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Ethyl carbamate induces cell death through its effects on multiple metabolic pathways

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ABSTRACT

Ethyl carbamate (EC), a multisite carcinogenic chemical causing tumors in various animal species, is probably carcinogenic to humans. However, information about the possible carcinogenic and toxicological effects of EC in humans is quite limited. Because EC is found in many dietary foods (such as fermented foods) and tobacco and its products, and exposure of humans to EC often occurs inevitably, its toxicological effects in humans need to be studied. This study was conducted to understand the metabolomic and transcriptomic changes in human hepatocellular carcinoma cells (HepG2) exposed to 100 mM EC for short term (4 h) and long term (12 h) period, respectively. The results revealed multiple influences of EC on the metabolome and transcriptome of HepG2 cells, which was exposure time-dependent and well correlated with the kinetic changes of cell viability and mortality. EC treatment affected multiple metabolic pathways, inducing oxidative stress, reducing detoxification capacity, depleting energy, decreasing reducing power, disrupting membrane integrity, and damaging DNA and protein. These metabolomic and transcriptomic biomarkers of EC on human cell metabolism identified in this study would facilitate further studies on the risk assessment and the mitigation of dietary EC.

1. Introduction

Ethyl carbamate (EC or urethane) is a by-product of food processing, and is commonly found in fermented foods, alcoholic beverages, breads and acidified milks with varying concentrations ranging from less than 1 ng/g in yogurt to more than 2000 ng/g in stone fruit brandies [1–4]. Tobacco and tobacco derived products also contain EC at levels comparable to that in foods [5]. With such widespread presence, exposure of humans to EC often occurs inevitably via routine dietary or through active/passive consumption of cigarettes [6].

EC had long been used as a hypnotic and antitumor drugs in humans or an anesthetic in laboratory animals before it was found to be toxic in 1940s. Nowadays, EC is a well-known multisite carcinogenic chemical that can cause tumors in various animal species, including mice, rats, hamsters and monkeys [7,8]. EC induces tumor formation in animal organs such as lung, liver and skin depending on its route of exposure, and it has been used for experimental induction of lung cancer in mice [9]. Based upon experimental and epidemiological data, EC has been classified as a

Abbreviations: EC, ethyl carbamate; GC-MS, gas chromatograph-mass spectrometry; GO, gene ontology; KEGG, Kyoto encyclopedia of genes and genomes; PPP, pentose phosphate pathway; ROS, reactive oxygen species; CA, tricarboxylic acid; UPLC-MS/MS, ultra-performance liquid-chromatography tandem mass spectrometry.

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probable human carcinogen (class 2A) by the International Agency for Research on Cancer [10,11], and also a probable human carcinogen by the US Environmental Protection Agency [12]. Countries such as Canada, Czech Republic, USA, France and Korea have set limits of EC in food products [6,13]. As a result, many studies have been conducted to understand EC metabolism and to develop methods preventing EC accumulation in foods [3,14]. Toxicological investigations into the mechanisms underlying the carcinogenic effects of EC have been carried out in various animal models [15–19]. These studies have revealed that EC can induce the production of reactive oxygen species (ROS), depurination of DNAs and mitochondrial dysfunction. In animal lungs, EC stimulated ROS production and activated extracellular signal regulated kinase (ERK), leading to the promotion of tumor formation [9], and repeated exposure to EC is an additional risk for lung cancer because of permanent mitochondrial dysfunction and increased cellular mitotic activity [20]. Toxicological studies of EC in human tissues or cells are rare, and related data is quite limited [20–22]. We found previously that EC reduces HepG2 cell viability in a concentration and exposure time dependent manner and the kinetics of EC accumulation is correlated well with the decline of cell viability, implying potential adverse effects of exposure to EC on human health [21]. Another study in human HepG2 cells confirmed the dose-dependent manner of EC inhibition of cell viability and revealed that high concentration of EC induces ROS, cell apoptosis and inhibits the transition of dividing cells from G1 to S phase [22]. The high occurrence of EC in food and tobacco products and the high exposure frequency to humans make it necessary to investigate further into potential carcinogenic effect of EC exposure in humans.

Due to concerns over animal welfare and cost efficiency, *in vitro* system have been increasingly used as an alternative to conventional toxicological studies using lab animals [23]. There is a particular interest in using liver cells for toxicological investigations since liver plays a critical role in the metabolism [detoxification or activation] of xenobiotic chemicals. In this study, we selected the human HepG2 liver cells as an *in vitro* model to investigate the potential toxic effects of EC. The objectives were to investigate the molecular responses of HepG2 cells to EC exposure using both transcriptomic and metabolomic approaches. The results revealed many interesting time-dependent metabolic and transcriptomic changes in EC treated HepG2 cells that are well correlated with the kinetic changes of cell viability and mortality, elucidating possible cellular responses through which EC exerts its adverse effects on normal cell function.

2. Materials and methods

2.1. Chemicals and reagents

Ethyl carbamate (EC) chemical standard was purchased from Sigma (St. Louis, USA). DMEM medium was purchased from Invitrogen (San Diego, USA). Fetal bovine serum was purchased from Biowest (Loire valley, France). Penicillin streptomycin solution was purchased from Solarbio (Beijing, China). All other reagents were analytical grade and purchased from qualified suppliers.

2.2. Cell culture and EC treatment

Cell culture and EC treatment was done as described previously [24]. HepG2 cells were cultured in DMEM medium containing 10% fetal bovine serum and 1% mixture of penicillin (100 IU/ml) and streptomycin (100 g/ml) at 37 °C in the presence of 5% CO_2. Cells at 80% confluent were treated with 100 mM (dissolved in DMEM medium) for different times and cell viability and mortality were timely examined. Cells without EC treatment were grown at the same time as control. For metabolomics and transcriptomic analyses, cells were collected at 4 h and 12 h after treatment, respectively, digested with trypsin and centrifuged in 2 mL Eppendorf tubes at 1000 rpm for 5 min in 4 °C. The cell pellets were washed with ice-cold PBS solution twice, frozen immediately in liquid nitrogen and stored at −80 °C until analysis. Each treatment included four replicates and each replicate was a pool of four individual wells.

2.3. Cell viability and mortality

The viability and mortality of cultured HepG2 cells were examined as described previously by MTT assay and trypan blue staining, respectively [21].

2.4. Metabolomic profiling

Metabolomic analysis of EC-treated and control HepG2 cells was performed in Shanghai Jiao Tong University-Metabolon Joint Metabolomics Laboratory as previously described [24,25]. Briefly, cell pellets were suspended in 200 μL ultrapure water and 100 μL of the suspension was extracted with 450 μL methanol for 2 min using a Geno Grinder 2000 homogenizer (Glen Mills Inc., Clifton, NJ, USA). After centrifugation at 3000 rpm for 5 min in 4 °C, the supernatants were recovered and aliquoted for UPLC-MS/MS and GC/MS analyses, respectively. Integrated peak areas of each sample were used to compare relative abundances of a metabolite. Missing values for a given metabolite [assuming that the metabolite was below the limits of detection] were first filled with the minimum detected value, and the resulting raw data was normalized to the median.

2.5. Transcriptomic profiling

Total RNA was isolated from HepG2 cells using Trizol Reagent (Invitrogen) according to the manufacturer's instructions. The concentration and purity of extracted RNA was measured using Bio analyzer 2200 (Agilent) by measuring absorption at 260 and 280 nm. RNA samples with a 260/280 nm absorption ratio of 1.6–1.8 and RIN >8.0 were used in subsequent RNA-Seq profiling. The complementary DNA (cDNA)] libraries for single-end sequencing were prepared using Ion Total RNA-Seq Kit v2.0 (Life Technologies). The cDNA libraries were then processed for the Proton Sequencing process according to the commercially available protocols. Samples were diluted and mixed, the mixture was processed on a OneTouch 2 instrument (Life Technologies) and enriched on a OneTouch 2 ES station (Life Technologies) for preparing the template-positive Ion PI™ Ion Sphere™ Particles (Life Technologies) according to Ion PI™ Template OT2 200 Kit v2.0 (Life Technologies). After enrichment, the mixed template-positive Ion PI™ Ion Sphere™ Particles of samples was loaded onto 1 P1v2 Proton Chip (Life Technologies) and sequenced on Proton Sequencers according to Ion PI Sequencing 200 Kit v2.0 (Life Technologies). Before mapping, clean reads were obtained from the raw reads by removing the following: adaptor sequences, reads with >5% ambiguous bases (noted as N), and low quality reads containing more than 20% of bases with qualities of <13. The clean reads were then aligned using the MapSplice program (v2.1.6).

2.6. Metabolomic data analysis

Significantly changed metabolites were determined by independent *t*-test as described previously [24,25]. The threshold for significance was $P < 0.05$, which were adjusted using the multiple testing procedures by controlling the false discovery rate (FDR).