Biochemical Mapping of Metabolic Alterations in Lungs of Rat Embryos

Oliver Fiehn; Dinesh Kumar; Gert Wohlgemuth; Jesse Joad; Carol Hood; Kent Pinkerton; Tobias Kind

UC Davis, Davis, CA

Introduction

GC-TOF or LC-MS unbiased surveys of biological samples yield hundreds of resolved peaks per chromatogram. Statistical significant differences between semi-quantitative peak intensities can be routinely assigned to the classes of study designs. Metabolite peaks are then often called ‘putative biomarkers’ which must be validated in subsequent studies and confirmed to be specific for a diagnostic case. Consequently, the biomarker peaks must be unambiguously re-detectable over months in subsequent studies. This can best be performed by establishing standardized mass spectrometric metabolome databases. Secondly, valid biomarkers require a clear route to annotation of novel compounds to be implemented in routine clinical screens. Thirdly, interpretation of differential regulation of the identified metabolites should be guided by biochemical mapping to be of biomedical relevance.

Methods

PBS-perfused rat lungs were fresh frozen prior to homogenization and extraction. GC-TOF mass spectrometry (Leco Pegasus IV) was performed using Gerstel automatic liner exchange and cold injection conditions. GC-TOF spectra were filtered by the in-house BinBase mass spectral database. Metabolites were identified by a retention index/MS library of 713 authentic standards including PubChem and KEGG identifiers. Quantitative results were statistically evaluated, and significant differences were mapped to biochemical and chemical databases by open-access tools. Unknown metabolites were annotated using chemical ionization GC-TOF MS (Waters GC-T) and database queries according to accurate mass and accurate isotope data. Lipid fingerprints complemented the survey using nanoelectrospray (Advion) coupled to FT-ICR MS (ThermoFisher LTQFT). Data were aligned by the Expressionist software (Genedata).

Results

Timed pregnant rats were subjected to environmental tobacco smoke daily at 1 mg/m^3 for 6 hours each day in controlled chambers from gestation day 5 to gestation day 20 (term is 21 days). After sample preparation, lung tissue GC-TOF chromatograms showed on average 852 deconvoluted peaks. However, many of these peaks were not consistently detectable in subsequent analysis of biological replicate samples. Consequently, this number of peaks was reduced to a much cleaner data set of 305 peaks using the in-house database BinBase by employing mass spectral metadata (peak purity, unique masses, s/n, apex masses). 155 of these metabolites were unambiguously identified by both retention index and mass spectral match criteria using a recently released mass spectral library and step-wise increase of similarity thresholds based on peak purity and peak abundance. Missing data were subsequently replaced from ion traces of unprocessed netCDF files, yielding a coherent result data sheet of 31,570 metabolic values (Fig. 1). Raw and processed data are publicly available for downloads from http://fiehnlab.ucdavis.edu:8080/m1/main_public.jsp.

Multivariate statistics clearly proved that metabolic phenotypes in developing lungs were altered by cigarette smoke. 46 metabolites were differentially regulated in fetal lungs which were exposed perinatally to environmental tobacco smoke, indicating changes in carbohydrate and lipid metabolism. Only six...
statistically significant differences were annotated as unknown biomarkers. These peaks were subjected to identification by GC-TOF under chemical ionization and altered derivatization schemes. Additionally, relative changes of polar membrane lipids were investigated by direct infusion nanoelectrospray LTQ-FTICR mass spectrometry, and identification was achieved by using LipidMaps as template to construct accurate mass and MS/MS lookup tables (fig. 2). Analysis of quantitative changes in ratios of polar lipids in lungs showed a tendency of up-regulation of lipids with higher degree of unsaturation and longer acyl carbon chain lengths under chronic exposure to environmental tobacco smoke.

Metabolic differences for small metabolites (<500 Da) for lungs of dams and fetuses were eventually mapped onto chemical similarity networks (Tanimoto distances) to enable straightforward interpretations of major metabolic disruptions (Fig. 3). Notably, fetus lungs were only indirectly smoke exposed through the dam’s blood streams. In contrast to lungs of dams, fetus lungs showed major down regulation (blue nodes) for free fatty acids which might lead to impairment of lung function and asthmatic conditions at later stages of life. Interestingly, a similarity of metabolic changes in lungs of fetuses and dams was observed for down regulation of purines such as adenosine and guanosine which may indicate lower rates of metabolic turnover in smoke-impaired lungs. Only few up-regulations of metabolites (red nodes) were observed such as for key energy metabolites like ketone bodies and sugar phosphates. Metabolites that were not altered at statistically significance levels of p<0.05 are displayed as small yellow nodes. Peaks that were not identified by the Fiehn mass spectral libraries are not displayed in these Cytoscape network graphs.

**Acknowledgments**
This research was funded by grant 5R01ES13932 of the U.S. National Institute of Environmental Health Sciences, granted to Oliver Fiehn.