Postprint: Metabolomics Study of the Cortex in Benzo[a]pyrene-Exposed Rats

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Abstract

Objective: To analyze changes in endogenous small molecule metabolites in the rat cortex following benzo[a]pyrene (B[a]P) exposure using a gas chromatography/mass spectrometry (GC/MS)-based metabolomics approach, and to investigate its neurotoxic mechanism. Methods: Five-day-old SD rats were randomly divided into a control group and a B[a]P exposure group (2 mg/kg), and were administered intragastrically for 7 consecutive weeks to establish a B[a]P exposure model. After the exposure period, spatial learning ability was assessed using the Morris water maze (MWM); cortical neuronal ultrastructure was observed by electron microscopy; cortical metabolic profiles were detected by GC/MS, and differential metabolites between the two groups were screened using partial least squares discriminant analysis (PLS-DA) and two independent samples t-test. Metabolic pathways associated with differential metabolites were analyzed using Cytoscape software. Results: Compared with the control group, B[a]P-exposed rats exhibited longer escape latencies ($P<0.05$) and shorter target quadrant dwell times ($P<0.05$). B[a]P-exposed rats also displayed widened synaptic clefts, thickened postsynaptic membranes, and cytoplasmic swelling. There were 18 differential metabolites in the cortex between the two groups (VIP>1, $P<0.05$). Analysis of these differential metabolites revealed 9 pathways associated with B[a]P neurotoxicity mechanisms, which involved amino acid metabolism, tricarboxylic acid cycle, and vitamin B3 (niacin and nicotinamide) metabolism. Conclusion: B[a]P can interfere with normal metabolism in the organism, and its neurotoxic mechanism may be related to disorders of amino acid metabolism, tricarboxylic acid cycle, and vitamin metabolism.

Full Text

Title and Authors

Changes of Cerebral Cortical Metabolomics in Rats Following Benzo[a]pyrene Exposure

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Abstract

Objective: To analyze changes in endogenous small molecule metabolites in the rat cerebral cortex following benzo[a]pyrene (B[a]P) exposure and explore the underlying neurotoxic mechanisms using a gas chromatography/mass spectrometry (GC/MS)-based metabolomics approach.

Methods: Five-day-old SD rats were randomly divided into a control group and a B[a]P exposure group (2 mg/kg). B[a]P was administered by gavage for 7 consecutive weeks to establish an exposure model. After exposure, spatial learning ability was assessed using the Morris water maze (MWM), ultrastructural changes in cortical neurons were examined by electron microscopy, and cortical metabolic profiles were detected by GC/MS. Differential metabolites between groups were identified using partial least squares discriminant analysis (PLS-DA) and independent samples t-test. Metabolic pathways associated with differential metabolites were analyzed using Cytoscape software.

Results: Compared with the control group, B[a]P-exposed rats exhibited significantly longer escape latencies ($P<0.05$) and shorter times spent in the target quadrant ($P<0.05$). Electron microscopy revealed widened synaptic clefts, thickened postsynaptic membranes, and cytoplasmic swelling in the B[a]P group. Eighteen differential metabolites (VIP>1, $P<0.05$) were identified in the cortex between the two groups. Network analysis of these metabolites revealed nine pathways associated with B[a]P neurotoxicity, involving amino acid metabolism, tricarboxylic acid (TCA) cycle, and vitamin B3 (niacin and nicotinamide) metabolism.

Conclusion: B[a]P can disrupt normal metabolism, and its neurotoxic mechanism may be related to disorders in amino acid metabolism, TCA cycle, and vitamin metabolism.

Keywords: benzo[a]pyrene; cortex; metabolomics; neurotoxicity

Introduction

Polycyclic aromatic hydrocarbons (PAHs), represented by benzo[a]pyrene (B[a]P), are environmental pollutants produced by incomplete combustion of organic matter. They are ubiquitous in the atmosphere and can enter the body through the respiratory tract, digestive tract, and skin, posing a serious health threat to residents in polluted areas. B[a]P and its metabolites are highly lipophilic and can readily cross the blood-brain barrier (BBB) to enter the brain, where they damage neuronal DNA, alter neuronal ultrastructure, and modify brain gene and protein expression profiles, ultimately impairing learning and memory. Due to the diverse and complex mechanisms of B[a]P-induced neurotoxicity, the specific pathways remain incompletely understood.

The cerebral cortex and hippocampus coordinate to perform various learning and memory tasks. The cortex encodes and retrieves episodic memories and participates in many higher cognitive functions, with cortical damage being closely associated with working memory deficits. However, most research on B[a]P neurotoxicity has focused on the hippocampus, with few studies examining the cortex, despite its critical importance. Metabolomics investigates metabolic responses to exogenous stimuli or genetic modifications and can elucidate changes in brain metabolites following neurotoxicant exposure. B[a]P can affect metabolism through numerous pathways, suggesting that disruption of cortical metabolism and homeostasis may be a mechanism underlying learning and memory impairment. Therefore, comprehensive analysis of small molecule metabolites in the cortex of B[a]P-exposed animal models can identify differential metabolites and provide metabolomic insights into B[a]P neurotoxic mechanisms.

Materials and Methods

Instruments

Morris water maze video tracking analysis system (Chengdu Taimeng Software Co., Ltd.); Hitachi-7500 transmission electron microscope (Japan); Vortex mixer and high-speed disperser (Germany IKA); Ultra-low temperature freeze dryer (USA Labconco); GC7890 gas chromatograph and MSD5975 mass spectrometer (USA Agilent).

Reagents

B[a]P (Sigma, USA, purity 95%); internal standard D4-alanine, methyl chloroformate (MCF), and pyridine (Sigma, USA); chloroform, methanol, potassium

hydroxide, sodium bicarbonate, and sodium hydroxide (Merck, Germany). All chemicals used were of analytical grade.

Animal Model and Exposure Protocol

Twenty male SD neonatal rats and two dams were purchased from the Animal Experiment Center of Chongqing Medical University (License No. SCXK(Yu)2012-0001). After 5 days of acclimation, neonatal rats were randomly divided into a control group (n=10) and a B[a]P exposure group (n=10). Rats from the same group were housed together with a dam (11 animals per cage). According to SD rat housing density requirements, pups were weaned at 21 days and redistributed into cages of 5 rats per group. Based on B[a]P exposure doses in residents near high-risk sites, the exposure group received 2 mg/kg B[a]P by gavage, while the control group received the same volume of corn oil, for 7 weeks to simulate continuous B[a]P exposure from infancy to adulthood. During exposure, rats had free access to food and water. The animal facility maintained a 12 h light/dark cycle (7:00-19:00), humidity of 40-60%, and temperature of 22-24°C. Successful establishment of the neurotoxicity model was verified by behavioral observation (reduced spatial learning ability) and histopathological examination (altered cortical neuronal ultrastructure).

Morris Water Maze Test

After B[a]P exposure, spatial learning and memory were assessed using the Morris water maze as described by Chen. On day 1, rats were trained to locate a clearly marked platform. On days 2-5, place navigation trials were conducted, recording the escape latency to find the platform. Four trials were performed daily, ending when the rat reached the platform. Rats failing to find the platform within 60 s were guided to it. After the final training session on day 5, the platform was removed and rats were placed randomly at pool edges. The time spent in the target quadrant (where the platform was previously located) during 120 s was recorded. Swimming patterns were tracked using video software.

Sample Collection and Electron Microscopy

After completing the Morris water maze test, rats were euthanized by cervical dislocation. Cortical tissue was rapidly dissected on ice. For electron microscopy, specimens were prepared as described by Chen and observed under transmission electron microscope to examine ultrastructural changes in cortical neurons. Remaining cortical tissue was frozen for subsequent analysis.

Metabolite Extraction

Cortical tissue (50 mg) was homogenized with 750 L of ice-cold methanol:water (1:1, v/v) and 20 L D4-alanine. After centrifugation at 15,000 r/min for 10

min, the supernatant was transferred to a clean EP tube. The pellet was re-extracted with 800 L of ice-cold chloroform:methanol (3:1, v/v), homogenized and centrifuged again. The two supernatants were combined and dried using an ultra-low temperature vacuum concentration system. The concentrated samples were stored at -80°C.

Derivatization

To the concentrated sample, 200 L NaOH was added and transferred to a clean glass tube. Then 167 L methanol and 34 L pyridine were added, vortexed for 10 s, followed by addition of 20 L MCF and vortexing for 30 s. Another 20 L MCF was added and vortexed for 30 s. Next, 400 L chloroform was added and shaken for 10 s, followed by 400 L sodium bicarbonate and shaking for 10 s. After centrifugation at 1500 r/min for 5 min, the aqueous layer was discarded. A small amount of anhydrous sodium sulfate was added, and the sample was transferred to a vial for GC/MS analysis.

GC/MS Analysis Conditions

Chromatographic conditions: ZB-1701 GC capillary column (30 m × 250 m, 0.15 m); injection port temperature 290°C. Temperature program: initial temperature 45°C held for 2 min, increased to 180°C at 9°C/min and held for 5 min, then increased to 220°C at 40°C/min and held for 2 min, finally increased to 280°C at 40°C/min and held for 5 min. Carrier gas was high-purity helium at 1 mL/min flow rate. Splitless injection with 1.0 L injection volume.

Mass spectrometry conditions: EI source, electron energy 70 eV, ion source temperature 230°C, full scan range 38-550 m/z, with a detection threshold of 100 ions.

Precision: The same sample solution was injected six times consecutively. Relative standard deviation (RSD) of peak areas was 2.5-6.12%, indicating good reproducibility.

Data Processing and Statistical Analysis

Morris water maze data were analyzed using SPSS 20.0 software with independent samples t-test; $P<0.05$ was considered statistically significant. Metabolomics data were processed using AMDIS software (version 2.66) and an in-house MCF mass spectral database. The processing included baseline correction, peak detection and matching, noise elimination, and metabolite identification. Endogenous markers with match quality >80% were selected as identified metabolites. Mass spectral data were imported into SIMCA-P 11.5 software for PLS-DA analysis to compare overall metabolic differences between groups. Metabolites with VIP (variable importance in the projection) >1 were further analyzed by independent samples t-test using SPSS 20.0 software; $P<0.05$ was considered statistically significant. Cytoscape software was used

to analyze metabolic pathways associated with the identified differential metabolites.

Results

Effects of B[a]P on Learning and Memory Behavior

With increasing training days, escape latencies to find the platform decreased in both groups. However, B[a]P-exposed rats showed significantly longer escape latencies (Figure 1A [Figure 1: see original paper], $P<0.05$) and shorter times spent in the target quadrant (Figure 1B, $P<0.05$) compared with controls.

Effects of B[a]P on Cortical Neuronal Ultrastructure

Electron microscopy revealed normal ultrastructure in control group cortical neurons, with typical synapses (Figure 2A [Figure 2: see original paper]) and normal neuronal morphology (Figure 2C). In contrast, B[a]P-exposed rats showed widened synaptic clefts, thickened postsynaptic membranes (Figure 2B), and cytoplasmic swelling (Figure 2D).

GC/MS Analysis of Cortical Metabolites

GC/MS analysis provided information on small molecule metabolites in rat cortex. PLS-DA, commonly used for classification and biomarker screening, completely separated the metabolic profiles of the two groups (Figure 3A [Figure 3: see original paper]), with model parameters R2X=0.57, R2Y=0.998, and Q2=0.925, demonstrating good robustness. Total ion chromatograms of representative samples from each group also showed obvious differences (Figure 3B, C).

Identification of Differential Metabolites and Pathway Analysis

Combining PLS-DA and independent samples t-test analysis identified 18 differential metabolites (VIP>1, $P<0.05$). Network analysis using Cytoscape software (Figure 4 [Figure 4: see original paper]) revealed nine pathways associated with neurotoxic mechanisms, involving amino acid metabolism, energy metabolism, and B vitamin metabolism (Table 1). Twelve differential metabolites participated in these pathways. Compared with controls, all these neurotoxicity-related metabolites were increased in B[a]P-exposed rat cortex except for cysteine, which decreased.

Discussion

The postnatal period of 5-11 days represents a critical window for rat brain development, with brain development continuing until adulthood (postnatal day 49). This study exposed rats during the brain development period to simulate continuous B[a]P exposure from infancy to adulthood. Consistent with our

previous findings, B[a]P-exposed rats showed impaired spatial learning in the MWM and ultrastructural changes in cortical neurons, indicating that B[a]P and its metabolites crossed the BBB and exerted neurotoxic effects, thus successfully establishing a neurotoxicity model.

Using GC/MS metabolomics, we detected cortical metabolites and performed PLS-DA analysis to explore potential neurotoxic mechanisms of B[a]P at the metabolic level. Eighteen differential metabolites were identified between B[a]P-exposed and control groups. Network analysis of these metabolites revealed nine pathways related to B[a]P neurotoxicity, involving amino acid metabolism, vitamin B3 (niacin and nicotinamide) metabolism, and the TCA cycle.

B[a]P exposure disrupted metabolism of methionine, valine, isoleucine, leucine, threonine, glutamate, aspartate, glycine, tyrosine, histidine, arginine, cysteine, alanine, serine, asparagine, and proline. Essential amino acids play crucial roles in growth, development, nutrition, and metabolism. In this study, several essential amino acids including valine, leucine, isoleucine, methionine, and threonine were increased in the cortex of B[a]P-exposed rats. These energy-supplying amino acids participate in brain energy supply, and their metabolic disorders can affect brain energy metabolism and subsequently impact learning and memory.

Non-essential amino acids regulate gene expression, antioxidant responses, neurotransmission, and immunity. Most non-essential amino acids and their derivatives also function as neurotransmitters involved in synaptic transmission and neural function regulation. Serine participates in neurotransmitter and protein synthesis in the brain and is closely related to brain development and neurological function. Tyrosine is involved in synthesis of biogenic amine neurotransmitters including dopamine (DA), norepinephrine (NE), and epinephrine (A), which play irreplaceable roles in learning, memory, and cognitive activities. Glutamate and aspartate are excitatory amino acid neurotransmitters (EAA), while glycine is an inhibitory amino acid neurotransmitter (IAA), all participating in learning and memory formation. After B[a]P exposure, increased levels of glutamate, aspartate, and glycine in the cortex disrupted the EAA/IAA balance, affecting normal excitatory and inhibitory processes in the central nervous system, which may contribute to learning and memory impairment. Thus, B[a]P may affect central nervous system function by disrupting cortical amino acid metabolism, leading to energy metabolism disorders, impaired neurotransmitter synthesis, and disturbed excitatory-inhibitory balance.

B[a]P exposure also interfered with the TCA cycle in the cortex, causing abnormal energy metabolism. ATP is the primary energy source for brain tissue and plays vital roles in active transport, protein synthesis and processing, membrane integrity, synaptic transmission, and neurotransmitter release. The TCA cycle is the main pathway for ATP production. Fumaric acid is a key intermediate in the succinate-propionate pathway, and its elevated level in B[a]P-exposed rat cortex suggests TCA cycle dysfunction. Advanced neural functions such as learning and memory are closely related to brain energy metabolism, making

energy metabolism disruption a likely mechanism of B[a]P neurotoxicity.

Disturbances in both vitamin B9 and B3 metabolism can affect cognitive function. Folate (vitamin B9) is converted to methyltetrahydrofolate by methylenetetrahydrofolate reductase and, together with homocysteine, is converted to methionine to ultimately synthesize S-adenosyl-L-methionine, a methyl donor involved in DA, 5-HT, and NE synthesis, which is closely associated with cognitive dysfunction. Niacinamide and niacin (vitamin B3) are interconvertible pyridine derivatives that, combined with ribose, phosphate, and adenine, form coenzymes I and II (CoI and CoII) for various dehydrogenases. Oxidative stress, mitochondrial changes, and inflammation are primary causes of apoptosis. CoII reduced by glucose-6-phosphate dehydrogenase generates NADPH, which participates in cellular antioxidant responses and may protect the brain from oxidative stress-induced damage.

In summary, this study demonstrates that disruption of amino acid metabolism, the TCA cycle, and vitamin metabolism are potential mechanisms of B[a]P neurotoxicity.

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