Inhibition of Glycolysis in the Denervated Dog Heart

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SUMMARY We measured glucose metabolism in five dogs before and 3 weeks after cardiac denervation; after this time myocardial norepinephrine is depleted. The discharge by the myocardium of $^{14}$CO$_2$ from infused $^{14}$C-D-glucose (U), decreased following denervation ($P = 0.05$). The ratio of $^{14}$CO$_2$ to total CO$_2$ production, which measured the proportion of glucose to total substrate oxidized, also decreased following denervation ($P = 0.05$). The inhibition of glucose oxidation by denervation was not due to an increase in arterial lactate concentration. There was an associated increase in myocardial content of fructose-6-phosphate in an additional seven dogs ($P < 0.01$). We postulate that myocardial tissue norepinephrine is one of the controllers of the activity of phosphofructokinase. Circ Res 47: 338-345, 1980

CHRONIC denervation of the heart leads to depletion of tissue catecholamines. Such denervation has been claimed to cause metabolic abnormalities (Barta et al., 1966, 1967; Barta and Pappova, 1968). However, it has not been possible to find any abnormality of enzyme content in hearts from dogs with selective cardiac denervation (Noble et al., 1972), in which general abnormalities of the whole body are avoided. In this study we have used this preferred method of denervation (Donald and Shepherd, 1963) to study directly the metabolism of glucose by infusion of $^{14}$C-D-glucose and measurement of $^{14}$CO$_2$. These measurements indicated an inhibition of glucose oxidation. Therefore, in an attempt to find the step in the glycolytic pathway at which this inhibition occurred, we made measurements of some glycolytic intermediates.

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Methods

Twelve male, mongrel dogs were studied. They were divided into two groups: series I, five dogs, weighing 15.0-17.0 kg for glucose oxidation studies, and series II, seven dogs, weighing 14.5-21.0 kg for tissue biopsy studies.

Series I (Glucose Oxidation Group)

The dogs were subjected to a standardized environment and diet for 3 weeks before as well as throughout the period of study. The first study was carried out 1 week prior to cardiac denervation. The dogs were anesthetized with intravenous methohexitone sodium (10 mg/kg for induction, wearing off in 5 minutes), followed by a standard dose of chloralose (Arfors et al., 1971).

The left jugular vein was exposed through a small incision and a cardiac catheter inserted. The catheter was advanced and positioned in the coronary sinus under fluoroscopic control. The position of the catheter was checked by visualizing the coronary sinus with a bolus injection of 5.0 ml of Uro-
To avoid any contamination with right atrial blood (Gregg et al., 1972), the catheter was advanced more than 15 mm inside the coronary ostium (Koberstein et al., 1969). Also, the sampling rate through the coronary sinus catheter was kept to 2.0 ml/min. A cannula was inserted into a branch of the femoral artery and connected to a 3-way tap. A cannula was also inserted into a peripheral vein and connected to a 3-way tap for the infusion of U. 

Arterial pressure was measured via the arterial cannula with an Elcomatic EM 750 pressure transducer, with a Hewlett-Packard 8850B carrier-amplifier. An electrocardiogram was obtained from a Medelec AA6 Mk. III AC amplifier. Variables were recorded on a Medelec MS6 recording oscilloscope.

A control pair of samples was taken for measurement of PO₂, PCO₂, pH, oxygen content, lactate, glucose, and free fatty acids (FFA). An infusion of sterile saline (0.9%) containing 125 μCi of ¹⁴C-D-glucose (Radiochemical Centre) was begun at a rate of 1.2 ml/min, after a bolus injection of 2.0 ml. After 10 minutes of infusion, four pairs of arterial and coronary sinus blood samples were taken at 4-minute intervals for measurement of PO₂, PCO₂, pH, oxygen content, lactate, glucose, total CO₂ and ¹⁴CO₂, and one pair of samples for FFA at the end of the experiment. One week later, the dog was subjected to cardiac denervation, using the technique of regional neural ablation (Donald and Shepherd, 1963, 1964a, 1964b, 1965; Donald et al., 1968; Donald and Samueloff, 1966). Evidence for denervation has been presented (Noble et al., 1972; Drake et al., 1978). The post-denervation study was carried out 3 weeks subsequent to this, an identical protocol to the control study being followed.

**Measurement of Total CO₂ and ¹⁴CO₂**

Total CO₂ was measured by the manometric method of Natelson (1951). Error of the measurement was about 1%. ¹⁴CO₂ was measured as described by Riemersma et al. (1971/72); the procedure was performed in triplicate. After 3 hours of absorption, the glass cup containing the ethanolamine methanol mixture was transferred to a clean glass counting vial and 10.0 ml of NE260 scintillant (Nuclear Enterprises) were added. Recovery of ¹⁴CO₂ from NaH¹⁴CO₃ by this method was 89.9 ± 2.0%. To determine the specific activity of the ¹⁴C-D-glucose in blood, 0.5 ml of the perchloric acid (PCA) supernatant was taken, to which 10.0 ml of NE260 were added. The CO₂ content of the PCA supernatant was less than 0.1 mM (i.e., below the limit of detection of total CO₂ measurement). All radioactive samples were counted in an NE8312 spectrometer and corrected for quenching with an external standard. Efficiency of counting was 36-40%.

**Calculations**

The results of the radioactive studies were calculated from the following formulas: (1) \( A-V¹⁴CO₂ = A-V¹⁴CO₂ \text{ counts} \times (\text{arterial glucose/arterial } ¹⁴C\text{-glucose}) \) where \( A-V¹⁴CO₂ = \) the difference between arterial and coronary sinus blood for \( ¹⁴CO₂ \) corrected for the specific activity of the injectate, in \( \mu\text{mol/ml} \). \( A-V¹⁴CO₂ \text{ counts} \) are in counts/min, arterial glucose in \( \mu\text{mol/ml} \), and arterial \( ¹⁴C\text{-glucose} \) in counts/min. (2) \( \text{CO₂ produced from glucose/CO₂ production} = [(6 \times \text{A-V¹⁴CO₂}) \times (\text{coronary blood flow})]/[(\text{A-V total CO₂}) \times (\text{coronary blood flow})] \), where \( \text{CO₂ produced from glucose} \) and \( \text{CO₂ production} \) are in \( \mu\text{mol/min} \) and \( \text{A-V¹⁴CO₂} \) is in \( \mu\text{mol/ml} \). Coronary blood flow is in ml/min, but because it cancels out, it was not measured. These results were expressed as percentages. Six \( \times \) \( A-V¹⁴CO₂ \) was necessary, as there are six carbon atoms per mole of D-glucose, and it was U-labeled with \( ¹⁴C \). The standard deviation of individual measurements of the pairs of samples was ± 4.6%. The mean of four such values was calculated for each study and these values are presented in Figure 1.

**Figure 1** A: The difference between arterial and coronary sinus \( ¹⁴CO₂ \) concentration, normalized for the specific activity of arterial glucose. Each line connects the pre- and post-denervation studies on individual dogs. There was a decrease following denervation in five dogs. B: \( \text{CO₂ produced from glucose as a percentage of total CO₂ production} \), i.e., the percentage of total substrate oxidized which was glucose. Presentation and results were similar to those in A.
All venous CO₂ and ¹⁴CO₂ values were higher than arterial values; the A-V differences were expressed as positive values.

Critique of the ¹⁴C-Glucose Method

The only assumption made in this method of assessing myocardial glucose oxidation is that the radioactivity in the PCA supernatant is due solely to ¹⁴C-glucose. The PCA expels all CO₂, but ¹⁴C-lactate produced from ¹⁴C-glucose by peripheral tissues (particularly brain and skeletal muscle) could have been present. The difference in counts between arterial ¹⁴C-glucose and arterial ¹⁴CO₂ was of the order of 200:1. Therefore, the ratio of ¹⁴C-glucose to ¹⁴C-lactate throughout the infusion period (25 minutes overall, including equilibration and sampling times) would have been related to peripheral lactate production rate and, therefore, to arterial lactate level. We have checked this in a separate series of dogs under the same anesthesia by measuring the ¹⁴CO₂-to-total CO₂ production rate ratios, at the various spontaneous arterial lactate levels. An inverse relationship between the ratio and arterial lactate was found (Drake et al., 1980) whereas, if a significant arterial level of ¹⁴C-lactate had been present, the opposite result would be obtained (Drake et al., 1980). Finally, Issekutz et al. (1965) infused ¹⁴C-glucose (U) into intact dogs for 50 minutes. Lactate was separated from glucose by elution on resin columns. When the glucose eluate carried 600 counts/min per 2 ml, no measurable radioactivity (less than 1%.) Therefore a small amount of peripheral tissues (particularly brain and skeletal muscle) as well as lactate present. Glucose eluate was neutralized with Tris-KOH (0.2 M Tris and 30% KOH, mixed 6:4). The extracts were centrifuged for 10 minutes at 2000 rpm at +4°C. The supernatant was decanted and analyzed that day, or stored overnight under liquid nitrogen until analysis.

Rectal temperature was measured during this procedure. When the biopsy procedure was complete, the anesthetic gases were changed to nitrous oxide and oxygen, and halothane was administered if necessary. The cannula was removed from the internal mammary artery, which was then tied off and the cardiac denervation procedure carried out, the left side being completed first. After successful surgery, the chest drains were removed on the first or second postoperative day. The dogs were left for 3 weeks to allow endogenous catecholamines to deplete.

At the time of the post-denervation study, the dogs were again anesthetized with methohexitone sodium (10 mg/kg) followed by chloralose (100 mg/kg). A left thoracotomy was performed and the heart exposed. We took biopsies, using the same procedure as described above, and these were stored under liquid nitrogen until analysis.

Tissue Analysis

Metabolites

The biopsies (approximately 10 mg) were extracted in 100 μl of a PCA:acetone extraction mixture at −10°C. This mixture was neutralized with 50 μl of Tris:KOH (0.2 M Tris and 30% KOH, mixed in the ratio 6:4). The extracts were centrifuged for 10 minutes at 2000 rpm at +4°C. The supernatant was decanted and analyzed that day, or stored overnight under liquid nitrogen.

Analysis of the extracts for adenosine triphosphate (ATP), creatine phosphate (CP), glucose-6-phosphate (G6P), and fructose-6-phosphate (F6P) was made fluorometrically with a Farrand Ratio Fluorometer Mark II (Kontron Instruments). ATP and CP were analyzed by means of a modification, for fluorometric analysis, of the method of Lam-
FFA pernatant (see above) was diluted with 0.3 M PCA until analysis. Lactate was measured enzymatically, supernatant was decanted and stored at —20°C elapsed between the two studies could have resulted performed here because the 3 weeks that had the in vivo conditions of the experiment. They were only by the amount of enzyme present and give no information about the activity of the enzyme under the in vivo conditions of the experiment. They were performed here because the 3 weeks that had elapsed between the two studies could have resulted in depletion of the enzyme protein.

Enzymes

Total enzyme activities were measured histochemically using the linkage of the reduced coenzyme nicotinamide adenine dinucleotide (NADH₂) to a hydrogen acceptor with the formation of an insoluble colored formazan which was precipitated in the section. Sections 10 µm thick were cut from the frozen biopsy on a Bright cryostate. The unfixed sections were incubated at 37°C in media saturated with nitrogen. Details of the method and incubation media for measurement of phosphofructokinase (PFK) levels have been published recently (Butcher and Papadoyannis, 1979; DE Papadoyannis, B Henderson, and RG Butcher, unpublished observations). Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) activity was measured by the method of Henderson (1976). Enzyme activities were quantified with a Vickers M85 microdensitometer, and the results were expressed as nmol H₂·cm⁻² per 10 minutes. These assays are similar to those carried out on myocardial extracts in vitro. Since the in vitro conditions are optimized, they are influenced only by the amount of enzyme present and give no information about the activity of the enzyme under the in vivo conditions of the experiment. They were performed here because the 3 weeks that had elapsed between the two studies could have resulted in depletion of the enzyme protein.

Measurement of Metabolites in Blood

Lactate

Approximately 1.0 ml of blood was injected into a preweighed tube containing 2.0 ml of 0.6 M PCA, kept in crushed ice. The tube was shaken well immediately. At the end of the experiment, all the tubes were reweighed and centrifuged at +4°C. The supernatant was decanted and stored at —20°C until analysis. Lactate was measured enzymatically, using a Boehringer kit (Boehringer Corporation London, Lactate, U-V method using lactate dehydrogenase only).

Glucose

One hundred microliters of the frozen PCA supernatant (see above) was diluted with 0.3 M PCA in the ratio of 1:2.8. This was assayed according to the BCL hexokinase U-V method for glucose.

FFA

Five milliliters of blood were chilled in ice immediately, centrifuged at +4°C, and the plasma stored at —20°C until analysis. The plasma was then thawed at room temperature and chilled immediately in crushed ice before assay. The FFA levels were measured by the microtitration method of Chlouverakis (1963), using the dye of Gordon (1957) and the extraction method of Dole and Mehnert (1960).

Gases

PO₂, PCO₂, and pH were measured in all samples with a Corning-165 pH/blood gas analyzer. Oxygen content measurements were made with a Lex O₂-ConTL.

Statistical Analysis

Statistical evaluation was made according to the method of Snedecor and Cochran (1973). The probability of difference in paired studies being due to chance was calculated by the Sign test (Dixon and Mood, 1946), with a correction for continuity (Snedecor and Cochran, 1973). Probability values of less than 0.05 were accepted as statistically significant, and values over 0.05 as not significant.

Results

Glucose Oxidation Studies (Series I)

The results of the paired studies of the oxidation of ¹⁴C-D-glucose are shown in Figure 1. There was a decrease in the myocardial arteriovenous difference for ¹⁴CO₂ (Fig. 1A; P = 0.05) and in ¹⁴CO₂ production as a percentage of total CO₂ production (Fig. 1B; P = 0.05) after denervation, indicating an inhibition of the oxidation of glucose (glycolysis). In four experiments there was a decrease in arterial lactate, whereas this level rose in the fifth experiment; mean values (±1 SD) were 1.13 ± 0.41 mmol/liter pre-denervation and 0.85 ± 0.27 mmol/liter post-denervation. Lactate consumption as a percentage of oxygen consumption was identical to that found previously in normal innervated hearts (Fig. 2).

Data for body weight, temperature, heart rate, mean arterial pressure, arterial pH, PaCO₂, PaO₂, arterial glucose, lactate, and FFA for the pre- and post-denervation studies are given in Table 1. There were no significant differences between the pre- and post-denervation studies of any of these variables.

Tissue Analysis (Series II)

The results of the paired tissue biopsy analyses of the dogs of this series are shown in Figure 3 and Table 2.

Figure 3A shows the F6P levels found pre- and post-denervation expressed as nmol/mg protein. In every dog there was an increase in F6P post-denervation (P < 0.01). The mean values are summarized in Table 2. The level of G6P in the same biopsies is shown in Figure 3B. In four of the dogs there was an increase, and in three a decrease. These changes were not significant. There was no overall significant change in the levels of ATP and CP from pre-
This conclusion is based on the following findings in the post-denervation data. There was (1) a decreased discharge of $^{14}$CO$_2$ from the myocardium during $^{14}$C-glucose (U) infusion (Fig. 1), (2) a decreased $^{14}$CO$_2$ production by the myocardium as a percentage of total CO$_2$ production (Fig. 1), (3) increased tissue levels of F6P (Fig. 3; Table 2), and (4) a much smaller increase in tissue levels of G6P which did not reach statistical significance (Fig. 3; Table 2).

An increased tissue level of F6P could be attributed to increased glycolytic flux but would then be associated with increased levels of G6P of the same magnitude. The increased levels of F6P therefore suggest a block in glycolysis between F6P and pyruvate. Do the $^{14}$C-glucose studies truly indicate an inhibition of glycolysis? It should be emphasized that lactate always is extracted from blood by the normal dog heart (i.e., the heart never produces lactate) (Griggs et al., 1966; Drake et al., 1980). Lactate metabolism in series I denervated hearts has been shown to be identical to that found in the normal hearts studied with $^{14}$C-lactate (Fig. 2). Therefore, we do not consider that the absence of measurements of $^{14}$C-lactate in the coronary sinus blood after $^{14}$C-glucose infusion introduces any error into the assumption that $^{14}$C-glucose conversion to $^{14}$CO$_2$ is a valid index of glycolysis.

It is possible that the decrease in $^{14}$CO$_2$ discharge found in this study (Fig. 1A) could have been outweighed by a greater increase in coronary blood flow so that absolute glucose oxidation was not decreased. However, coronary blood flow is not increased sufficiently in denervated hearts for this to occur, even at constant heart rate (Drake et al., 1978). It is also evident that the increase in metabolic rate found in denervated hearts (Drake et al., 1978) is insufficient to cause the decrease in glucose oxidation as a percentage of CO$_2$ production (Fig. 1). Another factor which could lead to a false indication of reduced glycolysis is a rise in arterial lactate. We previously found an inverse relationship between $^{14}$CO$_2$ production as a percentage of total CO$_2$ production and arterial lactate (Drake et al., 1980). However, the arterial lactate was lower in the denervated dogs (Table 1). This should have produced an increase in glucose oxidation as a percentage of CO$_2$ production (Drake et al., 1980) so that the reduction in that ratio found after denerv-
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viation (Fig. 1) is underestimated. Another consideration would be the possibility that glycogen breakdown was increased post-denervation, thus diluting the pathway with unlabeled metabolites. However glycogen stores are unaffected (Noble et al., 1972; total adrenergic drive is reduced (Noble et al., 1972; Drake et al., 1978) so that, if anything, less glycogen breakdown would be expected. From all these considerations, it seems clear that glucose oxidation is inhibited by cardiac denervation.

The three regulatory enzymes of glycolysis in the aerobic heart are hexokinase (HK), PFK, and pyruvate kinase (PK). Although it has been shown that G3PDH may have a regulatory effect in hearts in which the work-load is increased (Kobayashi and Neely, 1979; Mochizuki and Neely, 1978) these measurements were made in isolated rat hearts, perfused without red blood cells. The maximum arterial oxygen content possible under such circumstances is 2 vols%, whereas the normal arterial oxygen content is 18 vols%. The hearts in our intact dog experiments were completely aerobic as judged by arterial and coronary sinus PO$_2$ and oxygen content measurements in both the control and denervated condition. Moreover, the normality of the PCO$_2$ and pH in these samples indicate that there was no intracellular acidosis.

If the mass action ratios of all the enzymes in the glycolytic pathway are considered, HK, PFK, phosphoglycerate kinase (PGK), and PK are displaced by more than two orders of magnitude from the thermodynamic equilibrium. Reactions displaced from their equilibrium are predisposed to exhibit flux control of a pathway. The glycolytic pathway thus contains more than one of these reactions, and those operating at the beginning of the pathway are liable to exert more influence than those at the end. Therefore, HK and PFK can be expected to be more important rate-controlling enzymes than PGK or PK. HK activity is subject to feedback inhibition by the level of G6P (Sols and Crane, 1954) and the availability of glucose (Table 1). G6P is converted to F6P by the 6-phosphogluco-isomerase reaction (Hoffman, 1976), but there was no consistent increase in the level of G6P in this study. The activity of PFK is subjected to multiple control by a series of positive and negative effectors. It possesses an allosteric nature with the consequent cooperative kinetics, giving PFK the highest effector strength of all glycolytic enzymes (Rapoport et al., 1974).

It was not possible, in these small tissue samples, to measure fructose-16-diphosphate (F16P). If the rise in F6P had been accompanied by a decrease in F16P, an inhibition of PFK would have been implicated strongly. PFK activity is inhibited by the levels of adenosine triphosphate found in aerobic tissue (Mansour, 1963; Passonneau and Lowry, 1962). The levels of ATP in this study were identical pre- and post-denervation. F6P is a stimulator and F16P a stabilizer of PFK activity. Increased levels of F6P are important in reducing the inhibition of PFK by ATP (Mansour, 1972). The high F6P and

<table>
<thead>
<tr>
<th>Substance</th>
<th>Units</th>
<th>Control</th>
<th>Denervated</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>F6P</td>
<td>nmol/mg protein</td>
<td>0.126 ± 0.027</td>
<td>0.319 ± 0.181</td>
<td>0.025 &gt; P &lt; 0.01</td>
</tr>
<tr>
<td>G6P</td>
<td>nmol/mg protein</td>
<td>0.329 ± 0.140</td>
<td>0.461 ± 0.332</td>
<td>NS</td>
</tr>
<tr>
<td>ATP</td>
<td>nmol/mg protein</td>
<td>21.0 ± 5.8</td>
<td>22.3 ± 4.9</td>
<td>NS</td>
</tr>
<tr>
<td>CP</td>
<td>nmol/mg protein</td>
<td>27.1 ± 6.5</td>
<td>30.4 ± 9.4</td>
<td>NS</td>
</tr>
<tr>
<td>PFK</td>
<td>nmol H$_2$ cm$^{-2}$ per 10 min</td>
<td>23.3 ± 9.7</td>
<td>25.2 ± 3.4</td>
<td>NS</td>
</tr>
<tr>
<td>G3PDH</td>
<td>nmol H$_2$ cm$^{-2}$ per 10 min</td>
<td>22.5 ± 3.5</td>
<td>25.8 ± 9.2</td>
<td>NS</td>
</tr>
</tbody>
</table>

Values in columns 3 and 4 are expressed as mean ± 1 sd. NS = not significant.
normal ATP levels found should, in theory, stimulate PFK, but since glucose oxidation was inhibited, the large increase in F6P levels found must result from inhibition of an enzyme below this metabolite in the pathway and points, in particular, toward an inhibition of the PFK.

The levels of tricarboxylic acid cycle intermediates also influence the rate of glycolysis. Citrate exerts an inhibitory influence of PFK (Garland et al., 1963; Newsholme et al., 1977; Mansour, 1972) and could have been increased by denervation. However lactate consumption (Fig. 2) was normal. The influence on glycolysis (cytosolic) by the trichloracetic acid (TCA) cycle (mitochondrial) is via shuttle systems such as the malate-aspartate shuttle (Safer, 1975), which provides a rapid mechanism for linking the TCA cycle to the cytosolic compartment. This shuttle requires cytosolic NADH, which can be supplied by the conversion of lactate to pyruvate. PFK is also very sensitive to cyclic adenosine 3',5' monophosphate (cAMP) (Passonneau and Lowry, 1962; Mansour, 1972; Hoffman, 1976). The enzyme is activated by low concentrations of cAMP at the normal pH of the cell (6.9-7.0). cAMP has been shown to render PFK less susceptible to inhibition by ATP (Mansour, 1963). Fluctuations in the levels of cAMP, AMP, ATP, and inorganic phosphate would cause differences in the modulating ratios of these substances and, therefore, different degrees of activation of the enzyme. The levels of the various modulators of PFK activity in chronically denervated hearts would therefore be worthy of study.

The denervated heart is depleted of norepinephrine (Cooper et al., 1961; Noble et al., 1972; Drake et al., 1978) and uninfluenced by β-blockade (Noble et al., 1972; Drake et al., 1978). There is very little information available on any direct effect of norepinephrine on the metabolism of cardiac muscle. The local concentration of norepinephrine in myocardial tissue increases with sympathetic stimulation, which causes an increase in cAMP levels (Wastila et al., 1972). Such an increase in cAMP with adrenaline stimulation is well documented (Kaufman and Birnbaumer, 1974; Sutherland and Robinson, 1966). However, no metabolic consequences appear to have been demonstrated in the myocardium which are not secondary to the positive inotropic and chronotropic effects with attendant increase in metabolic rate (Mohrman and Fiegl, 1978).

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