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Methamphetamine induces heme oxygenase-1 expression in cortical neurons and glia to prevent its toxicity

Ya-Ni Huang [a], Ching-Hsiang Wu [a,b], Tzu-Chao Lin [c], Jia-Yi Wang [a,d,*]

[a] Graduate Institute of Life Sciences, National Defense Medical Center, Taipei, Taiwan 114, ROC
[b] Department of Biology and Anatomy, National Defense Medical Center, Taipei, Taiwan 114, ROC
[c] Graduate Institute of Medical Sciences, National Defense Medical Center, Taipei, Taiwan 114, ROC
[d] Department of Physiology, National Defense Medical Center, Taipei, Taiwan 114, ROC

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ABSTRACT

The impairment of cognitive and motor functions in humans and animals caused by methamphetamine (METH) administration underscores the importance of METH toxicity in cortical neurons. The heme oxygenase-1 (HO-1) exerts a cytoprotective effect against various neuronal injures; however, it remains unclear whether HO-1 is involved in METH-induced toxicity. We used primary cortical neuron/glia cocultures to explore the role of HO-1 in METH-induced toxicity. Exposure of cultured cells to various concentrations of METH (0.1, 0.5, 1, 3, 5, and 10 mM) led to cytotoxicity in a concentration-dependent manner. A METH concentration of 5 mM, which caused 50% of neuronal death and glial activation, was chosen for subsequent experiments. RT-PCR and Western blot analysis revealed that METH significantly induced HO-1 mRNA and protein expression, both preceded cell death. Double and triple immunofluorescence staining further identified HO-1-positive cells as activated astrocytes, microglia, and viable neurons, but not dying neurons. Inhibition of the p38 mitogen-activated protein kinase pathway significantly blocked HO-1 induction by METH and aggravated METH neurotoxicity. Inhibition of HO activity using tin protoporphyrine IX significantly reduced HO activity and exacerbated METH neurotoxicity. However, prior induction of HO-1 using cobalt protoporphyrine IX partially protected neurons from METH toxicity. Taken together, our results suggest that induction of HO-1 by METH via the p38 signaling pathway may be protective, albeit insufficient to completely protect cortical neurons from METH toxicity.

Introduction

Methamphetamine (*N*-methyl-*O*-phenylisopropylamine, METH), a psychostimulant abused worldwide, can cause neuropsychiatric and neurotoxic damage in humans and animals. Its relatively high lipid solubility allows the rapid transfer of the drug across the blood–brain barrier (Barr et al., 2006). After entering monoaminergic terminals via dopamine (DA) or serotonin (5-HT) transporters, METH displaces both vesicular and intracellular DA and 5-HT (Barr et al., 2006), which are oxidized to produce reactive oxygen species, thus resulting in neuronal death (Davidson et al., 2001). Despite the prominent toxic effect of METH in monoaminergic neurons (Cadet et al., 2003; Kita et al., 2003), this drug also has deleterious effects in widespread brain regions, such as the cortex (Eisch et al., 1998; Deng et al., 2001). Animal studies show that repeated administration of METH to rats leads to long-term degeneration of sensorimotor cortical neurons, which correlates with impairment of motor functions (Walsh and Wagner, 1992) or of recognition memory (Marshall et al., 2007). The METH-induced degeneration and altered cortical function underscore the importance of METH toxicity in cortical neurons. Although the mechanism implicated in METH toxicity in cortical neurons is not completely clarified, results from recent studies suggest that oxidative stress, excitotoxicity, and mitochondrial apoptosis pathways play important roles in the METH-induced neurotoxic damage to cortical neurons. For example, the superoxide radical (Jayanthi et al., 1998) and the hydroxyl radical (Giovanni et al., 1995) are involved in the neurotoxic effects of METH on cortical cells. Repeated administration of METH causes perturbation in the cellular antioxidant system. Consequently, the overproduction of free radicals may contribute to oxidative damage in cortical cells (Jayanthi et al., 1998). METH also induces excessive glutamate release, which damages cortical neurons via an excitotoxic mechanism (Eisch et al., 1996; O'Dell and Marshall, 2005). The induction of the expression of apoptotic genes after METH administration *in vivo* (Jayanthi et al., 2001) and *in vitro* (Stumm et al., 1999) also indicates the involvement of mitochondrial apoptosis pathways in METH-induced toxicity in cortical neurons.

Increasing evidence supports the contention that heme oxygenase-1 (HO-1) represents an important cellular cytoprotective mechanism

* Corresponding author. Department of Physiology, National Defense Medical Center, 161, Section 6, Ming-Chuan E. Road, Taipei, Taiwan 114, ROC. Fax: +886 2 87924893.
E-mail address: jywang@ndmctsgh.edu.tw (J.-Y. Wang).

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against oxidative damage (Ryter et al., 2002; Bishop and Cashman, 2003). HO catalyzes the rate-limiting step in the degradation of heme to carbon monoxide (CO), iron, and biliverdin-IX, which is subsequently converted to bilirubin (Ryter et al., 2002). The CO and bilirubin derived via the action of HO exhibit potent antioxidant (McDonagh, 1990), anti-inflammatory, and antiapoptotic properties (Brouard et al., 2000; Moon et al., 2003). Three isoforms of HO, which are encoded by different genes, have been described. HO-1, which is also known as heat shock protein 32 (Shibahara et al., 1987), is inducible in various cells under stress conditions, such as the presence of heavy metals (Elbekai et al., 2007) or hydrogen peroxide (Keyse and Tyrrell, 1987). In an ischemic model, transgenic mice overexpressing HO-1 in neurons exhibit higher Bcl-2 levels, lower lipid peroxidation, and smaller infarct volumes than wild-type mice (Panahian et al., 1999). The increased susceptibility of HO-1 knockout mice to *N*-methyl-D-aspartate (NMDA)-mediated neurotoxicity (Ahmad et al., 2006) and the increased resistance of neurons overexpressing HO-1 to oxidative stress-mediated cell death (Chen et al., 2000) further attest to the importance of HO-1 in the host/cellular defense mechanism against oxidant injury. In contrast, excessive HO activity and subsequent release of free iron result in neurotoxicity (Suttner and Dennery, 1999; Lee et al., 2006). Therefore, whether the role of HO-1 is protective or detrimental to neurons remains controversial. Moreover, the possible involvement of HO-1 in the METH-induced cytotoxicity in cortical neurons is also unclear. In the present study, we employed cortical neuron/glia cocultures, which mimic the *in vivo* situation, to delineate the role of HO-1 in METH-induced cytotoxicity.

Materials and methods

Primary neuron/glia cocultures from rat cerebral cortex. Cortical neuron/glia cocultures were prepared from one-day-old neonatal Sprague Dawley rats. Brains were quickly removed, and cerebral cortices were dissected and placed in ice-cold Hank's solution (without Ca^{2+} and Mg^{2+}). These procedures were performed according to the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and were preapproved by the Institutional Animal Care and Use Committee of the National Defense Medical Center, Taipei, Taiwan. Cells were then dissociated by trituration using a pipette. After centrifugation (1500 rpm for 5 min), cells were suspended in 10% FBS/DMEM (Gibco BRL, Grand Island, NY), plated at a density of 5×10^5 cells/ml, and then incubated at 37 °C in a humidified 5% CO_2/95% air atmosphere. Cell composition, as determined by cell counting after immunocytochemical staining, showed that our neuron/glia cocultures consisted of approximately 42.1 ± 0.87% neurons, 47 ± 1.6% astrocytes, and 9.9 ± 1.5% microglia. Other unstained cell types (which included oligodendrocytes, fibroblasts, and smooth muscle cells) represented less than 2 ± 0.1% of the cells present in the cocultures. The digital images of positively stained cells in each well (in five randomly selected fields) were analyzed and cells were counted. Data are mean ± SEM from 3 independent experiments. All experiments were performed in 13–14-day-old cultures.

Drugs. METH was purchased from the National Bureau of Controlled Drugs, Department of Health, Taipei, Taiwan. Tin protoporphyrin IX (SnPP, which is an HO inhibitor) and cobalt protoporphyrin IX (CoPP, which is an HO-1 inducer) were purchased from Frontier Scientific (Logan, UT) and were used to assess the functional role of HO-1 in the METH-induced cytotoxicity in neuron/glia cocultures. SB203580 (which is a p38 inhibitor), PD98059 (which is an extracellular-regulated kinase inhibitor), and SP600125 (which is a c-Jun N-terminal kinase inhibitor) were purchased from the Sigma-Aldrich Chemical Co (St. Louis, MO). These inhibitors were used to delineate the mitogen-activated protein kinase (MAPK) signaling pathways involved in the METH-mediated increase in HO-1 expression. The concentration of the MAPK inhibitors (20 μM) was selected according to previous studies (Elbirt et al., 1998; Alam et al., 2000).

3-(4,5-dimethylthianol-2-yl)-2,5 diphenyl tetrazolium bromide (MTT) reduction assay. A colorimetric MTT reduction assay, which measures cell viability, was performed as previously described (Mosmann, 1983; Wang et al., 2000). Each culture well was incubated in 0.5 mg/ml MTT culture medium, followed by 40 min incubation in 5% CO_2 at 37 °C. The culture medium was then aspirated, and cells were lysed with 50% DMSO. Quantification of MTT reduction was accomplished via the measurement of the absorbance at a test wavelength of 570 nm and a reference wavelength of 630 nm (the reading of which was subtracted from the 570 nm reading) using a microplate reader (Molecular Devices Corporation, Menlo Park, CA).

Measurement of lactate dehydrogenase (LDH) release. Cytotoxicity was quantified using an assay of LDH activity in the cultured media, as previously described (Wang et al., 2003). LDH activity (mAbs/min) was calculated from the slope of the decrease in optical density at 340 nm over a 3 min period.

DNA fragmentation assay. A DNA fragmentation assay was performed as previously described (Wang et al., 2003). Briefly, cell lysates were centrifuged at 12,000 *g* for 20 min at 4 °C. The supernatant was then treated with proteinase K (0.5 mg/ml) and DNAase-free RNase (20 μg/ml) for 60 min at 37 °C. DNA was extracted using phenol/chloroform, precipitated with ethanol, resuspended in Tris–EDTA buffer, and analyzed by electrophoresis run on 2% agarose gel. The gel was stained with ethidium bromide and visualized under UV light.

Morphological analysis of apoptotic nuclei. To visualize apoptotic nuclei, cells were fixed in 4% paraformaldehyde (PFA), stained with a fluorescent DNA-binding dye (Hoechst 33258, 1 μg/ml; Sigma-Aldrich Chemical Co, St. Louis, MO), as previously described (Monti et al., 2001), and observed using a fluorescent inverted microscope (IX70, Olympus, Japan).

Immunocytochemistry and immunofluorescence. Cultures were fixed in 4% PFA, as previously described (Wang et al., 2000). Background staining was reduced by blocking nonspecific binding sites with 10% goat serum for 1 h at room temperature (RT). After rinsing with phosphate-buffered saline (PBS), endogenous peroxidase activity was quenched by incubation with a 3% H_2O_2 in PBS solution for 10 min. Cultures were rinsed again and were then incubated overnight with the appropriate primary antibodies (mouse anti-NeuN, 1:500, Chemicon, Temecula, CA; mouse anti-GFAP, 1:1000, Chemicon; mouse anti-ED1, 1:500, Serotec, Bicester, UK) at 4 °C. Cells were then rinsed three times with PBS and visualized using the avidin–biotin peroxidase complex method (ABC Elite kit; Vector Laboratories, Burlingame, CA). To identify HO-1-expressing cells, we performed double-labeling immunofluorescence staining using antibodies against HO-1 (rabbit anti-HO1, 1:300, StressGen, Vancouver, Canada) and NeuN (1:500), GFAP (1:1000), or ED1 (1:500). Briefly, after incubation with the primary antibodies, cells were rinsed three times for 5 min in PBS and incubated in the presence of either a secondary antibody coupled to fluorescein isothiocyanate (FITC-conjugated anti-rabbit IgG; Jackson ImmunoResearch Laboratories, West Grove, PA) or a secondary antibody coupled to Texas red (Texas red-conjugated anti-mouse IgG; EY Laboratories, San Mateo, USA) for 1 h at RT. The immunofluorescence images were viewed using an inverted Olympus IX 70 microscope equipped with a cooled CCD camera and SPOT advanced software (Diagnostic Instruments INC., Sterling Heights, MI).

Cell-type identification and counting. The types of cells present in the coculture were identified by immunocytochemical staining using cell-specific markers: NeuN for neurons, GFAP for astrocytes, and ED1