Food and Chemical Toxicology 105 (2017) 61–72

Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

Methamphetamine induces hepatotoxicity via inhibiting cell division, arresting cell cycle and activating apoptosis: *In vivo* and *in vitro* studies

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ARTICLE INFO

Article history:
Received 16 November 2016
Received in revised form
7 March 2017
Accepted 20 March 2017
Available online 21 March 2017

Keywords:
METH
Hepatotoxicity
Microarray
Cell cycle arrest
Apoptosis

ABSTRACT

Methamphetamine (METH) resulted in acute hepatic injury. However, the underlying mechanisms have not been fully clarified. In the present study, rats were treated with METH (15 mg/kg B.W.) for 8 injections (i.p.), and the levels of alanine transaminase, asparatate transaminase and ammonia in serum were significantly elevated over those in the control group, suggesting hepatic injury, which was evidenced by histopathological observation. Analysis of the liver tissues with microarray revealed differential expressions of a total of 332 genes in METH-treated rats. According to the GO and KEGG annotations, a large number of down-regulated cell cycle genes were screened out, suggesting that METH induced cell cycle arrest and deficient of cell cycle checkpoint. Related genes and proteins were confirmed by RT-qPCR and western blotting in rat livers, respectively. Moreover, treatment of Brl-3A cells with METH caused significant cytotoxic response and marked cell cycle arrest. Furthermore, overexpressions of Cidea, cleaved caspase 3 and PARP 1 in METH-treated rats indicated activation of apoptosis, while its inhibition alleviated cell death in Brl-3A cells, suggesting that activation of apoptosis took an important role in METH-induced hepatotoxicity. Taken together, the present study demonstrates that METH induced hepatotoxicity via inducing cell cycle arrest and activating apoptosis.

1. Introduction

Methamphetamine (METH) is a highly addictive psychostimulant drug of abuse, commonly referred to as “speed”, “crystal”, “crank”, “go”, and “ice” (Carvalho et al., 2012; Kayagaki et al., 2011). METH was firstly synthesized in 1893 in Japan (Hamamoto and Rhodus, 2009). METH causes both physical and psychological alterations, such as euphoric, stimulant, and hallucinogenic effects (Panenka et al., 2013). Abuse of METH triggers significant societal problems (Hostetler et al., 2016) and causes intense attention widespread in the world.

Neurotoxic effects induced by METH could be attributed to induction of pro-inflammatory cytokines (Shah et al., 2012), oxidative stress (Shah et al., 2013), hyperthermia (Kiyatkin and Sharma, 2009) and ER stress-mediated apoptosis (Shah and Kumar 2016). METH may induce hyperthermia via complex integration of the subsequent hyperlocomotion, altered metabolism, changes in hypothalamic neurotransmission, and vasoconstriction (Brown et al., 2007). It has been proved that hyperthermia induced by METH contributes significantly to hepatic injury and increases ammonia in peripheral plasma (Halpin et al., 2013). Increased ammonia in peripheral blood and brain (Felipo and Butterworth, 2002), as well as acute hepatic injury caused by METH may also contribute to its neurotoxicity (Halpin and Yamamoto, 2012).

Previous studies demonstrated that hepatic injury induced by METH was related to oxidative stress and subsequent mitochondrial collapse in hepatocytes (Eskandari et al., 2014), and the resultant hepatocellular necrosis and apoptosis (da Silva et al., 2013). However, the exact cellular and molecular mechanisms involved in this process have not been completely illuminated.

In the present study, microarray was performed for a

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http://dx.doi.org/10.1016/j.fct.2017.03.030
0278-6915/

comprehensive analysis of the hepatic response to the treatment of METH. The alterations of differentially expressed genes were further confirmed in Brl-3A cell line. Therefore, this study may give evidence for the relevant genes and a clue of the underlying mechanisms for the hepatic injury caused by METH.

2. Materials and methods

2.1. Chemicals and cell line

Methamphetamine (METH, purity of 99.1%, identified by the National Institute for Food and Drug Control, Guangzhou, China) was obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Rat hepatocyte (Brl-3A) line was purchased from Typical Culture Preservation Commission Cell Bank, Chinese Academy of Sciences, Shanghai, China.

2.2. Animals and treatments

Adult male Sprague Dawley rats (6 weeks old) were purchased from the Laboratory Animal Center of Southern Medical University and were singly housed in tub cages on a 12 h light-12 h dark schedule with food and water available ad libitum. Animals were habituated to the animal facilities for 7 days before use. All animal procedure was conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals of the Southern Medical University.

METH was dissolved in saline. Rats were randomly divided into 2 groups (6 rats in each group) and received intraperitoneal (i.p.) injections of saline or METH (8 injections, 15 mg/ml/kg body weight/injection, at 12 h intervals). The exposure paradigm was chosen based on previous studies (Huang et al., 2015; Qiao et al., 2014). The average concentration of METH was 191.75 mg/L (about 1.3 μM) in the blood of METH-treated rats at 1 h after the last injection (Huang et al., 2015), which was consistent with the median value of METH in blood of METH abusers (Melega et al., 2007). Under deep anesthesia, rats were sacrificed 24 h after the last injection. Blood samples were collected from the coeliac artery for biochemical analysis. Livers were rapidly removed and were dissected on an ice cold glass plate. Half of the liver was fixed in 10% phosphate-buffered formalin for histopathological observation and the other half rapidly frozen in liquid nitrogen and stored at −80 °C for microarray and quantitative RT-PCR (RT-qPCR) analyses.

2.3. Biochemical analysis

For biochemical analysis, alanine transaminases (ALT) and aspartate transaminase (AST) levels in the serum were measured using ELISA kits (CUSABIO Biotechnology). Serum ammonia levels were measured using an automatic clinical analyzer (AMMONIA-CHECKERII).

2.4. Histopathological observation

After formalin fixation, liver tissue samples were embedded in paraffin, sectioned at 3-μm thickness, and stained with hematoxylin and eosin (H&E) for histopathological examination, according to the diagnostic criteria of Boorman et al. (1990).

2.5. Brl-3A cell culture

Brl-3A cell line was a rat normal hepatocyte line, which was first characterized by Coon (1968) as remaining diploid, typical in morphology, and producing at least five specific hepatic enzymes, including albumin, glutamic oxalic transaminase, glutamic pyruvic transaminase, lactic dehydrogenase, and alkaline phosphatase. The cells were subsequently found to secrete carboxypeptidase E, furin, and dynorphin converting enzyme (Petanceska et al., 1993). By microarray, regarding mRNA expression, liver slices exhibit the strongest similarity to liver tissue than primary rat hepatocytes and hepatic cell lines (Boess et al., 2003). Brl-3A cell line was found to express week cytochrome P450s, which were known as drug metabolic enzymes (Boess et al., 2003), therefore, it was not recommended for drug metabolic study. However, METH could induce obvious cytotoxicity in several different cells *in vitro* (Dias da Silva et al., 2013a; 2013b; Huang et al., 2015), implying that cell death induced by METH predominantly resulted from itself rather than its metabolites. Therefore, Brl-3A cells can be used to conduct the *in vitro* experiment in the present study. Cells were routinely cultured in 25 cm^2 canted-neck tissue culture flasks (Coning, NY, 14831, USA) in Dulbecco minimum essential medium (DMEM) with supplements of 3% fetal bovine serum (FBS) and 1% antibiotic (5000 U/ml penicillin, 5000 μg/ml streptomycin) in a thermostatic incubator (Thermo Fisher Scientific Inc, USA), at 37 °C, with 5% CO_2, in a humidified environment.

2.6. METH treatment, cell morphology and cell viability

METH was dissolved in phosphate-buffered saline (PBS) as a stock at the concentration of 100 mM. For cell viability examination, Brl-3A cells were seeded in the 96-well plates. 24 h later, the basal medium was replaced by medium with METH at the concentration of 0, 0.5, 1, 2 and 4 mM. The METH concentrations used in this study (0.5–4 mM) were based on the published study (da Silva et al., 2013), which reported that the median effect concentration of METH was 4.01 mM at 37 °C in primary rat hepatocytes. The cells were treated with METH alone or in combination with Z-VAD-FMK (10 μM, Selleck.cn) or with Z-VAD-FMK alone for 24 h followed by 6 h basal medium. Cell morphology was observed by an inverted microscope (TS100-F, Nikon, Tokyo, Japan). According to the operation manual of Cell Counting Kit-8 (CCK-8, Dojindo, Japan), 10 μl of CCK-8 solution were added to the basal medium and incubation was kept for 3 h. Optical densities (ODs) were read on a microplate reader (Bio-rad model 680, BIO-RAD, USA) with 450 nm wavelength.

2.7. Cell harvest

For RT-qPCR analysis, Brl-3A cells were seeded in the 6-well plates. 24 Hours later, the basal medium was replaced by medium with METH or the same volume of PBS. Cells were gently washed 3 times using PBS (PH 6.8) and were harvested using a sterile cell scraper (Corning, NY, USA) after 24 h-METH treatment followed by 6 h-basal medium. Then the harvested cells were centrifuged at 1000 rpm/min for 5 min, and the pellet was immediately frozen in liquid nitrogen and stored at −80 °C for use in the subsequent analysis.

2.8. Cell cycle analysis

Briefly, Brl-3A cells were cultured in 25 cm^2 canted-neck tissue culture flasks, harvested after 24 h-METH/METH + Z-VAD-FMK/Z-VAD-FMK treatment followed by 6 h-basal medium, washed with D-PBS (PH 7.4) twice and labeled with propidium iodide (PI) stain solution (A10798, Thermo Fisher Scientific Inc.), according to the manufacturer's protocol. Flow cytometric determination of DNA content was analyzed with a NovoCyte Flow Cytometer (ACEA Biosciences Inc., USA). The fractions of the cells in the G0-G1, S and G2-M phases were analyzed using Flow Jo software (NovoExpress