Protocols

Animal handling

According to the National Institutes of Health Guide for the Care and Use of Laboratory Animals, all animals involved in this study were cared for, and protocols were reviewed and approved by the Anhui Laboratory Animal Care Committee. The specific pathogen-free (SPF) seven-week-old male Sprague Dawley (SD) rats (weight 233 ± 5 g) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd and used in this study. The environmental conditions were set at 21-26°C a relative humidity of 50 ± 10% and a 12/12 h light/dark cycle. Food and tap water provided *ad libitum*, and body weights were recorded daily. After one week of acclimatization, rats were randomly assigned to the groups of non-stressed control (n 6) or the stressed (n = 7). For restraint stress, rats were individually placed in a ventilated plastic tube restrainer for 120 minutes, using a previously modified method. According to the general protocol, control rats were left undistributed in a home cage and allowed to contact each other without food and water.

Sample collection

Individual urine samples were collected in ice-cooled vessels containing 1% sodium azide (0.1 ml) for 2 h using a metabolic cage at 0, 24, and 48 h post-stress, respectively, and immediately frozen at -80°C. Animals were sacrificed by exsanguination under isoflurane anesthesia at 48 h post-stress. The blood sample was divided into two aliquots, one serum for biochemical analysis and the other heparinized plasma for NMR spectroscopic analysis. After weighing, brain, kidney, liver, lung, and spleen tissue were excised in duplicate: one being fixed in 10% formalin for histopathological examination, the other immediately snap-frozen in liquid nitrogen for tissue extraction. These samples were stored at -80°C until used.

Sample preparation and ^{1}H NMR spectroscopic analysis

Samples of plasma (255 μl) were mixed with 255 μl of phosphate D_2O buffer solution (NaH_2PO_4 and K_2HPO_4, 60 mM, pH 7.4). After centrifugation at 10000 × g at 4°C for 10 min to remove the precipitates, the supernatants were transferred to 5 mm NMR tubes and analyzed by NMR. ^{1}H NMR spectra were acquired on a Varian NMR System 500 spectrometer at 296 K. Standard one-dimensional (1D) ^{1}H NMR spectra were acquired with a CPMG pulse sequence. For each sample, 64 free induction decays (FIDs) were accumulated into 20 K data points over a spectral width of 10 kHz with a relaxation delay of 1 s.

Samples of urine (455 μl) were mixed with 55 μl of D_2O buffer solution (NaH_2PO_4 and K_2HPO_4, 1.5 M, including 0.1% TSP (sodium 3-(trimethylsilyl) propionate-2,2,3,3-d_4), pH 7.4) to minimize any gross variation in the pH of the urine samples. The mixture was left to stand for 10 min and centrifuged at 10000 × g at 4°C for 10 min to remove the precipitates. The supernatants were transferred to 5 mm

NMR tubes and analyzed by NMR. ^{1}H NMR spectra were acquired on a Varian NMR System 500 spectrometer at 296 K. Standard 1D ^{1}H spectra were acquired with NOESYPR1D pulse sequence. For each sample, 64 FIDs were accumulated into 40 K data points over a spectral width of 10 kHz with a relaxation delay of 1 s.

The polar metabolites in the rat tissue were extracted according to the protocol established in our previous work. In brief, pre-weighed brain, kidney, liver, lung, or spleen samples (100 mg) were homogenized in 400 μl of CH_3OH and 85 μl of H_2O at 4°C. The homogenates were transferred into a 2.5-ml tube, combined with 400 μl of $CHCl_3$ and 200 μl of H_2O, and then kept in a vortex for 60 s. After 10-min partitioning on ice, the samples were centrifuged for 5 min (10000 × g, 4°C). The upper supernatants were transferred into 1.5-ml tubes and lyophilized to remove CH_3OH and H_2O. The extracts were reconstituted in 0.5 ml D_2O containing 1 mM TSP, then transferred into 5-mm NMR tubes and analyzed by NMR spectroscopy. ^{1}H NMR spectra were acquired on a Bruker-AV600 spectrometer at 296 K. Standard 1D ^{1}H spectra were acquired with a "ZGPR" pulse sequence. 64 free induction decays (FIDs) were collected for each sample into 64 K data points over a spectral width of 12000 Hz with a relaxation delay of 6.5 μs and an acquisition time of 2.66 s.

Spectral processing and data transformation

The FIDs were multiplied by an exponential function corresponding to a 1 Hz line-broadening factor before Fourier transformation to increase the signal-to-noise ratio. The acquired NMR spectra were manually phased and baseline-corrected using MestReNova (V9.0, Mestrelab Research, Santiago de Compostela, Galicia, Spain) and referenced to the CH_3 resonance of lactate at δ 1.33 for plasma spectra and TSP at δ 0.00 for urine and tissue extract samples. The peaks observed were assigned based on their chemical shifts and signal multiplicity.

Each ^{1}H NMR spectrum of plasma, urine, or aqueous tissue extract was segmented into regions of 0.005 ppm using AMIX (v.3.8). The segments of δ 6.00-5.50 and δ 5.20-4.29 in the plasma spectra (δ 9.00-0.50) and of δ 6.02-5.45 and δ 5.35-4.24 in the urine spectra (δ 9.50-0.50) were removed to exclude the urea signal and the uncertainty of residual water signal. In the case of the chloroform/methanol tissue extract, the regions of δ 5.226-4.675 and δ 3.40-3.31 in the spectra were excluded to remove variation in residual water and methanol signal.

Metabolite identification

The peaks observed were assigned based on their chemical shifts and signal multiplicity.