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Appendix from Kudryashova et al., "Profiling the role of mammalian target of rapamycin in the vascular smooth muscle metabolome in pulmonary arterial hypertension" (Pulm. Circ., vol. 5, no. 4, p. 000)

Supplemental methods

Sample preparation for metabolomic profiling analysis

Sample preparation from frozen cell pellets used the automated MicroLab STAR (Hamilton, Reno, NV). Recovery standards were added before the first step in the extraction process for quality control (QC) purposes. Sample preparation was conducted with a proprietary series of organic and aqueous extractions to remove the protein fraction while allowing maximum recovery of small molecules. The resulting extract was divided into two fractions: one for analysis by liquid chromatography (LC) and one for analysis by gas chromatography (GC). Samples were placed briefly on a TurboVap (Zymark) to remove the organic solvent. Each sample was then frozen and dried under vacuum. Samples were then prepared for the appropriate instrument, either LC mass spectrometry (LC/MS) or GC/MS.

Quality assurance (QA) and QC

For QA/QC purposes, a number of additional samples were included with each analysis. A selection of QC compounds were added to every sample, including those under test. These compounds were carefully chosen so as not to interfere with the measurement of the endogenous compounds. These QC samples were primarily used to evaluate the process control for each study as well as aid in data curation.

LC/MS, LC/MS^2 (tandem MS)

The LC/MS portion of the platform was based on a Waters ACQUITY ultraperformance liquid chromatography (UPLC) system and a Thermo-Finnigan LTQ mass spectrometer, which consisted of an electrospray ionization (ESI) source and linear ion trap (LIT) mass analyzer. The sample extract was split into two aliquots, dried, and then reconstituted in acidic or basic LC-compatible solvents, each of which contained 11 or more injection standards at fixed concentrations. One aliquot was analyzed with acidic positive ion–optimized conditions and the other with basic negative ion–optimized conditions in two independent injections using separate dedicated columns. Extracts reconstituted in acidic conditions were gradient eluted with water and methanol, both containing 0.1% formic acid, while the basic extracts, which also used water/methanol, contained 6.5 mM ammonium bicarbonate. The MS analysis alternated between MS and data-dependent MS^2 scans using dynamic exclusion.

GC/MS

The samples destined for GC/MS analysis were redried under vacuum desiccation for a minimum of 24 hours before being derivatized under dried nitrogen using bistrimethyl-silyl-triflouroacetamide (BSTFA). The GC column was 5% phenyl, and the temperature ramp was from 40° to 300°C in a 16-minute period. Samples were analyzed on a Thermo-Finnigan Trace DSQ fast-scanning single-quadrupole mass spectrometer using electron impact ionization. The instrument was tuned and calibrated daily for mass resolution and mass accuracy. The information output from the raw data files was automatically extracted as discussed below.

Accurate mass determination and MS/MS fragmentation (LC/MS, LC/MS/MS)

The LC/MS portion of the platform was based on a Waters ACQUITY UPLC and a Thermo-Finnigan LTQ-FT mass spectrometer, which had an LIT front end and a Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer back end. For ions with counts greater than 2 million, an accurate mass measurement could be performed. Accurate mass measurements could be made on the parent ion as well as fragments. The typical mass error was less than 5 ppm. Ions with less than 2 million counts required a greater amount of effort to characterize. Fragmentation spectra (MS/MS) were typically generated in a data-dependent manner, but if necessary targeted MS/MS could be employed, as in the case of lower-level signals.

Data extraction and QA

The data extraction of the raw MS data files yielded information that could be loaded into a relational database and manipulated without resorting to BLOB manipulation. Once in the database, the information was examined and appropriate QC limits were imposed. Peaks were identified with Metabolon's proprietary peak integration software, and component parts were stored in a separate and specifically designed complex data structure.

Compound identification

Compounds were identified by comparison to library entries of purified standards or recurrent unknown entities. Identification of known chemical entities was based on comparison to metabolomic library entries of purified standards. More than 1,000 commercially available purified standard compounds have been acquired and registered into the Laboratory Information Management System (LIMS) for distribution to both the LC and GC platforms for determination of their analytical characteristics. The combination of chromatographic properties and mass spectra gave an indication of a match to the specific compound or an isobaric entity.

Reaction schemes

The reaction schemes depicted in Figures 3–6 are based on pathways from KEGG databases as follows:

1. Amino sugar and nucleotide sugar metabolism: http://www.genome.jp/kegg-bin/show_pathway?map00520+C00043 (Fig. 3).
2. Fructose and mannose metabolism: http://www.genome.jp/kegg-bin/show_pathway?map00051.
3. Biosynthesis of unsaturated fatty acids: http://www.genome.jp/kegg-bin/show_pathway?map01040 (Fig. 4).
4. Fatty acid biosynthesis: http://www.genome.jp/kegg/pathway/map/map00061.html.
5. Glycerophospholipid metabolism: http://www.genome.jp/kegg/pathway/map/map00564.html.
6. Glutathione metabolism: http://www.genome.jp/kegg/pathway/map/map00480.html (Fig. 5).
7. Cysteine and methionine metabolism: http://www.genome.jp/kegg/pathway/map/map00270.html.
8. Nicotinate and nicotinamide metabolism: http://www.genome.jp/kegg/pathway/map/map00760.html (Fig. 6).

Supplementary figures

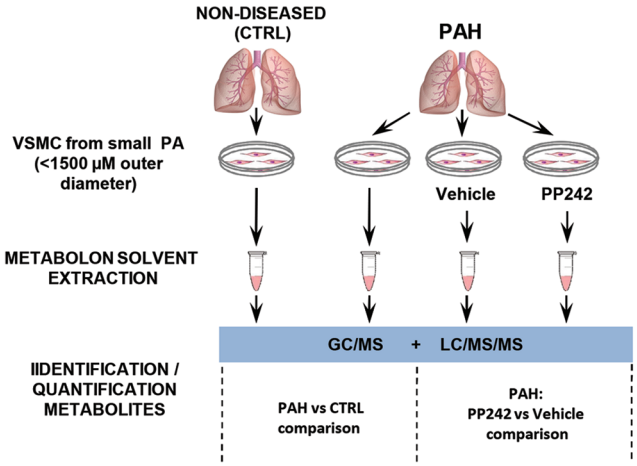

Figure S1. Experimental scheme of metabolomic profiling. Distal pulmonary artery (PA) vascular smooth muscle cells (VSMCs) from nondiseased (control) and idiopathic pulmonary arterial hypertension (PAH) subjects were snap-frozen, and cell pellets were subjected to liquid chromatography– and gas chromatography–based mass spectrometry analysis (LC/MS/MS and GC/MS, respectively). Comparisons between control and PAH groups and between PAH PAVSMCs treated with vehicle and those treated with PP242 were performed for each indicated metabolite.

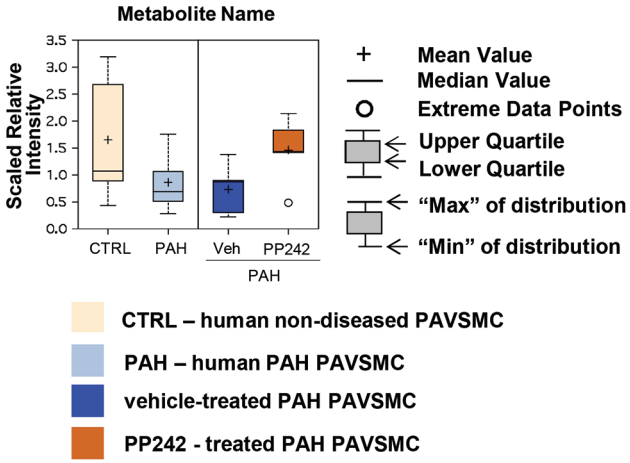

Figure S2. Metabolite box plots. Data for each metabolite obtained from pulmonary arterial vascular smooth muscle cells (PAVSMCs) from 5 subjects/group are presented as box-and-whiskers graphs. Data are quantity of metabolite in PAVSMCs, presented in arbitrary units specific to internal standards for each quantified metabolite and normalized by protein concentration. Light yellow: control (CTRL); light blue: pulmonary arterial hypertension (PAH) PAVSMCs; dark blue: vehicle-treated PAH PAVSMCs (Veh); orange: PP242-treated PAH PAVSMCs.