Impact of anesthesia and euthanasia on metabolomics of mammalian tissues: studies in a C57BL/6J mouse model

Experimental protocols

Materials and reagents

Carbon-13 stable isotope internal standards were purchased from Sigma-Aldrich (St. Louis, MO), Cambridge Isotope (Andover, MA), and Omicron Biochemicals (South Bend, IN). All other metabolite standards and other reagents were purchased from Sigma-Aldrich. A certified anesthetic delivery machine stocked with isoflurane was rented from the University of Michigan Unit for Laboratory Animal Medicine. Ketamine HCl (100 mg/mL) USP and sodium pentobarbital (50 mg/mL) USP were purchased from the University of Michigan Hospital Pharmacy.

Ethics statement

All procedures involving animals in this study were carried out in accordance with the recommendations set forth in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was reviewed and approved by the University Committee on Use and Care of Animals (UCUCA) of the University of Michigan, Ann Arbor, protocol #PRO00003797. Surgical tissue isolations were performed as terminal procedures under anesthesia as described below, and all precautions were taken to minimize suffering.

Animal handling

Male C57BL/6J mice, aged 20 weeks, weighing 26.8 ± 2.2 g (mean $\pm$ SD), were purchased from Jackson Laboratories (Bar Harbor, ME). The mice were maintained on a 12 h/12 h light/dark cycle and were provided with standard chow and water access ad libitum. On the day tissue collection was to be performed, mice were fasted beginning at 9 AM for a period of 5 hours, and tissue collection procedures were initiated at 2 PM.

Tissue collection

Six different methods of anesthesia or euthanasia were evaluated for tissue collection (n = 8 mice per method of anesthesia). In all cases, tissue collection procedures were initiated after animals had either been euthanized or were under deep anesthesia and unresponsive to all stimuli. The three methods of euthanasia were: 1) cervical dislocation: mice were removed from their cages and gently restrained while resting on the benchtop. Cervical dislocation was performed manually and resulted in euthanasia within approximately 10 seconds. 2) Carbon dioxide: euthanasia was performed by introduction of 100% carbon dioxide into a bedding-free cage initially containing room air with the lid closed at a rate sufficient to induce rapid anesthesia, with death occurring within 2.5 minutes. 3) Isoflurane overdose: mice were placed into a chamber filled with vapor of the anesthetic isoflurane until respiration ceased (within 2 minutes). The three methods of anesthesia were: 4) Continuous isoflurane: using a calibrated anesthetic delivery machine, mice were induced into anesthesia at a dose of 4% isoflurane, then maintained at a surgical plane by continuous inhalation of 2% isoflurane. Typical time to initiation of tissue collection was 1.5 minutes. 5) Ketamine: a 100 mg/mL solution of ketamine in sterile saline was

administered intraperitoneally (IP) at a dose of 120 mg/kg. Time to initiation of tissue collection was 20 minutes. 6) Pentobarbital: a 50 mg/mL solution of pentobarbital in sterile saline was administered IP at a dose of 60 mg/kg. Time to initiation of tissue collection was 15 minutes. Once anesthesia was induced or euthanasia was performed, collection of tissues was initiated immediately and was performed following the same procedure and timing regardless of anesthesia or euthanasia method. Tissues and blood were rapidly collected in the following order: gastrocnemius muscle, arterial blood (approximately 600 µL collected from descending aorta using a 25-gauge needle), liver, heart, and epididymal white adipose tissue. All tissues were rapidly rinsed in deionized water to remove excess blood, blotted dry and frozen by immersion in liquid nitrogen within 10 seconds of removal from the animal. Collection of all tissues was complete within 3 minutes. To prepare serum, blood was allowed to clot on ice for 15 minutes and was then centrifuged for 15 minutes at 3000 x g. The supernatant was withdrawn and frozen in liquid nitrogen. All samples were stored at -80°C until extraction.

Tissue extraction
Frozen gastrocnemius skeletal muscle, liver, heart and epididymal white adipose tissue samples from C57BL/6J mice were ground to a homogenous powder using a liquid nitrogen-chilled mortar and pestle. Approximately 30 mg (wet mass) of each pulverized tissue sample was rapidly transferred to a pre-weighed, dry-ice-chilled 1.5 mL microcentrifuge tube, which was returned to dry ice until addition of extraction solvent. The extraction solvent consisted of a single-phase mixture of 7 parts methanol: 2 parts water: 1 part chloroform, and contained ^{13}C-labeled internal standards at the concentrations specified in Table S1. To extract the samples, 1 mL of chilled (4°C) extraction solvent was added to the tube containing the frozen pulverized tissue, after which the contents were immediately homogenized by 20 seconds of pulsed sonication using a probe sonicator (Branson 450, output power setting 4, 40% duty cycle), with the tube in an ice bath. Samples were allowed to rest 5 minutes on ice, and were then centrifuged 10 minutes at 16,000 x g at 4°C. The supernatant was withdrawn and transferred to autosampler vials for LC-MS analysis. The residual tissue pellet was dried by vacuum centrifugation and its mass was measured for normalization.

Blood serum extraction
Serum was extracted according to a procedure described by Bruce et al. [1]. 50 µL of serum were extracted by addition of 200 µL of ice-cold 1:1:1 methanol:acetonitrile:acetone containing a mixture of ^{13}C internal standards (Table S1), followed by vigorous vortexing for 20 seconds. The samples were allowed to rest on ice for 5 minutes, and were then centrifuged for 10 minutes at 16,000 x g at 4°C. The supernatant was transferred directly to autosampler vials for LC-MS analysis.

LC-MS methods
Samples were analyzed by LC-MS using an Agilent 1200 LC system with an Agilent 6220 time-of-flight mass spectrometer. The chromatographic method was a modified version of the mixed-mode HILIC-anion exchange separation developed by Bajad et al. [2]. Briefly, the method employs a Phenomenex (Torrance, CA) Luna 3µ NH_2 column, 2.1 x 150 mm. Mobile phase A was acetonitrile and mobile phase B was 5mM ammonium acetate adjusted to pH 9.9 using ammonium hydroxide. The gradient was as follows: linear from 20 to 100% B over 15 minutes, 3 minute hold at 100% B, then return to 20% B at 18.1 minutes and re-equilibrate for 12 minutes.

The total run time was 30 minutes and the flow rate was 0.25 mL/minute. The column temperature was 25°C and sample vials were held at 4°C in the autosampler. The injection volume was 25 µL. Mass spectrometry was performed using electrospray ionization in negative ion mode with a dual spray source with Reference mass correction enabled. Full-scan mass spectra were acquired over the m/z range 50-1200 Da with a data acquisition rate of 1 scan / second. Source parameters were: drying gas temperature 350°C, drying gas flow rate 10 L/min, nebulizer pressure 30 psig, capillary voltage 3500 V. All samples from a given tissue type were extracted and analyzed on the same day, in randomized order. A pooled sample derived from the tissue extracts was injected every ninth run throughout the analysis to monitor peak area reproducibility.

Data analysis
Untargeted metabolite screening was performed using the metabolomics data analysis software package MZmine version 2.10 and the web-server based data processing package MetaboAnalyst version 2.0 [3,4]. Raw LC-MS data files were converted from Agilent .d format to mzXML format using Agilent Masshunter Qualitative Analysis (version B.04.00) and were then imported into MZmine. Mass detection was performed using the centroid mass detector with the noise level set at 1.0E3. The chromatogram builder was then used to generate peaks with a minimum time span of 0.2 min, minimum height of 1.0E3, an m/z tolerance of 0.002 m/z or 20 ppm. Chromatograms were smoothed with a filter width of 5. Chromatogram decovolution was performed using the noise amplitude algorithm with a minimum peak height of 5.0E3, a peak duration range of 0-25 minutes, and 2.0E3 as the amplitude of noise setting. Isotopic peaks were grouped using an m/z tolerance of 0.002 m/z or 20 ppm, a retention time tolerance of 0.1 minutes (absolute), a maximum charge of 2, and the representative isotope set as most intense. Retention time normalization was performed with an m/z tolerance of 0.002 m/z or 20 ppm, a retention time tolerance of 1.0 min (absolute), and a minimum standard intensity of 1.0E4. Chromatograms were then aligned into a peak list using the join aligner with an m/z tolerance of 0.005 m/z or 50.0 ppm, a weight for m/z of 50, a retention time tolerance of 1.5 min (absolute) and a weight for RT of 50. Gap-filling was performed using the peak finder algorithm with an intensity tolerance of 25%, a m/z tolerance of 0.002 m/z or 20 ppm, a retention time tolerance of 1.0 min (absolute) and RT correction enabled. A duplicate peak filter was applied to remove peaks within an m/z tolerance of 0.01 m/z or 50.0 ppm, and an RT tolerance of 0.5 min (absolute). Subsequently, a peak list rows filter was applied to keep only peaks appearing in 75% of all samples, with 1 peak minimum per isotope pattern, m/z range set automatically, retention time range 1.0-25.0 minutes, and peak duration range of 0.1-2.0 minutes. The resulting peak list was then displayed as a table within MZmine and the peak shapes of all features were visually inspected, and artifact features were discarded. The data were then exported to MetaboAnalyst for multivariate statistical analysis. Data were uploaded as a peak intensity table, with missing data points handled according to default parameters. Data were filtered by interquartile range and were then normalized by median intensity and log-transformed. Principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) were then performed. The PLS-DA model were validated by random permutation of the Y variable and by comparing of the goodness of fit (R2Y and Q2). For random permutation tests, 100 models were calculated and the goodness of fit was compared with the original model in a validation plot. Two-dimensional score plots were generated to visually assess separation between sample groups.

Targeted metabolite analysis was performed using Agilent Masshunter Quantitative Analysis software (version 4.0). Metabolite identification was accomplished by comparison of accurate mass and retention time with that of authentic standards analyzed using the same method. For relative quantitation, each metabolite was quantitated by peak area. For absolute quantitation of selected metabolites, peak areas were measured relative to the peak areas of ^{13}C-labeled internal standards added to the extraction solvent. Six-point calibration curves for these compounds were generated using non-labeled authentic standard solutions spiked with the same concentrations of ^{13}C-labeled internal standards as in the extraction solvent. Visual network mapping of detected metabolites was performed using Metscape 2.0 [5]. For comparisons between CD and other modes of anesthesia and euthanasia, a p-value was calculated using Student's t-test and corrected for multiple comparisons by false discovery rate correction [6].

References

1. Bruce SJ, Tavazzi I, Parisod V, Rezzi S, Kochhar S, et al. (2009) Investigation of human blood plasma sample preparation for performing metabolomics using ultrahigh performance liquid chromatography/mass spectrometry. Anal Chem 81: 3285-3296.
2. Bajad SU, Lu W, Kimball EH, Yuan J, Peterson C, et al. (2006) Separation and quantitation of water soluble cellular metabolites by hydrophilic interaction chromatography-tandem mass spectrometry. Journal of Chromatography A 1125: 76-88.
3. Pluskal T, Castillo S, Villar-Briones A, Oresic M (2010) MZmine 2: modular framework for processing, visualizing, and analyzing mass spectrometry-based molecular profile data. BMC Bioinformatics 11: 395.
4. Xia J, Mandal R, Sinelnikov IV, Broadhurst D, Wishart DS (2012) MetaboAnalyst 2.0--a comprehensive server for metabolomic data analysis. Nucleic Acids Res 40: W127-133.
5. Gao J, Tarcea VG, Karnovsky A, Mirel BR, Weymouth TE, et al. (2010) Metscape: a Cytoscape plug-in for visualizing and interpreting metabolomic data in the context of human metabolic networks. Bioinformatics 26: 971-973.
6. Benjamini Y, Hochberg Y (1995) CONTROLLING THE FALSE DISCOVERY RATE - A PRACTICAL AND POWERFUL APPROACH TO MULTIPLE TESTING. Journal of the Royal Statistical Society Series B-Methodological 57: 289-300.