ELSEVIER

Brain Research Bulletin 67 (2005) 466–475

BRAIN RESEARCH BULLETIN

www.elsevier.com/locate/brainresbull

Metallothionein provides zinc-mediated protective effects against methamphetamine toxicity in SK-N-SH cells

Amornpan Ajjimaporn [a,c,d], John Swinscoe [a], Shaik Shavali [b], Piyarat Govitrapong [a,c,d], Manuchair Ebadi [a,*]

[a] *Departments of Pharmacology, Physiology and Therapeutics, University of North Dakota, School of Medicine and Health Sciences, 501 N. Columbia Road, Grand Forks, ND 58203, USA*

[b] *Department of Pathology, University of North Dakota, School of Medicine and Health Sciences, 501 N. Columbia Road, Grand Forks, ND 58203, USA*

[c] *Center for Neuroscience and Department of Pharmacology, Faculty of Science, Mahidol University, Bangkok, Thailand*

[d] *Neuro-Behavioural Biology Center, Institute of Science and Technology for Research and Development, Mahidol University, Salaya, Nakornpathom, Thailand*

Received 9 January 2005; received in revised form 16 June 2005; accepted 5 July 2005
Available online 30 August 2005

Abstract

Methamphetamine (METH) is a drug of abuse and neurotoxin that induces Parkinson's-like pathology after chronic usage by targeting dopaminergic neurons. Elucidation of the intracellular mechanisms that underlie METH-induced dopaminergic neuron toxicity may help in understanding the mechanism by which neurons die in Parkinson's disease. In the present study, we examined the role of reactive oxygen species (ROS) in the METH-induced death of human dopaminergic SK-N-SH cells and further assessed the neuroprotective effects of zinc and metallothionein (MT) against METH-induced toxicity in culture. METH significantly increased the production of reactive oxygen species, decreased intracellular ATP levels and reduced the cell viability. Pre-treatment with zinc markedly prevented the loss of cell viability caused by METH treatment. Zinc pre-treatment mainly increased the expression of metallothionein and prevented the generation of reactive oxygen species and ATP depletion caused by METH. Chelation of zinc by CaEDTA caused a significant decrease in MT expression and loss of protective effects of MT against METH toxicity. These results suggest that zinc-induced MT expression protects dopaminergic neurons via preventing the accumulation of toxic reactive oxygen species and halting the decrease in ATP levels. Furthermore, MT may prevent the loss of mitochondrial functions caused by neurotoxins. In conclusion, our study suggests that MT, a potent scavenger of free radicals is neuroprotective against dopaminergic toxicity in conditions such as drug of abuse and in Parkinson's disease.

Keywords: Methamphetamine; Zinc; Metallothionein; Reactive oxygen species; Neurotoxicity

1. Introduction

The central nervous system (CNS) is highly susceptible to damage by a variety of neurotoxins. Methamphetamine (METH) is a well-known drug of abuse and neurotoxin that may cause long-lasting changes in the dopaminergic pathways of central nervous system [10]. METH treatment is known to cause nigrostriatal damage in experimental animals and also in humans [50,54,60]. Therefore, METH toxicity is frequently cited as a potential model of drug-induced parkinsonism [23] similar to MPTP, a parkinsonism-inducing environmental toxin. METH-induced massive release of dopamine (DA) from vesicles to cytosol with resulting formation of toxic reactive oxygen species (ROS) are well documented both in vivo [25] and in vitro [14] studies.

Two mechanisms have been proposed for METH toxicity: (i) formation of extracellular free radicals from DA and/or (ii) oxidative stress driven by increased levels of DA in the cytosol [40]. However, the oxygen-based free radical theory suggests that formation of toxic radicals from DA might be the main determinant of neurotoxicity induced by METH [9]. Furthermore, it is suggested that METH can also cause mitochondrialy-mediated oxidative stress that leads to mitochondrial dysfunction and finally cell death via apoptosis [59].

* Corresponding author. Tel.: +1 701 777 2284; fax: +1 701 777 4158.
E-mail address: mebadi@medicine.nodak.edu (M. Ebadi).

0361-9230/$ – see front matter © 2005 Published by Elsevier Inc.
doi:10.1016/j.brainresbull.2005.07.012

Many studies have reported that reactive oxygen species can be neutralized by an enzyme defense mechanisms comprising of superoxide dismutase (SOD) and catalase (CAT), and non-enzymatic mechanisms including the scavenging action of metallothionein (MT) [19]. MTs are a group of low-molecular weight (about 6000 Da), cysteine rich (30%) intracellular proteins with high affinity for both essential (zinc and copper) and nonessential (cadmium and mercury) metals [34,35]. The mammalian MT family consists of four similar but distinct isoforms of MTs designated as MT-I, MT-II, MT-III and MT-IV. MT-I and MT-II isoforms are expressed in many tissues, however, the MT-III isoform which is also known as a growth inhibitory factor is known to be expressed primarily but not solely in the brain. The MT-IV is widely expressed in stratified squamous epithelia [36,46,48]. Recently, a growing body of interest has been focused on MT as a potential free radical scavenger, because of its high thiol content which is involved exclusively in the formation of metal-thiolate clusters [53]. Several studies indicated that MTs can scavenge reactive oxygen species [31,58] and provide cellular protection against DNA-damage induced by chemicals or radiation [28]. In mammalian cells, MT-I and MT-II genes (but not MT-III and MT-IV) are highly inducible by many heavy metals including Zn, Cd, Hg, Cu and bismuth (Bi) [45]. Zinc and cadmium are the most potent inducers of MT transcription and protein synthesis [29]. In vitro studies with cultured astrocytes suggested that induction of MTs by zinc treatment afforded neuroprotection against diethyldithiocarbamate (DDC) toxicity [61]. Moreover, pre-treatment with either cadmium or dexamethasone provided a significant protection against the neurotoxic effects of MPTP in mice brain [51].

In the present study, we investigated the mechanisms of METH-induced neurotoxicity by studying the changes in reactive oxygen species and ATP levels following METH treatment in dopaminergic SK-N-SH cells in culture. Our studies demonstrated that METH causes dopaminergic cell death via increasing the production of ROS and by depleting the cellular ATP level. The induction of free radicals by METH may be critical for dopaminergic cell death. Furthermore, our study also suggests that metallothionein mediates zinc protective effects against METH toxicity.

2. Materials and methods

2.1. Reagents

Dulbecco's modified eagle's medium (DMEM), Hanks balanced salt solution (HBSS), F-12 medium, fetal bovine serum, penicillin and streptomycin were all purchased from Gibco Invitrogen (Carlsbad, CA). High protein binding polystyrene multiwell plates, MTT (Thiazolyl blue), bovine serum albumin, CaEDTA, metallothionein, (+) methamphetamine were each obtained from Sigma (St. Louis, MO). 2′,7′-Dichlorodihydrofluorescein diacetate (H_2DCF-DA) was purchased from Molecular Probes (Eugene, OR) and ATPLite kit was secured from Packard (USA). Monoclonal antibody to metallothionein was purchased from DAKO (Carpinteria, CA) and $ZnCl_2$ was obtained from Fisher Scientific (Fair Lawn, NJ). Nunclon™ culture flasks were obtained from Nalge Nunc International (Rochester, NY). Tissue culture plates (flat bottom cell+) were obtained from Sarstedt Inc. (Newton, NC). The human dopaminergic neuroblastoma SK-N-SH cell line was purchased from American Type Culture Collection (ATCC) (Manassas, VA). All other chemicals used in this study were analytical grade and obtained essentially either from Sigma or Fisher Scientific (USA).

2.2. SK-N-SH cell culture

SK-N-SH cells were grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with high levels of glucose, glutamine and 3.7 g/l sodium bicarbonate, containing 10% fetal bovine serum. The cell culture flasks were placed in a humidified incubator with 5% CO_2 in air at 37 °C.

2.3. Metallothionein induction with zinc

MT protein expression in SK-N-SH cell line was studied after treatment with various concentrations of $ZnCl_2$ (25, 50 and 75 μM) for 12–48 h. MT was measured by a competitive ELISA [11]. Total protein was measured with the Bradford assay [7]. MT concentrations in samples were calculated using a MT standard curve and normalized to sample protein values.

2.4. Enzyme linked immunosorbent assay (ELISA) for metallothionein

Metallothionein isoforms (MT-I and MT-II) were measured by ELISA method (J. Swinscoe, M.M. Meyer and E.C. Carlson, unpublished) using a monoclonal anti-metallothionein antibody (DAKO-MT, E9) which equally cross reacts with both MT-I and MT-II isoforms. SK-N-SH cells were treated with different concentrations of methamphetamine and incubated for 24 h. Methamphetamine was added either with or without pre-treatment with 50 μM $ZnCl_2$. In other experiments, we used CaEDTA, a zinc chelator to study the specific effects of zinc and MT. After incubation period, the cells were washed twice with PBS, sonicated and centrifuged at 14,000 × *g* for 10 min. The supernatants were used to determine MT. Briefly, wells were coated with 50 ng of MT using Tris buffer (0.1 M, pH 10.4) in high-protein binding 96-well microtiter plates for 6 h at room temperature. After washing the wells for three times with washing buffer, 100 μl of sample or MT standards (MT-I) were added to the wells and incubated with 10 μl of MT antibody (1:1000) for 24 h at 4 °C. The wells were then washed and incubated with secondary antibody conjugated to alkaline phosphatase (1:2500) for 3 h at 37 °C in assay buffer. After washing, the wells were incubated with *p*-nitrophenyl phosphate substrate in 1% diethanolamine, pH 9.8 for 30 min at 37 °C. The color was read in a microtiter plate reader (Molecular Device, Sunnyvale, CA) at 405 nm. MT concentration in samples was calculated using a MT standard curve and normalized to their protein values.

2.5. MTT assay

SK-N-SH cells were seeded in 96-well plates at a density of 0.5×10^6cells/well. The cultures were grown for 24 h followed by adding new medium containing various concentrations of methamphetamine (10 μM to 1.0 mM). Methamphetamine was added to the cells which were pre-treated either with or without 50 μM $ZnCl_2$ for 12 h. Experiments were also conducted using CaEDTA (150 μM), a zinc chelator to study the specific effects of zinc on cell viability. Cell viability was determined by MTT assay [42]. Briefly, after incubation for 24, 48 or 72 h, 30 μl of MTT reagent (0.5 mg/ml MTT in PBS containing 10 μM HEPES) was added to each well and incubated in a CO_2 incubator for 2 h. The medium was aspirated from each well and the culture plate was dried at 37 °C for 1 h. The resulting formazan dye was extracted with 100 μl of 0.04N HCl in isopropanol and the absorbance was measured in a microplate reader (Molecular Device, Sunnyvale, CA) at 570/630 nm.

2.6. Fluorescence microscopy

For morphological studies, SK-N-SH cells were grown in glass bottom culture dishes (MatTek, Ashland, MA, USA). Methamphetamine was added to the cells that were pre-treated either with or without 50 μM $ZnCl_2$ for 12 h and continued incubation for additional 24 h. After treatment, the dishes were