Toxicology in Vitro 35 (2016) 163–168

Contents lists available at ScienceDirect

Toxicology in Vitro

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

Necroptosis contributes to methamphetamine-induced cytotoxicity in rat cortical neurons

Kun Xiong [b], Huidan Liao [a], Lingling Long [a], Yanjun Ding [a], Jufang Huang [b], Jie Yan [a,*]

[a] Department of Forensic Science, School of Basic Medical Science, Central South University, Changsha, Hunan 410013, China
[b] Department of Anatomy and Neurobiology, School of Basic Medical Science, Central South University, Changsha, Hunan 410013, China

ARTICLE INFO

Article history:
Received 14 February 2016
Received in revised form 7 June 2016
Accepted 7 June 2016
Available online 8 June 2016

Keywords:
Methamphetamine
Necroptosis
Embryonic cortical neurons
Neurotoxicity
Necrosis

ABSTRACT

Necroptosis, a programmed necrosis, is involved in various types of neurodegenerative diseases. In this study, we investigated whether necroptosis contributed to neuronal damage in a methamphetamine injury model. **Methods**: Primary cultures of embryonic cortical neurons from Sprague-Dawley rats were subjected to different doses of methamphetamine with/without pre-treatment with a specific necroptosis inhibitor, Necrostatin-1. Necrosis was assessed by determining lactate dehydrogenase release and by Annexin V/propidium iodide double staining, while the neuronal ultra-structure was examined by electron microscopy. Tumor necrosis factor-α protein levels were determined by enzyme-linked immunosorbent assay. **Results**: At early stages (12 h) of post-treatment with methamphetamine, significant necrosis occurred and the viability of neurons decreased in a dose- and time-dependent manner in this model of acute neuronal injury. Pretreatment with Necrostatin-1 led to significant neuronal preservation compared with the methamphetamine-treated groups. Furthermore, tumor necrosis factor-α expression increased in a dose-dependent manner following methamphetamine exposure. **Conclusion**: Methamphetamine induced necrosis in rat cortical neurons *in vitro*, both time and dose dependently, and necroptosis may be an important newly identified mode of cortical neuronal death caused by single high-dose methamphetamine administration.

1. Introduction

Programmed necrosis, known as necroptosis, which differs from traditional necrosis and apoptosis, has attracted considerable attention over recent years. Necroptosis has been indicated to occur following tumor necrosis factor (TNF)-α-induced injury of tubular epithelial cells in donor kidneys and murine fibrosarcoma L929 cells, including others (Lau et al., 2013; Zhong et al., 2014). Furthermore, the potential biological relevance of necroptosis has recently been shown in several neurodegenerative or neurosurgical disease models, including Parkinson's disease (PD), Huntington's disease (HD), craniocerebral trauma, brain tumors, and cerebrovascular diseases (Hartmann et al., 2001; Zhu et al., 2011; Fricker et al., 2013; Jiang et al., 2014; Liu et al., 2015), indicating that it may be an important pathway of cell death in neuronal injury. However, it still remains unclear if necroptosis contributes to neuronal injury induced by methamphetamine (METH) neurotoxicity. As one of the top three illicitly used drugs worldwide, the overall negative effects of METH include irreversible brain damage that can cause neurologic and psychiatric abnormalities, and its abuse is associated with increased risk of neurodegenerative diseases, such as PD (Thompson et al., 2004; Callaghan et al., 2012). It was demonstrated that METH triggers a neuro-inflammatory response resulting in increased production of proinflammatory cytokines, such as TNF-α, and induces parietal cortical neuronal damage in rats (Eisch and Marshall, 1998). Experimental evidence indicates that apoptosis, necrosis, and autophagy are involved in acute METH neurotoxicity (Deng et al., 2001; Riddle et al., 2006; Chang et al., 2007,); however, the inhibition of apoptosis-related factors only partially inhibited METH-induced cell death, indicating that other factors may play a role in the associated neurotoxicity (Kanthasamy et al., 2011). To date, there has been no direct evidence elucidating the effects of necroptosis in METH-induced neuronal death. Therefore, we aimed to address this problem by examining the effects of Necrostatin-1 (Nec-1, a specific and potent small-

* Corresponding author.
E-mail address: wills212156@gmail.com (J. Yan).

http://dx.doi.org/10.1016/j.tiv.2016.06.002
0887-2333/

molecule inhibitor of necroptosis, Degterev et al., 2008; He et al., 2009) in an *in vitro* acute METH-induced neuronal cortex injury model, in which Nec-1 rescued necrotic cells that regarded as those that were undergoing necroptosis (Ding et al., 2015; Shang et al., 2014; Li et al., 2008; Rosenbaum et al., 2010).

2. Methods

2.1. Cell culture and treatment

Animal use was in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals. All procedures in the present study were approved by the Animal Care and Use Committee of Central South University (Hunan, China). Every effort was made to avoid unnecessary use of laboratory animals. Female Sprague-Dawley rats (weighing 400–600 g, specific pathogen free), at days 16–18 of pregnancy, were obtained from the Animal Center of Hunan SJA Laboratory Animal Co., Ltd., for use in the present study. Embryonic cortical neurons were prepared from rat embryos at days 16–18 of gestation. Primary cultures of cortical neurons were performed similar to previously described methods (Brewer et al., 1993; Brewer, 1995). Neurons were cultured for 7–9 days for the subsequent experiments and were treated with METH (applied by Changsha City Public Security Bureau, Hunan Province, China) at concentrations of 0.5, 1.0, 2.0, 4.0, and 8.0 mM for 4, 12, 24, and 36 h. To determine the protective effect of Nec-1 against neurotoxicity induced by METH, neurons were exposed to Nec-1 (Sigma, St Louis, MO, USA) at the concentrations of 10, 20, 50, 100, and 200 μM respectively for 12 h before METH treatment (METH concentration was selected as the one that caused the remarkably significant necrosis). The samples were divided into eight groups: normal serum-free medium (NS group), METH treatment alone, and METH plus five different doses of Nec-1 (10, 20, 50, 100, and 200 μM), as well as a DMSO plus normal serum-free medium group (vehicle group).

2.2. Lactate dehydrogenase (LDH) leakage assay

Primary cultures of cortical neurons were seeded in 96-well plates at a density of 1×10^4 cells/well, and then treated with METH according to the different experimental groups. LDH release rate was detected using the LDH cytotoxicity assay kit (Beyotime, Jiangsu, China) after METH treatment. The LDH assay is a non-radioactive colorimetric assay, measuring LDH release from necrotic cells into the extracellular space/supernatant upon the rupture of plasma membrane. Cell-free culture supernatants were collected from each well and incubated with the appropriate reagent according to the manufacturer's instructions at room temperature for 30 min. The intensity of red color formed in the assay, measured at a wavelength of 490 nm, is proportional to both LDH activity and the percentage of necrotic cells. The total LDH release was determined in the cell cultures treated. Cells were harvested at a fixed time and cell damage was assessed by determining the release of LDH by using the LDH Detection Kit according to the manufacturer's instructions (Zhou et al., 2014). All tests were repeated three times.

2.3. Electron microscopy

Neuronal cells were treated for 12 h with DMSO, 4 mM METH, or 4 mM METH plus Nec-1 (Nec-1 concentration was selected as the moderate one that inhibited necrosis) and were observed using electron microscopy. Then, fetal brain neurons were fixed in 2.5% glutaraldehyde in 0.1 M phosphate-buffered saline at pH 7.4, post-fixed in 1% osmium tetroxide phosphate-buffered saline, and embedded. Ultrathin sections were double stained with uranyl acetate and lead citrate and observed under an electron microscope (H-7500; Hitachi, Tokyo, Japan).

2.4. Annexin V/propidium iodide (PI) assay and flow cytometry

Cortical neurons were seeded in 6-well plates at a density of 5×10^5 cells/well, followed by METH treatment according to the different experimental groups. Cell death was determined by flow cytometry (Becton Dickinson, San Jose, CA, USA) using the Annexin V-FITC Apoptosis Detection kit (KeyGEN Biotech, Jiangsu, China) (Zhou et al., 2014), according to the manufacturer's instructions. Cells were then washed and analyzed by FACSCalibur™ (Becton Dickinson, Franklin Lakes, NJ, USA). The percentages of cells in each quadrant were analyzed by ModFit software (Verity Software House, Topsham, ME, USA). All tests were repeated three times.

2.5. TNF-α ELISA array

Primary cultures of cortical neurons were seeded in 6-well plates at a density of 5×10^5 cells/well, and then treated with METH according to the different experimental groups. The levels of TNF-α in cell culture supernatants were determined at 12 h post-administration of METH using ELISA kits purchased from Cusabio Biotech Co., Ltd. (Wuhan, China), according to the manufacturer's instructions (Zhang et al., 2013a). All tests were repeated three times.

2.6. Statistical analysis

One-way analysis of variance was performed to test differences in the average values between groups and the data were analyzed using SPSS 19.0 software (SPSS Inc., Chicago, IL, USA). All results were presented as mean ± SD. A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. METH-induced neurotoxicity in cortical neurons

LDH is a stable cytoplasmic enzyme present in all cells and is rapidly released following plasma membrane damage, making its release a good indicator of necrotic cell death (Abdel-Latif et al., 2006). To demonstrate whether necrosis is important in METH-induced cell death, samples were collected at 4, 12, 24, and 36 h after treatment with 0.5, 1.0, 2.0, 4.0, and 8.0 mM of METH, and neuronal injury was determined by LDH leakage assay. The percentage of necrotic cell death was calculated by the color intensity of METH-treated cells minus NS (normal serum-free group)/LDH releasing reagent-treated cells minus NS cells, according to the manufacturer's instructions. As shown in Fig. 1, the viability of neurons in primary cultures was reduced after METH

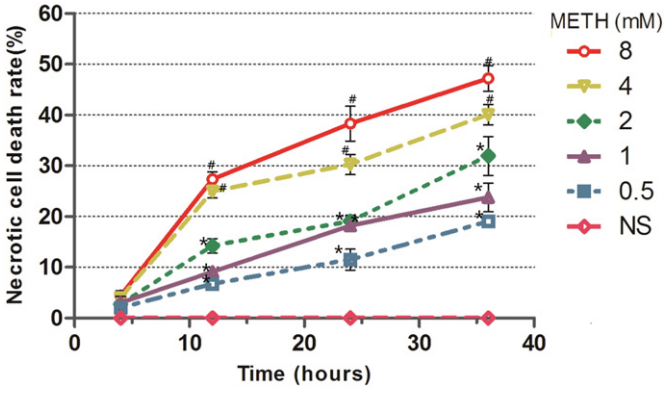

Fig. 1. Lactate dehydrogenase release in cultured cells was measured at indicated times post-treatment with different doses of methamphetamine. Data (Mean ± SD, n = 3 independent experiments and all samples were triplicated in each experiment) were analyzed using one-way ANOVA to detect significant differences between each group and NS group in the same time point (*, $p < 0.05$; #, $p < 0.01$).