Proteomic approaches in search for the protein markers for methamphetamine-associated neurotoxicity and dependence (2/3)
Proteomic approaches in search for the protein markers for methamphetamine-associated neurotoxicity and dependence

計畫編號：NSC 92-2413-H-006-008
執行期限：92年08月01日至93年07月31日
主持人：游一龍 國立成功大學行為醫學研究所

Abstract

Lately, protein synthesis has been shown involved in the methamphetamine (MA)-induced nigrostriatal dopaminergic neurotoxicity. We used two-dimensional electrophoresis in an attempt to comprehensively examine the related protein expression in such neurotoxicity. We have established that three and four cumulative doses (10 mg/kg each) of methamphetamine at 2-hr intervals produced a reliable and long-lasting nigrostriatal dopamine depletion. After a series of experiment, we started to notice that the MA-induced neuronal cell apoptosis could induce protein synthesis. To avoid the confounds, we decided to sacrifice the animals 30 minutes following the third dose of MA (10 mg/kg/injection). We, so far, have completed the two-dimensional display for the striatal samples both in MA-treated and saline-treated mice. We completed the spot matching and gel digest for candidate spots followed by identification with mass spectrometry. Increases in alpha-synuclein and alpha-fetoprotein levels while a decrease in F-actin capping protein has been observed associated with the MA-induced nigrostriatal neurotoxicity.

Keywords: Methamphetamine, Dopamine, Neurotoxicity, Protein
poly(ADP-ribose) synthase. Not surprisingly, a recent study demonstrated that the protein synthesis inhibitor blocked the MA-induced nigrostriatal dopamine neurotoxicity. Thus, we decided to simultaneously examine the related proteins involved in this dopaminergic neurotoxicity by using the proteomic approach. Specifically, the experiments were aimed for identifying, selecting, and characterizing the potential protein markers associated with the MA-induced nigrostriatal dopaminergic neurotoxicity.

三、結果與討論

Due to the fact that protein synthetic process could be also involved in the methamphetamine-induced cell apoptotic process, we proposed to profile the protein expression in mouse striatal samples 30 min following the three cumulative doses of methamphetamine treatment. We established reliably consistent differences in protein expression profile between methamphetamine-treated and saline-treated-treated animals (as indicated in attached article submitted). Moreover, we have demonstrated that increases in alpha-fetoprotein and alpha-synuclein, while a decrease in F-actin capping protein were reliably seen in the methamphetamine-treated striatal samples under the current protocol.

四、計畫成果自評

Our progress is promising and on schedule.

Neurotoxic Proteins Involved in
Methamphetamine-induced Nigrostriatal Dopamine Degeneration in Mice

Heng-Chia Hsu\textsuperscript{1}, Yu-min Kuo\textsuperscript{2}, Lung Yu\textsuperscript{1}

\textsuperscript{1}Institute of Behavioral Medicine, \textsuperscript{2}Department of Cell Biology and Anatomy, National Cheng Kung University College of Medicine, Tainan, Taiwan 701, ROC

All correspondences should be addressed to Dr. L. Yu at Institute of Behavioral Medicine, College of Medicine, National Cheng Kung University 1 University Rd., Tainan 70101, Taiwan, ROC Email: lungyu@mail.ncku.edu.tw Phone: (06)235-3535 ext.5114 (Lab) ext. 5106(O) Fax: (06)209-5616

Abstract

Methamphetamine (MA)-induced nigrostriatal dopamine neurotoxicity can be reversed by protein synthesis inhibitor treatment, indicating the pivotal role of protein synthesis in this MA-induced dopaminergic neurotoxicity paradigm. This study aimed to identify the specific neurotoxic proteins responsible for such nigrostriatal dopaminergic toxicity. Employing the two-dimensional gel electrophoresis (2-DE) technique, followed by mass spectrometry (MS), we obtained those proteins involved in such toxicity by contrasting the protein expression profiles of striatal samples dissected from MA-treated against them from saline-treated mice. To avoid necrotic and apoptotic confounds, striata were dissected out thirty minutes after the third dose of MA (10mg/kg for each dose) or saline administration. Approximately 300 spots were visualized by silver stain and analyzed on an ImagerMaster Labscan software for the quantitation of protein expression. Comparing the striatal protein expression profiles, five protein spots exhibited a significant increase in intensities and fifteen spots revealed a decrease in intensities in MA-treated group. Subsequent MS analysis results revealed and identified three out of them, alpha-synulein (up-regulated), alpha fetoprotein (up-regulated), F-actin capping protein beta subunit (down-regulated). By using this approach we are capable of identifying the candidates of neurotoxic and neuroprotective proteins involved in the MA-induced nigrostriatal dopamine neurotoxicity.

Introduction

Parkinson’s disease (PD) is an age-related neurodegenerative disorder characterized by neuronal death in selected areas of brain, particularly in the nigrostriatal dopaminergic (DA) neurons. One of the frequently-used experimental paradigms to mimic the primary pathology of PD is the cumulative methamphetamine (MA)-induced nigrostriatal neuronal deficits in mice. Methamphetamine is known to induce nigrostriatal DA damage by destroying dopamine nerve fibers (7).
and producing long-lasting dopamine neurochemical alterations. Many studies have addressed the critical roles of DA and glutamatergic systems in this MA-induced DA pathology paradigm, as evidenced by the protective effects of both NMDA and DAT antagonists against such toxicity (6, 9, 10). Interestingly, this MA-induced DA neuropathology seemed to be completely mitigated by pretreatment with protein synthesis inhibitors, including cycloheximide and anisomycin (2).

Given the pivotal roles of protein synthesis in this paradigm, we decided to employ the two-dimensional gel electrophoresis (2-DE) technique in an attempt to search for the key proteins by contrasting the protein expression profiles of striatal samples dissected from MA-treated and them from saline-treated control mice. Furthermore, we proposed to identify those proteins, especially neurotoxic proteins, responsible for such MA-induced dopamine toxicity by utilizing mass spectrometry (MS) identification. To minimize the confounding effects of degraded cellular proteins induced by cell apoptosis after MA treatment, we dissected out the striatal samples thirty minutes following three consecutive doses of MA. It was of importance to note that our preliminary results have demonstrated that this dosing regimen was sufficient to induce nigrostriatal DA neurotoxicity in our mouse model.

Materials and methods

Drugs
Methamphetamine hydrochloride was purchased from National Bureau of Controlled Drugs (Taipei, Taiwan) and was fully dissolved in 0.9% saline before use; injection doses of MA were expressed as the free base (mg/kg). Urea, dithiothreitol (DTT), and sodium dodecyl sulfate (SDS) were from USB Corporation (Cleveland, OH, USA). Thiourea and iodoacetamide (IAA) were obtained from Amersham Biosciences UK Limited (Little Chalfont, Buckinghamshire, UK). CHAPS, sodium thiosulfate, sodium acetate, and sodium carbonate were purchased from J.T.Baker Inc. (Phillipsburg, NJ, USA). IPG buffer pH 3-10, bromophenol blue (BPB), ammonium persulfate (APS), and glycerol were obtained from Pharmacia Biotech AB (Uppsala, Sweden). AgNO₃ was from Mallinckrodt Baker Inc. (Paris, Kentucky). Formaldehyde was from Nihon Shiyaku Industries Ltd. (Osaka, Japan). Acetic acid was from Fluka Chemie AG (Buchs, Switzerland).

Animals
Male C57BL/6NCrj mice from NCKU Lab Animal Center, at 10-13 weeks of age, were used in the present study. They were allowed free access to both food and water and were kept at temperature- and humidity-controlled colony room on a 12 h light/dark cycle.

**Drug treatment**
Mice were randomly divided into two groups. Each group of mice were injected subcutaneously with either three doses of MA (10 mg/kg for each injection separated by a 2-hr interval, n = 12) or equivalent volume of saline (n=12).

**Preparation of tissue proteins**
Thirty min after the last injection, the whole brains were removed by rapid decapitation, and a portion of striatum from both hemispheres was dissected out. The tissues were stored in liquid nitrogen before homogenization. Each sample (with bilateral striata of two mice, weighing 6.4-10.6 mg) was homogenized with 500 µL of a rehydration buffer solution, consisting of 7 M urea, 2 M thiourea, 2% CHAPS, 0.5% IPG buffer pH 3-10, 20 mM DTT, and trace amount of BPB. After homogenization, the solution was subjected to centrifugation at 200,000 x g, 4°C for 2 h. The protein concentration of the supernatant solution was determined (see below).

**Quantification of the protein concentration**
The protein concentration was determined with the dye reagent from Bio-Rad Laboratories (Hercules, CA, USA) using bovine serum albumin (Pierce Biotechnology, Rockford, IL, USA) as standard. The sample concentration for the two-dimensional electrophoresis (2-DE) was adjusted to 340 µg/mL (110 µg protein per gel).

**Two-dimensional electrophoresis**
2-DE was carried out in a horizontal electrophoresis system, IPGphor™ (Amersham Pharmacia Biotech) for the first-dimensional isoelectric focusing (IEF) using Immobilized pH gradient strip (IPG strip, 18 cm long, linear gradient between pH 4-7; Amersham Pharmacia Biotech, Uppsala, Sweden), and by a PROTEAN II xi system (Bio-Rad Laboratories) for the second-dimensional SDS-PAGE.

**Rehydration of immobilized pH gradient strip:** The IPG strip was rehydrated by the protein solution (Section 3.4) in the slot of a strip holder (18 cm long; Amersham Pharmacia Biotech) at 20°C, and applied 30V for 16 h.
**IEF:** After rehydration, the IPG strip was successively subjected to isoelectric focusing at 500 V for 1 h, 1000 V for 1 h, and finally 8000 V for 4 h. The current limit was adjusted to 50 µA per strip, and the run was carried out at 20°C. Upon the end of the isoelectric focusing, the IPG gel was equilibrated at room temperature for 15 min, with 10 mL of an equilibration buffer, consisting of 50 mM Tris pH 8.8, 6 M urea, 30% glycerol, 0.2% SDS, trace amount of BPB, and 1% DTT, and then equilibrated for another 15 min with equilibration buffer which 1% DTT was substituted with 2.5% IAA.

**SDS-PAGE:** The second-dimensional SDS-PAGE was performed with a fresh-made 12% polyacrylamide gel. The equilibrated IPG gel strip was placed on top of the gel and then sealed with 5% agarose solution. Electrophoresis was carried out at a constant voltage of 300 V and the temperature was controlled at 10°C by a cooling system (Panchum Scientific Corp., Taipei, Taiwan) before BPB reached the bottom of the gel.

**Silver staining of 2-DE gel**
Silver stain was performed on an orbital shaker with gentle shaking. The gel was first submerged in fixing solution, consisting of 40% ethanol and 10% acetic acid, for 30 min. The gel was then placed in a sensitizing solution, composed of 30% ethanol, 0.312% sodium thiosulfate, and 6.8% sodium acetate, for 30 min and then rinsed with distilled water for three times, each for 5 min. The gel was then transferred to silver staining solution, consisting of 0.25% AgNO₃ and 0.04% formaldehyde for 20 min and rinsed twice with water before placed in a developer solution, consisting of 2.5% sodium carbonate and 0.02% formaldehyde. When spots became visible, 250 mL of 5% acetic acid were added to terminate the developing step. After the whole staining process was done, the gel was washed with water and subjected to computer image analysis.

**Computer image analysis of 2-DE gel**
The silver-stained gel was scanned with an image scanner (PowerLook III, UMAX, Taiwan) and digitized using ImageMaster Labscan Version 3.01 (Amersham Biosciences). Data were analyzed using ImageMaster™ 2D software Version 2002.01 (Amersham Biosciences) on a Windows 2000 platform (Microsoft).

**In-gel digestion and Mass spectrometry**
The interested protein spots were excised manually and subjected to in-gel digestion as described before (8) with minor modifications. The excised gel slice was de-stained in 1:1 solution of 30 mM potassium ferricyanide and 100 mM sodium
thiosulfate, washed twice in 25 mM ammonium bicarbonate and in 25 mM ammonium bicarbonate with 50% acetonitrile. The protein in the gel slice was reduced in 10 mM DTT for 60 min and alkylated in 55 mM iodoacetamide for 45 min in the dark. The gel slice was then washed twice again as described above, air-dried after adding acetonitrile, and subjected to tryptic digestion. The gel slice was suspended and ground into small pieces in digestion buffer containing 1.0 µg TPCK-treated porcine trypsin (Promega, Madison, WI) in 25 mM ammonium bicarbonate and the digestion was carried out at 37° C for 16 h. At the end of digestion, the sample was centrifuged and the supernatant containing tryptic peptides were transferred, mixed with 5.0 % formic acid in 1:1 water/acetonitrile, diluted with an equal volume of water, and submitted to mass spectrometry analysis for protein identification.

The tryptic digests were injected into an LCQ Deca XP plus ion trap mass spectrometer (ThermoFinnigan, San Jose, CA) equipped with a high performance liquid chromatography (HPLC) system (LC Packings, Amsterdam, Netherlands). Before introduced into the nano-electrospray ionization (nano-ESI) source of the mass spectrometer, the peptides were fractioned by a reverse phase capillary C18 column (75 µm i.d. x 15cm, LC Packings) with a linear 5-60% solvent gradient (buffer A: 95:5 water/acetonitrile in 0.1% formic acid; buffer B: 20:80 water/acetonitrile in 0.1% formic acid) in 50 min at a flow rate of 200 nl/min. The electrospray voltage was 1.2 kV and tandem mass spectrometry was performed for the peptides eluted from liquid chromatography. The resulting tandem mass spectral data were used to search through NCBI GenBank sequence database in FASTA format using SEQUEST software (Bioworks 3.0, ThermoFinnigan, San Jose, CA) to reveal the identity of the protein.

Results

In excess of 700 spots were detected following silver stains. Approximately 300 spots were reliably matched in every gel and were analyzed on ImagerMaster™ 2D software for the quantitation of intensity of staining. Six pairs of computer-generated images were created for statistic analysis and each pair was composed of one image (n=2) from MA-treated group and the other (n=2) from saline-treated control group. Images of the 2-DE gels are presented as Figure 1. Comparing the striatal protein expression profiles by a statistic test (details described elsewhere), the intensities of five spots revealed statistically
significant increase and those of fifteen spots showed significant decrease following MA treatment ($p < .05$) (Figure 2). A total of 10 spots (five from increased expression and five from decreased expression) were selected for MS analysis (Figure 2). The five spots from decreased expression were selected based on the significance level. Following mass spectrometry analysis, we identified three proteins and the characteristics of each protein including the identified protein names, accession numbers, as well as the sequence coverages, and the theoretical $M_r$ and $pI$ values are listed in Table 1.

Table 1

<table>
<thead>
<tr>
<th>Spot No.</th>
<th>Protein name</th>
<th>Accession No.</th>
<th>Sequence coverages</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>Unnamed protein product</td>
<td>gi</td>
<td>2 638 454 8</td>
</tr>
<tr>
<td>5</td>
<td>F-actin capping protein beta subunit</td>
<td>gi</td>
<td>1 345 668</td>
</tr>
<tr>
<td>9</td>
<td>Alpha-synulein</td>
<td>gi</td>
<td>6 678 047</td>
</tr>
</tbody>
</table>
Figure 1. Images of the 2-DE gels are arranged as six pairs. Each pair is composed of one image from MA-treated group and the other from saline-treated group.
The pathological findings of methamphetamine (MA)-induced nigrostriatal dopamine (DA) neurotoxicity are similar to the primary pathology of Parkinson’s disease (PD) and most of the evidences were derived from animal models using rodents and non-human primates as subjects. However, the etiology of PD remains unclear despite the cumulative knowledge about the mechanism responsible for MA toxicity. In recent years, it has been suggested that both environmental and genetic factors may play
important roles in the incidence of PD. For example, mutations in α-synulein gene, which results in altered function of α-synulein, can cause familial PD (5). Furthermore, in one transgenic mouse model, increased expression of wild-type α-synuclein produced loss of dopaminergic terminals in the basal ganglia and motor impairments (4). Surprisingly, this key toxic protein in PD was found overexpressed in our acute MA-treated striata. This data further support the notion that the MA-induced dopaminergic neurotoxicity is an appropriate paradigm for studying both the pathology and etiology associated with Parkinson’s disease.

Furthermore, both microtubules and actin filaments play a significant role in maintaining the three-dimensional structure of neurons (1). Under normal conditions, the F-actin capping proteins bind to the fast growing ends of actin filaments (barbed end) thereby preventing the addition or loss of actin subunits from these ends and helps to stabilize the cytoskeletal network (3). Our data showed that F-actin capping protein beta subunit (CapZ beta) was down-regulated in the MA-treated striata, suggesting the role of the axonal transport disruption in the MA-induced dopaminergic neurotoxicity.

References


