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DNA damage-inducible transcript 4 (DDIT4) mediates methamphetamine-induced autophagy and apoptosis through mTOR signaling pathway in cardiomyocytes

Rui Chen [a,d,1], Bin Wang [a,1], Ling Chen [a], Dunpeng Cai [a], Bing Li [a], Chuanxiang Chen [a], Enping Huang [a], Chao Liu [b], Zhoumeng Lin [c], Wei-Bing Xie [a,*], Huijun Wang [a,*]

[a] Department of Forensic Medicine, School of Basic Medical Science, Southern Medical University, Guangzhou 510515, People's Republic of China
[b] Guangzhou Forensic Science Institute, Guangzhou 510030, People's Republic of China
[c] Institute of Computational Comparative Medicine and Department of Anatomy and Physiology, College of Veterinary Medicine, Kansas State University, Manhattan, KS 66506, USA
[d] Department of Forensic Medicine, Guangdong Medical University, Dongguan 523808, People's Republic of China

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ABSTRACT

Methamphetamine (METH) is an amphetamine-like psychostimulant that is commonly abused. Previous studies have shown that METH can induce damages to the nervous system and recent studies suggest that METH can also cause adverse and potentially lethal effects on the cardiovascular system. Recently, we demonstrated that DNA damage-inducible transcript 4 (DDIT4) regulates METH-induced neurotoxicity. However, the role of DDIT4 in METH-induced cardiotoxicity remains unknown. We hypothesized that DDIT4 may mediate METH-induced autophagy and apoptosis in cardiomyocytes. To test the hypothesis, we examined DDIT4 protein expression in cardiomyocytes and in heart tissues of rats exposed to METH with Western blotting. We also determined the effects on METH-induced autophagy and apoptosis after silencing DDIT4 expression with synthetic siRNA with or without pretreatment of a mTOR inhibitor rapamycin in cardiomyocytes using Western blot analysis, fluorescence microscopy and TUNEL staining. Our results showed that METH exposure increased DDIT4 expression and decreased phosphorylation of mTOR that was accompanied with increased autophagy and apoptosis both in vitro and in vivo. These effects were normalized after silencing DDIT4. On the other hand, rapamycin promoted METH-induced autophagy and apoptosis in DDIT4 knockdown cardiomyocytes. These results suggest that DDIT4 mediates METH-induced autophagy and apoptosis through mTOR signaling pathway in cardiomyocytes.

1. Introduction

Methamphetamine (METH) is an illegal, but widely abused, psychostimulant derived from amphetamine. Adverse effects of METH on human health have been well documented (Greenwell and Brecht, 2003), but the majority of available studies focus on METH's neurotoxicity (Qiao et al., 2014; Wu et al., 2014; Huang et al., 2015; Chen et al., 2016). In recent years, increasing reports have shown that METH can also cause adverse and potentially fatal effects on the cardiovascular system (Turdi et al., 2009; Tomita et al., 2011; Funakoshi-Hirose et al., 2013a). METH abuse, ranging from episodes of binge abuse to chronic abuse over several years, can cause a variety of myocardial damages in humans (Tomita et al., 2013). Cardiovascular pathology, typically coronary artery atherosclerosis, was detected in 54% of 371 METH-related deaths occurring between July 2000 and June 2005 in Australia, highlighting the role of cardiotoxicity in METH-induced death (Kaye et al., 2009). Our previous study showed that METH exposure induced apoptosis in cardiomyocytes (Leung et al., 2014). However, the mechanisms underlying METH-induced apoptosis in cardiomyocytes remain to be elucidated.

Our recent study demonstrated that METH exposure elicited autophagy that might contribute to METH-induced apoptosis in neuronal cells; increased expression of DDIT4 (DNA damage-inducible transcript 4, also known as REDD1 and Dig2) was associated with METH-induced autophagy and inhibition of DDIT4 prevented from METH-caused autophagy in vitro and in vivo (Li et al., 2016). DDIT4 is a protein with a molecular weight of 35 kD that is ubiquitously expressed in various human tissues (Canal et al., 2014). Previous studies suggest that increased expression of DDIT4 is the result of a series of cellular stress, such as hypoxia, DNA damage, and energy deprivation (Brugarolas et al., 2004; Tang et al., 2006). mTOR (mammalian target of rapamycin) is a highly-conserved modulator of many biological functions, including

* Corresponding authors.
E-mail addresses: xieweib@126.com (W.-B. Xie), hjwang711@yahoo.cn (H. Wang).
[1] These authors contributed equally to this study.

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cell growth, metabolism, etc. It is also an inhibitor of autophagy induction (Li et al., 2012). DDIT4 participates in mTOR signaling pathway by inhibiting the phosphorylation of mTOR, leading to up-regulated autophagy. It has been shown that exposure to another neurotoxicant alcohol increases DDIT4 expression in heart (Lang et al., 2008) and DDIT4 mediates the activation of mTOR when the heart undergoes ischemia/reperfusion injury (Hernandez et al., 2011). Additionally, DDIT4 has been shown to play a critical role in the process of phenylephrine-induced cardiac hypertrophy (Liu et al., 2014). However, the role of DDIT4 in the cardiotoxicity of METH has not been reported.

The objective of this study was to investigate the mechanisms of METH-induced autophagy and apoptosis in cardiomyocytes, focusing on the role of DDIT4 in this process. We hypothesized that DDIT4 may mediate METH-induced autophagy and apoptosis in cardiomyocytes and blockade of DDIT4 expression could partially protect against METH-induced autophagy and apoptosis. To test the hypothesis, we measured DDIT4 protein levels in cardiomyocytes and in heart tissues of METH-treated rats. We also evaluated the effects on METH-caused autophagy and apoptosis after silencing DDIT4 expression with synthetic siRNA with or without pretreatment of a mTOR inhibitor rapamycin in cardiomyocytes. Our results showed that METH exposure increased autophagy and apoptosis that were associated with upregulated DDIT4 expression and reduced phosphorylation of mTOR in cardiomyocytes both in vitro and in vivo. These effects were normalized after inhibiting DDIT4, but rapamycin promoted METH-induced autophagy and apoptosis in DDIT4 knockdown cardiomyocytes. We concluded that DDIT4 regulates METH-induced autophagy and apoptosis through mTOR signaling pathway in cardiomyocytes.

2. Materials and methods

2.1. Materials

METH (>99% purity) was obtained from the National Institutes for the Control of Pharmaceutical and Biological Products (Beijing, China). Cell culture reagents, including fetal bovine serum (FBS), DMEM/F12 medium and trypsin, were purchased from GIBCO (Carlsbad, CA, USA). Rapamycin was purchased from Sigma-Aldrich (St. Louis, MO, USA). Anti-DDIT4, anti-cleaved caspase-3 and anti-PARP were purchased from Abcam (Cambridge, UK). Anti-Beclin-1, anti-mTOR, anti-LC3-I/II, anti-p-mTOR (S2481), anti-β-actin and chloroquine were purchased from Cell Signaling Technology (Boston, MA, USA). Rhodamine phalloidin was purchased from Cytoskeleton (Denver, CO, USA). Other chemicals or reagents, unless specifically described below, were obtained from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Animal protocol

Healthy adult male Sprague–Dawley (SD) rats (180–220 g, 6–8 weeks old) and neonatal rats (from 0 to 3-day-old) were purchased from Laboratory Animal Center of Southern Medical University (Guangzhou, China). Adult rats were housed singly in tub cages in a temperature-controlled (~22 °C) room on a 12 h light–12 h dark schedule with food and water available ad libitum. Animal care and experimental procedures were in compliance with the NIH guidelines for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee at the Southern Medical University.

Adult animals were habituated to the animal facilities for 7 days before use. Rats were divided randomly into three groups (n = 3/group): saline control, 4-day exposure, and 14-day exposure groups. METH was dissolved in saline. Rats in the 4-day exposure group received 8 intraperitoneal (i.p.) injections of METH at 15 mg/kg/injection and 12 h intervals. The 14-day exposure group rats were injected i.p. with METH for 14 days following the schedule in Table 1. The saline control group (vehicle group) rats received similar volume of physiological 0.9% saline to the 4-day exposure group. These 4-day and 14-day exposure paradigms were selected based on previous studies (Kobeissy et al., 2012; Huang et al., 2015) where the rationale has been described in detail. Briefly, the 4-day exposure paradigm is relevant to human exposure because the measured blood METH concentrations in rats at 1 h after the last injection are in the range of reported blood concentrations in METH abusers (Huang et al., 2015). Additionally, it has been shown that the 14-day exposure paradigm can mimic long-term human METH abuse (Danaceau et al., 2007; Tokunaga et al., 2008). Rats were sacrificed 24 h after the last injection. Heart samples were rapidly removed, frozen and stored at −86 °C until analysis.

2.3. Cell culture

Neonatal rat ventricular myocytes (NRVMs) were prepared from 0 to 3-day-old neonatal Sprague–Dawley rats based on a published protocol (Cui et al., 2012). In brief, rats were sacrificed by immersion in 75% alcohol. Whole hearts were excised and immediately transferred into ice-cold PBS. The ventricles were excised in a sterile 60 mm Petri dish, and the auricles were carefully removed. The myocardial cells were dispersed by incubating with 0.25% trypsin EDTA, which was then mixed by intermittent pipetting along with stirring at 37 °C in a water bath for 8 min. The cell suspension was allowed to stand for 1 min. The supernatant containing individual cells was collected into a 15 ml falcon tube and kept on ice. Two milliliters DMEM/F12 (1:1) medium supplemented with 20% FBS was added to the tube. This digestion procedure was repeated for 4 times. Cells in the supernatant were isolated by centrifugation for 10 min at 2000 rpm at room temperature. The cells were plated on 100 mm dishes and incubated at 37 °C in a humidified atmosphere containing 5% CO_2 for 2 h to allow differential attachment of non-myocardial cells. The supernatant was aspirated gently, and cells were plated in six-well plates (5×10^5 cells/well). Cells were incubated at 37 °C in a humidified atmosphere containing 5% CO_2 throughout the experiments.

2.4. siRNA and transient transfection

DDIT4 siRNA (siDDIT4, sequence: 5′-GCAAGAGCUGCCAUAGUGUTT-3′) was synthesized by GenePharma (Shanghai, China). Nonspecific control siRNA (siNC) sequence was: 5′-UUCUCCGAACGUGUCACGUTT-3′. After 6–7 days of incubation of primary cardiomyocytes, 5 μl Lipofectamine 2000 (Invitrogen, Carlsbad, CA) reagent and 20 μmol siDDIT4 or siNC were added to Opti-MEM medium (Gibco BRL, Paisley, UK). The

Table 1
Dosing schedule of METH in the 14-day exposure group (mg/kg).

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Dose level × dosing times	1 × 1	1 × 2	1 × 2	1 × 4	1.5 × 4	1.5 × 4	2 × 4
	Day 8	Day 9	Day 10	Day 11	Day 12	Day 13	Day 14
Dose level × dosing times	2 × 4	2.5 × 4	3 × 4	3.5 × 4	4 × 4	4.5 × 4	5 × 4

Note: METH was injected once on day 1, twice at 6 h intervals on days 2–3, four times at 2 h intervals from day 4 on. The first injection was at 8 a.m.