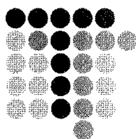

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The Gene Expression Profile of LPS-stimulated Microglial Cells

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Abstract

This study was conducted to evaluate the inflammatory mechanisms of LPS-stimulated BV-2 microglial cells. The inflammation mechanism was evaluated in BV-2 cells with or without LPS treated using the Affymetrix microarray analysis system. The microarray analysis revealed that B cell receptor signaling pathway, cytokine-cytokine receptor interaction, Jak-STAT signaling pathway, MAPK signaling pathway, Neuroactive ligand-receptor interaction, TLR signaling pathway, and T cell receptor signaling pathway-related genes were up-regulated in LPS stimulated BV-2 cells. Selected genes were validated using real time RT-PCR. These results can help an effective therapeutic approach to alleviating the progression of neuro-inflammatory diseases.

Key words: Gene expression profile, Inflammatory effects, LPS, Microglial cell

Lipopolysaccharide (LPS) is a potent activator of innate host immune response. Upon stimulation by LPS, monocytic phagocytes express a variety of mediators including proinflammatory cytokines, chemokines, and reactive oxygen species (ROS)[1,2]. Microglia have been proposed to play a role in host defense and tissue repair in the central nervous system[3,4]. Indeed, these cells are a class of brain mononuclear phagocytes that carry out the secretion of cytokine and the production of inflammatory mediators such as ROS, nitric oxide (NO) and excitatory amino acids[5-9]. NO plays an important role in a variety of physiological processes, including smooth muscle relaxation, platelet inhibition, neurotransmission, immune responses and inflammation. In addition, NO release from microglial cells is known to induce neurotoxicity[10,11]. The generation of NO following an inflammatory stimulation, its regulation depends on the formation of a multiple intracellular signaling complex composed of Janus kinases (JAKs), protein tyrosine kinases, mitogen-activated protein kinases (MAPKs) and protein kinase C[12].

Microarray analysis is a molecular technique that enables the parallel analysis of gene expression by a very large number of genes encompassing a significant fraction of the human genome. This method is both qualitative and quantitative because it is able to detect changes in the levels of expression in treated cells based on comparison with control samples[13,14]. Therefore, the use of microarray analysis allows development of more advanced therapies for prevent of inflammatory disease.

The goal of this study was to determine the inflammatory mechanisms of LPS-stimulated BV-2 microglial cells. The results of these tests and the possible mechanisms by which they occurred are discussed herein.

Gene Expression Profiles in BV-2 Microglial Cells

Gene expression profiles were significantly up- or down-regulated in the experimental groups (LPS-treated BV-2 microglial cells) when compared with the control (non-treated BV-2 microglial cells). In total, 452 differentially expressed genes (389 up- and 63 down-regulated) were detected in the experimental group using approximately 45,100 oligonucleotide probes. For the experimental group, genes showing highly altered expression levels were aligned according to the magnitude of the altered expression. Most of the differentially expressed genes (50) are listed in Table 1, which shows a comparison of the expression levels for a variety of genes between the experimental

Table 1. Up-regulation of genes based on comparison of gene expression in LPS stimulated BV-2 microglial cells.

Genes (50)	Symbol	Pathway	*P*-value	Regulation profile and average log2 ratio	
				NC	LPS*
<Inflammatory & immune response-related genes>					
Interleukin 6	Il6	CC, Toll	0.00	1	5.9
Chemokine (C-X-C motif) ligand 10	Cxcl10	CC	0.00	1	5.8
interferon-induced protein with tetratricopeptide repeats 1	Ifit1	–	0.00	1	5.4
Chemokine (C-C motif) ligand 5	Ccl5	CC, Toll	0.00	1	5.0
Chemokine (C-X-C motif) ligand 2	Cxcl2	CC	0.00	1	3.8
2′-5′ oligoadenylate synthetase-like 1	Oasl1	–	0.00	1	3.6
interferon activated gene 205	Ifi205	–	0.00	1	3.5
guanylate nucleotide binding protein 2	Gbp2	–	0.01	1	3.4
2′-5′ oligoadenylate synthetase-like 2	Oasl2	–	0.00	1	3.3
chemokine (C-C motif) ligand 2	Ccl2	CC	0.00	1	3.1
guanylate binding protein 6	Gbp6	–	0.00	1	2.9
interleukin 1 beta	Il1b	M, CC, Toll	0.00	1	2.9
macrophage activation 2 like	Mpa2l	–	0.00	1	2.8
chemokine (C-C motif) ligand 4	Ccl4	CC, Toll	0.00	1	2.7
interleukin 1 alpha	Il1a	M, CC	0.01	1	2.7
interferon induced with helicase C domain 1	Ifih1	–	0.00	1	2.7
Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta	Nfkbiz	–	0.00	1	2.7
chemokine (C-C motif) ligand 7	Ccl7	CC	0.00	1	2.5
C-type lectin domain family 4, member e	Clec4e	–	0.00	1	2.4
colony stimulating factor 3 (granulocyte)	Csf3	CC	0.00	1	2.4
guanylate nucleotide binding protein 3	Gbp3	–	0.00	1	2.2
nitric oxide synthase 2, inducible, macrophage	Nos2	–	0.01	1	1.9
CD40 antigen	Cd40	CC, Toll	0.00	1	1.7
<Signal transduction-related genes>					
Chemokine (C-C motif) receptor-like 2	Ccrl2	–	0.00	1	4.3
fibroblast growth factor receptor 3	Fgfr3	M	0.01	1	3.7
calcitonin receptor-like	Calcrl	NR	0.00	1	2.9
phosphodiesterase 4B, cAMP specific	Pde4b	–	0.00	1	2.4
Mitogen activated protein kinase 1	Mapk1	M	0.01	1	1.9
RAS p21 protein activator 4	Rasa4	–	0.04	1	1.6
RAS p21 protein activator 2	Rasa2	M	0.01	1	1.0
<Transcription-related genes>					
poly (ADP-ribose) polymerase family, member 14	Parp14	–	0.00	1	3.3
interferon regulatory factor 1	Irf1	–	0.00	1	2.4
signal transducer and activator of transcription 2	Stat2	JS	0.01	1	2.2
nuclear factor of kappa light polypeptide gene enhancer in B-cells 2, p49/p100	Nfkb2	M, Toll, BP, TP	0.01	1	1.1
<Apoptosis-related genes>					
Tnf receptor-associated factor 1	Traf1	–	0.00	1	3.4
nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, epsilon	Nfkbie	TP, BP	0.00	1	3.0
interferon activated gene 203	Ifi203	–	0.00	1	2.7
tumor necrosis factor, alpha-induced protein 3	Tnfaip3		0.00	1	2.3
interferon activated gene 204	Ifi204	–			
CASP8 and FADD-like apoptosis regulator	Cflar	–	0.00	1	2.0
Fas (TNF receptor superfamily member 6)	Fas	M, CC	0.00	1	1.9
B-cell leukemia/lymphoma 2 related protein A1a	Bcl2a1a	–	0.01	1	1.8
<Metabolism-related genes>					
prostaglandin-endoperoxide synthase 2	Ptgs2	–	0.00	1	2.9
<Cell adhesion-related genes>					
intercellular adhesion molecule	Icam1				

Table 1. Continued.

Genes (50)	Symbol	Pathway	P-value	Regulation profile and average log2 ratio	
				NC	LPS*
< The others>					
Radical S-adenosyl methionine domain containing 2	Rsad2	–	0.00	1	5.4
Thymidylate kinase family LPS-inducible member	Tyki	–	0.00	1	4.4
Receptor transporter protein 4	Rtp4	–	0.00	1	3.9
SAM domain and HD domain, 1	Samhd1	–	0.00	1	2.9
MARCKS-like 1	Marcksl1	–	0.00	1	2.7
three prime repair exonuclease 1	Trex1	–	0.01	1	2.7
serine (or cysteine) peptidase inhibitor, clade E, member 1	Serpine1	–	0.00	1	2.7

*Compared to untreated BV-2 microglial cells incubated for the same time. Abbreviation: BP: B cell receptor signaling pathway, CC: cytokine-cytokine receptor interaction, JS: Jak-STAT signaling pathway, M: MAPK signaling pathway, NR: Neuroactive ligand-receptor interaction, Toll: Toll-like receptor signaling pathway, TP: T cell receptor signaling pathway.

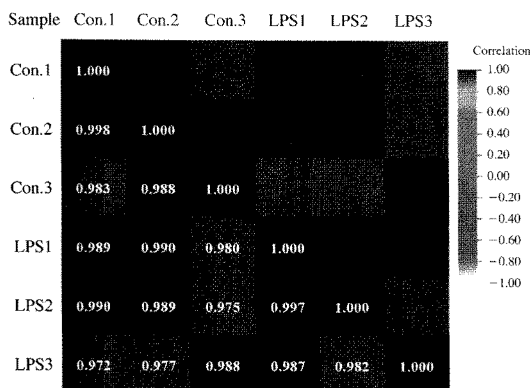

Figure 1. Correlation matrix plot analysis for calculate reproducibility between triplicates. The R^2 value calculation is based on intensity signals from all probe sets on the Affymetrix Mouse 430 2.0 array. Based on R^2, the microarray hybridization patterns were highly consistent among the sample. A perfect relationship among the samples would produce a slope of 1.

group and the control. All genes were grouped into functional categories and metabolic pathways based on the KEGG database. To assess the reliability of our microarray technique, we calculated the microarray reproducibility between triplicates. The correlation matrix plot from the control (non-treated), LPS-treated BV-2 cells showed in Figure 1. A good relationship between triplicates would be a slope of 1.

Validation of Selected Genes via Real-Time RT-PCR

The SYBR Green assay was used to confirm expression changes of 4 selected genes, Mapk1, NfkB2, RASA2, and FGFR3 identified by microarray analysis in the LPS-treated BV-2 cells. The real-time RT-PCR assay yielded results that showed qualitative agreement with the microarray results (Figure 2).

Discussion

Microglia is the major cell type involved in neuroinflammatory events in brain diseases such as encephalitis, stroke, and neurodegenerative disorders including Alzheimer's disease, Parkinson's disease, and multiple sclerosis[5,15,16]. Therefore, the inflammatory mechanisms of LPS-stimulated BV-2 microglial cells were evaluated in an effort to find new therapeutic strategy of neuroinflammation diseases. However, the early signaling events involved in LPS-induced microglial activation are not completely understood, therefore we evaluated the gene expression profiles of BV-2 microglial cells.

We found specific and significant alterations of the expression profile of LPS-treated BV-2 microglial cells (Table 1). The cellular processes represented by these genes include B cell receptor signaling pathway (nuclear factor of kappa light polypeptide gene enhancer in B-cells 2, p49/p100 (*Nfkb2*), and nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, epsilon (*Nfkbie*)), cytokine-cytokine receptor interaction (Interleukin 6 (*Il6*), *Il1a*, *Il1b*, Chemokine (C-X-C motif) ligand 2 (*Cxcl2*), *Cxcl10*, Chemokine (C-C motif) ligand 2 (*Ccl2*), *Ccl4*, *Ccl5*, *Ccl7*, colony stimulating factor 3 (*Csf3*), *Cd40*, and *Fas*), Jak-STAT signaling pathway (signal transducer and activator of transcription 2 (*Stat2*)), MAPK signaling pathway (*Il1a*, *Il1b*, fibroblast growth factor receptor 3 (*Fgfr3*), Mitogen activated protein kinase 1 (*Mapk1*), RAS p21

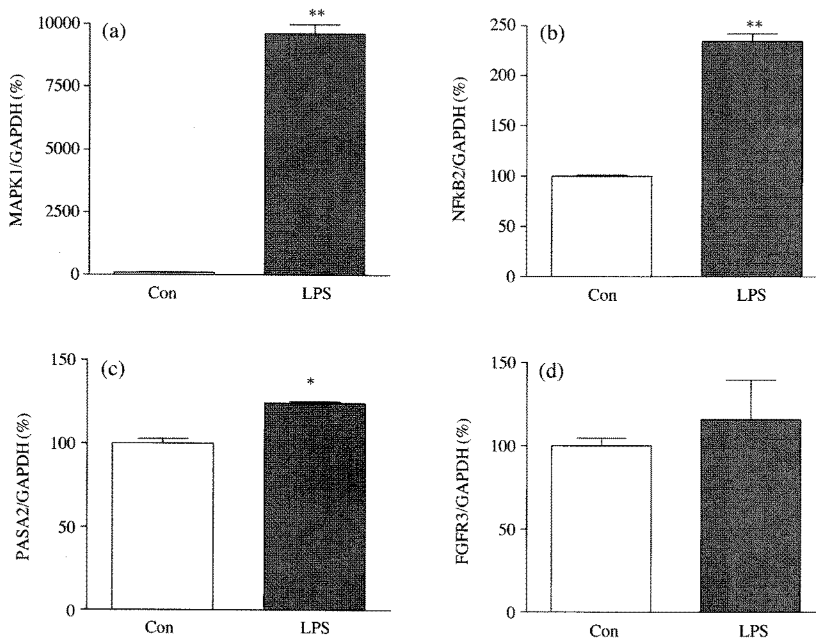

Figure 2. Effects of *Nelumbinis semen* on mRNA levels determined by real-time RT-PCR. (a) MAPK1, (b) NFkB2, (c) RASA2, and (d) FGFR3. Data are presented as mean $\pm$S.E.M. $*P<0.05$ and $**P<0.01$ compared to control; $n=3$ (each group).

protein activator 2 (*Rasa2*), *Nfkb2*, and *Fas*), Neuroactive ligand-receptor interaction (calcitonin receptor-like (*Calcrl*)), Toll-like receptor (TLR) signaling pathway (*Il6*, *Il1b*, *Ccl5*, *Ccl4*, *Cd40*, and *Nfkb2*), and T cell receptor signaling pathway (*Nfkb2*, and *Nfkbie*).

Engagement of TLRs induces the secretion of inflammatory cytokines[17]. In addition, Janus kinases (JAKs) have been implicated as playing crucial roles in cytokine signaling because they physically bind to the receptor cytoplasmic regions that appear to be critical in mitogenic signal transduction, and their kinase activity is rapidly stimulated by cytokine binding to these receptors[18]. In this study, we detected the up-regulation of *Il1a*, *Il1b*, *Il6*, *Cxcl2*, *Cxcl10*, *Ccl2*, *Ccl4*, *Ccl5*, *Ccl7*, *Csf3*, *Cd40*, *Fas*, *Cd40*, *Nfkb2*, and *Stat2* in LPS stimulated BV-2 microglial cells (Table 1). LPS signaling through the TLR4 to activate the NF-kB and MAPK pathways[19-21]. The MAPK pathways are deeply involved in signaling for various immune responses including apoptosis. Activation of the MAPK pathway often occurs in response to growth factor stimulation of receptor tyrosine kinases[2,22]. In this study, we detected the up-regulation of *Il1a*, *Il1b*, *Fgfr3*, *Mapk1*, *Rasa2*, *Nfkb2*, and *Fas* in LPS stimulated BV-2 microglial cells (Table 1, Figure 2 (a), (b), (c), and (d)). Each of the MAPKs has also been implicated in neuroinflammatory events, including mediation of many of the physiological responses to NO. NO is a signaling molecule, neurotransmitter, and immune effector[2,6,23]. NO is produced by the activity of the family of enzymes nitric-oxide synthases (NOSs). In this study, we found that *Nos2* was up-regulated in LPS stimulated BV-2 microglial cells (Table 1).

Taken together, the results of this study suggest that LPS may have induced inflammatory disease and other neurodegenerative diseases through neuroinflammation activity that occurs via B cell receptor signaling pathway, cytokine-cytokine receptor interaction, Jak-STAT signaling pathway, MAPK signaling pathway, Neuroactive ligand-receptor interaction, TLR signaling pathway, and T cell receptor signaling pathway.

Materials & Methods

Cell Culture

The immortalized murine BV-2 microglial cell line, which exhibits both the phenotypic and functional properties of reactive microglia cells, was grown and maintained in 100% humidity and 5% CO_2 at 37°C in Dulbecco's modified Eagles's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), streptomycin, and penicillin (Invitrogen Life Technologies, Rockville, USA). BV-2 microglial cells were then plated onto 100 mm, flat-bottom tissue culture plates at a density of 1×10^7 cells/mL in hormonally defined DMEM media as described previously. The medium was changed every 2 days until the cells became 80-

90% confluent, at which point they were used for experiments.

RNA Preparation

BV-2 microglial cells were initially cultured in a 100 mm dish (1×10^7 cells/mL) for 24 hr, after which 1 µg/mL LPS was added. Next, the cells were incubated at 37°C for 3 hr. The RNA was then isolated from the BV-2 microglial cells using an Rneasy® mini kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions, after which the RNA was quantified using a NanoDrop (NanoDrop Technologies, Inc ND-1000; Wilmington, DE, U.S.A).

Oligonucleotide Chip Microarray

Oligonucleotide chip microarray was performed using single round RNA amplification protocols, following the Affymetrix specifications (Affymetrix GeneChip Expression Analysis Technical Manual). Briefly, 3 micrograms of total RNA were used to synthesize first-strand complementary DNA (cDNA) using oligonucleotide probes with 24 oligo-dT plus T7 promoter as primers (Proligo LLC, Boulder, CO) and the Superscript Choice System (Life Technologies, Invitrogen, Milan, Italy). After double-stranded cDNA synthesis, the products were purified by phenol-chloroform extraction, and then biotinylated antisense complimentary RNA (cRNA) was generated through *in vitro* transcription using a BioArray RNA High-Yield Transcript Labeling Kit (ENZO Life Sciences Inc., Farmingdale, NY). The biotinylated labeled cRNA was then fragmented, and 10 µg of the total fragmented cRNA was hybridized to the Affymetrix Mouse 430 2.0 GeneChip array (P/N900470, Affymetrix Inc., USA). The Affymetrix Fluidics Station 400 was then used to wash and stain the chips, after which the nonhybridized target was removed. Next, the samples were incubated with a streptavidin-phycoerythrin conjugate to stain the biotinylated cRNA. The staining was then amplified using goat IgG as blocking reagent and biotinylated antistreptavidin antibody (goat), followed by a second staining step using a streptavidin-phycoerythrin conjugate. The fluorescence was detected using the Genechip System Confocal Scanner (Hewlett-Packard), and analysis of the data contained on each GeneChip was conducted using the GeneChip 3.1 software produced by Affymetrix, using the standard default settings. To compare different chips, global scaling was used, with all probe sets being scaled to a user-defined target intensity of 150.

Data Analysis

The MAS5 algorithm was used to evaluate the expression signals generated by the Affymetrix Mouse 430 2.0 array. Global scaling normalization was then performed and the normalized data were log-transformed using base 2. Next, fold change and a Welch t-test were applied to select the differentially expressed genes (DEGs) using a fold change threshold of 2.0-fold and a $P < 0.05$ to indicate significance. Each probe set used in the Affymetrix GeneChip produces a detection call, with P (present call) indicating good quality, M (marginal call) indicating intermediate quality and A (absent call) indicating relatively low reliability. Therefore, probe sets that resulted in A calls in the compared groups were removed to filter false positives. A volcano plot was used to better visualize and compare the two DEG methods. The 2.0-fold DEGs were clustered using the GenPlex™ v3.0 software (ISTECH Inc., Korea) using hierarchical clustering with Pearson correlation as a similarity measure and complete linkage as the linkage method). In addition, gene ontology significance analysis was conducted to investigate the functional relationships among the 2.0-fold DEGs using high-throughput GoMiner. The 2.0-fold DEGs were then mapped to relevant pathways using GenPlex™ v3.0 software (ISTECH Inc., Korea). The pathway resources were provided by the KEGG database. Samples were compared to extract regulated genes between LPS-treated and control (non-treated) BV-2 cells.

Real-Time RT-PCR Analysis

Microarray verification was performed by real-time RT-PCR analysis of selected genes using SYBR Green I Master Mix (Applied Biosystems, Foster City, CA, U.S.A) and primers (Genotech Inc., Korea). Complementary DNA (cDNA) was synthesized using 2 µg of RNA in a reverse transcription reaction. Real time-PCR quantitative mRNA analyses were performed with an Applied Biosystems 7300 Real Time PCR System using the SYBR Green fluorescence quantification system (Applied Biosystems, Foster City, CA, U.S.A) to quantify the amplicons. The PCR conditions were 40 cycles of 95°C (15 sec), 60°C (1 min), and a standard denaturation curve. The sequences of the mouse primers were as follows: Mapk1 (forward primer 5′-ggc tgg cct caa act cta cc-3′; reverse primer 5′-agc atc cca gca gca ata aa-3′), NfkB2 (forward primer 5′-gcg aga ata gag gag gag gc-3′; reverse primer 5′-gag gaa ggg cat cca ctt tg-3′), RASA2 (forward primer 5′-ggg gtt tgt ctg tgt ctc ca-3′; reverse primer 5′-gcg gag tcc atc ttt tag gc-3′), FGFR3 (forward primer 5′-atg atc atg cgg gaa tgt tg-3′; reverse primer 5′-agt act gct caa acg gca cg-3′), and GAPDH (forward primer 5′-ttc acc acc atg gag aag gc-3′; reverse primer 5′-ggc atg gac tgt ggt cat ga-3′). PCR conditions for each target were optimized according to the primer concentra-

tion, the absence of primer dimer formation, and the efficiency of amplification of both the target genes and the housekeeping gene control. PCR reactions were carried out in a total volume of 20 μL in PCR master mix containing 10 μL 2X SYBR Green, 5 μM each of sense and antisense primer, and 2 μL of 1 : 2 diluted cDNA to a final volume of 20 μL with DEPC-treated H_2O. Data were analyzed using GeneAmp 7300 SDS software (Applied Biosystems). The dissociation curves from all data showed only one distinct peak, implying that there is only one PCR product in the reaction (data not shown). Additionally, the standard curves also demonstrate that the R^2 values were between 0.98 and 0.999, which show reliability of the data (data not shown).

Statistical Analysis

Statistical analysis of the data was conducted using the Prism 3.02 software (GraphicPad Software Inc., CA, U.S.A). Data were analyzed by one-way ANOVA for multiple comparisons. Results with a $P < 0.05$ were considered statistically significant.

Acknowledgements

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