Metabolic pathways involved in Xin-Ke-Shu protecting against myocardial infarction in rats using ultra high-performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry

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ARTICLE INFO

Article history:
Received 7 September 2013
Received in revised form 31 October 2013
Accepted 2 November 2013
Available online 15 November 2013

Keywords:
Myocardial infarction
Metabolic pathways
Xin-Ke-Shu
Plasma metabolomics
Pharmacological action

ABSTRACT

Xin-Ke-Shu (XKS) is a patent drug used for coronary heart diseases in China. This study evaluated the protective effect of XKS against isoproterenol (ISO)-induced myocardial infarction (MI). For its underlying mechanism in rats with MI, a metabonomic approach was developed using ultra high-performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry (UPLC/QTOF-MS). Plasma metabolites were profiled in MI rats, pretreated orally with or without XKS. Two genres of metabolic biomarkers were used to elucidate the pharmacological action of XKS: pathological biomarkers and pharaco biomarkers. Fifteen metabolites significantly varying between MI rats and normal rats were characterized as potential pathological biomarkers related to MI, including t-acetylcarnitine (1), t-isoleucyl-t-proline (2), tyramine (3), isobutyryl-t-carnitine (4), phytosphingosine (5), sphinganine (6), t-palmitoylecarnitine (7), lypoPC(18:0)/8, uric acid (9), t-tryptophan (10), lypoPC(18:2) (11), lypoPC(16:0) (12), docosahexaenoic acid (13), arachidonic acid (14) and linoleic acid (15). Among them, eight (1-6, 9 and 10) were first reported as pathological biomarkers related to ISO-induced MI, which mainly involved into fatty acid β-oxidation pathway, sphingolipid metabolism, proteolysis, tryptophan metabolism and purine metabolism. The metabolites significantly varying between MI rats with and without XKS pretreatment were considered as pharaco biomarkers. A total of 17 pharaco biomarkers were recognized, including 15 pathological biomarkers (1-15), hexanoylcarnitine (16) and tetradecanoylcaritnine (17). The results suggested that pretreatment of XKS protected metabolic perturbations in rats with MI, major via lipid pathways, amino acid metabolism and purine metabolism, which also provided a promising approach for evaluating the pharmacodynamics and mechanism of traditional Chinese medicines (TCM) formulas.

1. Introduction

Myocardial infarction (MI) is a leading cause of clinic death. It is associated with ischemic necrosis of cardiac muscles due to a decrease in the supply of blood to apportion of the myocardium below a critical level necessary for viability and proper physiological function. Lipid peroxides due to oxidation has been demonstrated in MI pathogenesis [1]. Inflammatory mediators induced coronary arteriole spasm in impaired lipid homeostasis [2]. Western medications such as isosorbide dinitrate (vasodilator), verapamil (calcium antagonist), propranolol (beta-adrenergic blocker), captopril (angiotensin converting enzyme inhibitor), trimethazine (fatty acid oxidation inhibitor) have been used for the clinical treatment of MI. However, the efficacies of these most currently used drugs have always been limited by their adverse reactions for long-term administration. Accordingly, the search for new cardioprotective drugs is an area of intense research activity. More recently, Chinese medicines and their formulas have aroused much interest especially due to their superiority in the treatment of MI and low side effects [3].

XKS is a traditional Chinese patent medicine, composed of five commonly used Chinese herbs: i.e. the roots of Salviae miltiorrhizae Bge. (Dan-Shen), the roots of Pueraria lobata (Wild.) Ohwi. (Ge-Gen), the roots of Panax notoginseng (Burk.) F. H. Chen. (San-Qi), the fruit of Crataegus pinnatifida Bge. (Shan-Zha) and the roots of Aucklandia lappa Decne (Mu-Xiang). It has been widely used for
the treatment of coronary heart disease in China and has protective effects on myocardial ischemia and reperfusion injury [4]. The chemical constituents in XKS preparation were qualitatively and quantitatively investigated by an optimized LC–ESI–MS² method [5]. Our previous report has also demonstrated that XKS could rescue coronary endothelial injury in rabbits with atherosclerotic MI via regulating the expressions of endothelial nitric oxide synthase and vascular cell adhesion molecule [6]. This highlight connects the coronary arterial spasms and XKS protection, and reveals a systemic mechanism exploiting from metabolic products of soluble epoxide hydrolase.

Metabolomics, refers to the comprehensive analysis of small-molecule metabolites present in a biological system, is an emerging technology of systems biology in studies of either clinical diagnosis or pharmaceutical industry [7]. Successful applications of metabolomic approach have been located at active herb molecule, single herb preparation and multiple herb derived prescription [8]. Therefore, plasma metabolites are proposed available in XKS protection from MI rats.

The pathophysiological and morphological changes observed in isoproterenol (ISO) administered rats are similar to those observed in human MI. ISO-induced MI in rats has been demonstrated as multiple MI lesions with small sizes from a systemic perturbation on disease-causing metabolic network [9]. Thirteen lipid biomarkers including lysophosphatidylcholines (lysoPCs) and fatty acids have been identified to reveal the lipid disorders in ISO-induced MI [10]. Further investigation is still needed to find new biomarkers to achieve a global understanding of the biological process of MI and evaluate the pharmacological action of diverse drugs. In this study, the potential biomarkers and metabolic pathways underlying the pharmacological action of XKS were analyzed in the model rats with or without XKS pretreatment based on UPLC/QTOF-MS.

2. Experimental

2.1. Reagents and materials

XKS tablets were supplied by Wo Hua Pharmaceutical Co, CHN (batch No. 090629) and its quality control was performed as previously reported [5]. HPLC-grade acetonitrile was purchased from J.T. Baker (Phillipsburg, NJ, SA). Isoproterenol (ISO) was purchased from Sigma–Aldrich (St. Louis, USA). The assay kits for lactate dehydrogenase (LDH), creatinines (CK), aspartate transaminase (AST), superoxide dismutase (SOD) and malondialdehyde (MDA) were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Ultrapure water (18.2 MΩ) was prepared with a Milli-Q water purification system (Millipore, France). All other used chemicals were of analytical grade.

2.2. Experimental design

Wistar rats, male, weighing 196.4 ± 8.2 g from the institute of Laboratory Animal Science, CAMS & PUMC (Beijing, China), were housed individually in cages and maintained (20–25 °C and 40–60% humidity) under a standard 12-h light/dark cycle with free access to purified water and commercial diet in Specific Pathogen Free Laboratory. All experimental procedures were approved by the Ethics Committee of the Institute of Medicinal Plant Development, CAMS & PUMC. Rats were randomly divided into four groups (n = 6) and equalized with body weights. ISO was dissolved in normal saline and injected subcutaneously to rats at an interval of 24 h for 2 days to induce experimental MI. [11]. Group I (control group) received normal saline, group II (model group) received ISO (85 mg/kg s.c., on 27th and 28th day), group III (XKS treated group) received XKS (2.88 g/kg/day, for 4 weeks) and ISO (85 mg/kg s.c., on 27th and 28th day), group IV (positive group) received propranolol (0.10 g/kg/day for 4 weeks) and ISO (85 mg/kg s.c., on 27th and 28th day). After 12 h following the last dose of ISO, rats were sacrificed under anesthetized with urethane (1.3 g/kg). Blood samples were collected and centrifuged at 4000 rpm for 15 min at 4 °C and stored at −80 °C until being tested for cardiac injury and the metabolomic study. The myocardial tissues were quickly removed, and one part of myocardial tissues was cut and put into a flask containing 10% buffered formalin solution for the pathological analysis.

2.3. Biochemistry assays in plasma sample

MI was biochemically diagnosed with changed markers of cardiac injury and its oxidative mechanisms. The cardiac injury was evaluated by measuring plasma CK, LDH and AST activities, and the oxidative stress markers including activities of SOD and MDA in plasma were determined using standard kits (Nanjing Jiancheng Institute of Biotechnology) on a spectrophotometry (Mapada, UV-3100, China) according to the manufacturer’s instruction.

2.4. Pathology

Myocardial tissues from all the groups were subjected to pathological observations. The lower portion of myocardial tissue from each heart was fixed in 10% buffered formalin solution for 48 h, embedded in paraffin, 5 μm sectioned, and stained with hematoxylin-eosin (HE). Images were obtained and studied under light microscopy (Olympus, BX53, Japan).

2.5. Plasma metabolomics

Plasma analysis workflow was modified from nature protocol [12] according to Customer Familiarization Guide using a Waters SYNAPY G2 HDMS (Fig. 1).

2.5.1. Sample preparation

Plasma (200 μL) was added into acetonitrile (600 μL), vortex-mixing for 30 s, and centrifuged at 6000 rpm for 10 min to precipitate the proteins. 750 μL of protein free supernatant was collected and dried with nitrogen at 37 °C. The dried residue was reconstituted in 100 μL of acetonitrile–water (1:99, v/v); after centrifugation for 15 min at 13,000 rpm, an aliquot of 2 μL was injected for UPLC/MS analysis.

2.5.2. Data acquisition

Chromatographic separation was performed on an Acquity UPLC BEH C18 column (2.1 mm × 100 mm, 1.7 μm, Waters Corp., Milford, USA) using a Waters ACQUITY UPLC system, equipped with a binary solvent delivery system. The column was maintained at 40 °C and eluted at a flowing rate of 0.4 mL/min, using a mobile phase of (A) 0.1% (by volume) formic acid in water and (B) acetonitrile. The gradient program was optimized as follows: 0–0.5 min, 1% B; 0.5–3 min, 1% B to 60% B; 3–9 min, 60% B to 72% B; 9–11 min, 72% B to 99% B; 11–13 min, washing with 99% B, and 14–17 min, equilibration with 1% B. The eluent from the column was directed to the mass spectrometer without split.

A Waters SYNAPY G2 HDMS (Waters Corp., Manchester, UK) was used to carry out the mass spectrometry with an electrospray ionization source (ESI) operating in positive and negative ion mode. The capillary voltages were set at 3.0 and 2.5 kV, sample cone voltage 40 V, extraction cone voltage 4.0 V, respectively. Used drying gas nitrogen, the desolvation gas rate was set to 800 L/h at 450 °C, the cone gas rate at 40 L/h, and the source temperature at 120 °C. The scan time and inter scan delay were set to 0.15 and 0.02 s, respectively. Leucine-enkephalin was used as the lockmass in all analyses (m/z 556.2771 for positive ion mode and m/z 554.2615 for
negative ion mode) at a concentration of 0.5 μg/mL with a flow rate of 5 μL/min. Data was collected in centroid mode from m/z 100 to m/z 1500. The lock spray frequency was set at 5 s and the lock mass data were averaged over 10 scans for correction.

To ensure the stability of sequence analysis, a quality control (QC) sample was prepared by pooling the same volume (10 μL) from each plasma sample and then preparing the pooled QC sample in the same way as the samples. The pooled QC sample was analyzed randomly through the analytical batch. Twenty ions were extracted from the base peak intensity chromatography and selected for method validation, as retention time and m/z pairs of 0.60,204,1235, 1.63,188,0711, 3.95,453,1673, 4.26,268,3087, 4.55,494,3242, 5.60,496,3433, 6.03,522,3561, 7.33,524,3722, 7.24,758,5690 and 10.27,758,5684 in positive mode, 0.56,167,0207, 1.63,103,2804, 2.20,187,0070, 3.53,391,2850, 3.95,313,2385, 4.53,538,3133, 5.58,540,3298, 7.31,568,3629, 10.11,303,2323, and 10.85,281,2484 in negative mode. The relative standard deviations (RSD) of retention time and m/z of the selected ions were 0.00–0.72%, 0.00–0.00% in positive mode, and 0.02–0.45%, 0.00–0.00% in negative mode. Method repeatability was evaluated using six replicates by analyzing QC sample. The RSD of retention time, m/z and peak area of the selected ions were 0.01–0.55%, 0.00–0.00% and 5.23–11.34% in positive mode and 0.003–0.48%, 0.00–0.00% and 3.23–12.23% in negative mode, respectively (Table S1).

2.5.3. Multivariate analysis
The raw MS spectra were first analyzed using MarkerLynx Applications Manager version 4.1 (Waters Corp, Manchester, UK), which allowed deconvolution, alignment and data reduction to give a list of mass and retention time pairs with corresponding peak area for all the detected peaks from each file in the data set. The main parameters in MarkerLynx were set as follows: retention time range, 0–11 min; mass range, 100–1200 Da; XIC window, 0.02 min; automatically calculate peak width and peak–peak baseline noise; use the raw data during the deconvolution procedure; marker intensity threshold (count), 1000; mass tolerance, 0.02 Da; retention time windows, 0.2 min; noise elimination level, 6; retain the isotopic peaks. The processed data list was then exported and processed by the principal component analysis (PCA) and orthogonal partial least-squares-discriminant analysis (OPLS-DA) in the software package Simca-P software (v12.0, Umetrics, Umeå, Sweden). All the tested groups were discriminated in the PCA model. In the OPLS-DA model, samples from two groups were classified, and the results were visualized in the form of score plot to show the group clusters and S-plot to show variables contributing to the classification. To validate the model against overfitting, a typical 7-round cross-validation was carried out with 1/7 of the samples being excluded from the model in each round. This procedure was repeated in an iterative manner until each sample had been excluded once and the Q2 and R2 values were calculated from the results in Simca-P package.

2.6. Statistical analysis
All values were expressed as mean ± S.D. The significance of differences between the means of the treated and un-treated groups has been compared by a two-tailed Student’s t-test using the Statistical Package for Social Science program (SPSS 16.0, Chicago, IL, USA). The significance threshold was set at p < 0.05 for this test.

3. Results
3.1. Cardiac injury in MI rats
Plasma enzymes (CK, LDH and AST) are quantitative markers in MI model. Rats treated with ISO (model group) showed significant increase in the levels of CK, LDH and AST in the plasma compared with normal rats (control group) (p < 0.01 ) (as shown in Fig. 2, indicating ISO-induced acute cellular necrosis. The most changed percentage was CK for striated muscle special, indicating the plasma enzymes being released from the myocardial cell due to MI. Oral administration with XKS (XKS group) or propranolol (positive group) to ISO-induced rats significantly (p < 0.05) decreased the levels of CK, LDH and AST compared with model rats (p < 0.01), indicating both pretreatments capable of ameliorating
the acute cellular necrosis. However, the cardioprotective effect of XKS was better on regulating CK than that of propranolol. Meanwhile, propranolol actually showed better results than XKS on LDH and AST.

3.2. Oxidative pathogenesis in MI rats

Oxidative pathogenesis has been expressed here with SOD or MDA levels. ISO-induced MI rats showed a significant decrease in the level of SOD in the plasma compared with control rats (\(p < 0.01\)). Pretreatment with propranolol significantly increased the circulatory antioxidants near to normal levels (\(p < 0.01\)), indicating propranolol at this dosage could markedly inhibit ISO-induced SOD decrease. XKS restored partially the change of plasma SOD induced by ISO (\(p < 0.01\)). MDA represents total levels of lipid peroxidation. The level of plasma MAD was significantly increased in ISO-induced MI rats compared with control rats (\(p < 0.01\)). Pretreatment with XKS or propranolol to ISO-treated rats failed to meliorate MAD level (\(p > 0.05\)), indicating that the therapeutic effects of both drugs were independent from the oxidative process in rats with ISO-induced MI (Fig. 2).

3.3. Pathological changes in ISO-induced MI infracted rats

Pathological changes in model rats were located at the subendocardia from left ventricle, being compared with those in control rats in light microscopic images. Fig. 3A showed the normal architecture of the rat myocardium. Cardiac changes in model rats showed classic pathological alterations (Fig. 3B), including watery degeneration of myocytes, coagulative necrosis within single myocyte, capillary edema and scattered bleeding. Obviously, a lot of neutrophilic granulocytes appeared in or out blood vessels, sometime located in myocyte sarcoplasma (a necrotic cell), indicating an acute infiltration of neutrophilic granulocytes. The intravascular resident neutrophilic granulocytes significantly enlarged vascular volumes, especially in capillaries. All myocardial damages were moderated obviously in propranolol group, such as widen sarcoplasma, ghost cell, stainless area, red blood cells out of vessels, and infiltrated neutrophilic granulocytes. Moderated coagulative necrosis and acute infiltration caused a relieved thick wall in left ventricle in gross, especially. The protective effect of XKS appeared to be greater than that of propranolol (Fig. 3C and D). All pathological abnormalities in ISO-induced MI infracted rats, such as watery degeneration, coagulative necrosis, edema, bleeding, and acute infiltration, were markedly ameliorated by XKS pretreatment. Less edema and infiltration lead to left ventricle wall thin in gross.

3.4. Plasma metabolomics

3.4.1. Multivariate analysis of UPLC/QTOF-MS data

Plasma samples collected on the 28th day in each groups were analyzed by UPLC/QTOF-MS in positive and negative modes. The base peak intensity chromatograms of all experimental groups were showed in Fig. S1. PCA was firstly carried out on the preprocessing of raw data from the spectra to find out their metabolic distinction. The data were combined into a single matrix by aligning peaks with the exact mass/retention time pair together from each data file in the data set, along with their associated peak areas. In our experiment, the X variables between model group and control group in positive and negative modes were 1959 and 351, respectively. Meanwhile, the noise levels were 39% and 45%. If the value is above 50% here, the summary plot (score plot) shows less than half of the information (variation) in the data and the risk to miss important information is substantial. OPLS-DA was more focused on the actual class discriminating variation in the data compared to the unsupervised approach (PCA). In the OPLS-DA model, samples from model group and control group or drug-pretreatment group were classified, and the results were visualized in the form of score plots to show the group clusters and S-plots to show variables contributing to the classification.

3.4.2. Plasma metabolic profiles of the MI rats

PCA (Fig. 4A and B) analysis indicated that the metabolic profile of rat in the model group deviated from the control, suggesting that significant biochemical changes were induced by MI. The metabolic profile of rats in XKS treated group fairly differed from the MI group and closed to the control, indicating the deviations induced by MI were significantly improved after treatment of XKS. Similar results were observed in propranolol treated group except the mean center of spots in XKS treated group was much closer to control group than that of propranolol treated group, which was consistent with the results of biochemical and pathological ethological study.
Similar results were observed in propanolol treated group except in negative mode, which was more near the model group without apparent classification.

3.4.3. Identification of potential pathological biomarkers related to MI

The OPLS-DA method was employed to sharpen an already established separation between the MI and control groups in PCA. The integrity of the mathematical model was evaluated firstly before being used for further interpretation. Clear separation of the control and model rats could be observed, indicating that significant biochemical changes were induced by MI (Fig. 4C and E). The S-plots of OPLS-DA (Fig. 4D and F) indicated the variables responsible for the differentiation, and the variable importance for projection (VIP) value signifies the influence of the metabolites on the classification. Variables far from the origin in the S-plots with VIP values ≥ 1 contributed significantly to the clustering. Only those variables with significant difference between model and control rats (p < 0.05) using an independent t-test were selected as potential biomarkers.

The S-plots based on plasma metabolic profiles between control group and model group indicated the possible biomarkers with retention time and m/z pairs of 0.60,204.1233, 0.65,229.1547, 1.00,160.0762, 1.62,232.1544, 3.73,318.2999, 4.59,302.3050, 7.24,400.3416 and 7.83,524.3712 in positive mode and 0.56,167.0204, 1.62,203.0820, 4.93,564.3296, 5.58,540.3297, 7.83,568.3609, 9.81,327.2327, 10.09,303.2325 and 10.25,279.2325 in negative mode. As a result, fifteen metabolites were recognized as potential pathogenic biomarkers related to ISO-induced MI, and their structures were identified by analyzing their accurate molecular weights and the collected MS^E spectra (Table 1). Databases, such as Biofluid Metabolites Database (http://metlin.scripps.edu) and Human Metabolome Database (http://www.hmdb.ca) were used for confirmation. They are L-acetylcarnitine (1), L-isoleucyl-L-proline (2), tyramine (3), isobutyryl-L-carnitine (4), phytosphingosine (5), sphinganine (6), L-palmitoylcarnitine (7), lysoPC(18:0) (8), uric acid (9), L-tryptophan (10), lysoPC(18:2) (11), lysoPC(16:0) (12), docosahexanoic acid (13), arachidonic acid (14) and linoleic acid (15).

Here, an identified biomarker at m/z 400.3416 (the retention time was 7.24 min in the positive mode) was taken as an example to illustrate the identification process. First, the low-energy mass spectrum of the peak of m/z 400.3416 at 0.62 min in its MS^E spectra (Fig. S2B in the Supporting information) in the positive ion mode gave the accurate molecular weight of the quasi-molecular ion at m/z 400.3416, suggesting a molecular formula with [C_{23}H_{45}NO_{4} + H]^+ (calculated to be 400.4327). Candidates are obtained in searching molecular weight at 400.3416 Da (Positive mode, MW tolerance ± 10 ppm) from database METLIN (http://metlin.scripps.edu). As a result, there are two candidates with molecular formula of [C_{23}H_{45}NO_{4} + H]^+ , which are described as L-palmitoylcarnitine and palmitoylcarnitine, respectively. As shown in the high energy spectrum of MS^E spectra, the ion at m/z 400.3416 (Fig. S2A) generated the ions at m/z 341.2709, 144.1022 and m/z 85.0290 by loss of a group of C_{16}H_{32}O_{2} and C_{10}H_{14}N + C_{16}H_{22}O_{2}, continuously. The fragmentation pathways matched with the report of database METLIN (Fig. S2C). So the ion of m/z 400.3416 was tentatively identified as L-palmitoylcarnitine (2).

The varied tendencies of the identified pathological biomarkers related to MI were shown in Table 1. The concentrations of eight metabolites (1–7, 14) were significantly increased and seven (8–13, 15) decreased in MI group compared with normal control. XKS pretreatment corrected the variations of all pathogenic biomarkers, while propanolol did not show any effects on some metabolites (2, 4, 6, 7, 12 and 13).

3.4.4. Identification of potential pharmaco biomarkers associated with XKS mediations

Besides the pathological biomarkers, the protection of XKS has great potential to mediate other metabolites involved in the progress of MI. Thus, the OPLS-DA method was also employed to analysis the differences of metabolic profiles between ISO-induced MI and XKS pretreatment obtained from the UPLC/QTOF-MS data.
The score plots of OPLS-DA (Fig. 4G and I) showed that the MI model group and XKS pretreated group can be successfully differentiated in both positive and negative modes. Seventeen metabolites were responsible for discrimination of two groups and considered as potential pharmaco biomarkers associated with XKS mediations (Fig. 4H and J). They were identified as l-acetylcarnitine (1), l-isoleucyl-l-proline (2), tyramine (3), isobutyryl-l-carnitine (4), phytosphingosine (5), sphinganine (6), l-palmitoylcarnitine (7), lysoPC(18:0) (8), uric acid (9), l-tryptophan (10), lysoPC(18:2) (11), lysoPC(16:0) (12), docosahexaenoic acid (13), arachidonic acid (14), linoleic acid (15), hexanoylcarnitine (16) and tetrade-canoylcarnitine (17), respectively. There was no doubt that fifteen of the identified pathological biomarkers (1–15) related to MI are included, because XKS could prevent deviations of these
Table 1
Potential biomarkers detected by UPLC-QTOF-MS in positive and negative modes.

<table>
<thead>
<tr>
<th>No.</th>
<th>Compound</th>
<th>RT (min)</th>
<th>m/z</th>
<th>Adduct ion</th>
<th>Formula</th>
<th>VIP_M</th>
<th>VIP_N</th>
<th>M/C</th>
<th>XKS/M</th>
<th>P/M</th>
<th>Pathway</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>l-Acetylcarnitine</td>
<td>0.60</td>
<td>204.1233</td>
<td>[M+H]+</td>
<td>C$_7$H$_9$NO$_4$</td>
<td>1.31</td>
<td>7.68</td>
<td>b</td>
<td>b</td>
<td></td>
<td>Fatty acid β-oxidation pathway</td>
</tr>
<tr>
<td>2</td>
<td>l-Isoleucyl-l-proline</td>
<td>0.65</td>
<td>229.1547</td>
<td>[M+H]+</td>
<td>C$<em>{11}$H$</em>{20}$NO$_3$</td>
<td>1.72</td>
<td>7.16</td>
<td>b</td>
<td>b</td>
<td></td>
<td>Proteolysis</td>
</tr>
<tr>
<td>3</td>
<td>Tyramine</td>
<td>1.00</td>
<td>160.0762</td>
<td>[M+Na]$^+$</td>
<td>C$_6$H$_5$NO</td>
<td>1.53</td>
<td>8.86</td>
<td>b</td>
<td>b</td>
<td></td>
<td>Tyrosine metabolism</td>
</tr>
<tr>
<td>4</td>
<td>Isobutyryl-l-carnitine</td>
<td>1.62</td>
<td>232.1545</td>
<td>[M+H]+</td>
<td>C$_7$H$_9$NO$_4$</td>
<td>1.86</td>
<td>3.77</td>
<td>b</td>
<td>b</td>
<td></td>
<td>Fatty acid β-oxidation pathway</td>
</tr>
<tr>
<td>5</td>
<td>Phytosphingosine</td>
<td>3.73</td>
<td>315.2899</td>
<td>[M+H]+</td>
<td>C$<em>{12}$H$</em>{24}$NO$_3$</td>
<td>3.40</td>
<td>7.11</td>
<td>b</td>
<td>b</td>
<td></td>
<td>Sphingolipid metabolism</td>
</tr>
<tr>
<td>6</td>
<td>Sphinganine</td>
<td>4.59</td>
<td>302.3050</td>
<td>[M+H]+</td>
<td>C$<em>{12}$H$</em>{20}$NO$_4$</td>
<td>5.01</td>
<td>1.15</td>
<td>b</td>
<td>b</td>
<td></td>
<td>Sphingolipid metabolism</td>
</tr>
<tr>
<td>7</td>
<td>7-l-Palmitylcarnitine</td>
<td>7.24</td>
<td>400.3416</td>
<td>[M+H]+</td>
<td>C$<em>{12}$H$</em>{22}$NO$_4$</td>
<td>5.96</td>
<td>1.40</td>
<td>b</td>
<td>b</td>
<td></td>
<td>Fatty acid β-oxidation pathway</td>
</tr>
<tr>
<td>8</td>
<td>LysoPC</td>
<td>7.83</td>
<td>524.3712</td>
<td>[M+H]+</td>
<td>C$<em>{20}$H$</em>{43}$NO$_5$</td>
<td>14.78</td>
<td>6.23</td>
<td></td>
<td></td>
<td></td>
<td>Glycerophospholipid</td>
</tr>
<tr>
<td>9</td>
<td>(C18:0)</td>
<td>7.83</td>
<td>568.3609</td>
<td>[M+HCOO]$^-$</td>
<td>C$<em>{20}$H$</em>{45}$NO$_4$</td>
<td>2.64</td>
<td>3.23</td>
<td></td>
<td></td>
<td></td>
<td>metabolism</td>
</tr>
<tr>
<td>10</td>
<td>Uric acid</td>
<td>0.56</td>
<td>167.0204</td>
<td>[M–H]$^-$</td>
<td>C$_5$H$_8$NO$_3$</td>
<td>4.04</td>
<td>3.59</td>
<td>b</td>
<td>b</td>
<td></td>
<td>Purine metabolism</td>
</tr>
<tr>
<td>11</td>
<td>7-Tryptophan</td>
<td>1.62</td>
<td>203.0820</td>
<td>[M–H]$^-$</td>
<td>C$_5$H$_8$NO$_3$</td>
<td>3.26</td>
<td>3.17</td>
<td>b</td>
<td>b</td>
<td></td>
<td>Tryptophan metabolism</td>
</tr>
<tr>
<td>12</td>
<td>LysoPC (C18:2)</td>
<td>4.93</td>
<td>563.3926</td>
<td>[M+HCOO]$^-$</td>
<td>C$<em>{20}$H$</em>{45}$NO$_4$</td>
<td>2.40</td>
<td>2.29</td>
<td>b</td>
<td>b</td>
<td></td>
<td>Glycerophospholipid metabolism</td>
</tr>
<tr>
<td>13</td>
<td>LysoPC (C16:0)</td>
<td>5.58</td>
<td>540.3297</td>
<td>[M+HCOO]$^-$</td>
<td>C$<em>{20}$H$</em>{45}$NO$_4$</td>
<td>5.33</td>
<td>2.30</td>
<td>b</td>
<td>b</td>
<td></td>
<td>Glycerophospholipid metabolism</td>
</tr>
<tr>
<td>14</td>
<td>Docosahexaenoic acid</td>
<td>9.61</td>
<td>327.2327</td>
<td>[M–H]$^-$</td>
<td>C$<em>{22}$H$</em>{36}$O$_2$</td>
<td>5.95</td>
<td>4.13</td>
<td>b</td>
<td>b</td>
<td></td>
<td>Glycerophospholipid metabolism</td>
</tr>
<tr>
<td>15</td>
<td>Arachidonic acid</td>
<td>10.07</td>
<td>303.2325</td>
<td>[M–H]$^-$</td>
<td>C$<em>{22}$H$</em>{36}$O$_2$</td>
<td>5.11</td>
<td>4.02</td>
<td>b</td>
<td>b</td>
<td></td>
<td>Arachidonic acid metabolism</td>
</tr>
<tr>
<td>16</td>
<td>Linoleic acid</td>
<td>10.25</td>
<td>279.2325</td>
<td>[M–H]$^-$</td>
<td>C$<em>{18}$H$</em>{32}$O</td>
<td>3.26</td>
<td>1.99</td>
<td>b</td>
<td>b</td>
<td></td>
<td>Fatty acid β-oxidation pathway</td>
</tr>
<tr>
<td>17</td>
<td>Hexanoylcarnitine</td>
<td>2.29</td>
<td>260.1853</td>
<td>[M–H]$^-$</td>
<td>C$<em>{13}$H$</em>{20}$NO$_4$</td>
<td>0.43</td>
<td>3.90</td>
<td>b</td>
<td>b</td>
<td></td>
<td>Fatty acid β-oxidation pathway</td>
</tr>
<tr>
<td>18</td>
<td>Tetradecanoylcarnitine</td>
<td>5.16</td>
<td>372.2103</td>
<td>[M–H]$^-$</td>
<td>C$<em>{15}$H$</em>{26}$NO$_4$</td>
<td>0.91</td>
<td>3.57</td>
<td>b</td>
<td>b</td>
<td></td>
<td>Fatty acid β-oxidation pathway</td>
</tr>
</tbody>
</table>

The VIP$_M$ and VIP$_N$ values representing for the variables responsible for model group differentiation from control group or XKS group, respectively; M/C representing for the change trend in model group compared to control group, XKS/M and P/M representing for the changes trend in XKS group or positive group compared to model group, respectively.

* p < 0.05
b p < 0.01
#: no significant differences.

metabolites induced by ISO. Meanwhile, there are two more metabolites (16 and 17) responsible for the differentiation between XKS treated group and model group. Both of them decreased significantly in XKS treated rats compared with the MI rats, and probably represent comprehensive mediation of XKS on ISO-induced MI.

3.4.5. Perturbed metabolic pathways in response to MI rats

According to the KEGG PATHWAY database, the metabolic pathways involved in ISO-induced MI and regulation of XKS were depicted based on the identified potential biomarkers (Fig. 5A). Eight metabolic pathways were disturbed by MI, including fatty acid β-oxidation pathway, sphingolipid metabolism, glycerophospholipid metabolism, arachidonic acid metabolism, tyrosine metabolism, tryptophan metabolism, proteolysis and purine metabolism. XKS pretreatment mediated the disturbances of all metabolic pathways induced by MI with additional regulation on the levels of hexanoylcarnitine (16) and tetradecanoylcarnitine (17), both of them potential accelerating the β-oxidation of fatty acids to meet the decreased energy supply induced by high dose of ISO.

3.5. Fatty acid β-oxidation pathway

It has long been known that most of the energy production in the heart is derived from the oxidation of fatty acids. In heart failure, the chief myocardial energy substrates switch from fatty acids to glucose, with a down-regulation of the enzymes involved in fatty acid oxidation (FAO). During FAO, l-carnitine is an essential cofactor required for mitochondrial fatty acid oxidation, in which the fatty acyl CoA enters the mitochondria as fatty acylcarnitines via carnitine transport. Acylcarnitines generated during this carnitine-dependent pathway play the role of fatty acid carrier between the mitochondrial outer membrane and the matrix side of the inner membrane in which are located carnitine palmitoyltransferases types (CPT I and II, respectively. It has been reported that inhibition of FAO resulted in the accumulation of toxic intermediates such as long-chain acylcarnitine derivatives. Increased myocardial accumulations of long-chain acyl coenzyme A (CoA) and acylcarnitine may exert several deleterious effects including uncoupling of oxidative phosphorylation, alteration of mitochondrial permeability, and impaired sarcosomal function and intracellular Ca$^{2+}$ handling.[13]. In this study, the increased levels of l-acetylcarnitine (1), isobutyryl-l-carnitine (4) and l-palmitylcarnitine (7) participating into the FAO were observed in MI rats. XKS pretreatment alleviated the changes of acylcarnitines and satisfied the energy requirement. Additionally, hexanoylcarnitine (16) and tetradecanoylcarnitine (17) are also the longer-chain length acyl carnitines related to the β-oxidation of medium-chain fatty acids [14] and long-chain fatty acids [15], respectively. Although accumulation of both of them was not observed in MI treated rats, XKS pretreatment could prevent the potential accumulation of them in the progress of MI due to acceleration of FAO to meet the decreased energy induced by ISO. Moreover, the elevation of the unsaturated fatty acid linoleic acid (15) in MI rats could also indicate that the β-oxidation of the unsaturated fatty acid was inhibited, contributing to the lipotoxicity in myocardium [16]. Both XKS and propranolol could impair the lipid disorders in the MI formation.

3.6. Glycerophospholipid metabolism

Glycerophospholipids serve as structural components of cell membranes. LysoPCs degradation indicates that an underlying lipid disturbance is well associated with the formation of ISO-induced MI [17]. The levels of lysoPCs in the plasma (8, 11 and 12) were decreased in the ISO-induced MI rats compared with the normal rats, which is in agreement with the breakdown of membrane in the MI rats detected in the physiology study. XKS pretreatment could maintain the normal levels of those glycerophospholipids, while propranolol did not regulate the decrease of 12.

3.7. Arachidonic acid metabolism

The metabolism of arachidonic acid (14, AA) may follow multiple and interrelated pathways, leading to the generation or release of a wide variety of inflammation substances (bradykinins, serotonin, histamine, prostaglandins, and cytokines) [18]. The increased AA is associated with increased risk of non fatal acute myocardial infarction, and AA is also a crucial biomarker for inflammatory involved at the onset of MI [19]. Therefore, this pathological metabolism involved into ischemic cardiac cells, infiltrated neutrophils, and coagulated platelets in rats with ISO-induced MI, matching with the pathological observation in this work (Fig. 3).
The pretreatment with XKS could more ameliorate the abnormal change of AA than propranolol.

3.8. Sphingolipid metabolism.

Sphingolipids play significant physiological roles in cell growth, cell differentiation and critical signal transduction pathways [20]. Phytosphingosine (5) and sphingasine (6) are two important metabolites involved in sphingolipid metabolism. Phytosphingosine (5), metabolized from sphingasine (6), prevents opening of the mitochondrial permeability transition pore (MPT), with subsequent release of Cytochrome C and activation of Caspase 3, which lead to the cell apoptosis and play a crucial role in the development of MI [21]. The accumulations of phytosphingosine (5) and sphingasine (6) in MI rats might accelerate the apoptosis of the myocardial cell in progress of MI. XKS significantly decreased the levels of phytosphingosine (5) and sphingasine (6), suggesting the protection of XKS might due to the inhibition of subsequent cell apoptosis.

3.9. Proteolysis

l-Isoleucyl-l-proline (2) was a proteolytic breakdown product of larger proteins and could inhibit the expression of Cathepsin B [22], which may play a role in the progression of intracellular damage and might be an indicator of myocardial cell death [23]. The increased l-isoleucyl-l-proline in MI group suggested autophagic digestion of proteins and myocardial cell injury involved into the process of MI induced by ISO. And the rats pretreated with XKS could effectively ameliorate those damages.

3.10. Tryptophan metabolism

L-Tryptophan (10), one type of α-amino acids, is an important energy metabolism precursor and can be transformed into some biomolecules, such as pyruvate and fumarate, to enter into citrate cycle [24]. In our study, L-tryptophan was decreased in the plasma of MI rats, most likely because ischemia and oxygen deficiency lead to the metabolic remodeling to meet energy requirement in myocardium [25]. XKS could reverse the decreasing level of L-tryptophan and satisfy the energy supply in the organism.

3.11. Purine metabolism

Uric acid (9) is the final oxidation product of purine metabolism. It is difficult to establish whether or not an independent association between uric acid and cardiovascular disease [26], where oxidative stress plays an important pathophysiological role [27]. High plasma level of uric acid was observed in the MI rats and down-regulated to normal with XKS pretreatment.
3.12. Tyrosine metabolism.

Tyramine (3) is a monoamine compound derived from tyrosine, which is associated with the development of cardiac hypertrophy in the formation of MI [28]. The plasma level of tyramine (3) was increased in MI rats, which was in response to increased stress on the heart induced by high dose of ISO. Down-regulation of tyramine (3) by XKS indicated the XKS pretreatment could maintain normal cardiac function to normalize heart stress.

4. Discussion

Traditional Chinese Medicine (TCM) was a unique medical system which can hit multiple targets with multiple components. It has been pursued an overall therapeutic effect on cardiovascular diseases. In our study, an ISO-induced rat MI model was applied to evaluate the pharmacodynamics and mechanism of XKS. The plasma biochemical study demonstrated that the elevated enzyme activities of plasma CK, LDH and AST induced by ISO were decreased by pretreatment of XKS, which might partially ascribe to its increased antioxidant capacity (SOD). Additionally, XKS also could ameliorate neutrophil recruitment and endothelial injury in the histopathological study.

To understand the multi-targeted effects of XKS against MI in depth, a metabolomic approach was carried out in this study. With the multivariate analysis of UPLC/MS data, the deviations induced by MI were significantly improved after treatment of XKS and prananol, suggesting that their cardioprotective effects are most likely that they ameliorated the metabolic disorders induced by ISO. Eighteen pathological biomarkers (1–18) were identified for differentiating the metabolic profiles of MI rats from normal rats. Among them, eight (1–6, 9, 10 and 15) were for the first time indentified as pathological biomarkers related to ISO induced MI. They have also been found linking to metabolic disturbances of MI rats performed by ligating the left ventricular coronary artery [29]. XKS pretreatment not only mediated the changes of 15 pathological biomarkers, but also affected two other metabolites (hexanoylcarnitine (16) and tetradecanoicarnitine (17)). All of those metabolites were considered as potential pharmaco biomarkers for differentiating the metabolic profiles of MI rats with and without XKS pretreatment. Interesting, XKS protected myocardial infarction by mediating the metabolic pathways of all pharmaco biomarkers, while prananolol has no effect on the metabolic disorders of seven biomarkers (2–4, 6, 7, 12 and 13).

Based on the identified potential pathological biomarkers, perturbation in ISO-induced MI were involved in eight metabolic pathways including fatty acid β-oxidation pathway, glycerophospholipid metabolism, arachidonic acid metabolism, sphingolipid metabolism, tryptophan metabolism, proteolysis, tyrosine metabolism and purine metabolism. The pretreatment with XKS could ameliorate the abnormal changes of these pathways. Moreover, XKS inhibited the increases of other two pharmaco biomarkers involved in potential acceleration of fatty acid β-oxidation, hexanoylcarnitine (16) and tetradecanoicarnitine (17). Lipid pathways would result in the alterations of energy metabolism, inflammation as well as endothelial dysfunction, which significantly impact on the formation of MI [30]. Thirteen of the identified pharmaco biomarkers (1, 4–8, 11–17) involved into lipid metabolism including fatty acid β-oxidation pathway, glycerophospholipid metabolism, arachidonic acid metabolism and sphingolipids metabolism. Among them, the degradation of glycerophospholipids (8, 11 and 12) and the increasing of arachidonic acid (14) were the major pathological disorders in rats with ISO-induced MI, being related to the disruptions of cell membrane and the occurrence of inflammation [16] (Fig. 5B). Pretreatment of XKS could effectively improve the abnormalities of all identified biomarkers related to lipid pathways in ISO-induced MI rats, suggesting that the targeted interventions of XKS on disorders of lipid pathways played a key role in its protective effects against MI.

Amino acid metabolites (2, 3 and 10) participated into the process of protein degradation, energy supply and cardiac hypertrophy in the physiological changes of MI. XKS treatment could inverse the changes of these metabolites near to normal levels to maintain the homeostasis.

The increased level of uric acid (9) was associated with oxidative stress, which plays a vital role in the formation of MI. XKS pretreatment could effectively inhibit the evaluated uric acid (9), matching with the effects of XKS on plasma oxidative pathogenesis induced by ISO.

5. Conclusion

In the present work, the protective effect of XKS was evaluated in ISO-induced MI rats using a metabonomic approach. Two genres of metabolic biomarkers including pathological biomarkers and pharmaco biomarkers were used to construct the metabolic pathways underlying the pharmacological action of XKS. 15 metabolites significantly varied between MI rats and normal rats were characterized as potential pathological biomarkers related to MI, while 17 metabolites for differentiating between MI group and XKS treated group were characterized as potential pharmaco biomarkers. The involved metabolic pathways included fatty acid β-oxidation pathway, sphingolipid metabolism, glycerophospholipid metabolism, arachidonic acid metabolism, tyrosine metabolism, tryptophan metabolism, proteolysis and purine metabolism. The perturbations of those pathways related to the disorders of energy metabolism, inflammation, disruption of myocardium structures, cardiac hypertrophy as well as oxidation stress in the development of ISO-induced MI. XKS pretreatment mediated the metabolic disturbances induced by MI with additional regulation on the levels of hexanoylcarnitine (16) and tetradecanoicarnitine (17), both of them potentially accelerating the β-oxidation of fatty acids to meet the decreased energy supply induced by high dose of ISO.

Among the identified metabolites, eight (1–6, 9 and 10) were first reported as pathological biomarkers related to ISO-induced MI. They mainly involved into perturbed energy metabolism, amino acids metabolism, proteolysis as well as purine metabolism, which not only provided new insights into the ISO-induced MI network in vivo but also revealed the mechanism of XKS for the prevention or treatment of MI.

Acknowledgements

This work has been financially supported by National S & T Major Special Project on Major New Drug Innovation (2013ZX095080104), National Natural Science Foundation of China (no. 81073021), Beijing National Science Foundation (no. 7112093) and Program for Innovative Research Team in IMPLAD.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jpba.2013.11.008.

References


Y.-t. Liu et al. / Journal of Pharmaceutical and Biomedical Analysis 90 (2014) 35–44


