발생과 생식 제 15권 제 2호, 173~178 (2011)
Dev. Reprod. Vol. 15, No. 2, 173~178 (2011)

MDMA (Ecstasy) Induces Egr-1 Expression and Inhibits Neuronal Differentiation

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ABSTRACT : The amphetamine derivative 3,4-methylenedioxymethamphetamine (MDMA) is a potent monoaminergic neurotoxin with the potential to cause serotonergic neurotoxicity, but has become a popular recreational drug. Little has been known about the cellular effects induced by MDMA. This report shows that MDMA inhibits neuronal cell growth and differentiation. MDMA suppressed neuronal cell growth. The results of quantitative real-time PCR analysis showed that Egr-1 expression is elevated in mouse embryo and neuroblastoma cells after MDMA treatment. Transiently transfected Egr-1 interfered with the neuronal differentiation of neuroblastoma cells such as SH-SY5Y and PC12 cells. These findings provide evidence that the abuse of MDMA during pregnancy may impair neuronal development via an induction of Egr-1 over-expression.

Key words : Egr1, MDMA, Neuron, SH-SY5Y

INTRODUCTION

The amphetamine derivative 3,4-methylenedioxymethamphetamine (MDMA, ecstasy) has become a popular recreational drug, as humans consume it for its acute psychotropic effects, which include euphoria, a reduction in negative ideation, and increased sociability and energy (Cole & Sumnall, 2003). MDMA is known to cause neurotoxic effects, which result into depression, enhanced impulsivity, impaired decision-making, anxiety disorders, and memory impairment. It also induces long-term changes in several markers of the integrity of the serotonin neurotransmitter system (Sumnall et al., 2004). Whereas, the neurotoxicity, pharmacokinetics, and neurobehavioral consequences of MDMA dosage have been studied extensively (Campbell et al., 2006), the detailed effects in the offspring of MDMA-using mice, as well as the relevant cellular mechanisms, have yet to be systematically addressed.

MDMA exposure is toxic to the developing human fetus. One study reported that babies exposed to Ecstasy in utero showed significantly increased risks for congenital defects, including cardiovascular and musculoskeletal anomalies (McElhatton et al., 1999). In addition, several preclinical studies in animals and tissue culture suggest that in utero exposure to MDMA can cause damaging effects (Broening et al., 2001; Bronson et al., 1994). In addition, histological studies have demonstrated that MDMA can produce neurodegeneration in the striatum and cortex of rats and axonal loss in nonhuman primates (Wilson et al., 1989). It will also be necessary to decipher the cellular and molecular mechanisms that are involved in causing these changes.

Previously, we reported that mouse embryo development upon treatment with MDMA had impaired brain development. And also MDMA repressed the neuronal differentiation of neuroblastoma cells (Chae et al., 2009). Early growth response-1 (Egr-1) also known as nerve growth factor 1-A, krox24 can function in different cellular processes such as growth and apoptosis by modulating p21, p53, TIMP1 and transforming growth factor β1 expression (Ragione et al., 2003; Baron et al., 2005). It has also been shown that Egr1 promotes apoptosis of neuroblastoma cells (Pignatelli et

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al., 2003).

In this study, we aimed to determine whether MDMA treatment inhibits mouse development and induces the expression of the Egr-1 gene. We also investigated to show that MDMA increases the expression of Egr-1, interfering with the neuronal differentiation of neuroblastoma cell, SH-SY5Y.

MATERIALS AND METHODS

1. Animals and MDMA Treatment

In this study, we used 6- to 7- week old C57BL/6 mice. The mice were maintained under standard experimental conditions at a normal room temperature (20-23℃), humidity (50%) with a 12:12-hr light/dark cycle and fed a pelleted diet and water. MDMA (Sigma) were dissolved in filtered saline. MDMA (20 ㎎/㎏/day) was injected intra-orally to female mice from 7 days before mating. And MDMA treated female mice were randomly cohabited with male mice and the mating was confirmed by the presence of virginal plugs. The day when mating was confirmed, termed as gestation day (GD) 0. MDMA (20 ㎎/㎏/day) were injected intra-orally to the mated mice at GD1 to GD8. After that the mice were euthanized and fetuses were collected used for quantitative RT-PCR. Images were captured by dissecting microscope at equal magnification (8×).

2. Cell Culture and Neuronal Differentiation

SH-SY5Y cells were grown in MEM medium supplemented with 10% fetal bovine serum. Transfections into SH-SY5Y cells were conducted using Fugene HD transfection reagent (Roche, USA), in accordance with the manufacturer's instructions. In order to differentiate the neuroblastoma cells, retinoic acid was added to SH-SY5Y cells on the day after plating at a final concentration of 2 ㎛M in MEM. Cells exhibiting one or more neuritis of a length more than twice the diameter of the cell body were considered differentiated (Kim et al., 2004). In order to quantify cell differentiation, at least 300 cells were counted, and the average percentage of differentiated cells was calculated from triplicate experiments.

3. Cell Growth Assay

Cell growth was determined using WST-1 reagent (Roche). Cells were seeded at 24 h before treatment in a final volume of 100 ㎕ in 96 well plate. After treatment (48 h), WST-1 reagent (10 ㎕, 1:10 final dilution) was added and the cells were incubated in CO_2 incubator at 37℃ for 1 hr. The absorbance was measured at 440/670 nm using Benchmark plus (Bio-Rad).

4. Real Time Quantitative RT-PCR

Total RNA was isolated from the fetus or cultured cells using easy-spin kit (iNtRON, Korea) in accordance with the manufacturer's instructions. First-strand cDNA was prepared from total RNA and oligo dT using I Script cDNA synthesis kit (Bio-Rad, Hercules, CA). Real-time PCR was conducted with a Mini Opticon System (Bio-Rad) and SYBR Green (Bio-Rad). The forward and reverse primers for mouse Egr-1 were CCGAGCGAACAACCCTATGAG and TGATGGGAGGCAACCGAGTC, and the primers for human Egr-1 were AGCCCTACGAGCACCTGA and GGCAGTCGAGTCGTTTGG. Human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was utilized to calibrate the original concentration of mRNA. The concentration of mRNA in the cell was defined as the ratio of target mRNA copies versus GAPDH mRNA copies. Data from three separate experiments were averaged.

5. Immunoblotting

The cells were harvested and lysed in lysis buffer (150 mM NaCl, 50 mM HEPES [pH 8.0], 0.5% NP-40) containing a protease inhibitor cocktail (Roche, Germany). For the protein immunoblots, polypeptides in the whole cell lysates were resolved via SDS-PAGE and transferred to nitrocellulose membrane. Immunoblot detection was conducted with a 1:1,000 dilution of primary antibody with an enhanced chemiluminescence (ECL) system. Antibody for Egr-1 was purchased

from MBL International (Woburn, MA).

RESULTS

1. MDMA Inhibits Mouse Embryo Growth

Previously we tested MDMA effects on mouse development by *in vitro* whole embryo culture. MDMA resulted in severe teratogenesis and fetal damage (Chae et al., 2009). Next, we attempted to determine whether MDMA also influences neuronal cell growth or differentiation. First, to confirm the growth inhibitory effect of MDMA we cultured human neuroblastoma, SH-SY5Y cells in the MDMA (200 μM) treated culture media. MDMA inhibited the growth of SH-SY5Y cells by dose-dependent manner (Fig. 1).

2. MDMA Inhibits Retinoic Acid-induced Neuronal Cell Differentiation

Because MDMA inhibited embryo development, we attempted to determine whether MDMA also influences neuronal differentiation. We utilized human neuroblastoma, SH-SY5Y cells for the neuronal differentiation assay, because retinoic acid (RA) evokes the neuronal differentiation of SH-SY5Y cells. SH-SY5Y cells were treated with 2 μM RA with or without MDMA for 5 days. RA induced morphologic differentiation with neurite extension, however, the SH-SY5Y cells treated with MDMA inhibited outgrowth of neurite (Fig. 2A). These results suggest that the neuronal differentiation of neuroblastoma cells was inhibited by MDMA.

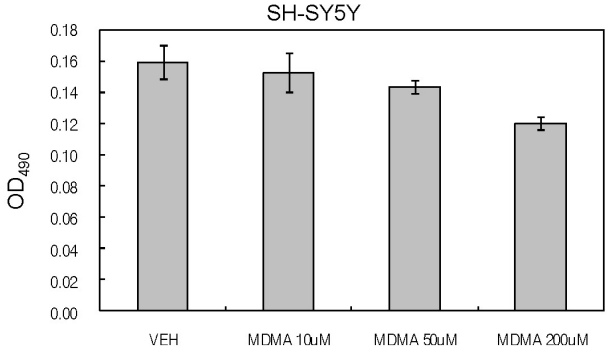

Fig. 1. MDMA effects on neuroblastoma cell growth. SH-SY5Y cells were treated with MDMA. Cell growth was assessed 72 h after MDMA treatment. Values are means±S.D. from triplicate samples and are representative of two independent experiments. $p<0.05$ compared with control.

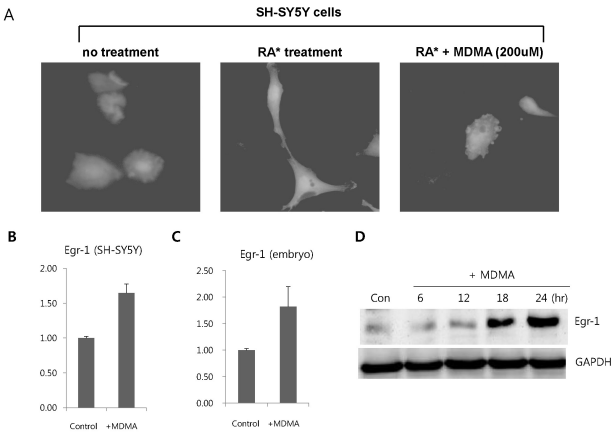

Fig. 2. The inhibition of neuronal cell differentiation by MDMA and the induction of Egr-1 expression. A. SH-SY5Y cells were treated 2 μM RA and 200 μM MDMA for 5 days. B. MDMA was administered to the SH-SY5Y cells and then the Egr-1 mRNA levels were evaluated via real-time quantitative RT-PCR. $p<0.05$ compared with control. C. MDMA was injected intra-orally to female mice after which the level of Egr-1 mRNA was determined. $p<0.05$ compared with control. D. MDMA was administered to the SH-SY5Y cells and then the Egr-1 protein levels were determined by western hybridization.

3. MDMA Induces Egr-1 Expression in SH-SY5Y Neuroblastoma Cells

To understand the molecular changes by MDMA during embryo development, we profiled differentially expressed genes by cDNA microarray. As the result, we found that Egr-1 expression was increased significantly by MDMA (data not shown). To confirm the increased expression of Egr-1 gene in the MDMA-treated SH-SY5Y cells and embryos, we evaluated Egr-1 mRNA expression via real-time RT-PCR analysis. From MDMA treated SH-SY5Y cells and mouse embryos mRNAs were extracted, and cDNAs were synthesized and subjected to real-time RT-PCR analysis. The result showed that Egr-1 expression was increased

by 1.6-fold in SH-SY5Y and by 1.8-fold in 8 day embryos (Fig. 2B and 2C). And western blot analysis was done in MDMA- treated SH-SY5Y cells. Fig. 2D shows that Egr-1 expression was induced within 18 h of treatment of MDMA. These results show that MDMA induces Egr-1 expression in SH-SY5Y neuroblastoma cells.

4. Egr-1 Suppresses Differentiation of Neuroblastoma Cells by Mediating MDMA Effect

As the levels of Egr-1 in the neuronal cells were increased upon MDMA treatment, we hypothesized that Egr-1 might modulate neuronal differentiation. To evaluate our hypothesis, we examined the neuronal differentiation of SH-SY5Y and PC12 cells. SH-SY5Y and PC12 cells were transfected with Egr-1 and 2 μM RA was add for 5 days. As shown in Fig. 3A, RA treated SH-SY5Y cells induced morphologic differentiation with neurite extension. However, as the MDMA effect in the neuronal cells, Egr-1 inhibited neurite extension. SH-SY5Y/PC12 cells were differentiated by over 50%, whereas less than 15% of the SH-SY5Y/PC12 cells transfected with the plasmid encoding for Egr-1 were differentiated after 5days. These results indicated that the Egr-1 protein strongly inhibited the neuronal differentiation of neuroblastoma cells.

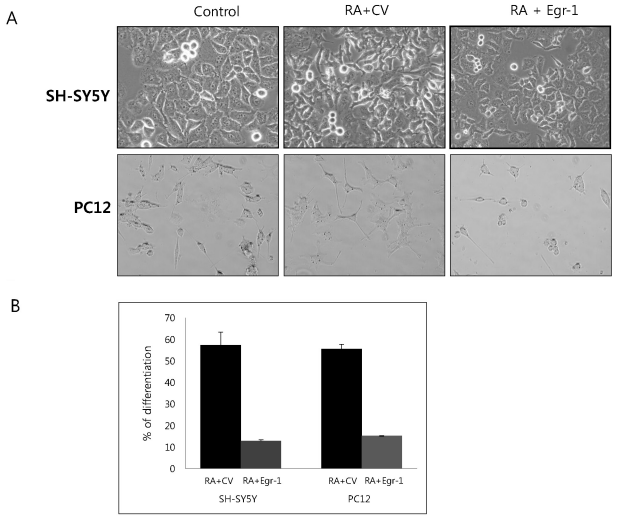

Fig. 3. Effects of Egr-1 on neuronal differentiation. A. SH-SY5Y and PC12 cells were transfected with Egr-1 expressing vector and 2 μM RA was administered for 5 days. B. SH-SY5Y and PC12 cells were transfected with GFP vector in the absence or presence of the plasmid expressing Egr-1. Differentiation rate were determined as described in materials and method. $p<0.05$ compared with control GFP vector.

DISCUSSION

MDMA is popular recreational drug and exposure to MDMA produces acute and long-lasting neurotoxic effects in animals. Recently, we demonstrated that MDMA interfered with neuronal diffenentiation of mouse embryo and neuroblastoma cells (Chae et al., 2009) and prenatal MDMA exposure in rats induced persistent alterations in the developing brain, including changes in dopamine and serotonine metabolism (Galineau et al., 2005). In this report, we investigated the effects of MDMA on the developing fetus in both pregnant mouse and neuroblastoma cells.

Egr-1 is a transcriptional factor that is induced by many different growth factors and stress signals. Induction of Egr-1 is implicated cell proliferation, differentiation and survival. Transcription of Egr-1 is induced by different signaling pathway depending on the cell type. Mostly, Egr-1 is increased by the mitogen activated protein-kinase (MAP-K) ERK-1 and ERK-2 (extracellular regulated protein kinases) and p38/c-Jun N-terminal kinase (JNK) (Silverman & Collins, 1999; Thiel & Cibelli, 2002). In several types of tumor cell lines, they expressed little or no Egr-1 and loss of Egr-1 function may play a role in tumorigenesis (Huang et al., 1997). Previous reports have shown that EGR-1 induce apoptosis in neuroblastoma cells (Pignatelli et al., 2003). The SUMO (small ubiquitin-related modifier) ylation of Elk-1 facilitates the recruitment of HDAC (histone deacetylase) to the egr-1 promoter and resulting repression of Egr-1 promoter, thereby evade apoptosis (Demir & Kurnaz, 2008). MDMA-treated pregnant female mice showed delayed embryo growth and inhibited neuroblastoma cell growth. Via real-time PCR analysis and western blot analysis, we

showed that MDMA-treated SH-SY5Y cells increased Egr-1 gene expression, which was similar with microarray results. These results demonstrated that the increased levels of Egr-1 expression in the neuroblastoma cells were induced by MDMA and that inhibited neuronal differentiation. To investigate the ability of Egr-1 on neuronal differentiation, we overexpressed Egr-1 in differentiated SH-SY5Y cells and PC12 cells by RA. The differentiated cells evidenced morphologic changes, with neurite extension and a profuse neuritic arborization forming extensive networks (Encinas et al., 2000; Kim et al., 2002), whereas the cells transfected with Egr-1 showed dramatically reduced differentiation rate. These results indicated that the Egr-1 inhibited the neuronal differentiation of neuroblastoma cells.

In this study, we observed that MDMA inhibited tube formation of endothelial cells (data not shown) and retinoic acid induced neuronal cell differentiation. We also showed that Egr-1 inhibited retinoic acid-induced neuronal cell differentiation by mediating MDMA activity. Fahmy et al. suggested that microvascular endothelial cell growth, neovascularization, tumor angiogenesis, and tumor growth are processes, critically depending on Egr-1 (Fahmy et al., 2003). Our results suggest a possibility that MDMA-induced Egr-1 inhibits vascular development in the early development stage.

In conclusion, this study provides a mechanism that MDMA inhibits neuronal differentiation in neuroblastoma cells by inducing Egr-1 expression. And MDMA consumption could impair the early development.

ACKNOWLEDGEMENTS

This research was supported by a grant from Korea Food and Drug Administration (KFDA) in 2007-2009.

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(Received 29 April 2011, Received in revised form 22 May 2011, Accepted 16 June 2011)