05) than hearts perfused with n-Ins, but glucose oxidation and glycogen synthesis were not different. s-Ins did not affect glycolysis, glucose oxidation, or H⁺ production. Glycogen synthesis was increased by s-INS (Fig. 1F), but the absence of changes in glycogen content (Table 2) or glycolysis suggests that glycogen turnover was accelerated.

Effect of insulin-drug combinations. To identify insulin-drug combinations that optimize glucose utilization and, subsequently, could be tested for their ability to alter postischemic LV function, we first measured the effects of s-Ins in combination with agents shown previously to affect glycolysis or glucose oxidation in nonischemic hearts under conditions of stable and comparable LV work (Fig. 2A). Rates of glycolysis, glucose oxidation, and H⁺ production from glucose metabolism were similar in each group before drug treatments (see pooled metabolic data for baseline in Fig. 2, B–D). Although s-Ins per se did not stimulate glucose oxidation, s-Ins + DCA (3 mmol/l) + OXF (1 mmol/l) + CHA (0.5 μmol/l) substantially increased glucose oxidation (2.6-fold, n = 6, P < 0.001) relative to n-Ins (n = 6). Inasmuch as glycolysis was unaffected, this four-drug “metabolic cocktail” (s-Ins + DCA + OXF + CHA) improved glycolysis-glucose oxidation coupling and decreased the rate of H⁺ production from glucose metabolism by 74% (P < 0.001). s-Ins + DCA + OXF also stimulated glucose oxidation (2.5-fold, n = 6, P < 0.001), and again glycolysis was not significantly affected. This three-drug combination (s-INS + DCA + OXF) caused a smaller inhibition of H⁺ production (by 30%, P < 0.05) than the four-drug combination.

Table 1. Effect of perfusate glucose concentration on glucose uptake, glycogen synthesis, glycogen content, glycolysis, glucose oxidation, and H⁺ production

<table>
<thead>
<tr>
<th>Perfusate Glucose Concentration</th>
<th>11 mmol/l (n = 5)</th>
<th>5.5 mmol/l (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose uptake, μmol glucose·g dry wt⁻¹·min⁻¹</td>
<td>5.4 ± 0.2</td>
<td>4.4 ± 0.3*</td>
</tr>
<tr>
<td>Glycogen synthesis, μmol glucose·g dry wt⁻¹·min⁻¹</td>
<td>1.5 ± 0.05</td>
<td>1.06 ± 0.1*</td>
</tr>
<tr>
<td>Glycogen content, μmol glucose units/g dry wt</td>
<td>150.9 ± 8.6</td>
<td>105.6 ± 7.6*</td>
</tr>
<tr>
<td>Glycolysis, μmol·g dry wt⁻¹·min⁻¹</td>
<td>3.9 ± 0.3</td>
<td>3.3 ± 0.3</td>
</tr>
<tr>
<td>Glucose oxidation, μmol·g dry wt⁻¹·min⁻¹</td>
<td>1.14 ± 0.05</td>
<td>0.69 ± 0.11*</td>
</tr>
<tr>
<td>H⁺ production, μmol·g dry wt⁻¹·min⁻¹</td>
<td>5.6 ± 0.5</td>
<td>5.3 ± 0.7</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, number of hearts. Hearts were perfused with Krebs-Henseleit solution containing palmitate (1.2 mmol/l), normal insulin (n-Ins, 50 μU/ml), and glucose at 11 or 5.5 mmol/l. Glucose uptake, glycogen synthesis, glycolysis, glucose oxidation, and H⁺ production were measured during the 45-min aerobic baseline period, and glycogen content was measured in hearts frozen at the end of the 45-min aerobic baseline period. *Significantly different from 11 mmol/l glucose.
Instead, when OXF was removed from the four-drug combination, the increase in glucose oxidation was still demonstrable (2.5-fold, \( n = 11005 \), \( P = 0.001 \)). Glycolysis was unaffected, but \( H^+ \) production was inhibited by 48% (\( P = 0.001 \)).

Postischemic Reperfusion

**Effect of glucose.** When hearts were subjected to 15 min of ischemia, measurable LV work ceased. After 15 min of ischemia, the recovery of LV work was significantly lower in the low-glucose group [21 ± 14% (\( n = 6 \)) vs. 63 ± 5% (\( n = 10 \)), \( P < 0.005 \)]. Coronary flow in the low-glucose group was reduced to 32 ± 17% of preischemic values (from 22.3 ± 2.5 to 8.8 ± 5.5 ml/min, \( n = 6 \)), while in the 11 mmol/l glucose group it was only reduced to 85 ± 4% (from 26.5 ± 2.4 to 22.0 ± 1.9 ml/min, \( n = 10 \)). During reperfusion, rates (\( \mu \text{mol} \cdot \text{g dry wt}^{-1} \cdot \text{min}^{-1} \)) of glucose oxidation were lower in hearts...
Table 2. Glycogen content of hearts frozen after aerobic perfusion

<table>
<thead>
<tr>
<th>Duration of Working Mode Aerobic Perfusion</th>
<th>n-Ins</th>
<th>0-Ins</th>
<th>s-Ins</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 min</td>
<td>113.5±7.4</td>
<td>150.9±8.6</td>
<td>150.9±8.6</td>
</tr>
<tr>
<td>45 min</td>
<td>150.9±8.6</td>
<td>150.9±8.6</td>
<td>150.9±8.6</td>
</tr>
<tr>
<td>80 min</td>
<td>158.9±13.0</td>
<td>164.8±6.4</td>
<td>164.8±6.4</td>
</tr>
</tbody>
</table>

Values (μmol glucose units/g dry wt) are means ±SE of number of hearts in parentheses. Glycogen content of hearts frozen at the start of working mode perfusion (time 0) or after 45 min or 80 min of aerobic perfusion with Krebs-Henseleit solution (11 mmol/l glucose and 1.2 mmol/l palmitate) containing n-Ins (from 0 to 80 min), zero insulin (0-Ins, from 0 to 80 min), or supplemental insulin (1,000 mU/l, s-Ins; from 45 to 80 min after n-Ins from 0 to 45 min). *Significantly different from n-Ins.

Effect of insulin on hearts subjected to 15 min of ischemia.
During reperfusion following 15 min of ischemia, LV work (Fig. 3A) of untreated hearts recovered to 57 ± 4% (n = 9) and coronary flow to 91 ± 5% (n = 9) of preischemic values. However, in hearts perfused with 0-Ins, recovery of LV work after 15 min of ischemia was depressed (26 ± 13%, n = 6, P < 0.05), and coronary flow was reduced to 53 ± 17% (n = 6, P < 0.05). Similarly, in hearts exposed to s-Ins during reperfusion, recovery was also depressed (22 ± 11%, n = 6, P < 0.05) relative to n-Ins (Fig. 3A), with a reduction of coronary flow to 46 ± 6% (n = 6, P < 0.05). Thus, although removal of insulin is deleterious, supplemental insulin is also deleterious.

Effect of insulin on hearts subjected to 30 min of ischemia.
After more prolonged ischemia (30 min), LV work (Fig. 3C) in the untreated group recovered during reperfusion to 20 ± 7% (n = 7, P < 0.01) of preischemic values, which was less than that in n-Ins hearts subjected to 15 min of ischemia. Coronary flow was reduced during reperfusion to 53 ± 18% of baseline values. In hearts perfused throughout ischemia with 0-Ins, recovery of LV work was similar (19 ± 10%, n = 6; Fig. 3C). When hearts in which n-Ins was present during baseline and...
ischemic periods were reperfused with s-Ins, recovery of LV work was also not different (29 ± 10%, n = 5; Fig. 3C) from that in n-Ins hearts. CHA (0.5 μmol/l, present only during reperfusion) increased recovery of LV work to 54 ± 6% (n = 5, P < 0.05; Fig. 3C). Thus, under conditions when CHA was beneficial, reperfusion with s-Ins did not improve recovery of LV work. If s-Ins was introduced immediately before ischemia and present during ischemia and reperfusion, LV work recovered very poorly (6 ± 6%, n = 6). Coronary flow in this group was reduced to 30 ± 17% (from 21.6 ± 1.3 to 7.2 ± 4.4 ml/min, n = 6) but was sufficient for metabolic measurements.

In hearts reperfused after 15 or 30 min of ischemia in the absence or presence of n-Ins or s-Ins, rates (μmol·g dry wt⁻¹·min⁻¹) of glycolysis were similar and remained uncoupled from glucose oxidation (Table 3). Calculated rates of H⁺ production in all groups during reperfusion were unchanged (Fig. 3, B and D). However, administration of CHA at reperfusion inhibited H⁺ production by 36% (from 5.9 ± 0.5 to 3.8 ± 0.5 μmol·g dry wt⁻¹·min⁻¹, n = 5, P < 0.05).

**Effects of insulin-drug combinations.** After 30 min of ischemia, the four-drug combination (s-INS + DCA + OXF + CHA) caused a modest improvement in recovery of LV work from 20 ± 7% in n-Ins hearts to 54 ± 9% of preischemic values (n = 7, P < 0.01; Fig. 4A). Coronary flow also improved from 53 ± 18% (n = 7) in n-Ins hearts to 80 ± 14% (n = 7, P < 0.01). A three-drug combination (s-INS + DCA + OXF) depressed recovery of LV work to 8 ± 6% (n = 7, P < 0.01; Fig. 4C).

**Table 3. Effects of insulin on metabolism of exogenous glucose during reperfusion following 15 or 30 min of ischemia**

<table>
<thead>
<tr>
<th></th>
<th>Glycolysis, μmol·g dry wt⁻¹·min⁻¹</th>
<th>Glucose Oxidation, μmol·g dry wt⁻¹·min⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>15 min of ischemia</strong></td>
<td></td>
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<tr>
<td>n-Ins</td>
<td>3.7±0.7 (9)</td>
<td>0.67±0.14 (9)</td>
</tr>
<tr>
<td>0-Ins</td>
<td>4.2±0.9 (6)</td>
<td>0.32±0.13 (6)</td>
</tr>
<tr>
<td>s-Ins</td>
<td>4.1±0.4 (6)</td>
<td>0.32±0.06 (6)</td>
</tr>
<tr>
<td>s-Ins (I + R)</td>
<td>2.5±0.7 (6)</td>
<td>0.22±0.18 (6)</td>
</tr>
<tr>
<td><strong>30 min of ischemia</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n-Ins</td>
<td>3.1±0.3 (7)</td>
<td>0.19±0.06 (7)</td>
</tr>
<tr>
<td>0-Ins</td>
<td>2.4±0.5 (6)</td>
<td>0.15±0.08 (6)</td>
</tr>
<tr>
<td>s-Ins</td>
<td>2.8±0.5 (5)</td>
<td>0.40±0.13 (5)</td>
</tr>
<tr>
<td>s-Ins (I + R)</td>
<td>2.5±0.7 (6)</td>
<td>0.22±0.18 (6)</td>
</tr>
</tbody>
</table>

Values are means ± SE of number of hearts in parentheses. Hearts were perfused with Krebs-Henseleit solution containing palmitate (1.2 mmol/l) and glucose (11 mmol/l), and s-Ins was added at reperfusion or during both ischemia and reperfusion [s-Ins (I + R)].
Recovery of coronary flow was also reduced (to 26 ± 11%, n = 7) but was sufficient for metabolic measurements. Hearts treated with a combination lacking OXF (s-INS/DCA/CHA) exhibited recovery of LV work (78 ± 5%, n = 7, P < 0.001) and coronary flow (101 ± 5%, n = 7). During reperfusion, rates of glycolysis were similar in all groups (Fig. 4B). Glucose oxidation was markedly depressed in the untreated group (to 19% of baseline), but rates were higher [7-fold (P < 0.01) and 6-fold (P < 0.05), respectively] in the four-drug (s-INS + DCA + OXF + CHA) and three-drug (s-INS + DCA + CHA) groups (Fig. 4C). As a consequence, H⁺ production was 1.6-fold higher (P < 0.05) in hearts treated with the combination lacking CHA (s-INS + DCA + OXF). In contrast, hearts treated with the four-drug combination (s-INS + DCA + OXF + CHA) or the combination lacking OXF (s-INS + DCA + CHA) showed similar 50% reductions in the rates of H⁺ production (P < 0.01; Fig. 4D).

Effects of DCA, CHA, and DCA + CHA. Inasmuch as s-INS or OXF showed no marked benefit and the ability of CHA to inhibit glycolysis was impaired in the presence of s-INS, the effectiveness of DCA, CHA, and DCA + CHA was compared in hearts perfused with n-Ins (Fig. 5). During reperfusion after 25 min of ischemia, untreated hearts recovered to 34 ± 13% (n = 9) of preischemic values (Fig. 5A). DCA (administered at reperfusion) improved recovery of LV work (58 ± 9%, n = 9, P < 0.05, unpaired t-test). CHA (administered immediately before ischemia and present throughout ischemia and reperfusion) also improved recovery (77 ± 11%, n = 8, P < 0.05). However, hearts treated with DCA + CHA showed almost complete recovery (93 ± 2%, n = 9, P < 0.001; Fig. 5A). Coronary flows in each of these three treatment groups recovered to 92 ± 12%, 91 ± 13%, and 99 ± 2%, respectively, which were greater than in the untreated group (63 ± 19%, P < 0.05). Rates of glycolysis during reperfusion were lower [DCA by 43% (P < 0.05), CHA by 56% (P < 0.01), and DCA + CHA by 47% (P < 0.01)] than in n-Ins hearts. Although DCA stimulated glucose oxidation threefold (P < 0.05), CHA had no affect per se (Fig. 5C). When given in combination with DCA, CHA not only increased glucose oxidation sixfold (P < 0.001), but it also increased the rate twofold (P < 0.001) beyond that observed with DCA alone (Fig. 5C). Although the DCA-mediated stimulation of glucose oxidation and the CHA-mediated inhibition of glycolysis each caused an equivalent decrease in H⁺ production (by ∼70%, P < 0.01), their combination caused a total
suppression of H+ production from glucose metabolism (P < 0.001), an effect that was accompanied by a near-complete return of postischemic LV work.

To examine the relationship between H+ production and the percent recovery of LV work, data from each of the eight groups of hearts reperfused after prolonged ischemia (Figs. 4 and 5) were analyzed. There was a significant inverse relationship (P < 0.0001, n = 63), suggesting that recovery was improved in hearts with lower rates of H+ production from glucose metabolism (Fig. 6).

**DISCUSSION**

The present study indicates that when hearts are perfused with glucose and palmitate as energy substrates, the removal of insulin or lowering of glucose reduces glycogen content and glucose utilization and impairs recovery of postischemic LV function. However, relative to hearts perfused with a physiological concentration of insulin, the presence of supplemental insulin during reperfusion does not accelerate rates of glucose uptake or utilization and exerts no beneficial actions on the recovery of postischemic function. Indeed, under some conditions, supplemental insulin impairs recovery. Supplemental insulin in combination with other classes of metabolic modulators selected to accelerate rates of glucose oxidation reduces H+ production from glucose metabolism under nonischemic conditions. These drug combinations also reduce H+ production during reperfusion and improve postischemic function. Although supplemental insulin, DCA, OXF, and CHA have each been shown previously (14, 23, 25, 31) to enhance partially the recovery of postischemic function by different mechanisms, their combinations unexpectedly did not always result in an improved cardioprotective profile. The greatest beneficial effect, an increase in recovery from ~34% to >90%, was achieved in hearts perfused with n-Ins and a combination of agents that, via the simultaneous stimulation of glucose oxidation (DCA) and partial inhibition of glycolysis (CHA), caused a complete coupling of glycolysis to glucose oxidation. These results indicate that, rather than attempting to promote glucose uptake with insulin supplementation, a more effective strategy is to optimize glucose utilization in the presence of normal concentrations of insulin. Improvement in coupling of glycolysis to glucose oxidation suppresses H+ production and, therefore, limits intracellular acidosis dur-
solution containing 11 mmol/l glucose along with 1.2 mmol/l palmitate as energy substrates.

Insulin supplementation has been shown previously to elicit modest improvement in the recovery of posts ischemic contractility (9, 15, 49), but comparisons have often been made with hearts perfused in the absence of insulin. In the present study, when n-Ins was used as control, 0-Ins depressed glucose uptake and utilization in aerobic hearts, whereas s-Ins had no effect. The present demonstration of the deleterious consequence of 0-Ins, as well as s-Ins, on the recovery of LV function after 15 min of ischemia confirms earlier work (9, 15) and indicates that optimal insulin-induced benefits are achieved at close to physiological concentrations. When the duration of ischemia was increased to 30 min, there was, as expected, a greater depression of posts ischemic recovery, and the similar recovery in n-Ins, 0-Ins, and s-Ins groups provides no evidence for a deleterious or beneficial action of insulin. The failure of insulin to enhance recovery is not due to irreversible injury or to cell death, inasmuch as the adenosine A1 receptor agonist CHA, when given at the onset of reperfusion, elicits a significant improvement in posts ischemic LV function. Thus, although the complete absence of insulin is deleterious, possibly by limiting glycogen synthesis and pres ischemic glycogen content, insulin supplementation above physiological levels provides no additional benefit.

It should be noted that when heart perfusate contains both glucose and fatty acids, rates of acetyl-CoA production are not depressed in the posts ischemic period (25), indicating that impaired recovery of function is not due to limitation of energy supply. Consequently, any insulin-mediated acceleration of glucose utilization and any associated increase in ATP generation may be less important under these conditions. In support of this notion, insulin-mediated activation of glucose utilization in mouse hearts enhances recovery of posts ischemic LV function, but only when perfusate is devoid of fatty acids and the heart is reliant solely on glucose metabolism for ATP generation (16). When mouse heart perfusate contains a concentration of fatty acid (1.2 mmol/l) equivalent to that in patients following myocardial ischemia (27), insulin is no longer beneficial and then, paradoxically, impairs recovery of posts ischemic LV function. Similarly, in normal (nondiabetic) mouse hearts perfused in the presence of pulminate (0.7 mmol/l), insulin (300 mU/l) does not improve recovery of posts ischemic function (20). Clearly, fatty acid availability, in addition to having marked effects on ATP generation, glycogen homeostasis, and recovery of posts ischemic LV function (26), can also influence the balance between beneficial and detrimental effects of insulin (16).

Interestingly, the poorer recovery of LV work in hearts exposed to s-Ins during ischemia and reperfusion suggests that higher-than-normal insulin may exert deleterious actions during the actual ischemic period. This conclusion is supported by data showing detrimental effects of insulin supplementation during ischemia on cardiomyocyte viability (19) and on infarct size (22). Also, increased morbidity and mortality have been noted in some clinical studies (18). These adverse effects of s-Ins appear unrelated to changes in myocardial glucose utilization and require further investigation. Inasmuch as nonischemic function was unaffected by s-Ins, a direct negative inotropic effect (38) is unlikely to be involved. Insulin activates Na+–H+ exchange (36), and this effect may elicit further

The experimental approach using working rat hearts provided direct measurements of glucose uptake and utilization under physiological workload and energy demand (17). Rates of H+ production calculated from steady-state rates of glycolysis and glucose oxidation measured directly using dual-labeled (3H/14C) glucose provide an important index of the potential for intracellular acidosis and Na+ and Ca2+ overload (7, 44), a mechanism hypothesized to contribute to posts ischemic LV contractile dysfunction (4). Measurements of glucose metabolism in the absence of ischemia (normal aerobic workload) were performed to facilitate interpretation of whether alterations in glucose metabolism during reperfusion are a cause or a consequence of changes in LV work and energy demand.

Krebs-Henseleit solution normally contains 11 mmol/l glucose, but many heart perfusion studies now use 5.5 mmol/l glucose, presumably because this lower concentration approximates the normal human plasma level in the fasted state. However, normal rat values are intermediate (~8 mmol/l), and glucose concentrations may be much higher during periods of stress (45). Although LV work under aerobic conditions was slightly lower in hearts perfused with the lower glucose concentration (5.5 mmol/l), recovery of posts ischemic function was markedly depressed in these hearts. The lower recovery in the 5.5 mmol/l group cannot be attributed to differences in rates of glycolysis or H+ production, and the lower rate of glucose oxidation during reperfusion was likely related to the lower workload and energy demand. Instead, the poorer recovery of function may be related to the significantly lower rates of glucose uptake and glycogen synthesis before ischemia, which were unable to replenish glycogen to normal in vivo values (48). Glycogen is markedly depleted during the process of euthanasia and heart extraction, and, to reestablish normal baseline rates of glycogen turnover and glucose metabolism, it must be replenished during baseline perfusion (34). Inasmuch as glycogenolysis is an important source of substrate for glycolysis and ATP generation during ischemia and can affect energy availability (33), as well as glucose uptake (34), hearts in all subsequent groups were perfused with Krebs-Henseleit

Fig. 6. Correlation between recovery of LV work (expressed as percentage of preischemic baseline) and rate of H+ production from uncoupled glucose metabolism. Regression line was calculated from data obtained from individual hearts in 6 treated and 2 control groups (see Figs. 4 and 5; n = 63). For clarity, only means ± SE are shown for each group.
increases in postischemic Na\(^+\) accumulation and Ca\(^{2+}\) overload during reperfusion. Systemic, noncardiac actions may also influence the effects of insulin on postischemic LV function in vivo. Clearly, fatty acid availability is an important consideration (28, 41), and insulin-mediated decreases in lipolysis (12) and fatty acid plasma concentrations (30) may underlie some of its indirect beneficial effects. Similarly, suppression of hyperglycemia may also provide benefit in vivo (24, 42).

Inasmuch as insulin supplementation does not exert beneficial effects directly on myocardial glucose metabolism or H\(^+\) production, it was important to determine whether any beneficial effects could be achieved with insulin in combination with other metabolic modulators. Drugs representative of three previously well-documented classes that separately enhance postischemic function but have not previously been examined in combination were investigated in nonischemic and ischemic hearts. Although s-Ins per se (relative to n-Ins) does not accelerate glucose oxidation, “metabolic cocktails” comprising s-Ins in combination with DCA, OXF, or CHA exerted a marked (−2.5-fold) acceleration of glucose oxidation. That DCA is mainly responsible for this stimulation of glucose oxidation is supported by previous reports that 1) DCA per se elicits a severalfold acceleration of glucose oxidation in a similar model (31), and 2) CHA per se (14), s-Ins per se (present study), or removal of OXF from the four-drug combination (present study) does not alter glucose oxidation. Interestingly, in contrast to previous studies in CHA-treated hearts perfused with n-Ins (14) and by an as yet unexplained mechanism, glycolysis was not inhibited by CHA in the presence of s-Ins. Nevertheless, H\(^+\) production was partially inhibited, and recovery of postischemic LV work was partially improved. Similar benefits of modulation of glucose metabolism have been noted in a previous study (47) in which acceleration of glucose oxidation with a combination of high insulin (1,000 nU/l) and high glucose (30 mmol/l) increased the rate of recovery of postischemic function. However, because the treatment conditions in this study (47) were different during the preischemic period, it is not possible to determine whether the accelerated rate of recovery of function was due to an effect of insulin before ischemia, such as greater glycogen repletion after heart extraction.

Taken together, our data with s-Ins-drug combinations support the hypothesis that drug-induced inhibition of H\(^+\) production may be an effective approach to enhance recovery of postischemic function. This is further supported by data from the drug combination lacking CHA, which also caused a marked increase in glucose oxidation but inhibited H\(^+\) production to a lesser extent and was not protective. It is interesting that a greater benefit was observed for the drug combination lacking OXF, an agent with a cardioprotective action linked to inhibition of fatty acid oxidation (23, 39), and that cardioprotection was lost in the presence of OXF. Clearly, inasmuch as the heart derives most of its ATP production from fatty acid oxidation, excessive inhibition of this source of energy will limit the overall rate of acetyl-CoA production; hence, LV workload may become compromised. This may occur if the Randle cycle (35) is inoperative or if glucose oxidation has been maximally accelerated and cannot increase further to maintain energy availability. Indeed, deleterious effects of OXF on postischemic function (delay in recovery accompanied by increase in lactate release) have been noted previously (29).

Further support for the importance of glycolysis-glucose oxidation coupling is derived from n-Ins-perfused hearts in which graded reductions in H\(^+\) production from glucose metabolism were elicited in response to DCA or CHA, either alone or in combination. DCA and CHA each reduce H\(^+\) production by different mechanisms (14, 31). However, it has not been established whether combinations of these agents might have greater benefit. In the present study, their combination was highly effective in improving glycolysis-glucose oxidation coupling and eliminated H\(^+\) production from glucose metabolism. This effect translated into an almost complete recovery of postischemic LV work, an effect that may have contributed to the higher rate of glucose oxidation with the drug combination. It would be of interest to determine whether drug interactions were additive or synergistic, but this requires many more experimental groups and examination of a wider range of drug concentrations.

Since CHA and DCA each alter glucose metabolism in the absence of ischemia or alteration in workload (energy demand) (14, 31), the significant correlation between the recovery of LV work and the reduction in H\(^+\) production during reperfusion cannot be simply a consequence of improved contractility. Rather, inhibition of H\(^+\) production during the critical early period of reperfusion improves recovery of function. The clear relationship between H\(^+\) production from glucose metabolism and the recovery of LV function in hearts perfused with the various combinations of drugs that altered the coupling of glycolysis and glucose oxidation supports the hypothesis that the recovery of postischemic function is limited by glycolysis uncoupled from glucose oxidation and that optimization of glucose metabolism is a potential target to enhance recovery.

In summary, the concentration-response relationship for insulin is an important consideration in experimental evaluation of its potential to enhance recovery of postischemic function. In working rat hearts supplied with glucose and fatty acids as energy substrates, n-Ins appears optimal for the recovery of postischemic function. Improved recovery during reperfusion with optimized glycolysis-glucose oxidation coupling indicates that drug-induced alteration of glucose metabolism is a more effective approach to limit postischemic LV mechanical dysfunction.

GRANTS

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REFERENCES


