Metabolic Characterization of Myocardial Infarction Using GC-MS-Based Tissue Metabolomics

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Summary

Understanding the metabolic features of myocardial infarction (MI) is critical to its prevention and treatment. Here, we aimed to characterize the metabolic features of early MI using a tissue metabolomics method based on gas chromatography-mass spectrometry (GC-MS). Thirty-four pairs of infarcted myocardia and their matched non-infarcted myocardia were collected from 34 rats that underwent coronary artery ligation (CAL); their metabolic profiles were compared by GC-MS-based tissue metabolomics to characterize the metabolic features of MI. On the basis of differential metabolites, their diagnostic potential for MI was analyzed, and MI-related metabolic pathways were investigated. Serum samples before and post MI were used to validate the results obtained in myocardia. The metabolic profile of the infarcted myocardia was obviously different from that of the non-infarcted myocardia, as indicated by partial least squares discriminate analysis (PLS-DA) plots. Twenty-two metabolites were identified to be different between the infarcted myocardia and non-infarcted myocardia. These metabolic alterations reflect energy deficit, acidosis, oxidative stress, ionic imbalance, and cardiac injury post MI. Glutamine, glutamate, and lactate were confirmed to jointly confer a favorable potential for diagnosing MI, which can be well validated in serum.  (Int Heart J 2017; 58: 441-446)

Key words: Myocardial ischemia, Metabolic feature, Glutamate, Self-control study

Myocardial ischemia is a leading cause of morbidity and mortality, accounting for approximately 12 million deaths annually worldwide, and is expected to continue to be a serious problem all over the world. Metabolism is among the first area affected post myocardial ischemia, which can then lead to different deleterious consequences, such as arrhythmia, myocardial infarction (MI), and heart failure, and the latter is an irreversible myocardial injury secondary to persistent myocardial ischemia. Therefore, understanding metabolic features of MI is critical to its prevention and control.

Several clinical studies concerning metabolic changes in plasma of MI have been carried out. The metabolic profiles of MI in plasma or serum of MI patients are increasingly being determined. Several potential biomarkers of MI or myocardial ischemia, such as phytosphingosine, sphinganine, acetylcarnitine, adenine, and inosine have been suggested. However, circulating biomarkers may be easily influenced by diverse tissues which may affect their specificity.

Thus, we should elucidate the global metabolic changes of myocardia per se, which serve as a source of systemic metabolic alterations post MI, as they are not yet fully understood. Such an understanding not only helps to explore the pathophysiological mechanisms of MI, but can also validate the results of clinical studies. Therefore, it needs to be retrotranslated from the clinical study stage to the animal study stage. Coronary artery ligation (CAL) rat models are widely used to study related pathophysiological alterations of MI, or to explore the effect of drug treatment. However, CAL animal studies are easily influenced by the operation itself, even if a control group has been established.

Thus, we designed a self-control study here. Thirty-four pairs of matched infarcted myocardia and non-infarcted myocardia, from rats that underwent CAL, were collected. The metabolic characteristics of MI were explored with a tissue metabolomics strategy based on gas chromatography-mass spectrometry (GC-MS), by comparing the metabolic profile of the infarcted myocardia with that of their matched non-infarcted myocardia. On the basis of differential metabolites, their synergistic diagnostic potential for MI was analyzed by receiver operating characteristic curve (ROC) analysis and binary logistic regression, and the MI-related metabolic pathways were...
investigated. Serum samples before and after MI were collected, and quantitative analysis of the differential metabolites was performed with the GC-MS system to validate the diagnostic value of the potential metabolic biomarkers found in myocardia.

**METHODS**

**Animals:** Thirty-eight adult male Sprague-Dawley rats (weighing 250-500 g) were obtained from the Animal Research Center of Shantou University Medical College. All rats were kept at room temperature and 55 ± 10% relative humidity with a 12-hour light-dark cycle, and had access to tap water and rodent chow ad libitum. All animal experiments were approved by the Medical Animal Care & Welfare Committee of Shantou University Medical College and conducted in accordance with the Guidelines for Animal Experimentation. Blood was drawn from the tail vein the day before the CAL operation.

**MI rat model:** MI was induced by CAL as previously reported. Briefly, after anesthetization by peritoneal injection of 3% pentobarbital sodium (90 mg/kg), the rats were monitored by a lead II electrocardiogram (ECG). Artificial ventilation by endotracheal intubation through a small animal ventilator was then established. A thoracotomy was performed by tearing open the cardiac pericardium, exposing the heart outside the chest, and then ligating the left anterior descending coronary artery from the root as soon as possible. The heart was immediately placed back into the thoracic cavity. After CAL, the ST segment of the ECG rose substantially, which verified the success of the ligation and the occurrence of MI. Those rats which did not die during the experiment were euthanized by clamping the aorta 70 minutes after the ligation. Within 3 minutes after death, the abdominal vein blood was withdrawn to separate the serum; the heart was retrieved, washed with pre-cooled saline, and stored at -80°C.

**Triphenyltetrazolium chloride TTC and hematoxylin-eosin (HE) staining:** TTC staining was performed to investigate the morphological changes in the myocardium after MI. Conventional HE staining was performed to investigate the infarcted myocardia and non-infarcted myocardia according to prior reference. Briefly, the whole heart was frozen at -80°C for 20 minutes, and then cut into slices of 3-4 mm thickness. After that, they were incubated in TTC (Sigma, 1% in PBS) solution at 37°C for 30 minutes.

Conventional HE staining was performed to investigate the morphological changes in the myocardium after MI.

**Measurement of cardiac troponin (cTnT):** cTnT is an important indicator in serum for evaluating MI clinically. In this study, cTnT content in the serum before and after CAL was determined using an Agilent 6890N GC system, connected to an Agilent 5975c single quadrupole MSD. A DB-5MS capillary column (Agilent) was used for the separation of metabolites. High-purity helium (99.9996%) was used as the carrier gas at a constant flow rate of 1 mL/minute. The GC oven temperature was programmed with an initial temperature at 60°C for 2 minutes, which was then increased to 285°C at 5°C/minute, and maintained for 10 minutes. The temperatures of the injection port, transfer interface, and EI source were set to 230, 290, and 230°C, respectively. The selected mass range was set to 50-600 m/z with electron impact ionization (70 eV).

Each acquired chromatogram was analyzed using ChemStation software (Agilent). Metabolites were identified in the 2.0 NIST library. Peak area of the chromatogram in every sample was normalized using the internal standard peak area so as to identify the alterations of metabolites.

Before detection, precision and stability were evaluated to ensure the quality of analysis as described in a previous paper by our team.

**Data processing:** 1) Multivariate statistics analyses; the GC-MS raw data were processed by XCMS in R language. First, a CDF format file was converted from the chromatogram in each test (Agilent). After peak matching, retention time correction and peak filling, a txt format file was established and imported into the SIMCA-P program (version 13.0 Umetrics) for multivariate analysis. Partial least squares discriminate analysis (PLS-DA), a kind of discriminant multivariate analysis method, was applied with unit variance (UV) scaling to explore the differences in the global metabolic signature of the infarcted myocardia and their controls. The parameters of the model, such as $R^2_X$, $Q^2_Y$, and the $R^2_Y$ and $Q^2_Y$ intercepts, were analyzed to ensure the quality of the multivariate models, and to avoid the risk of over-fitting. Important projection values greater than 0.75 (VIP > 0.75) were used as a criterion of differential metabolites in multivariate analysis. 2) Univariate statistics analysis; a paired $t$-test was performed to compare the relative amount (peak area) of identified metabolites between the infarcted myocardia and their controls, and $P < 0.05$ was considered statistically significant. 3) Diagnostic potential analyses: MedCalc statistical software was used to calculate the area of the receiver operating characteristic curve (AUC) of differential metabolites, both from multivariate and univariate statistics. Binary regression was performed to investigate the synergistic diagnostic value of the core differential metabolites on MI.
**RESULTS**

**MI rat model:** Thirty-eight SD rats underwent CAL. All had a T wave elevation of lead II ECG (Figure 1C). Four CAL rats were randomly selected for TTC and HE staining. TTC staining revealed pale areas in the left ventricular myocardia, indicating the occurrence of MI (Figure 1A). HE staining showed the cardiac cell swelling characteristic of infarcted myocardia (Figures 1D2, D3). Thirty-four rats were used for metabolic profiling. Of these, 14 developed and died from ventricular tachycardia (VT) and ventricular fibrillation (VF), 7 died from severe atrioventricular block, and 13 maintained a relatively normal heart rate and were euthanized by clamping the aorta at 70 minutes post-operation. The serum cTnT content post CAL was 65-fold higher than that of the pre-operation controls (2.63 ng/mL versus 0.04 ng/mL, respectively ($P < 0.05$), C: Lead II ECGs. C1: Normal ECG before treatment, C2: High T wave after CAL. D: Pathological features after CAL. HE staining showed cardiac cell swelling characteristic for the infarcted myocardia (D2, D3 is the local amplification of D2), and no significant morphological change was observed in the non-infarcted areas (D1).

**Metabolic profile of MI:** The metabolic profile of MI was investigated with a GC-MS-based tissue metabolomics approach by comparing the infarcted areas with their matched non-infarcted areas. Validations of the GC-MS detection, including precision and stability, showed the relative metabolomics was reliable. To analyze the differences in metabolic profile, the GC-MS data were imported into a SIMCA-P 13.0 software package. Subsequently, PLS-DA was performed to classify the infarcted myocardia and their controls. Each plot in PLS-DA represents a global metabolic feature of the related myocardia. PLS-DA plots illustrate the infarcted myocardia were clearly separated from their controls. In the PLS-DA model, the cumulative $R^2$Y and $Q^2$ were 0.557 and 0.667, respectively, when two components were calculated. No over-fitting was observed according to the results of the 200 random permutations. The $R^2$Y-intercepts and $Q^2$-intercepts were 0.342 and -0.429, respectively (Figures 2A, B).
Differential metabolites between the infarcted myocardia and non-infarcted myocardia: A total of 44 metabolites were identified according to the NIST library, from all detected spectra. Metabolites that significantly facilitated the discrimination and clustering were selected according to a threshold of VIP greater than 0.75 in the PLS-DA model. Fourteen metabolites met this criterion. The paired t-test was then used to select different metabolites from univariate statistics. As a result, 17 metabolites were found to be significantly different. In total, 22 metabolites that met at least one of the above screening criteria (VIP > 0.75 or \( P < 0.05 \)) were selected as potential different metabolites between infarcted myocardia and their controls (Table).

**Table.** List of Differential Metabolites Between Infarcted Myocardia and Non-Infarcted Myocardia, Validation in Serum

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>VIP</th>
<th>( P )</th>
<th>Fold change</th>
<th>Metabolic pathways</th>
<th>Serum validation</th>
<th>Fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Elaidic acid</td>
<td>NS</td>
<td>0.011</td>
<td>0.66</td>
<td>Fatty acid degradation</td>
<td>↓</td>
<td>0.34</td>
</tr>
<tr>
<td>Linoleic acid</td>
<td>1.16</td>
<td>0.012</td>
<td>0.70</td>
<td>Fatty acid degradation</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Hexadecanoic acid</td>
<td>1.18</td>
<td>NS</td>
<td>0.92</td>
<td>Fatty acid degradation</td>
<td>↓</td>
<td>0.79</td>
</tr>
<tr>
<td>Octadecanoic acid</td>
<td>0.87</td>
<td>NS</td>
<td>0.98</td>
<td>Fatty acid degradation</td>
<td>↑</td>
<td>1.19</td>
</tr>
<tr>
<td>Pantothenic acid</td>
<td>NS</td>
<td>0.012</td>
<td>1.43</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Lactate</td>
<td>1.08</td>
<td>0.001</td>
<td>1.28</td>
<td>Glycolysis and gluconeogenesis</td>
<td>↑</td>
<td>3.58</td>
</tr>
<tr>
<td>Succinate</td>
<td>1.27</td>
<td>0.000</td>
<td>0.68</td>
<td>Oxidative phosphorylation</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Fumarate</td>
<td>NS</td>
<td>0.001</td>
<td>0.68</td>
<td>Oxidative phosphorylation</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Glutamine</td>
<td>1.76</td>
<td>0.000</td>
<td>1.57</td>
<td>Alanine, aspartate and glutamate metabolism</td>
<td>↑</td>
<td>5.75</td>
</tr>
<tr>
<td>Glutamate</td>
<td>1.06</td>
<td>0.014</td>
<td>1.21</td>
<td>Alanine, aspartate and glutamate metabolism</td>
<td>↑</td>
<td>5.16</td>
</tr>
<tr>
<td>Lysine</td>
<td>NS</td>
<td>0.015</td>
<td>1.31</td>
<td>Valine, leucine and isoleucine degradation</td>
<td>↑</td>
<td>1.78</td>
</tr>
<tr>
<td>Leucine</td>
<td>NS</td>
<td>0.022</td>
<td>1.13</td>
<td>Valine, leucine and isoleucine degradation</td>
<td>↑</td>
<td>0.20</td>
</tr>
<tr>
<td>Valine</td>
<td>NS</td>
<td>0.025</td>
<td>1.18</td>
<td>Valine, leucine and isoleucine degradation</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Alanine</td>
<td>0.87</td>
<td>NS</td>
<td>0.93</td>
<td>D-Alanine metabolism</td>
<td>↑</td>
<td>3.98</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>0.91</td>
<td>NS</td>
<td>1.02</td>
<td>Tyrosine metabolism</td>
<td>↓</td>
<td>0.88</td>
</tr>
<tr>
<td>Proline</td>
<td>0.98</td>
<td>NS</td>
<td>1.01</td>
<td>Arginine and proline metabolism</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>NS</td>
<td>0.006</td>
<td>1.49</td>
<td>Valine, leucine and isoleucine degradation</td>
<td>↑</td>
<td>2.91</td>
</tr>
<tr>
<td>Glycine</td>
<td>0.77</td>
<td>0.008</td>
<td>0.77</td>
<td>Glutathione metabolism</td>
<td>↑</td>
<td>3.35</td>
</tr>
<tr>
<td>Creatinine</td>
<td>0.80</td>
<td>0.014</td>
<td>1.17</td>
<td>Arginine and proline metabolism</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Urea</td>
<td>NS</td>
<td>0.002</td>
<td>1.29</td>
<td>Arginine and proline metabolism</td>
<td>↓</td>
<td>0.60</td>
</tr>
<tr>
<td>Phosphatidylethanolamine</td>
<td>0.77</td>
<td>0.005</td>
<td>1.09</td>
<td>Oxidative phosphorylation</td>
<td>↑</td>
<td>1.65</td>
</tr>
</tbody>
</table>

VIP indicates variable importance in the projection; \( P \), significance of the paired \( t \)-test between infarcted myocardia and non-infarcted myocardia; Fold change, ratio of relative content of metabolite in infarcted myocardia to that of their matched non-infarcted myocardia; Metabolic pathways, from the KEGG metabolic pathway, –metabolite was not detected in serum; ↑, metabolite had an increasing or decreasing trend, respectively, in serum post MI. The change is significant \( (P < 0.05) \).

**Diagnostic potential of differential metabolites for MI:** Potential biomarkers were screened by a metabolomics strategy. Those metabolites that simultaneously fulfilled the criteria of having VIP > 1 and \( P < 0.05 \), specifically linoleic acid, lactate, succinate, glutamine, glutamate and biotin (Table), were selected. The tissue metabolome can more directly represent metabolic deregulation than the serum metabolome, but the biomarkers for diagnosis should enter the serum from the tissues. Therefore, quantitative analysis of these potential biomarkers in the serum pre and post MI was performed on the GC-MS system. Their diagnostic potential for MI was investigated by ROC curve analysis. We found that lactate, glutamine, and glutamate had the same dramatic increase in the serum post MI. Importantly, both lactate, glutamine, and glutamate in the serum had AUC greater than 0.95. Collectively, they displayed an AUC of 1.0, identical to that of cTnT; their combined diagnostic potential is illustrated in Figures 3A, B.

**Discussion**

In this study, 3 types of data were used to confirm the occurrence of MI. The first evidence was ECG; the T wave was clearly elevated in all experimental rats after the operation (Figure 1C). The second indicator was TTC staining, which is widely used to confirm heart and brain ischemia in animal studies. The third evidence was the striking increase in the blood biochemistry indicator cTnT post operation; cTnT is an important clinical indicator to evaluate myocardial injury, especially from ischemia.

In our study design, the infarcted myocardia, from the left ventricle and their matched non-infarcted myocardia, were
used to compare their metabololome and to explore the pathophysiological pathways of MI. Such a self-control animal study allows us to specifically focus on the effect of MI itself on myocardial metabolism, avoiding potential interventions of the CAL operation per se, individual differences, time course differences, and complications of MI on the myocardial metabololome.

**Metabolic characteristics of MI:** The current results revealed MI induces changes in myocardial metabolism. The PLS-DA score plots demonstrate a clear separation between the infarcted myocardia and non-infarcted myocardia (Figure 2), which probably reflected obvious MI-related pathophysiological alterations in myocardia. Most ischemia-related differential metabolites are involved in the metabolic processes related to myocardial energy metabolism, including the TCA cycle, glycolysis, fatty acid beta oxidation, and ATP production. The current tissue metabolomics confirms several previous results in clinical or animal MI studies using serum metabolomics, that most fatty acids and TCA intermediate metabolites are significantly decreased, and lactate is increased post MI. The heart has an abundant source of fuels, which mainly include fatty acids, glucose, and keto-bodies. Fatty acids are one of the principal sources of fuels, and they and pyruvate contribute to approximately 97% of ATP formation in the normal heart. Under ischemic conditions, myocardial blood flow is inadequate to meet the oxygen demand. It would reduce the formation of ATP via aerobic mechanisms and accelerate anaerobic ATP production by glycolysis, leading to an enhancement of glycolysis and an increase in lactate. The latter has been considered to be a marker of MI and a sign of acidosis. Moreover, lower oxygen inhibits aerobic oxidation, and relatively fewer metabolites would enter the TCA cycle, leading to a lower production of its intermediate metabolites, such as succinate and fumarate. Despite the fact that lower oxygen post ischemia can suppress fatty acid oxidation, fatty acids are still greatly consumed under ischemia because they are the major energy substances of the heart and would be given a priority for usage. Additionally, phosphate is elevated in ischemic myocardia, which implies more decomposition and less synthesis of ATP, and dysfunction of mitochondria post MI.

In addition to energy metabolism, amino acids also serve a variety of biological functions. Eight amino acids are simultaneously disturbed in the infarcted myocardia and the serum after MI, which might be related to the pathophysiological mechanisms of MI. For example, glutamate was increased in the infarcted myocardia (Table), in a way similar to that in a previous study that found stress-induced myocardial ischemia is accompanied by greater uptake of glutamate. In the infarcted myocardia, due to injury to the cardiomyocyte membrane, more glutamic oxaloacetic transaminase (GOT) will be released into the serum, prohibiting the transamination from glutamate to α-ketoglutaric acid in myocardia and leading to the increase of glutamate in infarcted myocardia. In addition, necrotic cardiomyocyte means more proteins are decomposed, which may also contribute to the increased level of glutamate in the infarcted myocardia. Previous studies showed that a higher level of glutamate was correlated with ongoing myocardial ischemia and that it could activate the generation of NO, inducing the production of free radicals and leading to cardiomyocyte death by mediating inflammatory and cytotoxic actions. Therefore, the increase of glutamate in the infarcted myocardia is detrimental; this suggests a consequence of myocardial injury and poor prognosis after MI. In the infarcted areas, as a result of ischemia and acidosis, glutaminase activity is reduced, resulting in a decrease in glutamine hydrolysis; this can partly explain the higher level of glutamine in these areas. The level of alanine was decreased in the infarcted myocardia, while that in the serum was elevated (Table). These results indicate more alanine was released into the serum during myocardial ischemia and MI, following which it may enter the liver for enhanced gluconeogenesis (alanine-glucose cycle), and the latter was proven to occur during stress and sympathetic excitement. Additionally, glycine is lower in the infarcted myocardia; it has been found to be able to protect cardiac cells under myocardial ischemia by several teams. The decline of glycine in infarcted myocardia might be a decomposition or terminated state of myocardial function post MI.

Taken together, the perturbed metabolites in the infarcted myocardia post MI represent an energy deficit, ionic imbal-
ance, oxidative stress, acidosis, and cardiac injury in myocardia.

**Diagnostic potential of differential metabolites:** Biomarkers could help to explore determining factors of MI and uncover related preventive and therapeutic targets for clinical applications. In this study, linoleic acid, lactate, succinate, glutamine, glutamate, and biotin are considered to be potential metabolic markers of MI in myocardia. These combinations mainly reflect hypoxia-related metabolic alterations, oxidative stress, and cardiac injury during ischemia. The potential markers found in the myocardia can be used to investigate their diagnostic potential using the serum as samples. Among them, lactate, glutamine, and glutamate show the same trend of increase, and have a collaborative AUC of 1.0, equal to that of cTnT (Figure 3B), indicating that they are effective in diagnosing MI, which may serve as an adjudant diagnostic avenue of cTnT to confirm the occurrence of MI.

**Study limitations:** In this study, the controls were retrieved from the right ventricles, which might be slightly different to the left ventricles in terms of metabolic circumstances. Therefore, for a more accurate understanding of the metabolic signature of MI, further studies should compare the metabolome differences of infarcted areas and non-infarcted areas that were retrieved within the left ventricle in a MI model using a relatively bigger animal, such as rabbits or dogs.

**Conclusions:** In this study, we described a global metabolic feature of MI in myocardia, which reflects MI-related pathophysiological processes of energy deficit, ionic imbalance, oxidative stress, acidosis, and cardiac injury. Lactate, glutamine, and glutamate in the myocardia collaboratively display a good potential to diagnose MI, which can be well validated in the serum, and may serve as an adjudant diagnostic avenue of cTnT for MI.

**REFERENCES**


**SUPPLEMENTAL FILES**

Supplemental Figure
Please see supplemental files; https://www.jstage.jst.co.jp/article/ihj/58/3/58_16-432/_article/supplement