cDNA Array Analysis of Gene Expression Profiles in Brain of Mice Exposed to Manganese

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Abstract: This study is performed to detect changes of gene expression in substantia nigra (SN) and striatum in manganese (Mn)-exposed mice brain. The cDNA array is a recently developed molecular biological method that can detect the differential expression of several hundreds of genes simultaneously and is therefore advantageous in the study of trace metal intoxication effect at the genetic level. Using this technology, we discovered 5 genes in the mouse striatum and 9 genes in SN changed by more than 50% following Mn exposure. Depression were observed in two genes (neural cell adhesion protein BIG2, heavy neurofilament subunit genes) in striatum and three genes (light neurofilament subunit, brain acyl-CoA synthetase II, heavy neurofilament subunit genes) in the SN. However three genes (N-acetylglucosaminyltransferase I, S100β, and synaptonemal complex protein I genes) in striatum and six genes (noggin, striatin, Ost oncogene, S100β, calcium/calmodulin-dependent protein kinase kinase beta, and N-acetylglucosaminyltransferase I genes) in SN were elevated following Mn exposure. Immunohistochemical study revealed that protein levels of S100β also increased following Mn treatment. Activated astrocytes overexpressing S100β are invariably and intimately associated with decreased expression of heavy and light neurofilament subunits which is a distinguishing feature of neurodegeneration by Mn exposure. All our findings suggested that neuronal degenerations occur in SN as well as striatum of mice exposed to Mn.

Key words: cDNA array, Gene expression, Mouse, Brain, Manganese

Introduction

Manganese (Mn) is an essential element for metalloproteins, such as glutamate synthetase, mitochondrial superoxide dismutase1, 2. However excessive Mn is also toxic to brain and can produce an irreversible syndrome resembling Parkinson’s disease3, 4. Oxidative stress has been suggested as the underlying mechanism of Mn neurotoxicity. Mn may stimulate dopamine autoxidation within the dopaminergic neuron, a process accompanied by an increase in formation of reactive oxygen species and quinones. The pursuant oxidative stress has been proposed as being responsible for the neuronal damage. But there is few reports about changes in gene levels by Mn intoxication in mammals. There are several techniques that can be used to examine gene transcription, such as reverse-transcriptase coupled to the polymerase chain reaction (RT-PCR), differential display, and serial analysis of gene expression (SAGE), however, these procedures are labor intensive and are limited in the number of genes that can be monitored simultaneously. In
contrast, monitoring the expression level of thousands of genes is feasible when using cDNA array-based techniques\(^*\). The use of cDNA arrays to identify gene modulation events associated with toxicity may enable to detect toxicant exposure at an earlier time than the methods to identify protein products or terminal cellular processes. In addition, the highly sensitive cDNA arrays can detect gene expression events associated with low-level toxicity\(^*\). Although microarrays have been used to examine differential gene expression in static systems, such as cultures of healthy and diseased cells, the ability of this tool to identify gene modulation events reproducibly is unclear.

In the present study, a mouse model was used to examine the effects of Mn exposure on gene expression in the striatum and substantia nigra (SN) of mice brain.

**Materials and Methods**

**Animals**

These experiments were carried out using a previously reported regimen of Mn exposure\(^*\). Male C57BL/6 mice (10 wk old, body weight 25–30 g) were caged in an air-conditioned room maintained at 22 ± 2°C, relative humidity 50 ± 10%, with a 12/12 h light/dark cycle. The animals had free access to tap water and were fed a conventional rat chow diet *ad libitum*. The animals acclimated for 2 wk prior to the beginning of the study. All procedures related to animal care were in accordance with the guidelines for the Care and Use of Laboratory Animals of College of Medicine, Pusan National University. The mice were allocated randomly into two groups: Mn exposure group (n=7) and control group (n=7). Three out of seven were used for cDNA array, and the others for immunohistochemical analysis. Following acclimation, the exposure group received 0.2 ml of MnCl\(_2\)·4H\(_2\)O (Sigma) (2 mg Mn/kg body weight) once a day for 3 wk. The control group received the same volume of saline intraperitoneally.

**Animal dissection and tissue handling/storage**

Animals were anesthetized with ether and killed by decapitation at the next day of last Mn injection. Brains were quickly removed and sliced in a contour-fit brain matrix and dissected for striatum including caudate-putamen and globus pallidus, and ventral midbrain region including SN.

**RNA isolation**

Total RNA was isolated from dissected brain tissue using Trizol reagent, following the procedure described by the reagent’s manufacture (Invitrogen-Life Technologies). Isolated RNA sample were dissolved in RNase-free water and treated with DNase to remove any genomic DNA contamination. The optical densities of the samples were measured at 260 nm (A260) and 280 nm (A280) wavelengths and ratios were calculated. Only samples with A260/A280 ratios >1.7 were used for further study. The integrity of each sample was examined by running a small fraction (~2 μg) of the total amount on a 1% agarose gel (100 V, 1 h) and staining the gel with ethidium bromide to determine the relative intensities of the 28S and 18S rRNA bands.

**cDNA array probe preparation and hybridization, and array analysis**

An Atlas Mouse 1.2 Array Kit (lot No.:110360) (Clontech, Palo Alto, CA, USA) was used according to the protocol outlined by the manufacturer. The experiment was performed on the Mn-treated RNA samples (n=4) concurrently with their corresponding control RNA samples (n=4) in duplicate. One array was used for each mouse RNA sample (i.e. samples within each experimental group were not mixed) for a total four duplicate array hybridizations per experimental group. \(^{32}\)P-labeled cDNA probes were generated from each RNA sample through reverse transcription using Moloney Murine Leukemia Virus (MMLV) reverse transcriptase and primers specific for the gene sequences contained in the kit’s cDNA array. Each array consists of 1,176 known mouse genes. Only probes with specific activity of between 2 and 10 × 10\(^6\) cpm/sample were used in subsequent hybridization steps. Pre-hybridization was performed on the kit’s cDNA arrays using the ExpressHyb solution provided. Probes were then hybridized to the cDNA arrays in roller bottles overnight at 68°C in a hybridization chamber. Following stringent washes, the arrays were exposed to phosphor storage screens overnight and then viewed on a phosphorimager. The resulting images were analyzed for differential gene expression using AtlasImage 2.0 software (Clontech).

**Immunohistochemistry and image analysis**

Mn-treated animals (n=3) and control animals (n=3) were anesthetized with pentobarbital sodium (50 mg/kg) at the next day of last Mn injection. Animals were killed by intracardiac perfusion with 4% paraformaldehyde in a 0.1 M phosphate buffer, pH 7.4 (PB). Brains were postfixed for 5 h at 4°C and cryoprotected with 30% sucrose. Tissue was frozen with an OCT compound-embedding medium in dry ice powder. Thirty micrometer coronal sections were cut using cryostat and processed for immunohistochemistry. Immunohistochemical reaction was performed every 6th section of the striatum and midbrain. Sections were incubated...
in a blocking buffer (0.3% Triton X-100 and 10% goat serum in PBS) for 1 h, followed by overnight incubation with primary antibodies to S100β (rabbit polyclonal anti-S100β 1:5000; Swant, Switzerland) diluted in an incubation buffer (0.1% Triton X-100, 1% goat serum and 1% bovine serum albumin in PBS) at 4°C. Sections were then washed 3 times for 10 min each with PBS and incubated in biotinylated goat antirabbit IgG (1:200, Vector laboratories, Burlingame, CA, USA) for 2 h at room temperature. Sections were washed and processed with 0.05% 3,3’-diaminobenzidine tetrahydrochloride (Sigma-Aldrich) with 0.01% H2O2. For all incubation and rinse steps, the sections were agitated on a shaker table. After processing, the sections were washed, mounted on coated slides, dried, dehydrated through graded alcohols, cleared in xylene, and coverslipped with an Entellan mountant medium (Merck).

Forebrain sections containing the rostral, middle and caudal levels of striatum, were chosen for image analysis. We measured the optical density of S100β-immunoreactivity in the striatum of Mn-treated and saline-treated control animals using the Metamorpho 4.7 Image program (Universal Image Corporation, USA) on PC computer connected to a digital camera with constant illumination. To estimate the specific staining density, the optical density reading was corrected for non-specific background density, as measured from regions with no S100β immunoreactivity. Data were presented as a percentage area of the total measured area (% area).

**Statistical analyses**

The statistical significance of the difference in the means of variables between the Mn-exposed and the control group was determined using Student’s t tests.

**Results**

No significant changes were observed in body weights and diet amounts between Mn-exposed and control group during the experiments.

We considered the positive signals as genes showing an expression change of more than 50% between control and Mn-treated groups in each striatal and SN. Based on these criteria, 5 array genes in striatum and 9 array genes in SN were differentially expressed at RNA transcript level. Of these genes, two were down-regulated, and three were up-regulated in the striatum (Table 1), while three were down-regulated, and six were up-regulated in the SN (Table 2). No significant differences in expression of the considered genes were found between mice of the same experimental group. Figure 1 represents the images of the hybridized

| Table 1. Genes showing > 50% change in expression by Mn treatment in striatum |
|-----------------------------|---------------------------------|-----------------------------|
| Gene                        | GenBank Accession No. | Relative expression change (Mn/control) |
| Neural cell adhesion protein BIG2 | U35371              | 0.14 ± 0.08                 |
| Heavy neurofilament subunit (NF-H) | M21964              | 0.40 ± 0.05                 |
| N-acetylglycosaminyltransferase I | D16302              | 1.70 ± 0.12                 |
| S100 calcium-binding protein, beta | M54919              | 4.93 ± 0.50                 |
| Synaptosomal complex protein I   | X67805              | 6.00 ± 0.37                 |

| Table 2. Genes showing > 50% change in expression by Mn treatment in substantia nigra |
|-----------------------------|---------------------------------|-----------------------------|
| Gene                        | GenBank Accession No. | Relative expression change (Mn/control) |
| Light neurofilament subunit (NF-L) | AF031880              | 0.02 ± 0.01                  |
| Brain acyl-CoA synthetase II | D30666               | 0.20 ± 0.08                  |
| Heavy neurofilament subunit (NF-H) | M21964              | 0.48 ± 0.12                  |
| Noggin                      | U31203               | 1.60 ± 0.09                  |
| Striatin                    | X99326               | 1.81 ± 0.02                  |
| Ost oncogene                | Z35654               | 2.12 ± 0.12                  |
| S100 calcium-binding protein, beta | M54919              | 3.26 ± 0.47                  |
| Calcium/calcmodulin-dependent protein kinase kinase beta (CAM kinase kinase beta; CAMKKB) | AB018081 | 6.90 ± 0.22 |
| N-acetylglycosaminyltransferase I | D16302              | 7.52 ± 0.57                  |
The phosphorimaging signals of selected genes have been enlarged to provide a clearer depiction of the variance in gene expression observed in the array study. There were no significant changes in such as genes associated with carbohydrate metabolism, transcription factors, ion channel proteins, and immune system proteins in this array study.

Among these genes the S100β mRNA transcript was up-regulated in the striatum and the SN by 4.93 folds and 3.26 folds, respectively, following Mn treatment.

Immunohistochemistry was used to determine whether striatal S100β protein level concurrently increased by Mn treatment. Activated astrocytes, immunoreactive for S100β, increased in number and enlargement of their cell body size which are distinguishing features of astrocyte activation was compared with saline-treated control animals (Fig. 2). The optical density of S100β immunoreactivity in the striatum was significantly increased in the Mn-treated animals in comparison to the saline-treated control animals (1.28 ± 0.22 vs 0.79 ± 0.15; P<0.001).
Discussion

This is the first cDNA array analysis of gene expression profiles in mouse brain exposed to Mn to our best knowledge. We identified several genes in the mouse striatum and SN whose expressions are affected by Mn treatment through the use of the cDNA array. Most interesting observation is that the S100β mRNA transcript is up-regulated following treatment. This finding is significant because overexpression of S100β of the astrocyte-derived cytokine has been shown to be involved in neurodegenerative diseases8–10). The immunohistochemical study revealed that striatal protein level of S100β also increased significantly after treatment, indicating that Mn-induced S100β mRNA transcript up-regulation was translated. This Mn-induced increase in S100β overexpression is reminiscent of increases in S100β expression that accompany normal aging in both humans11) and experimental animals12). In Alzheimer’s disease, there are elevated tissue levels of both biologically active S100β protein and S100β mRNA13), and these increases correlated with overexpression of S100β by plaque-associated astrocytes8, 10, 14). S100β is a neurite growth-promoting cytokine15), and promotes astrocytic activation8). The numbers of S100β-overexpressing astrocytes correlated with the extent of dystrophic neurite formation in amyloid plaques of Alzheimer’s disease10). Mn-induced S100β overexpression, as shown here, supports the idea that activated astrocytes overexpressing S100β are important and necessary factors in the genesis of neuritic pathology in neurodegenerative disease.

Down-regulation of the heavy neurofilament subunit (NF-H) genes in striatum and light neurofilament subunit, NF-H genes in SN shown in the present study mean the neuronal degeneration in each region. Mn induced the specific degeneration of neuronal cytoskeletons, which is the common effect of neurodegenerative disease, including Parkinson’s disease and Alzheimer’s disease. N-acetylglucosaminyltransferase I gene which is related to transcript the protein modification enzyme increased in striatum and SN. This means increased transcription of Mn-related or binding proteins such as transferrin in the striatum and SN. Synaptonemal complex protein I is functionally unknown gene.

It is generally assumed that the substantia nigra pars compacta (SNpc) remains intact, and postsynaptic region is damaged in Mn intoxication16–18). However, all our findings suggested that neuronal degenerations might occur in SN as well as striatum of mice exposed to Mn. The present findings were compatible with some human pathologic findings10, 21), animal pathologic finding24), and animal biochemical findings25, 26). The present finding was also in accordance with our recent brain MRI studies on humans showing that Mn frequently deposits in midbrain as well as globus pallidus27, 28) and the hypertrophy of astrocytes by Mn intoxication in mouse brain finally7).

However, the present findings do not necessarily mean that Mn also damage dopaminergic pathway. The present findings have limitations of preliminary studies, and further studies need to be performed.

In summary, using cDNA array method, the present study showed several genes in the SN as well as striatum of mice exposed to Mn. Among them the S100β gene, was up-regulated following Mn treatment. Furthermore, we showed that Mn exposure increased the expression of S100β at protein level in the astrocytes.

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References


