

GC–MS-based metabolic profiling reveals metabolic changes in anaphylaxis animal models

Xia Hu · Gong-ping Wu · Meng-hui Zhang ·
Shan-qing Pan · Rong-rong Wang · Jie-hu Ouyang ·
Jun-ge Liu · Zi-yuan Chen · Hong Tian · Dong-bo Liu

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Abstract Clinical definition and appropriate management of anaphylaxis is a clinical challenge because there is large variability in presenting clinical signs and symptoms. Monitoring of the metabolic status of anaphylaxis may be helpful in understanding its pathophysiological processes and diagnosis. The purpose of this study was to conduct GC–MS serum metabolic profiling of anaphylaxis animal models and search for potential biomarkers of anaphylaxis. Thirty-six guinea pigs were randomly divided into an ovalbumin group ($n=12$), a cattle albumin group ($n=12$), and a control group ($n=12$). The IgE level in the serum of the guinea pigs was evaluated by use of ELISA kits and the major metabolic changes in serum were detected by gas chromatography–mass spectrometry. Typical clinical symptoms appeared

after the animals had been challenged with ovalbumin or cattle albumin. The IgE levels in serum of both model groups were significantly higher than those of the control group. Clustering trend of the three groups based on variables was observed and nine out of 858 metabolomic features were found to be significantly different between control group and model groups. Among the nine features, six features were tentatively identified as metabolites related to energy metabolism and signal transduction in anaphylaxis. In conclusion, GC–MS-based metabolic profiling analysis might be an effective auxiliary tool for investigation of anaphylaxis.

Keywords Anaphylaxis · Allergen · ELISA · Gas chromatography-mass spectrometry · Metabolic profiling

Hu and Wu contributed equally to the article

X. Hu · J.-g. Liu · D.-b. Liu (✉)
Hunan Agricultural University,
Changsha, Hunan 410128, People's Republic China
e-mail: chinasaga@163.com

X. Hu · D.-b. Liu
State Key Laboratory of Subhealth Intervention Technology,
Changsha, Hunan 410128, People's Republic China

G.-p. Wu
School of Public Health, Central South University,
Changsha, Hunan 410078, People's Republic China

G.-p. Wu · S.-q. Pan · R.-r. Wang · J.-h. Ouyang · Z.-y. Chen ·
H. Tian (✉)
Hunan Provincial Institute for Drug Control,
Changsha, Hunan 410001, People's Republic China
e-mail: 770719582@qq.com

M.-h. Zhang
State Key Laboratory of Microbial Metabolism
(Shanghai Jiao Tong University),
Shanghai 200240, People's Republic China

Introduction

Anaphylaxis is an acute multi-organ hypersensitivity reaction that can rapidly progress to a severe life-threatening reaction. It occurs upon exposure to allergens, for example food, aeroallergens, venom, drugs, and immunizations. The incidence of anaphylaxis has been increasing over the past few decades [1–3]. Recent estimates suggest a lifetime prevalence of 0.05 % to 2.0 % with food anaphylaxis being the most common cause [1, 4, 5]. Currently, the clinical definition of anaphylaxis is controversial, because of the non-specificity of symptoms and the variability of presentation. Routine diagnostic methods of anaphylaxis are based on indirect detection tools using several laboratory markers [6]. Immunochemical and biochemical mediators released during anaphylaxis are detected as diagnostic adjuncts, for example IgE antibodies, histamine, prostaglandin D₂, carboxypeptidase, CD63, interleukins 4 and 6, C-reactive

protein (CRP), and tryptase [6–8]. However, these methods have limitations because of the complexity and diversity of anaphylaxis. Simultaneous determination of metabolic change in anaphylaxis might be helpful in understanding its pathophysiological processes and diagnosis.

Metabolomics is a method of investigating metabolic pathways and identifying unique biochemical molecules generated in a living system [9]. It may provide an overview of the dynamic metabolic status of the organism and yield new biomarkers which can be a clinic tool for diagnosis of disease [9]. Metabolic profiling is a complementary approach for metabolomic investigations [10]. It has been applied in studies investigating the metabolic response to various diseases, for example pre-eclampsia [11], Huntington's disease [12], Parkinson's disease [13], prostate cancer [14], human hepatocellular carcinoma [15], diabetes [16], intrauterine growth restriction (IUGR) [17], but not anaphylaxis.

The most commonly used techniques in metabolomic analysis are nuclear magnetic resonance (NMR) spectroscopy, Fourier-transform infrared spectroscopy (FT-IR), and mass spectrometry (MS) [18]. MS-based metabolomics, including gas chromatography–mass spectrometry (GC–MS), gas chromatography–tandem mass spectrometry (GC–MS–MS), liquid chromatography–mass spectrometry (LC–MS), liquid chromatography–tandem mass spectrometry (LC–MS–MS), and capillary electrophoresis–mass spectrometry (CE–MS) enable highly efficient separation, sensitive quantitative analysis, and use of MS databases for metabolite identification [10, 19]. Among these, GC–MS has been widely used in metabolomics because of its widespread availability, high resolution, and reproducibility [19].

The purpose of this study was to generate GC–MS serum metabolic profiles for anaphylaxis animal models and search for differences between control and model groups by use of pattern-recognition techniques. This would be helpful in identifying potential biomarkers for application in clinical diagnosis and monitoring of anaphylaxis.

Materials and methods

Chemicals

Compounds and reagents were all of analytical grade and purchased from Sigma, except where stated otherwise. *N,O*-bis(Trimethylsilyl)trifluoroacetamide (BSTFA) and 1 % trimethylchlorosilane (TMCS) were purchased from Tokyo Chemical Industry (Tokyo, Japan). *O*-Methoxyamine hydrochloride and pyridine (silylation-grade) were obtained from Aladdin Chemistry (Shanghai, China). Acetonitrile (HPLC-grade) was from Sinopharm Chemic Reagent (Shanghai, China). Ultrapure water was produced by a Milli-Q Reagent Water System (Millipore, USA).

Animals and sensitization

All guinea pigs weighed 300–350 g initially, and received humane care in compliance with the Guide for the Care and Use of Laboratory Animals of Hunan Provincial Institute for Drug Control (HPIDC). On the basis of previous work on anaphylaxis models [20], guinea pigs were randomly divided into three groups:

1. control group ($n=12$): untreated, nonsensitized;
2. ovalbumin group ($n=12$): guinea pigs sensitized to intraperitoneal ovalbumin (0.1 mg mL^{-1}); and
3. cattle albumin group ($n=12$): guinea pigs sensitized to intraperitoneal cattle albumin (100 mg mL^{-1}).

Animals in the ovalbumin and cattle albumin groups were sensitized by intraperitoneal injection with the corresponding allergen on the 1st, 3rd, and 5th days. The sensitized guinea pigs were then challenged intravenously with ovalbumin (0.1 mg mL^{-1}) or cattle albumin (100 mg mL^{-1}), respectively, on the 14th day. Blood samples were collected for analysis by cardiac puncture 1 h after challenge. The samples were clotted, centrifuged at 12,000 rpm for 10 min, and stored in aliquots at -80°C .

Assessment of systemic anaphylactic symptoms

The extent of anaphylactic responses induced by ovalbumin or cattle albumin was evaluated on the basis of the occurrence of typical clinical symptoms of anaphylaxis, which were graded as follows: no symptom, $-$; nose rubbing and/o fur erection, $\pm$; weakness, dyspnea, coughing, sneezing, vomiting and/or wheezing, $+$; convulsion, cyanoses and/or collapse, $++$; death, $+++$.

Measurement of IgE

IgE levels in guinea pig serum were investigated by use of ELISA kits (R&D Systems, USA and RapidBio Lab, Calabasas, USA). Data were analyzed by analysis of variance (ANOVA) with SPSS 13.0 software.

Sample treatment for GC–MS

Serum was thawed and vortex-mixed. Each 150- μL aliquot of the serum sample was deproteinated by addition of 450 μL acetonitrile followed by vortex mixing and centrifugation (12,000 rpm, 15 min, 4°C). The supernatant (150 μL) was then transferred to a GC vial and volatilized to dryness with nitrogen by use of an SE812 pressured gas-blowing concentrator (Shuaien Technology, Beijing, China). Methoxyamine hydrochloride (20 mg mL^{-1} , 25 μL) in dry pyridine was added to the dried sample, followed by vortex mixing for 5 min and incubation at 40°C for 90 min.

Finally, 25 μL BSTFA with 1 % TMCS was added, followed by vortex mixing for 5 min and incubation for 60 min at 70 °C.

GC–MS method

The analytical method was a modified version of the method published by Sieber [21]. Samples were analyzed by GC–MS with a QP2010 gas chromatograph–mass spectrometer (Shimadzu, Japan) equipped with a Agilent DB5-MS column (30 m $\times$ 0.25 mm $\times$ 0.1 μm film). Helium was used as carrier gas at a flow rate of 1 mL min^{−1}. Samples (1 μL) were injected in split mode (1:10); the injector temperature was 280 °C. The oven temperature was increased from 60 to 85 °C at 3 ° min^{−1}, then from 85 to 280 °C at 10 ° min^{−1}, and at 280 °C for 8 min. The total run time was 35.8 min. The ion-source temperature and interface temperature were 230 and 250 °C, respectively. The detector operated in scan mode from m/z 60 to 750.

Data analysis

Initial pretreatment of the raw chromatographic data included baseline correction, chromatogram alignment, noise reduction, and normalization by use of GCMSsolution software. The metabolite peaks were then tentatively identified from their retention characteristics and mass fragmentation patterns, by use of the NIST mass spectrum database. The metabolite peaks were tentatively quantified on the basis of the corresponding peak intensity.

Data analysis including principal-components analysis (PCA), analysis of variance (ANOVA), and multivariate analysis of variance (MANOVA) were performed in a Matlab environment.

Results

Anaphylactic symptoms emerging in sensitized animals challenged by ovalbumin or cattle albumin

After challenge with ovalbumin or cattle albumin, the two model groups expressed typical clinical symptoms of anaphylaxis, for example sneezing, rubbing, and death, whereas

the control group had no active systemic anaphylaxis symptoms (ASA) (Table 1). Because three guinea pigs died in each model groups after challenge, only 30 serum samples were obtained for metabolomic analysis.

IgE levels markedly increased in animals after challenging

Levels of IgE in the serum of challenged guinea pigs were measured by ELISA. IgE was detected in 23 of the 30 samples (the other seven samples appeared hemolytic and were removed from the test). After challenge, IgE levels in the serum of animals in the model groups had increased approximately fourfold (Table 2).

Metabolomic analysis revealed that nine variables were different between control and model groups

In the 30 samples, 858 metabolomic features in total were detected by GC–MS and were used to form a matrix of size 30 (samples) $\times$ 858 (variables). No significant difference was observed among the three groups when 858 variables were used in MANOVA analysis or PCA analysis. However, when each variable was considered alone in turn by ANOVA, comparing either of the model groups with the control group, significant differences of both comparisons were observed. Twenty-two variables were found significantly different at $p<0.05$ between the cattle albumin group and the control group (Table 3) and 19 variables differed sharply between the ovalbumin and control groups (Table 4). Among these, nine variables significantly changed in both model groups compared with the control group. By using these nine variables in a new MANOVA analysis, significant differences appeared between control group and model groups (Fig. 1a). The clustering between the control group and the model groups also became clear in PCA when the matrix was reduced to 30 (samples) $\times$ 9 (variables) with only the selected nine variables kept and the other 849 variables removed (Fig. 1b).

Function analysis suggested that anaphylaxis might be linked to energy metabolism and inositol signal transduction

Information about the biological function and pathways of the selected metabolites was obtained from the Kyoto

Table 1 Results from the active systemic anaphylaxis (ASA) test

–, no symptom; $\pm$, nose rubbing and/or fur erection; +, weakness, dyspnea, coughing, sneezing, vomiting and/or wheezing; ++, convulsion, cyanosis, and/or collapse; +++, death

Group	Animal no.											
	1	2	3	4	5	6	7	8	9	10	11	12
Control	–	–	–	–	–	–	–	–	–	–	–	–
Cattle albumin-induced	++	++	+	++	+++	+++	++	++	++	+	+++	++
Ovalbumin-induced	++	+	+++	++	++	+++	+++	++	++	++	++	++

Table 2 IgE levels in serum after challenge

Group	Amount of sample	IgE level
Control	8	0.06±0.02
Ovalbumin	8	0.31±0.08*
Cattle albumin	7	0.28±0.04*

IgE levels are given as mean value ±SEM

* $p < 0.05$ compared with control

Encyclopedia of Genes and Genomes (KEGG) database. As shown in Table 5, among the nine variables significantly changed in both model groups, six were tentatively identified as ionic species, namely glucose, fumaric acid, L-arabitol, *n*-pentadecanoic acid, (*R*)-3-hydroxybutyric acid, and *myo*-inositol. Among these, amounts of glucose, fumaric acid, and L-arabitol had decreased and those of the other three metabolites had increased in the model groups. This result implied that anaphylaxis might be related to glucose metabolism, lipid metabolism, and inositol phosphate metabolism.

Table 3 Variables responsible for the classification of the cattle albumin group and the control group

Retention time (min)	<i>m/z</i> ^a	<i>p</i> value
11.573	73, 116, 147, 190	0.005
12.095	73, 115, 133, 147, 245	0.028
12.200	73, 131, 147, 174, 205	0.007
12.856	73, 117, 147, 191, 233	0.004
13.267	73, 115, 147, 184, 258	0.03
14.392	66, 73, 103, 117, 147	0.024
14.788	103, 147, 205, 218, 319	0.005
15.183	73, 117, 130, 147, 219	0.048
15.362	63, 77, 124, 155, 247	0.004
15.558	71, 85, 99, 115, 141	0.032
18.204	73, 133, 147, 189, 233	0.046
20.374	73, 103, 147, 217, 307	0.002
20.954	73, 179, 192, 209, 281	0.048
21.007	73, 103, 147, 205, 217	0.017
21.257	73, 117, 129, 147, 160	0.016
21.750	73, 103, 129, 147, 191	0.01
23.665	73, 147, 205, 307, 319	0.000
23.875	73, 117, 129, 145, 299	0.013
25.289	73, 147, 217, 305, 318	0.036
25.666	73, 103, 147, 205, 319	0.000
27.150	73, 315, 330	0.004
29.041	70, 98, 124, 138, 153	0.037

^aTypical ion fragmentation for the variables

p values were calculated on the basis of ANOVA (significance at $p < 0.05$)

Table 4 Variables significantly different between the ovalbumin group and the control group

Retention time (min)	<i>m/z</i> ^a	<i>p</i> value
9.820	69, 73, 147, 159	0.025
10.775	66, 73, 117, 147, 191	0.017
12.095	73, 115, 133, 147, 245	0.044
12.856	73, 117, 147, 191, 233	0.015
14.788	103, 147, 205, 218, 319	0.013
15.183	73, 117, 130, 147, 219	0.045
15.362	63, 77, 124, 155, 247	0.038
15.558	71, 85, 99, 115, 141	0.037
16.015	73, 117, 147, 203, 220	0.045
17.651	70, 85, 113, 141, 169	0.015
18.204	73, 133, 147, 233, 245	0.032
21.007	73, 103, 147, 205, 217	0.023
22.600	147, 191, 205, 217, 319	0.030
23.875	73, 117, 129, 145, 299	0.001
24.336	73, 129, 191, 217, 265	0.003
25.289	73, 147, 217, 305, 318	0.048
27.832	73, 129, 143, 173, 225	0.007
27.989	67, 73, 91, 106, 129	0.003
28.903	63, 75, 103, 195, 242	0.028

^aTypical ion fragmentation for the variables

p values were calculated on the basis of ANOVA (significance at $p < 0.05$)

Discussion

In this study, anaphylaxis models were successfully established in guinea pigs, which expressed typical clinical symptoms of anaphylaxis and high IgE level in serum after challenge with allergen on the 14th day of sensitization. Serum metabolic profiling was performed by GC–MS analysis. The GC–MS method was validated by measuring repeatability, and good repeatability (RSD < 10 %) was observed for six tested metabolites tentatively identified in serum samples. From metabolic profiling, metabolic differences between the control group and the model groups were characterized by multivariate pattern-recognition. Variables with significant differences between the control group and the model groups were observed (Tables 3 and 4). The metabolites predominantly responsible for the differences ($p < 0.05$) might range from amino acids, simple hydroxyacids, and fatty acids to sugars, sugar alcohols, and polyols. In addition, metabolic change of these metabolites can be putatively interpreted in a functional way.

Glucose is a major source of energy for living organisms. Fumaric acid is an intermediate in the tricarboxylic acid cycle (TCA). The lower levels of both in the model groups might be a result of high energy expenditure in the anaphylaxis reaction, which indicates energy metabolism is a

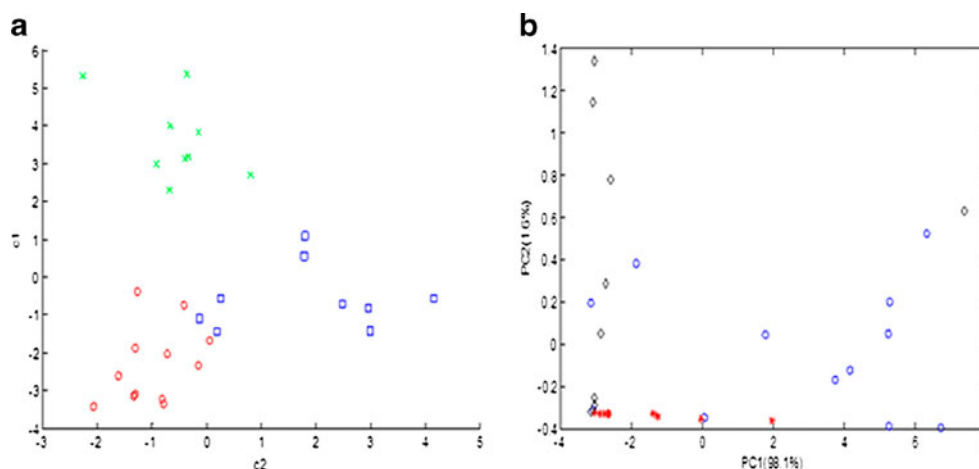


Fig. 1 Loading plot of the three groups by MANOVA based on $p < 0.01$ (circles, control; crosses, cattle albumin group; squares, ovalbumin group). (a) Scattering plot of the three groups by MANOVA based on nine selected

metabolite peaks. (b) PCA score plot of the three groups based on nine selected metabolite peaks. The three groups were obviously separated by MANOVA and PCA when only nine selected variables were used

significant aspect of anaphylaxis. As we observed, the anaphylaxis occurring in the model groups is IgE-dependent; IgE may induce smooth-muscle contraction and increase vascular contractility [22]. Anaphylaxis is an acute multisystem syndrome. It may affect multiple systems and organs including the respiratory system, cardiovascular system, and smooth muscle [22]. These reactions occurring during anaphylaxis lead to sudden energy expenditure. As a result, there might be a sharp increase of glucose consumption [23] with acceleration of energy metabolism and increased consumption of intermediate metabolites.

In contrast, higher levels of *n*-pentadecanoic acid and (*R*)-3-hydroxybutyric acid were observed in the model groups. Usually, when blood glucose and adenosine triphosphate (ATP) are low, the organism will burn fat to produce energy. As a result, the fat was degraded to free fatty acids, for example *n*-pentadecanoic acid. The fatty acids are then oxidized by the β -oxidation pathway to form acetyl CoA, which leads to the formation of ketone bodies [24]. (*R*)-3-Hydroxybutyric acid (beta-hydroxybutyrate) is one of these ketone bodies, and is an important source of energy for tissues such as the brain, heart, and skeletal muscle [24, 25].

Table 5 Tentatively identified metabolites related to anaphylaxis

Metabolite	Change, as multiple		Pathway	Key enzyme
	Cattle-albumin/ control	Ovalbumin/ control		
(<i>R</i>)-3-Hydroxybutyric acid	1.74	2.10	ko00072 Synthesis and degradation of ketone bodies	3-Hydroxybutyrate dehydrogenase [EC:1.1.1.30]
Fumaric acid	-2.78	-4.17	ko00020 Citrate cycle (TCA cycle)	Succinate dehydrogenase flavoprotein subunit [EC:1.3.99.1] succinate dehydrogenase (ubiquinone) flavoprotein subunit [EC:1.3.5.1]
Glucose	-3.70	-4.7	ko00010 Glycolysis/gluconeogenesis ko00030 Pentose phosphate pathway	Glucose dehydrogenase (acceptor) [EC:1.1.99.10]
<i>myo</i> -Inositol	1.55	1.58	ko00562 Inositol phosphate metabolism ko04070 Phosphatidylinositol signaling system	<i>myo</i> -Inositol-1(or 4)-monophosphatase [EC:3.1.3.25]
L-Arabitol	-1.67	-1.75	ko00040 Pentose and glucuronate interconversions	Aldehyde reductase [EC:1.1.1.21]
<i>n</i> -Pentadecanoic acid	7.51	10.06	ko00071 Fatty acid metabolism	Long-chain acyl-CoA synthetase [EC:6.2.1.3] lipases

Metabolites were identified by use of NIST commercial compound libraries

“–” means reduced level

The corresponding pathway and enzyme possibly involved in anaphylaxis were obtained from KEGG

A rapid decrease of glucose and fumaric acid with release of a large amount of *n*-pentadecanoic acid and synthesis of a large amount of (*R*)-3-hydroxybutyric acid are suggestive of depletion of cellular energy stores in anaphylaxis. This could partly explain why irreversible anaphylaxis shock and death developed so rapidly in model groups after challenge.

myo-Inositol is a carbocyclic polyol which is the structural basis for several secondary messengers. Phosphorylated *myo*-inositol derivatives have essential second messenger functions in many immunocytes. Antigen/IgE-mediated mast cell activation can be markedly enhanced by generation of inositol 1,4,5-trisphosphate (IP₃), which also has an important function in T cell activation [26, 27]. SHIP-1 (Src homology region 2 domain-containing inositol 5'-phosphatase) negatively regulates cytokine immunoreceptors and amplifies signaling pathways in different cell types, including T cells, B cells, mast cells, basophils, and neutrophils [28, 29]. It has been reported recently that SHIP-1 is critically involved in the adaptive Th2 immune response to aeroallergens [29]. The increased level of inositol detected in the model groups indicates inositol is involved in signal transduction during the allergic response. The last tentatively identified metabolite, L-arabitol, is a sugar alcohol metabolite involved in the pentose and glucuronate interconversions pathway. The detailed mechanism of involvement of L-arabitol in anaphylaxis needs further study.

In summary, this is the first report of comprehensive metabolic profiling analysis in anaphylaxis. Samples from a control group and model groups were successfully classified by GC–MS-based metabolomics analysis, and nine variables accounting for the classification were observed. Among the nine variables, glucose, fumaric acid, L-arabitol, (*R*)-3-hydroxybutyric acid, and *myo*-inositol closely associate anaphylactic reaction with the mechanisms of energy metabolism and signal transduction. These results suggest that GC–MS-based metabolic profiling analysis might be an effective tool for investigating metabolic regulation of anaphylaxis, and anaphylaxis could be diagnosed at the level of a small molecule in serum.

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Conflict of interest The authors declare that there are no conflicts of interest

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