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ORIGINAL PAPER

Apelin-13 Protects PC12 Cells Against Methamphetamine-Induced Oxidative Stress, Autophagy and Apoptosis

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Abstract

Methamphetamine (METH) is a potent psychomotor stimulant that has a high potential for abuse in humans. In addition, it is neurotoxic, especially in dopaminergic neurons. Long-lasting exposure to METH causes psychosis and increases the risk of Parkinson's disease. Apelin-13 is a novel endogenous ligand which studies have shown that may have a neuroprotective effect. Therefore, we hypothesized that Apelin-13 might adequately prevent METH-induced neurotoxicity via the inhibition of apoptotic, autophagy, and ROS responses. In this study, PC12 cells were exposed to both METH (0.5, 1, 2, 3, 4, 6 mmol/L) and Apelin-13 (0.5, 1.0, 2.0, 4.0, 8.0 µmol/L) in vitro for 24 h to measure determined dose, and then downstream pathways were measured to investigate apoptosis, autophagy, and ROS responses. The results have indicated that Apelin-13 decreased the apoptotic response post-METH exposure in PC12 cells by increasing cell viability, reducing apoptotic rates. In addition, the study has revealed Apelin-13 decreased gene expression of Beclin-1 by Real-Time PCR and LC3-II by western blotting in METH-induced PC12 cells, which demonstrated autophagy is reduced. In addition, this study has shown that Apelin-13 reduces intracellular ROS of METH-induced PC12 cells. These results support Apelin-13 to be investigated as a potential drug for treatment of neurodegenerative diseases. It is suggested that Apelin-13 is beneficial in reducing oxidative stress, which may also play an important role in the regulation of METH-triggered apoptotic response. Hence, these data indicate that Apelin-13 could potentially alleviate METH-induced neurotoxicity via the reduction of oxidative damages, apoptotic, and autophagy cell death.

Keywords Methamphetamine · Apelin-13 · Neurotoxicity · Apoptosis · Autophagy · Reactive oxygen species

Introduction

Methamphetamine (METH) and related compounds are the second most commonly used illegal substance worldwide after cannabis. METH is a highly addictive and neurotoxic psychostimulant that can produce euphoria and stimulatory effects such as other stimulants including cocaine [1]. METH has a chemical structure similar to amphetamine and has similar effects on the central nervous system (CNS). This drug increases motor activity, satisfaction, happiness, consciousness, respiration, and decreases appetite [2]. In addition, METH can easily be produced by chemical available at low cost in illegal laboratories, unlike narcotics such as marijuana or heroin derived from plants [3]. These characteristics have led to extensive use throughout the world. According to a global drug report in 2015, METH users have risen by 158% over the past 5 years, with predominant areas of use expanding, including North America, Europe [4, 5]. In addition, the use of narcotics in Iran has changed considerably over the last several years and has turned from traditional opium into opioids with newer forms (such as heroin, crackers) and synthetic substances (such as METH). Iran is the fifth country in the world after Mexico, the United States, China, and Thailand for METH misuse [6, 7]. Deaths associated with METH are one of the most important problems in Iran. On the other hand, these

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behaviors may lead to criminal behavior, resulting in one of the social concerns, which suggests that the misuse of METH should be considered as a global health problem.

Neurotoxicity is one of the symptoms in the CNS that cause by misusing METH. Long-lasting exposure to METH causes psychosis and increases the risk of Parkinson's disease [1, 8, 9]. According to evidence of METH neurotoxicity, during a series of oxidation processes, it produces reactive oxygen species (ROS), which develops in the cortex or other regions of the brain [10, 11]. On the other hand, METH can increase inflammatory factors such as TNF-α and apoptosis induces by the expression of apoptotic genes and activation of apoptotic pathways [12, 13]. Recent studies have shown that autophagy induced by METH through Akt/mTOR-dependent pathways [14, 15]. Understanding the mechanisms involved in the neurotoxicity of METH can lead to the discovery of new strategies to prevent or counteract the neurotoxic and neurodegenerative process.

Apelin is one of the many types of adipokines. It is a prepropeptide and its gene translates into 77 amino acids in humans. Apelin-13 is one of the most important functional peptides of this gene. The biological effects of Apelin have shown that it is involved in cardiovascular activities, neuroangiogenesis, immunological integration and homeostasis in body fluids [16–18]. Recently, the Apelin-APJ system has been studied as a neurological regulator for various neurological disorders. These include inhibition of apoptosis and reduction of neurological deficits in brain ischemia. Additionally, treatment with Apelin-13 results in the reduction of brain damage and cerebral edema caused by ischemia via blocking cellular death [19-21]. There is also a positive correlation between TNF-α and Apelin in mouse and human tissues. In addition, studies have shown that Apelin-13 regulates mechanisms that reduce the activity of enzymes associated with autophagy, including reduction of expression levels of LC3-II and p62, which can be attributed to factors. It is important to induce autophagy. Apelin also reduces the capacity of macrophages, inhibits the pro-inflammatory cytokines and activates chemokines, which suggests that the Apelin peptide is involved in anti-inflammatory [22–25]. Due to the lack of effective therapeutic strategies to deal with neurotoxic agents such as METH, to investigate them to counteract these adverse effects seems to be necessary. In the present study, we examined the neuroprotective activity and potential mechanisms of Apelin-13 in PC12 cells treated with METH.

Methods and Materials

Reagents

RMPI-1640 medium, fetal bovine serum (FBS), Trypsin-EDTA were purchased from Gibco (Carlsbad, CA, USA). Fluorometric intracellular ROS kit, Autophagy Assay Kit, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Horse serum (HS), and PBS were purchased from Sigma Chemical Crop (St Louis, MO, USA). The Annexin V-FITC Apoptosis detection kit was obtained from Invitrogen (Carlsbad, CA, USA). PARIS™ Kit was purchased from Life Technologies, Thermo Fisher Scientific Inc. (Waltham, MA, USA). The PrimeScript RT-PCR Kit and BCA assay kit were purchased from TaKaRa Biotechnology (Shiga, Japan). Apelin-13, MAP LC3β and β-actin antibody were purchased from Santa Cruz biotechnology, Inc. (Dallas, TX, USA).

Cell Culture and Drug Treatments

Rat differentiated PC12 cell line was used in this study. The cells were purchased from Buali (Avicenna) Research Institute (Mashhad, Iran). PC12 cells were cultured in RPMI1640 medium supplemented with 10% heat inactivated fetal bovine serum and 5% horse serum, 100 U/mL penicillin/streptomycin (PAN-Biotech GmbH, Aidenbach, Germany) at 37 °C in an incubator (BINDER, Tuttlingen, Germany) containing a humidified atmosphere of 5% CO_2. In addition, 0.25% Trypsin/EDTA was used for detaching cells and they were passaged every 2–3 days. PC12 cells were exposed the indicated determined concentrations of METH for 24 h. Apelin-13 was added to cell culture medium at indicated determined concentrations 1 h prior to METH treatment.

Cell Viability Assay and Morphological Changes

PC12 cells were seeded into 96-well plates at an initial density of 10^4 cells/well. METH was then added to achieve a final concentration of 0, 0.5, 1.0, 2.0, 3.0, 4.0, or 6.0 mM to determine the appropriate METH concentrations for using in subsequent studies. 0.5, 1, 2, 4, or 8 μM Apelin-13 were added to cells 1 h prior 4 mM METH exposure to observe the protective role of Apelin-13 in METH-induced neurotoxicity. The morphology of PC12 cells were taken by using an inverted microscope (Olympus IX71, Central Valley, PA, USA). MTT was dissolved in PBS water to obtain a 5 mg/mL concentration, filter sterilized, added in a volume of 10 μL to each well (0.5 mg/mL final concentration), and incubated for 4 h. The MTT-containing medium was replaced by 100 μL of dimethylsulfoxide (DMSO, Santa Cruz, Dallas, TX, USA), and the plates were gently agitated for 10 min. Absorbances were obtained at 570 nm with background subtraction at 690 nm using Biotek Cytation 5 imaging reader (Biotek Instruments, Winooski, VT, USA). All of the experiments were performed in triplicate.

RNA Extraction and cDNA Synthesis

RNA of samples were isolated using PARIS™ Kit (Life Technologies, Thermo Fisher Scientific Inc., Waltham, MA, USA) using manufacturer's instructions. The quantity and quality of isolated RNA samples were determined by Picodrop microliter spectrophotometer (OEM, UK) and electrophoresis on a 0.8% agarose gel. Afterwards, 1 µg of total RNA was converted into cDNA using PrimeScript™ RT reagent kit (TaKaRa Bio, Shiga, Japan) according to the manufacturer's instruction.

Real-Time PCR

Real-Time qPCR was performed using the Bio-Rad CFX96™ Real-Time System (Bio-Rad, Foster City, CA). The 25 µL reaction contained 12.5 µL SYBER Master Mix without ROX (Ampliqon, 5230 Odense M, Denmark), 10.5 µL ddH_2O, 1 µL cDNA template, 5 µM of each primer (forward and reverse). Sequences of *Beclin-1* primers were as follows: forward, 5′-ATCCTGGACCGAGTGACCATTC-3′ and reverse, 5′-TCTCCTGAGTTAGCCTCTTCCTCC-3′. Sequences of *GAPDH* primers were as follows: forward, 5′-GGCTGCCTTCTCTTGTGACAA-3′ and reverse, 5′-TGC CGTGGGTAGAGTCATACTG-3′. Conditions for amplification were 95 °C for 15 min, followed by 40 cycles of 95 °C for 5 s, 60 °C for 30 s. Melting curves were obtained by slow heating (0.5 °C/s) at temperatures in the range of 65 °C to 95 °C. All samples were run in duplicate. Relative expression of target genes was normalized to *GAPDH* as internal control. Relative gene expression and fold changes were calculated using the $2^{-\Delta\Delta Ct}$ method [26].

Western Blotting

Protein and total RNA were isolated simultaneously from the same samples using PARIS™ Kit (Life Technologies, Thermo Fisher Scientific Inc., Waltham, MA, USA). The total protein were corrected for their protein levels based on the reference manual from BCA assay kit (TaKaRa, Shiga, Japan). Protein extraction samples were boiled for 10 min with loading buffer and separated by 15% SDS-PAGE. Proteins were transfer onto PVDF membrane. Membrane were subsequently blocked for 1 h at room temperature in blocking buffer (3% BSA in TBST). Then, the membranes were incubated overnight at 4 °C on an orbital shaker with anti-LC3B and anti-beta-actin antibody (1:1000 dilution, Santa Cruz, Dallas, TX, USA). After washed three times with TBST, membrane were incubated with secondary antibody (1:1000 dilution, peroxidase-labeled Mouse IgGκ) at room temperature for 2 h, washed with TBST for three times, visualize with ECL immunoblotting detection system. The intensity of each band was quantitatively determined by the Image J software (NIH, Bethesda, MD, USA). β-Actin was used as a loading control. Therefore, the density ratio indicated the relative intensity of each band against β-actin.

Autophagosome Assay

PC12 cells were seeded at a density of 10^4 cells per well of a 96-well plate. After 24 h drug incubation, the autophagy assay kit (Sigma-Aldrich #MAK 138) was used to determine the formation of autophagosomes to measure fluorescence intensity at $\lambda_{ex} = 360/\lambda_{em} = 520$ nm using Biotek Cytation 5 imaging reader.

Determination of Intracellular ROS Production

PC12 ($\approx 10^4$) cells/well/90 µL were plated in growth media at a 96 well plate. Then, cells were treated in desired wells and added 100 µL/well of Master Reaction Mix into the cell plate (Sigma-Aldrich; #MAK143-KT) following manufactures protocol. Finally, after adding METH to the wells, the plate was incubated in a 5% CO_2 at 37 °C for 24 h. Then, we measured the fluorescence intensity at $\lambda_{ex} = 490/\lambda_{em} = 525$ nm by by using Biotek Cytation 5 imaging reader.

Measurement of Cell Apoptosis

PC12 cells were seeded on 6-well plates at a density of 5×10^5 cells/well and incubated overnight at 37 °C. After 24 h drug incubation, Cells were centrifuged to remove the medium, washed twice with 4 °C PBS and stained with Annexin V-FITC and propidium iodide (PI) according to the Annexin V apoptosis detection kit instructions. The percentage of apoptotic cells was quantified by flow cytometry (Attune NxT, Thermo Fisher Scientific, Eugene, OR, USA).

Statistical Analysis

Statistical analyses were performed using GraphPad Prism 6 software (GraphPad Software Inc., San Diego, CA, USA). Data were presented as the means ± standard error of the mean (mean ± SEM) and compared by one-way ANOVA followed by Turkey's post hoc analysis, was applied to all the experiments to examine the significance of the data. $P < 0.05$ was considered statistically significant. All of the experiments were replicated at least three times.

Results

The Effect of Apelin-13 on the Viability of METH-Induced PC12 Cells

We firstly determined the effect of METH at various concentration (0.5, 1, 2, 3, 4, 6 mM) on the PC12 cell viability and the protective role of Apelin-13 by using MTT. As shown in Fig. 1a, METH dose-dependently inhibited the proliferation of PC12 cells when incubated for 24 h. The selected concentration value of METH was about 4 mM such that concentration was adopted in some experiments [27, 28]. Then, we examined whether Apelin-13 protects against METH-induced PC12 cells viability loss. As shown in Fig. 1b, when Apelin-13 (0.5, 1, 2,4, 8 μM) was added in the cell culture medium 1 h prior to 4 mM METH treatment, the survival of PC12 cells significantly increased in a dose-dependent manner. The maximal rescue occurred at a concentration of 4 and 8 μM Apelin-13, Which we have chosen 4 μM for downstream techniques which was similar to the referenced experiment [29]. In addition, treatment of Apelin-13 (4 μM) alone showed no effect on PC12 cell viability.

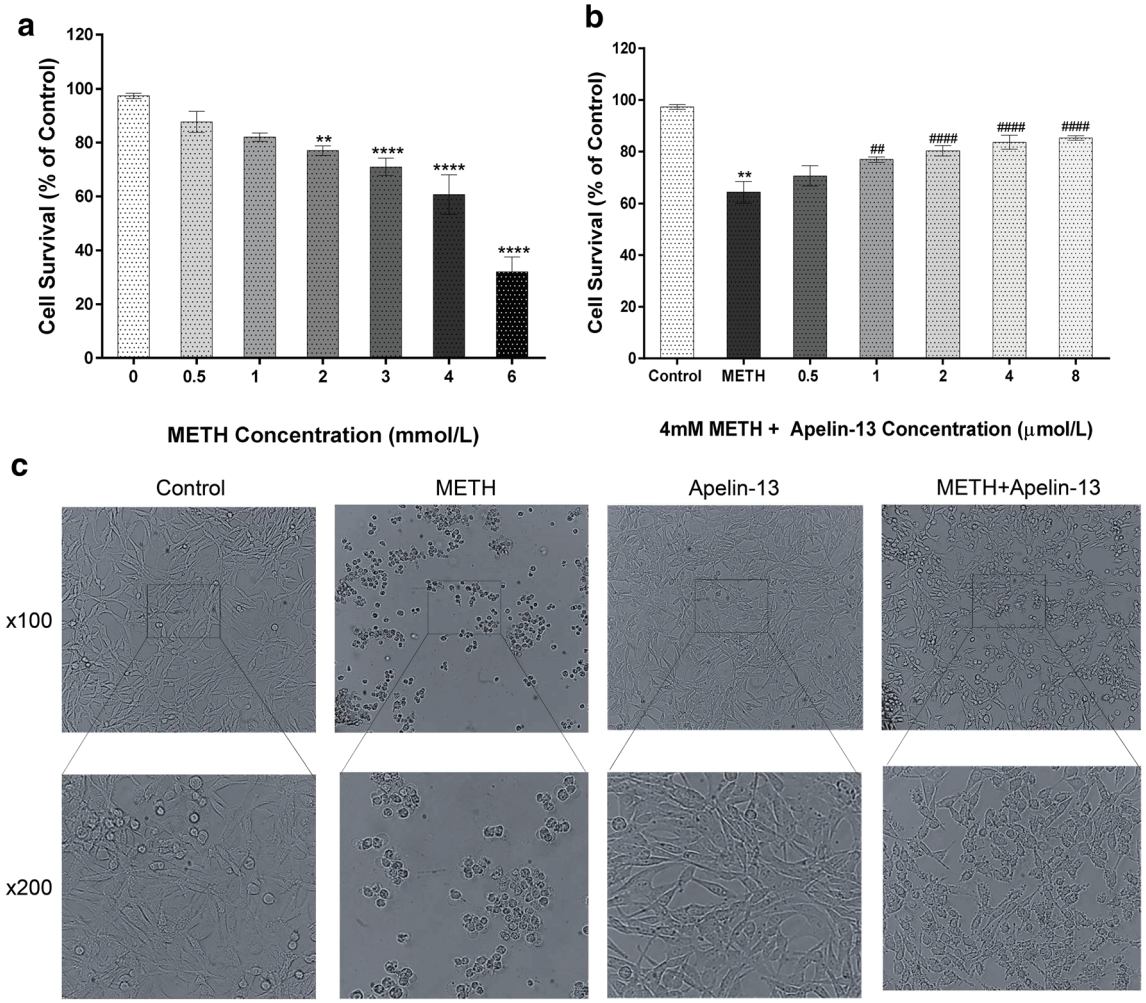

Fig. 1 Apelin-13 protect against METH-induced cells viability loss in PC12 cells. **a** Effects of METH on cell viability. PC12 cells were treated with the indicated concentration of METH (0.5, 1, 2, 3, 4, 6 mM) and the cell viability was measured after treatment of 24 h by MTT assay (n = 9). **b** Effects of Apelin-13 on METH-induced cell viability loss. Cells were treated with 4 mM METH for 24 h in the absence or presence of Apelin-13 (0.5, 1, 2, 4, 8 μM) treatment at the indicated concentrations 1 h prior to METH treatment, and a dose-dependent effect of Apelin-13 was assessed by MTT assay (n = 9). **c** The cells were treated with 4 mM METH, 4 mM METH plus 4 μM Apelin-13 or 4 μM Apelin-13 alone for 24 h. Morphological changes have shown in response to Apelin-13 treatment. Results are expressed as percentage of value in untreated control cultures and are mean ± SEM of three replicate values obtained from three independent experiments. $^{**}P < 0.01$ and $^{****}P < 0.0001$ versus control; $^{\#\#}P < 0.01$, $^{\#\#\#\#}P < 0.0001$ versus METH treatment alone

Morphological Changes of METH-Induced PC12 Cells in Response to Apelin-13 Treatment

For investigation of morphological changes of PC12 cells after treatment of it with METH and Apelin-13, the cells were observed after 24 h under inverted microscope. Figure 1c. has shown the morphological appearance of METH-induced PC12 cells in response to Apelin-13 treatment. Morphological results have shown that the METH-induced PC12 cells have changed compared to the control samples. The shape of the cells under the influence of METH has been rounded, which shows a major change in the normal state of the cell membrane and decreased cell volume. In addition, a number of cells were singled from the flask bottom and floated. While 1 h pre-treatment with Apelin-13 cause in resistance of the cells to be rounded. In addition, there is no change in the morphology of the cells in the control group and the group with Apelin-13 alone.

The Effect of Apelin-13 on the Expression of Beclin-1 and LC3-II in METH-Induced PC12 Cells

To evaluate the autophagy response in METH-induced PC12 cells and protective effect of Apelin-13, we measured mRNA expression levels of Beclin-1 using Real-Time PCR. Our result has shown that Beclin-1 mRNA expression in METH-induced PC12 cells were significantly increased compared with the control group (1.618 ± 0.325, $P < 0.0286$) as shown in Fig. 2a. While, 1 h pre-treatment with Apelin-13 significantly decreased Beclin-1 mRNA expression in METH-induced cells (1.097 ± 0.1893, $P < 0.05$), On the other hand, 4 µM Apelin-13 alone decreased the mRNA expression level of Beclin-1 (0.906 ± 0.1893).

In addition, we measured protein expression of LC3-II using Western Blot. The result has shown protein expression of LC3-II in METH-induced PC12 cells were significantly increased compared with that the control group (6.713 ± 1.705, $P < 0.01$). In contrast, pre-treatment with Apelin-13 with 4 µM concentration significantly decreased protein expression of LC3-II in METH-induced PC12 cells (2.644 ± 0.892, $P < 0.05$), While 4 µM Apelin-13 significantly decreased the protein expression level of LC3-II the most, to (1.288 ± 0.330, $P < 0.05$), compared with the METH-only treated group (Fig. 2b).

The Effect of Apelin-13 on METH-Induced Autophagosomes in PC12 Cells

Autophagy assay kit was used to investigate the effect of Apelin-13 on METH-induced autophagosomes in PC12 cells. According to Fig. 3a, b, results were determined that the METH group significantly increased fluorescence intensity compared to the control group ($P < 0.01$). On the other hand,

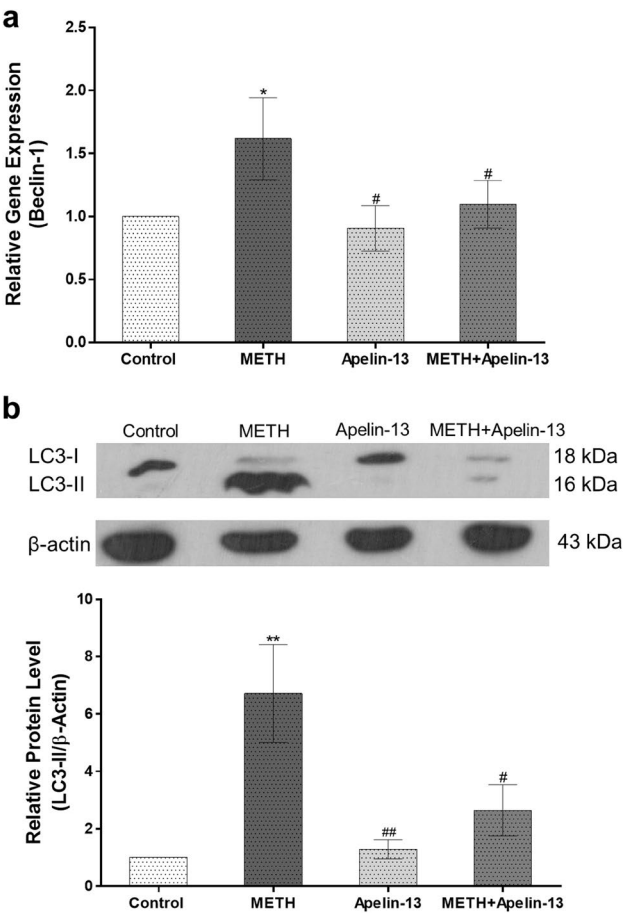

Fig. 2 Effect of Apelin-13 on autophagy in METH-induced PC12 cells. The cells were treated with 4 mM METH, 4 mM METH plus 4 µM Apelin-13, or 4 µM Apelin-13 alone for 24 h. RNA and Proteins were extracted and analyzed by Real-Time and western blot **a** Real-time PCR analysis for Beclin-1 gene expression (n = 8). **b** Western blot analysis for LC3-II gene expression (n = 4). Values are means ± SEM. Our results were analyzed by one-way ANOVA followed using a post hoc test of Turkey. Statistical significance: $^{*}P < 0.05$, and $^{**}P < 0.01$ compared to the control group, $^{\#\#}P < 0.01$ compared to the METH group

the Apelin-13 group alone and the METH-Apelin-13 group have shown they significantly decreased fluorescence intensity level compared to the METH group ($P < 0.01$ and $P < 0.05$, respectively). As illustrated in Fig. 3a, images taken from the inverted fluorescence microscope confirmed the results which obtained from using Biotek Cytation 5 imaging reader. In addition, METH group exhibits higher levels of fluorescence than other groups, and the group, which has been treated with Apelin-13 reduced fluorescence intensity.

Effect of Apelin-13 on Intracellular ROS Level in METH-Induced PC12 Cells

As regards, the METH-induced cytotoxicity is known to be mediated mainly by oxidative stress; we used Fluorometric

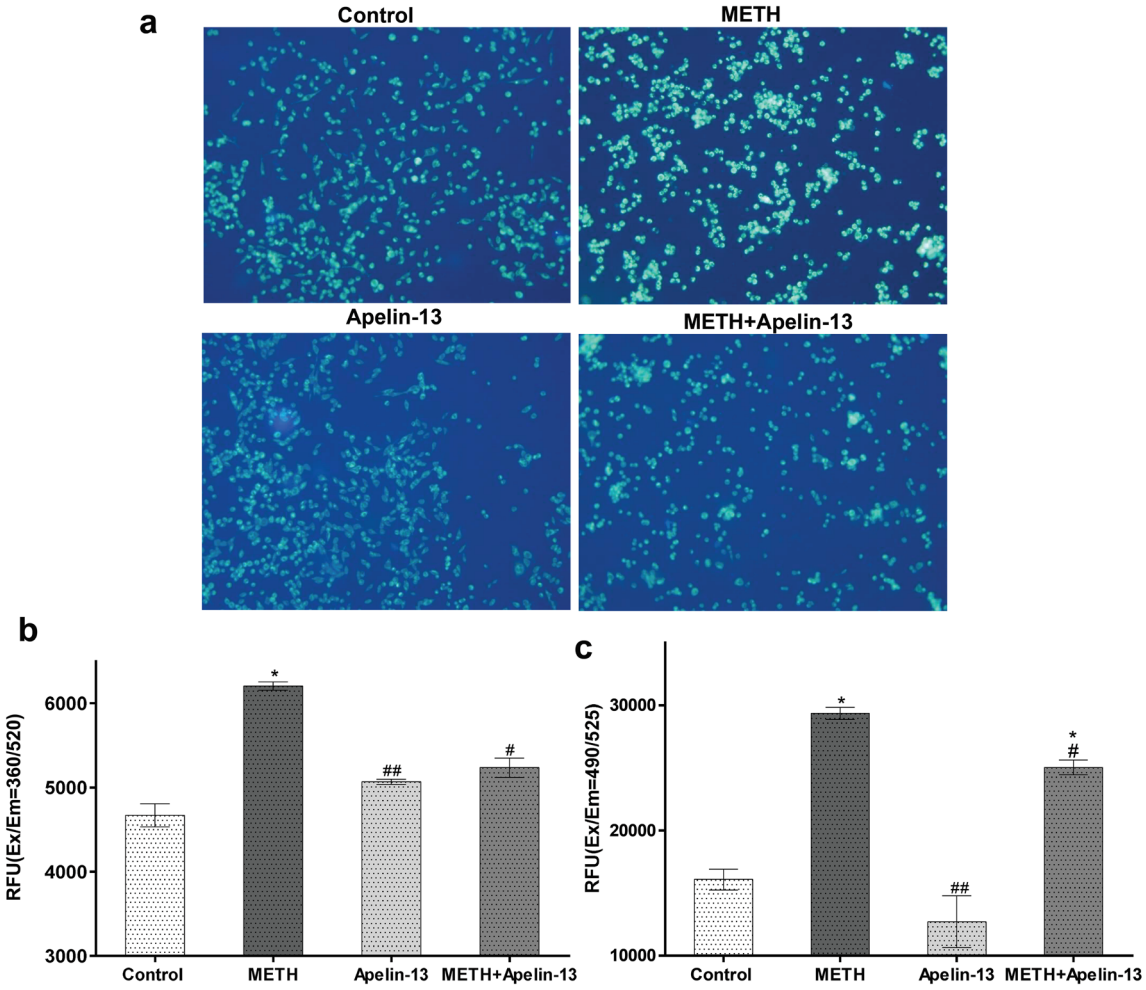

Fig. 3 The effect of Apelin-13 on METH-induced autophagosomes and intracellular ROS level in PC12 cells. **a** Autophagic vacuole formation was observed by fluorescence microscope. **b** The mean fluorescence intensity was measured by Biotek Cytation 5 imaging reader (n = 4) **c** intracellular ROS level determined by ROS kit (n = 3). The results shown is presented as the mean ± SEM of three independent experiments. * $P < 0.05$ compared to the control group, $^{\#}P < 0.05$, and $^{\#\#}P < 0.01$ compared to the METH group

Intracellular ROS Kit to investigate whether Apelin-13 affected intracellular ROS formation by METH exposure of PC12 cells. As shown in Fig. 3c, results have shown that exposure to 4 mM METH significantly increased the level of intracellular ROS production ($P < 0.01$). While, pre-treatment with 4 μM prior to METH-induced PC12 reduced intracellular ROS production ($P < 0.05$).

Apelin-13 Protect Against METH-Induced PC12 Cell Apoptosis

We used Annexin V-FITC/PI kit to determine effect of Apelin-13 on apoptosis of METH-induced PC12 cells. As shown in Fig. 4a, b, the control group has 7.587 ± 0.443 percent early apoptosis, 0.780 ± 0.858% late apoptosis, and 1.917 ± 0.858% necrosis. While METH group has 19.6 ± 3.722% early apoptosis, 5.257 ± 0.959% late apoptosis, and 3.993 ± 0.965% necrosis. On the other hand, PC12 treated with Apelin-13 has 7.733 ± 1.242% early apoptosis, 1.2 ± 0.17% late apoptosis, and 2.783 ± 1.377% necrosis. However, when the cells were pre-treatment with Apelin-13 prior to exposure to 4 mM METH, the observed apoptosis rate was significantly attenuated and the apoptosis rate was 11.06 ± 2.352 percent early apoptosis, 2.497 ± 0.864 late apoptosis, and 2.323 ± 1.036 percent necrosis.

Results have shown that cell death generally increased in the METH group compared with the control group ($P < 0.01$) and as indicated in Fig. 4b, this experiment have proved that Apelin-13 reduced cell apoptosis on METH-induced PC12 cells ($P < 0.01$). Partial analyses revealed that the early apoptosis of METH group compared to control group was significantly higher that control group ($P < 0.001$), while the early apoptosis of Apelin-13 group and METH-Apelin-13

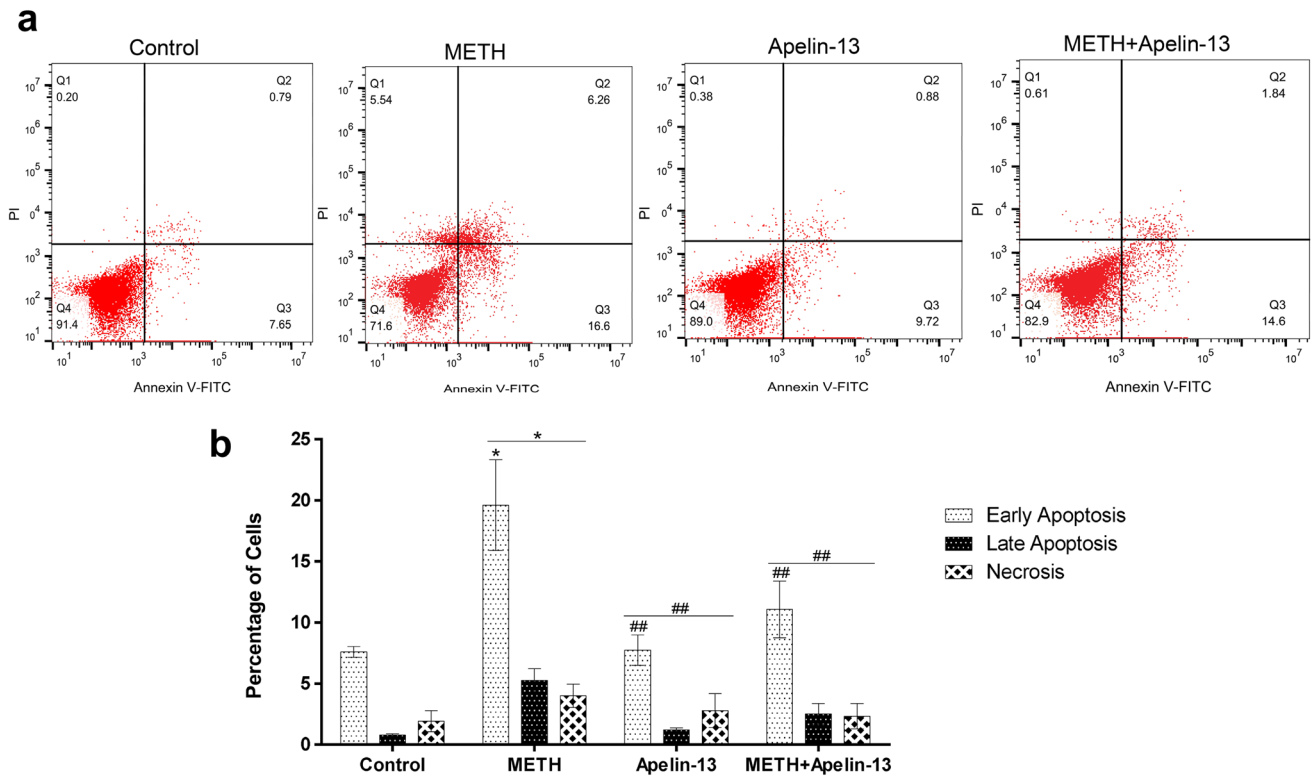

Fig. 4 Apelin-13 Protect Against METH-Induced PC12 Cell Apoptosis. Cells were treated with 4 mM METH for 24 h in the absence or presence of 4 μM Apelin-13 treatment 1 h prior to METH treatment. **a** Apoptosis results are shown in Logarithmic flurescence intensity with the x-axis (annexin V-FITC) versus y-axis (PI). Four quadrants represent necrosis cells (Q1), late apoptotic cells (Q2), viable cells (Q3) and early apoptotic cells (Q4). **b** The percentage of apoptotic PC12 cells in each group. Data are presented as mean ± SEM (n = 3). $^{*}P < 0.05$ compared to the control group, $^{\#\#}P < 0.01$ compared to the METH group

group significantly decreased compared to METH group ($P < 0.001$ and $P < 0.01$, respectively).

Discussion

Cell death and neurological damage have been associated with oxidative stress and the imbalance between the production of free radicals and antioxidant. Heretofore, several studies have explored the role of oxidative stress in cellular damage in various disease, including neurodegenerative disease. ROS, such as superoxide anion and hydroxyl radicals damages biological molecules, including proteins and nucleic acids, and eventually leads to oxidative cell death. Recently, many efforts have been made by researchers to obtain composites with neuroprotection potential, in particular, those with the capacity to cross the blood–brain barrier [27, 30]. PC12 cells are suitable models for neurological and neurobiological studies due to multiple features such as nerve growth factors. In the present study, we investigated the protective effects of Apelin-13 on METH-induced neurotoxicity. Our results indicate that Apelin-13 treatment enhance cell viability of PC12s. Moreover, Apelin-13 reduces intracellular ROS, apoptosis and autophagy elevated by METH neurotoxicity.

METH is well known as a neurotoxic model, and recent studies have reported that it is involved in inflammatory-neurological processes and brain dysfunction due to abuse of the drug. In fact, glial cells appear to be activated in response to METH, although its effects on microglial cells are not well understood. In addition, recent reports have shown that cytokines, normally released by activated microglia, may have a dual role in brain injury [31]. This had led researchers to pursuit at the toxic effects of METH and the study of compounds that reduce its toxic effects.

One of the most recently considered compounds is Apelin-13, a neuropeptide that some studies have demonstrated that this peptide has a neuroprotective effect [21]. Several studies have shown that Apelin-13 protects the brain from damage of ischemia–reperfusion and cerebral edema in a transient model of focal cerebral ischemia [32, 33]. On the other hand, Bao et al. have reported that Apelin-13 reduces injuries from brain damage by controlling autophagy [34]. Xin and et al. have displayed that Apelin-13 protects the ischemic stroke-induced damage by suppression of inflammation [35]. Another study by Kasai et al. have suggested

that Apelin deficiency promotes the development of amyotrophic lateral sclerosis (ALS) in the mouse model SOD1 (G93A) [36]. Some other studies have shown that Apelin protects hippocampal neurons against excitotoxic damage via over-activation of the NMDA receptor [37, 38]. According to the studies, our current study confirmed the protective effects of Apelin-13 and has shown that it could reduce the neurotoxic effect of METH.

Prior studies have displayed that overexposure to METH may lead in decreased cell viability. Pitaksalee explained that cell viability of SH-SY5Y cells decreased significantly post 24 h METH exposure in a dose-dependent manner [39]. In the current study, MTT results showed that METH decreased cell viability significantly compared with the control group, while cell death could be blocked when the concentrations of Apelin-13 were between 0.5 and 8 μM. Therefore, our data determined that a METH-induced decrease in cell viability can be rescued by apelin-13.

Autophagy is a cell-based mechanism for controlling the suitable level of proteins and intracellular organelles. This mechanism can also be activated against physiological stresses such as lack of nutrients, protein and cytokines accumulation. Autophagy, like the antioxidant system, somewhat resists oxidative stress, eventually, cell death occurs in the event of the inability of these defense mechanisms [37]. Previous observations have shown that many drugs such as cocaine, heroin, morphine, tetrahydrocannabinol, and nicotine increase autophagy [40, 41]. In addition, another study has proved that METH induces autophagy as a survival response to the death of apoptotic endothelial cells through the kappa opioid receptor. Ma et al. showed that autophagy is an early response to the stress induced by METH and may have a protective role during exposure to METH. However, exposure to METH for prolonged exposure to flushing autophagy and condemning the cell to apoptotic death [42]. In the current study, to determine the rate of autophagy, Real-Time PCR is used to measure the expression of Beclin-1 gene and Western blot technique to determine the expression of the LC3-II protein. These studies revealed that exposing PC12 cells to METH resulted in a significant increase in the expression of Beclin-1 and LC3-II autophagy genes, following in increased autophagy in this group. However, the expression of these proteins, as well as autophagy in METH-induced cells, can be reduced through Apelin-13. This evidence demonstrates the protective effect of apelin-13 against oxidative stress in the pathway of autophagy.

Increasing intracellular ROS by destroying antioxidant defense can be detrimental to cells and tissues. ROS functions at a low physiological level as an oxidation–reduction reaction in the signaling and intracellular regulation, while at high levels in macromolecular cells, they perform oxidative changes and inhibit the function of proteins and promote cell death [43]. Lau and Jang have proposed that METH leads to an increase in ROS in the cell line of animal models [11, 44]. On the other hand, Zhang et al., have represented that Apelin-13 directs to inhibition of ROS via Akt and MAPK signaling pathways [45]. Moreover, the results of current study confirmed previous studies that intracellular ROS increases in the presence of METH, while Apelin-13 can lead to reduce intracellular ROS in METH-induced PC12 cells.

Apoptosis or programmed cell death involves various pathways leading to a change in the structure of the biomolecules, including lipids, proteins, and nucleic acids. One of the important changes during apoptosis is the alteration of the membrane structure and consequently, the permeability of the membrane to the different materials, which is based on the assay of apoptosis assay of Annexin V-FITC/PI in determination the percentage of apoptosis in cells [46]. Previous studies have shown that apoptotic cell death may be associated with the neurotoxicity of drug and psychotropic drugs. For example, Oliveria et al. showed that heroin was able to induce apoptosis in PC12 cells and rat neurons [47]. In addition, METH has been shown to produce neuronal apoptosis in vivo and in vitro. In addition, Tian and colleagues reported that the amount of apoptosis in PC12-treated cells with METH and heroin alone and their combination significantly increased apoptosis [48]. In current study, the results of the apoptosis assay using the Annexin V-FITC/PI kit showed that exposing PC12 cell to METH significantly increase cell apoptosis, while the apoptosis of METH-induce neurotoxicity can be reduced by Apelin-13. This evidence demonstrates the protective effect of Apelin-13 against oxidative stress in the pathway of apoptosis.

Conclusion

Together, our investigation demonstrates that Apelin-13 is effective in protecting PC12 cells against METH induced neurotoxicity. Apelin-13 is a neuroprotective peptide and it prevents neuronal death by affecting multiple enzymatic and molecular pathways and reduces intracellular ROS, apoptosis, and autophagy. Given the role of METH and Apelin-13 implicates in psychosis and neurodegenerative disease, these findings in vitro may support to future investigations of other pathways in related models.

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Compliance with Ethical Standards

Conflict of interest The authors declare no conflict of interests related to this work.

Ethical Approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Research Involving Human and Animal Participants This article does not contain any studies with human participants performed by any of the authors.

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