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Changes of gene expression profiles in the cervical spinal cord by acupuncture in an MPTP-intoxicated mouse model: Microarray analysis

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ABSTRACT

It has been shown that acupuncture at acupoints GB34 and LR3 inhibits the degeneration of nigrostriatal neurons in a 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model of Parkinson's disease. The degeneration of spinal cord was reported to be induced in the MPTP-treated pre-symptomatic mouse. In this study, the gene expression profile changes following acupuncture at the acupoints were investigated in the cervical spinal cord of an MPTP-induced parkinsonism model using a whole transcript array (Affymetrix GeneChip mouse gene 1.0 ST array). It was shown that 8 of the probes up-regulated in MPTP, as compared to the control, were down-regulated after acupuncture at the acupoints. Of these 8 probes, 6 probes (4 annotated genes in 6 probes: *Ctla2a*, *EG383229*, *Ppbp* and *Ube2l6*) were exclusively down-regulated by acupuncture at the specific acupoints except for 2 probes as these 2 probes were commonly down-regulated by acupuncture at both the acupoints and the non-acupoints. In addition, 11 of the probes down-regulated in MPTP, as compared to the control, were up-regulated by acupuncture at the acupoints. Of these 11 probes, 10 probes (5 annotated genes in 10 probes: *EG665033*, *ENSMUSG00000055323*, *Obox6*, *Pbp2* and *Tmem150*) were exclusively up-regulated by acupuncture at the specific acupoints except for the *Fut11* because the *Fut11* was commonly up-regulated by acupuncture at both the acupoints and the non-acupoints. The expression levels of the representative genes in the microarray were validated by real-time RT-PCR. These data suggest that the expression of these exclusively regulated 16 probes (9 genes) may be, at least in part, affected by acupuncture at the acupoints in the cervical spinal cord which can be damaged by MPTP intoxication.

1. Introduction

Parkinson's disease (PD) is a common neurodegenerative disease characterized by the progressive loss of striatal dopamine and dopaminergic neurons within the substantia nigra (Sit, 2000). Animal models of parkinsonism are known to be produced by loading neurotoxic compounds such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), 6-hydroxydopamine (6-OHDA), pesticides and other parkinsonism agents (Emborg, 2004; Emborg, 2007). It was especially reported that MPTP induces the degeneration of spinal cord in a mouse model of MPTP intoxication (Samantaray et al., 2008). Pharmaceutical studies have been conducted using these animal models of PD-like neuropathological features as well as using actual PD patients (Dutta et al., 2008; Etminan et al., 2003; Hamaue 2000; Kasture et al., 2009; Liang et al., 2007; Neef and van Laar, 1999; Yano et al., 2009). However, these studies have not yet led to the development of satisfactory anti-parkinsonism drugs with long-term therapeutic effects for PD patients; for example, long-term treatment with Levodopa (a drug used to treat PD) is known to cause serious side effects such as dyskinesia (Calon et al., 2000). The lack of a breakthrough in clinical and therapeutic PD research has led the search for a cure to explore alternative forms of medicine, like oriental medicine, and particularly acupuncture. A recent report announced that adenosine and adenosine A1 receptors mediate the local analgesic effect of acupuncture (Goldman et al., 2010). It has also been reported that long-term synaptic plasticity in the spinal dorsal horn might contribute to the anti-nociceptive effects of electroacupuncture, revealing a previously unknown effect of electroacupuncture on synaptic transmission in the spinal dorsal horn (Xing et al., 2007). Acupuncture has also been applied, although to a limited extent, to PD patients (Rabinstein and Shulman, 2003). Some previous studies have shown that acupuncture at the acupoints GB34 and LR3 clearly inhibits the nigrostriatal degeneration of parkinsonism models

Abbreviations: DTT, dithiothreitol; DAT, dopamine transporter; 6-OHDA, 6-hydroxydopamine; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; PD, Parkinson's disease; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SNpc, substantia nigra pars compacta; TBS, Tris-buffered saline; TH, tyrosine hydroxylase.

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induced by 6-OHDA and MPTP (Choi et al., 2009; Kang et al., 2007; Park et al., 2003). These observations suggest the potential application of acupuncture to help ameliorate the symptoms of PD patients.

As the genes which may be related to the inhibition of nigrostriatal neurodegeneration by acupuncture at the acupoint GB34 in the MPTP model need to be further elucidated (Hong et al., 2010) and the spinal cord can be damaged by MPTP intoxication (Samantaray et al., 2008), this study investigated the gene expression profile changes in the cervical spinal cord using the whole transcript GeneChip microarray after acupuncture at the acupoints GB34 and LR3 in the MPTP-induced parkinsonism model. The results of this study suggest that acupuncture at the acupoints may affect the expression of the target genes in the cervical spinal cord which can be damaged by MPTP intoxication (Samantaray et al., 2008).

2. Materials and methods

2.1. MPTP model of parkinsonism

Six-week-old male inbred C57BL/6 mice (20–22 g, Samtaco, Korea) were divided into four experimental groups: control (CTL), MPTP-treatment only (MPTP), MPTP- and acupuncture-treatment at acupoints GB34 and LR3 (MPTP-A), and MPTP- and acupuncture-treatment at non-acupoints (MPTP-NA, $n=9$ in each group). MPTP-HCl (20 mg/kg of free base) dissolved in 0.9% saline (100 μl) was intraperitoneally administered for 4 weeks at 24-intervals to produce the parkinsonism model (Bezard et al., 1997a, 1997b; Choi et al., 2009), while 0.9% saline was intraperitoneally administered (100 μl) to the control group following the same time schedule. The mice were anesthetized using 16.5% urethane on the day after the last MPTP treatment and then perfused with a cold 0.05 M sodium phosphate buffer for immunohistochemistry. The Kyung Hee University Animal Experimentation Committee approved all animal protocols used in this study. Reagents used but not mentioned were purchased from Sigma (USA).

2.2. Acupuncture administration

Acupuncture was performed by hand 2 h after the first MPTP injection and then at 2-day intervals (14 trials in total). The acupuncture procedure (acupoint GB34 (Yanglingquan) and acupoint LR3 (Taichong) for acupoints; both sides of the hips for non-acupoints) was performed as previously reported (Choi et al., 2009; Kang et al., 2007; Park et al., 2003). Mice in the acupoints group were immobilized by hand 2 h after MPTP administration. Acupuncture needles were inserted bilaterally to depths of 3 mm at the acupoint GB34 and 1 mm at the acupoint LR3, and then turned at a rate of two spins per second for 15 s as reported in a previous study (Kang et al., 2007). For the non-acupoint group, the needles were inserted to depths of 3 mm at both sides of the hips instead of at the specified acupoints and the same needle-turning was performed as with the acupoints.

2.3. Immunohistochemistry

After 4 weeks of the MPTP and the acupuncture treatments, the mice brains were removed, postfixed in a 0.05 M sodium phosphate buffer containing 4% paraformaldehyde, and then cryosected. Coronal sections of the brains (30 μm thick) were cut using a cryomicrotome, and immunohistochemical analysis was carried out using an ABC kit and a M.O.M. immunodetection kit (Vector Laboratories, CA) following a modification of the avidin–biotin–peroxidase method. Striatal or substantia nigra (SN) sections were incubated with 3% H_2O_2 in PBS (pH 7.4) and treated with an avidin/biotin blocking kit (Vector Laboratories, CA) to block the endogenous avidin and biotin. When using the mouse anti-tyrosine hydroxylase (TH) antibody, the sections were treated with an M.O.M. mouse Ig blocking reagent (Vector Laboratories, CA). Afterwards, each section was stained with mouse anti-TH (1:1000, SantaCruz Biotechnology, USA) and goat anti-dopamine transporter (DAT, 1:500, SantaCruz Biotechnology, USA) for dopaminergic neurons. The sections were then sequentially treated with a biotinylated anti-mouse (or anti-goat) IgG, followed by an avidin–biotin–peroxidase complex, and then developed with a diaminobenzidine-hydrogen peroxide solution (0.003% 3,3-diaminobenzidine and 0.03% hydrogen peroxide in 0.05 M Tris, pH 7.0). The densities of TH and DAT positivities in the ST were evaluated by measuring the densitometry in the same area (500 μm^2) from three brains of each group, and the number of cells of TH positivity in the substantia nigra pars compacta (SNpc) was calculated in the same area (500×200 μm) from three brains of each group.

2.4. Western blot

The bilateral striatal and SN regions were homogenized in 20 mM HEPES-KOH (pH 7.5) with 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate and a protease inhibitor cocktail. After centrifugation, supernatant soluble samples of equal protein concentration (30 μg of total proteins) were separated by a 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to the nitrocellulose membranes (Pharmacia Biotech, USA). The membranes were blocked and then incubated with mouse anti-TH (1:5000), goat anti-DAT (1:3000) and mouse anti-actin (1:1000, SantaCruz Biotechnology, USA). After incubation with the anti-mouse (or anti-goat) IgG-peroxidase antibody (1:3000, Bio-Rad, USA), the antigen–antibody complexes were visualized with a SuperSignal West Pico (Thermo Scientific, USA).

2.5. RNA extraction and microarray

In the next stage, the total RNA in the cervical spinal cord of each group ($n=2$ per group) was extracted using the RNeasy Plus Mini kit (QIAGEN, USA). The isolated RNA was quantified by a NanoDrop ND-1000 (NanoDrop Technologies, USA). The labeled single-stranded cDNA was produced using the total RNA (300 ng) according to the GeneChip Whole Transcript (WT) Sense Target Labeling Assay manual as previously reported (Hwang et al., 2009; Lin et al., 2010), and then hybridized to an Affymetrix GeneChip® Mouse Gene 1.0 ST Array (WT expression profiling chip, 28,853 genes of 35,557 probes, Affymetrix, USA). The staining, washing and scanning of the arrays were conducted using a Fluidics 450 station, GeneChip® Operating Software and GeneChip® Scanner 3000 7G (Affymetrix, USA).

2.6. Microarray data analysis

The quality control of the scanned data was first estimated by confirming the order of the signal intensities of the Poly-A and Hybridization controls using Expression Console Software (Affymetrix, USA). The microarray data was analyzed using GenPlex V3.0 (ISTECH, Korea) which includes the Preprocessing, Differentially Expressed Genes (DEGs) Finding, Clustering, Classification, and Pathway modules (An et al., 2009; Hwang et al., 2009). A total of eight CEL files (2 CEL files generated from each group×4 experimental groups) were uploaded and normalized in PM (perfect match)-only conditions as a PM intensity adjustment. A Robust Multichip Analysis (RMA) quantification method was used as a probe set summarization algorithm for log transformation with base 2 (log2) and the Quantile normalization method was chosen to evaluate the preliminary data quality in the Preprocessing module, which functions as a data quality control through the Affymetrix Expression Console Software. The mean signal intensities of all genes (28,853 genes) were obtained using 2 chips from each group.

After normalization, the differentially expressed genes satisfying the conditions of the fold change cutoff 1.3 and the Student's t-test $p<0.05$ from all of the genes probed in the GeneChip were selected in