NeuroToxicology 57 (2016) 31–38

Contents lists available at ScienceDirect

NeuroToxicology

Full Length Article

Cholecystokinin-8 inhibits methamphetamine-induced neurotoxicity via an anti-oxidative stress pathway

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ARTICLE INFO

Article history:
Received 15 February 2016
Received in revised form 7 June 2016
Accepted 15 August 2016
Available online 23 August 2016

Keywords:
Methamphetamine
CCK-8
CCK2 receptor
Cytotoxicity
Oxidative stress

ABSTRACT

As a powerful addictive psychostimulant drug, coupled with its neurotoxicity, methamphetamine (METH) abuse may lead to long-lasting abnormalities in brain structure and function. We found that pretreatment of cholecystokinin-8 (CCK-8) inhibited METH-induced brain cellular dopaminergic (DA) damage in the striatum and substantia nigra, and related behavioural deficits and hyperthermia. However, the mechanism of CCK-8 action on METH-induced toxicity is not clear. The aim of this study was to explore whether the possible protective effect of CCK-8 on METH-induced neurotoxicity involved anti-oxidative stress mechanisms. The subtypes of CCK receptors mediating the regulatory action of CCK-8 were also investigated. The present results revealed that CCK-8 dose-dependently inhibited METH-induced cytotoxic effect by activating the CCK2 receptor subtype in PC12 cells and CCK2 receptor stable transfected-HEK293 cells. Pre-treatment of CCK-8 before METH stimulation significantly attenuated the generation of reactive oxygen species and NADPH oxidase activation in PC12 cells. In conclusion, our study demonstrated a protective effect of CCK-8 on METH-induced neurotoxicity *in vitro* and suggested that a possible mechanism of this action was dependent on the activation of the CCK2 receptor to reduce the neurotoxicity and oxidative stress induced by METH stimulation.

1. Introduction

Methamphetamine (METH) is one of the most frequently used illicit drugs due to its strong euphoric effects, wide availability, relatively uncomplicated production, and low price in comparison with heroin or morphine. As a powerful addictive psychostimulant drug, coupled with its neurotoxicity, the abuse of METH has resulted in increased numbers of associated medical complications and fatalities.

Long-term or high dose use of METH leads to long-lasting abnormalities in brain structure, chemistry and function, and results in psychotic symptoms, aggressiveness, and potential neurotoxicity (Gouzoulis-Mayfrank and Daumann, 2009; Yamamoto et al., 2010). METH causes a massive release of dopamine (DA) in the brain, and DA then reacts with molecular oxygen to activate NADPH oxidase and form reactive oxygen species (ROS) by degenerating dopaminergic terminals and damaging dopaminergic neurons. (Cadet and Krasnova, 2009; Chauhan et al., 2014; Ferrucci et al., 2008). NADPH oxidase is a complex enzyme consisting of two membrane-bound components ($p22^{PHOX}$ and $gp91^{PHOX}$) and three components in the cytosol ($p40^{PHOX}$, $p47^{PHOX}$ and $p67^{PHOX}$), as well as rac 1 or rac 2. The phosphorylation of $p47^{PHOX}$ is vital to the activation of NADPH oxidase. NADPH oxidase plays a crucial role in the production of ROS within neurons. Under pathological conditions, ROS are overproduced and result in oxidative stress. A common underlying mechanism of METH-induced damage to neurons is intimately related to ROS and oxidative stress (Ferrucci et al., 2008).

New treatment challenges have arisen with the increased use of METH. Several studies have shown that the initiation of METH-induced changes involves the interaction of several neurotransmitters, neuropeptides, neurotrophic factors, and their associated receptor signalling pathways. Cholecystokinin (CCK), a gut-brain peptide, was initially identified as a gastrointestinal hormone (Dockray et al., 2005). Endogenously, CCK also may play a role as a neurotransmitter or neuromodulator in the central and peripheral nervous systems (Beinfeld et al., 1981; Crawley, 1985; Vanderhaeghen et al., 1981). CCK has been identified to exist in several different peptide sizes, including lengths of 4, 8, 33, 39, and 58 amino acid, and CCK-8 is the predominant form in the central

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http://dx.doi.org/10.1016/j.neuro.2016.08.008
0161-813X/

nervous system (Raiteri et al., 1993). It has been suggested that alteration in CCK mRNA levels in the frontal cortex and the hippocampus of METH-treated rats persisted for at least 2 weeks and might be involved in the expression of METH-induced long-lasting behavioural sensitization. Previously, we found that pretreatment of CCK-8 inhibited METH-induced brain cellular dopaminergic damage (loss of nerve terminal tyrosine hydroxylase and DA transporters) in the striatum and substantia nigra, and related behavioural deficits and hyperthermia (Gou et al., 2015). However, the mechanism of CCK-8 action on METH-induced neurotoxicity is not clear. Psychostimulant drugs activate molecular signalling in the dopaminergic pathways. A targeted mutation of the CCK receptor gene induced significant changes in the activity of the dopaminergic system (Koks et al., 2003, 2001). Stimulation of mouse pancreatic acinar cells with CCK-8 leads to the generation of ROS (Granados et al., 2004). However, a previous study has shown that CCK-8 can protect human retinal pigment epithelial cells against oxidative injury (Liu et al., 2014). Based on the pharmacological properties and distribution of its specific ligand binding sites, CCK receptors have been classified as CCK1 receptor and CCK2 receptor subtypes (Wank, 1995). The two CCK receptor subtypes have opposing effects on different neuronal activities. Studies have demonstrated that CCK potentiates dopamine-dependent behaviour in a CCK1 receptor-mediated manner, but inhibits the behaviour *via* the CCK2 receptor subtype (Crawley, 1985; Hirosue et al., 1992; Weiss et al., 1988). Thus, the ability of CCK-8 to prevent METH-induced oxidative stress and neurotoxicity needs to be clarified.

The goal of this study was to investigate the possibility that CCK-8 serves as a protective agent against METH-induced neurotoxicity. We performed the experiments *in vitro* to examined the effect of CCK-8 on METH-induced cell damage and then explore whether an anti-oxidative stress mechanism was involved in the action of CCK-8. The subtypes of the CCK receptor mediating the regulatory effect of CCK-8 were also investigated.

2. Materials and methods

2.1. Drugs and regents

DL-METH (purity > 95%) was provided by the Beijing Municipal Public Security Bureau, China, and was dissolved in a saline solution in a concentration of 10 mg/ml or 1 mol/l. CCK-8 was purchased from Sigma, Ltd (MA, USA), and was resuspended to a concentration of 1 mg/ml or 100 mmol/l in a 1% ammonia saline solution.

Lipofectamine 2000, pcDNA^{TM}3.1/Zeo(+), siRNAs targeting CCK1R and CCK2R, Zeocin and DMEM-High Glucose medium were purchased from Invitrogen Corporation (Invitrogen^{TM}, Carlsbad, CA, USA). Feotal bovine serum was purchased from PAA Laboratories (PAA, Strasse, Pasching, Austria).

2.2. Cell culture

PC12 and HEK293 cells were obtained from Shanghai Bioleaf Biotec (Shanghai, China). The cells were grown in DMEM medium supplemented with 10% fetal bovine serum, 100 U/ml of penicillin and 100 U/ml of streptomycin. All of the cultures were maintained at 37 °C in a humidified atmosphere consisting of 95% air and 5% CO_2.

2.3. Methyl thiazolyl tetrazolium (MTT) cell viability assay

Cells were seeded onto 96-well plates and pretreated with various concentrations of CCK-8 for 24 h before stimulation with or without MA. After drug treatments, cells were washed with PBS and incubated with 0.5 mg/mL MTT (Sigma, Ltd., MA, USA) dissolved in DMED medium. After 3 h, the medium was removed, and the formazan crystals were dissolved in DMSO by incubation at 37 °C for 30 min. The absorbance was measured by a photometer at 570 nm by subtracting background at 630 nm. The data were normalized to the control, and three independent experiments were conducted.

2.4. Small interfering RNA (siRNA) transfection

The siRNA duplex targeting rat CCK1R mRNA (5′-UACGGAGUCAUGA UGGUAAAGGAGA-3′) and CCK2R mRNA (5′-UCAGCAAGUGGAUGAAAGAGAUAGG-3′) were synthesized from Invitrogen (Carlsbad, CA, USA). Scrambled siRNA (si-Control) served as a negative control. The siRNA was transfected into PC12 cells using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Twenty-four hours after transfection, the PC12 cells were treated with drugs.

2.5. Stable cell transfection

The plasmids of human CCK1 receptor (EX-A0232-M02) and CCK2 receptor (EX-A0966-M02) were the production of Genecopoeia^{TM} (Guangzhou, China). A cDNA encoding CCK1R or CCK2R was inserted into a pcDNA^{TM}3.1/Zeo(+) expression vector, and transfected into HEK293 cells by Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Cells transfected with CCK1R or CCK2R cDNA were isolated and cloning cultured under the selection pressure of 300 g/ml Zeocin.

2.6. Measurement of ROS level

There were two independent approaches for ROS measurement. Superoxide in PC12 cells on the chamber slides was detected using 2 μM dihydroethidium (DHE) at 37 °C, and then visualized using a fluorescence microscope (Olympus, Japan). Quantitative analysis of the DHE relative fluorescence intensity was performed on 1×10^5 cells of each group from the samples of experiments using flow cytometry (FACS Calibur, BD Bioscience, Heidelberg, Germany). The other method was an assay for malondialdehyde (MDA), a marker of lipid peroxidation, using a thiobarbituric acid (TBA) Assay kit (Beyotime Technology, China) according to the manufacturer's protocol.

2.7. Western blotting

PC12 cells were lysed in an ice-cold RIPA Lysis Buffer System (Solarbio, Beijing, China). Equal amounts of protein from each sample were separated using 10% SDS-PAGE. The separated proteins were transferred onto nitrocellulose membranes. After blocking in 5% skim milk, the membranes were incubated with primary antibodies against P47 (diluted 1:1000; Cell Signaling), gp91(diluted 1:1000; Cell Signaling) and phospho-P47 (diluted 1:1000; Epitomics) over-night at 4 °C, followed by appropriate secondary antibodies for 1 h at 37 °C (diluted 1: 15000; Rockland). The secondary antibodies were bound to the fluorophore which underwent excitation by light (700 or 800 nm). The emitted light of the blot was then detected using an imager (LI-COR, Inc., Lincoln, NE, USA). β-Actin expression was analysed in the same blots using a monoclonal antibody (diluted 1:1000; Bioworld Technology). The experiments were replicated three times.

2.8. Co-immunoprecipitation assay

Cells were harvested after MA treatment for 3 min with or without CCK-8 pre-treatment. After cell lysis and centrifugation,