Nagoya J. Med. Sci. **57.** 35 ~ 42, 1994

MYOCARDIAL METABOLIC MARKERS OF TOTAL ISCHEMIA IN VITRO

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ABSTRACT

The influence of total ischemia on the formation of products of anaerobic metabolism was investigated in canine hearts in vitro. Contents of organic acids were quantitatively analyzed by gas chromatography-mass spectrometry, and high energy phosphate compounds were determined using the luciferin-luciferase enzyme system. During a 10-min period of total ischemia, myocardial creatine phosphate significantly decreased, while ATP remained unchanged as compared with preischemic values. Both 10 min and 60 min of ischemia induced significant increase in tissue succinic acid levels. In contrast, a significant increase in myocardial lactic acid content was only induced with 60 min of ischemia. The data obtained indicate that the accumulation of succinic acid may be regarded as a potential metabolic marker of myocardial total ischemia.

Key Words: Total ischemia in vitro, Succinate, Lactate, High energy phosphate

INTRODUCTION

It is well recognized that various metabolic changes occur when ischemia appears in the myocardium, and investigation has been conducted from such aspects as the depletion of high energy phosphates (HEP) and the accumulation of metabolites. A number of studies have evaluated how tissue HEP contents correlate with the severity of myocardial ischemic injury.[1–4]

Synthesis of HEP continues in the ischemic myocardium but at a much reduced rate compared with aerobic conditions, because anaerobic glycolysis may be the only source of new HEP, which are thus derived from the breakdown of glycogen to lactate. Accumulation of lactate has therefore been considered a sensitive marker of ischemic changes in myocardial metabolism.[4–6] On the other hand, a depletion of HEP in cardiac muscle during oxygen deprivation is accompanied by alteration in the operation of the tricarboxylic acid (TCA) cycle and in metabolism of certain amino acids.[7,8] Some of these metabolites can probably be regarded as potential markers that reflect the severity of myocardial ischemia.

It has been described that myocardial metabolic alterations during ischemia induced by coronary occlusion in vivo were related not only to the duration of ischemia but also to the degree of decrease in myocardial blood flow, the myocardium being not always damaged uniformly.[9–11] Jennings et al. reported that in ischemic dog hearts both the rate of HEP production and degradation were much slower in vitro than in vivo, and indicated that total ischemia in vitro is a good model of the events occurring in zones of severe ischemia in vivo.[12] Therefore, in the present study we assayed myocardial HEP and organic acid contents during total ischemia in vitro to help clarify the metabolic markers of myocardial ischemia.

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Accepted for Publication in June 21, 1993

MATERIALS AND METHODS

Total ischemia in vitro

Thirteen adult mongrel dogs of both sexes, weighing 7 to 13 kg, were used in this investigation. They were anesthetized by intraperitoneal administration of 50 mg/kg of sodium pentobarbital. The left side of the chest was opened and the heart was quickly excised. One block of muscle from the anterior wall of the left ventricle (approximately 0.2 g) was cut from the beating heart using a biopsy drill for use as control tissue. The remaining heart was immediately placed in a pre-warmed jar, which then was closed tightly and immersed in a 37°C water bath.[12,13] After 10 and 60 min of total ischemia, ischemic myocardium was briefly removed from the water bath and sampled. Control and ischemic muscles were rapidly frozen by liquid nitrogen for determination of organic acids and HEP.

Organic acid determinations

Samples of control and ischemic myocardium from five hearts were analyzed by gas chromatography-mass spectrometry (GC-MS) to obtain organic acid profiles. The samples were homogenized in 0.5 ml of physiological saline solution using a Potter homogenizer. As an internal standard, 10 μg of p-(n-amyl)benzoic acid were added to the homogenized samples, deproteinized by addition of 3 ml of ethanol. The collected supernatant was then evaporated at 40°C for 5 min in a rotary evaporator to eliminate the ethanol. After addition of 3 ml of distilled water, the sample solution was adjusted to pH 1.0 with hydrochloric acid. Organic acid fractions were obtained by repeated extraction (twice) with 3 ml of ethyl acetate. Organic solvent extracts (6 ml) were dried with anhydrous sodium sulphate (2 g) and then evaporated to dryness at room temperature for 30 min under a stream of nitrogen. The dried extracts were trimethylsilylated by adding 0.2 ml of N,O-bis(trimethylsilyl) trifluoroacetamide (Tokyo Kasei Co., Tokyo) to the residues. The mixtures were then heated to 60°C for 1 h in glass tubes. Aliquots of solution were subjected to GC-MS analysis.[14,15]

A Hewlett-Packard 5710A gas chromatograph (MA, USA) was used in combination with a JMS D-300 double-focusing mass spectrometer (JEOL, Tokyo). The data were stored and processed with a JMA 2000 data system (JEOL, Tokyo). The gas chromatograph was equipped with an OV-101 open-tubular glass capillary column (30 m × 0.25 mm I.D.) and a splitless injector. The injection temperature was 250°C and the column temperature was programmed to change from 70°C to 260°C at a rate of 3 °C/min. Electron-impact ionization (EI) mass spectra were recorded under the following conditions: ionizing energy, 70 eV; ionizing current, 300 μA; ion source temperature, 200°C; accelerating voltage, 3 kV.[14,16]

Fig. 1 demonstrates the typical gas chromatogram profile of organic acids from canine myocardium after 60 min of total ischemia in vitro. The results for ratios of peak heights to internal standards showed significant differences in lactic acid and succinic acid among control and 10-min and 60-min-ischemia groups.

Therefore, samples from eight canine hearts were homogenized with known quantities of stable analogues of lactic acid and succinic acid (10 μg of sodium D,L-lactate-1,2,3-$^{13}C_3$ and 20 μg of succinic acid-d_6) and subjected to GC-MS analysis. Fig. 2 demonstrates EI mass spectra of succinic acid and succinic acid-d_6, with respective fragment ions at *m/Z* 247 and *m/Z* 251. Quantitative determinations were performed to give peak height ratio between succinic acid and its stable analogue obtained from mass chromatograms (Fig. 3). In this way lactic acid was quantitatively determined.

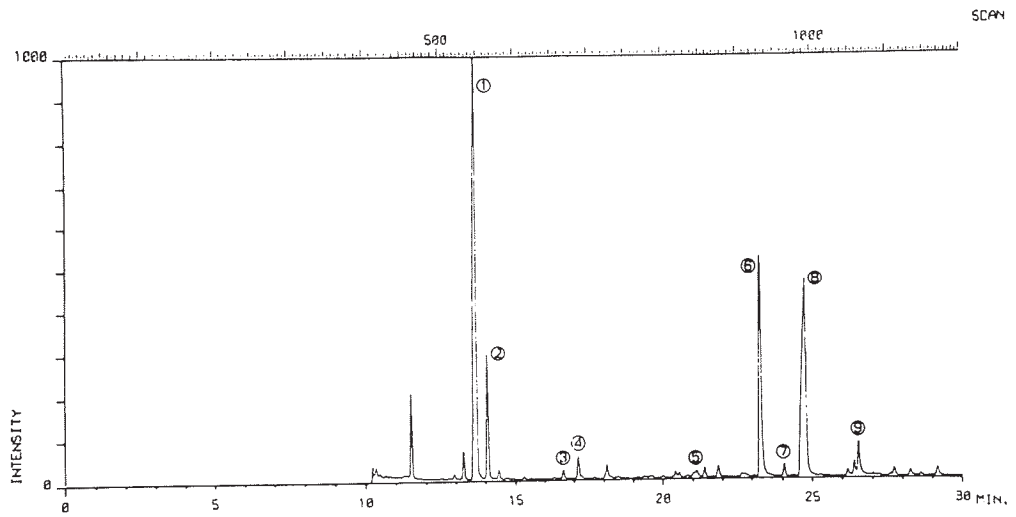

Fig. 1. Typical gas chromatogram profile of organic acids obtained from canine myocardium after 60 min of total ischemia in vitro. The peaks were identified as follows: ①, lactic acid; ②, glycolic acid; ③, 2-hydroxybutyric acid; ④, 3-hydroxypropionic acid; ⑤, benzoic acid; ⑥, phosphoric acid; ⑦, glycerol; ⑧, succinic acid; ⑨, fumaric acid.

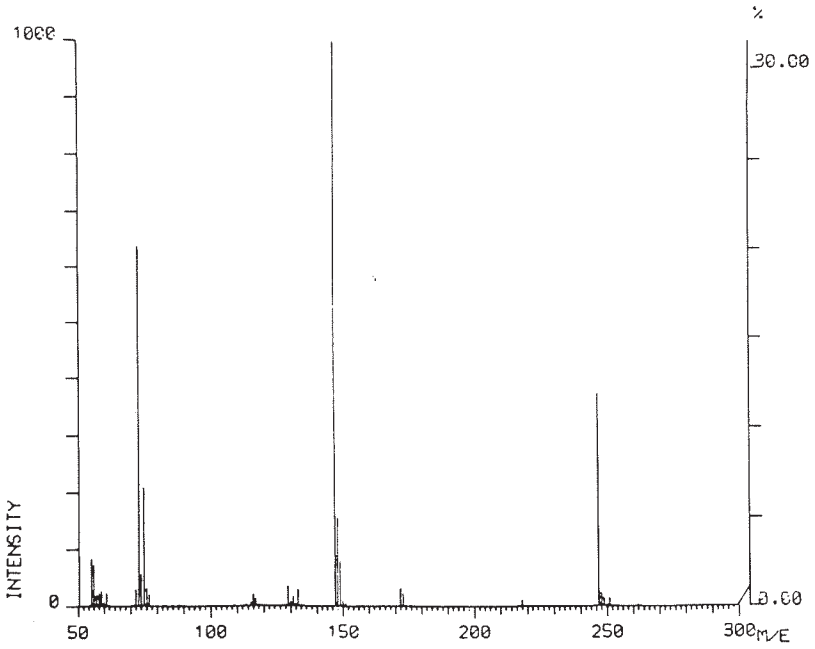

Fig. 2-a. Electron-impact ionization mass spectra of succinic acid.

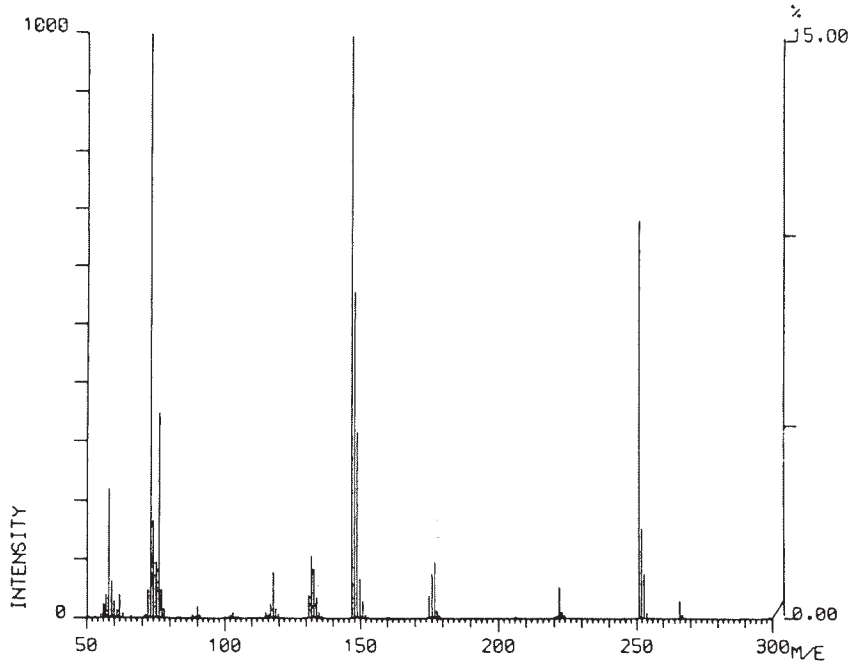

Fig. 2-b. Electron-impact ionization mass spectra of succinic acid-d_6.

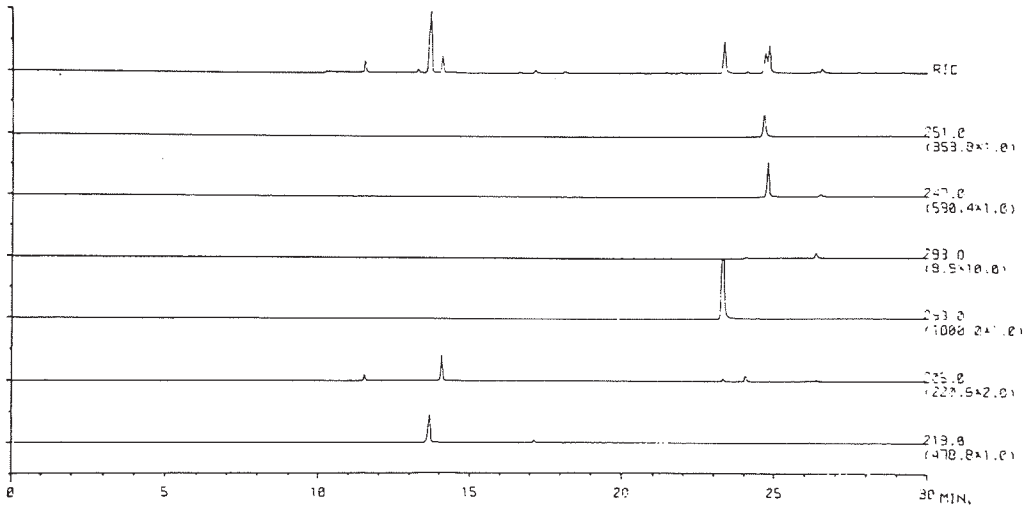

Fig. 3. Mass chromatogram. Ions: *m/Z* 247 for the detection of succinic acid, *m/Z* 251 for succinic acid-d_6.

High energy phosphate determinations

Frozen myocardial samples from eight dogs were used for determination of tissue ATP and creatine phosphate (CP). Each was weighed and homogenized with the addition of 2.5 ml of 7% perchloric acid per 100 mg tissue at 0°C. After deproteinization, tris buffer containing 4N-KOH was added to the homogenate to neutralize the perchloric acid, and the samples were centrifuged at 3000 rpm for 10 min. ATP contents were determined by a modification of McElroy-Strehler's firefly luminescence method using a firefly lantern extract (FLE 50, Sigma, MO, USA),[17,18] measurements being made using a bioluminescence reader (BLR-101C, Aloka, Tokyo). In the same way, CP contents were assayed with conversion of CP to ATP using adenosine diphosphate and creatine kinase.[19,20]

Statistical analysis

The results presented are mean ± SE values. Statistical analysis was carried out by analysis of variance with Scheffe's test for multiple comparisons. The probability was considered significant if less than 0.05.

RESULTS

Fig. 4 shows the time course of changes in myocardial lactic acid and succinic acid contents (μmol/g wet weight). Lactic acid in the 60-min ischemia group (19.90 ± 2.45) was significantly increased as compared with that in the control group (2.50 ± 0.38). Succinic acid, in contrast, was significantly higher in both the 10-min (1.94 ± 0.14) and the 60-min (5.37 ± 0.38) ischemia groups than in the control group (0.16 ± 0.05). The time courses of changes in myocardial HEP levels are shown Fig. 5. The ATP value in the 60-min ischemia group was significantly decreased as compared with the control value. CP was significantly lower in both the 10-min and the 60-min ischemia groups than in the control group.

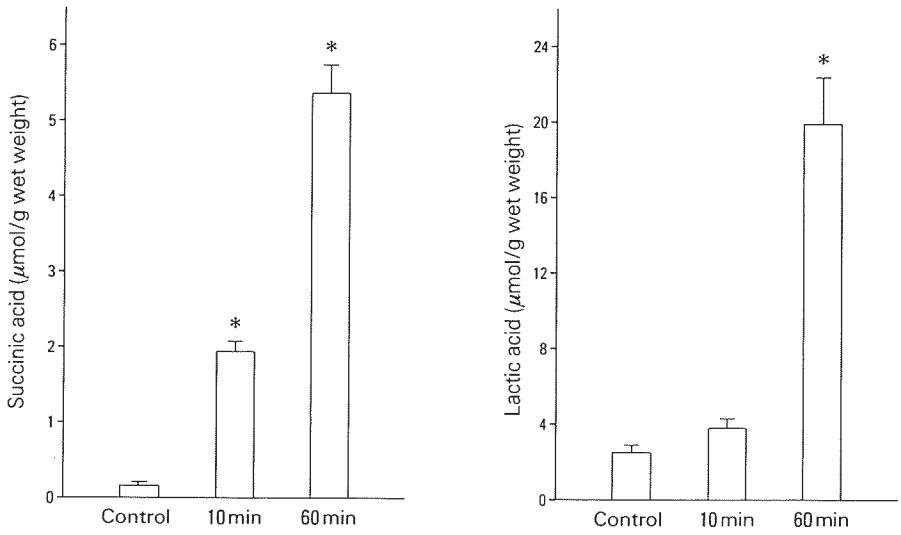

Fig. 4. Histograms showing time course of changes in myocardial succinic acid (left) and lactic acid (right) contents during total ischemia in vitro. Values are means ± SE.
10 min, 10 min of ischemia; 60 min, 60 min of ischemia
*$p < 0.01$ vs control

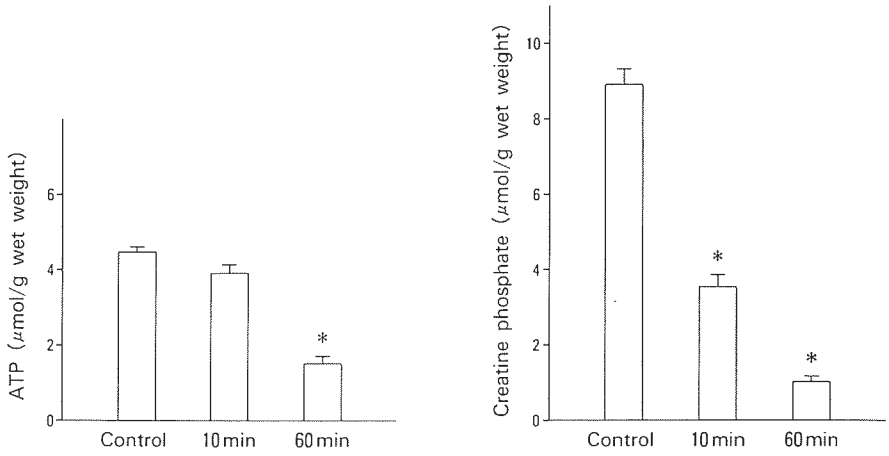

Fig. 5. Histograms showing time course of changes in myocardial high energy phosphate contents (left, ATP; right, creatine phosphate). Values are means ± SE.
10 min, 10 min of ischemia; 60 min, 60 min of ischemia
*$p < 0.01$ vs control

DISCUSSION

The present investigation, designed to evaluate the time course of changes in myocardial organic acids and HEP during total ischemia in vitro, revealed tissue succinate to be a potential marker of myocardial ischemia.

Within seconds of the onset of severe ischemia induced by coronary occlusion in vivo, myocardial HEP begin to decrease, and after a minute the initially preserved ATP stores begin to decline.[4,21] The degree of damage during regional ischemia is not always uniform in the myocardium, being related to ischemic duration and flow deprivation.[9–11] Jennings et al. indicated that the model of total ischemia in vitro can therefore be used to study metabolic pathways occurring in zones of severe regional ischemia in vivo.[12] The larger quantities of uniformly ischemic tissue in the in vitro model and the slower time course of metabolic changes should permit better resolution of the relationship between metabolic and structural events during the early phases of severe ischemic injury. The present investigation of metabolic alterations in the ischemic myocardium during total ischemia in vitro was made from this standpoint.

GC is suitable for rapid separation and assay of volatile and low molecular substances and is often used for detecting trace amounts of substances in the body. Moreover, GC in combination with MS is highly effective in identifying substances. Thus GC-MS has been used to screen a number of metabolites in blood, urine, and several tissues.[14–16] Therefore, we applied this approach to organic acids associated with a depletion of HEP in ischemic myocardium, such as lactic acid and TCA cycle-related compounds.

Although amino acids have not been considered active metabolites in the normal myocardium, in recent years it has been reported that myocardial ischemia leads to accumulation of alanine and certain TCA cycle intermediates in the cardiac tissue.[7,8,22] During ischemia, anaerobic glycolysis of glucose cannot produce adequate HEP compounds to maintain critical cellular function, and the result is a breakdown of cellular organelles. However, amino acids, through transamination to TCA cycle metabolites and subsequent anaerobic substrate phosphorylation, could provide for energy needs during ischemia and protect the ischemic myocardium

from damage.[23,24] Citrate concentration has been reported to increase in the anaerobic zones of ischemic rat myocardium,[25] although Williamson et al. did not found any changes.[26] Malate has also been shown to increase during ischemia in the isolated perfused rat heart.[8] With ischemic conditions in the rat brain, tissue citrate, malate and fumarate contents have been demonstrated to decrease and succinate and alanine contents to increase.[27]

Hochachka et al.[28] and Taegtmeyer et al.[7,23] earlier postulated that succinate production from amino acid during hypoxia might generate HEP via amino acid substrate level phosphorylation, in line with our present results concerning myocardial succinate increase in total ischemia. Although our data do not confirm simultaneous conversion to succinate, augmented succinate formation suggests that anaerobic mitochondrial energy production includes transamination of glutamate with pyruvate to alanine and α-ketoglutarate, with subsequent conversion to succinate, and transamination of aspartate to oxaloacetate, with subsequent conversion to malate, fumarate, and then to succinate.[23,24,29] The physiological significance of these pathways is that exogenous glutamate may be used as an anaerobic fuel through conversion to succinate coupled with GTP formation.[30,31]

Changes in cellular energy metabolism have long been regarded as sensitive indicators of ischemic injury and many researchers have used the tissue contents of ATP, CP and/or lactate in the assessment of tissue injury and protection.[4,6,32] In the present investigation, it should be noted that, after 10 min of total ischemia in vitro, myocardial succinic acid content was markedly increased, whereas lactic acid remained unchanged. Therefore, tissue succinate can possibly be regarded as the better marker of myocardial ischemia under the present experimental conditions. Further investigations of its potential appear warranted.

ACKNOWLEDGEMENT

We thank Miss Yoshie Mano for secretarial help.

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