Crossover Plot Study of Glycolytic Intermediates in the Ischemic Canine Heart

Kazuo Ichihara, Ph.D. and Yasushi Abiko, M.D.

Summary

Effects of regional ischemia on myocardial glycolysis were studied by measuring the levels of glycolytic intermediates in the endo- and epicardial layers of the left ventricle in dogs anesthetized with pentobarbital. Regional ischemia was induced by ligating a small branch of the left anterior descending coronary artery. The myocardial tissue samples were removed before and 1.5, 3, 7, or 30 min after coronary artery ligation. Based on a crossover plot study of the glycolytic intermediates, it is suggested that the activity of glycogen phosphorylase was accelerated, while that of phosphofructokinase was inhibited in ischemic myocardium samples removed 1.5, 3, 7, and 30 min after ligation. When the frozen myocardium was allowed to stand at room temperature for 10 min, the crossover plot study revealed acceleration of phosphofructokinase activity.

The metabolic response to regional ischemia of the endocardial layers was more marked than that of the epicardial. During ischemia the levels of adenine nucleotides did not change significantly, but those of citrate and hydrogen ions increased significantly. It appears that inhibition of myocardial phosphofructokinase activity during ischemia is partly due to an increase in the levels of citrate and hydrogen ions in the ischemic tissue.

Additional Indexing Words:
Glycogen phosphorylase    Phosphofructokinase    Adenine nucleotides    Citrate    Myocardial pH

A NOXIA or ischemia of the heart produces acceleration of myocardial glycolysis. In the previous papers we have demonstrated that ligation of a small branch of the canine left coronary artery accelerated glycogenolysis in the ischemic region, and that the acceleration of myocardial...
glycogenolysis after coronary ligation was prevented by pretreatment with propranolol.\textsuperscript{6)} The effect of anoxia or ischemia on myocardial glycolysis, however, is still a point of controversy. In the isolated perfused rat heart, anoxia accelerates myocardial glycolysis,\textsuperscript{9,10} but ischemia may either accelerate or inhibit myocardial glycolysis depending upon the extent of and/or time after reduction of coronary flow.\textsuperscript{11,12} In the dog heart \textit{in situ}, Opie\textsuperscript{13} demonstrated that glycolytic flux increased within 1 min after coronary artery ligation, thereafter decreasing to the control level or even less, and suggested that activity of phosphofructokinase decreased when the glycolytic flux was reduced.

The present study was undertaken to examine the effect of ligation of a small branch of the coronary artery on myocardial glycolysis. The activities of glycolytic enzymes were estimated by crossover plots, which were constructed by plotting percent changes of the myocardial levels of glycolytic intermediates after ischemia. A similar study was performed in the "thawing" myocardium (autolysis) resembling the anoxic heart, to compare the metabolic responses of the anoxic heart and ischemic heart. Since the metabolic response to ischemia might differ for the endocardial and epicardial layers of the myocardium,\textsuperscript{7,14} the effect of ischemia on both layers was studied.

\textbf{Materials and Methods}

\textit{Experimental design and animal preparation}

The following experiments were conducted using 61 mongrel dogs, weighing 8–15 Kg, anesthetized with intravenous sodium pentobarbital (30 mg/Kg):

1. effect of ischemia on myocardial glycolysis (40 dogs),
2. effect of "thawing" frozen myocardium on glycolysis (6 dogs),
3. effect of ischemia on the myocardial citrate level (10 dogs),
4. effect of ischemia on myocardial pH (5 dogs).

Under artificial respiration, the chest was opened to permit free access to the left ventricular wall. "Coronary artery ligation" was performed by ligating one of the small branches of the left anterior descending coronary artery with a silk thread to induce regional ischemia in the left ventricle. Blood pressure and electrocardiogram (limb lead II) were recorded throughout the experiments. In most of the experiments (except for the myocardial pH experiment), the region of myocardium that had been perfused by the ligated coronary artery was rapidly removed with scissors before, or 1.5, 3, 7, or 30 min after coronary artery ligation, and immediately pressed and frozen with freezing clamps previously chilled with liquid nitrogen. The time for removing and freezing the myocardium was less than 10 sec. The ischemic
region was estimated by tissue cyanosis and changes in epicardial ECG induced by coronary artery ligation. The ischemic tissue sample was removed from the area of cyanosis. Thus prepared, the frozen myocardium was carefully cracked into fragments with a chisel chilled with liquid nitrogen, so that the fragments originating in the endo- and epicardial layers could be collected separately. Details of the method for separating the myocardium into the endo- and epicardial portions have been described earlier.7) The frozen endo- and epicardial tissue samples (fragments) were then plunged into liquid nitrogen and stored in it until extraction.

In experiments on the "thawing" myocardium, the frozen endo- and epicardial tissue samples were obtained from nonischemic normal hearts by the method described above, and each sample was divided into two parts. A part of the frozen endo- and epicardial samples was allowed to stand at room temperature for 10 min ("thawing" myocardium), and the other was not allowed to thaw but kept frozen at liquid nitrogen temperature. The "thawing" myocardium was frozen again with liquid nitrogen after 10 min of standing at room temperature. Both frozen and "thawing" myocardial samples were stored in liquid nitrogen until extraction.

In experiments on the citrate level, the endo- and epicardial levels of citrate, fructose-6-phosphate (F6P), and fructose-1,6-diphosphate (FDP) were measured in both nonischemic and ischemic (3 min after coronary ligation) myocardium.

In experiments on myocardial pH, a micro glass pH electrode (MI-410, Microelectrodes, Inc) was inserted into the region of myocardium which was expected to become ischemic during coronary artery ligation as described previously.15) The diameter of the pH electrode tip was about 0.8 mm. The tip of the electrode was inserted into the myocardium at about the middle of the ventricular wall. In order to measure the depth to which the tip of the electrode had been inserted, the ventricular wall was taken and cut transmurally by scissors after the experiment was done. The left ventricular wall thickness of the canine heart was about 10-15 mm, and the depth to which the tip had reached was about 6-8 mm from the surface of the left ventricular wall. The pH electrode was connected to a pH meter (F-7, Horiba Co, Ltd), and calibrated with standard pH solution (pH 6.84 and 7.384) before each experiment. Myocardial pH was recorded continuously on a pen recorder (Model VP-653B, Matsushita Communication Industrial, Co, Ltd) before, during, and after regional ischemia, in which a small branch of the left anterior descending coronary artery was ligated for 30 min.

**Analysis of tissue intermediates**

Each myocardium stored in liquid nitrogen was divided into two parts.
One part was pulverized in a mortar chilled with liquid nitrogen and extracted in ice-cold 1 N perchloric acid to determine the tissue levels of glucose-1-phosphate (G1P), glucose-6-phosphate (G6P), F6P, FDP, dihydroxyacetone phosphate (DHP), glyceraldehyde-3-phosphate (G3P), 3-phosphoglycerate (3PG), 2-phosphoglycerate (2PG), phosphoenolpyruvate (PEP), pyruvate, lactate, adenosine triphosphate (ATP), adenosine diphosphate (ADP), adenosine monophosphate (AMP), and citrate. The level of lactate was measured in the neutralized perchloric acid extract by the method of Barker and Summerson,16) and the levels of other metabolites were measured in the neutralized acid extract by the standard enzymatic method described by Bergmeyer.17)

The other part of the divided tissue sample was used for determination of the tissue level of glycogen according to the method of Seifter et al.18)

Statistical analysis
All values are expressed as mean±SEM, and a p value of 0.05 or less was considered significant.

RESULTS

1. Effect of ischemia on myocardial glycolysis

Dogs were divided into 5 groups according to the duration of ischemia: a control group (in which hearts were removed before coronary artery ligation), and 1.5, 3, 7, and 30 min groups (in which hearts were removed 1.5, 3, 7, and 30 min after coronary ligation, respectively). Eight dogs were used for each of the 5 groups. Before coronary artery ligation, mean arterial blood pressure and heart rate were 115.6±3.6 mmHg and 142.3±2.6 beats·min⁻¹, respectively, and there were no significant differences in blood pressure and heart rate between before and 1.5, 3, 7, or 30 min after coronary ligation. Table I shows the levels of glycolytic intermediates in the ischemic myocardium (3 min group) and the nonischemic myocardium (control group). In the control group, the levels of glycogen, G6P and lactate in the endocardial layers were significantly (p<0.05) higher than those in the epicardial, but the levels of other intermediates in the endocardial layers were not significantly different from those in the epicardial. In the 3 min group, the levels of G1P, G6P, F6P, and lactate in the endocardial layers were significantly (p<0.01) higher than those in the epicardial, but the level of FDP in the endocardial layers was significantly (p<0.05) lower than in the epicardial. The levels of glycogen and FDP in both layers were low in the 3 min group, and the levels of hexose monophosphates (G1P, G6P, and F6P) and lactate were high as compared to those in the control group. The levels of the other glycolytic intermediates in the 3 min group did not significantly differ from those in the control group,
Table 1. Changes in Myocardial Glycolytic Intermediates during Coronary Artery Ligation for 3 Min

<table>
<thead>
<tr>
<th>Glycolytic intermediates (nmol·g wet wt⁻¹)</th>
<th>Control group (n=8)</th>
<th>3 min group (n=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Endocardial layers</td>
<td>Epicardial layers</td>
</tr>
<tr>
<td>Glycogen</td>
<td>61279±4163a</td>
<td>51287±3330</td>
</tr>
<tr>
<td>G1P</td>
<td>18±4</td>
<td>9±2</td>
</tr>
<tr>
<td>G6P</td>
<td>313±38a</td>
<td>192±20</td>
</tr>
<tr>
<td>F6P</td>
<td>33±7</td>
<td>24±6</td>
</tr>
<tr>
<td>FDP</td>
<td>61±16</td>
<td>51±8</td>
</tr>
<tr>
<td>DHP</td>
<td>59±13</td>
<td>58±9</td>
</tr>
<tr>
<td>G3P</td>
<td>16±5</td>
<td>17±7</td>
</tr>
<tr>
<td>3PG</td>
<td>132±18</td>
<td>121±13</td>
</tr>
<tr>
<td>2PG</td>
<td>10±3</td>
<td>13±2</td>
</tr>
<tr>
<td>PEP</td>
<td>19±3</td>
<td>20±3</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>110±23</td>
<td>91±14</td>
</tr>
<tr>
<td>Lactate</td>
<td>3200±270a</td>
<td>2240±237</td>
</tr>
</tbody>
</table>

Hearts (left ventricular myocardium) were removed before (control group), or 3 min after ligation of a small branch of the left anterior descending coronary artery (3 min group). Analytical methods and abbreviated names of intermediates are described in Materials and Methods. Values are expressed as mean±SEM.

a p<0.05; b p<0.01. Significant difference from epicardial layers in the same group.

c p<0.05; d p<0.01. Significant difference from values in the control group.

except for the endocardial 3PG level. Similar results were obtained in hearts that were removed 1.5, 7, or 30 min after coronary ligation.

The levels of glycolytic intermediates in the 1.5, 3, 7, and 30 min groups are illustrated in Fig. 1, in which values of the intermediates obtained in the ischemic groups are expressed as a percent of those in the control group (crossover plots). The general pattern of crossover plots revealed increases in the levels of G1P, G6P, F6P, and lactate, and decreases in the levels of glycogen, FDP, DHP, and 3PG. The order of intensity of increase in hexose monophosphate levels was F6P>G6P>G1P. The decrease in FDP was indisputably evident when the ischemic period was prolonged. It is clear that there is a positive crossover point between glycogen and G1P, and a negative crossover point between F6P and FDP.

The levels of ATP, ADP, and AMP were also determined in this series of experiments. Representative data in the ischemic group (3 min group) and control group are shown in Table II. The levels of ATP, ADP, and AMP in the 3 min group were not significantly different from those in the control group.

2. Effect of "thawing" the frozen myocardium on glycolysis

In this series of experiments, the effect of "thawing" the frozen myo-
cardium on the glycolytic intermediate levels was studied to define the difference between anoxia and ischemia. In Fig. 2, changes in the levels of glycolytic intermediates and adenine nucleotides in the "thawing" myocardium are expressed as a percent of those in the frozen myocardium. The levels of intermediates and adenine nucleotides in nonischemic frozen myocardium in this series of experiments were similar to those in the control group in the preceding series of experiments. The myocardial glycogen and hexose monophosphates levels in both layers decreased after the frozen myocardium was al-
Table II. Changes in Myocardial Adenine Nucleotides during Coronary Artery Ligation for 3 Min

<table>
<thead>
<tr>
<th></th>
<th>ATP (μmoles·g wet wt⁻¹)</th>
<th>ADP (μmoles·g wet wt⁻¹)</th>
<th>AMP (μmoles·g wet wt⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Endocardial layers</td>
<td>Epicardial layers</td>
<td>Endocardial layers</td>
</tr>
<tr>
<td>Control group (n=8)</td>
<td>3.41±0.19</td>
<td>3.48±0.20</td>
<td>0.86±0.11</td>
</tr>
<tr>
<td>3 min group (n=8)</td>
<td>3.18±0.09</td>
<td>3.40±0.09</td>
<td>1.00±0.10</td>
</tr>
</tbody>
</table>

Hearts were removed before (control group), or 3 min after coronary ligation (3 min group). Heart samples used in this experiment are those in the experiment of Table I. Values are expressed as mean±SEM.

Fig. 2. Crossover plots of the endo- (○) and epicardial (●) glycolytic intermediates and adenine nucleotides in the “thawing” myocardium. Each of the frozen nonischemic endo- and epicardial samples was divided into two. One part was allowed to stand at room temperature for 10 min (“thawing” myocardium), and the other was not allowed to thaw but kept frozen. Details are described in Materials and Methods. Values in the frozen nonischemic myocardium are taken as 100% (control), and those in the “thawing” myocardium are expressed as percent of the control values. See text for abbreviated names. * p<0.05, ** p<0.01 compared to values in the frozen myocardium.
allowed to stand at room temperature for 10 min, while the level of FDP and lactate increased. The pattern of changes in intermediate levels in the "thawing" myocardium was entirely different from that in the ischemic frozen myocardium, in which hexose monophosphates increased and FDP decreased.

When the frozen myocardium was allowed to thaw at room temperature for 10 min, the endocardial ATP level decreased to 49% of that in the frozen myocardium and the epicardial ATP level also decreased to 67% of that in the frozen myocardium. On the other hand, the levels of ADP and AMP in both layers increased when the frozen myocardium was allowed to thaw.

3. Effect of ischemia on the myocardial citrate level

In this series of experiments, endo- and epicardial levels of citrate, F6P and FDP were determined in the frozen myocardium. Five out of 10 dogs were used for the control (nonischemic) group, and 5 other dogs were used for the ischemic (3 min) group. The results are shown in Table III. The F6P level of both layers in the ischemic group was significantly (p<0.05) higher than that in the control group, and the FDP level of both layers in the ischemic group was significantly lower (p<0.01, endocardial; p<0.05, epicardial). A negative crossover point was observed between F6P and FDP in both layers as shown in Fig. 1; the endo- and epicardial citrate levels increased significantly (p<0.05).

4. Effect of ischemia on myocardial pH

The effect of regional ischemia on myocardial pH is illustrated in Fig. 3. The regional pH in the nonischemic myocardium (before ligation) was 7.35±0.09. After a small branch of the left anterior descending coronary artery was ligated, the myocardial pH decreased markedly. The decrease in pH of ischemic myocardium became significant after 3 min of coronary ligation; the pH value 30 min after ischemia was 6.42±0.13. The myocardial pH, how-

| Table III. Changes in Myocardial F6P, FDP, and Citrate during Coronary Artery Ligation for 3 Min |
|--------------------------------------------------|--------------------------------------------------|--------------------------------------------------|
|                                                        | F6P (nmole•g wet wt⁻¹) | FDP (nmole•g wet wt⁻¹) | Citrate (nmole•g wet wt⁻¹) |
|                                                        | Endocardial layers | Epicardial layers | Endocardial layers | Epicardial layers | Endocardial layers | Epicardial layers |
| Control group (n=5)                                   | 39±9               | 16±4               | 62±10              | 56±11              | 132±6              | 126±9              |
| 3 min group (n=5)                                     | 90±16*             | 32±5*              | 29±5**             | 41±7*              | 164±8*             | 159±6*             |

Hearts were removed before (control group), or 3 min after coronary ligation (3 min group). Values are expressed as mean±SEM.

* p<0.05; ** p<0.01. Significant difference from values in the control group.
ever, increased immediately after release of the occluded coronary artery, and returned to the pre-ligation level. The pH value 10 min after the release was 7.30±0.10. Cardiac arrest did not occur after release of the ligated coronary artery.

**Discussion**

The results of the present study indicate that there are two crossover points in the glycolytic pathway of ischemic myocardium; one is a positive crossover point between glycogen and G1P, and the other is a negative crossover point between F6P and FDP. The former corresponds to acceleration of glycogen phosphorylase activity and the latter inhibition of phosphofructokinase activity (PFK). Thus the acceleration of glycogen phosphorylase during ischemia was confirmed. Usually anoxia of the heart increases the PFK activity of the myocardium, but our results indicate that ischemia of the heart decreases the PFK activity. Since the order of intensity of the accumulated hexose monophosphate levels in the ischemic myocardium was F6P >G6P>G1P, and since the level of FDP progressively decreased along with prolongation of the ischemic period, it is very likely that PFK in the ischemic myocardium is inhibited. Opie suggested that PFK should be inhibited within 1 min after coronary artery ligation, supporting the results obtained in the present study with PFK activity.

The results of experiments with the “thawing” myocardium (autolysis)
indicate that PFK activity increases during "thawing" of frozen myocardium. This metabolic situation resembles that in myocardial anoxia, in which glycogen and hexose monophosphate decreased, and FDP and lactate increased.19) Therefore, it is likely that PFK activity increases during anoxia while it decreases during ischemia. Nevertheless there is a possibility that the PFK activity may increase within 1.5 min after ischemia and then decrease.

The question remains, however, whether apparent inhibition of PFK, even when detected by a crossover plot study, actually represents suppression of PFK itself, because increased rate of glycolytic flux along with the accumulation of F6P may mimic PFK inhibition. However, it can be mentioned that the "relative" activity of PFK is inhibited even when the rate of glycolytic flux is accelerated. In vitro determination of the activity of PFK extracted from the myocardium does not make any sense in this kind of experiment, because there are many effectors that modify the activity of PFK in vivo; a change of myocardial PFK activity produced by altered concentrations of the effectors in the ischemic heart cannot be detected in vitro. Therefore, the glycolytic flux cannot be measured accurately in this particular type of experiment as suggested by Opie.13)

In the isolated perfused rat heart, anoxia accelerates myocardial PFK activity,1)19) but ischemia (whole-heart ischemia) does not.12) Whole-heart ischemia of the rat heart accelerates glycolysis when the coronary flow is reduced to 60% of the control, but it inhibits glycolysis at the level of glyceraldehyde-3-phosphate dehydrogenase (G3P dehydrogenase) when the coronary flow is further reduced.20) In the present experiments, the coronary artery branch was completely ligated. This does not mean, however, that peripheral blood flow through collaterals is constant. The reason why regional myocardial ischemia of the dog heart produces inhibition of PFK while whole-heart ischemia of the rat heart produces inhibition of G3P dehydrogenase remains to be clarified. Differences exist, however, between myocardial ischemia in the rat heart and dog heart experiments, consequently making it difficult to directly extrapolate the results obtained in the dog heart to those in the rat heart.

By what mechanisms is PFK inhibited during coronary artery ligation? There are three major factors affecting PFK activity: adenine nucleotides,9)21) citrate,22) and hydrogen ions.21)–24) The levels of ATP, ADP, and AMP in the ischemic myocardium did not differ from those in the nonischemic myocardium as shown in Table II. Accordingly, it appears that adenine nucleotides are not primary factors inhibiting PFK. The myocardial level of citrate was elevated in the ischemic myocardium (Table III), suggesting that increased levels of citrate may inhibit PFK activity.22) In the present experi-
ment, however, the increase in citrate level was about 24%. It is not certain whether a 24% increase in citrate level is enough to inhibit PFK activity in the ischemic myocardium. The myocardial pH measured by a micro glass pH electrode decreased markedly after coronary artery ligation (Fig. 3). A decrease in pH inhibits the activity of PFK in vitro.\textsuperscript{21,23,24} The decrease in myocardial pH during ischemia seems to be responsible for inhibition of PFK, although the percent contribution of acidosis to the PFK inhibition has not yet been determined.

The metabolic response of endocardial layers to coronary artery ligation was greater than that of epicardial layers. This result accords with the findings of our previous paper,\textsuperscript{7} and suggests that the endocardial layers are more sensitive to coronary artery ligation.

In conclusion, when the coronary artery is ligated, myocardial glycolysis is inhibited at the level of PFK probably because of increases in the levels of citrate and hydrogen ions.

**ACKNOWLEDGMENTS**

The authors gratefully acknowledge the helpful suggestions of Dr. Michio Ui, Professor of Pharmaceutical Science, Hokkaido University. The technical assistance of Mrs. Mikiko Ichihara and Mr. Tadahiko Yokoyama is deeply appreciated.

**REFERENCES**

2. Bing RJ: Cardiac metabolism. Physiol Rev 45: 171, 1965
24. Ui M: Multiple inhibitor sites for ATP on muscle phosphofructokinase as influenced by a change of pH: a computer analysis of "nonlinear" kinetic data. Biochim Biophys Acta 159: 50, 1968