INTRODUCTION

N-methyl-D-aspartate (NMDA) receptor antagonists have been prescribed to patients with stroke, brain trauma, and epilepsy. They are also considered promising candidates for the treatment of several pain-associated diseases such as multiple sclerosis. On the other hand, NMDA receptor antagonists such as MK-801 have also been found to induce neuronal vacuolation in the retrosplenial cortex (RSC), identified as dilation of mitochondria and endoplasmic reticulum, following single administration in rats (Olney et al., 1989; Fix et al., 1995). Novel NMDA receptor antagonists should therefore be evaluated for induction of neuronal vacuolation. However, to our knowledge, little is known concerning the molecular mechanisms of neuronal vacuolation. We performed a proteomic analysis to investigate changes in proteins in the RSC region accompanying MK-801-induced neuronal vacuolation in rats.

MATERIALS AND METHODS

This study was performed in compliance with Laboratory Animal Policy at Eisai and with the approval of Laboratory Animal Care and Use Committee of Eisai Co., Ltd.

Subjects

Sprague-Dawley rats [Crl:CD(SD)] (8 weeks of age; N = 5 per each group) were obtained from Charles River Japan Inc. (Shiga, Japan). Rats were housed in a room with 12-hr light: dark cycle and were allowed free access to a standard chow (CRF-1; Oriental Yeast Co., Ltd., Tokyo, Japan) and sterilized water. Female rats were used because they are known to have higher sensitivity than males to the neurotoxic effects of NMDA receptor antagonists (Olney et al., 1989; Fix et al., 1995).

Experimental design

Rats were given a single subcutaneous (sc) injection of (+)-MK-801 hydrogen maleate (9 mg/kg; Sigma-Aldrich Corp., Saint Louis, MO, USA). The dose level of 9 mg/kg has been reported to consistently produce vacuolation (Carliss et al., 2007). Rats in the control group were given the same volume of saline. The rats were sacrificed 6 hr post-dose and their brains were collected and fixed in a 10% phosphate-buffered formaldehyde solution. The paraffin-embedded brains were sectioned at about bregma -5.8 mm (Paxinos and Watson, 1997), and stained with hematoxylin and eosin. This time of sacrifice and region of the brain were reported to be adequate for consistent detection of vacuolation in the RSC (Auer and Coulter, 1994; Colbourne et al., 1999; Fujimura et al., 2000).

Correspondence: Motohiro Shiotani (E-mail: m2-shiotani@hhc.eisai.co.jp)
Sample preparation for proteomics

After confirming neuronal vacuolations on histological examination in the RSC in MK-801-treated rats, 3-4 adjacent sections were obtained at 10-μm from all embedded brains including those of the control group. The RSC regions were subsequently collected using a laser micro dissection tool (P.A.L.M. Microlaser Technologies, Bernried, Germany) with a total area of 8 mm², and the proteins were solubilized and digested with trypsin using the Liquid Tissue MS protein Prep Kit (AMR, Tokyo, Japan).

Proteomic Analysis

Peptide mixtures were applied to high-performance liquid chromatography (HPLC), which was performed with a 100 x 0.1 mm C18 capillary column, and the eluent was directed to an Orbitrap-XL mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) at a flow rate of 0.5 μl/min. MS/MS spectra were obtained in a data-dependent mode in which the highest intensity peaks in each MS scan were chosen for collision-induced dissociation. Peptides and proteins were identified with automated database searching using X!Tandem.

Statistical analysis

The Student t-test was used to examine differences in Exponentially Modified Protein Abundance Index (emPAI) between the MK-801 and control groups. The emPAI can be used to estimate absolute protein contents in complex mixtures (Ishihama et al., 2005). Because the emPAI indicates semi-absolute protein content, only proteins with statistically significant differences between groups were considered. A probability (P) of less than 0.05 was considered significant.

RESULTS AND DISCUSSION

The histopathological changes were limited to neuronal vacuolation in the RSC; no changes were observed in other regions of the brain including those reported to be affected by MK-801.

The proteins with a statistically significant difference in emPAI values between the MK-801 and control groups are summarized in Table 1. The emPAI values for vesicle-fusing ATPase, triosephosphate isomerase, and fructose-bisphosphate aldolase C were increased (× 1.5-2 vs. control). On the other hand, emPAI values (× 0.5-0.6 vs. control) were decreased for putative adenosylhomocysteinase 3, nucleoside diphosphate kinase A and glyceraldehydes-3-phosphate dehydrogenase. Meloni et al. (2005) reported the changes in proteins in cortical neuronal cells induced by MK-801 exposure at 1 μM for 30 min. Among the proteins with changes in expression in the present study, findings similar to those in Meloni’s report (2005) were observed for fructose-bisphosphate aldolase, nucleoside diphosphate kinase, and glyceraldehyde-3-phosphate dehydrogenase, while other proteins that exhibited change in expression in the present study did not in Meloni’s study.

Vesicle-fusing ATPase is known to be involved in the heterotypic fusion of membrane vesicles with target membranes. An increase in vesicle-fusing ATPase in the RSC may reflect the fact that MK-801 induced excessive release of acetylcholine in the RSC (Kim et al., 1999). Triosephosphate isomerase, fructose-bisphosphate aldolase C, and glyceraldehydes-3-phosphate dehydrogenase are involved in glycolysis. Notably, decrease in glyceraldehyde-3-phosphate dehydrogenase may induce a decreased supply of pyruvic acid to mitochondria and is reported to indirectly facilitate glutamate-mediated neuronal injury in rat brain (Camacho et al., 2007). Adenosylhomocysteinase is related to the pathway of methionine metabolism, thus, its reduction may result in a decreased synthesis of cysteine, followed by a decreased supply of pyruvic acid. Nucleoside diphosphate kinase A is known as a metastasis.

Table 1. Proteins exhibiting statistically significant differences in expression between the MK-801 and control groups

<table>
<thead>
<tr>
<th>Proteins</th>
<th>Control group</th>
<th>MK-801 group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vesicle-fusing ATPase</td>
<td>0.123 ± 0.053</td>
<td>0.251 ± 0.059**</td>
</tr>
<tr>
<td>Triosephosphate isomerase</td>
<td>0.135 ± 0.055</td>
<td>0.263 ± 0.109*</td>
</tr>
<tr>
<td>Fructose-bisphosphate aldolase C</td>
<td>0.303 ± 0.089</td>
<td>0.463 ± 0.111*</td>
</tr>
<tr>
<td>Putative adenosylhomocysteinase 3</td>
<td>0.092 ± 0.033</td>
<td>0.056 ± 0*</td>
</tr>
<tr>
<td>Nucleoside diphosphate kinase A</td>
<td>0.333 ± 0.127</td>
<td>0.194 ± 0*</td>
</tr>
<tr>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
<td>0.357 ± 0.139</td>
<td>0.174 ± 0.045*</td>
</tr>
</tbody>
</table>

Means ± S.D. (N = 5), *:P < 0.05, **:P < 0.01.
Proteomic analysis of neuronal vacuolation induced by MK-801 in rat

sis suppressor, but, the biological meaning of its reduction in the present study is unknown.

The present study provides findings useful for determination of the molecular mechanisms of neuronal vacuolation induced by MK-801 in rats in vivo.

ACKNOWLEDGMENTS

The authors would like to thank Mr. Ikuo Mori and Mr. Norio Akaogi for their support in the in vivo portion of the study and histological processing, respectively.

REFERENCES


Vol. 36 No. 1