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Environmental Tobacco Smoke Alters Metabolic Systems in Adult Rats

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Abstract

Human exposure to environmental tobacco smoke (ETS) is associated with an increased incidence of pulmonary and cardiovascular disease and possibly lung cancer. Metabolomics can reveal changes in metabolic networks in organisms under different physio-pathological conditions. Our objective was to identify spatial and temporal metabolic alterations with acute and repeated subchronic ETS exposure to understand mechanisms by which ETS exposure may cause adverse physiological and structural changes in the pulmonary and cardiovascular systems. Established and validated metabolomics assays of the lungs, hearts, and blood of young adult male rats following 1, 3, 8, and 21 days of exposure to ETS along with day-matched sham control rats ($n = 8$) were performed using gas chromatography time-of-flight mass spectrometry, BinBase database processing, multivariate statistical modeling, and MetaMapp biochemical mapping. A total of 489 metabolites were measured in the lung, heart, and blood, of which 142 metabolites were identified using a standardized metabolite annotation pipeline. Acute and repeated subchronic exposure to ETS was associated with significant metabolic changes in the lung related to energy metabolism, defense against reactive oxygen species, substrate uptake and transport, nucleotide metabolism, and substrates for structural components of collagen and membrane lipids. Metabolic changes were least prevalent in heart tissues but abundant in blood under repeated subchronic ETS exposure. Our analyses revealed that ETS causes alterations in metabolic networks, especially those associated with lung structure and function and found as systemic signals in the blood. The metabolic changes suggest that ETS exposure may adversely affect the mitochondrial respiratory chain, lung elasticity, membrane integrity, redox states, cell cycle, and normal metabolic and physiological functions of the lungs, even after subchronic ETS exposure.

Graphical Abstract

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Supporting Information

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Metabolite descriptors and cytoscape node attributes (XLSX)

Figures of network graphs of heart and blood tissues and the full results of statistical analysis (PDF)

The authors declare no competing financial interest.

INTRODUCTION

Environmental tobacco smoke (ETS) is a product of the approximately 1.2 billion worldwide smokers. ETS is generated by smoke exhaled from a smoker's lungs, along with smoke that comes from the smoldering end of the cigarette, cigar, or pipe between puffs. ETS, also referred to as second-hand smoke, passive smoke, or involuntary smoke, is a health hazard associated with an elevated incidence of pulmonary, cardiac, and neural disorders in nonsmokers.¹ An estimated 126 million nonsmokers in the United States are exposed to ETS in homes, vehicles, workplaces, and public places.² In 2004, it was estimated that ETS exposure caused 603,000 deaths (63% from ischemic heart disease, 27% from lower respiratory infections, 6% from asthma, and 3.5% from lung cancer) worldwide, a figure representing 1.0% of all global mortality.¹ Exposure to ETS has been associated with compromised lung function in children, coronary atherosclerosis,³ ischemic heart disease,⁴ pulmonary morbidity in children with asthma,⁵ increased risk of coronary heart disease,⁶ and lung cancer^{7,8} among nonsmokers. The chemical composition of ETS is formed by over 4000 chemicals, many of which have been identified as potential carcinogens, toxicants, and irritants.^{2,9} Polycyclic hydrocarbons, nitrosamines, and heterocyclic aromatic amines are among the most studied carcinogenic compounds in tobacco smoke.^{10–12} These compounds induce different cascades of cellular events, including immunological reactions,¹³ mutagenesis,¹⁴ and metabolic alterations.

Comprehensive profiling of metabolites, known as metabolomics, can reveal changes in metabolic networks in organisms under different physio-pathological conditions.^{15–18} This technology has been used to demonstrate the effect of mainstream cigarette smoke on the metabolome of human lung epithelial cells grown in vitro.¹⁵ Although, an entire metabolome cannot be studied using a single analytical methodology, use of gas chromatography time-of-flight mass spectrometry (GC-TOF-MS) and efficient algorithms¹⁹ can yield reliable quantitative data for a majority of the metabolites in primary metabolic

pathways likely to be affected by exposure to ETS. Such data can be used to begin to understand disease pathogenesis, especially over a known period of time.

In this study, the lungs, hearts, and blood of male adult rats were collected following 1, 3, 8, and 21 days of progressive daily exposure to ETS. The biological samples were analyzed using GC-TOF-MS and BinBase database processing.²⁰ Metabolomics data were mapped into context-specific metabolic networks using MetaMapp²¹ and visualized in Cytoscape²² as network graphs overlaid with statistical outputs. Our analyses revealed that ETS causes alterations in metabolic networks, especially those associated with lung structure and function and found as systemic signals in the blood.

EXPERIMENTAL SECTION

Animal Exposure to ETS, Organ Collection, and Preparation.

Pathogen-free male adult (2.5 months old) Sprague–Dawley rats were purchased from Zivic Laboratories (New Castle, PA). A total of 64 rats were divided into 2 groups. One group was exposed to ETS for up to 21 days, while the second group was exposed only to filtered air (controls). Animals were necropsied after 1, 3, 8, or 21 days of exposure. For each time-point, 8 controls were used, along with 8 animals exposed to ETS. The rats were quarantined for 1 week prior to ETS exposure in plastic cages with pelleted bedding during which time they became acclimated to a 12 h light/12 h dark cycle. Animals had access to water and laboratory rodent diet 5001 ad libitum before, during, and after exposure. The rats were housed two per cage. Rats were exposed to filtered air or ETS (1 mg/m³ for 6 h each day) from a smoking apparatus housed at the University of California, Davis, Center for Health and the Environment. Humidified 3R4F research cigarettes (www.3r4f.com; Lexington, KY) were used. An automatic, metered puffer smoked the cigarettes under U.S. Federal Trade Commission conditions (35 mL puff volume, 2 s duration, and 1 puff per minute). The sidestream smoke from the smoldering end of each cigarette along with the puff volume were passed through a conditioning chamber, aged, and diluted with fresh filtered air and delivered to the rats in whole body exposure chambers.

Rats were euthanized with an intraperitoneal injection of pentobarbital. Whole blood was collected, and the lung and heart tissues were perfused in situ in the thoracic cavity with phosphate saline buffer (PBS) and then removed. Approximately, 20 mg of lung and heart was flash-frozen and pulverized in 2 mL Eppendorf tubes (2 min, 25 spins per seconds, with 20 mm i.d. metal balls in a MM300 ball mill (Retsch, Germany)). Subsequent extraction was done by adding 1 mL of a one phase mixture of degassed isopropanol/acetonitrile/water (3:3:2) and mixing and shaking at –20 °C for 5 min. Tubes were centrifuged for 30 s at 12,800g, and the supernatant was collected and concentrated to complete dryness in a vacuum concentrator.

Metabolomics Analysis.

The samples were derivatized in two steps with methoxyamine hydrochloride followed by a silylation reaction with *N*-methyl-*N*-trimethylsilyl trifluoroacetamide (MSTFA) for GC-TOF-MS analysis, as previously described.¹⁶ A mixture of 50 compounds with known

concentration was used as a positive control, and a reagent blank was used as a negative control. A fatty acid methyl ester (FAME) mixture of C8 through C30 was added to the derivatized samples as retention indices markers. Derivatized samples were analyzed by gas chromatography–mass spectrometry (GC-MS) on an Agilent 6890 GC-LECO Pegasus III TOF equipped with a Cooled Injection System (CIS4), an Automated Linear Exchange system (ALEX), and a Multi Purpose Sampler (MPS, all Gerstel). The injector was run with an initial temperature of 50 °C and ramped to 275 °C at a rate of 12 °C/s. The injection volume was 0.5 μ L, and injector mode was splitless with a purge time of 25 s. GC conditions were set with a programmed oven temperature of 50 °C, held here for 1 min, then ramped to 330 °C at a rate of 20 °C/min, and held at 330 °C for 5 min with the carrier gas flow rate at 1 mL/min. The GC column was RTX-5 MS, 30 m long, 0.25 mm i.d. $\times$ 0.25 μ m film with a 10 m integrated guard column. The transfer line and ion source temperature were set at 280 and 250 °C, respectively. Solvent delay was adjusted to 4.5 min, and MS acquisition was optimized to 17 spectra per scan, using a mass range of 80 to 500 m/z . The multichannel plate (MCP) detector voltage was adjusted to 1850 V. Data processing: Chromatogram acquisition, data handling, automated peak deconvolution, and export of spectra were automatically performed by the Leco ChromaTOF software (v2.32). Peak picking was achieved in ChromaTOF (v2.32) at signal/noise levels of 5:1 throughout the chromatogram, with baseline subtraction just above the noise level, no smoothing, 3-s default peak widths, automatic mass-spectral deconvolution and peak detection, and export of result spectra as *.csv files, in addition to export of raw data in open-access *.cdf formats. Data were further processed using the algorithms in the open-source BinBase metabolome database.²³ This algorithm used the following settings: validity of chromatogram (<10 peaks with intensity >107 counts s⁻¹), unbiased retention index marker detection (MS similarity >800 and exceeding thresholds for ion-ratio abundances for high m/z marker ions), and retention index calculation by fifth-order polynomial regression. Spectra were cut to 5% base peak abundance and matched to database entries from most- to least-abundant spectra, using the following matching filters: retention index window $\pm$ 2000 units (equivalent to about $\pm$ 2 s retention time), validation of unique ions and apex masses (unique ion must be included in apex masses and present at >3% of base-peak abundance), mass spectrum similarity that must fit criteria dependent on peak purity and signal/noise ratios, optional ion-ratio settings to distinguish peaks with high similarity, and a final isomer filter (annotating the isomer spectrum with the closest RI fit). Signal intensities were reported as peak heights, using the unique ion as default, unless an alternative quantification ion was manually set in the BinBase administration software Bellerophon. All known artifact peaks such as internal standards, column bleed, plasticizers, or reagent peaks were assigned by BinBase but not exported for further statistical calculations.

Metabolites were identified using the Fiehnlib libraries consisting of >1,000 authentic compounds and referenced using PubChem identifiers.²⁴ Daily quality controls were used comprising method blanks and five-point calibration curve samples of 31 pure reference compounds, which were repeatedly analyzed over the full analytical sequence, in addition to injection of one QC sample for every 10 biological samples. A quantification report table was produced for all database entries that were positively detected in >50% of the samples of a study design class (as defined in the SetupX database). This procedure results in

10–30% missing values, which could be caused by true negatives (compounds below the detection limit) or false negatives (compounds present but did not match quality criteria in the BinBase algorithm). A subsequent postprocessing module was employed to automatically replace missing values from the *.cdf files with the following parameters: for each positively detected metabolite, the average retention time was calculated for the day of analysis. Subsequently, for each chromatogram and each missing value, intensity of the quantification ion at this retention time was extracted by seeking its maximum value in a retention-time region of ± 1 s and subtracting the minimum (local background) intensity in a retention-time region of ± 5 s around the peak maximum. The resulting report table, therefore, did not contain any missing values. Replaced values were labeled as “lower confidence” by color-coding. Data normalization: Results were normalized by calculating the sum intensities of all identified compounds for each sample and dividing all data associated with a sample by the corresponding metabolite sum. The resulting data were multiplied by the average sum of all identified metabolites detected in the study (total average metabolome transformation), disregarding unknown metabolites because these might potentially also represent artifacts. Intensities of identified metabolites with more than one peak (e.g., for the syn- and antiforms of methoximated reducing sugars or amino acids with different derivatization status of amine groups) were summed to only one value in the transformed data set. The original nontransformed data set was retained for retrospective analysis. When comparing classes of samples with biologically different sum concentrations of identified metabolites ($P < 0.05$), these class averages were used for mean transformations. Result files were exported and processed by metabolomics BinBase database.²³

Statistical Analysis and MetaMapp Mapping.

The data matrix was normalized to the sums of total signals in each chromatogram. Data were normalized to the sum of all identified metabolites. StatSoft Statistica data miner version 8 (Tulsa, OK) was used for calculations of analysis of variance. Fold changes were calculated by dividing the median of metabolites in the ETS group by median of metabolites in the filter aired group. Using a cutoff of <0.5 p -value, significantly altered metabolites were identified. Metabolites were mapped into a biochemical network using the MetaMapp software.²¹ MetaMapp software enables mapping and visualization of detected metabolites in the context of metabolic networks generated using biochemical and chemical similarities among metabolites with a similarity cutoff of a 0.70 Tanimoto score and visualized in Cytoscape using an organic layout algorithm. Fold changes were mapped to node size, and direction was mapped to node color, where blue means down-regulated and red means up-regulated metabolites. For clarity, labels of the unchanged metabolites are not displayed.

RESULTS

At the concentrations of filtered environmental tobacco smoke used in this study, there were no overt or statistically significant differences for physiological parameters such as body weight differences or tissue weight differences and no differences in inflammatory status. Metabolic phenotypes precede such overt physiological differences, including differences in

lung function. For this reason, we utilized metabolomic tools to investigate differences that may eventually lead to respiratory problems.

Description of Pulmonary and Cardiovascular Metabolomics Data Sets.

A total of 489 high quality metabolic peaks were detected in the heart, lung, and blood samples (Figure 1) analyzed at four time-points. From these time-points, a total of 142 peaks were annotated as structurally identified metabolites by the BinBase peak annotation algorithm.²⁰ 101 peaks were specifically detected only in blood but not in the other two organs (Figure 1a). This specificity analysis is validated by detecting structurally identified metabolites, such as hippuric acid and cholic acid (Figure S1), that have been previously reported for plasma but not for lung or heart tissues (Human Metabolome Database). Other identified metabolites, such as glycine and taurine, are detected across heart, lung, and blood samples but with drastically different relative abundance for each sample type (Figure S1).

Exposure to environmental tobacco smoke caused statistical differences to control animals in each organ. A summary of all of detected metabolites is provided in Table S1. Interestingly, lung metabolism showed the highest number of significantly altered metabolites ($p < 0.05$) at 1, 3, and 8 days of exposure (Figure 1b–e). In order highlight biochemical modules affected by ETS exposure, we have mapped the metabolomics results onto organ-specific MetaMapp network graphs. MetaMapp clusters represent canonical metabolic pathways but also map metabolites (by chemical structure similarity) that lack annotation in biochemical pathway databases.

Lung metabolism went through a dynamic response along the 28 days of exposure, yielding large differences in number and magnitude of differentially altered metabolites (Figure 2). In comparison, plasma metabolites only showed a strong metabolic response after subchronic ETS exposure but much less pronounced at acute, short-term exposure (Figure S3). Heart muscle metabolism (Figure S2) was impacted much less than lung metabolites and almost fully recovered to normal conditions under subchronic exposure. Surprisingly, all three organs showed different metabolic modules being affected under acute and subchronic ETS exposure. Table 1 shows fold-changes of metabolites that were significantly altered in at least two comparisons ($p < 0.05$).

Acute Response: Days 1 and 3.

Lung. Immediately following exposure to ETS (1 day), a range of energy, lipid, and nucleotide metabolites showed significant changes in the lung compared to the control (Figure 2). For example, levels of TCA cycle intermediates, lactic acid, and 3-hydroxybutanoic acid (a ketone body) were increased, indicating a profound effect of ETS exposure on energy metabolism. Membrane lipid components, such as glycerol-3-galactoside, and redox-response metabolites, such as glutathione, a major reactive oxygen species (ROS) scavenging disulfide, were also increased. Xanthine and uric acid, indicators of nucleotide metabolism, were increased under ETS exposure, whereas hypoxanthine was found to be decreased, indicating the differential activity of xanthine oxidase, an ROS generating enzyme. This induction was associated with altered levels of intermediates of the pyrimidine salvage pathway.

After 3 days of ETS exposure, the lung showed lower metabolic changes compared to those of the control, indicating an adaptive stress response. The majority of the metabolites altered at 1 day of ETS exposure returned to normal levels, with notable exceptions in the carbohydrate cluster of metabolites. Significant, but small increases in the levels of glycolytic intermediates and cytidine-diphosphate were observed after 3 days of ETS exposure.

Cardiovascular System (Heart Muscle and Blood).

At acute ETS exposure time-points (days 1 and 3), carbohydrate and lipid metabolism were found dysregulated in heart muscle tissues (Figure S2). Strikingly, carbohydrates, especially sugar phosphates, were decreased on day 1, and a majority of lipids were increased on day 3. Plasma levels showed changes in lipid, organic acid, carbohydrate, and amino acid metabolism under acute exposure to ETS. After 1 day of acute exposure, blood metabolites elaidic acid (lipid cluster), threonate, and dehydroascorbate (organic acid cluster) and fructose (carbohydrate cluster) were decreased, and only urea was increased. On 3 days of exposure, only 2-ketoisocaproic acid, glutamate, tryptophan, and dihydro-3-coumaric acid, all in the amino acid metabolite cluster, were significantly altered (Figure S3).

Chronic Response: Days 8 and 21.

Lung. At 8 days of ETS exposure, the lung displayed alteration of antioxidant metabolism. Intermediates of the pentose phosphate pathway (that produces NADPH) were increased, while levels of glutathione and glutamate; threonic acid, an indicator of ascorbate oxidation; and taurine (antioxidant) were decreased (Figures 2 and 3). Energy metabolism, as reflected by the TCA cycle intermediates malate, citrate, and fumarate, continued to be increased from 1 day to 8 days of ETS exposure. Accumulation of squalene, an intermediate of cholesterol biosynthesis, coincided with lower levels of cholesterol. Similarly, the product of leucine degradation, 2-ketoisocaproic acid, was increased along with a decrease in the levels of leucine. Furthermore, the levels of five additional amino acids were found to be down-regulated in ETS-exposed lungs on day 8. There were still metabolic alterations in the lung after 21 days of ETS exposure, although not as dramatic as after 8 days of exposure. Importantly, pentose phosphate pathway metabolites were still up-regulated after 21 days of ETS exposure, similar to the pyrimidine salvage pathway. Cholesterol biosynthesis was still down-regulated. Low levels of hypoxanthine suggest that xanthine oxidase was still induced, but levels of xanthine and uric acid were not changed. A decrease in the levels of fructose-6-phosphate coincided with an increase in the levels of n-acetyl-D-hexosamine indicating that long-term ETS exposure can promote glycosaminoglycan accumulation in the lung.

Cardiovascular System.

The metabolic responses observed in the heart at acute time-points (days 1 and 3) were not observed at repeated, subchronic time-points (days 8 and 21). On day 8 of ETS exposure, no sugar phosphates or organic acids were significantly altered, but six fatty acids along with two monooliens were increased. Additionally, in the amino acid cluster of metabolites, only oxoproline and pantothenic acid were increased on day 8. Similar to the lung, the heart refocused energy metabolism toward the pentose phosphate pathway on day 8. This was accompanied by decreased levels of citric acid and 2-hydroxyglutamate, suggesting a low

activity level of isocitrate dehydrogenase. On day 21, hypoxanthine was increased, pointing toward xanthine oxidase being activated in the heart when chronically exposed to ETS.

Valine, isoleucine, alanine, sucrose, fructose, alpha ketoglutarate, and urea were significantly altered on exposure day 8 in the blood. The most striking response was observed on exposure day 21, when a large number of metabolites representing almost every metabolic cluster were decreased or increased in blood samples. Various saturated fatty acids, glycerate, alpha ketoglutarate, sucrose, and galactose-6-phosphate were all decreased, whereas unsaturated fatty acids, ketone bodies, glucose, and nine different amino acids had accumulated to higher levels in blood plasma. Elevation of unsaturated fatty acids could be an adaptive mechanism to lower the burden on cardiac muscles that were under stress at acute time-points but scarcely showed metabolic alterations at subchronic time-points.

DISCUSSION

Metabolic Dysregulation with Time.

Over the course of the ETS exposure, every metabolic module was dysregulated at least once in at least one organ (Figures 2 and 3). Biochemical and physiological roles of the altered metabolites in the lung, heart, and blood are summarized in Figure 3 and Tables S2 and S3. Drastic differences were observed in the magnitude of metabolic dysregulation at different time-points. As expected, more metabolites were altered in lung tissue (after 1 and 8 days of ETS exposure) than in the heart and blood. The dysregulations can be categorized into two distinct temporal phases, acute and subchronic responses. Interestingly, at 21-day subchronic ETS exposure, the most changes were found in plasma metabolites, indicating that many organs may have been affected by that time that contributed systemically to changes in plasma levels. Another example for large temporal differences was found for lung metabolism. A range of lung compounds were differentially regulated on days 8 and 21 but not on days 1 and 3 (Figure 3). In fact, most lung metabolites that were found dysregulated after 1 day ETS exposure were regulated back to normal levels at 3 days of exposure, indicating a strong adaptive response against short-term stresses that could not be maintained for longer time periods. We also found clear differences for heart muscle metabolism for which initial acute responses were noted but that were regulated back to minimal metabolic changes under subchronic exposure to ETS.

Interpretation of Biological Processes after ETS Exposure.

ETS disturbs intracellular redox balance and causes oxidative stress in the lung. Reactive oxygen species (ROS) in ETS interact with airway epithelial cells and can initiate a cascade of cellular events that can adversely affect respiratory physiology.²⁵ ROS are involved in the pathogenesis of respiratory diseases such as asthma, COPD, or hypersensitivity through impaired signaling pathways.²⁶ Increased glutathione, a disulfide in the lungs, which can scavenge ROS originating from smoke,²⁷ demonstrated fluctuating patterns with reduced levels following 1 day and elevated levels at 8 days of ETS exposure. These changes are likely to reflect an acute reaction to tobacco smoke with an adaptive response over time at an elevated level of expression. Altered levels of hypoxanthine, xanthine, and uric acid indicate that xanthine oxidase activity is increased to produce uric acid, which is a known

antioxidant.²⁸ However, increased xanthine oxidase activity also results in accumulation of intracellular ROS and imbalance in the redox state in the lung, which may result in toxic mechanisms, such as impaired mitochondrial respiration, oxidation of lipids, and apoptosis.²⁶ Subchronic response in lungs to ETS exposure can be characterized by the activation of antioxidant mechanisms (Figure 3) to scavenge the accumulating reactive oxygen species and byproducts of cell-repair.

At later time-points, primary metabolic pathways in the lung shifted from ATP generation toward NADPH generation (via increase in levels of pentose phosphate metabolites), a possible reflection of an adaptive, protective response. Biochemically, NADPH is utilized in the glutathione cycle, likely to scavenge ROS generated by ETS exposure. Accumulated TCA intermediates and increased PPP intermediates (Figure 3) support the shift in metabolic pathways. Cigarette smoke causes increased expression of pentose phosphate pathway (PPP) genes.²⁹ Exposure to ETS can hamper the electron transport chain (ETC) in mitochondria,³⁰ which can cause accumulation of TCA intermediates and lactate.³¹ Increased levels of lactate support a low cellular respiration rate due to a lower activity of the ETC. Accumulation of the TCA cycle intermediate was additionally supported by finding an increase in the ketone body beta-hydroxybutyrate under acute ETS exposure. Ketone bodies are converted into acetyl CoA³² which can lead to both increased TCA cycle intermediates and increased levels of fatty acids (used for phospholipid surfactant production). These processes had been proposed as preferential use of ketone bodies for rat lungs.³³

Tobacco smoke lowers the expression of the gene encoding peroxisome-proliferator-activated receptor gamma (PPAR gamma),³⁴ which regulates the beta oxidation of fatty acids.³⁵ The lung synthesizes surfactant phospholipids and proteins and connective tissue proteins (collagen and elastin) and removes toxic substances from the blood. These biochemical processes may be affected by ETS as they require high energy input by well-coordinated energy metabolism in the lung.

ETS exposure dysregulates arachidonate metabolism, which may result in an altered immune response. Cigarette smoke induces destruction of membrane lipids in lung epithelial cells,¹⁵ and ROS can attack membrane lipids and polyunsaturated fatty acids.^{36,37} The linings of rat airways contain very high concentrations of PUFA-containing phospholipids that generate lipid mediators, such as leukotrienes.³⁸ Increased arachidonate (Figure 3) may be used to produce inflammatory factors, such as prostaglandin and leukotrienes, as inflammatory cells (e.g., leukocytes) are recruited to the airways with ETS exposure.³⁹ The immune-modulatory role of arachidonate can be supported by the observation that cigarette smoke induces PLA2 (phospholipase 2) to release free fatty acids from membrane phospholipids and induce COX-2 (cyclo-oxygenase), which initiates an inflammation response.^{40,41}

Increase in the levels of PUFA suppresses the cholesterol biosynthesis in ETS exposed lungs. Cholesterol is a major component of lipids in the lung surfactant system.⁴² Sterol regulatory-element-binding proteins (SREBP) regulate cholesterol biosynthesis,⁴³ and PUFAs are known to suppress the expression of SREBP-1 and lower cholesterol levels.^{44,45} Low levels of cholesterol and cholesterol biosynthesis intermediate squalene (Figure 3) were

observed in the lung at all the time-points of exposure. ETS-mediated lipid destruction may, therefore, lead to down-regulation of cholesterol biosynthesis.

Our data in the study indicate that ETS alters the DNA salvage pathway activity, even after a relatively short exposure. Those alterations could also, in turn, affect DNA replication and repair mechanisms. The alterations we observed in pyrimidine metabolism intermediates suggest that ETS may specifically change the activity of the cytosolic enzyme 3-ureidopropionase. Epithelium and endothelium cell layers are recycled at a high rate requiring active cell division and DNA metabolism.⁴⁶ Thus, by impairing DNA metabolism, ETS can disturb the maintenance and repair of the alveolar epithelium.

Our results suggest that lung elasticity may be compromised following acute and repeated, subchronic exposure to ETS. Lung elasticity is required for normal respiratory gas exchange,⁴⁷ and decreased elasticity is seen in the progression of several lung disorders (e.g., asthma or emphysema)⁴⁸ in combination with altered surfactant protein metabolism, further complicating lung function, even under moderate conditions of ETS exposure. The mechanical properties of lung parenchyma are determined by collagen, elastin, and proteoglycans.⁴⁹ Cigarette smoke causes small airway remodeling by inducing precollagen biosynthesis and growth factors.⁵⁰ Increased hydroxyproline in ETS-exposed lungs indicates collagen remodeling, which may alter the mechanical properties of the lung parenchyma.

Besides the effects on primary metabolic pathways, ETS also impairs substrate uptake in the lung, which removes toxic substances.⁵¹ Taurine is a metabolite downstream of the disulfide compound cystamine, which is selectively transported into lung tissues by a dedicated substrate system⁵² and has several protective roles in pulmonary tissues.^{53–58} We observed a sharp decrease in the level of taurine in the lung following prolonged ETS exposure, which may result in lung injury.

Biological Processes in the Heart and Blood after ETS Exposure.

Acute ETS exposure alters glycolytic metabolism in the heart, which signifies a shift in preferred energy source for cardiomyocytes. GLUT4 transports hexose sugars into cardiomyocytes,⁵⁹ and ETS may suppress GLUT4 and hexokinase: decreased levels of a majority of sugar phosphates in the heart were observed at early time-points. In addition, due to low sugar availability, transport of fatty acids is elevated in heart muscles, as supported by increased levels of free fatty acids. However, increased levels of fatty acids cause lipotoxicity in heart muscles.⁶⁰ This finding may indicate that exposure to ETS might cause lipotoxicity in heart muscles.

For early time-points, the metabolomic composition of the blood did not change. However, repeated, subchronic exposure to ETS in our model led to massive changes in the levels of blood metabolites. This indicates that repeated or long-term exposure to ETS may affect multiple organ systems of the body. Consistent with a previous study,⁶¹ we have found that serum ascorbate levels are significantly lowered in blood plasma, along with a high rate of lipid peroxidation and accumulation of LDL cholesterol in nonsmokers exposed to ETS.⁶² It was also observed that plasma in children exposed to secondhand smoke contains low levels of antioxidants.⁶³ These data link the exposure to increased risk of coronary heart disease.

Previous studies have reported the effect of individual compounds in the tobacco smoke on few metabolic pathways in lung cells. For example, free radicals such as superoxide and hydroxyl radicals in the gaseous phase can induce ROS formation in lung tissues as well as lipophilic compounds such as phenolics, aldehydes, and polycyclic aromatic hydrocarbons in the particulate phase of the tobacco smoke.^{64–66} Metabolic effects observed in our study were likely caused by the sum of the toxic smoke chemicals but cannot be easily attributed to one or even a few specific compounds. Further metabolomics studies of lung cells exposed to the individual chemicals in the ETS would be required to find out the common and specific metabolic pathways altered by the chemicals.

CONCLUSIONS

ETS causes significant alterations to lung, heart, and blood metabolomics that change cellular, anatomical, and physiological homeostasis. Our metabolomics data of the pulmonary, cardiac, and blood (vascular) systems in the rat reveal local and systemic metabolic processes affected by ETS exposure. Since the metabolome is a representation of organismal phenotype, the evidence and mechanisms proposed herein assist in establishing the causal links between exposure to ETS and the genesis of respiratory and cardiovascular disorders.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

ETS	environmental tobacco smoke
GCTOFMS	gas chromatography and time of flight mass spectrometry
ROS	reactive oxygen species
PPP	pentose phosphate pathway
TCA	tricarboxylic acid
PUFA	polyunsaturated fatty acids
ETS	electron transport chain
COPD	chronic obstructive pulmonary disease

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Figure 1. Metabolomics data sets and differentially altered metabolomes in ETS exposed organs. (a) Total metabolites detected in lungs, hearts, and blood. Numbers represent the metabolites that were detected with high confidence in each sample type. (b–e) The magnitude and dynamics of altered metabolomes in lungs, hearts, and blood. The number represents altered metabolites in ETS exposed organs compared to a filtered air treated organ ($p < 0.05$). Days 1, 3, 8, and 21 represent the time-points of sample collection during the time course experiment. (B = blood, H = heart, and L = lungs).

Figure 2.

Metabolic dysregulation in lungs of rats under ETS stress. Each node is a structurally identified metabolite. See Experimental Section for details about network graph construction and visualization. The graphs show five major clusters of compounds representing sugars and sugar phosphates, fatty acids, amino acids, aromatics, and organic acids. Red edges are KEGG reactant pair annotation, and blue edges are chemical similarity annotations. The graph was visualized using organic layout in Cytoscape. Red nodes are increased metabolites, and blue nodes are decreased metabolites. The size of nodes reflects fold changes. For clarity, only altered metabolites ($p < 0.05$) are labeled.

Metabolites	Biological roles (structural and reactants)	Effect of exposure (day)			
		1	3	8	21
fumarate, citrate, malate	TCA cycle intermediates				
glucose-6-phosphate, fructose-6-phosphate	glycolysis				
lactate	Energy metabolism				
3-hydroxybutanoic-acid (ketone bodies)	Energy metabolism				
ribose, ribose-5-phosphate, ribulose-5-phosphate,	Pentose phosphate pathways				
oleic acid, linoleic acid, arachidonic acid and palmitoleic acid	Unsaturated free fatty acids				
1-mono-olein, 2 mono-olein, glycerol-3-galactoside, glycerol	Membrane lipids (galactolipids)				
cholesterol, squalene	Cholesterol biosynthesis/surfactant				
leucine and 2-ketoisocaproic acid	Leucine degradation				
threonic acid	Ascorbate metabolism (Oxidative. Stress)				
cytidine di-phosphate	Lipids in lung surfactant/Nucleotides				
beta-alanine	Pyrimidine salvage				
hypoxanthine, xanthine, uric acid, Inosine	Nucleotide metabolism, xanthine-oxidase,				
alanine, valine, phenylalanine, tryptophan, aspartate	Amino acid, protein synthesis, elastin components				
oxoproline, glutamate, glutathione	Glutathione metabolism				
taurine	Taurine uptake, antioxidant				
hydroxyproline	Collagen component				
inositol, inositol-monophosphate	inositol phosphatase activity, surfactant system component				
n-actyl hexosamine	Glycosaminoglycan component				

Figure 3.

Biological interpretation of the metabolic dysregulations in ETS-exposed lungs on acute and subchronic time-points. Altered pathways and biological mechanisms are highlighted as filled cells.

Fold Changes of Significantly Altered Metabolites in the Cardiovascular and Respiratory Systems of Adults Male Rats Exposed to ETS, in Comparison to Control Groups^a

metabolite name	blood				heart muscles				lungs			
	day 1	day 3	day 8	day 21	day 1	day 3	day 8	day 21	day 1	day 3	day 8	day 21
alpha ketoglutaric acid	-1.1	1.3	-1.4	1.4	-1.2	-1.3	-1.3	1	-1.6	2.5	3.0	1.3
2-monoolein	-1.6	1	-1.4	-1.8	4.0	3.4	1.2	1.2	2.7	1.4	2.2	-1.3
hypoxanthine	-1.7	1.1	-1.1	-1.6	1.0	1	1.1	1.4	-1.9	-1.4	-3.8	-4.1
tryptophan	1.0	1.3	-1.2	1.2	1.0	1.2	1.1	1.1	1.3	-1.2	-1.7	-1.8
ribose	-1.1	-1.8	-1.5	-1.3	-1.8	1.1	2.8	1.1	-1.3	1.2	16.9	8.8
glyceric acid	-1.1	1	1.2	1.6	-1.2	1.4	1.8	-1.1	1.3	1	-1.3	-1.4
3-phosphoglycerate	-1.2	-1.1	1.5	-1.8	1.0	1.5	3.2	1	4.3	-1.1	-1.4	1.1
squalene	-1.3	1.5	-1.2	-1.5	-1.2	1.3	1.1	-2.0	-1.7	1.2	1.7	1.5
fructose	-1.3	-1.1	1.3	1.0	-3.2	1.5	1.1	1.2	1.3	-1.3	-1.2	2.7
threonic acid	-1.4	1.2	1.4	-1.3	-1.5	-1.3	-1.1	-1.1	1.1	1.2	-2.0	-2.2
glutamic acid	-1.4	-1.1	-1.2	-1.1	-1.3	-1.8	-1.3	1	1.3	1	-38.7	-1.9
1-monoolein	-1.5	1.0	1.4	-1.5	1.4	3.4	1.4	1	5.7	1.7	2.2	-1.5
ribulose-5-phosphate	-1.6	1.1	-1.1	-1.4	-1.3	1.1	3.5	-1.1	-1.5	1.1	13.9	2.3
sucrose	2.1	-1.3	-2.7	-4.2	-1.1	1.4	-1.4	-2.0	-1.3	1.1	-1.1	1.3
oleic acid	1.4	-1.2	-2.6	1.9	1.3	1.8	1.3	1	3.4	-1.1	-1.1	-1.2
linoleic acid	1.3	-1.4	-1.6	2.2	1.3	1.6	1.2	1	3.4	-1.1	-1.1	-1.3
2-deoxyerythritol	1.2	1.3	-1.1	-1.1	-1.1	1.2	1	1.1	-1.4	2.1	1.6	1.3
hydroxylamine	1.2	1.3	-1.2	-1.4	-1.2	-1.1	-1.7	1.6	-2.0	1.3	1.2	1
aminomalonate	1.1	1.3	-1.1	-1.3	1.1	-1.1	-1.1	1.0	1.2	-1.2	-2.0	-1.6
fumaric acid	1.1	1.1	-1.4	1	-2.7	-1.2	1.4	-1.2	1.8	1.3	-1.1	-1.9
valine	1.0	1.1	-1.3	1.3	-1.1	-1.1	-1.1	1.2	1.2	-1.1	-2.4	1.0
malate	1.0	-1.1	1.2	1.0	-3.0	-1.3	1.7	-1.3	1.4	-1.2	-1.6	1
glucose-1-phosphate	1.0	1	-1.4	-1.3	-1.2	1.2	1	-1.1	1.4	-1.1	-1.5	-1.2
butane-2,3-diol	1	1	-1.6	-1.3	-3.5	1.4	1.6	1.2	-1.6	1.5	2.5	1.9
oxoproline	-1.1	1.3	-1.3	1	1	1.4	1.2	1	1.4	1.2	2.5	1.5
alanine	-1.2	1.0	-1.4	1.6	1.2	-1.2	-1.1	1	1.2	-1.1	-2.9	-1.2

	blood			heart muscles			lungs					
adenosine	-1.2	-1.3	-1.4	-1.4	1	1	-1.7	1	1.2	1.9	1.5	-1.3
lyxitol	-1.3	-1.3	1.2	-1.4	1.5	-1.1	1.6	1.4	-11.9	1.9	1.2	1.7
methanolphosphate	-1.3	2.2	-1.4	-1.6	-8.3	-1.1	1.3	-1.4	-24.6	1.5	-1.2	-1.6
shikimic acid	-1.3	1.5	1.1	-1.4	-1.3	-2.0	-1.2	1.2	1.3	1.1	-1.2	-1.9
cytidine-5'-diphosphate	-1.4	1.0	1.5	-1.1	1.1	1.1	1.4	1.3	2.5	2.9	-2.5	-1.7
fructose-6-phosphate	-1.4	-1.3	-1.1	-1.1	-48.0	2.7	19.4	-4.5	1.3	-1.4	-1.9	-1.4
azelaic acid	-1.5	1.1	-1.2	-1.4	-1.1	1.7	2.3	-1.4	1.0	1.2	2.0	1.4
galactose-6-phosphate	-1.6	1.2	-1.2	-1.4	-10.2	1.2	4.5	-2.3	1.1	-1.5	-1.4	-1.1
mannose-6-phosphate	-1.7	-1.1	-1.1	-1.4	-5.9	-1.3	2.0	-1.3	-8.4	2.0	-1.2	1.1
3-aminoisobutyric acid	-2.3	1.2	1	1.0	-1.4	-1.2	-1.1	1.1	-1.9	1.4	2.9	1.9
glutathione	-2.7	1.9	1	-1.1	1.1	-1.2	-1.2	1.2	2.9	1.0	-3.0	-1.4
urea	1.5	1.1	-1.2	1.0	1.4	1	-1.2	-1.1	1.4	1.1	-3.2	1.0
2-hydroxyvaleric acid	1.2	1.3	1.0	-1.2	-1.1	1.0	-1.2	1.2	-1.9	1.6	1.8	1.5
hippuric acid	1.1	-1.1	-1.1	1.4	1	1	1.0	-1.1	1.7	1.3	1.3	1.2
conduritol-beta-epoxide	1.1	-1.1	-1.3	1.3	1.5	1.5	1.2	1.2	1.5	1.2	1.3	1.0
citric acid	1.1	1.0	1	1.2	1.1	-1.1	-2.5	-1.3	1.4	-1.1	-2.5	1.1
2-hydroxyglutaric acid	1.1	1.1	-1.6	1	1	-1.1	-1.4	-1.1	1.1	1.2	1.7	-1.1
phenylalanine	1.1	1.0	-1.1	1.2	1	-1.1	1.2	-1.1	1.0	-1.1	-1.2	-1.3
glucose	1.0	1.2	1.1	2.0	-1.8	1.2	1.3	1.3	1.1	-1.6	1.2	1.2
myo-inositol	1.0	-1.1	-1.5	1.1	1	1.3	1	1	1.3	1.2	1.3	-1.1
creatinine	1	-1.4	-1.1	1.3	1.4	-1.1	-1.1	1	1.0	-1.3	-8.1	-2.1
ethanolamine	1	1.1	-1.3	-1.5	-1.2	1.2	-1.1	1.3	-1.3	1.0	1.4	1.5
leucine	1	1	-1.2	1.6	1.0	-1.1	1.0	1.2	1	-1.1	-3.3	-1.1
2-ketoisocaproic acid	1	1.6	-1.1	1.0	-1.4	-1.1	-1.4	1.1	-1.9	1.7	3.8	1.3
glucose-6-phosphate	1	-1.1	-1.1	1.1	-53.1	3.3	22.6	-5.8	1.3	-1.5	-1.7	1.1
4-hydroxyproline	1	1.1	-1.2	1.1	1	1.3	1.1	1	-2.5	1.0	1.5	1.2
maleic acid	-1.1	1.1	-1.3	-1.5	-1.1	1.1	-1.1	1.0	-1.3	1.2	2.0	1.3
erythronic acid lactone	-1.1	1.3	-1.2	-1.3	-1.2	-1.1	-1.1	-1.1	1.0	1.5	1.7	-1.3
3-hydroxypropionic acid	-1.1	1.1	-1.1	-1.2	-1.1	1.1	1.0	1.1	-1.3	1.2	2.2	1.9
arachidonic acid	-1.1	1.0	1	1.6	1.1	-1.1	1.1	1.1	1.9	-1.1	1.1	-1.1
xylulose	-1.1	-1.2	1.0	-1.6	-1.2	1.1	2.4	1.2	-1.4	1.7	1	-2.1

	blood			heart muscles			lungs					
<i>trans</i> -4-hydroxyproline	-1.2	1	-1.5	-1.2	1	1.0	1.1	-1.3	1.6	1.2	1.4	-1.5
aspartic acid	-1.2	-1.2	-1.1	1	1.1	1.1	-1.1	-1.1	1	1.0	-1.6	-1.3
thymidine	-1.2	-1.2	1.1	1.6	-1.2	1.1	-1.2	1.2	1.9	-1.2	1.5	1
icosanoic acid	-1.3	1.1	-1.4	1.2	1.2	1.7	1.3	-1.1	2.0	1.1	1.1	1.0
ascorbic acid	-1.3	-1.1	1.1	-1.2	-1.4	1.2	-1.5	1.3	-1.4	4.6	1.2	2.1
beta-alanine	-1.3	1.1	-1.1	-1.2	1.1	-1.1	1	-1.1	1.3	1	1.1	-1.3
cholesterol	-1.3	1.4	1	1.1	1.0	1.1	1.2	1.1	-1.4	-1.2	-2.5	-1.7
lactic acid	-1.3	-1.3	1.0	1.3	-1.1	-1.2	-1.2	1.1	1.4	1.3	-4.0	-1.2
adipic acid	-1.4	-1.3	-1.2	-1.7	-1.3	-1.2	-1.2	1.1	-1.6	1.8	1.2	1.2
arachidic acid	-1.4	1.3	1.0	-1.2	1.9	-1.5	1.2	-1.3	-1.4	1.1	1.3	1.4
benzoic acid	-1.5	-1.3	-1.1	-1.6	-1.2	1.2	-1.2	-1.8	-2.1	1.1	-1.6	1.0
inosine	-1.6	1	1.0	-1.3	1.1	1.2	1.1	1.2	-1.4	2.3	-1.3	-2.4
maltotriose	-1.6	-1.2	1	-1.3	-1.4	-1.4	-1.5	1.4	-2.0	1.4	1.1	1.2
terephthalic acid	-1.6	1.1	-1.1	-1.7	-1.1	-1.2	1	1.1	-1.4	1.8	2.3	1.1
glycerol-3-galactoside	-1.6	1.4	1.3	-1.4	-2.2	-1.2	1.3	-1.2	1.8	-1.5	-1.1	1.3
palmitoleic acid	-1.7	1.5	-2.3	-1.6	1.1	1.8	1.0	1.1	2.7	-1.2	1.5	-1.2
elaidic acid	-4.6	-1.2	1.2	1.2	1.3	1.5	1.3	1.0	5.1	1.0	1.3	-1.9

^aValues having a *p*-value (Student's *t*-test) of less than 0.05 are highlighted (bold). Only the metabolites that were significantly altered in at least two comparisons are shown in this table. Detailed results are provided in Table S2. Compounds are sorted by how many times they were significantly altered.