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

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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.

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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 (Turdit 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 (Bragarolas et al., 2004; Tang et al., 2006). mTOR (mammalian target of rapamycin) is a highly-conserved modulator of many biological functions, including
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-induced cardiomyocytes 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-Beclin-1, anti-mTOR, anti-LC3-I/II, and β-actin antibodies were purchased from Abcam (Cambridge, UK). Anti-Beclin-1, anti-mTOR, anti-LC3-I/II, and anti-β-actin antibodies 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 (NRVs) were prepared from 0 to 3-day-old newborn 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% CO2 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 × 105 cells/well). Cells were incubated at 37 °C in a humidified atmosphere containing 5% CO2 throughout the experiments.

2.4. siRNA and transient transfection

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

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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.
mixed solution was incubated at room temperature for 20 min prior to incubation with cells. After 6 h incubation at 37 °C, the siRNA/Lipofectamine 2000 complex medium was replaced with the same volume of regular FBS-supplemented culture medium. After 48 h, the medium was changed to non-serum medium prior to the treatment of METH (1.0 mM) for another 24 h.

2.5. Western blot analysis

Heart tissues from rats and primary cardiomyocytes exposed to vehicle or METH were lysed in ice-cold RIPA buffer with protease inhibitors and phosphatase inhibitors. Protein concentrations were measured with the BCA-100 Protein Quantitative Analysis kit (Biocolors, Shanghai, China). Protein samples (10 μg) were separated by 6–12% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). The membranes were incubated at room temperature for 2 h in blocking buffer (5% BSA or 5% nonfat dry milk in TBST buffer), followed by incubation with primary antibodies [anti-DDIT4, anti-Beclin-1, anti-LC3-I/II, anti-mTOR, anti-p-mTOR(S2481), anti-caspase3, anti-β-actin (1:1000 for all)] overnight at 4 °C. After washing the membranes three times with TBST, they were incubated with an anti-rabbit IgG-horseradish-peroxidase (1:10,000, Jackson, MI, USA) for 1 h at room temperature. The membranes were developed with Chemiluminescence ECL Plus Western Blotting detection reagents and the signal of band intensities was quantitated with Gel-Pro analyzer. Expression of the housekeeping gene β-actin was used as a reference control. Western blot experiments for each protein of interest were repeated three times. Only one representative band for each protein is shown in this manuscript.

2.6. Fluorescence microscopy

siNC or siDDIT4-transfected cardiomyocytes were transfected with GFP-LC3 plasmid (a gift provided by Dr. Jingbo Xiong from Southern Medical University, China) using Lipofectamine 2000 reagent (Invitrogen). After transfection, cells were exposed to 1.0 mM METH in the presence and absence of chloroquine (a lysosomal degradation inhibitor) for 24 h. At the end of exposure, cells were fixed with 4% paraformaldehyde (Solarbio, China) for 5 min. Rhodamine phalloidin was used together with DAPI (4′,6′-diamidino-2-phenylindole) nuclear labeling. The coverslip was blocked with blocking buffer. Autophagosome formation was observed using a fluorescence microscope (A1+/A1R+, Nikon, Tokyo, Japan). The number and percentage of GFP-LC3 puncta-positive cells were analyzed using Image J software (NIH).

2.7. TUNEL staining

Cardiomyocytes (5×10⁶/well) were pretreated with siRNA as described above followed by METH (1.0 mM) treatment for 24 h. DNA fragmentation was analyzed using the fluorometric TUNEL system (Roche Applied Science, USA) according to the manufacturer’s instructions. Briefly, cells were fixed in 4% paraformaldehyde in fresh PBS (pH 7.4) at room temperature for 15 min and then incubated with fluorescein-conjugated TdT enzyme at 37 °C for 1 h in the dark. Cells were mounted with DAPI for nuclear counter staining. For frozen heart tissues, 5 μm tissue sections were sliced using a Leica Rotary microtome (CM1900, Leica, Germany). The tissue sections were placed on a Fisher Superfrost slide and dried overnight at room temperature. The slides were fixed by immersion into cold acetone (−20 °C) for 2 min and then air-dried at room temperature. The tissue section pretreatment procedures, including deparaffinizing, rehydration, retrieval with reconstituted protease K, equilibration, and slide washing, were conducted according to manufacturer’s instructions. Briefly, tissue slides were incubated with Nucleotide Mix and fluorescein-conjugated rTdT buffer solution at 37 °C for 60 min to allow sufficient reaction. The reaction was terminated by adding 2× SSC solution (a concentrated solution of sodium chloride–sodium citrate in distilled/deionized water), followed by 15 min incubation. The control incubation buffer was prepared without the rTdT enzyme; all other steps were similar. Samples were stained with DAPI to determine total number of nuclei. Cross-sections were photographed (20× and 40× objectives) using a fluorescence microscope (A1+/A1R+, Nikon, Tokyo, Japan). Both TUNEL-positive and DAPI-positive cells were counted. Data are expressed as TUNEL index, which represents the total number of TUNEL-positive cells.

2.8. Statistical analysis

Data are summarized as mean ± standard error (SE) of at least 3 independent replicates. Parametric and nonparametric tests (as appropriate) were used to analyze the data using SPSS20.0 software. The parametric test was one-way ANOVA, followed by LSD post hoc test. The nonparametric test included Mann Whitney U in two-independent sample test or Kruskal–Wallis H in K independent samples test and the post hoc test was Bonferroni method. p < 0.05 was considered statistically significant.

3. Results

3.1. METH increases DDIT4 protein expression in cardiomyocytes

To assess the role of DDIT4 in the METH-induced cardiotoxicity, firstly we determined DDIT4 protein expression in primary cardiomyocytes treated with a dose range (0–1.5 mM) of METH for 24 h and in the heart tissues of rats after 4-day or 14-day METH exposure. The results showed that DDIT4 protein expression in primary cardiomyocytes was increased by ~1.75–3.5 fold after 0.6–1.5 mM METH exposure in a dose-dependent manner (Fig.1A, 1B). The maximum increase was ~3.5-fold at 0.9 mM dose group. Additionally, DDIT4 protein level was 5.0-fold and 5.5-fold higher in the 4-day and 14-day exposure groups, respectively, than in the control group (Fig.2A, 2B). These results suggest that METH exposure induces DDIT4 protein expression in vivo and in vitro.

3.2. METH induces autophagy and apoptosis in cardiomyocytes

To evaluate whether METH induces autophagy of cardiomyocytes, the expression of autophagy-related protein markers (Beclin-1, LC3-I/II) after METH exposure was measured. The results showed that the expression of Beclin-1 and LC3-II was increased dose-dependently, in a pattern similar to the increase of DDIT4 (Fig.1A, 1D). For example, Beclin-1 protein expression was upregulated by ~1.4-fold at 0.3 mM METH dose group and this increase reached maximum of ~2.5-fold at 1.2 mM dose group. The maximal increase of LC3-II was ~4.5-fold at 1.2 mM dose group. The increase of Beclin-1 and LC3-II protein expression was also observed in the heart tissues of rats after 4-day or 14-day exposure (Fig.2A, 2D).

mTOR is an important inhibitor of autophagy induction. To confirm the effect of METH on cardiomyocyte autophagy, we determined the effect of METH on p-mTOR expression. Western blot analyses showed that in the METH-treated cardiomyocytes, p-mTOR/mTOR protein expression ratio was decreased by ~55%–77% after 0.6–1.5 mM METH exposure in a dose-dependent manner (n = 3, p < 0.01; Fig.1A, 1C). We also found that p-mTOR/mTOR protein expression ratio was decreased significantly by ~76% in the 4-day and 14-day METH exposure groups (n = 3, p < 0.01; Fig.2A, 2C). Based on these results, we concluded that METH exposure induces autophagy in cardiomyocytes both in vitro and in vivo, probably by inhibiting mTOR signaling pathway. Besides the autophagy-related markers, we also determined the expression of apoptotic markers, including cleaved caspase-3 and cleaved PARP. We observed that METH exposure increased the
protein levels of these markers in primary cardiomyocytes in a pattern similar to the increase of DDIT4 and autophagy-related markers (Fig. 1A, 1E). Similar effects were also observed in vivo. Cleaved PARP and cleaved caspase-3 protein expression in the heart tissues of METH-treated rats was increased by 2–4 fold after 4-day or 14-day exposure (Fig. 2A, 2E).

To confirm that METH induces apoptosis of cardiomyocytes, DNA fragmentation, a typical feature of apoptotic cells, in the heart tissues of METH-treated rats was evaluated with TUNEL staining. The results showed that the number of TUNEL-positive cardiomyocytes was increased by ~5-fold after 4-day or 14-day METH exposure (24.7 ± 0.32% and 22.7 ± 0.3% vs. 4.6 ± 0.26%, p < 0.01, Fig. 3A, 3B). Collectively, the aforementioned results suggest that METH exposure induces autophagy and apoptosis of cardiomyocytes, and increased DDIT4 protein expression may contribute to METH-induced autophagy and apoptosis in cardiomyocytes.

3.3. Synthetic siDDIT4 knockdown DDIT4 protein expression in cardiomyocytes

To confirm that DDIT4 is involved in METH-induced autophagy and apoptosis, we transfected cardiomyocytes with synthetic siDDIT4 or

![Image of Western Blot and quantification graphs](image-url)
control siNC prior to METH exposure for 24 h. We found that siDDIT4 significantly decreased DDIT4 protein expression in cardiomyocytes regardless of whether cells were exposed to METH or not (Fig. 4A, 4B). Specifically, at basal level in the absence of METH, the expression of DDIT4 was relatively low. Although the inhibiting effect of siDDIT4 on DDIT4 expression was statistically significant, the extent of reduction was only by 25%. Note that METH exposure increased DDIT4 protein expression by ~2.7-fold in siNC + METH group compared to siNC + vehicle group; this effect was significantly reduced by ~80% after co-exposure of siDDIT4. These data demonstrated that synthetic siDDIT4 could efficiently block DDIT4 expression in cardiomyocytes independent of METH exposure.

3.4. Silencing of DDIT4 protects cardiomyocytes from METH-induced autophagy and apoptosis

To explore whether inhibition of DDIT4 expression reduces METH-induced autophagy and apoptosis in cardiomyocytes, we determined
Beclin-1, LC3-II, cleaved PARP and cleaved caspase-3 protein expression in cardiomyocytes with or without silencing DDIT4 and treated with or without 1.0 mM METH. Western blot results showed that Beclin-1 and LC3-II proteins were increased by ~2-fold in METH-treated cells compared with the vehicle-treated cells transfected with siNC; this effect was significantly decreased by ~70% after transfection with siDDIT4 (Fig. 4A, 4C). Similar response was observed for apoptotic markers. The cleaved PARP and cleaved caspase-3 proteins were increased after siNC + METH treatment; and this effect was decreased apparently after transfection with siDDIT4 (Fig. 4A, 4D).

To confirm that knockdown of DDIT4 protects against METH-induced autophagy, we used fluorescence microscopy to monitor the formation of autophagosomes. According to earlier reports, activated LC3-I can be converted into membrane-bound LC3-II, which leads to autophagosome formation. As shown in Fig. 5A, A1, the number of GFP-LC3 puncta-positive cells was significantly increased in METH-exposed cells compared to the control group (transfection efficiency was around 30–35%); and this number was markedly decreased in METH-treated cells transfected with siDDIT4. These results demonstrated that silencing of DDIT4 gene can reduce METH-induced autophagy in cardiomyocytes.

Inhibition of lysosomal degradation can also result in an accumulation of autophagosomes and increased expression of LC3-II (Funakoshi-Hirose et al., 2013a). To evaluate whether METH impairs lysosomes, we exposed cardiomyocytes transfected with siNC or siDDIT4 to vehicle or 1.0 mM METH for 24 h in the presence and absence of chloroquine, a lysosomal degradation inhibitor. As shown in Fig. 5, METH treatment or chloroquine treatment increased GFP-LC3 puncta compared to PBS-treated group in cardiomyocytes. In addition, METH + chloroquine exposure increased GFP-LC3 puncta compared to PBS-treated group, chloroquine-treated group, or METH-treated group in cardiomyocytes. Silencing DDIT4 expression reduced METH + chloroquine-induced GFP-LC3 puncta substantially. However, in the absence of METH, silencing DDIT4 expression had minimal effect on chloroquine-induced GFP-LC3 puncta. These data indicate that DDIT4-mediated LC3-II increase is not via the pathway by inhibiting lysosomal degradation, but via the pathway of converting LC3-I to LC3-II.

To determine whether silencing of DDIT4 expression also reduces METH-induced apoptosis in vitro, TUNEL assays were performed to evaluate DNA damage in cardiomyocytes after METH exposure. The number of TUNEL-positive cells was increased by >6.9-fold in METH-treated cardiomyocytes transfected with control siRNA compared to vehicle-treated cells (25.3 ± 0.27% vs. 3.6 ± 0.23%, p < 0.05, Fig. 5B, 5B1). This number was reduced by ~72.4% in METH-treated siDDIT4-transfected cardiomyocytes (Fig. 5B, 5B1). These results suggest that knockdown of DDIT4 expression can reduce apoptosis induced by METH in cardiomyocytes.

3.5. Rapamycin promotes METH-induced autophagy and apoptosis in DDIT4 knockdown cardiomyocytes

We used rapamycin, a mTOR inhibitor, to further confirm that DDIT4 is involved in METH-induced autophagy and apoptosis. Cardiomyocytes were transfected with siNC or siDDIT4 for 48 h, and then exposed to rapamycin (1 μM) for 1 h followed by treatment with or without METH (1.0 mM) for 24 h. Western Blot results showed that METH decreased the expression of p-mTOR compared to the control, but the level of p-mTOR was increased when silencing DDIT4 expression. Pretreatment with rapamycin reversed this effect (Fig. 6A, 6B). Moreover, Beclin-1 and LC3-II protein expression was increased (2.2-fold and 3.7-fold, respectively) after rapamycin administration, compared with siDDIT4 + METH group (Fig. 6A, 6C). Additionally, cleaved PARP and cleaved caspase-3 protein expression was increased (2.7-fold and 2.6-fold, respectively) after rapamycin treatment, compared with siDDIT4 + METH group (Fig. 6A, 6D). These results suggest that DDIT4-mTOR pathway is involved in METH-induced autophagy and apoptosis.

4. Discussion

In the present study, the main findings can be summarized as follows: (1) DDIT4 expression in cardiomyocytes is increased after METH exposure in vivo and in vitro, (2) DDIT4 can mediate METH-induced autophagy and apoptosis in cardiomyocytes, (3) DDIT4 silencing can activate the phosphorylation of mTOR and protect cardiomyocytes against...
METH-caused autophagy and apoptosis, and (4) the effect of DDIT4 silencing in METH-induced autophagy and apoptosis could be reversed after exposure to the mTOR inhibitor rapamycin. These findings indicate that DDIT4 plays a critical role in METH-induced autophagy and apoptosis in cardiomyocytes.

The present study shows that METH exposure induces apoptosis of cardiomyocytes both in vitro and in vivo. The finding that METH induces cardiomyocyte apoptosis in vitro is consistent with our earlier study (Leung et al., 2014). The present study extends our previous in vitro finding (Leung et al., 2014) to in vivo and demonstrates that METH also causes apoptosis of cardiomyocytes in the heart tissues from METH-treated rats. This new in vivo finding is important as it may, in part, account for the reported cardiotoxicity, such as cardiac fibrosis, hypertrophy, and disarray observed in METH abusers (Islam et al., 2009). Multiple studies have shown that METH exposure can cause apoptosis of other cell types, including endothelial cells (Ma et al., 2014), astrocytes (Shah et al., 2014), and dopaminergic cells in particular (Lin et al., 2012; Huang et al., 2015; Nam et al., 2015). Thus, the apoptotic effect of METH is not specific to brain cells, but also to cardiovascular cells. The relative sensitivity of various cell types to the apoptotic effect of METH needs to be investigated. This is important because it will provide important information regarding which organ system may be the most sensitive target to METH and the underlying mechanism of METH-caused death.

Besides apoptosis, this study also demonstrates that METH exposure induces autophagy of cardiomyocytes in vitro and in vivo. It is widely known that METH can induce autophagy in dopaminergic cells (Kanthasamy et al., 2006; Pasquali et al., 2008; Pitaksalee et al., 2015). However, to the authors’ knowledge, there is only one published study that has investigated the effect of METH on autophagy in a HL-1 murine atrial cardiomyocyte cell line (Funakoshi-Hirose et al., 2013a). Thus, the present study is the first study that examines METH’s effect...
on autophagy in primary cardiomyocytes and heart tissues from METH-treated rats. Using HL-1 cardiomyocyte cell line, Funakoshi-Hirose et al. (2013a) showed that METH induced autophagy of cardiomyocytes by impairing the autophagy-lysosome protein degradation system. However, the present study showed that DDIT4 up-regulated induced by METH mediated autophagy of primary cardiomyocytes, probably not by inhibiting lysosomal degradation, but via the pathway of converting LC3-I to LC3-II. These data indicate that there are at least two pathways to explain the formation of autophagy induced by METH, one is by impairing the autophagy-

Fig. 5. Effects of synthetic siDDIT4 on METH-caused autophagy and apoptosis in cardiomyocytes. Cardiomyocytes were transfected with siNC or siDDIT4 for 48 h prior to treatment, pretreated with or without chloroquine (CQ) (3 μM) for 1 h, and then co-incubated with or without METH (1.0 mM) for another 24 h. Effects of synthetic siDDIT4 on METH-caused autophagy in cardiomyocytes were assessed by calculating the number of GFP-LC3 puncta-positive cells. METH exposure increased the number of GFP-LC3 puncta-positive cells, and inhibition of DDIT4 decreased this number (A, A1). Actin patches were visualized with phalloidin conjugated with rhodamine. Effects of suppressing DDIT4 expression in METH-treated cardiomyocytes were assessed with TUNEL assays (B, B1). Apoptotic cells were stained with TUNEL (green). Nuclei were counterstained with DAPI (blue). The number of positive cells is presented as mean ± SE of 3 experiments. *p < 0.01 vs. siNC + vehicle group. Δp < 0.01 vs. siNC + METH group.
lysosome protein degradation system, the other is by promoting LC3-I conversion to LC3-II.

Previous studies have shown that METH-induced autophagy is a pro-survival factor at low concentrations, but it becomes a pro-apoptotic factor at high concentrations in dopaminergic cells (Kongsuphol et al., 2009; Nopparat et al., 2010; Chandramani et al., 2012; Pitaksalee et al., 2015a). In cardiomyocytes, the relationship between METH-induced apoptosis and autophagy remains to be investigated.

It is important to note that DDIT4 is involved in METH-induced autophagy and apoptosis in cardiomyocytes. DDIT4 is a stress-responsive protein and its expression can be increased in various stressful scenarios, such as hypoxia, oxidative stress, DNA damage, exposure to toxicants, diet-induced obesity, and ischemia/reperfusion (Lang et al., 2008; Hernandez et al., 2011; Lang and Korzick, 2014; Pieri et al., 2014). It has long been recognized that DDIT4 can promote tumor cell apoptosis, such as in esophageal cancer EC9706 cells (Lin et al., 2014), human osteosarcoma cell line (Hsia et al., 2015), human myeloid leukemia cells (Cheng et al., 2013), K562 chronic myeloid leukemia cell line (Kim et al., 2010), and human prostate cancer cells (Liu et al., 2015). However, the role of

**Fig. 6.** Rapamycin promotes METH-induced autophagy and apoptosis in DDIT4-silenced cardiomyocytes. Cardiomyocytes were transfected with siNC or siDDIT4 for 48 h, and then exposed to rapamycin (Rap, 1 μM) for 1 h followed by treatment with or without METH (1.0 mM) for 24 h. Western Blot (A) and quantitative analyses (B-D) were performed to determine p-mTOR/mTOR, Beclin-1, LC3-II, cleaved PARP and cleaved caspase-3 protein expression in cardiomyocytes. β-Actin was used as a loading control. Fold induction relative to vehicle-treated cells is shown. The experiment was performed in triplicate and the average fold change is shown. *p < 0.01 vs. siNC + vehicle group. #p < 0.01 vs. siNC + METH group. △p < 0.01 vs. siDDIT4 + METH group.
DDIT4 in METH-caused cardiotoxicity and the related mechanisms remain unknown. This study showed that DDIT4 is induced by METH significantly in vivo and in vitro. These results indicate that DDIT4 plays a critical role in METH-induced autophagy and apoptosis in cardiomyocytes and it may also be a therapeutic target for gene therapy of METH abuse.

Another major finding is that METH-induced autophagy through mTOR signaling pathway in cardiomyocytes. Autophagy (lysosome-dependent macroautophagy) is responsible for eliminating dysfunctional substrates and thereby maintaining cellular homeostasis (Feng et al., 2014). mTOR is an inhibitor of autophagy induction (Jung et al., 2010; Sudarsanam and Johnson, 2010). Our findings indicate that DDIT4 can mediate METH-induced autophagy in cardiomyocytes by inhibiting the phosphorylation of mTOR. Furthermore, our data confirm that DDIT4 is an upstream inhibitor of mTOR in METH-caused autophagy. A schematic depicting the role of mTOR in the METH-induced autophagy in cardiomyocytes is provided in Fig 7. Interestingly, we found that DDIT4 silencing can decrease the apoptosis-related proteins in cardiomyocytes. We also observed reversible changes after rapamycin retreatment. It is likely that DDIT4-mediated METH-induced autophagy may stimulate apoptosis of cardiomyocytes. However, further studies are needed to test this hypothesis.

In conclusion, the present study demonstrates that DDIT4 is induced by METH significantly in vivo and in vitro, and knockdown of DDIT4 significantly decreased METH-induced autophagy and apoptosis in cardiomyocytes. DDIT4 can mediate METH-induced autophagy in cardiomyocytes by inhibiting the phosphorylation of mTOR. However, the relationship between METH-induced apoptosis and autophagy in cardiomyocytes and the role of DDIT4 in this interaction still need further research.

Conflicts of interest statement
The authors declare that there are no conflicts of interest.

Transparency document
The Transparency document associated with this article can be found, in the online version.

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References


