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An increase in the redox state during reperfusion contributes to the cardioprotective effect of GIK solution

I. W. Suranadi,1* L. Demaison,2,3,5† V. Chaté,3,5 S. Peltier,3,5 M. Richardson,4 and X. Leverve,3,5‡
1Udayana University Faculty of Medicine, Denpasar, Bali, Indonesia; 2Institut National de la Recherche Agronomique, Unité de Nutrition Humaine, Clermont Université, Clermont-Université, Clermont-Ferrand, France; 3Laboratoire de Bioénergétique Fondamentale et Appliquée, Université Joseph Fourier, Grenoble, France; 4Department of Population Health Sciences, School of Medicine and Public Health, University of Wisconsin, Madison, Wisconsin; and 5Laboratoire de Bioénergétique Fondamentale et Appliquée, INSERM, U1055, Grenoble, France

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An increase in the redox state during reperfusion contributes to the cardioprotective effect of GIK solution. J Appl Physiol 113: 775–784, 2012. First published July 12, 2012; doi:10.1152/japplphysiol.01153.2011.—This study aimed at determining whether glucose-insulin-potassium (GIK) solutions modify the NADH/NAD+ ratio during postischemic reperfusion and whether their cardioprotective effect can be attributed to this change in part through reduction of the mitochondrial reactive oxygen species (ROS) production. The hearts of 72 rats were perfused with fluids known to increase the cytosolic NADH/NAD+ production. The cardioprotection was also observed when the hearts were perfused with fluids (control and pyruvate groups). The hearts with a high mechanical dysfunction during reperfusion (45 min). From the beginning of ischemia, the perfusate was subjected to various changes: enrichment with GIK solution, enrichment with lactate (2 mM), enrichment with pyruvate (2 mM), enrichment with pyruvate (2 mM) plus ethanol (2 mM), or no change for the control group. Left ventricular developed pressure, heart rate, coronary flow, and oxygen consumption were monitored throughout. The lactate/pyruvate ratio of the coronary effluent, known to reflect the cytosolic NADH/NAD+ ratio and the fructose-6-phosphate/dihydroxyacetone-phosphate (F6P/DHAP) ratio of the reperfused myocardium, were evaluated. Mitochondrial ROS production was also estimated. The GIK solution improved the recovery of mechanical function during reperfusion. This was associated with an enhanced cytosolic NADH/NAD+ ratio and reduced mitochondrial ROS production. The cardioprotection was also observed when the hearts were perfused with fluids known to increase the cytosolic NADH/NAD+ ratio (lactate, pyruvate plus ethanol) compared with the other fluids (control and pyruvate groups). The hearts with a high mechanical recovery also displayed a low F6P/DHAP ratio, suggesting that an accelerated glycolysis rate may be responsible for increased cytosolic NADH production. In conclusion, the cardioprotection induced by GIK solutions could occur through an increase in the cytosolic NADH/NAD+ ratio, leading to a decrease in mitochondrial ROS production.

cytosolic NADH/NAD+ ratio; ischemia-reperfusion; isolated rat heart; glucose/insulin/potassium; mechanical dysfunction

GLUCOSE-INSULIN-POTASSIUM (GIK) solutions have been shown to protect the heart in several pathological situations such as acute myocardial infarction, cardioplegia, cardiac surgery, and septic shock (26, 37). In acute myocardial infarction, they are efficient when administrated at the beginning of reperfusion (15, 17, 29, 47) and the ischemic event is moderately severe (53). They decrease the mortality rate by 28% in patients with a reperfused myocardial infarction, particularly in diabetics (44). The beneficial effect of GIK is also observed in animal models (2, 4, 36). Several proposals were made to explain this effect (6): activation of ATP-sensitive potassium (KATP) channels (34), restoration of the metabolic pools resulting from glucose oxidation (50), reduced reactive oxygen species (ROS) production (10, 27), and insulin-mediated antiapoptotic activity (21, 28). Except for the restoration of the metabolic pools, the other potential metabolic effects of GIK solutions have not been well studied. Several investigators have shown that GIK solutions shift myocardial energy metabolism from long-chain fatty acids toward glucose as a fuel (18, 33, 41, 48). This could increase the cytosolic NADH/NAD+ ratio, since complete glucose degradation to CO2 leads to the production of NADH in the cytosolic space (glycolysis). The high amount of cytosolic reduced equivalent could then enter the mitochondria and favor the reduction of oxidized glutathione to its reduced form through glutathione reductase. This could thus increase the activity of glutathione peroxidase and improve the ROS scavenging activity of the mitochondria. The role of a high cytosolic NADH/NAD+ ratio on the recovery of mechanical activity during postischemic reperfusion has not been fully investigated yet.

The aim of this study was to examine first whether the cardioprotective effect of GIK solutions during reperfusion is associated with an increase in the cytosolic NADH/NAD+ ratio, then if this beneficial action is associated with a reduction of the mitochondrial ROS production, and, finally, if alterations of the cytosolic NADH/NAD+ ratio lead to the expected changes in the severity of reperfusion. To answer these questions, we first had to show that the cardioprotective effect of GIK solutions was associated with an increased cytosolic NADH/NAD+ ratio. This was performed in the isolated perfused rat heart subjected to global low-flow ischemia (0.5% of the preischemic coronary flow for 20 min) followed by reperfusion (45 min). The cytosolic NADH/NAD+ ratio was assessed by determining the lactate/pyruvate ratio of the coronary effluent (43). Second, we had to show that the GIK solution-induced increase in the cytosolic NADH/NAD+ ratio was associated with a decrease in the mitochondrial ROS production. This was carried out in isolated mitochondria purified from the postischemic myocardium of hearts perfused with or without a GIK solution. Third, we had to demonstrate that

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different substrates known to modulate the cytosolic NADH/NAD\(^+\) ratio triggered the expected changes in the recovery of mechanical function during reperfusion. On one hand, the addition of pyruvate (2 mM) to the basal perfusion medium containing glucose (5.5 mM) and hexanooate (0.5 mM) could allow the reduction of the cytosolic NADH/NAD\(^+\) ratio without decreasing glucose oxidation and the mitochondrial production of NADH. If so, this was expected to be associated with a worse mechanical recovery compared with the control group. On the other hand, the addition of lactate (2 mM) or pyruvate (2 mM) plus ethanol (2 mM) to the basal medium could allow the increase in the cytosolic NADH/NAD\(^+\) ratio, perhaps favoring the recovery of mechanical function during reperfusion. Because the cytosolic NADH/NAD\(^+\) ratio mainly depends on the glycolysis rate, the fructose-6-phosphate/dihydroxyacetone-phosphate (F6P/DHAP) ratio was estimated in the five experimental groups.

**Materials and Methods**

**Animal care.** The experiments followed the European recommendation guidelines for the use of laboratory animals and were approved by the local ethics review board (authorization number: 380537). Seventy-two 3-mo-old male Wistar rats (250–300 g) were housed in an animal facility with controlled temperature (24°C), hygrometry (60%), and 12:12-h light-dark cycle. They were fed a standard commercial diet (A04; Safe, Gannat, France) and received water ad libitum.

**Heart perfusion.** All the hearts were perfused during the active phase of the rats (from 12:00 PM to 8:00 AM). Animals were anesthetized with an intraperitoneal injection of pentobarbital sodium (50 mg/kg) and heparinized (1,000 IU/kg) through the saphenous vein. After a rapid thoracotomy, the heart was quickly collected and immediately immersed in a large volume of cold (4°C) Krebs-Heinselet buffer. The heart was then perfused according to the Langendorff mode (35) with a control Krebs-Heinselet buffer containing (in mM) 129 NaCl, 2.4 MgCl\(_2\)-6H\(_2\)O, 21 NaHCO\(_3\), 4.5 KCl, 2.5 CaCl\(_2\)-2H\(_2\)O, and various substrates according to the experiments performed. The time between chest opening and heart mounting on the Langendorff apparatus never exceeded 1 min, thus limiting the risk of cellular damage or preconditioning. During the perfusion, the Langendorff apparatus never exceeded 1 min, thus limiting the time between chest opening and heart mounting on the Langendorff apparatus.

**Experimental protocol.** In a first set of experiments, 50 rats were randomly divided into 5 groups of 10 animals, and their hearts were perfused with the control Krebs-Heinselet buffer containing glucose (5.5 mM) and hexanooate (0.5 mM) as a hydroxysoluble fatty acid for 30 min. After that period, the thermostatized chamber was filled with control Krebs-Heinselet buffer maintained at 37°C, and the hearts were subjected to a 20-min global low-flow ischemia at 0.5% of the preischemic coronary flow. It is known that this level of residual coronary flow is adequate to produce an ischemia of low severity in our experimental conditions. A 45-min reperfusion then followed. At the beginning of the reperfusion, the thermostatized chamber surrounding the heart was emptied. During the ischemic and reperfusion periods, the hearts were perfused with one of the five following buffers: (1) control Krebs-Heinselet buffer (C group), (2) control Krebs-Heinselet buffer supplemented with lactate (2 mM; L group), (3) control Krebs-Heinselet buffer supplemented with pyruvate (2 mM; P group), (4) control Krebs-Heinselet buffer supplemented with pyruvate (2 mM) and ethanol (2 mM) (PE group), and (5) GIK-like buffer similar to the control buffer except that the KCl and glucose concentrations were adjusted to 5.6 and 11 mM, respectively, and insulin (10 IU/l) was added.

Aliquots of the coronary effluent were collected throughout (just before ischemia and after 5, 15, 30, and 45 min of reperfusion). They were then stored at −80°C for determination of the lactate and pyruvate concentrations. At the end of the reperfusion period, the hearts were freeze-clamped at the temperature of liquid nitrogen and stored at −80°C until the biochemical analyses were performed. In a second set of experiments, 12 rats were randomly divided into 2 groups of 6 animals, and their hearts were subjected to the same ischemia-reperfusion protocol except that the reperfusion duration was limited to 30 min. The two media used during the ischemia and reperfusion periods were the control medium with glucose (5.5 mM) and hexanooate (0.5 mM) and the GIK-like buffer previously described. At the end of the 30th minute of reperfusion, the hearts were collected and mitochondria were immediately prepared to determine their oxidative properties and capacities of ROS production.

Finally, in a third set of experiments, 10 hearts were perfused with three control media with glucose (11 mM) for 20 min. Thereafter, the medium was changed three times by three other media containing one of the following substrates: hexanooate (0.5 mM), lactate (2 mM), or pyruvate (2 mM). Each substrate was perfused for a period of 10 min. The rank in which the substrates were perfused was changed regularly to allow good comparison of the effect of each substrate on the cardiac function. At the end of each period, the heart rate, LVDP, RPP, coronary flow, oxygen consumption, and cardiac metabolic efficiency (RPP-to-oxygen consumption ratio) were evaluated.

**Mitochondria preparation.** After the 30-min reperfusion, the atria and remaining aorta were cut off from the heart. Myocardium was minced with scissors in a cold isolation buffer composed of (in mM) 150 sucrose, 75 KCl, 50 Tris-HCl, 1 KPO\(_4\), 5 MgCl\(_2\), 1 EGTA, pH 7.4, and fatty acid-free serum albumin (0.2%). The pieces of myocardium were rinsed several times on a filter and put in a Potter-Elvehjem homogenizer containing 15 ml of isolation buffer. A protease (0.02% subtilisin) was added to digest myofibrils for 1 min at ice-cold temperature, and the totality was then homogenized (300 rpm, 3–4 transitions). Subtilisin action was stopped by the addition of isolation buffer (30 ml). The homogenate was then centrifuged (800 g, 10 min, 4°C), and the resulting supernatant was collected and filtered. Mitochondria were then washed through two series of centrifugation (8000 g, 10 min, 4°C). The last pellet of mitochondria was resuspended in sucrose (250 mM), Tris-HCl (10 mM), and EGTA (1 mM), pH 7.4, at the approximate concentration of 20 mg/ml.

**Respiration measurements.** The mitochondrial oxidative phosphorylation was determined by evaluating the rate of mitochondrial oxygen consumption at 30°C with a Clarke-type O\(_2\) electrode. The
respiration medium contained (in mM) 125 KCl, 20 Tris·HCl, 3 KH₂PO₄, 0.05 EDTA, 0.01 CaCl₂, pH 7.2, and fatty acid-free bovine serum albumin (0.15%). All measurements were performed on mitochondria (0.2 mg mitochondrial protein/ml) incubated with freshly prepared pyruvate (5.5 mM)/malate (2.5 mM) or DL-palmitoylcarnitine (50 μM)/malate (2.5 mM), without or with ADP (100 mM; state III) and then oligomycin (1 μM; state IV). The incubation medium was constantly stirred with a built-in electromagnetic stirrer and bar flea. Coupling of the mitochondrial oxidative phosphorylation was assessed by the state III/state IV ratio, which measures the degree of control imposed on oxidation by phosphorylation (respiratory control ratio, RCR).

Mitochondrial ROS release. The rate of mitochondrial production of H₂O₂ was measured at 30°C following the linear increase in fluorescence (excitation at 560 nm, emission at 584 nm) due to enzymatic oxidation of Amplex red by H₂O₂ in the presence of horseradish peroxidase on a F-2500 computer-controlled Hitachi fluorometer. Reaction conditions were 0.25 mg/ml mitochondrial protein, 5 U/ml horseradish peroxidase, and 1 μM Amplex red, with pyruvate (5.5 mM)/malate (2.5 mM) and/or DL-palmitoylcarnitine (50 μM). Values are means ± SE. The number of experiments was 10 per group; C, control group; L, lactate group; G, glucose-insulin-potassium (GIK) group; P, pyruvate group; PE, pyruvate + ethanol group. LVDP, left ventricular developed pressure; heart rate; RPP, rate-pressure product; CF, coronary flow; ANOVA, analysis of variance; NS, not significant.

![Graphs showing the effects of glucose-insulin-potassium (GIK) solution on heart rate (A), diastolic pressure (B), left ventricular developed pressure (LVDP; C), and rate-pressure product (RPP; D) during perfusion with control medium (C) or medium enriched with GIK solution (G). The number of experiments was 10 per group. S ef., substrate effect; T ef., effect of reperfusion duration; CI, cross-interaction between those 2 factors; NS, not significant.](http://jap.physiology.org/)
μM)/malate (2.5 mM) added to start the reaction in the same incubation media used for measurements of mitochondrial oxygen consumption. Mitochondrial ROS were measured in the absence of ADP (state II respiration rate). Rotenone (1 μM) and antimycin A (0.5 μM) were sequentially added to determine the maximum rate of H₂O₂ production of complexes I and III, respectively, of the respiratory chain.

Biochemical analysis. Lactate and pyruvate in the coronary effluent were evaluated spectrophotometrically according to the method of Bergmeyer (5) by using the transformation of each compound in the other by lactate dehydrogenase and estimating the appearance or disappearance of NADH.

F6P and DHAP were assayed in the heart after perchloric acid extraction. The two compounds were evaluated fluorimetrically according to the methods described by Bergmeyer (5).

Statistical analysis. Results are means ± SE. The data describing the cardiac functioning and those representing the fluxes of lactate and pyruvate in the coronary effluent were subjected to a repeated-measures analysis of variance with the composition of the ischemic and reperfusion buffer as external factor and the reperfusion time as internal factor. This analysis described the effect of the composition of the perfusion buffer (group effect), that of the reperfusion time (time effect), and the cross-interaction between these two factors. When necessary, the means were compared using a two-way Fisher’s least significant difference test. The results of the myocardial F6P and DHAP contents, mitochondrial function, and perfusion in aerobic conditions with the different substrates were submitted to one-way analysis of variance, describing the effect of the composition of the perfusion buffer (group effect). The means were compared using a one-way Fisher’s least significance difference test. P < 0.05 was considered significant. All the calculations were performed using NCSS 2007 software.

RESULTS

Cardiac function during the stabilization period. In preischemic conditions, all the hearts were perfused with the control buffer. Since the cardiac weight and the temperature of the perfusion fluid were similar in the different groups (general mean = 1.17 ± 0.02 g), the cardiac mechanical activity was similar in all the groups (Table 1). Because slight differences existed, however, the results describing the cardiac function during the reperfusion are expressed as percentages of the preischemic value.

Effect of GIK solution on ischemia and reperfusion dysfunctions. During ischemia, the systolic pressure rapidly dropped to the level of the diastolic pressure until the end of ischemia. From the 5th minute of ischemia, the diastolic pressure then progressively increased to a value that was maximal at the end of the
The results of state II, III, and IV respiration rates are expressed in ng atoms control; G, hearts perfused with GIK solution during ischemia and reperfusion. The lactate/pyruvate ratio, reflecting the cytosolic NADH/NAD⁺ (16% at the 45th minute of reperfusion). Interestingly, the higher from the 15th minute until the end of reperfusion lower in the G group during the first 4 min of reflow, it became B consumption behaved differently (Fig. 2). Although it was

Effect of GIK solution on the function of mitochondria purified from reperfused myocardium. Mitochondria were collected from the myocardium of the C and G groups after 30 min of postischemic reperfusion. Their capacities to consume oxygen and to produce ROS are presented in Table 2. As shown by the high RCR value observed with pyruvate (~8.5), they were perfectly functioning. The GIK solution did not alter their respiratory parameters. ROS production was also generally unaltered, but a GIK solution-induced decrease in the maximal rate of ROS production at the level of complex III of the respiratory chain (~20%) was observed when palmitoylcarnitine was used as a substrate. Furthermore, the yield of ROS production normalized to the oxygen consumption during state II respiration rate was interestingly reduced by 29% when pyruvate was used as a substrate. This was not the case with palmitoylcarnitine. Similar results were obtained when the yield of ROS production was calculated with state IV respiration rate (data not shown).

Effect of changes in the cytosolic redox state on ischemic contracture. At the end of ischemia, the diastolic pressure reached levels ranging from ~600 to 1,200% of the preischemic value, depending on the group studied (Fig. 4). Interestingly, the composition of the perfusion buffer modulated the ischemic contracture. As soon as the 5th minute of ischemia, the P and PE groups displayed higher diastolic pressures than that measured in the C group. This difference was roughly maintained during all the ischemic period. The diastolic pressure of the L group was close to that evaluated in the C group and was generally lower than those of the P and PE groups.

Effect of changes in the cytosolic redox state on reperfusion dysfunctions. As shown in Fig. 5, the cardiac mechanical function partly recovered during reperfusion. The recovery of RPP (Fig. 5D) was different according to the group studied. In the L and PE groups, the RPP was almost as high after stabilization during reperfusion as at the end of the stabilization period. This was not the case for the C group, which displayed a RPP during reperfusion never exceeding 60% of the preischemic value. Thus the recovery of the RPP in the L and PE

Table 2. Function of the mitochondria extracted from 30-min-reperfused myocardium

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Parameter</th>
<th>C</th>
<th>G</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyr/mal</td>
<td>State II</td>
<td>12.8 ± 1.4</td>
<td>16.9 ± 2.0</td>
</tr>
<tr>
<td></td>
<td>State III</td>
<td>129.6 ± 20.7</td>
<td>156.7 ± 18.8</td>
</tr>
<tr>
<td></td>
<td>State IV</td>
<td>14.8 ± 2.3</td>
<td>18.4 ± 1.2</td>
</tr>
<tr>
<td></td>
<td>RCR</td>
<td>8.9 ± 1.1</td>
<td>8.4 ± 0.5</td>
</tr>
<tr>
<td>PC/mal</td>
<td>State II</td>
<td>18.9 ± 5.0</td>
<td>15.4 ± 1.9</td>
</tr>
<tr>
<td></td>
<td>State III</td>
<td>108.3 ± 23.3</td>
<td>93.3 ± 16.4</td>
</tr>
<tr>
<td></td>
<td>State IV</td>
<td>16.3 ± 3.6</td>
<td>15.1 ± 1.0</td>
</tr>
<tr>
<td></td>
<td>RCR</td>
<td>6.3 ± 0.5</td>
<td>6.1 ± 0.9</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>ROS production</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyr/mal</td>
</tr>
<tr>
<td>State II</td>
</tr>
<tr>
<td>+ Rotenone</td>
</tr>
<tr>
<td>+ Antimycin A</td>
</tr>
<tr>
<td>State II</td>
</tr>
<tr>
<td>+ Rotenone</td>
</tr>
<tr>
<td>+ Antimycin A</td>
</tr>
</tbody>
</table>

| PC/mal         |
| State II       | 156 ± 10 |
| + Antimycin A  | 9.1 ± 0.8* |
| State II       | 8.2 ± 1.2 |
| + Antimycin A  | 9.1 ± 1.1 |

Values are means ± SE. The number of experiments was 6 per group: C, control; G, hearts perfused with GIK solution during ischemia and reperfusion. The results of state II, III, and IV respiration rates are expressed in ng atoms O₂/min mg mitochondrial protein⁻¹, reactive oxygen species (ROS) production is expressed in pmol·min⁻¹·mg mitochondrial protein⁻¹, and the ROS/state II ratio is expressed in pmol/ng atoms O₂. Pyr/mal, pyruvate/malate; PC/mal, palmitoylcarnitine/malate; RCR, respiratory control ratio. *P < 0.05 indicates a significant difference.

ischemic period (Fig. 1B). The ischemia-induced increase in diastolic pressure was similar in the G and C groups. Conversely, during reperfusion, the diastolic pressure of the G group was lower compared with that of the C group. This was true from the beginning to the end of the reperfusion with a 53% decrease at the 45th minute of reperfusion. Despite this difference, the recovery of the LVDP did not significantly differ in the two groups, although it tended to be enhanced in the G group (Fig. 1C). The recovery of the heart rate was improved in the G group whatever the duration of reperfusion (+46% at the 45th minute of reperfusion, Fig. 1A). Consequently, the RPP (Fig. 1D) was significantly higher in the G group throughout the duration of reperfusion (+67% at the 45th minute of reperfusion compared with the C group). This improved recovery was not associated with a better coronary flow (Fig. 2A). Instead, this parameter was higher in the C group during the first 6 min of reperfusion (+20% compared with the G group). Thereafter, the difference in coronary flow progressively diminished until it became nil. The oxygen consumption behaved differently (Fig. 2B). Although it was lower in the G group during the first 4 min of reflow, it became higher from the 15th minute until the end of reperfusion (+16% at the 45th minute of reperfusion). Interestingly, the lactate/pyruvate ratio, reflecting the cytosolic NADH/NAD⁺ ratio, followed roughly the progression of oxygen consumption (Fig. 3). It was lower in the G group at the 5th minute of reperfusion but progressively increased thereafter to reach a significantly higher value from the 15th minute of reperfusion (+97% at the 45th minute of reperfusion compared with the C group).

Fig. 4. Progression of the diastolic pressure during low-flow ischemia in the control, lactate, pyruvate, and pyruvate plus ethanol groups. The number of experiments was 10 per group: L, lactate-rich medium; P, pyruvate-rich medium; PE, medium enriched with pyruvate plus ethanol. a,b,c,d,e P < 0.05, different letters indicate significant differences as represented.

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groups was higher than that measured in the C group. The RPP of the P group ranged between those of the three other groups but never differed significantly from them.

The better recovery of the RPP in the L and PE groups compared with the C group was due to an enhancement of recovery of the heart rate (Fig. 5A) and LVDP (Fig. 5C), but also to a reduced diastolic pressure (Fig. 5B), which was particularly low in the L group. As for the RPP, the recovery of the heart rate and LVDP of the P group was intermediary compared with those of the PE, L, and C groups.

Figure 6A shows that the recovery of the coronary flow was not responsible for the effect of the perfusion conditions on the restoration of mechanical function. Indeed, the coronary flow was close to the values measured before ischemia in the four groups and never differed from one group to another during postischemic reperfusion. Conversely, the oxygen consumption of the reperfused hearts (Fig. 6B) was different in the four groups. At the 2nd minute of reperfusion, the oxygen consumption was higher in the L group and lower in the P group compared with those determined in the PE and C groups. Between the 6th and 10th minutes of reperfusion, the high oxygen consumption of the L group normalized compared with that of the PE and C groups, but that of the P group remained lower. Thereafter, for the remaining duration of reperfusion, the oxygen consumption of the L group became higher again and that of the P group remained normal.

Effects of various interventions on glycolytic intermediates. The levels of F6P and DHAP were evaluated in the myocardial tissue frozen at the end of the reperfusion period (Fig. 7, A and B). F6P levels were similar in the P, PE, L, and G groups and lower compared with that measured in the C group (62%, 74%, 63%, and 68% in the P, PE, L, and G groups, respectively). Conversely, DHAP levels were similar in the five groups studied (Fig. 7B). Interestingly, the F6P/DHAP ratio (Fig. 7C) was high in the C and P groups and low in the L, PE, and G groups.

Quality of various substrates to sustain cardiac mechanical activity. The effects of perfusion with glucose (11 mM), hexanoate (0.5 mM), lactate (2 mM), or pyruvate (2 mM) on
cardiac function are presented in Table 3. Each substrate similarly sustained the cardiac mechanical activity. The coronary flow and myocardial oxygen consumption were also similar in general, except for hexanoate perfusion, which slightly increased these parameters. However, the cardiac metabolic efficiency was never significantly altered by the various substrates.

DISCUSSION

The aim of this study was to determine whether an increase in the cytosolic NADH/NAD\(^+\) ratio contributed to the beneficial action of the GIK solutions during ischemia-reperfusion. The beneficial action of GIK solutions is supposed to occur through different mechanisms, including activation of K\(_{\text{ATP}}\) channels, restoration of the metabolic pools resulting from glucose oxidation, reduced ROS production, and insulin-mediated ant apoptotic activity. In the present study, we showed that our GIK solution increased the cytosolic NADH/NAD\(^+\) ratio during postischemic perfusion, leading to cardioprotection through reduced mitochondrial ROS production.

Strengths and limitations of the study. In our study, we performed 20-min low-flow ischemia. According to the literature and our own experience, this leads, through ischemia and reperfusion, to cellular damage of low severity. This is probably a requirement to observe the influence of GIK solutions and that of changes in the cytosolic NADH/NAD\(^+\) ratio on the severity of ischemia-reperfusion. Indeed, with acute levels of severity, the oxidative stress and cellular death (13) would be high and changes in the cytosolic redox potential would not be able to alter the resulting low cardiac function, a fact that constitutes a limitation of our study.

The experiments performed in this study were carried out in rat hearts collected during the active phase of the animals (dark period). In humans, myocardial ischemia, myocardial infarction, and cardiac death mainly occur during the first hours of the light period (45, 55), when heart rate, blood pressure, and myocardial oxygen consumption are increased compared with what can be measured at nighttime (46). This is explained by the disequilibrium between a restricted blood supply and the increased myocardial oxygen demand. In the rat, the rhythm is different. Because of the nocturnal activity of this animal, the increase in the heart rate and blood pressure occurs during the dark period (3) and seems related to changes in endocrine and neural influences. The heart itself has an internal circadian clock that is associated with modifications of gene expression, metabolism, and contractile performance (56). We therefore decided to perform our animal study during the dark period, which has the advantage of mimicking the situation occurring during the active phase of humans.

Biochemical assessments of cardiac NADH and NAD\(^+\) are possible, but these two molecules are mainly located in the mitochondria. Estimation of NADH and NAD\(^+\) thus reflects mainly the mitochondrial pools. To estimate the cytosolic NADH/NAD\(^+\) ratio, we therefore had to use the lactate/pyruvate ratio of the coronary effluent. This last parameter reflects the functional equilibrium of lactate dehydrogenase, which depends on the cytosolic redox potential. That is why it is usually used as a marker of the cytosolic NADH/NAD\(^+\) ratio in different organs, including the heart (43). However, it can be estimated only when lactate and pyruvate are not added to the perfusion fluid. In the present study, this was the case for the C and G groups. However, the L, P, and PE groups did contain lactate or pyruvate, making the estimation of the cytosolic NADH/NAD\(^+\) ratio impossible through the measurement of the lactate/pyruvate ratio. To discuss the effects of the enrichment with lactate, pyruvate, or pyruvate plus ethanol, we were only able to hypothesize the expected effects of each substrate. This is a limitation of our study.

Effects of the GIK solution. We showed that the GIK solution is cardioprotective during ischemia-reperfusion. Its effect was characterized by an improved recovery of the RPP, mainly due to an enhanced heart rate. Indeed, in the C group, but not in the G group, severe arrhythmias occurred and estimation of the heart rate was impossible. In this situation when the developed pressure was almost nil, the heart rate and RPP were considered to be zero. Severe arrhythmias are known
to result from heterogeneity between the different cardiomyocytes, which can be healthy or severely damaged. The effect was associated with increased oxygen consumption and reduced F6P/DHAP ratio. This strongly suggests that the conversion of glucose to CO₂ was increased, perhaps through the effect of insulin. This reaction is able to produce NADH in the cytosol through the glycolytic pathway. As a matter of fact, the lactate/pyruvate ratio of the coronary effluent was progressively increased during reperfusion until it reached values much higher than those measured in the C group. This strongly suggests that the GIK solution increased the cytotoxic NADH/NAD⁺ ratio (43). Such an improvement could act locally at the level of key enzymes such as creatine kinases, favoring the energy transfer and restoration of the adequate ionic equilibrium. Moreover, cytotoxic NADH can enter the mitochondria, where it can be converted into NADPH via several enzymes (49). The surplus mitochondrial NADPH can then improve the detoxification of ROS, particularly those produced during fatty acid oxidation, by increasing the reduced glutathione pool. Indeed, we reported that our GIK solution reduced ROS production by mitochondria purified from reperfused myocardium. This was true for palmitoyl carnitine as an oxidative phosphorylation substrate for the maximal rate of ROS production at the level of complex III of the respiratory chain. This was also true for pyruvate during state II respiration rate, since the yield of ROS production normalized to the oxygen consumption was reduced. This GIK solution-induced low mitochondrial ROS production could prevent the opening of the PTP (permeability transition pore) and could account for an antiapoptotic effect previously attributed to the effect of insulin (21, 28).

**Effects of lactate addition in the control medium.** We tested the effects of a medium expected to increase the cytotoxic NADH/NAD⁺ ratio. This medium contained the same oxidative substrates as the control medium (5.5 mM glucose and 0.5 mM hexanoate), but it was also enriched with 2 mM lactate. It was expected to increase the cytotoxic NADH/NAD⁺ ratio through the conversion of lactate to pyruvate by cytosolic lactate dehydrogenase. Unfortunately, the high level of lactate added to the perfusion fluid prevented the estimation of that ratio through the assessment of the lactate/pyruvate ratio. Lactate addition to the medium significantly improved the recovery of the heart rate, diastolic pressure, LVDP, and RPP compared with the control group. Yet, lactate does not seem to have had a direct effect on the myocardium, since lactate per se in the perfusion fluid sustains the cardiac function in aerobic conditions as well as glucose. The effect of that substrate on the NADH/NAD⁺ ratio was thus rather involved in the cardioprotection.

The literature reports interesting information concerning the action of lactate. In fact, the beneficial effect of lactate observed in our study was surprising compared to what has been published in the literature. Indeed, lactate has a reputation for being either inefficient (22, 54) or even deleterious (9, 11, 12, 14) during ischemia-reperfusion. Only one study has reported the beneficial effect of lactate during posts ischemic reperfusion in the isolated perfused heart (23). However, all the studies available in the literature have considered the effect of lactate in the absence of free fatty acids in the perfusion buffer, which does not constitute a physiological situation. In vivo studies testing the effect of lactate during reperfusion were in favor of a positive action of this substrate (51, 52). In our study, we

**Table 3. Myocardial function during heart perfusion with various substrates**

<table>
<thead>
<tr>
<th></th>
<th>Glucose</th>
<th>Lactate</th>
<th>Pyruvate</th>
<th>Hexanoate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart rate, beats/min</td>
<td>274 ± 14</td>
<td>282 ± 15</td>
<td>280 ± 7</td>
<td>270 ± 7</td>
</tr>
<tr>
<td>LVDP, mmHg</td>
<td>129 ± 6</td>
<td>130 ± 6</td>
<td>130 ± 6</td>
<td>136 ± 6</td>
</tr>
<tr>
<td>RPP, mmHg/min</td>
<td>35,602 ± 2,741</td>
<td>36,846 ± 2,764</td>
<td>36,225 ± 1,423</td>
<td>36,618 ± 1,654</td>
</tr>
<tr>
<td>CF, ml·min⁻¹·g wet wt⁻¹</td>
<td>12.1 ± 0.9</td>
<td>12.3 ± 0.6</td>
<td>12.0 ± 0.7</td>
<td>13.5 ± 0.6*</td>
</tr>
<tr>
<td>O₂ consumption, µmol·min⁻¹·g wet wt⁻¹</td>
<td>8.3 ± 0.9</td>
<td>8.9 ± 0.8</td>
<td>9.0 ± 0.6</td>
<td>9.8 ± 0.6*</td>
</tr>
<tr>
<td>Metabolic efficiency, mmHg/µmol</td>
<td>4,098 ± 503</td>
<td>3,876 ± 390</td>
<td>3,953 ± 328</td>
<td>3,534 ± 275</td>
</tr>
</tbody>
</table>

Values are means ± SE. The number of experiments was 10 per group. *P < 0.05 indicates a significant difference.
added hexanoate, a short-chain fatty acid, as a substrate for myocardial energy production. We believe that the presence of hexanoate in the perfusion buffer was essential in the observed beneficial effect of lactate. Excessive lipid oxidation has adverse effects during reperfusion (1, 7, 24, 32, 39, 40, 42, 57). Lactate could partly suppress the deleterious effect of excessive fatty acid oxidation. In favor of this theory, we observed that the F6P/DHAP ratio was reduced in the lactate group compared with the control group. This suggests an acceleration of the glycolysis and, perhaps, an activation of the glucose oxidation. Perfusion of the lactate-rich medium also increased the myocardial oxygen consumption compared with that obtained with the control buffer. This is in agreement with the increased recovery of the RPP observed in that group during reperfusion and also with a higher glucose oxidation rate, since increasing the cardiac mechanical work is known to boost the rate of glucose oxidation (31).

Effects of a solution expected to decrease the cytosolic NADH/NAD⁺ ratio. We also tested the supplementation with pyruvate (2 mM). The addition of that substrate could increase the supply of substrate directly oxidizable by the mitochondrial pyruvate dehydrogenase. Yet, pyruvate per se sustained cardiac function similarly to lactate, indicating comparable energizing capacities. However, it could decrease the cytosolic NADH/NAD⁺ ratio through the action of lactate dehydrogenase. Compared with the control buffer, the supplementation with 2 mM pyruvate did not improve the recovery of the diastolic pressure, heart rate, LVDP, and RPP during reperfusion. This confirms that the extra carbon moieties supplied by the addition of pyruvate had no effect. Furthermore, in contrast with the media expected to increase the cytosolic NADH/NAD⁺ ratio, the buffer containing pyruvate augmented the F6P/DHAP ratio, suggesting that the glycolytic pathway was not activated. In past years, pyruvate was considered to be beneficial during ischemia-reperfusion (8, 14, 16, 19), although some investigators reported no effect of this substrate (9, 22, 25). In the present study, we did not see any beneficial action of pyruvate addition. As mentioned by Johnston and Lewandowski (30), the presence of hexanoate, a lipid substrate directly utilizable by the mitochondrial β-oxidation pathway (38), in the perfusion media may have contributed to attenuate the beneficial effect of pyruvate.

Effect of ethanol addition in the pyruvate-rich medium. We finally increased the cytosolic NADH/NAD⁺ ratio by supplementing the pyruvate-rich medium with ethanol (2 mM). Ethanol is known to be oxidized by the cytosolic alcohol dehydrogenase and to produce reduced equivalents through that reaction. The beneficial effect of pyruvate plus ethanol was slightly different from that observed with lactate. Indeed, the increased recovery of the RPP was also associated with a reduction of the F6P/DHAP ratio, but the diastolic pressure and oxygen consumption were not improved. The difference might be due to the type of reduced equivalent produced. In the heart, it seems that the alcohol dehydrogenase forms NADPH rather than NADH (20). That reduced equivalent could also enter the mitochondria and reinforce the enzymatic antioxidative properties. However, it could also have a local action by preventing the oxidative stress-induced inhibition of key enzymes in the cytosol. For example, creatine kinase is known to be reversibly inhibited when the redox ratio is low. Since this enzyme plays a crucial role in the energy transfer, the restoration of its activity through an adequate redox potential could favor the recovery of the cardiac cells to a normal physiology.

Conclusion. Our study indicates that the GIK solutions increased the cytosolic NADH/NAD⁺ ratio during postischemic reperfusion. As indicated by the addition of various substrates modulating this ratio, this effect could be partly responsible for the cardioprotective action of GIK solutions. The high amount of NADH produced could enter the mitochondria and stimulate the enzymatic defenses against ROS overproduction, leading to an antiapoptotic effect. However, other mechanisms including stimulation of KATP channels and recovery of the metabolic pools could also be involved.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

I.W.S., L.D., V.C., and S.P. performed experiments; L.D. and X.M.L. interpreted results of experiments; L.D. prepared figures; L.D. drafted manuscript; L.D. edited and revised manuscript; L.D. and M.R. approved final version of manuscript.

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