Substrate-Induced Changes in the Lipid Content of Ischemic and Reperfused Myocardium

Its Relation to Hemodynamic Recovery

Monique J.M. de Groot, Will A. Coumans, Peter H.M. Willemsen, and Ger J. van der Vusse

To investigate the effect of lactate, pyruvate, and glucose on the endogenous levels of lipids in the normoxic, ischemic, and reperfused myocardium, isolated working rat hearts were exposed to various grades of ischemic insult (15, 30, or 45 minutes). Glucose was present as the basal substrate in the perfusion medium, and lactate (5 mM) or pyruvate (5 mM) was added as the cosubstrate. Lipid metabolism was evaluated by fatty acid accumulation, triacylglycerol turnover, and phospholipid homeostasis. Exogenous lactate significantly increased fatty acid content above preischemic levels after 45 minutes of ischemia. In glucose-perfused hearts, fatty acid levels were even slightly higher than in lactate-perfused hearts, whereas pyruvate-perfused hearts demonstrated less accumulation of fatty acids. By reperfusion, fatty acid levels in glucose-perfused hearts returned to control values. In lactate- and pyruvate-perfused hearts, fatty acid accumulation was further enhanced by reperfusion. When the fatty acid content exceeded 400 nmol/g dry wt during reperfusion, hemodynamic function was impaired, whereas fatty acid levels below 400 nmol/g dry wt did not correlate with hemodynamic recovery. The total triacylglycerol content did not change during ischemia and reperfusion. However, accumulation of glycerol was remarkable during the first 15 minutes of ischemia in all hearts, and release of glycerol by reperfusion was considerable in lactate-perfused hearts after 30 minutes of ischemia and in all groups of hearts after 45 minutes of ischemia. Release of glycerol in association with maintained levels of triacylglycerols suggests turnover of the triacylglycerol pool. The rate of triacylglycerol cycling correlated poorly with hemodynamic recovery. Accumulation of arachidonic acid revealed disturbances in phospholipid turnover. Arachidonic acid accumulation during reperfusion demonstrated a strong relation with impairment of cardiac function. Hence, derangements in phospholipid homeostasis during reperfusion might be involved in myocardial damage, which is influenced by the substrates available. (Circulation Research 1993;72:176–186)

KEY WORDS • lactate • pyruvate • lipids • ischemia • reperfusion

Myocardial ischemia and reperfusion are associated with specific pathological changes that may lead to decline in function and acceleration of cell death. Disturbance of lipid metabolism has been recognized as an important contributor to myocardial damage.1–3 Accumulation of fatty acids has been detected in flow-deprived tissue by several studies.4–7 Additional fatty acid release has been found in reperfused hearts.3–7 High levels of fatty acids and acyl derivatives are thought to exert detrimental effects on membrane function.1,8 Moreover, a relatively high increase of arachidonic acid, a fatty acid almost exclusively incorporated in the phospholipid pool, indicates that membrane phospholipids are degraded and thus that the integrity of the cell might be impaired.6–9,12

Release of glycerol from ischemic tissue3,13 might imply hydrolysis of the triacylglycerol pool. So far, glycerol release has been generally accepted as a valid index of hydrolysis of triacylglycerols, since other sources of glycerol (i.e., glycerol-3-phosphate and phospholipids) are reported to be of minor importance.14–16 Trach and colleagues17 referred to "futile cycling" of triacylglycerols. Reesterification of fatty acids into the triacylglycerol pool with glycolytically produced glycerol-3-phosphate may stimulate the turnover process. This process gives rise to enhanced ATP consumption and may further aggravate ischemia-induced damage.

The extent of cardiac injury and the recovery of hemodynamic function after ischemia have been shown to be influenced by substrates exogenously present. In this context, pyruvate, in addition to glucose, was found to improve cardiac recovery after mild ischemia.18–20 Interestingly, we have recently demonstrated that lactate (5 mM) impairs cardiac function after ischemia.21 Bünger and coworkers19 also reported functional impairment by exogenous lactate (15 mM). At present, no information is available as to whether pyruvate- and lactate-influenced postischemic functional outcome is related to alterations in lipid homeostasis. Recent experiments in our laboratory have indicated that exoge-

From the Department of Physiology, Cardiovascular Research Institute Maastricht, University of Limburg, Maastricht, The Netherlands.

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Address for correspondence: M.J.M. de Groot, PhD, Department of Physiology, Cardiovascular Research Institute Maastricht, University of Limburg, P.O. Box 616, 6200 MD Maastricht, The Netherlands.

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nous lactate stimulates the turnover of the endogenous neutral fat pool accompanied by elevated levels of fatty acids under normoxic conditions. In contrast, no cycling of triacylglycerols was found in glucose-perfused hearts, whereas in pyruvate-perfused hearts, the triacylglycerol cycling rate was very low.22,23

It was the aim of the present study to investigate the effect of lactate, pyruvate, and glucose on alterations of the cardiac lipid pool under ischemic and reperfused conditions. In addition, the relation between lipid changes on the one hand and hemodynamic recovery during reperfusion and loss of cellular integrity, as measured by release of myocardial enzymes, on the other was delineated.

Materials and Methods

Animals

Hearts were obtained from male Lewis rats (10 weeks old; body weight, 250–350 g). The animals were fed ad libitum (Diet SRM-A, Hope Farms), had free access to water, and were kept under an artificial light cycle of 12 hours.

Experimental Setup

Under light ether anesthesia, hearts were rapidly excised and immediately immersed in an ice-chilled perfusion medium (see below). Lung and fat tissue were removed. Subsequently, hearts were ligated to the aortic cannula of the perfusion system, and retrograde perfusion was started at a perfusion pressure of 8 kPa. A second cannula was connected to the left atrium to allow antegrade perfusion of the hearts. The perfusion system has been described previously.24,25 The composition of the perfusion medium was as follows (mmol·L⁻¹): NaCl 130.0, KCl 5.6, CaCl₂ 2.2, MgCl₂ 1.2, NaH₂PO₄ 1.2, and NaHCO₃ 25.0. (±)Glucose (11 mM) was added as substrate. Where indicated, (±)lactate (5.0 mM) or pyruvate (5.0 mM, sodium salt) was added as cosubstrate. The pH of the lactate buffer was adjusted by NaOH. The buffer was continuously gassed with a mixture of 95% O₂, 5% CO₂ (PO₂ > 75 kPa) and filtered (1.2 μm-pore filter, Millipore Corp., Bedford, Mass.) during perfusion. The final pH range varied from 7.35 to 7.45. Temperature was kept at 37°C throughout the experiment.

Protocol

After a stabilization period of 10 minutes in retrograde mode, hearts were subjected to antegrade perfusion for 30 minutes. Left atrial filling pressure was 1 kPa, and diastolic aortic pressure was 8 kPa. Then, hearts were subjected to 15, 30, or 45 minutes of normothermic no-flow ischemia and subsequently reperfused for 35 minutes (retrogradely during the first 5 minutes and antegrade thereafter). Since hearts were perfused in the assisted mode,20 coronary perfusion pressure (8 kPa) was maintained even when reperfused hearts were unable to provide their own coronary flow. Aortic pressure was continuously monitored at the entrance of the aortic cannula, with a catheter connected to an external pressure transducer (Century Technology Co., Inglewood, Calif.). Mean aortic flow was measured with an electromagnetic flow probe (Skalar 601, Utrecht, The Netherlands) connected to a “sine wave” electromagnetic flowmeter (Transflow 601). Coronary flow was measured by timed collection of the coronary perfusate dripping from the heart. Cardiac output was calculated by adding aortic flow and coronary flow. Platinum electrodes were attached to the surface of the right atrium and ventricle to record the electrogram. Heart rate was calculated by using the electrogram registrations. Stroke volume was defined as cardiac output divided by heart rate.

Collection of Tissue and Effluent Samples

At the end of the preischemic, ischemic (15, 30, or 45 minutes), or reperfusion phase, ventricular tissue was rapidly dissected from atrial tissue with a surgical blade, immediately freeze-clamped with the use of aluminum tongs, and cooled in liquid nitrogen. Coronary effluents were sampled during preischemic perfusion and during reperfusion. Samples were stored at −80°C until analysis.

Biochemical Analysis of Tissue

Fatty acids, triacylglycerols, and phospholipids. Part of the frozen tissue was powdered at the temperature of liquid nitrogen and subsequently extracted with chloroform/methanol (2:1 [vol/vol]).26 Neutral and polar lipids were separated by silica gel column chromatography.26 The neutral lipid fraction was further separated using thin-layer chromatography. The lipid spots, corresponding to fatty acids and triacylglycerols, were scraped from the plates. After elution from the silica gel powder and methylation, the fatty acid methyl esters were quantitated by gas chromatography.4,26

Glycerol. Analysis of glycerol was performed in small parts of the frozen ventricles that were freeze-dried at −30°C. The compound was extracted by use of a mixture of perchloric acid (3 M) and dithiothreitol (5 mM) as described previously.24 Glycerol was determined fluorometrically according to Laurell and Tibbling.27

Biochemical Analysis of Coronary Effluent

Glycerol was determined fluorometrically in deproteinized (perchloric acid) and neutralized (KHCO₃) effluent samples according to the method of Laurell and Tibbling.28 Lactate dehydrogenase (LDH) was assessed spectrophotometrically by use of a Cobas Bio Autoanalyser.28

Normalization of Data

Measurements in tissue samples are expressed in moles per gram dry weight. The dry weight of cardiac tissue was determined by freeze drying overnight. Data concerning release of glycerol in the coronary effluent are converted to moles per gram dry weight. Therefore, it is accepted that glycerol release per heart corresponds to glycerol release per gram wet weight, because earlier findings demonstrated that hearts of rats of this age weigh approximately 1 g (0.97±0.07, mean±SD). For conversion of glycerol release per gram wet weight to glycerol release per gram dry weight, an average factor 6.25 is used, as calculated by the ratio of preischemic dry and wet weight values.

Statistical Analysis

Data are presented as median values and 95% confidence limits throughout. The number of experiments...
FIGURE 1. Bar graphs showing the content of fatty acids in myocardial tissue of glucose (11 mM)-perfused hearts (top panel), glucose (11 mM) plus lactate (5 mM)-perfused hearts (middle panel), and glucose (11 mM) plus pyruvate (5 mM)-perfused hearts (bottom panel) before ischemia (Pre-I), after 15, 30, and 45 minutes of ischemia (15'I, 30'I, and 45'I, respectively), or after subsequent reperfusion (/R). Data represent median values and 95% confidence limits. The number of experiments per group varies from six to eight. The amount of fatty acids is expressed as nanomoles per gram dry weight. Hatched bars represent preischemic values; dotted bars represent ischemia; and open bars represent reperfusion. *p < 0.05 vs. preischemic value; †p < 0.05 vs. preceding ischemic interval; ‡p < 0.05 for ischemia vs. reperfusion; ‡p < 0.05 vs. glucose-perfused hearts at corresponding times; ‡p < 0.05 vs. lactate-perfused hearts at corresponding times.
varied from six to eight. Differences within groups were evaluated for significance using Wilcoxon’s matched-pairs signed-rank test. Differences between groups were tested by the Mann-Whitney U test. Values of $p<0.05$ were considered to be statistically significant. Correlations were calculated using Spearman’s rank correlation.

**Results**

**Fatty Acids**

The preischemic fatty acid content was significantly higher in lactate- and pyruvate-perfused hearts than in glucose-perfused hearts (Figure 1). During early ischemia (15 minutes), these contents were reduced to approximately 35% of the preischemic level. In contrast, the content of fatty acids in glucose-perfused hearts remained unchanged. When the duration of ischemia was extended to 30 minutes, fatty acids started to accumulate in hearts of the lactate and glucose group. In pyruvate-perfused hearts, fatty acid levels were maintained at the 15-minute ischemia level. After 45 minutes of ischemia, fatty acid levels were also increased in the pyruvate group. At this time point, the fatty acid content tended to be lower in lactate- than in glucose-perfused hearts ($0.05<p<0.10$), whereas fatty acid levels in pyruvate-perfused hearts were appreciably lower than in lactate- or glucose-perfused hearts ($p<0.05$).

During reperfusion, fatty acids produced by ischemia were partly removed in glucose-perfused hearts. In contrast, lactate- and pyruvate-perfused hearts showed additional fatty acid accumulation by reperfusion. In lactate-reperfused hearts, fatty acid levels were significantly increased above preischemic values when the previous time of ischemia was 30 or 45 minutes. Pyruvate-reperfused hearts reached values above the preischemic content when the previous time of ischemia was 45 minutes. End-reperfusion fatty acid values were higher in the lactate and pyruvate group compared with the glucose group, although not significantly for the pyruvate group subjected to 30 minutes of ischemia. In the lactate group, values were significantly higher than in the pyruvate group when the preceding duration of ischemia was 30 or 45 minutes.

**Triacylglycerols**

Preischemic triacylglycerol values were lower in glucose- than in lactate- ($0.05<p<0.10$) or pyruvate-perfused hearts ($p<0.05$, Table 1). During ischemia, no pronounced changes were found in the total triacylglycerol content of the three substrate groups. Upon reperfusion, tissue triacylglycerol levels also remained constant (Table 1).

An appreciable change in the fatty acyl composition of triacylglycerols was found for the arachidonic acid moiety (Table 1). In pyruvate-perfused hearts, the content of arachidonic acid chains significantly increased during 15 minutes of ischemia. The increase was approximately 0.3 µmol/g dry wt. After 45 minutes of ischemia, arachidonic acid incorporation amounted to 0.4 µmol/g dry wt. In lactate- and glucose-perfused hearts, the increase was slightly less than in pyruvate-perfused hearts. In this respect, both the lactate- and glucose-perfused hearts reached a significant incorporation of arachidonic acid of 0.1–0.2 µmol/g dry wt after 30 minutes of ischemia. By reperfusion, the arachidonic acid content of triacylglycerols continued to rise in the lactate-perfused hearts subjected to 45 minutes of ischemia (i.e., 0.24 µmol/g dry wt above the preischemic value). In pyruvate- and glucose-perfused hearts, the amount of arachidonic acid incorporation in triacylglycerols did not increase further.

**Glycerol**

Although no decrease of the total triacylglycerol content was observed, glycerol accumulated in tissue of all the hearts during ischemia (Table 2). The three substrate groups showed the highest rate of glycerol increase during the first 15 minutes of ischemia, and levels were nearly constant thereafter. At the end of 45 minutes of ischemia, the total glycerol content was not significantly different in the three groups. By reperfusion, the glycerol content decreased toward preischemic values in all hearts. Only in lactate-perfused hearts previously exposed to 45 minutes of ischemia was the end-reperfusion level still significantly elevated above the preischemic value.

The extent of glycerol decrease in tissue after 15 minutes of ischemia and subsequent reperfusion corresponded closely to the amount of glycerol released in coronary effluent when lactate was present as cosubstrate or glucose was present as sole substrate (Table 2). When hearts were perfused by pyruvate, cumulative glycerol release was slightly above the amount expected on the basis of glycerol decrease in tissue ($0.05<p<0.10$). After 30 minutes of ischemia, the lactate-perfused hearts demonstrated significantly elevated levels of glycerol release by reperfusion compared with the decrease in tissue levels. By 45 minutes of ischemia, all three substrate groups showed more release of glycerol than was expected on the basis of the difference between end-ischemic and end-reperfusion tissue contents of glycerol. The observed discrepancy was most pronounced in lactate-perfused hearts (Table 2).

**Phospholipids**

The total content of phospholipids in lactate-, pyruvate-, and glucose-perfused hearts (approximately 250 µmol fatty acid equivalents/g dry wt) did not change during ischemia and reperfusion (data not shown). However, changes in the content of arachidonic acid, a fatty acid predominantly incorporated in the phospholipid pool of normoxic tissue, followed a pattern qualitatively comparable to alterations in the total amount of fatty acids (Table 1). In glucose-perfused hearts, arachidonic acid significantly accumulated after 30 minutes of ischemia and reached levels 35 times the preischemic content after 45 minutes of flow cessation. In contrast, arachidonic acid levels initially decreased at 15 minutes of ischemia in lactate- and pyruvate-perfused hearts. Prolongation of the ischemic time to 30 minutes resulted in a marked increase of the arachidonic acid accumulation in lactate-perfused hearts and a further elevation at 45 minutes of ischemia. In pyruvate-perfused hearts, ischemia-induced accumulation of arachidonic acid was somewhat retarded. Both in lactate- and pyruvate-perfused hearts, the arachidonic acid content significantly exceeded preischemic values after 45 minutes of ischemia.

Reperfusion dramatically reduced the tissue arachidonic acid content in glucose-perfused hearts when the
preceding ischemic time lasted 30 and 45 minutes. On the other hand, reperfusion enhanced the tissue arachidonic acid content under all circumstances in lactate- and pyruvate-perfused hearts (Table 1).

Hemodynamic Recovery

In addition to the duration of ischemia, hemodynamic recovery during reperfusion, expressed as stroke volume, was dependent on substrates available in the perfusion buffer (Figure 2). The recovery of stroke volume in glucose-perfused hearts was found to be unaffected after 15 minutes of ischemia. By addition of lactate or pyruvate to the perfusion medium, the recovery of stroke volume was also not influenced after a short interval of ischemia (15 minutes). However, recovery of stroke volume was severely depressed after 30 minutes of ischemia when lactate was present in the perfusion medium. In contrast, pyruvate in combination with glucose significantly improved cardiac function of postischemic hearts compared with glucose as the sole substrate. After 45 minutes of ischemia, both lactate and pyruvate were detrimental for hemodynamic performance during reperfusion. Only glucose-reperfused hearts returned stroke volume to 19% of its preischemic value (i.e., median recovery when each heart serves as its own control).

Lethal Cell Damage

To assess the extent of myocardial damage, the release of LDH into the coronary perfusate was measured during reperfusion. Preischemic LDH release was very low (approximately 25 milliunits/min) and did not differ between the lactate-, pyruvate-, and glucose-perfused hearts. Postischemic LDH release was influenced by the preceding period of ischemia and the type of substrate present (Table 3). Differences between the

<p>| Table 1. Content of Arachidonic Acid, Arachidonic Acid Molecules in Triacylglycerols, and Total Triacylglycerols Before Ischemia, After 15, 30, or 45 Minutes of Ischemia, or After Subsequent 35 Minutes of Reperfusion in Glucose-, Glucose Plus Lactate-, and Glucose Plus Pyruvate-Perfused Hearts |</p>
<table>
<thead>
<tr>
<th>AA (nmol/g dry wt)</th>
<th>AA in triacylglycerols (μmol AA/g dry wt)</th>
<th>Triacylglycerols (μmol FA/g dry wt)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Median</td>
<td>CI</td>
</tr>
<tr>
<td>Pre-1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>2.9</td>
<td>0.0-14.5</td>
</tr>
<tr>
<td>P</td>
<td>34.3*</td>
<td>4.4-74.7</td>
</tr>
<tr>
<td>L</td>
<td>27.8*</td>
<td>18.5-75.4</td>
</tr>
<tr>
<td>15'1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>13.1</td>
<td>0.0-15.3</td>
</tr>
<tr>
<td>L</td>
<td>10.2†</td>
<td>0.0-12.0</td>
</tr>
<tr>
<td>P</td>
<td>7.9†</td>
<td>0.0-15.4</td>
</tr>
<tr>
<td>30'1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>35.7†‡</td>
<td>12.0-51.0</td>
</tr>
<tr>
<td>L</td>
<td>22.8‡</td>
<td>17.5-50.8</td>
</tr>
<tr>
<td>P</td>
<td>9.7‡†</td>
<td>6.3-16.6</td>
</tr>
<tr>
<td>45'1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>100.3‡†</td>
<td>47.3-145.4</td>
</tr>
<tr>
<td>L</td>
<td>81.6‡†</td>
<td>32.0-103.4</td>
</tr>
<tr>
<td>P</td>
<td>53.0*‡†</td>
<td>22.1-82.0</td>
</tr>
<tr>
<td>15'1/R</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>10.4†</td>
<td>8.8-15.8</td>
</tr>
<tr>
<td>L</td>
<td>21.1‡</td>
<td>6.9-27.4</td>
</tr>
<tr>
<td>P</td>
<td>17.2‡†</td>
<td>11.3-86.3</td>
</tr>
<tr>
<td>30'1/R</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>7.4</td>
<td>1.8-140.6</td>
</tr>
<tr>
<td>L</td>
<td>53.0*‡</td>
<td>20.5-115.3</td>
</tr>
<tr>
<td>P</td>
<td>26.3‡</td>
<td>9.1-66.3</td>
</tr>
<tr>
<td>45'1/R</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>29.7†‡</td>
<td>14.3-69.7</td>
</tr>
<tr>
<td>L</td>
<td>118.2*‡†</td>
<td>105.9-147.7</td>
</tr>
<tr>
<td>P</td>
<td>96.9*‡†</td>
<td>44.8-100.8</td>
</tr>
</tbody>
</table>

AA, arachidonic acid; CI, 95% confidence interval; FA, fatty acid equivalents; Pre-1, preischemic value; G, glucose (11 mM)--perfused hearts; L, glucose (11 mM) plus lactate (5 mM)--perfused hearts; P, glucose (11 mM) plus pyruvate (5 mM)--perfused hearts; 15', 30', and 45', values measured at the end of 15, 30, and 45 minutes of ischemia, respectively; 15'/R, 30'/R, and 45'/R, values measured at the end of 35 minutes of reperfusion preceded by 15, 30, and 45 minutes of ischemia, respectively.

*p<0.05 vs. G value; †p<0.05 vs. corresponding Pre-1 value; ‡p<0.05 vs. preceding ischemic interval; §p<0.05 vs. L value; ||p<0.05 for ischemic vs. reperfused value.
three substrate groups were most pronounced after 30 minutes of ischemia. In this respect, lactate-perfused hearts showed augmented leakage of LDH compared with glucose-perfused hearts, whereas pyruvate-perfused hearts demonstrated less release of LDH. Interestingly, the pattern of LDH release was not consistent. After 15 minutes of ischemia, LDH release by pyruvate- and glucose-perfused hearts reached maximum values during the first 5 minutes of reperfusion and rapidly declined to normal values thereafter (data not shown). In lactate-perfused hearts, a peak value between 10 and 15 minutes was observed. After 30 minutes of ischemia, only pyruvate-perfused hearts showed maximal release of LDH during the first 5 minutes, whereas in glucose-perfused hearts, the peak value was found between 10 and 15 minutes of reperfusion. Lactate-perfused hearts also showed maximal release of LDH during the time interval of 10-15 minutes, but LDH kept leaking at a high level throughout the postischemic period. After 45 minutes of ischemia, LDH release continued at a high level throughout reperfusion in all hearts.

**Discussion**

**Lipid Changes During Ischemia**

The present study demonstrated that at the onset of ischemia fatty acid levels were elevated in lactate- and pyruvate-perfused hearts compared with glucose-perfused hearts. Increased fatty acid levels in lactate- and pyruvate-perfused hearts most likely reflect the preference of lactate or pyruvate over endogenous fatty acids during preischemic perfusion. Earlier studies in our laboratory have shown that both lactate and pyruvate are alternative substrates for the heart when the concentration of lactate and pyruvate in the perfusion medium is increased (5 mM).22,23 In this respect, it was found that by perfusion with lactate or pyruvate the endogenous triacylglycerol pool was preserved, whereas perfusion with glucose as the sole substrate caused depletion of the endogenous lipid pool (as well as the glycopeen).22,23

During the ischemic phase, the tissue content of fatty acids changed differently in the three substrate groups. In glucose-perfused hearts, the tissue content of fatty acids remained constant during the first 15 minutes of ischemia and markedly increased thereafter. In contrast, lactate- and pyruvate-perfused hearts showed a decrease of fatty acids at the onset of ischemia. After 15 minutes of ischemia, fatty acids started to accumulate in lactate-perfused hearts, whereas in pyruvate-perfused hearts, accumulation of fatty acids was delayed; i.e., after 30 minutes of ischemia, fatty acid levels started to increase. Burton and colleagues29 reported that, during the initial phase of ischemia, fatty acids are reesterified into the triacylglycerol pool. In the present study, increased incorporation of arachidonic acid moieties in triacylglycerols of lactate-, pyruvate-, or glucose-perfused hearts during early ischemia may support the findings of Burton and coworkers.

Because no changes in the total triacylglycerol content were observed and release of glycerol (a presumed marker of lipolysis14–17) was considerable, cycling of the triacylglycerol pool was suggested. The total amount of glycerol accumulated during ischemia in, for example, lactate-perfused hearts would implicate a triacylglycerol breakdown of 13 µmol fatty acid equivalents/g dry wt. However, no net degradation was found; hence, the rate of degradation is fully compensated by the rate of triacylglycerol synthesis. During ischemia, glycerol accumulation was noticeable in all groups during the first 15 minutes (approximately 0.2 µmol glycerol/g dry

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### Table 2. Content of Glycerin in Tissue Before Ischemia, After 15, 30, or 45 Minutes of Ischemia, and After Subsequent Reperfusion and Amount of Glycerin Released in Coronary Effluents During Reperfusion of Glucose-, Glucose Plus Lactate-, and Glucose Plus Pyruvate–Perfused Hearts

<table>
<thead>
<tr>
<th>Duration of Ischemia</th>
<th>Tissue Glycerol before Ischemia (µmol/g dry wt)</th>
<th>Tissue Glycerol at the End of Ischemia (µmol/g dry wt)</th>
<th>Tissue Glycerol after Reperfusion (µmol/g dry wt)</th>
<th>Glycerol Release during Reperfusion (µmol/g dry wt)</th>
<th>Expected Glycerol Release (µmol/g dry wt)</th>
<th>ΔGlycerol (µmol/g dry wt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15 Minutes</td>
<td></td>
<td></td>
<td></td>
<td>A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>1.21</td>
<td>0.00–1.43</td>
<td>4.72*</td>
<td>4.02–6.75</td>
<td>1.06†</td>
<td>0.00–4.84</td>
</tr>
<tr>
<td>L</td>
<td>0.22‡</td>
<td>0.00–0.92</td>
<td>2.87*</td>
<td>0.41–14.02</td>
<td>0.30</td>
<td>0.00–1.23</td>
</tr>
<tr>
<td>P</td>
<td>0.06</td>
<td>0.00–1.42</td>
<td>3.10‡</td>
<td>0.97–4.24</td>
<td>0.00†</td>
<td>0.00–0.70</td>
</tr>
<tr>
<td>30 Minutes</td>
<td></td>
<td></td>
<td></td>
<td>A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>1.21</td>
<td>0.00–1.43</td>
<td>5.44*</td>
<td>1.24–17.28</td>
<td>2.87</td>
<td>0.00–5.99</td>
</tr>
<tr>
<td>L</td>
<td>0.22‡</td>
<td>0.00–0.92</td>
<td>4.00*</td>
<td>0.34–6.56</td>
<td>0.18‡</td>
<td>0.00–1.30</td>
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<tr>
<td>P</td>
<td>0.06</td>
<td>0.00–1.42</td>
<td>3.30*</td>
<td>1.87–5.91</td>
<td>0.06‡</td>
<td>0.00–0.73</td>
</tr>
<tr>
<td>45 Minutes</td>
<td></td>
<td></td>
<td></td>
<td>A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>1.21</td>
<td>0.00–1.43</td>
<td>4.55*</td>
<td>2.14–8.28</td>
<td>1.40†</td>
<td>0.00–4.14</td>
</tr>
<tr>
<td>L</td>
<td>0.22‡</td>
<td>0.00–0.92</td>
<td>4.52*</td>
<td>2.25–9.72</td>
<td>2.16†</td>
<td>0.21–5.50</td>
</tr>
<tr>
<td>P</td>
<td>0.06</td>
<td>0.00–1.42</td>
<td>3.88*</td>
<td>2.32–4.18</td>
<td>0.56‡</td>
<td>0.00–2.28</td>
</tr>
</tbody>
</table>

CI, 95% confidence interval; G, glucose (11 mM)–perfused hearts; L, glucose (11 mM) plus lactate (5 mM)–perfused hearts; P, glucose (11 mM) plus pyruvate (5 mM)–perfused hearts.

Release values are corrected for basal values measured during the preischemic phase. Expected glycerol release is the amount of glycerol accumulation during ischemia minus residual amount in cardiac tissue at the end of reperfusion. Glycerol balance (ΔGlycerol) is the actual amount of glycerol released minus the expected amount. Data of expected glycerol release and glycerol balance represent calculated values (by median).

*P < 0.05 vs. preischemic value; †P < 0.05 for ischemic vs. reperfusion value; ‡P < 0.05 vs. G value; §§P < 0.05 for glycerol release during reperfusion vs. expected glycerol release; ||p < 0.05 vs. L value.
stroke volume (ml/beat)

FIGURE 2. Bar graph showing the recovery of stroke volume during reperfusion after 15, 30, or 45 minutes of ischemia in the absence or presence of either lactate or pyruvate. Data represent median values and 95% confidence limits. The number of experiments per group varies from six to eight. Stroke volume is expressed as milliliters per beat. Pre-I refers to the preischemic value; 15'I/R, 30'I/R, and 45'I/R refer to values measured at the end of reperfusion preceded by 15, 30, and 45 minutes of ischemia, respectively. *p<0.05 vs. preischemic value; +p<0.05 vs. glucose-perfused hearts at corresponding times; pp<0.05 vs. lactate-perfused hearts at corresponding times.

TABLE 3. Cumulative Release of Lactate Dehydrogenase During 35 Minutes of Reperfusion After 15, 30, or 45 Minutes of Ischemia in the Absence or Presence of Lactate or Pyruvate

<table>
<thead>
<tr>
<th>Lactate dehydrogenase release (units/g wet wt)</th>
<th>15'I/R</th>
<th>30'I/R</th>
<th>45'I/R</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median</td>
<td>CI</td>
<td>Median</td>
<td>CI</td>
</tr>
<tr>
<td>G</td>
<td>0.4</td>
<td>0.1-1.2</td>
<td>6.0</td>
</tr>
<tr>
<td>L</td>
<td>1.0</td>
<td>0.2-2.0</td>
<td>15.1*</td>
</tr>
<tr>
<td>P</td>
<td>0.1</td>
<td>0.0-1.1</td>
<td>2.5*+</td>
</tr>
</tbody>
</table>

15'I/R, 30'I/R, and 45'I/R, values measured at the end of 35 minutes of reperfusion preceded by 15, 30, and 45 minutes of ischemia, respectively; CI, 95% confidence interval; G, glucose (11 mM)-perfused hearts; L, glucose (11 mM) plus lactate (5 mM)-perfused hearts; P, glucose (11 mM) plus pyruvate (5 mM)-perfused hearts.

Lactate dehydrogenase is corrected for basal release as measured during the preischemic phase.

*p<0.05 vs. G value; +p<0.05 vs. L value.

wt · min, as calculated by ischemic values corrected for preischemic values; Table 4) and leveled off thereafter. Correspondingly, triacylglycerol cycling has been observed in Langendorff-perfused rat hearts during low-flow ischemia and anoxia as well as in isolated myocytes during hypoxic incubation. In the present study, the rate of triacylglycerol cycling during the ischemic phase did not markedly differ between the three substrate groups (Table 4).

Cycling of triacylglycerols is associated with the consumption of ATP required for activation of fatty acids to acyl coenzyme A. The question can be raised whether activation of triacylglycerol cycling poses a burden on the energetic state of the heart during ischemia. The release of 0.2 µmol glycerol/g dry wt · min during the first 15 minutes of ischemia corresponds to a fatty acid release of 0.6 µmol/g dry wt · min. Since the fatty acid content of the total triacylglycerol pool is approximately 20 µmol/g dry wt in the lactate- and pyruvate-perfused hearts, it can be calculated that during each minute of early ischemia approximately 3% of the triacylglycerol pool is cycled. In glucose-perfused hearts, approximately 5% of the triacylglycerol pool is cycled per minute, because the content of the total triacylglycerol pool is 11 µmol fatty acid equivalents/g dry wt. For conversion of three fatty acyl moieties into three acyl coenzyme A, 6 mol ATP is used. Hence, it can be
inferred that 6 mol ATP is charged for each mole of glycerol hydrolyzed. The production of 0.2 μmol glycerol/g dry wt · min corresponds with the consumption of 1.2 μmol ATP/g dry wt · min. The total amount of high-energy phosphate bond delivery during the first 15 minutes of ischemia is approximately 265, 269, and 259 μmol/g dry wt in lactate-, pyruvate-, and glucose-perfused hearts, respectively, as estimated by degradation of ATP and creatine phosphate and the production of lactate. (In lactate-perfused hearts, ATP decreases by 4.8 μmol/g dry wt, creatine phosphate decreases by 12.2 μmol/g dry wt, and lactate production amounts to 81.2 μmol/g dry wt during the first 15 minutes of ischemia; in pyruvate-perfused hearts, ATP and creatine phosphate decrease by 8.8 and 13.7 μmol/g dry wt, respectively, and lactate production amounts to 79.3 μmol/g dry wt; in glucose-perfused hearts, ATP and creatine phosphate decrease by 7.9 and 9.3 μmol/g dry wt, respectively, and lactate production amounts to 78.0 μmol/g dry wt [data not shown]. Further, it is assumed that hydrolysis of 1 mol ATP delivers 2 mol high-energy phosphate bonds, hydrolysis of 1 mol creatine phosphate delivers 1 mol high-energy phosphate bonds, and 1 mol lactate production from glycerol delivers 1.5 mol ATP.) These calculations indicate that approximately 7% of the energy stored in high-energy phosphate bonds during the first 15 minutes of ischemia is used for futile triacylglycerol cycling. Trach et al.17 found that in a low-flow ischemia model the energy loss by triacylglycerol cycling was slightly less, i.e., approximately 2.5%. Schoonderwoerd et al.30 suggested that 3.3–4.4% of the glycolytically produced energy was consumed in triacylglycerol cycling. The small differences between the results in the present study and the results in the study of Trach et al or Schoonderwoerd et al can be explained by a higher ATP production in a low-flow ischemic model than in a global ischemic model. The assumption that triacylglycerol cycling poses a burden on the ischemic heart from an energetic point of view cannot be confirmed by the data presented.

Several investigators4,6,32 have demonstrated that accumulation of arachidonic acid during ischemia and reperfusion most likely indicates disturbed phospholipid homeostasis, because this fatty acid is mainly incorporated in the cellular phospholipid pool (over 99%).4 Like fatty acids, accumulation of arachidonic acid was manifest after 30 and/or 45 minutes of ischemia and more pronounced in glucose- than in pyruvate-perfused hearts but not significantly different from the accumulation in lactate-perfused hearts. It is of interest that in the three substrate groups arachidonic acid was found reincorporated into triacylglycerols during early ischemia. Reincorporation of arachidonic acid moieties into triacylglycerols along with the accumulation of free arachidonic acid implies a higher extent of phospholipid breakdown than would be suggested by free arachidonic acid accumulation only.

### Lipid Changes During Reperfusion

During reperfusion, the fatty acid content was profoundly reduced in glucose-perfused hearts. In contrast, in the lactate- and pyruvate-perfused hearts, fatty acid levels were raised above ischemic levels. The fatty acid accumulation was more pronounced in the lactate- than in the pyruvate-perfused hearts at the end of reperfusion that was preceded by 30 and 45 minutes of ischemia. The decrease of accumulated fatty acids in glucose-perfused hearts may be due to either oxidation or reincorporation into lipid pools. Release of fatty acids via coronary effluents is excluded in the present experimental setup because of the lack of circulating albumin. If oxidation of fatty acids is responsible for their decrease in glucose-perfused hearts, the availability of alternative sources may explain limited fatty acid consumption in lactate- or pyruvate-perfused hearts. Interestingly, the fatty acid accumulation did continue in these hearts upon reperfusion. The contribution of arachidonic acid to the fatty acid increase during reperfusion implies prolongation of the phospholipid breakdown.

The average tissue content of triacylglycerols did not significantly change by reperfusion in the three substrate groups. Concomitant release of glycerol indicated an operative triacylglycerol/fatty acid cycle during reperfusion (Table 4). In lactate-reperfused hearts, considerable rates of cycling were observed after 30 minutes of ischemia, whereas in glucose- and pyruvate-reperfused hearts, cycling rates were enhanced after 45 minutes of ischemia. Increased turnover of the triacylglycerol pool under reperfused circumstances did not coincide with elevated incorporation of arachidonic acid in triacylglycerols of pyruvate- and glucose-perfused hearts. Lactate-perfused hearts, though, demonstrated enhanced incorporation of arachidonic acid in triacylglycerols when preceded by 45 minutes of ischemia.

The consumption of ATP due to cycling during reperfusion as percentage of total energy production cannot be calculated, since no data are available to estimate ATP production during reperfusion. However, the rate of triacylglycerol cycling under reperfused circumstances did not exceed the rate of cycling under ischemic circumstances, even in hearts with the highest rates of cycling (lactate-reperfused hearts subjected to 45 minutes of ischemia). Hence, the ATP consumption by cycling during reperfusion will not rise above the ATP consumption during ischemia (<1.2 μmol ATP/g dry wt · min). Moreover, the ATP production is most likely higher during reperfusion than during ischemia, since oxidative pathways deliver more energy than the
nonoxidative pathway. Thus, it is very unlikely that the energy loss due to cycling during reperfusion will exceed 7% of the total energy production, as calculated under ischemic conditions.

**Hemodynamic Function and Lethal Cell Damage**

The choice of exogenous substrates markedly influenced the recovery of stroke volume. In this respect, hemodynamic function (expressed as stroke volume) was compromised more by lactate than by glucose after 30 minutes of ischemia, whereas pyruvate protected the ischemic hearts. After 45 minutes of ischemia, pyruvate also impaired recovery, whereas glucose as the sole substrate delayed severe cardiac depression. In the same line, Bünger and colleagues reported a detrimental effect of lactate on hemodynamic function after 45 minutes of low-flow ischemia by increasing the exogenous lactate concentration from 5 to 15 mM. Pyruvate, though, has been found to be a beneficial substrate in several studies. The present findings clearly demonstrate the importance of the duration of ischemia.

The release of LDH into the coronary perfusate supports the diverse effects of the three substrates after 30 minutes of ischemia, but no marked differences were found after 15 or 45 minutes of ischemia. Since the average LDH content is approximately 350 units per heart, the release of LDH after 30 minutes of ischemia and reperfusion indicates that in lactate-perfused hearts at least 4.3% of the myocardial cells lost their integrity. In glucose- and pyruvate-perfused hearts, the amount of LDH released represented 1.7% and 0.7% of total LDH activity, implicating less damage of cardiac cells after this time interval. Moreover, LDH release in lactate-perfused hearts was maintained at a high level throughout reperfusion after 30 minutes of ischemia (i.e., no maximum value accompanied by a rapid decline thereafter). The characteristic pattern of persisting LDH release may result from local underperfused areas after restoration of flow.

**Lipid Changes and Myocardial Damage**

The extent of fatty acid accumulation at the end of ischemia does not unequivocally correlate with the recovery of stroke volume during reperfusion. Glucose-perfused hearts showed the highest levels of fatty acids by ischemia, whereas recovery of stroke volume was better than in lactate-perfused hearts after 30 minutes of ischemia and prevailed over both lactate- and pyruvate-perfused hearts after 45 minutes of ischemia. However, an inverse relation between the tissue fatty acid content at the end of reperfusion and the recovery of stroke volume is found (Figure 3, Spearman's rank correlation \( r_s = -0.76 \)). The strength of this relation is not merely determined by the duration of ischemia, since a similar relation is observed when the three substrate groups subjected to 30 minutes of ischemia and subsequent reperfusion are considered \( r_s = -0.68 \). Figure 3 shows that the relation between the hemodynamic recovery and tissue fatty acid content at the end of reperfusion is not linear. There seems to be a threshold value of approximately 400 nmol fatty acids/g dry wt above which reperfused hearts do not recover. Accordingly, in the lactate-perfused hearts, the critical fatty acid threshold was surpassed after 30 and 45 minutes of ischemia and subsequent reperfusion, and hemodynamic function was impaired. In the pyruvate-perfused hearts, the threshold value was reached after 45 minutes of ischemia and subsequent reperfusion, and hemodynamic recovery was impaired. In contrast to the lactate-perfused hearts, the pyruvate-reperfused hearts did not demonstrate increased fatty acid levels above the critical threshold when the preceding time of ischemia was 30 minutes. In this group of pyruvate-perfused hearts, hemodynamic recovery was substantial. Moreover, the critical fatty acid threshold was not exceeded in either one of the glucose groups after reperfusion, whereas postischemic hemodynamic recovery was obvious in all glucose groups. Interestingly, when the fatty acid content is below the threshold value of approximately 400 nmol/g dry wt, no strong relation between tissue fatty acid content and hemodynamic recovery can
be observed. This statement is substantiated by the observation that nearly similar fatty acid levels at the end of reperfusion were found for glucose-perfused hearts subjected to 15, 30, and 45 minutes of ischemia, pyruvate-perfused hearts subjected to 15 and 30 minutes of ischemia, and lactate-perfused hearts subjected to 15 minutes of ischemia, whereas their hemodynamic recoveries were very diverse. Factors other than lipid changes are obviously responsible for the outcome of posts ischemic function below the critical fatty acid level. Excess production of oxygen free radicals, calcium overload, decreased production of (glycolytic) ATP, osmotic stress, and alterations of enzyme activities have been recognized as potential contributors to the degree of cardiac injury. Moreover, it has been suggested that pyruvate stimulates the activity of pyruvate dehydrogenase, which might explain the protective effect of exogenous pyruvate after 30 minutes of ischemia. However, this protective effect seems to be overruled as soon as the fatty acid threshold is surpassed.

Plotting data of triacylglycerol cycling during reperfusion against hemodynamic recovery indicate a weak negative relation between the extent of triacylglycerol cycling and recovery of hemodynamic function. Moreover, it has been suggested that pyruvate stimulates the activity of pyruvate dehydrogenase, which might explain the protective effect of exogenous pyruvate after 30 minutes of ischemia. However, this protective effect seems to be overruled as soon as the fatty acid threshold is surpassed.

A strong relation is obtained between arachidonic acid accumulation during reperfusion and hemodynamic function ($r=-0.77$); hence, breakdown of phospholipids may be involved. Although the fatty acid increase up to 1,000 nmol/g dry wt reflects less than 0.4% phospholipid breakdown when the fatty acids totally come from the phospholipid pool (on fatty acid base), the cell's integrity may be well compromised when breakdown of phospholipids is confined to damaged cells or more selectively reflects sarcolemma breakdown. In this respect, a positive, but weak, relation between tissue fatty acid accumulation and enzyme release during reperfusion ($r=-0.50$) is observed. It seems contradictory that there is no relation between arachidonic acid accumulation (and hence membrane damage) during ischemia and recovery of hemodynamic function after restoration of flow. A possible explanation might be that arachidonic acid accumulation is compartmentalized at different sites during ischemia and reperfusion. Schrijvers and colleagues have demonstrated that ischemia induces morphological changes in predominantly mitochondrial membranes, whereas reperfusion induces changes in sarcolemal membranes. It is unclear whether the relation between arachidonic acid accumulation and hemodynamic recovery is causal.

Recent findings indicate that phospholipid breakdown is not merely a consequence of membrane rupture by osmotic stress but an independent process. Further, an earlier study by Van Bilsen and colleagues has shown that treatment of ischemic hearts with mepacrine, a putative phospholipase inhibitor, results in a significant reduction of fatty acid accumulation during reperfusion and, concomitantly, a better recovery of hemodynamic function. Hence, breakdown of phospholipids and accumulation of fatty acids during reperfusion (in a sufficiently severe degree) may be well responsible for cardiac dysfunction.

To summarize, no direct correlation between ischemic lipid changes and posts ischemic function was found. However, when the fatty acid content increased above a critical threshold of approximately 400 nmol fatty acids/g dry wt during reperfusion, posts ischemic function was impaired. The fatty acid threshold was surpassed in the presence of either lactate or pyruvate in the perfusion buffer but not in the presence of glucose only. Moreover, lactate augmented fatty acid accumulation during reperfusion compared with pyruvate. Triacylglycerol cycling did not importantly contribute to the extent of cardiac recovery. Accumulation of arachidonic acid during reperfusion demonstrated a strong relation with cardiac recovery after restoration of flow, whereas arachidonic acid accumulation during ischemia did not relate to posts ischemic cardiac function. Hence, derangements in phospholipid homeostasis during reperfusion might be involved in myocardial damage. The specific effect of lactate (compared with pyruvate and glucose) on membrane function (or in particular on the sarcolemma) remains to be elucidated.

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Substrate-induced changes in the lipid content of ischemic and reperfused myocardium. Its relation to hemodynamic recovery.

M J de Groot, W A Coumans, P H Willemsen and G J van der Vusse

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