Exploration of the amino acid metabolic signature in anthracycline-induced cardiotoxicity using an optimized targeted metabolomics approach based on UPLC-MS/MS

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Abstract
Although anthracyclines improve the long-term survival rate of patients with cancer, severe and irreversible myocardial damage limits their clinical application. Amino acid (AA) metabolism in cardiomyocytes can be altered under pathological conditions. Therefore, exploring the AA metabolic signature in anthracycline-induced cardiotoxicity (AIC) is important for identifying novel mechanisms. We established mouse and cellular models of Adriamycin (ADR)-induced cardiac injury. We observed a decreased expression of troponins I (cTnI) after ADR treatment and ADR accelerated the degradation of cTnI, implying that AA metabolism could be altered in AIC. Using a targeted AA metabolomics approach based on ultra-performance liquid chromatography–tandem mass spectrometry (UPLC-MS/MS), the AA metabolic signatures in the sera of AIC mice and supernatant samples of ADR-treated H9c2 cardiomyocytes were analyzed. The levels of 14 AA metabolites were altered in ADR-treated mice (p < 0.05). Via bioinformatics analysis, we identified nine differential AA metabolites in mice and five differential AA metabolites in ADR-treated H9c2 cardiomyocytes. Three AAs with increased levels (l-glutamate, l-serine, and l-tyrosine) overlapped in the two models, suggesting a possible mechanism of AA metabolic impairment during AIC. The metabolic pathways perturbed by AIC involved aminoacyl-tRNA biosynthesis and alanine, aspartate, and glutamate metabolism. Our data suggests that ADR perturbed AA metabolism in AIC models. Moreover, the targeted AA metabolomics approach based on UPLC-MS/MS can be a unique platform to provide new clues for the prevention and treatment of AIC.

Keywords Targeted metabolomics · Anthracycline-induced cardiotoxicity · Amino acid metabolism · Signature

Introduction
Anthracyclines feature a tetracyclic ring structure (aglycone) attached by sugars or amino sugars through glycosidic bonds, and anthracyclines differ in their ligands or sugars (Mele et al. 2016). Anthracyclines remain the cornerstones of chemotherapy for malignancies including lymphoma, sarcoma, breast cancer, and pediatric leukemia because of their significant anti-cancer effects. According to available data, anthracyclines are used to treat approximately 30% of breast cancers, up to 70% of lymphomas in older adults, and 60% of pediatric cancers (Sallustio and Boddy 2021). Although anthracyclines improve the long-term survival rate of patients with cancer, they cause severe and irreversible myocardial damage, thereby limiting their clinical application (Sawicki et al. 2021). The observation of a relationship between the anthracycline dose and cardiovascular side effects can be traced back four decades (Middleman et al. 1971). When the cumulative dose of doxorubicin exceeds 250–300 mg/m², the risk of cardiotoxicity
rises dramatically (Tantawy et al. 2021). Cardinale et al. found that anthracycline-induced cardiotoxicity (AIC) occurred in 9% of adults, and the incidence of AIC is highest (98%) during the first year after the completion of chemotherapy (Cardinale et al. 2015). To date, anthracyclines remain the major cause of chemotherapy-induced cardiotoxicity (Lenneman and Sawyer 2016; Mudd et al. 2021). AIC, the mechanism of which has not been fully clarified, is a broad term encompassing both changes in resting cardiac parameters and dynamic functional assessments of the cardiovascular system such as diminished left ventricular ejection fraction, cardiac cell and structural damage, conduction abnormalities, vascular abnormalities, and other adverse effects that perturb normal cardiac function. Therefore, identifying new insights into the mechanism of AIC is critical for clinical prevention and treatment.

AAs are essential for protein synthesis and the maintenance of normal heart structure, but they also can be catabolized as substrates for energy generation (Miyajima 2020). Therefore, an imbalance of AA metabolism induces myocardial contractile dysfunction and eventually heart failure (De Jong and Lopaschuk 2017; Du et al. 2018). Murashige et al. measured more than 270 metabolites using liquid chromatography–mass spectrometry (MS) in patients with heart failure and reported a high level of AA secretion from the heart, indicating that AA metabolic disorder may contribute to cardiac injury (Murashige et al. 2020). In comparison with glucose and fatty acids as metabolic fuels for the heart, the role of AA metabolic pathways in the heart has received little attention. Emerging evidence suggests that AAs are closely related to AIC (Xue et al. 2016; Tedesco et al. 2020), and it is of great significance to explore the role of AA metabolism in AIC. However, it is unclear whether AA metabolism is altered in AIC. Serum metabolomics data showed great potential in development highly specific biomarkers for AIC (Li et al. 2015). Previous studies have proved that conditioned medium from cultured cardiomyocytes is suitable for metabolic footprint analysis in cardiotoxicity (Strigun et al. 2011). Therefore, we collected the sera of AIC mice and conditioned medium from ADR-treated H9c2 cardiomyocytes for the UPLC-MS/MS detection. In the present study, we established Adriamycin (ADR)-induced cardiac injury models. Using an optimized targeted AA metabolomics method based on ultra-performance liquid chromatography–tandem MS (UPLC-MS/MS), we obtained the AA metabolic signature of AIC.

Materials and methods

Chemicals and reagents

Doxorubicin hydrochloride (ADR) was obtained from Solarbio (Beijing, China). L-2-Aminoadipic acid (2-AA) was purchased from Aladdin (Shanghai, China). HPLC-grade formic acid, acetonitrile, and methanol were purchased from Thermo Fisher Scientific (Waltham, MA, USA). AR-grade K2HPO4·3H2O and NaH2PO4·2H2O were purchased from Sinopharm Chemical Reagent Co., Ltd. (China). AR-grade boric acid, N-ethylmaleimide (NEM), 4-tert-butylbenzenethiol (tBBT), dimethyl sulfoxide (DMSO), 5-aminoisooquinoline (5-AIQ), N,N’-disuccinimidyl carbonate (DSC), ascorbic acid (Vc), ethylenediaminetetraacetic acid (EDTA), and phosphate hydrochloride (TCEP) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The 126 amino analyte standards were purchased from Sigma-Aldrich and J&K Scientific (China), 5-Aminoisoquinolinyl-N-hydroxysuccinimidyl carbamate (5-AIQC) for tagging amino groups was synthesized via the drop-wise addition of 5-AIQ solution (2 mmol in 50 mL ACN) to DSC solution (3 mmol in 40 mL ACN) over approximately 2 h at ambient temperature with magnetic stirring. After further stirring for 24 h and removal of acetonitrile by rotary evaporation, 5-AIQC was obtained as crystals from the concentrated solution through filtration (650 mg, 82% yield). Phosphate buffer and borate buffer were prepared in a normal manner with their pH adjusted to 7.0 and 8.8, respectively, using sodium hydroxide solution. Phosphate buffer (0.1 M) contained 10 mM Vc and 10 mM EDTA, whereas borate buffer (0.2 M) contained 20 mM TCEP and 1 mM Vc. Cell Counting Kit-8 (CCK-8) was purchased from Dojindo (Kumamoto, Japan).

Animal experiments

All animal protocols were approved by the Ethics Committee of Peking University People’s Hospital (No. 2018PHC051). Six-to-eight-week-old male C57BL/6 J mice (Vital River Laboratories, Beijing, China) were maintained on a 12-h/12-h light/dark cycle and permitted free access to food and water. Sixteen mice were weighed and randomly allocated to the ADR (n = 8) and control groups (n = 8); however, one mouse accidentally died during echocardiography, and it was excluded from the study. The AIC model was constructed as previously described (Shioji et al. 2002). In brief, mice in the ADR group were intraperitoneally injected with 15 mg/kg ADR on the first day of the experiment. An equal volume of normal saline was injected into the mice of the control group. Echocardiography was performed to determine the cardiac structure and function on the sixth day of the experiment. At least three measurements were conducted, and the values were averaged for each parameter. All mice were fasted for 12 h prior to subsequently euthanasia, and the serum samples were collected. In order to ensure that the collected tissues uncontaminated by chemical agents, we used cervical dislocation method for euthanasia. Hearts were rapidly harvested and transected, and the upper parts were fixed with 4% paraformaldehyde and embedded in paraffin.

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Histological cross-sections (5–6 μm thickness) of the hearts were stained with hematoxylin and eosin (H&E) staining for morphometric analysis and cardiomyocyte cross-sectional area (CSA) measurement.

**Cell culture and treatments**

H9c2 (National Infrastructure of Cell Line Resource, Beijing, China) cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM, HyClone) containing 10% fetal bovine serum (FBS, BioInd, Israel). AC16 cells (National Infrastructure of Cell Line Resource) were cultured in DMEM/F-12 medium (Gibco) supplemented with 10% FBS (Gibco). H9c2 and AC16 cells were maintained in a 37 °C humidified atmosphere containing 5% CO2 and 95% O2. The cells were seeded onto 12-well plates at a density of 5 × 10^5 cells/mL for 24 h before the addition of ADR. For the UPLC-MS/MS analysis, H9c2 cells were treated with 1 μM ADR for 24 h, and cells treated with an equal volume of normal saline were used as a control. There were six parallel wells in each group. After treatment, the conditioned medium samples were collected and centrifuged at 12,000 rpm to remove the precipitates, and the supernatants were immediately stored at −80 °C. To measure the half-life of cTnI, H9C2 and AC16 were treated with 50 μM cycloheximide (CHX) and then lysed at different time points (0, 1, 3, 6, 12, 24 h).

**CCK-8 assay**

The viability of H9c2 cells after ADR treatment at concentrations of 0.5, 1, 2, 4, 8, and 16 μM was detected using the CCK-8 assay. H9c2 cells were seeded (5 × 10^3 cells/mL) into 96-well plates and incubated with various concentrations of ADR for 24 h at 37 °C (0 μM used as a control). Subsequently, 10 μL of CCK-8 reagent was added to each well according to the manufacturer’s protocol and incubated for 2 h at 37 °C. The absorbance of each well at 450 nm was measured using a microplate reader, and the relative viabilities were analyzed.

**UPLC-MS/MS analysis**

Serum samples (20 μL) or conditioned medium samples (20 μL) were each mixed with 60 μL of pre-cooled methanol for protein precipitation. Samples were vortexed and centrifuged at 12,000 rpm for 10 min at 4 °C. Then, 10 μL of the supernatant was vortex-mixed with 10 μL of NEM solution (20 mM) in phosphate buffer for 1 min. Ten microliters of 1BBT solution (0.23 M in DMSO) was added followed by incubation at 55 °C for 10 min. The mixture was cooled to ambient temperature and mixed with 2 μL of formic acid, and the solution was filtered through a 0.22-μm membrane filter before UPLC-MS/MS analysis.

Quantitative detection of amino analytes was performed by UPLC-MS/MS using a previously reported optimized method (Wang et al. 2017). The UPLC-MS/MS system consisted of an Agilent 1290 UPLC coupled to an Agilent 6470 triple quadrupole mass spectrometer equipped with an electrospray ionization (ESI) source (Agilent Technologies, USA). The 5-AIQE-tagged samples (1 μL) were individually injected into an UPLC column (Agilent ZORBAX RRHD Eclipse XDB C18 column, 2.1 × 100 mm, 1.8 μm particles) with its temperature set to 50 °C. The mobile phase consisted of solvent A (water) and solvent B (methanol containing 0.1% formic acid), and the flow rate was 0.5 mL/min. An optimized gradient elution was performed using the following scheme: 1% B (0–2 min), 1–3.8% B (2–4 min), 3.8–14% B (4–7.3 min), 14–22% B (7.3–10.7 min), 22–24% B (10.7–14.7 min), 24–30% B (14.7–16 min), 30–60% B (16–16.3 min), 60–70% B (16.3–17.3 min), 70–95% B (17.3–17.31 min), and 95% B (17.31–20 min). ESI was performed in the positive ion mode under the following conditions: nebulizer pressure, 50 psi; sheath gas temperature, 350 °C (flow rate, 10 L/min); dry gas temperature, 315 °C (flow rate, 10 L/min); and capillary voltage, 4000 V. Multiple reaction monitoring was used for the quantification of screening fragment ions. Quality control samples were prepared using no fewer than 5% of the total number of samples divided into three parallel parts throughout the whole process of sample preparation, detection, and analysis.

**Western blot analysis**

The mice myocardial tissue and cellular samples were lysed and homogenized in RIPA lysis buffer (Solarbio, Beijing, China), and total protein was extracted. Their concentrations were quantified using BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). Equal amounts of proteins were separated on SDS-PAGE gels and then transferred to nitrocellulose membranes. The membranes were blocked with 5% nonfat milk for 1 h at room temperature. Subsequently, they were incubated with specific primary antibodies against GAPDH (Santa Cruz, Dallas, TX, USA) and cTnI (Proteintech, Wuhan, China) overnight at 4 °C. Next, they were incubated with HRP-conjugated goat anti-rabbit or mouse secondary antibodies (ZSGB-BIO, Beijing, China) for 1 h at room temperature. After washing with TBS-T for four times, the reactive bands were developed using Super ECL Plus luminescence reagent (Applygen Technologies, Beijing, China) and scanned using Quantity One software V 4.6.2 (Bio-Rad, Hercules, CA). GAPDH was used as a loading control.
Fig. 1 Adriamycin (ADR) induces cardiac injury in mice. a Cardiac function and structure were detected by echocardiography, and representative images are presented. The ejection fraction (EF%), fractional shortening (FS%), left ventricular posterior wall thickness at end diastole (LVPWd) and systole (LVPWs), and left ventricular anterior wall thickness at end diastole (LVAWd) and systole (LVAWs) were analyzed. Data are presented as the mean ± SD. ****p < 0.0001, ***p < 0.001, **p < 0.01, ns = not significance. ADR group (n = 8) vs. control group (n = 7). b Heart tissues from the control (a) and the ADR-treated mice (b–d) were stained with hematoxylin and eosin (H&E), scale bars: 100 μm. The red arrows in b myocardial fiber fracture; c pericardium inflammatory cell infiltration; d myocardial scattered bleeding points. e Left ventricular cardiomyocyte cross-sectional area (CSA) in control (n = 7) and ADR-treated mice (n = 8), scale bars: 50 μm, data is presented as the mean ± SD. ****p < 0.0001. f ADR decreases H9c2 cell viability. H9c2 cells were treated with a range of ADR concentrations for 24 h. Cell viability was determined using the cell counting kit-8 assay. Data are presented as the mean ± SD, ****p < 0.0001 vs. 0 μM, n = 6 per group.

Data preprocessing and statistical analysis

Peak determination and peak area integration were performed using MassHunter Workstation software (Agilent, Version B.08.00). Standard curves were constructed by least-squares linear regression analysis using the peak area ratio of the derivatized individual standard against the nominal concentration of the calibrator. Quantification of samples was performed identically. Statistical analysis was performed using SPSS 22.0 software. AA levels were compared between the ADR and control groups using Student’s t-test, and p < 0.05 denoted statistical significance. Multivariate statistical analysis was performed using Simca-P 14.1. Principal component analysis (PCA) was used to gain an overview of all samples and find possible outliers. Orthogonal partial least squares discriminant analysis (OPLS-DA) modeling was used to screen differential metabolites that contributed significantly to the discrimination of samples from each group. The criteria for identifying significant differential metabolites were variable importance in projection (VIP) > 1.0 and p < 0.05 in the OPLS-DA model. Meanwhile, the relevant metabolic pathways were enriched by MetaboAnalyst 5.0 (http://www.metaboanalyst.ca), as well to discover the significant pathways in the two models.

Results

ADR induces cardiac injury

To establish experimental AIC in mice, 15 mg/kg ADR was injected intraperitoneally (Shioji et al. 2002). Echocardiographic analysis was performed on the sixth day after ADR treatment. The representative echocardiographic images are presented in Fig. 1a. We found that ADR induced a significant decrease of the ejection fraction (EF%), demonstrating that the mouse model was successfully established. In addition, we observed that the left ventricular fractional shortening (LVFS) was significantly lower in the ADR group than in the control group, suggesting that ADR treatment impaired cardiac systolic function (Fig. 1b). The left ventricular posterior wall thickness at end diastole (LVAWd) and systole (LVAWs) and the left ventricular anterior wall thickness at end diastole (LVAWd) and systole (LVAWs) were remarkably lower in the ADR group than in the control group, indicating that cardiac structural remodeling was induced by ADR (Fig. 1b). Moreover, we compared histopathological changes of myocardia in ADR-treated mice. We found that the cardiomyocytes in the control group were orderly arranged, closely connected and normal in morphology, while the cardiomyocytes in ADR group showed a variety of pathological changes, such as myocardial fiber fracture, pericardium inflammatory cell infiltration, and myocardial scattered bleeding points (Fig. 1c). The left ventricular cardiomyocyte CSA was measured in each animal. As shown in Fig. 1d, the CSA in ADR group was decreased (211.4 ± 17.8 μm²) compared with the control group (343.3 ± 29.0 μm²), demonstrating that ADR treatment may cause cardiomyocyte atrophy.

H9c2 cardiomyocytes were treated with ADR at a series of concentrations for 24 h. Data from the CCK-8 assay indicated that treating H9c2 cells with 1 μM ADR significantly induced cytotoxicity (Fig. 1e). Therefore, this concentration was selected for the following in vitro experiments.

ADR induces the downregulation of cTnI in cardiomyocytes

Troponins I (cTnI), one of the most crucial proteins of the contractile myofibrils in myocardial cells, is a well-known cardiomyocytes injury biomarkers in clinical practice (Semeraro et al. 2021). Previous studies indicated that substantial cTnI degradation occurred during myocardial ischemia (Palmer et al. 2004). Therefore, we examined the expression of cTnI in ADR-induced cardiac injury models. We observed that the expression of cTnI at protein levels was significantly decreased, demonstrating that cTnI can be downregulated in AIC.

Increased degradation of intracellular proteins can be observed in the failing heart (Murashige et al. 2020). Since the total cTnI protein amounts reflect a balance between cellular synthesis and degradation (Willis and Patterson 2006), we speculate the decreased cTnI levels might be associated with the increase of degradation. To investigate the degradation status of cTnI in the presence of ADR, a CHX chase assay was performed. H9c2 (Fig. 2d) and AC16 (Fig. 2e)
Fig. 2 Adriamycin (ADR) treatment decreased cTnI expression in cardiomyocytes. a The mice were treated as indicated, and the heart tissues were harvested after echocardiographic analysis. The expression of the cTnI and GAPDH was measured using Western blotting and the normalized expression intensity of cTnI is shown. Each experiment was performed three times. Data are expressed as mean ± SD (n = 4). *p < 0.05. b, c H9c2 and AC16 cells were treated with ADR (1 μM) for 24 h. The expression of the cTnI and GAPDH were measured using Western blotting and the normalized expression intensity of cTnI is shown. Each experiment was performed three times. Data are expressed as mean ± SD (n = 3). *p < 0.05, **p < 0.01. d Effect of ADR on the degradation rate of cTnI protein in H9c2 cells. Cells were lysed at different time points (0, 1, 3, 6, 12, 24 h). e ADR accelerated the degradation of cTnI in AC16 cells. The cTnI protein half-life was observed shorter (12 h) under ADR treatment than CHX only (longer than 24 h).
cardiomyocytes were treated with 50 μM CHX, an inhibitor of de novo protein synthesis to block translation, in the presence or absence of ADR (1 μM) for 0, 1, 3, 6, 12, and 24 h, respectively. We found that the degradation of cTnI was increased in the presence of ADR.

Identification of differential AA metabolites in mice

ADR accelerates protein degradation can cause AA metabolism altered in cardiomyocytes. Thus, exploring the AA metabolic signature in AIC is important for identifying novel mechanisms. AA profiles were obtained using validated and robust methodology, enabling the quantitation of 62 analytes from serum samples. In all analyzed samples, there were 51 detectable analytes. The concentrations of the remaining nine analytes were below the limit of quantitation (LOQ) or they exceeded the LOQ for some of the samples; thus, they were excluded from statistical analyses. The AA levels were compared between the ADR and control groups using Student’s t-test (Supplementary Table S1). We identified 14 differential metabolites \( p < 0.01 \) in mice, including 10 metabolites with increased levels (L-lysine, L-serine, 5-aminovaleric acid, L-asparagine, O-phosphorylethanolamine, L-glutamic acid, L-methionine, L-histidine, L-tyrosine, and L-tryptophan) and 4 metabolites with decreased levels (ethanolamine, cystathionine, 2-AA, L-glutathione oxidized) (Fig. 3).

Bioinformatic analysis of AA metabolites in mice

We then processed the 51 analyzed metabolites using PCA to obtain an overview of the data and identify potential severe outliers between the ADR and control groups regarding the metabolic profiles (Fig. 4a; principal component 1 (PC1), 49.9%; PC2, 18.3%). The distribution of metabolites in the control group was more compact, whereas that in the ADR group was more dispersed. However, the PCA score plot failed to reveal clear separation between the analyzed groups. The results demonstrated that the PCA-X model could not completely distinguish the ADR and control groups. A supervised OPLS-DA model was then established to acquire clustering information and differential metabolites to differentiate the ADR and control groups. The variables significant at VIP > 1.0 and \( p < 0.05 \) in the OPLS-DA model were considered as biomarker candidates. We used the parameters R2 and Q2 to assess the fitness and prediction capabilities of the OPLS-DA model, respectively. The OPLS-DA model resulted in two predictive components with R2X (cum) = 0.742, R2Y (cum) = 0.931, Q2 (cum) = 0.776. Meanwhile, coefficient variability analysis of variance (CV-ANOVA) and permutation testing were further used to validate the OPLS-DA model. The \( p \) value of CV-ANOVA in this established model was 0.025, and the plot of permutation testing with 200 permutations is presented in Fig. S1a. The OPLS-DA score plots of serum samples are presented in Fig. 4b, in which clear separation between the two groups is observed. The result suggests that the model has good practicability and predictability, and the separation reveals fundamental metabolic differences between the two groups. The plot of the predictive VIP values is presented in Fig. S2a. Metabolites in the serum samples that satisfied both VIP > 1.0 and \( p < 0.05 \) are listed in Table 1. Nine metabolites including L-glutamic acid, L-lysine, L-serine, L-tryptophan, L-methionine, L-histidine, L-asparagine, L-tyrosine, and O-phosphorylethanolamine comprised the signature of AIC in ADR-treated mice. Furthermore, Metabo Analyst 5.0 was applied to analyze the data of differential metabolites to find the potential metabolic pathways based on Kyoto Encyclopedia of Genes and Genomes database, and the result is presented in Fig. 4c. Multiple metabolic pathways were perturbed by AIC, especially D-glutamine and D-glutamate metabolism; histidine metabolism; alanine, aspartate, and glutamate metabolism; aminoacyl-tRNA biosynthesis; and arginine biosynthesis. Detailed pathway results are summarized in Table S2.

The differential AA metabolites in the in vitro model

Considering that the in vivo model might reflect the global AA metabolic status, we established an in vitro model using ADR-treated cardiomyocytes to specifically focus on the AA metabolic signature in AIC. Using UPLC-MS/MS, we identified 15 AA metabolites with significantly different levels among 44 detected metabolites in the conditioned medium of ADR-treated H9c2 cells (Table S3). The levels of 10 AA metabolites (hypotaurine, D-homoserine, 2-AA, ethanolamine, taurine, L-asparagine, L-glutamic acid, L-serine, L-glutamine, L-tyrosine) were increased in the ADR group, whereas those of five AA metabolites (cadaverine, L-homocysteine, L-aspartic acid, L-ornithine, L-alanine) were decreased (Fig. 5). The 44 analyzed metabolites were processed by PCA to characterize the metabolic profile of the cellular model (PC1, 60.5%; PC2, 24.6%, Fig. 6a). The PCA score plot revealed clear separation between the analyzed data groups, indicating that two groups had different metabolic profiles.

Supervised OPLS-DA was also performed within the in vitro model. The OPLS-DA score plots of culture medium supernatant samples are presented in Fig. 6b, and the plot of the predictive VIP values is presented in Fig. S2b. The OPLS-DA model revealed clear separation between the analyzed data groups, indicating that significant changes of AA metabolism occurred after ADR treatment. Five metabolites (L-tyrosine, L-alanine, L-glutamine, L-serine, L-glutamic acid) satisfying both VIP > 1.0 and \( p < 0.05 \) were identified as the signature of AIC in the cellular injury model (Table 2). Five important pathways including phenylalanine, tyrosine, and tryptophan...
Fig. 3  Concentrations of 14 amino acid (AA) metabolites changed in mouse serum samples. Data are presented as the mean ± SD, and the levels of all 14 AA metabolites were significantly different ($p < 0.05$) between the ADR and ($n = 8$) control groups ($n = 7$)
biosynthesis; alanine, aspartate, and glutamate metabolism; glycine, serine, and threonine metabolism; aminoacyl-tRNA biosynthesis; and tyrosine metabolism were perturbed in the in vitro AIC model (Fig. 6c and Table S4).

Pathway analysis
Via an overlap analysis, we found that the levels of three AAs, namely L-glutamate, L-serine, and L-tyrosine, were increased in both the in vivo and in vitro models (Fig. 7a), suggesting AA utilization impairment in AIC. Furthermore, we found the aminoacyl-tRNA biosynthesis and alanine, aspartate, and glutamate metabolism were both involved in the two models (Fig. 7b), suggesting the two pathways might be associated with AIC.

Table 1 Amino acid metabolic signature of Adriamycin-induced cardiotoxicity in mice

| Amino acid          | VIP value | p value |
|---------------------|-----------|---------|
| L-Glutamic acid     | 3.70606   | <0.0001 |
| L-Lysine            | 2.87705   | 0.039   |
| L-Serine            | 1.82099   | 0.023   |
| L-Tryptophan        | 1.80749   | 0.024   |
| L-Methionine        | 1.57079   | 0.003   |
| L-Histidine         | 1.36099   | 0.010   |
| L-Asparagine        | 1.16729   | 0.012   |
| L-Tyrosine          | 1.1004    | 0.028   |
| O-Phosphorylethanolamine | 1.08842 | <0.0001 |

Variable importance for projection (VIP) from the orthogonal partial least-squares discriminant analysis model constructed with the control and model groups.
Fig. 5 The concentrations of 15 amino acid (AA) metabolites were changed in the H9c2 cell conditioned medium. The levels of hypo-
taurine, d-homoserine, L-2-aminoadipic acid, ethanolamine, taurine, 
L-asparagine, L-glutamic acid, L-serine, L-glutamine, and L-tyros-
ine were increased in the ADR group, whereas those of cadaver-
ine, L-homocystine, L-aspartic acid, L-ornithine, and L-alanine were 
decreased. Data are presented as the mean ± SD, and 15 AA metabo-
lites were significantly different at \( p < 0.05 \) between the ADR and 
control groups (\( n = 6 \) per group)
Discussion

In the present study, a new parameter-optimized UPLC-MS/MS method based on 5-AIQC derivatization-assisted sensitivity enhancement for the simultaneous quantification of amino-containing metabolites was developed. Using an NEM click reaction followed by the addition of anti-oxidants (TCEP and Vc), our method enabled the simultaneous quantification of amino analytes in a one-pot manner (and in a single run). This 5-AIQC-based method had high sensitivity for an extensive array of analytes including 20 proteinogenic AA, more than 10 modified AAs, more than 50 non-proteinogenic AAs, more than 20 sulfur-containing analytes, more than 10 monoamine neurotransmitters (e.g., catecholamines), a variety of small peptides, and aliphatic and aromatic amines. This method enabled the simultaneous quantification of more than 100 important functional metabolites involved in more than 20 metabolic pathways such as protein biosynthesis/degradation; catecholamine, arginine, and glutathione biosynthesis; and homocysteine and taurine metabolism. Therefore, the present method offers many advantages for metabonomic analysis, such as wide

Table 2 Amino acid metabolic signature in Adriamycin-induced H9c2 cell injury models

| Amino acid     | VIP value | p value   |
|----------------|-----------|-----------|
| l-Tyrosine     | 3.63705   | <0.0001   |
| l-Alanine      | 2.76496   | <0.0001   |
| l-Glutamine    | 2.60996   | 0.025     |
| l-Serine       | 2.54653   | <0.0001   |
| l-Glutamic acid| 2.11724   | <0.0001   |

Fig. 6 Amino acid (AA) metabolic signature in the in vitro models. a Principal component analysis was performed using 44 metabolites from the conditioned medium samples. The first two principal components (PCs) explained 85.1% of the total variance (PC1 = 60.5%, PC2 = 24.6%). b Orthogonal partial least-squares discriminant analysis (OPLS-DA) was performed to analyze the in vitro model. The score plot of the OPLS-DA model is presented (R2X [cum] = 0.877, R2Y [cum] = 0.992, Q2 = 0.98). The coefficient variability analysis of variance p value for the OPLS-DA model was 4.97356×10⁻⁵. The supervised model was validated by a permutation test (N=200, presented in Figure S1B). c Metabolic pathway analysis of differential metabolites (a phenylalanine, tyrosine, and tryptophan biosynthesis; a alanine, aspartate, and glutamate metabolism; a glycine, serine, and threonine metabolism; d aminoacyl-tRNA biosynthesis; e tyrosine metabolism)
metabolite coverage, excellent precision, accuracy, linearity repeatability, and capability in biomarker discovery, making it useful for both basic and clinical metabolic research (Wang et al. 2017).

The incidence of cancer-related diseases has increased without a consequent rise in cancer-related mortality, which is attributable to the remarkable progress in cancer treatment (Herrmann 2020). With this change, people have increasingly recognized the importance of the adverse effects of cancer therapies, including chemotherapy-induced cardiotoxicity. Anthracyclines remain the cornerstones of cancer chemotherapy. Unfortunately, severe and irreversible myocardial damage limits their clinical application. Despite more than five decades of research, the mechanism underlying AIC is not completely understood (Middleman et al. 1971). The metabolites of endogenous compounds play an important role in diagnosing and treating patients since many of them are laboratory biomarkers and targets for therapeutic agents. CTnI is one of the most critical biomarkers to diagnose cardiovascular diseases, including AIC (Semeraro et al. 2021). In the present study, we observed ADR accelerated cTnI protein degradation, implying AA metabolism may be altered in AIC. Previous studies suggest that substantial cTnI degradation occurs during myocardial ischemia (Palmer et al. 2004). However, the decrease of cTnI in ADR-induced cardiac injury models has not been reported. Therefore, we assessed the effect of ADR on the stability of endogenous cTnI protein using the CHX chase assay. The results showed that DOX enhanced the instability of cTnI in both H9c2 and AC16 cells, which indicated that ADR may cause the instability of contraction related proteins such as cTnI in cardiomyocytes. However, it is unclear whether the decrease of cTnI is due to increased release or enhanced protein degradation. In any event, these results hint an AA metabolism alteration may occur in AIC due to the protein instability. Thus, we established ADR-induced cardiac injury models. Using an optimized targeted AA metabolomics method based on UPLC-MS/MS, we explored the AA metabolic signature to investigate whether AA metabolism is altered in AIC.

“Omics” technologies such as proteomics, metabolomics, and genomics have emerged as promising tools for discovering novel biomarkers associated with cardiotoxicity (Ngo et al. 2016; Serie et al. 2017; Wells et al. 2017; Asnani et al. 2020). These techniques have the potential to uncover novel pathways and mechanisms of AIC. Among these modern techniques, metabolomics appears extremely promising for biomarker research because it can reflect changes in endogenous substances in different physiological or pathological states. Untargeted metabolomics fails to provide information about the absolute concentration of analyzed compounds, and it is limited by unsatisfactory repeatability.

Fig. 7 Overlap analysis between the in vivo and in vitro models a Venn diagram of differential metabolites screened in the two models. The numbers in the figure represent the same metabolites between the corresponding two models.
AA metabolites, as the most important basic substances in the life movement of organisms, participate in various energy and substance metabolic pathways. In the present study, significant changes in AA analytes were identified as signatures in AIC models based on targeted AA metabolomic analysis. Regarding the AA metabolite profiling, we found that the levels of most of the metabolites were increased in the ADR groups, implying that prominent metabolite accumulation may occur in AIC. A metabolomic study based on gas chromatography-MS identified 21 metabolites in an ADR-induced toxicity rat model and found an upward trend of many AAs (Geng et al. 2021). Another metabolomic analysis reported that the levels of many AAs were significantly increased in the heart tissues and plasma of ADR-treated B6C3F1 mice (Schnackenberg et al. 2016). Sansbury et al. also reported extensive and pervasive increases in the levels of branched-chain AAs in the failing heart (both pressure-overloaded and infarcted mouse hearts) (Sansbury et al. 2014). These findings indicated that the alteration of metabolism is a hallmark of dysfunctional hearts, and the disturbance of AA metabolism contributes to AIC (Yi et al. 2018; Yuan et al. 2020; Mamic et al. 2021).

Although previous studies have applied metabolomics technology to obtain highly specific metabolites panels in cardiotoxicity (Li et al. 2015), they usually carried out a non-targeted metabolite analysis to analyze different types of metabolites, which can only detect limited essential AAs. In this study, we used the optimized targeted metabolomics method enabling the simultaneous quantification of more than 100 amino metabolites. It obtained a more comprehensive and representative AA metabolic signature in AIC in vivo and in vitro models. For the first time, we found that most AA metabolites showed an increased level in AIC models and three differential metabolites 3-glutamate, L-serine, and L-tyrosine can be considered as AA metabolic signature of AIC. Meanwhile, it suggests that the disorder of AA metabolic pathways can be considered as a potential access point of AIC mechanism. In the present study, we identified 14 differential AA metabolites in the in vivo model and 15 metabolites in the in vitro model. Through OPLS-DA, the AA metabolic signatures of the two models were drawn to depict the effects of AIC on AA metabolism, and many of these individual metabolites have been reported to be associated with cardiac injury. Previous studies illustrated that alterations in the glutamate–glutamine cycle with an increase in glutamic acid levels and decrease in glutamine levels are associated with an unfavorable cardiometabolic status (Papandreou et al. 2020). L-Tyrosine has been reported to be associated with heart failure (Cheng et al. 2021). In addition, tyrosine can be metabolized into p-cresyl sulfate, whereas tryptophan can be metabolized into indoxyl sulfate. These two metabolites contribute to adverse cardiac remodeling through their direct pro-fibrotic, pro-hypertrophic, and pro-inflammatory effects (Fujii et al. 2009; Lekawannijit et al. 2010, 2012). L-Arginine is an essential AA with numerous functions. An earlier study linked L-arginine to mitochondrial function (Geiger et al. 2016). Another study reported that L-arginine can upregulate the serine biosynthesis pathway, which has been demonstrated to fuel the tricarboxylic acid cycle and oxidative phosphorylation, and L-arginine can be converted into intermediates for energy generation (Possemato et al. 2011). Taurine is a sulfur-containing AA present abundantly in the heart, and it plays protective roles through the regulation of the intracellular Ca2+ concentration and through its anti-oxidant, anti-inflammatory, anti-apoptosis, and membrane-stabilizing properties (Kim et al. 2006; Ghosh et al. 2009; Schaffer et al. 2010; Samadi et al. 2021).

Energetic metabolism in cardiomyocytes is a crucial feature of myocardial contractile function (Neubauer 2007), and balanced AA metabolism is required. Using both in vivo and in vitro models, we found a dominant upward trend of AA metabolite levels under ADR treatment, indicating that abnormal AA metabolism associated with energetic utilization impairment or protein degradation may occur in AIC (Murashige et al. 2020). Through overlap pathway analysis, we identified significant differences in L-glutamate, L-serine, and L-tyrosine levels in the two models. Furthermore, we found that aminoacyl-tRNA biosynthesis and alanine, aspartate, and glutamate metabolism were involved in both the in vitro and in vivo models, suggesting that the two pathways are associated with AIC. A previous study revealed that aminoacyl-tRNA synthetases are involved in heart failure in humans and pathologic cardiac remodeling in mice. Aminoacyl-tRNA synthetases activate pro-fibrotic genes in cardiac fibroblasts in translational control and augment pathological cardiac remodeling (Wu et al. 2020). Alanine, aspartate, and glutamate metabolism has been recognized as an important pathway associated with doxorubicin-induced nephropathy (Li et al. 2020). In any event, these results indicated an underlying AA disturbance exists in our ADR-induced cardiac injury model that is associated with AIC. Previous studies proposed that ADR-induced cardiotoxicity was associated with a variety of pathways and mechanisms, including reactive oxygen species formation, cardiomyocyte apoptosis, intracellular calcium dysregulation, and DNA damage (Henriksen 2018; Tewey et al. 1984; Arai et al. 2000; Nitobe et al. 2003; Ichikawa et al. 2014). In the present study, we found that aminoacyl-tRNA biosynthesis pathway was perturbed in both in vivo and in vitro models. Recently, emerging evidence showed that aminoacyl-tRNA synthetase is
closely related to the occurrence and development of cardiovascular diseases (Zou et al. 2021). Aminoacyl-tRNA synthetase is an essential enzyme for normal metabolism of organisms. It catalyzes AAs and corresponding tRNA to the formation of aminoacyl-tRNA, providing raw materials for protein translation. Our data suggested that ADR treatment led to contraction associated protein (e.g., cTnl) abnormal expression. Meanwhile, the aminoacyl-tRNA biosynthesis pathway was enriched in AIC models through bioinformatics analysis. The above results suggest that aminoacyl-tRNA biosynthesis pathway is the potential target of ADR in cardiomyocytes and may have a bright prospect in the clinical prevention and treatment in AIC.

Conclusion

In conclusion, we explored the signature of AIC using targeted AA metabolomics techniques, which may provide new clues for the early and rapid diagnosis of AIC in patients under anthracycline therapy. Further studies using more specific methods are needed to reveal the exact mechanism in AIC involving key differential AA metabolites.

Abbreviations

ADR: Adriamycin; AIC: Anthracycline-induced cardiotoxicity; AA: Amino acid; cTnl: Cardiac troponin I

Supplementary Information

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Author contribution

M. J., W. Q., and M. S. conceived and designed the study, W. Q. and M. S. provided financial supports, W. L. and S. L. performed most of the experiments, analyzed the data, and wrote the manuscript. Z. C. performed the cell experiments. Y. S. assisted with performing the animal experiments. All authors have read and approved the final manuscript. The authors declare that all data were generated in-house and that no paper mill was used.

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Data availability

All data generated or analysed during this study are included in this published article and its supplementary information files.

Declarations

Ethics approval

All animal protocols were approved by the Ethics Committee of Peking University People’s Hospital (No. 2018PHC051).

Consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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