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Expression Profiling of Neuroinflammatory Genes in LPS and LPS/IFNγ-Stimulated BV-2 Microglia Cells

In Su Kim[1], Ji-Hwan Park[1], Jin Hwan Do[2], Hyun Myung Ko[1], Eun Jung Park[3], Kyoungho Suk[4] & Dong-Kug Choi[1]

[1]Department of Biotechnology, Konkuk University, Cheongju 380-701, Korea
[2]Human Genome Center, Institute of Medical Science, University of Tokyo, Tokyo 108-8639, Japan
[3]Immune and Cell Therapy Branch, National Cancer Center, Goyang 410-769, Korea
[4]Department of Pharmacology, Kyungpook National University School of Medicine, Daegu 700-422, Korea
Correspondence and requests for materials should be addressed to D.-K. Choi (choidk@kku.ac.kr)

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Abstract

A large body of evidence indicates that microglia, resident innate immune cells in the brain, play a role in host defense and function as active contributors to neuron damage in neurodegenerative disease. Microglia are crucial in the immune response within the central nervous system (CNS), and function in protecting the brain against injury. In contrast, activated microglia can secrete numerous reactants that damage neurons. The pathogenesis of various neurodegenerative diseases has been associated with microglia activation. Elucidation of molecular mechanisms underlying microglia activation could aid in controlling neuroinflammation and subsequent neurodegeneration. To increase the understanding of microglia activation, a cDNA microarray was presently used to profile genome-wide expression in activated BV-2 microglia cells. The analysis revealed that genome expression was differentially modulated for 1.2% of the total cellular transcripts in lipopolysaccharide/interferon-γ (LPS/IFNγ)-activated BV-2 microglia cells. Comparison of array data between LPS- and LPS/IFNγ-activated cells showed that the transcriptional signature was similarly regulated. This common expression pattern of stimulation was statistically significant, with >2-fold change evident for 38 genes. To evaluate the functional significance of the altered gene expression in microglia cells, PBEF1, an induced gene both LPS and LPS/IFNγ-activated BV-2 microglia, was further analyzed in a 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-intoxicated animal model.

Keywords: Neuroinflammation, Microglia, Microarray analysis, Interferon gamma, Lipopolysaccharide

Introduction

Microglial cells are ubiquitously distributed throughout the nervous system. They are present as a dominant proportion of the total glial cell population in the brain and are considered to be the resident innate immune cells of the central nervous system (CNS)[1,2]. Microglia become activated in response to environmental alterations and brain injury, and undergo marked morphological changes into activated amoeboid microglia[3,4]. Inflammatory responses in the brain are thought to be mainly associated with activity of glial cells. Inflammation in the brain, characterized by the activation of microglia and astroglia, has been closely associated with the pathogenesis of many neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease (PD) and several other neurological disorders[5-7]. Moreover, activated microglia exert cytotoxic effects by releasing various inflammatory mediators and neurotoxic factors, such as nitric oxide (NO), arachidonic acid metabolites, reactive oxygen species and reactive nitrogen species that are believed to induce neuronal cell death[8-11]. Other neurotoxic compounds that may exert their neuronal damaging effects through activation of microglia include 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)[12,13], Gram-negative bacteria outer membrane component lipopolysaccharide (LPS) and methamphetamine[14,15]. The signaling pathways that mediate microglial activation might be diverse depending on the activating stimuli. BV-2 microglial cells are activated by LPS, which activate mitogen-activated protein kinases, nuclear factor-kappa B (NF-κB), protein kinase C and tyrosine kinases, and the cell activation has been implicated in the release of immune-related cytotoxic factors, such as nitric oxide (NO) and proinflammatory cytokines[16-18]. These microglial products are thought to be responsible for neuroglia-mediated neurotoxicity[19]. The combination of two agents, LPS and interferon-γ (IFNγ) potently activates microglia cells[20].

Identification of genes specifically induced or suppressed in activated microglia may provide an alternative approach to clarify the molecular mechanisms

Table 1. Selected significant ($P < 0.05$) mRNA changes in LPS- and LPS/IFNγ-induced microglia activation.

GenBank accession No.	Gene symbol	Expression pattern	Gene name
AA185052	Rsad2	Induced	Radical S-adenosyl methionine domain containing 2
AA250238	Usp18	Induced	Ubiquitin specific protease 18
AA289657	Oasl1	Induced	2′-5′ oligoadenylate synthetase-like 1
AA172456	Ccl12	Induced	Chemokine (C-C motif) ligand 12
AA153021	Gbp2	Induced	Guanylate nucleotide binding protein 2
AI390695	UnKnown	Induced	Similar to very large inducible GTPase-1
AA178155	Ccl4	Induced	Chemokine (C-C motif) ligand 4
AA178100	Oas2	Induced	2′-5′ oligoadenylate synthetase 2
AA174620	H2-Eb1	Induced	Histocompatibility 2, class II antigen E beta
AA098149	Cybb	Induced	Cytochrome b-245, beta polypeptide
AA286393	Ccl12	Induced	Chemokine (C-C motif) ligand 12
AA388607	Cybb	Induced	Cytochrome b-245, beta polypeptide
AA276912	UnKnown	Induced	cDNA sequence BC006779
AA250385	Arhgef12	Induced	RIKEN cDNA B430007K19 gene
AI322749	Tyki	Induced	Thymidylate kinase family LPS-inducible member
AA170598	Ms4a4c	Induced	Membrane-spanning 4-domains, subfamily A, member 4C
AA451385	Actr2	Induced	ARP2 actin-related protein 2 homolog (yeast)
AA245451	Mrps16	Induced	Mitochondrial ribosomal protein S16
AA123007	Oasl2	Induced	2′-5′ oligoadenylate synthetase-like 2
AA145466	UnKnown	Induced	Similar to very large inducible GTPase-1
AA023159	Stat1	Induced	Signal transducer and activator of transcription 1
AA087193	Lcn2	Induced	Lipocalin 2
AI893126	Rab6	Induced	RAB6, member RAS oncogene family
W82276	UnKnown	Induced	EST
AI510217	Epsti1	Induced	Epithelial stromal interaction 1 (breast)
AA146363	Parp9	Induced	Poly (ADP-ribose) polymerase family, member 9
AA240404	Gbp4	Induced	Guanylate nucleotide binding protein 4
AA177731	UnKnown	Induced	EST
W11882	Tac2	Induced	Tachykinin 2
AA216991	Hck	Induced	Hemopoietic cell kinase
AA174447	Ifi203	Induced	Interferon activated gene 203
AA087123	Ppp2r4	Suppressed	Protein phosphatase 2A, regulatory subunit B (PR 53)
AA498495	Epb4.1	Suppressed	Erythrocyte protein band 4.1
AA048111	Tbcc	Suppressed	Tubulin-specific chaperone c
AA080231	Man2b1	Suppressed	Mannosidase 2, alpha B1
AI450494	Asxl1	Suppressed	Additional sex combs like 1 (Drosophila)
AA051561	Cdc42se2	Suppressed	CDC42 small effector 2
W66730	6530402N02Rik	Suppressed	Anterior pharynx defective 1a homolog (C. elegans)

of neuroinflammation. Therefore, to increase the understanding of the process of neuroinflammation, specifically the global picture of pathway, which could aid in the identification of novel regulators during neuroinflammation, a complimentary DNA (cDNA) microarray was presently utilized to analyze and compare the inflammation-related transcriptome between LPS- and LPS/IFNγ-activated BV-2 microglia. This analysis provides insight into those elements of the microglia transcriptome that contribute to neuronal damage through the process of activation.

Results and Discussion

Gene Expression Profile of Activated Microglia

To investigate the responses of BV-2 microglia cells exposed to activator, cells were stimulated with LPS or LPS/IFNγ. Microarray analysis revealed that 258 genes were differentially regulated (induced or suppressed in expression by $\geq$2-fold, $P < 0.05$, ANOVA) in LPS/IFNγ-activated BV2 microglia. Of these genes, the expression of 167 was up-regulated and 91 genes were down-regulated. These genes included the following inflammatory response- and immune response-related genes: NF-κB-related genes (Nfkb2), interferon-related genes (Gbp2, Irf1, Iigp1, Ifi203, Iigp1, Igtp, Ifi203, Icsbp1 and Iigs1) and chemokine ligand (Ccl12, Ccl4 and Ccl19).

Comparative microarray analysis of gene expression between LPS and LPS/IFNγ showed that a number of transcripts were similarly regulated in the activation of microglia, even though a dichotomous expression pattern of some genes was observed. The

common expression pattern of stimulation of the LPS- and LPS/IFNγ-activated cells was statistically significant and >2-fold different for 38 of these genes ($P < 0.05$, Table 1). These genes were selected based on the biological processes and molecular functions of their gene ontology. Gene expression results from microarray analysis were used as input to the software program Pathway Assist (Agilent Technologies, Santa Clara, CA, USA), which uses Kyoto Encyclopedia of Genes and Genomes (KEGG) databases as well as analysis using PubMed to define connectivity among genes, to delineate a functionally-related network. The gene included the following signal transduction pathway (Ccl4, 6530402N02Rik, Stat1), cell communication (Ppp2r4, Epb4.1), cell motility (Arhgef12) and metabolism (Man2b1).

Validation of Microarray Results

We selected seven genes from the list of genes and submitted them to internal validation by reverse transcription-polymerase chain reaction (RT-PCR). The results from this analysis are presented in Figure 1. The expression patterns of these genes agreed very well with the microarray results. Some of the identified genes were of interest from the context of inflammation. In particular, Lipocalin 2 (Lcn2) has been recently identified as a microglial secretory protein that plays a dual role in determining morphological and functional fate of activated microglia[21]. Additionally, Lcn2 can be induced by oxidative stress and protects from oxidative stress-induced injury[22]. In the immune system, Lcn2 suppresses cytokine production induced by LPS in macrophages[23]. A homology search using various databases revealed that five novel transcripts (AI390605, AA276912, AA145466, W82276 and AA177731).

Expression of PBEF1 in an Animal Model of Neuroinflammation

The injection of the MPTP neurotoxin has become a widely-used method to investigate the pathogenic role of glia cells. Mice intoxicated with MPTP show a strong microglial reaction, which reaches its maximum before most of the dopaminergic neurons have died, suggesting a participation of microglia in the neurodegenerative process[24,25].

Although PBEF1 was presently identified as a molecule that was induced by both LPS and LPS/ IFNγ, its role, especially linked on neuroinflammation and neurodegeneration, was not evident in both *in vitro* and *in vivo* models. We examined the expression pattern of PBEF1 mRNA and immunolocalization in MPTP-intoxicated mice. The PBEF1 mRNA level was significantly increased after MPTP injection, compared

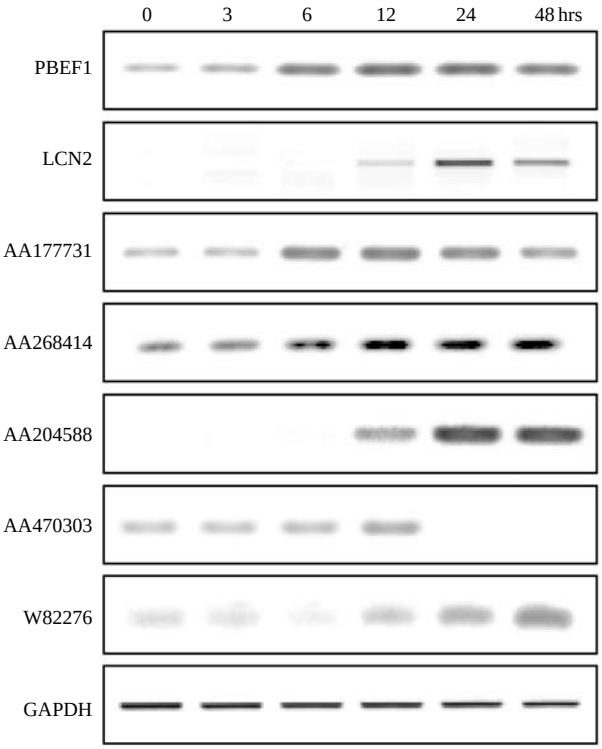

Figure 1. Expression patterns for seven transcripts identified by the microarray analysis. The results of semi-quantitative RT-PCR assays are shown.

with that of saline controls (Figure 2). Expression of the proinflammatory mediators interleukin (IL)-1, IL-1ra and TNF-α were increased following MPTP administration compared with control cells (Figure 2). Immunohistochemical staining revealed the presence of PBEF1-positive cells after MPTP treatment (Figure 3). It is interesting to note that PBEF that is expressed and localized to lung tissue may induce NF-κB activation and subsequent induction of genes that are involved in acute lung injury via augmentation of the inflammatory process[26]. Taken together, our observations suggest that PBEF1 might play role in glia activation during the process of neuroinflammation.

Conclusions

Microglia undergo inflammatory activation in most CNS pathologies, and activated microglia exert cytotoxic effects that are believed to induce neuronal cell death. We investigated a global pattern of gene expression analysis in LPS- and LPS/IFNγ-activated BV-2 microglia cells. Microarray analysis revealed that the expression pattern was differentially regulated for 1.2% of the cellular transcripts in the LPS/IFNγ-

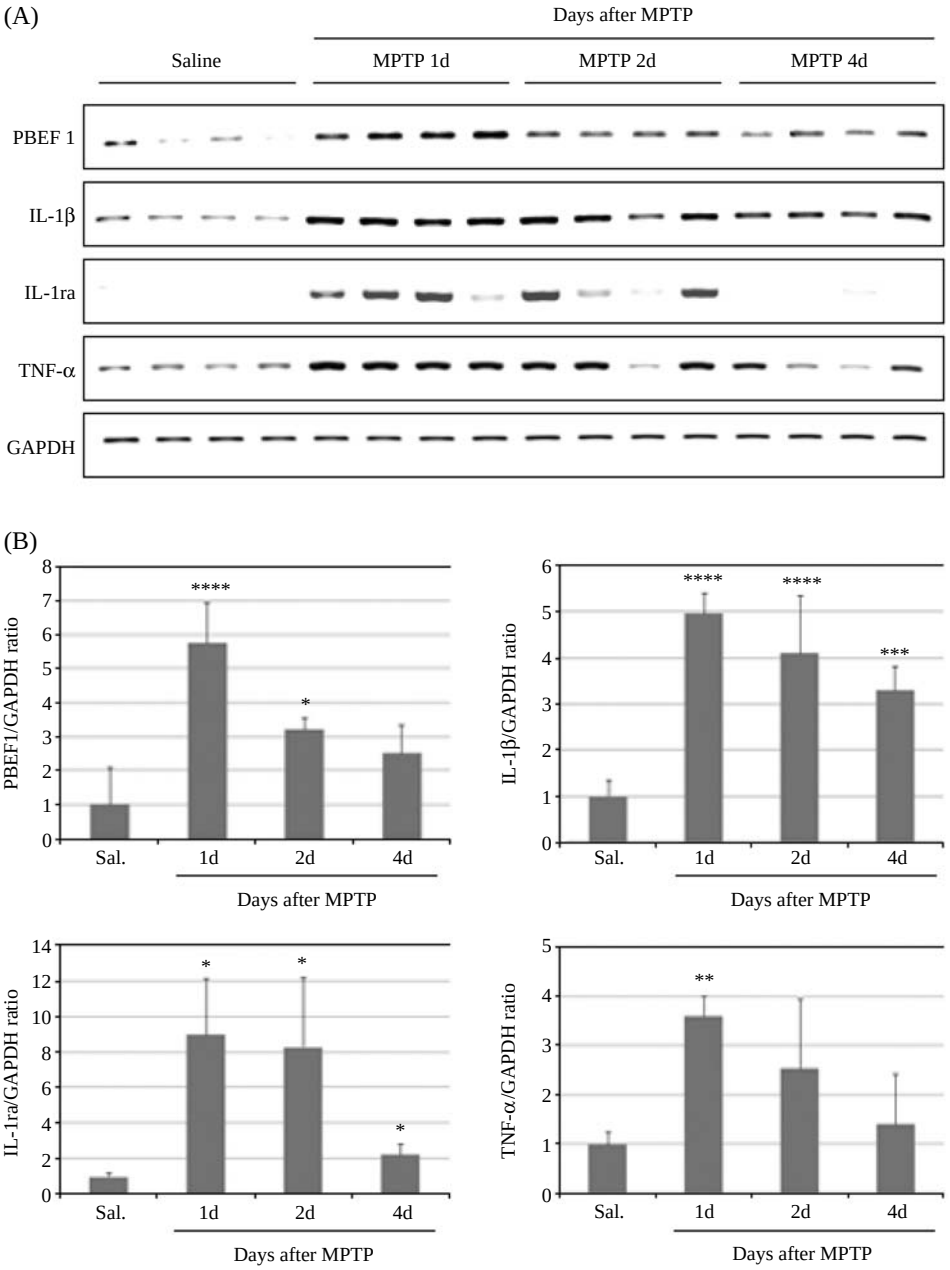

Figure 2. Expression analysis of PBEF1 mRNA in ventral midbrain of MPTP-intoxicated mice. IL-1, IL-1ra and TNFα mRNA levels were increased after MPTP acute injection, compared with controls. $*P < 0.05$, compared with saline (post hoc Bonfferoni multiple comparison).

activated cells. The effects of LPS and LPS/IFNγ on microglial gene expression were similar, as shown by comparison using two models. The common expression pattern of LPS- and LPS/IFNγ-mediated stimulation were statistically significant for 38 of these genes ($P < 0.05$, $\geq$2-fold). Analysis using seven differentially-modulated species confirmed that the expression pattern of the corresponding transcripts was faithfully reflected on the microarray analysis patterns, confirming the reliability of the approach. The PBEF1 gene that was A induced in both LPS and LPS/ IFNγ-activated microglia cells was further analyzed in a MPTP-intoxicated mouse model. Expression of PBEF1 was acutely increased after MPTP injection compared to saline-injected controls, and PBEF1-positive cells were evident by immunostaining following MPTP administration, supporting a possible role of PBEF1 in the process of neuroinflammation.

Materials and Methods

Reagents and Cells

Escherichia coli 0111 : B4 LPS, Tween-20, bovine

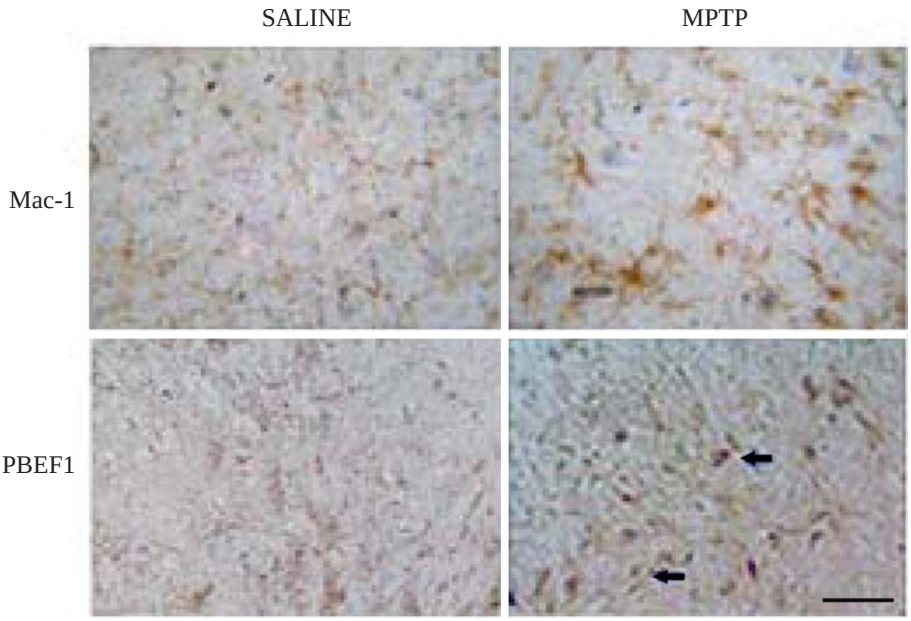

Figure 3. Immunolocalization of PBEF1 in MPTP-intoxicated mice. PBEF1 positive cells by immunostaining are shown after MPTP treatment (arrow).

serum albumin (BSA) and dimethyl sulfoxide (DMSO), were purchased from Sigma-Aldrich (St. Louis, MO, USA). Recombinant mouse IFNγ was purchased from R & D Systems (Minneapolis, MN, USA). Six-well and 96-well tissue culture plates and 100 mm-diameter culture dishes were purchased from Nunc (Rochester, NY, USA). Dulbecco's modified Eagle's medium (DMEM), phosphate buffered saline (PBS) and other cell culture reagents were obtained from Gibco (Carlsbad, CA, USA).

The BV-2 mouse microglial cell line originally developed by Dr. V. Bocchini (University of Perugia, Perugia, Italy) was generously provided by Dr. K. Suk (Kyung-Pook National University, Daegu, Korea). Immortalized murine BV-2 that exhibited phenotypic and functional properties of reactive microglia cells[5] were grown and maintained in DMEM supplemented with 5% heat-inactivated fetal bovine serum (FBS) and 50 μg/mL gentamicin, and were maintained in a humidified incubator with 37˚C, 5% CO_2. Cells were incubated in medium containing 100 ng/mL LPS alone or LPS and INFγ.

Microarray Analysis

The experiment was performed using a mouse 7.4 K cDNA chip (GenoCheck, Ansan, Korea) harboring a mouse 7426 cDNA microarray. Total RNA was isolated by extraction with TRIzol (Invitrogen, Carlsbad, CA, USA). Microarray hybridization was done according to the manufacturer's instructions. Extracted total RNA was inspected by use of a Bioanalyzer 2100 (Agilent Technologies). Fluorescence-labeled cDNA probes were prepared from 5 μg of total RNA by RT primer (Genisphere, Hatfield, PA, USA)-primed polymerization using Super-Script II reverse transcriptase (Invitrogen) in a total reaction volume of 10.5 mL. After reverse transcription, the sample RNA was degraded by adding 1 mL of stop solution (0.5 M NaOH/ 50 mM EDTA) and by incubating 65 μL for 10 min. After two labeled cDNAs were mixed and the mixture was denaturized at 95˚C for 2 min and incubated in a 45˚C water chamber for 20 min. The cDNA mixture was then placed on the cDNA 7.4 K chip and covered by a hybridization chamber. The slides were hybridized for 12 h in a 62˚C hybridization oven. Hybridized slides were scanned with a GenePix 4000B scanner (Molecular Devices, Sunnyvale, CA, USA) and the scanned images were analyzed using GenePix Pro 5.1 software (Molecular Devices) and GeneSpring GX 7.3.1 (Agilent). To allow for algorithmic eliminate of bad spots, no data points were eliminated by visual inspection from the initial GenePix image. For signal normalization, positive control genes (*Arabidopsis thaliana* genes and Amp genes) were spotted onto each slide. The signals of these spots were used for normalization. To determine the background signal intensity, spotting solution was spotted on each slide. To filter out the unreliable data, spots with a signal-to-noise ratio (signal background-background, standard deviation) below 100 were not included in the data. Data were normalized by global, lowess, print-tip and scaled normalization for data reliability. Genes of interest were those that exhibited a 2-fold change between test and control sample. Data were clustered in groups

Table 2. Nucleotide sequences of the primers used for RT-PCR.

Gene target	Genebank accession no.		Primer sequences
GAPDH	BC083149	Forward primer	5′-GCAGTGGCAAAGTGGAGATTG-3′
		Reverse primer	5′-TGCAGGATGCATTGCTGACA-3′
IL-1β	BC011437	Forward primer	5′-CATATGAGCTGAAAGCTCTCCA-3′
		Reverse primer	5′-GACACAGATTCCATGGTGAAGTC-3′
TNF-α	NM_013693	Forward primer	5′-TTCGAGTGACAAGCCTGTAGC-3′
		Reverse primer	5′-AGATTGACCTCAGCGCTGAGT-3′
IL-1ra	NM_031167	Forward primer	5′-CCATAGACACTGCCTGGGTG-3′
		Reverse primer	5′-GCACAAGACAGGCACAGCTT-3′
PBEF1	NM_021524.1	Forward primer	5′-TGTCTCCTTCGGTTCTGGTG-3′
		Reverse primer	5′-ACGTCCTGCTCGATGTTCAG-3′
AA470303	AA470303	Forward primer	5′-CAACAAAAGGCTGGGTCATT-3′
		Reverse primer	5′-TTCACCCCCATGTATCCTGT-3′
AA177731	AA177731	Forward primer	5′-TTGGTGTGCACCATAAAAAGA-3′
		Reverse primer	5′-TCACCAAAACACATGTTCTTCA-3′
AA268414	AA268414	Forward primer	5′-TTCCTGAAGCATCCATTTCC-3′
		Reverse primer	5′-GAAAGCTCTGGTCCATCAGG-3′
W82276	W82276	Forward primer	5′-TAAGCCTTTTCCTTTGGGTG-3′
		Reverse primer	5′-CAAAATTGGGCAACCTCCTA-3′
AA204588	AA204588	Forward primer	5′-TCTTGCGAAAGACCAGGATT-3′
		Reverse primer	5′-TCATCTTCCCTAGTTCTGGCA-3′

of genes that behaved similarly across an experiments using GeneSpring 7.3.1 (Agilent). An algorithm based on Euclidean distance was utilized to separate genes having similar patterns. The distance cutoff was considered statistically significant when there was a 1.5-fold change between time course experiments the correlation cutoff was 0.95.

Isolation of Total RNA and RT-PCR Analysis

BV-2 cells (5×10^4 cells/mL) were cultured in six well plates, and the total RNA was isolated by extraction with TRIzol (Invitrogen). For the reverse transcription-polymerase chain reaction (RT-PCR), 2.5 μg of total RNA from BV-2 cells was used for RT-PCR using a First Strand cDNA synthesis kit (Invitrogen). PCR was performed using the cDNA as a template. Nucleotide sequences of the primers used for RT-PCR are shown in Table 2. Glyceraldehyde-3-phosphate dehydrogenase was used as an internal control to evaluate the relative expressions.

Animals

Ten to twelve-week-old male C57BL/6J mice weighing 23-28 g were housed at a constant temperature on a 12 h light-dark cycle with free access to food and water. All animal treatments and care were performed according to the Konkuk University Health Guide for the Care and Use of Laboratory Animals, and the experiments were approved by the Konkuk University Institutional Animal Care and Use Committee. For acute MPTP injection, mice received four intraperitoneal injections of MPTP-HCl (20 mg/kg of free base; Sigma-Aldrich) dissolved in saline at 2 h intervals in 1 day. Control mice received saline only. Total RNA from ventral midbrain of saline or acute MPTP-injected mice prepared as detailed above was used for expression analyses.

Immunohistochemistry

Animals were anaesthetized with pentobarbital and transcardially perfused with 0.4% paraformaldehyde in 0.1 m phosphate buffer. Brains were removed, post-fixed and cryoprotected. Immunohistochemistry was performed as described previously[27]. The substaintia nigra coronal sections (30 μm) were cut with a cryostat. For immunostaining, sections were first washed with phosphate buffered saline three times andthen quenched peroxidase with 3% hydrogen peroxide for 10 min. The sections were blocked with 10% goat serum for 1 h and incubated with one of the following primary antibodies at 4˚C overnight: rat anti-MAC-1 (1 : 200; Serotec, Raleigh, NC, USA) or goat anti-PBEF1 (1 : 250, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Sections were then treated with biotinylated secondary antibody (Vector Laboratories, Burlingame, CA, USA) for 1 h at room temperature and subsequently incubated with avidin-biotinylated horseradish peroxidase complex for 1 h. Bound peroxidase was revealed by incubation with 0.05% 3,3′-diaminobenzidine tetrahydrochloride containing 0.015% hydrogen per-

oxide.

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