Online Methods Supplement

PAH Clinical Measures

PAH patients were evaluated by our multidisciplinary team according to current guidelines (1). New York Heart Association functional class (NYHA), 6-minute walk distance, N-terminal pro B-type natriuretic peptide (NT-proBNP) and right heart catheterization data were extracted from our registry.

Metabolic Control Procedures

Stringent control procedures were implemented to minimize the influence of diet and physical activity on metabolic testing. For 3 days prior to metabolic testing, subjects were counseled by a registered dietitian to eat a balanced diet that contained at least 200 g of carbohydrate daily to stabilize muscle and liver glycogen stores (2). Subjects were asked to refrain from physical activity beyond typical activities of daily living for 48 hours prior to metabolic testing. Finally, subjects fasted overnight for at least 10 hours prior to metabolic testing. This approach has been used successfully to control for the influence of diet and physical activity in metabolic research (3-5).

Clamp Study Exclusion Criterion

Participants were excluded for the following: body mass index (BMI) $<25 kg/m^2$, nicotine use, known causes of pulmonary hypertension including left heart disease, pulmonary disease, sleep apnea, thromboembolic disease, connective tissue disease, congenital heart disease, portal hypertension, and/or chronic renal failure, or if they were diagnosed with type 2 diabetes or presented with clinical values indicative of type 2 diabetes as defined by the American Diabetes Association (HbA1c >6.5%, fasting blood glucose >126 mg/dl, 2-hour OGTT glucose >200 mg/dl).

Quantification of Insulin and C-peptide

We specifically utilized a radioimmunoassay approach to quantify insulin and C-peptide, which offers a methodological advantage over commercially available ELISA methods due to contamination by unintended quantification of the insulin precursor, proinsulin.

Quantitation of Free Fatty Acids in Cell Lysate Using HPLC Online Tandem Mass Spectrometry (LC/MS/MS)

All the fatty acid (FA) standards from 16 to 22 carbons including the saturated and unsaturated were purchased from Cayman Chemical (Ann Arbor, Michigan). A 20 µl sample of human plasma was mixed with 80 µl of methanol containing 2.5 ug/ml internal standard heneicosapentaenoic acid (FA(21:5); HPA) and vortexed for 30 sec. After centrifuging at 18,000 rcf for 10 min, 40 µl of supernatant was transferred into a vial for FA analysis by LC/MS/MS. A triple quadrupole mass spectrometer (Thermo Quantiva) was used for analysis of FA. A volume at 2 µl was injected onto a C18 column (Gemini, 3 µm, 2 x 150mm, Phenomenex) for the separation of FA species. Mobile phases were A (water containing 0.1% acetic acid) and B (methanol/acetonitrile (50/50) containing 0.1% acetic acid and 0.06% ammonium hydroxide). The run started with 75% mobile phase B from 0 to 2 min at the flow rate of 0.3 ml/min. Solvent B was then increased linearly to 100% B from 2 to 8 min and held at 100% B from 8 to 18 min. The column was finally re-equilibrated with 75% B for 8 min. The HPLC eluent was directly injected into the triple quadrupole Thermo Quantiva and the FA species were ionized using electrospray ionization at negative mode. All the fatty acids were analyzed using Selected Reaction Monitoring (SRM) and the SRM transitions (m/z) were their precursor to precursor ions (m/z) expressed as 255 > 255 for FA(16:0), 253 > 253 for FA(16:1), 283 > 283 for (18:0), 279 > 279 for FA(18:2), 277 > 277 for FA(18:3), 275 > 275 for FA(18:4), 311 > 311 for FA(20:0), 309 > 309 for FA(20:1), 307 > 307 for FA(20:2), 305 > 305 for FA(20:3), 303 > 303 for FA(20:4), 301 > 301 for FA(20:5), 339 > 339 for FA(22:0), 333 > 333 for FA(22:3), 331 > 331 for FA(22:4) 329 > 329 for FA(22:5), 327 > 327 for FA(22:6) and 315 > 315 for HPA (internal standard). Peak areas for all the FA species and the internal standards were integrated using the software Xcalibur. Internal standard calibration curves were used for quantitation of FA species in human plasma.

Quantification of plasma free- and acylcarnitines by LC/MS/MS

UPLC coupled to triple quadrupole mass spectrometer (LCMS-8050, Shimadzu Corporation, Kyoto, Japan) was used to quantify plasma free carnitine, acetylcarnitine, priopionylcarnitine and butyryl carnitine. Four volumes of cold methanol containing isotope labeled internal standards (10 µM each) was added to 1 volume of plasma to precipitate protein. 0.2 µl of the supernatant after centrifugation at 20,000 g, 4°C for 10 minutes was analyzed by injection onto a silica column (Luna® 5 µm Silica) 100 Å, LC column 150 x 2 mm, Phenomenex, #00F-4274-B0) at a flow rate of 0.35 ml/min using 2 Shimadzu LC-30AD pump system, Sil-30AC autosampler, interfaced with a Shimadzu 8050 mass spectrometer. A discontinuous gradient was generated to resolve the analytes by mixing solvent A (0.1% propanoic acid in water) with solvent B (0.1% acetic acid in methanol). Analytes and their respective isotope labeled internal standards were monitored using ESI in positive-ion mode with multiple reaction monitoring (MRM) of precursor and characteristic product ion transitions. The parameters for the ion monitoring were individually optimized. Various concentrations of analytes were mixed with internal standard mix to prepare the calibration curves for quantification of each analyte.

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