



Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma



Integrative analysis of proteomics and metabolomics of anaphylactoid reaction induced by Xuesaitong injection

Yubin Xu, Deqiang Dou^{*}, Xiaoku Ran, Chunyan Liu, Jing Chen

College of Pharmacy, Liaoning University of Traditional Chinese Medicine, 77 Life One Road, DD port, Dalian 116600, China

ARTICLE INFO

Article history:

Received 20 June 2015

Received in revised form 7 August 2015

Accepted 7 September 2015

Available online xxx

Keywords:

Anaphylactoid reaction

Proteomics

Metabolomics

Mechanism

ABSTRACT

Injection with natural compounds is an important method in the application of natural medicine, but its adverse drug reactions (ADRs) occur frequently, particularly the anaphylactoid reaction, which accounts for more than 77% of all reactions and has become a serious threat to public health. Here, the Xuesaitong injection (XSTI) was employed as an example to elucidate its anaphylactoid mechanism and look for potential biomarkers to assay the anaphylactoid reaction of herbal medicine injection by proteomics and metabolomics. These results disclosed that 13 differential proteins and 28 metabolites, which were further approved using the ELISA method and reference standards, respectively, were suggested as potential biomarkers to examine the anaphylactoid mechanism. The up-regulated expression of Gpx1, Sc5b9, C4d and down-regulated expression of F12, Kng1, C2 and C6 revealed that the XSTI-induced anaphylactoid reaction occurs via direct stimulation, complement and the kallikrein–kinin pathway. In addition, substances that induce an anaphylactoid effect include histamine, LTB4, uric acid and other drugs, which have been confirmed to be involved in arginine and proline metabolism, histidine metabolism, arachidonic acid metabolism purine metabolism and the TCA cycle. Furthermore, separation experiments have indicated that 10-kDa molecules of XSTI are the main allergenic factor inducing an anaphylactoid reaction.

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1. Introduction

Injections of natural compounds with the superiority of rapid action and high bioavailability are an important method in the application of natural medicine, such as vitamin and ginkgo leaf injections [1–3]. However, epidemic exploration has indicated that their adverse drug reactions (ADRs) occur frequently, particularly the anaphylactoid reaction, which accounts for over 77% of the events [4]. An anaphylactoid reaction, which is also known as a pseudo-allergic or idiosyncratic reaction, whose symptoms resemble generalized anaphylaxis and conform in Coombs and Gell's Type I category, are not truly initiated or mediated by pre-existing IgE antibodies and occur after the first exposure to antigen [3–5]. Current research studies have predominantly concentrated on in vitro studies, animal behavioral symptoms or histamine, which has been used to evaluate the anaphylactoid reaction. Moreover, only injection additives of injections have been engaged [6–8]. The anaphylactoid reaction is mainly related to proteins and small molecules; thus, proteomics and metabolomics are important methods used to elucidate the anaphylactoid mechanism. In

addition, due to the rare research studies performed in the anaphylactoid reaction induced by herbal medicine injections, it is imperative to identify potential anaphylactoid biomarkers to systematically indicate the occurrence of the mechanism.

Xuesaitong injection (XSTI) is one of the bestselling prescriptions of herbal medicine and consists of over 85% ginsenosides attributed to an extract obtained from *Panax notoginseng* (Burk.) F. H. Chen (Chinese Sanqi) [9]. In China, XSTI has good therapeutic effects on the cardiovascular and cerebrovascular system, blood system and nervous system and it is extensively used in the treatment of cardio-cerebrovascular diseases, such as myocardial infarction, cerebral infarction, thrombosis and coronary heart disease [10–14]. Recently, reports on its ADRs have increased [2]; thus, XSTI was selected as an example to study the anaphylactoid reaction of herbal medicine. In addition, a brown norway (BN) rat animal model has been previously established for the anaphylactoid reaction [15]. And the anaphylactoid constituents of XSTI was elucidated by a RBL-2H3 cell model in previous study [2], but the mechanism and biomarkers for the anaphylactoid reaction induced by XSTI in vivo remains unclear due to the outstanding characteristic of the complicated ingredients.

In a continuing study, this is the first study to explore the mechanism and search for biomarkers of anaphylactoid reaction induced by XSTI via proteomics and metabolomics, and the objective of the

^{*} Corresponding author.

E-mail address: deqiangdou@126.com (D. Dou).

work presented here was to address the following problems: (1) identification of differential proteins and small molecules of blood related to the anaphylactoid reaction induced by XSTI; and (2) the mechanism underlying the anaphylactoid reaction induced by XSTI. In addition, this study is more systematic compared to previous studies [4,16], and the method also establishes for the first time a way to explore the mechanism of anaphylactoid reaction induced by XSTI.

2. Experimental procedures

2.1. Reagents and materials

HPLC-grade methanol, acetonitrile and formic acid were purchased from Merck (Merck, Germany). The assay kits for histamine were purchased from Usen Life Science Inc. (Wuhan, China). These assay kits for immunoglobulin E (IgE), glutathione peroxidase 1 (Gpx1), coagulation factor 13 (F13), kininogen-1 (Kng1), complement factor Bb (Bb), complement factor C4d (C4d) and terminal complement complex (Sc5b9) were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). XSTI was purchased from Harbin Zhenbao Pharmaceutical Co. (Harbin, China), and the following compounds were obtained from Sigma–Aldrich (St. Louis, MO, USA): leucine-enkephalin, histamine, adenosine, adenine, uric acid, glutathione, leukotriene B4 (LTB4), citric acid, hippuric acid, valine, alanine, arginine, tryptophan, serine, aspartic acid, arachidonic acid, α -ketoglutaric acid and octadecanoic acid. Ultrapure water was prepared using a Milli-Q water purification system (Millipore, France).

2.2. Animal experiments and sample collection

Male BN rats, 200 ± 20 g in weight, were purchased from WeiTongLiHua Co. (Beijing, China), laboratory animal license, SCXK (Army): 2012-0001. The animals were maintained under SPF laboratory conditions (Centre for Animal Experiment of Liaoning University of Traditional Chinese Medicine, Shenyang, China) and provided with a standard laboratory diet and filtered tap water ad libitum. All animal procedures were approved by the Liaoning Provincial Animal Welfare and Care Guidelines and were performed according to the National Institute of Health's guidelines with regard to the principles of animal care (2004). The experimental animals were housed under the above conditions for a 1-week acclimation period.

According to the results of the preliminary experimental studies, BN rats were randomly divided into 4 groups, with 8 rats in each group: control groups (Con.1 and Con.2, saline), XSTI groups (XSTI.1 and XSTI.2, 3 mL/kg), Con.2 and XSTI.2 were biological replicates. Subsequently, the test substances were administered intravenously into BN rats and the injection was completed in 20 s. The anaphylactoid symptoms of BN rats were observed 30 min after the injection. Symptoms were evaluated using a scoring system, which has been previously described by Li [17]. The immune toxicity of traditional Chinese medicine, natural medicine (allergic, light allergic reaction) technology guidelines for research were scored as the following: (1) 0: no abnormal reaction, (2) 1: trembling, scratching and rubbing around the nose, (3) 2: sneezing, coughing, puffiness around the eyes and the mouth, shortness of breath, (4) 3: dyspnea, wheezing, unsteady gait, cyanosis around the mouth and the tail, myasthenia of limbs, convulsions, spasm, rotation, tidal breathing and (5) 4: death.

Thirty minutes after injection, blood samples were obtained and collected into chilled tubes containing EDTA-K2 and then centrifuged at $3000 \times g$ (10 min, 4°C) to obtain plasma. Next, 200 μL plasma of each rat was moved to separate tubes, respectively, for

the analysis of metabolomics. Next, 1.5 mL plasma of each rat in the same group was collected in a separate tube for the analysis of proteomics, and all plasma was then stored at -80°C until further analysis.

2.3. Proteomics

2.3.1. Protein preparation

Plasma samples were ground into powder in liquid nitrogen, then 20 mg plasma samples were weighed and extracted with 200 μL lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 40 mM Tris–HCl, pH 8.5) containing 1 mM PMSF and 2 mM EDTA (final concentration) on the ice. After 5 min, 10 mM DTT (final concentration) was added to the samples. The suspension was sonicated at 200 W for 15 min and then centrifuged at 4°C , $30,000 \times g$ for 15 min. The supernatant was mixed well with 5 $\times$ volume of chilled acetone containing 10% (v/v) TCA and incubated at -20°C overnight. After centrifugation at 4°C , $30,000 \times g$, the supernatant was discarded. The precipitate was washed with chilled acetone three times. The pellet was air-dried and dissolved in lysis buffer (7 M urea, 2 M thiourea, 4% NP40, 20 mM Tris–HCl, pH 8.0–8.5). The suspension was sonicated at 200 W for 15 min and centrifuged at 4°C , $30,000 \times g$ for 15 min. The supernatant was transferred into another tube. To reduce disulfide bonds in proteins of the supernatant, 10 mM DTT (final concentration) was added and incubated at 56°C for 1 h. Subsequently, 55 mM IAM (final concentration) was added to block the cysteines and incubated for 1 h in the darkroom. The supernatant was mixed well with 5 $\times$ volume of chilled acetone for 2 h at -20°C to precipitate proteins. After centrifugation at 4°C , $30,000 \times g$, the supernatant was discarded, and the pellet was air-dried for 5 min, dissolved in 500 μL 0.5 M TEAB (Applied Biosystems, Milan, Italy) and sonicated at 200 W for 15 min. Finally, the samples were centrifuged at 4°C , $30,000 \times g$ for 15 min. The supernatant was transferred into a new tube and quantified, using the Bradford method. Proteins in the supernatant were stored at -80°C until further analysis.

2.3.2. iTRAQ labeling and SCX fractionation

Total protein (100 μg) was obtained from each sample solution and the protein was then digested with Trypsin Gold (Promega, Madison, WI, USA) at a ratio of protein: trypsin = 30:1 at 37°C for 16 h. After trypsin digestion, peptides were dried by vacuum centrifugation. Peptides were reconstituted in 0.5 M TEAB and processed according to the manufacturer's protocol for the 8-plex iTRAQ reagent (Applied Biosystems). Briefly, one unit of iTRAQ reagent was thawed and reconstituted in 24- μL isopropanol. Samples were labeled with iTRAQ tags as follows: Sample Con.1 (113 tag), Sample Con.2 (115 tag), Sample XSTI.1 (116 tag) and Sample XSTI.2 (118 tag). The peptides were labeled with isobaric tags and incubated at room temperature for 2 h. The labeled peptide mixtures were then pooled and dried by vacuum centrifugation.

SCX chromatography was performed using a LC-20AB HPLC Pump system (Shimadzu, Kyoto, Japan). The iTRAQ-labeled peptide mixtures were reconstituted with 4 mL buffer A (25 mM NaH_2PO_4 in 25% ACN, pH 2.7) and loaded onto a 4.6 mm $\times$ 250 mm Ultramex SCX column containing 5- μm particles (Phenomenex). The peptides were eluted at a flow rate of 1 mL/min with a gradient of buffer A for 10 min, 5–60% buffer B (25 mM NaH_2PO_4 , 1 M KCl in 25% ACN, pH 2.7) for 27 min, 60–100% buffer B for 1 min. The system was then maintained at 100% buffer B for 1 min before equilibrating with buffer A for 10 min prior to the next injection. Elution was monitored by measuring the absorbance at 214 nm, and fractions were collected every 1 min. The eluted peptides were pooled into 20 fractions, desalted with a Strata X C18 column (Phenomenex) and vacuum-dried.

2.3.3. LC-ESI-MS/MS analysis based on Triple TOF 5600

Each fraction was resuspended in buffer A (5% ACN, 0.1% FA) and centrifuged at $20,000 \times g$ for 10 min. The final concentration of peptide was approximately $0.5 \mu\text{g}/\mu\text{L}$ on average. Next, $10 \mu\text{L}$ supernatant was loaded onto a LC-20AD nanoHPLC (Shimadzu, Kyoto, Japan) using an autosampler onto a 2-cm C18 trap column. Next, the peptides were eluted onto a 10-cm analytical C18 column (inner diameter $75 \mu\text{m}$) packed in-house. The samples were loaded at $8 \mu\text{L}/\text{min}$ for 4 min, and the 35-min gradient was run at $300 \text{ nL}/\text{min}$ starting from 2 to 35% B (95% ACN, 0.1% FA), followed by a 5-min linear gradient to 60%, followed by a 2-min linear gradient to 80%, maintained at 80% B for 4 min and, finally, returned to 5% in 1 min.

Data acquisition was performed using a TripleTOF 5600 System (AB SCIEX, Concord, ON) equipped with a Nanospray III source (AB SCIEX, Concord, ON) and a pulled quartz tip as the emitter (New Objectives, Woburn, MA). Data were acquired using an ion spray voltage of 2.5 kV, curtain gas of 30 psi, nebulizer gas of 15 psi, and an interface heater temperature of 150°C . The MS was operated with a RP greater than or equal to 30,000 FWHM for TOF MS scans. For IDA, survey scans were acquired in 250 ms and as many as 30 product ion scans were collected if a threshold of 120 counts per second (counts/s) and with a 2+ to 5+ charge-state was exceeded. The total cycle time was fixed to 3.3 s. The Q2 transmission window was 100 Da for 100%. Four time bins were summed for each scan at a pulsar frequency value of 11 kHz by monitoring via a 40-GHz multichannel TDC detector with four-anode channel detect ion. A sweeping collision energy setting of $35 \pm 5 \text{ eV}$ coupled with iTRAQ adjust rolling collision energy was applied to all precursor ions for collision-induced dissociation. Dynamic exclusion was set for 1/2 of peak width (15 s), and the precursor was then refreshed off the exclusion list.

2.3.4. Data analysis

Raw data files acquired from the Orbitrap were converted into MGF files using Proteome Discoverer 1.2 (PD 1.2, Thermo) [5600 ms converter] and the MGF file was searched. Proteins identification was performed using Mascot search engine (Matrix Science, London, UK; version 2.3.02) against Uniprot database containing 34,908 sequences.

For protein identification, a mass tolerance of 0.05 Da was permitted for intact peptide masses and 0.1 Da for fragmented ions, with an allowance for one missed cleavage in the trypsin digests: Gln→pyro-Glu (N-term Q), oxidation (M), deamidated (NQ) as the potential variable modifications and carbamidomethyl (C), iTRAQ8plex (N-term), iTRAQ8plex (K) as fixed modifications. The charge states of the peptides were set to +2 and +3. Specifically, an automatic decoy database search was performed in Mascot by selecting the decoy checkbox in which a random sequence of database was generated and tested for raw spectra as well as the real database. To reduce the probability of false peptide identification, only peptides at the 95% confidence interval by Mascot probability analysis greater than “identity” were considered as identified. Each confident protein identification involves at least one unique peptide.

For protein quantitation, it was required that a protein contain at least two unique peptides. The quantitative protein ratios were weighed and normalized by the median ratio in Mascot. Ratios with P -values <0.05 and fold changes of >1.2 were considered as significant.

Functional annotations of the proteins were performed using the Blast2GO program against the non-redundant protein database (NR; NCBI). The KEGG database (<http://www.genome.jp/kegg/>) and COG database (<http://www.ncbi.nlm.nih.gov/COG/>) were used to classify and group these identified proteins.

Table 1
MS condition.

MS condition	ESI(+)(T3)	ESI(-)(T3)	ESI(+)(amide)
Capillary voltage	3.0 kV	2.5 kV	3.0 kV
Sampling cone	20.0 kV	35.0 kV	20.0 kV
Extraction cone	3.0 kV	4.0 kV	3.0 kV
Source temp.	110°C	110°C	110°C
Desolvation temp.	450°C	450°C	450°C
Cone gas	20.0 L/h	20.0 L/h	20.0 L/h
Desolvation gas	800.0 L/h	800.0 L/h	800.0 L/h
Collision energy	6.0 V	6.0 V	6.0 V
Mass range	50–1200 Da	50–1200 Da	50–1200 Da
Scan rate	0.2 s	0.2 s	0.2 s
Data collection	MS ^E centroid with lock mass applied	MS ^E centroid with lock mass applied	MS ^E centroid with lock mass applied
LockSpray™	556.2771 m/z	554.2615 m/z	556.2771 m/z

2.4. Metabolomics

2.4.1. Sample preparation and UPLC/Q TOF-MS analysis

Plasma ($200 \mu\text{L}$) was added into acetonitrile ($500 \mu\text{L}$), vortex-mixed for 30 s and centrifuged at $11,600 \times g$ for 3 min to precipitate the proteins. Next, $500 \mu\text{L}$ of protein-free supernatant was collected and dried with vacuum drying centrifuged at room temperature. The dried residue was reconstituted in $130 \mu\text{L}$ of acetonitrile–water (1:1, v/v), after centrifugation for 3 min at $11,600 \times g$, an aliquot of $3 \mu\text{L}$ was injected for UPLC/Q-TOF MS analysis.

Chromatographic separation was performed on Acquity UPLC HSS T3 column ($2.1 \text{ mm} \times 100 \text{ mm}$, $1.7 \mu\text{m}$, Waters Corp., Milford, USA) and BEH Amide column ($2.1 \text{ mm} \times 100 \text{ mm}$, $1.7 \mu\text{m}$, Waters Corp., Milford, USA) using a Waters ACQUITY UPLC system, equipped with a binary solvent delivery system. The column was maintained at 45°C and eluted at a flow rate of $0.3 \text{ mL}/\text{min}$, using mobile phase of (A) 0.1% (by volume) formic acid in water and (B) methanol, mobile phase of (A) water and (B) acetonitrile separately. The gradient programs were optimized as follows: T3 column, 0–1 min, 0% B; 1–7 min, 0% B to 60% B; 7–10 min, 60% B to 100% B; 10–14 min, 100% B; 14–14.1 min, 100% B to 0% B; 14.1–16 min, 0% B and amide column, 0–0.5 min, 5% B; 0.5–6 min, 5% B to 30% B; 6–8 min, 30% B to 50% B; 8–10 min, 50% B; 10–10.1 min, 50% B to 5% B; 10.1–12 min, 5% B separately; curve: 6. The column eluent was directed to the mass spectrometer without split.

Mass spectrometry was performed on a Waters Xevo G2 QTOF quadrupole accelerated time-of-flight mass spectrometer (Waters Corp., Manchester, USA) with an electrospray ionization source (ESI) operating in positive and negative ion mode (T3 column), positive ion mode (amide column) separately. The MS condition was described as Table 1.

2.4.2. Data processing

All data analysis was controlled by MarkerLynx software.

2.5. ELISA assay

BN rats were randomly divided into the following 2 groups, with 10 rats for each group, i.e., control group and XSTI group. The experimental operation was the same with 2.2, and the plasma of each rat was collected into tubes, respectively, and stored at -80°C until further analysis. Next, histamine, IgE, Gpx1, F13, Kng1, Bb, C4d and Sc5b9 level were determined using ELISA kits following the manufacturer's instructions, respectively.

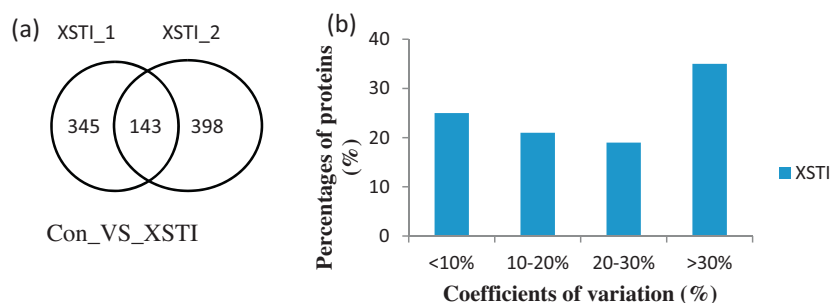


Fig. 1. Reproducibility of identified proteins.

Table 2
The symptom scores of BN rats.

Group	Dosage (mg/kg)	Judgment of the typical symptom score/count					Symptom scores
		0	1	2	3	4	
Control	–	10	0	0	0	0	0
XSTI	10	0	0	0	9	1	3.1 ± 0.32

2.6. Statistical analysis

All values were expressed as the mean ± S.D. The significance of differences between the mean of XSTI and control groups has been compared using the two-way ANOVA test with the Statistical Package for Social Science program (SPSS 20.0, Chicago, IL, USA). The significance threshold was set at $P < 0.05$ for this test.

3. Results and discussion

3.1. Animal behavioral study

Symptoms of the BN rats in the control group were regarded as normal. Symptoms of the BN rats in the XSTI group included sneeze, dyspnea, unsteady gait, myasthenia of limbs, convulsions, spasm and death. Scores for each group are shown in Table 2, and the results indicated that rats in the XSTI group exhibited a severe anaphylactoid reaction.

3.2. Proteomics study

Data obtained from Con.1, XSTI.1 and biological replicates (Con.2 and XSTI.2) were analyzed using Mascot. The total number of distinct peptides identified by Mascot in the run was 10,585, which yielded 1677 proteins (Supplemental Table S1).

Table 3
A list of proteins identified and quantified with iTRAQ technology.

N	%	Cov	Score	Peptide	Unique peptide	Uniprot accession #	Gene name	Protein name	Con-VS-XSTI	KEGG pathway
122.7	833	12	12			Q07968	F13b	Coagulation factor XIII B chain	1.09*	Complement and coagulation cascades
214.8	291	8	8			D3ZTE0	F12	Coagulation factor XII	0.709*	Complement and coagulation cascades
330.8	288	6	6			P35704	Prdx2	Peroxioredoxin-2	1.387*	–
411.6	172	5	5			Q66HG3	Cndp1	Beta-Ala-His dipeptidase	0.46*	Arginine and proline metabolism/histidine metabolism
557.4	165	7	7			P02608	MYLPF	Myosin regulatory light chain	2.446*	–
635.5	124	4	4			P81605	DCD	Dermcidin	0.706*	–
729.8	697	11	8			P08934	Kngr1	Kininogen-1	0.875*	Complement and coagulation cascades
863.2	611	10	10			P04041	Gpx1	Glutathione peroxidase 1	2.164*	Arachidonic acid metabolism
957.5	1445	25	23			P12928	Pklr	Pyruvate kinase isozymes R/L	2.15*	Purine metabolism
1020.2	821	16	16			Q811M5	C6	Complement component C6	0.781*	Complement and coagulation cascades
1116	517	10	5			P21180	C2	Complement C2	0.75*	Complement and coagulation cascades
1242	575	15	15			P09034	Ass1	Argininosuccinate synthase	5.804*	Arginine and proline metabolism
1317.6	122	8	8			P20673	Asl	Argininosuccinate lyase	1.769*	Arginine and proline metabolism

* Compared to control group, $P < 0.05$.

Only proteins present in Experiment 1 (Con.1 and XSTI.1 groups) and biological replicate Experiment 2 (Con.2 and XSTI.2 groups) with their ratios ($P < 0.05$) and fold change (> 1.2) were considered as differential proteins. Comparing the 345 proteins identified in Con.1.VS.XSTI.1 with the 398 proteins identified in the biological replicate (Con.2.VS.XSTI.2), there were 143 differential proteins identified in the XSTI group (Fig. 1a). In the biological replicate experiment, among the 143 differential proteins in the XSTI group, there were 25%, 21% and 19% of proteins that had CV values under 10%, 10–20% and 20–30%, respectively, and 35% of proteins exceeded 30% (Fig. 1b).

Among the 143 differential proteins, 13 differential proteins were further subjected to functional analysis with reference to the KEGG database (<http://www.genome.jp/kegg/>). Table 3 exhibited detailed information, including the UniProt accession number, description, gene name, ratio and the KEGG pathway. Intriguingly, 5 of the 13 proteins, including F13b, F12, Kngr1, C2 and C6 are involved in complement and coagulation cascade pathways. Furthermore, F13b, Prdx2, Gpx1, Pklr, Ass1 and Asl were up-regulated in the XSTI group; and F12, Cndp1, MYLPF, DCD, Kngr1, C2 and C6 were down-regulated in the XSTI group.

To confirm the results obtained from proteomics and to reveal the anaphylactoid action routes of XSTI, 7 additional proteins (F13, Kngr1, Gpx1, IgE, Sc5b9, C4d and Bb), which are regarded as key biomarkers of the anaphylactoid reaction, were selected and assayed using the ELISA method in further replicate experiments. Simultaneously, histamine was also measured to indicate the anaphylactoid degree of the rats. Student *t*-test was used to analyze the data and these results showed that the histamine levels were significantly increased in the XSTI group ($P < 0.01$) and 10-kDa group ($P < 0.05$) compared to the control group, indicating the occurrence of anaphylactoid reactions in BN rats. The result of proteomics showed that F12 and Kngr1 was down-regulated, but F13b levels were not regulated, and F13 levels showed no

Table 4

The histamine, IgE, F13, Kng1, Gpx1, Sc5b9, C4d and Bb levels of BN rats using ELISA assay.

Group	Dosage (mL/kg)	Histamine (ng/mL)	IgE (U/mL)	F13 (ng/mL)	Kng1 (ng/mL)	Gpx1 (ng/mL)	Sc5b9 (ng/mL)	C4d (ng/mL)	Bb (ng/mL)
Control	3.0	0.90 ± 0.23	2.70 ± 0.33	23.47 ± 1.88	92.15 ± 10.64	13.82 ± 1.14	118.12 ± 15.89	29.94 ± 4.01	81.09 ± 12.69
XSTI	3.0	3.67 ± 1.70**	2.38 ± 0.56	23.01 ± 5.11	67.69 ± 24.15**	15.52 ± 1.84*	135.57 ± 19.06	33.48 ± 3.39*	69.29 ± 15.7
10 kDa	3.0	3.64 ± 0.52**	2.29 ± 0.51	22.32 ± 3.6	96.53 ± 20.4	10.81 ± 2.04**	152.21 ± 25.64**	35.32 ± 3.14**	89.81 ± 23.21

* Compared to control group, $P < 0.05$ ** Compared to control group, $P < 0.01$.

changes in both groups compared to the control group as assessed using the Elisa method. In addition, Kng1 levels were significantly decreased in the XSTI group ($P < 0.01$), but it showed no obvious change in the 10-kDa group compared to the control

group, indicating that XSTI causes an anaphylactoid reaction via the kallikrein–kinin pathways but not the coagulation cascade pathway. Moreover, small molecules, such as ginsenosides, can decrease the levels of Kng1 [18,19]. Furthermore, Gpx1 levels were signifi-

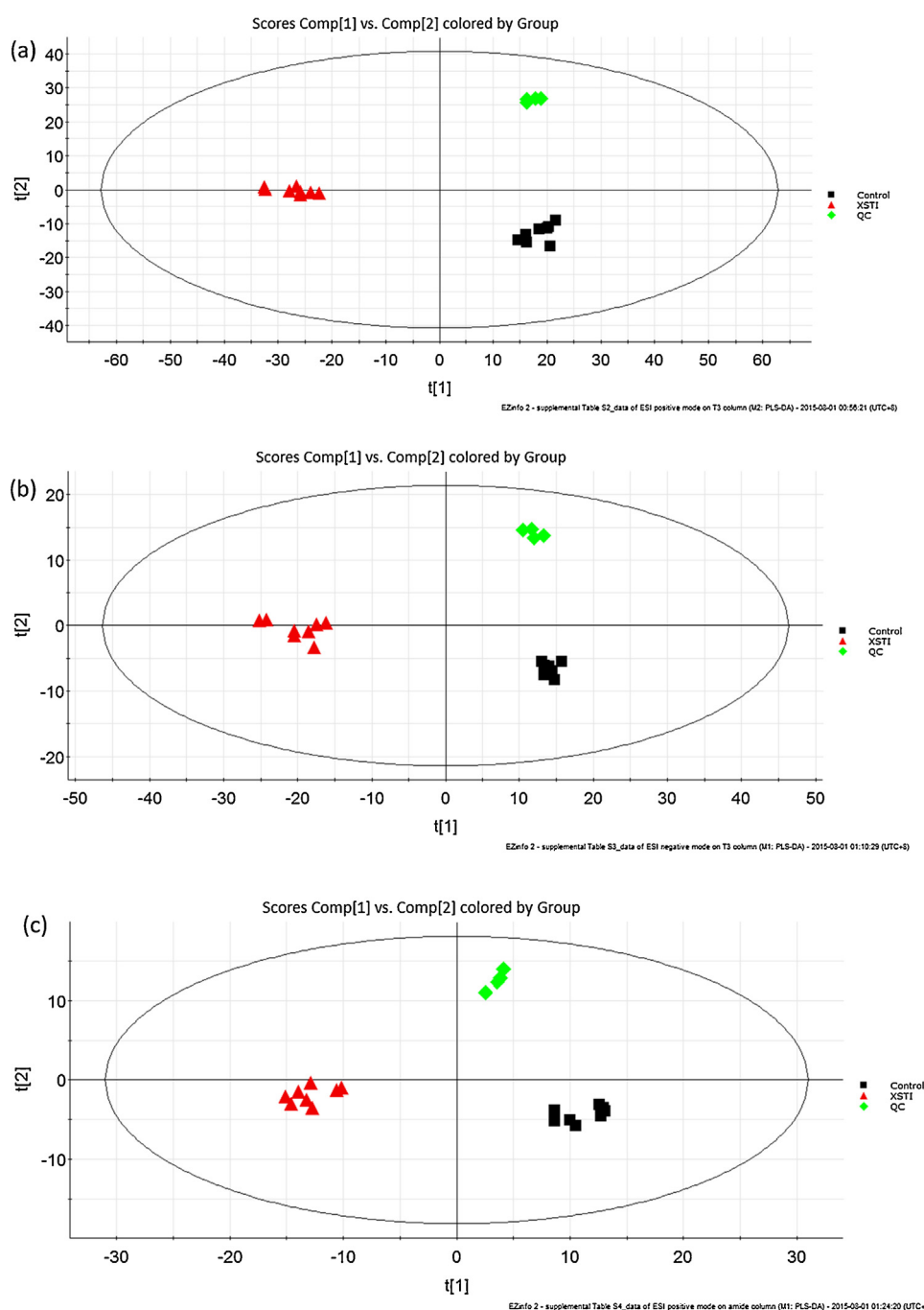
**Fig. 2.** PLS-DA scores plots of rat plasma data.

Table 5
Mass databases of 28 metabolites.

No.	R.T.(min)	m/z	Ion mode	Elemental composition	Identification results	Control group	XSTI group	P	XSTI group ^a	VIP
1	7.343	112.0872	[M+H] ⁺	C ₅ H ₉ N ₃	Histamine ^b	0.17 ± 0.03	1.56 ± 0.22	0.000	+ (↑)	2.0935
2	3.5848	268.1045	[M+H] ⁺	C ₁₀ H ₁₃ N ₅ O ₄	Adenosine ^b	0.21 ± 0.04	0.27 ± 0.05	0.021	+ (↑)	1.9651
3	3.523	136.0599	[M+H] ⁺	C ₅ H ₅ N ₅	Adenine ^b	0.23 ± 0.04	0.28 ± 0.06	0.045	+ (↑)	1.2350
4	1.1078	169.036	[M+H] ⁺	C ₅ H ₄ N ₄ O ₃	Uric acid ^b	2.02 ± 0.4	2.55 ± 0.5	0.034	+ (↑)	1.2073
5	1.0055	308.0926	[M+H] ⁺	C ₁₀ H ₁₇ N ₃ O ₆ S	Glutathione ^b	0.25 ± 0.05	0.57 ± 0.1	0.000	+ (↑)	1.2317
6	9.925	335.2219	[M–H] [–]	C ₂₀ H ₃₂ O ₄	Leukotriene B ₄ ^b	0.01 ± 0	2.42 ± 0.36	0.000	+ (↑)	1.2909
7	1.1128	191.0193	[M–H] [–]	C ₆ H ₈ O ₇	Citric acid ^b	9.42 ± 0.78	13.47 ± 2.68	0.003	+ (↑)	1.2210
8	5.1939	178.0505	[M–H] [–]	C ₉ H ₉ NO ₃	Hippuric acid ^b	15.57 ± 3.08	30.9 ± 5.92	0.000	+ (↑)	1.3151
9	4.1317	154.0977	[M+H] ⁺	C ₇ H ₁₁ N ₃ O	N-acetylhistamine	0.43 ± 0.06	0.9 ± 0.13	0	+ (↑)	1.9556
10	7.5913	241.13	[M+H] ⁺	C ₁₀ H ₁₆ N ₄ O ₃	N(alpha)-gamma-L-glutamylhistamine	0.58 ± 0.1	1.37 ± 0.27	0	+ (↑)	1.7754
11	5.3998	118.0866	[M+H] ⁺	C ₅ H ₁₁ NO ₂	Valine ^b	1.36 ± 0.15	1.67 ± 0.33	0.034	+ (↑)	1.2002
12	7.5961	170.0929	[M+H] ⁺	C ₇ H ₁₁ N ₃ O ₂	3-Methylhistidine	39.62 ± 5.33	48 ± 6.26	0.012	+ (↑)	1.5371
13	1.8079	191.0194	[M–H] [–]	C ₆ H ₈ O ₇	Isocitrate	0.77 ± 0.14	3.00 ± 0.20	0	+ (↑)	1.5427
14	1.8071	189.0005	[M–H] [–]	C ₆ H ₈ O ₇	Oxalosuccinate	0.11 ± 0.02	0.16 ± 0.03	0	+ (↑)	1.8125
15	1.094	115.0039	[M–H] [–]	C ₄ H ₄ O ₄	Fumarate	1.13 ± 0.19	2.74 ± 0.23	0	+ (↑)	1.3002
16	1.0841	133.0136	[M–H] [–]	C ₄ H ₆ O ₅	Malate	2.42 ± 0.47	4.76 ± 0.43	0	+ (↑)	1.4941
17	6.042	90.0554	[M+H] ⁺	C ₃ H ₇ NO ₂	Alanine ^b	5.79 ± 0.75	4.23 ± 0.63	0.000	+ (↓)	1.2847
18	7.6213	175.1194	[M+H] ⁺	C ₆ H ₁₄ N ₄ O ₂	Arginine ^b	115.01 ± 11.45	81.12 ± 5.16	0.000	+ (↓)	1.2663
19	4.6653	205.0975	[M+H] ⁺	C ₁₁ H ₁₂ N ₂ O ₂	Tryptophan ^b	37.61 ± 5.36	27.4 ± 6.78	0.005	+ (↓)	1.9346
20	6.7678	106.0502	[M+H] ⁺	C ₃ H ₇ NO ₃	Serine ^b	10.09 ± 0.9	7.19 ± 1.52	0.000	+ (↓)	1.74848
21	6.806	134.0452	[M+H] ⁺	C ₄ H ₇ NO ₄	Aspartic acid ^b	0.57 ± 0.13	0.21 ± 0.06	0.000	+ (↓)	1.4081
22	11.1785	303.2326	[M–H] [–]	C ₂₀ H ₃₂ O ₂	Arachidonic acid ^b	601.5 ± 34.76	448.77 ± 58.42	0.000	+ (↓)	1.0667
23	1.11	145.0142	[M–H] [–]	C ₅ H ₆ O ₅	α-Ketoglutaric acid ^b	0.59 ± 0.11	0.44 ± 0.04	0.003	+ (↓)	1.2784
24	11.6869	283.2637	[M–H] [–]	C ₁₈ H ₃₆ O ₂	Octadecanoic acid ^b	504.8 ± 15.45	317.64 ± 60.28	0.000	+ (↓)	1.5793
25	10.3807	317.2113	[M–H] [–]	C ₂₀ H ₃₀ O ₃	Leukotriene A ₄	12.6 ± 2.4	3.29 ± 0.35	0	+ (↓)	1.2995
26	9.8387	335.2217	[MH] [–]	C ₂₀ H ₃₂ O ₄	5-Hydroperoxyeicosatetraenoic acid	16.58 ± 2.47	3.4 ± 0.51	0	+ (↓)	1.3909
27	4.5779	188.0711	[M+H] ⁺	C ₁₁ H ₉ NO ₂	Indoleacrylic acid	82.03 ± 8.24	51.06 ± 10.08	0	+ (↓)	1.2123
28	6.8747	114.0552	[M+H] ⁺	C ₅ H ₇ NO ₂	1-Pyrroline-2-carboxylic acid	1.25 ± 0.2	0.74 ± 0.14	0	+ (↓)	1.2649

“↑” means a higher level of metabolites, whereas “↓” represents a lower level of metabolites. “+” means a statistically significant difference ($P < 0.05$), whereas “–” represents no statistically significant difference.

^a Compared to normal controls.

^b Confirmed with authentic standards.

cantly increased in the XSTI group ($P < 0.01$), but decreased in the 10-kDa group compared to the control group, indicating that XSTI can directly stimulate mast cells to release histamine, and the allergenic ingredients should be 10-kDa molecules, such as proteins. In

contrast, small molecules of XSTI may have an antagonistic effect due to Gpx1, which is involved in the regulation of prostaglandin synthesis and plays an important role in the anaphylactoid reaction [20]. Sc5b9 levels were up-regulated but had no statistical

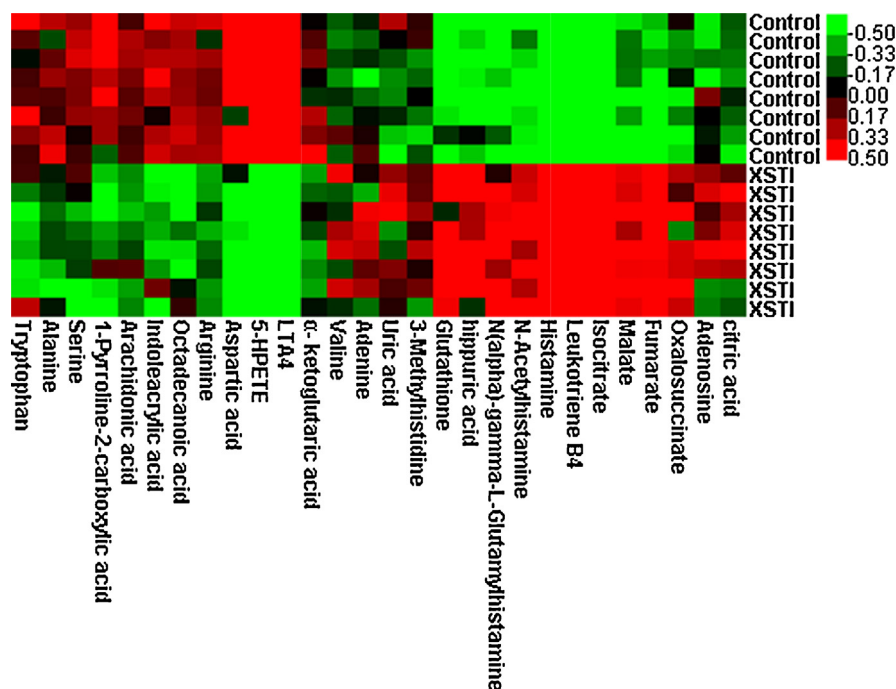


Fig. 3. Heatmap of the identified metabolites in the different groups.

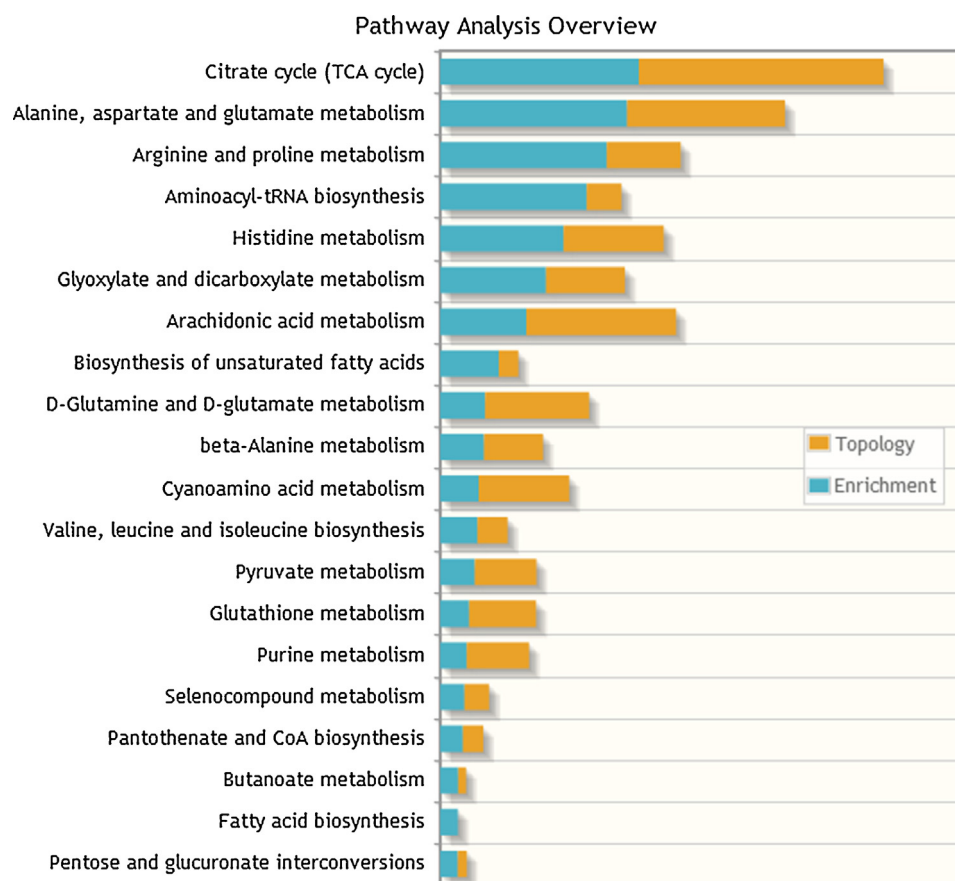


Fig. 4. The topology and enrichment of protein and metabolites data based on the pathway overrepresentation analysis.

significance in the XSTI group, and it was significantly increased in the 10-kDa group compared to the control group. In addition, C4d levels were significantly increased in the XSTI ($P < 0.05$) and 10-kDa groups ($P < 0.01$) compared to the control group, indicating that XSTI induced an anaphylactoid reaction via the classic complement pathway [21], and the main allergen are 10-kDa molecules. There were no significant differences of Bb and IgE levels in both groups compared with the control group (Table 4).

3.3. Metabolomics study

Reproducibility of the UPLC-Q-TOF-MS was determined from four replicated analyses of the same quality control sample (QC) interspersed throughout the analysis. The RSD of the peak area of all metabolites are below 20%, demonstrating good stability and reproducibility throughout the entire sequence of sample runs. Metabolic profiling was acquired in the positive and negative ion mode of different columns (Supplemental Figure S1). PLS-DA analysis was employed to determine the specific variation between control and XSTI groups. The QC samples clustered together tightly in the score plot of PLS-DA, which indicated that the system stability was accommodative for this metabolomics study (Fig. 2).

Variables (biomarkers) that significantly contributed to the clustering and discrimination were identified according to a threshold of variable importance in the projection (VIP) values ($VIP > 1$), which could be generated after PLS-DA processing using MarkerLynx software (Supplemental Tables S2–S4). The critical P -value was set to 0.05 for significantly differential variables in this study. Following the above criteria, 28 significantly differential biomarkers were identified on the basis of accurate elemental compositions and the context of retention time with the available database ([https://](https://metlin.scripps.edu)

metlin.scripps.edu) and selected for further study (Table 5). Due to the absence of fragmentation information of some biomarkers, as compensation, 17 of the markers were confirmed with available reference standards by matching their retention time and accurate mass measurement. To further understand the metabolic differences between different groups, a clustering heatmap was generated for all identified biomarkers, demonstrating the relative increase (red) or decrease (green) (Fig. 3).

Significantly increased histamine, adenosine, adenine, uric acid, glutathione, leukotriene B4 (LTB4), citric acid, hippuric acid, N-acetylhistamine, N(alpha)-gamma-L-glutamylhistamine, valine, 3-methylhistidine, isocitrate, oxalosuccinate, fumarate, malate and significantly decreased alanine, arginine, tryptophan, serine, aspartic acid, arachidonic acid (AA), α -ketoglutaric acid, octadecanoic acid, leukotriene A4 (LTA4), 5-hydroperoxyeicosatetraenoic acid (5-HPETE), indoleacrylic acid, 1-pyrroline-2-carboxylic acid were observed in the XSTI group compared with the control group (Table 5). These biomarkers were distributed in the related pathways of histidine metabolism, purine metabolism, energy metabolism, AA metabolism and amino acid metabolism.

Previous research studies have predominantly concentrated on behavioral symptoms or histamine levels of anaphylactoid reaction [22–24]. In the current study, it was found that purine and AA metabolism were also the most important metabolisms for the anaphylactoid reaction. Increased production of AA metabolites (LTA4, LTB4 and 5-HPETE) occurred at sites of inflammation and then contributed to inflammation by increasing blood flow, dilating vessels, increasing capillary permeability and pain sensitivity to other mediators, such as bradykinin and histamine [25,26]. In addition, purine metabolites (adenosine, adenine, uric acid) had been identified first as having a role in the development of the anaphylactoid

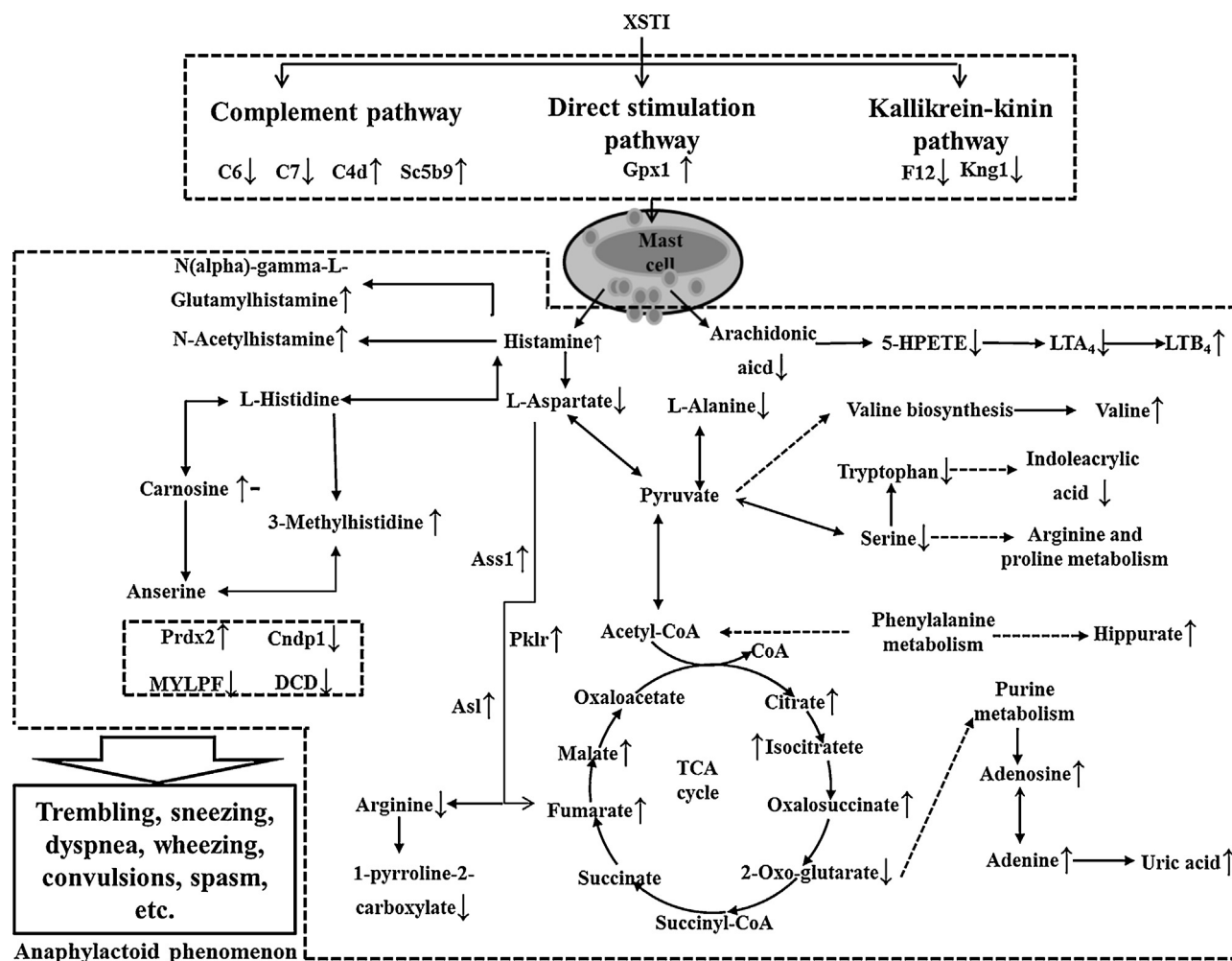


Fig. 5. The mechanism study of anaphylactoid reaction according to KEGG nomenclature using proteomics integrated with metabolomics.

reaction induced by XSTI. Previous reports have also indicated that uric acid is an inflammatory mediator of allergic asthma [27].

3.4. Integrating proteomics and metabolomics data

As discussed above, a number of proteins and metabolites were identified and validated as potential biomarkers. Moreover, for a more detailed pathway analysis, the integrated pathway enrichment was analyzed using MetaboAnalyst (<http://www.metaboanalyst.ca>), as shown in Fig. 4. These results indicated that the citrate cycle, arginine and proline metabolism, histidine metabolism and arachidonic acid metabolism play important roles in the anaphylactoid reaction induced by XSTI.

Current studies have concluded that compounds induce mast cells degranulation via several pathways, including direct stimulation, the complement pathway and other pathways [28,29]. We further analyzed the mechanism of anaphylactoid reaction induced by 10-kDa molecules of XSTI and XSTI to supplement the result of proteomics and metabolomics. The histamine, Sc5b9 and C4d of 10 kDa groups were regulated according to the XSTI group, and Gpx1 was down-regulated in the 10-kDa group, but up-regulated in the XSTI group. In addition, Kng1 was down-regulated in the XSTI group, indicating that XSTI induced mast cell degranulation to release the effect substances (such as histamine and purine metabolites) via direct stimulation and the complement and kallikrein–kinin pathways (Fig. 5). Gpx1, C4d and Kng1 could be used as biomarkers for the two pathways, respectively [21,30,31].

Furthermore, these results revealed that >10-kDa molecules are chiefly responsible for the anaphylactoid reaction of XSTI, and ginsenosides may antagonize anaphylactoid reactions. In addition, metabolomics have indicated that amino acids are substrates for metabolic energy to generate acyl-CoA derivatives, which is an important index in the TCA cycle.

4. Conclusion

We applied the proteomics and metabolomics approach to study the mechanism of the anaphylactoid reaction induced by XSTI, indicating that various proteins and metabolites are correlated with its anaphylactoid reaction. We concluded that the XSTI-induced anaphylactoid reaction can be caused via direct stimulation, and the complement and kallikrein–kinin pathways and the effect substances were confirmed to be involved in arginine and proline metabolism, histidine metabolism, arachidonic acid metabolism, purine metabolism and the TCA cycle. Furthermore, the separation experiment indicated that 10-kDa molecules of XSTI are the main allergenic ingredients inducing an anaphylactoid reaction.

Acknowledgments

These studies were supported by funding obtained from the Natural Science Foundation of China (81373939) and Innovation Team Project of Colleges and Universities in China (LT2013020).

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.chroma.2015.09.019>.

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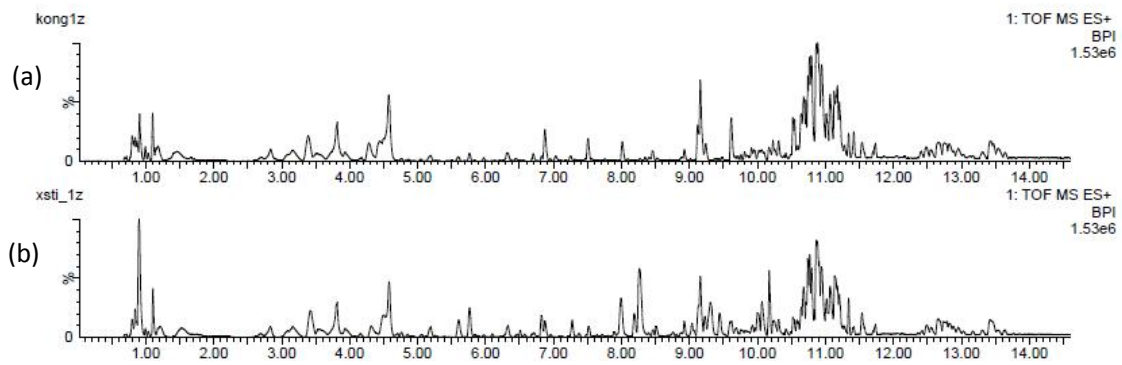


Figure 1S. Base peak chromatogram of plasma samples from three groups in ESI positive mode on T3 column. (a): control group; (b): XSTI group.

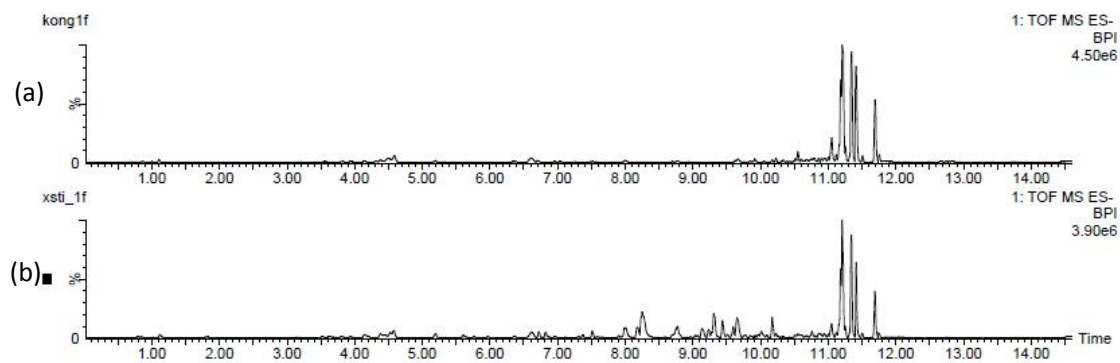


Figure 2S. Base peak chromatogram of plasma samples from three groups in ESI negative mode on T3 column. (a): control group; (b): XSTI group.

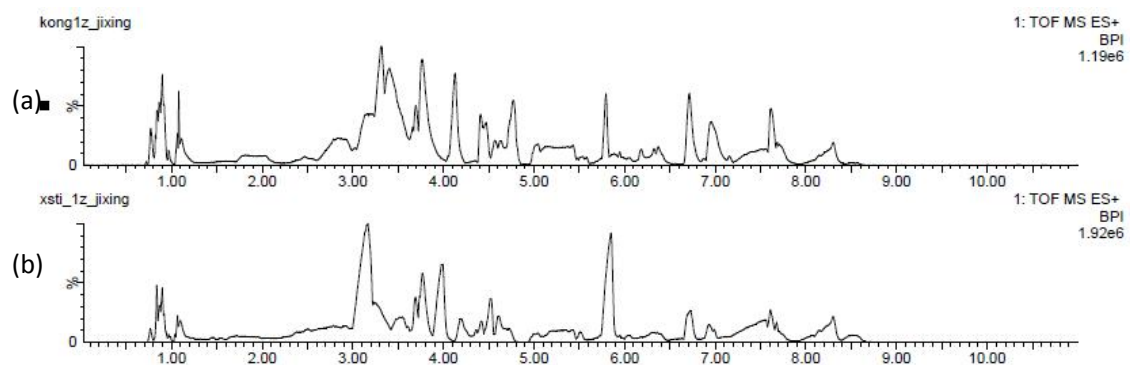


Figure 3S. Base peak chromatogram of plasma samples from three groups in ESI positive mode on amide column. (a): control group; (b): XSTI group.

In vitro study

1. Method

1.1 Sc5b9 determination

To study the Sc5b9 level influenced by the different incubation concentration of xuesaitong injection (XSTI), XSTI were incubated with the human serum at three ratios ($V_{\text{substance}}/V_{\text{serum}}=1:4, 1:2$ and $1:1$) at 37°C for 30min, then the Sc5b9 level was determined using ELISA kit following the manufacturer's instruction. [1]

1.2 Histamine determination

500 μL of RBL-2H3 cell suspension were plated at 1.0×10^5 cells/mL in 24-well plates, incubated for 48h. Then, the medium was removed and the wells were washed twice with PBS. The plates were divided into the total release group (TritonX-100, 0.3%, v/v), negative control group (PBS) and XSTI group, 500 μL of the corresponding solutions were added and incubated at 37°C for 1h. The plates were put into the ice-water bath (0°C) for 10min to stop reaction, and liquid was collected into 1.5ml eppendorf tube, centrifugated for 10min (2000 r/min, 4°C). The histamine level was determined using ELISA kit following the manufacturer's instruction. [2]

2. Results and discussion

The Sc5b9 level were significantly increased in the XSTI group ($V_{\text{substance}}/V_{\text{serum}}=1/2$ and $1/1$) ($P<0.01$), indicating the high dosage of XSTI can activate the complement pathway to induce anaphylactoid reactions in BN rats, this result was in accordance with the result of proteomics.

Table 1. The SC5b-9 level ($\mu\text{g/mL}$) influenced by XSTI ($\bar{x}\pm s$, $n=3$)

Group	Incubation Concentration ($V_{\text{substance}}/V_{\text{serum}}$)		
	1/4	1/2	1/1
PBS	7.75 ± 0.18	9.42 ± 0.08	11.78 ± 0.11
Xuesaitong injection	7.61 ± 0.27	$12.06\pm 0.10^{**}$	$14.51\pm 0.12^{**}$

Compared with PBS control, * $P<0.05$, ** $P<0.01$.

The histamine level were significantly increased in the XSTI group ($P<0.01$), indicating XSTI can induce anaphylactoid reactions in BN rats; this result was in

accordance with the result of metabolomics.

Table 2. The histamine level (ng/mL) influenced by XSTI ($\bar{x} \pm s$, n=3)

Substance	Vsubstance/VPBS	Histamin(ng/mL)
PBS	-	7.83±0.45
XSTI	1/120	10.67±0.12**
0.3%Triton X-100	3/1000	45.94±2.27**

Compared with PBS, * $P < 0.05$, ** $P < 0.01$.

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