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Microarray analysis of gene expression profiles in response to treatment with bee venom in lipopolysaccharide activated RAW 264.7 cells

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ABSTRACT

Aim of the study: The therapeutic application of bee venom (BV) has been used in traditional medicine to treat diseases such as arthritis, rheumatism and pain. Macrophages produce molecules that are known to play roles in inflammatory responses.
Material and methods: We performed microarray analysis to evaluate the global gene expression profiles of RAW264.7 macrophage cells treated with BV. In addition, six genes were subjected to real-time PCR to confirm the results of the microarray. The cells were treated with lipopolysaccharide (LPS) or BV plus LPS for 30 min or 1 h.
Results: 124 genes were found to be up-regulated and 158 were found to be down-regulated in cells that were treated with BV plus LPS for 30 min, whereas 211 genes were up-regulated and 129 were down-regulated in cells that were treated with BV plus LPS for 1 h when compared with cells that were treated with LPS alone. Furthermore, the results of real-time PCR were similar to those of the microarray. BV inhibited the expression of specific inflammatory genes that were up-regulated by nuclear factor-kappa B in the presence of LPS, including mitogen-activated protein kinase kinase kinase 8 (MAP3K8), TNF, TNF-α-induced protein 3 (TNFAIP3), suppressor of cytokine signaling 3 (SOCS3), TNF receptor-associated factor 1 (TRAF1), JUN, and CREB binding protein (CBP).
Conclusions: These results demonstrate the potent activity of BV as a modulator of the LPS-mediated nuclear factor-κB (NF-κB)/MAPK pathway in activated macrophages. In addition, these results can be used to understand other effects of BV treatment.

1. Introduction

Bee venom (BV), which is stored by bees within their venom sacs for self-defense, has traditionally been used in Asian countries to relieve pain and treat inflammatory diseases (Billingham et al., 1973; Kwon et al., 2001). Since the anti-inflammatory effects of BV were first discovered, many studies conducted using diverse methodologies have confirmed this effect. BV contains various peptides, including mellitin, apamin, adolapin and mast cell degranulation (MCD) peptide, as well as various enzymes, such as phospholipase A2, and non-peptide components, such as histamines, lipids and carbohydrates (Kwon et al., 2001). Although adolapin and MCD peptide have anti-inflammatory activities, these substances are only present in very small quantities in BV (Martin and Hartter, 1980). In addition, even though mellitin, a major component of BV, has been reported to inhibit the enzymatic activity of phospholipase A2, an inflammatory trigger (Saini et al., 1997), another study confirmed that injection of mellitin induced paw edema in mice (Hartman et al., 1991). Furthermore, injection of whole BV into the hind paw of mice has been shown to produce local inflammation (Lariviere and Melzack, 1996), however, in a previous study, we found that BV had an anti-inflammatory effect that occurred via the inhibition of inducible nitric oxide synthesis (iNOS) and cyclooxygenase (COX)-2 expression in lipopolysaccharide (LPS) activated RAW264.7 macrophage cells (Jang et al., 2005).

Microarray analysis is a technique that has been shown to be useful for the simultaneous profiling of global gene expression and

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Table 1
Sequence of primers used for real time PCR.

Gene	Forward primer	Reverse primer	Size
Gapdh	GGCATGGACTGTGGTCATGA	TTCACCACCATGGAGAAGGC	237 bp
Cxcl2	ATAGATGCAGTCGGATGGCT	GCACTGTGCCTTACGAGGA	200 bp
Egr1	TGGTTTGTTTGGTTGGGTTT	GGCACCAAGACGTGAAACTT	79 bp
Fyb	TCAACACGGGGAGTAACCC	CGAGCTTTGTCCTGCAACT	74 bp
Nfil3	GAGCTATGCATGGAGGAGGA	CACAAGGACACCCAGACAGA	109 bp
Nr4a2	TCGACATTTCTGCCTTCTCC	GGTAGTTGGGTCGGTTCAAA	162 bp
Irg1	CCACGCTGAATGTCTTCTGA	TTGTCAAATGGCTACCCACA	114 bp

uncovering new genes or new functions of known genes (Thornton et al., 2002). For these reasons, microarray analysis of gene expressions in HEK 293 cells following treatment with herbal medicines (Chen et al., 2006) and in chondrosarcoma cells following treatment with BV (Yin et al., 2005) have previously been conducted.

Macrophages are major sources of cytokines, such as tumor necrosis factor-alpha (TNF-α). Cytokine expression that is induced by LPS occurs primarily at the transcriptional level through the action of several transcription factors, including members of the nuclear factor-κB (NF-κB)/rel, C/EBP, Ets, and activating protein-1 (AP-1) protein families (Sweet and Hume, 1996), and binding of NF-κB to specific consensus DNA elements present on the promoter of target genes initiates the transcription of TNF-α, iNOS, COX-2 and interleukin (IL)-6 (Kuprash et al., 1995).

In this study, a microarray assay was conducted to determine which genes are differentially expressed in response to BV in LPS activated macrophages. The results of the microarray analysis were then confirmed by real time PCR.

2. Materials and methods

2.1. Materials

DMEM media, penicillin-streptomycin and acetylated BSA were purchased from Invitrogen Life Technologies (Carlsbad, CA, USA). A first strand cDNA synthesis kit was obtained from Roche applied Science (Roche, Mannheim, Germany). Bee venom and *Escherichia coli* LPS (Serotype 055:B5) were obtained from Sigma (St. Louis, MO, USA). Syringe filters (pore size: 0.2 μm) and 100-mm diameter dishes were purchased from Nunc (Naperville, IL, USA).

2.2. Preparation of bee venom extract

Dried extract of bee venom was dissolved in distilled water and then centrifuged at 10,000 × *g* for 10 min. The supernatant was then passed through a 0.22-μm filter and diluted to a final concentration of 25 mg/ml. The solution was then aliquoted and stored at 4 °C for future use.

2.3. Cell culture

RAW 264.7 cells were cultured in DMEM media with 10% fetal bovine serum (FBS), 1% penicillin and 1% streptomycin. The cells were then maintained in a humidified atmosphere that contained 5% CO_2 at 37 °C. Next, 20 μg/ml of BV was pre-treated for 30 min, and then 200 ng/ml of LPS was treated for either 30 min or 1 h.

2.4. RNA extraction

Total RNA was extracted from cultured cells using an RNeasy mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. The extract was then assayed to determine the quality and the concentration of the RNA using a ND-1000 spectrophotometer (Nanodrop Technologies). The extract was then stored at −20 °C until analysis.

2.5. Preparation of cRNA and array hybridization

Biotinylated cRNA was prepared from 500 ng of total RNA using an Illumina RNA Amplification Kit (Ambion, Austin, TX). The cRNA yields were quantified using a ND-1000 spectrophotometer (Nanodrop Technologies). cRNA (1500 ng) was then hybridized to Sentrix Mouse-6 Expression BeadChips (Illumina, San Diego, CA) using the manufacturer's hybridization solution. Washing, detection and scanning were then conducted following the BeadStation 500× system protocols (Illumina).

2.6. Data preprocessing

The MAS5 algorithm was used for expression summary and signal calculation of Mouse-6 Expression BeadChips data. Global scaling normalization was performed and then fold changes were calculated based on the relative signal intensity.

Table 2
Simultaneously up- or down-regulated inflammatory genes in cells that were treated with BV for 30 min or 1 h.

Symbol	Gene name	Accession No	Fold change	
			30 min	1 h
Fbxw5	F-box and WD-40 domain protein 5	NM_013908.4	11.88	15.11
Egr2	Early growth response 2	NM_010118.2	9.02	15.03
Egr3	Early growth response 3	NM_018781	6.01	15.37
Egr1	Early growth response 1	NM_007913.5	3.82	4.14
Dusp5	Dual specificity phosphatase 5	XM_904896.2	5.96	11.69
Nr4a2	Nuclear receptor subfamily 4, group A, member 2	NM_013613.1	5.06	10.45
Nr4a2	Nuclear receptor subfamily 4, group A, member 2	NM_013613	3.17	5.66
Ier3	Immediate early response 3	NM_133662.2	4.85	7.87
Rcan1	Regulator of calcineurin 1	NM_019466.3	4.50	6.42
Rcan1	Regulator of calcineurin 1	NM_001081549.1	3.22	4.03
Osm	Oncostatin M	NM_001013365.1	4.30	4.92
Il1b	Interleukin 1 beta	NM_008361.3	3.92	5.36
Cxcl2	Chemokine (C-X-C motif) ligand 2	NM_009140	3.61	8.87
Ccl7	Chemokine (C-C motif) ligand 7	NM_013654	3.43	4.75
gly96	gly96	X67644.1	2.75	2.66
Pms1	Postmeiotic segregation increased 1	NM_153556.1	2.36	2.40
Sgk	Serum/glucocorticoid regulated kinase	NM_011361.1	2.34	3.94
Golga2	Golgi autoantigen, golgin subfamily a, 2	NM_133852.2	0.33	0.32

Table 3
Simultaneously up-regulated inflammatory genes in cells that were treated with LPS for 30 min or 1 h.

Pathway	Symbol	Gene name	Accession No	Fold change	
				30 min	1 h
Toll-like receptor signaling	Il1b	Interleukin 1 beta	NM_008361	9.52	78.78
	Tnfaip3	Tumor necrosis factor, alpha-induced protein 3	NM_009397.2	14.97	17.75
	Tnf	Tumor necrosis factor	NM_013693	26.78	22.75
	Traf1	Tnf receptor-associated factor 1	AK089281	7.48	11.01
	Jun	Jun oncogene	NM_010591.1	5.19	5.06
	Nfkbia	Nuclear factor of kappa light chain gene enhancer in B-cells inhibitor, alpha	NM_010907	4.90	4.86
	Crebbp	CREB binding protein	XM_148699.3	3.07	2.68
	Ccl3	Chemokine (C-C motif) ligand 3	NM_011337.1	2.09	7.62
	Ccl4	Chemokine (C-C motif) ligand 4	NM_013652.1	2.78	19.14
MAPK signaling	Gadd45a	Growth arrest and DNA-damage inducible 45 alpha	NM_007836.1	8.20	17.44
	Dusp1	Dual specificity phosphatase 1	NM_013642.1	7.09	7.66
	Dusp2	Dual specificity phosphatase 2	NM_010090.2	15.26	19.64
	Dusp4	Dual specificity phosphatase 4	NM_176933	1.58	3.79
	Ddit3	DNA-damage inducible transcript	NM_007837.2	2.81	1.89
	Jund1	Jun proto-oncogene related gene d1	NM_010592.3	1.57	1.69
	Socs3	Suppressor of cytokine signaling 3	NM_007707.2	9.96	13.62
	Tnfaip2	Tumor necrosis factor, alpha-induced protein 2	NM_009396.1	3.67	8.84
	Pim1	Proviral integration site 1	NM_008842.2	1.89	5.25
Cytokine-cytokine receptor interaction	Cxcl2	Chemokine (C-X-C motif) ligand 2	NM_009140	29.34	61.34
	Csf3	Colony stimulating factor 3 (granulocyte)	NM_009971.1	1.81	26.18
	Lif	Leukemia inhibitory factor	NM_001039537.1	2.32	20.35
	Tnfsf9	Tumor necrosis factor (ligand) superfamily, member 9	NM_009404.1	2.01	3.97
	Ccl2	Chemokine (C-C motif) ligand 2	NM_011333.1	1.57	2.63
	Ccl7	Chemokine (C-C motif) ligand 7	NM_013654	1.77	2.54
	Il10ra	Interleukin 10 receptor, alpha	NM_008348.1	1.69	2.34
	Il11	Interleukin 11	NM_008350.1	1.70	2.79
	Vegfa	Vascular endothelial growth factor A	NM_009505.2	1.65	1.75
Cell adhesion molecules	Icam1	Intercellular adhesion molecule	NM_010493.2	2.73	4.55

The 1.5-fold differentially expressed genes were mapped to their relevant pathways using GenPlex v2.4 software (ISTECH Inc., Korea).The pathway resources are provided by the KEGG database.

2.7. Real-time PCR

Reverse transcription of the total RNA was performed by mixing 1 μg of RNA, DEPC-DW, 4 μl of transcriptor reverse transcriptase reaction buffer, 0.5 μl of protector RNase inhibitor, and 2 μl of deoxynucleotide mixture with 2.5 μM oligo (dT) primer and 0.5 μl of transcriptor reverse transcriptase. The solution was then incubated for 30 min at 55 °C, after which it was heated at 85 °C for 5 min to inactivate the transcriptor, reverse transcriptase. The samples were then stored at −20 °C until further use.

Real-time quantitative PCR was performed using an ABI 7900 HT Sequence detection system (Applied Biosystems, Foster City, CA, U.S.A.) employing SYBR Green I as the dsDNA-specific binding dye for continuous fluorescence monitoring. Amplification was carried out in a total volume of 20 μl containing 25 μM of each gene-specific primer (Table 1), 2× PCR Master Mix (Applied Biosystems, Foster City, CA, U.S.A.) and 0.5 μl of cDNA. The PCR reactions were then subjected to 40 cycles of denaturation (95 °C, 30 s) and annealing and extension at 62–67 °C, depending on the primers used, with the fluorescence being measured at the end of each cycle. After the

Table 4
Simultaneously down-regulated genes in cells that were treated with LPS for 30 min or 1 h.

Symbol	Gene name	Accession No	Fold change	
			30 min	1 h
Itga4	Integrin alpha 4	AK041096	0.35	0.37
Abcd4	ATP-binding cassette, subfamily D, member 4	AK049489	0.46	0.48
Mitf	Microphthalmia-associated transcription factor	AB009397	0.56	0.44
Casp2	Caspase 2	AK052396	0.40	0.62
Atpi	ATPase inhibitor	AK011886	0.54	0.54
Tia1	Cytotoxic granule-associated RNA binding protein 1	AK009502	0.50	0.57
Tcf12	Transcription factor 12	AK078415	0.54	0.56
Hoxb5	Homeo box B5	NM_008268.1	0.64	0.47
Tnpo1	Transportin 1	NM_178716	0.46	0.66
Fbxw5	F-box and WD-40 domain protein 5	NM_013908.1	0.57	0.58
Skp2	S-phase kinase-associated protein 2	AK037002	0.53	0.63
Pgls	6-Phosphogluconolactonase	AK054257	0.55	0.64
Nfatc3	Nuclear factor of activated T-cells, cytoplasmic 3	AK039929	0.63	0.60
Arhgef1	Rho guanine nucleotide exchange factor 1	AK040093	0.58	0.65
Hist1h2ah	Histone 1, H2ah	NM_175659.1	0.58	0.66
Hist1h2ak	Histone 1, H2ak	NM_178183.1	0.59	0.66
Atp6v1g2	ATPase, H+ transporting, V1 subunit G isoform 2	NM_023179.2	0.65	0.62
Hsf2	Heat shock factor 2	AK053976	0.63	0.66
Pole	DNA polymerase epsilon	AK031352	0.66	0.64

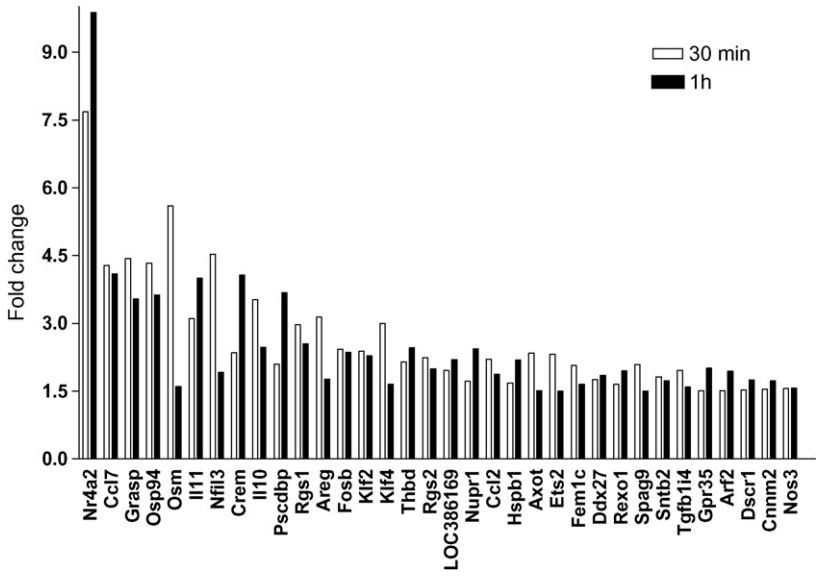

Fig. 1. Simultaneously up-regulated genes in cells that were treated with BV plus LPS for 30 min and 1 h.

cycles were terminated, the signal of each temperature between 60 and 95 °C was also collected for generation of a dissociation curve. All reactions were performed in triplicate to confirm reproducibility, and included a negative control (without template) to verify that no primer–dimers were being generated. The standard curve for each primer was constructed using serial dilutions of cDNA, and the amount of target mRNA in each sample was normalized using that of the mean GAPDH levels.

3. Results

3.1. Gene expression profiles of BV-treated cells

Since the injection of BV has been reported to evoke tonic pain and hyperalgesia (Lariviere and Melzack, 1996), we evaluated gene expression profiles in BV only treated cells compared with non-treated cells. As shown Table 2, BV treatment up-regulated some of inflammatory genes such as Nr4a2, Osm, IL1b, chemokine (CXC motif) ligand 2 (CXCL2) and chemokine (CC motif) ligand 7 (CCL7). These results indicated BV could evoke inflammatory responses by these genes expression in macrophages.

3.2. Gene expression profiles of LPS-treated cells

To investigate the responses of RAW 264.7 macrophages to LPS, cells were stimulated with 200 ng/ml of LPS for either 30 min or 1 h. One-hundred and fifty eight and 383 genes (>1.5 fold) were up-regulated in the cells that were treated with LPS for 30 min or 1 h, respectively. Of these genes, 127 were simultaneously up-regulated at both time points, including the following inflammation related genes (Table 3): CXCL2, IL1b, TNF, TNF-α-inducible protein (TNFAIP) 3, suppressor of cytokine signaling 3 (SOCS3), CCL4, TNF receptor-associated factor 1 (TRAF1) and nuclear factor of kappa light chain gene enhancer in B-cells inhibitor, alpha (NFKBIA). However, only 20 genes were simultaneously down-regulated by LPS treated cells at both time points (Table 4). These results were similar to those of a previous study conducted by Huang et al. (2006), in which 320 genes were up-regulated in RAW 264.7 cells in response to LPS treatment but only 32 genes were down-regulated. Taken together, these results suggest that inflammatory mediated genes are expressed early during LPS treatment.

3.3. Gene expression profiles of BV plus LPS treated cells

To investigate the effects of BV on genes that were up- or down-regulated by LPS treatment, the gene expression profile from the group treated with LPS alone was compared with those obtained from groups treated with BV plus LPS. Thirty-four genes were simultaneously up-regulated and eleven genes were simultaneously down-regulated after treatment with BV plus LPS for 30 min and 1 h when compared to cells treated with LPS alone (Figs. 1 and 2). Among these simultaneously up-regulated genes, NR4A2, CCL7, OSM, IL11, AREG, FOSB, KLF2, NUPR1 and CCL2 were also up-regulated in cells that were treated with LPS when compared to un-treated cells.

When cells that were treated with BV plus LPS were compared with those that were only treated with LPS for 30 min, treatment with BV was found to reverse the LPS-induced up-regulation of mitogen-activated protein kinase kinase kinase 8 (MAP3K8), myeloid differentiation primary response gene 116 (MYD116), TNF, TNFAIP3, SOCS3, TRAF1, JUN, NFKBIA, TNFAIP2, IRG1, and CREB-binding protein (CBP) (Table 5). These genes are inflammatory mediated genes, therefore these results indicate that BV suppressed inflammatory mediated genes soon after treatment. In addition,

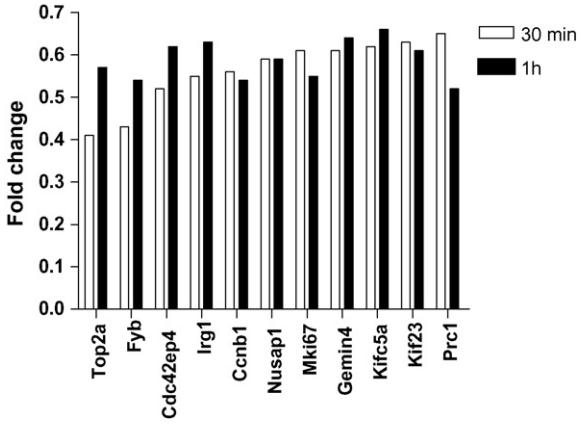

Fig. 2. Simultaneously down-regulated genes in cells that were treated with BV plus LPS for 30 min and 1 h.

Table 5
Gene expression profiles that were reversed when cells treated with BV plus LPS were compared with those that were treated with LPS alone for 30 min.

Symbol	Gene name	Accession No	LPS (30 min), BV (30 min)	
			Up	Down
Myd116	Myeloid differentiation primary response gene 116	NM_008654.1	32.01	0.55
Tnf	Tumor necrosis factor	NM_013693	26.78	0.37
Arc	Activity regulated cytoskeletal-associated protein	NM_018790.1	15.11	0.50
Tnfaip3	Tumor necrosis factor, alpha-induced protein 3	NM_009397.2	14.97	0.38
Cias1	Cold autoinflammatory syndrome 1 homolog	NM_145827.1	10.57	0.48
Socs3	Suppressor of cytokine signaling 3	NM_007707.2	9.96	0.43
Gadd45a	Growth arrest and DNA-damage-inducible 45 alpha	NM_007836.1	8.20	0.38
Traf1	Tnf receptor-associated factor 1	AK089281	7.48	0.59
Cybb	Cytochrome b-245, beta polypeptide	AK079926	7.34	0.40
Ybx3	Y box protein 3	AK029441	5.56	0.64
Jun	Jun oncogene	NM_010591.1	5.19	0.59
Nfkbia	Nuclear factor of kappa light chain gene enhancer in B-cells inhibitor, alpha	NM_010907	4.90	0.52
Junb	Jun-B oncogene	NM_008416.1	4.64	0.62
Tnfaip2	Tumor necrosis factor, alpha-induced protein 2	NM_009396.1	3.67	0.45
Irg1	Immunoresponsive gene 1	XM_127883.4	3.09	0.55
Crebbp	CREB binding protein	XM_148699.3	3.07	0.35
Dnajb1	DnaJ homolog, subfamily B, member 1	NM_018808.1	2.96	0.57
Gpr84	G protein-coupled receptor 84	NM_030720.1	2.91	0.57
Ddit3	DNA-damage inducible transcript 3	NM_007837.2	2.81	0.59
Zbtb7	Zinc finger and BTB domain containing 7	NM_010731.1	2.74	0.43
Icam1	Intercellular adhesion molecule	NM_010493.2	2.73	0.51
Map3k8	Mitogen activated protein kinase kinase kinase 8	NM_007746.1	2.59	0.46
Ifrd1	Interferon-related developmental regulator 1	NM_013562	2.48	0.61
H2-Q5	Histocompatibility 2, Q region locus 5	NM_010393.1	2.47	0.66
Cdc42ep4	CDC42 effector protein 4	NM_020006.1	2.41	0.52
Irf1	Interferon regulatory factor 1	NM_008390.1	2.28	0.45
Ccnl	Cyclin L	AK051380	2.24	0.57
Lcp2	Lymphocyte cytosolic protein 2	AK037970	2.17	0.40
Ddx6	DEAD box polypeptide 6	NM_007841.2	2.11	0.64
Slfn2	Schlafen 2	NM_011408.1	2.04	0.53
Itpkc	Inositol 1,4,5-trisphosphate 3-kinase C	NM_181593.2	2.04	0.66
Msn	Moesin	AK087001	2.03	0.53
Tnfsf9	Tumor necrosis factor superfamily, member 9	NM_009404.1	2.01	0.65
Myh9	Myosin heavy chain IX	NM_181327.2	1.89	0.57
Saa3	Serum amyloid A 3	NM_011315	2.00	0.60
Itgav	Integrin alpha V	AK036485	1.65	0.43
Ptpn11	Protein tyrosine phosphatase, nonreceptor type 11	NM_011202.2	1.52	0.65
Kpnb3	Karyopherin beta 3	AK017701	1.52	0.66

when cells that were treated with BV plus LPS were compared to those that were only treated with LPS for 1 h, 13 of the genes that were up-regulated following LPS treatment were found to be down-regulated following BV treatment (Table 6). We categorized the genes in which reversed expression was observed, and, as shown in Tables 7 and 8, BV affected genes that were involved in the following pathways: Toll-like receptor signaling, MAPK signaling, Jak-STAT signaling, cytokine–cytokine receptor interaction, and cell adhesion molecules.

3.4. Real-time RT-PCR

Using the real-time quantitative PCR technique, we verified the microarray results obtained for the six genes that are known to be involved in inflammation.

Although the fold-change reported by the array is an underestimate, the results presented in Figs. 3 and 4 show that the expression patterns obtained by real-time PCR were similar to the results of the microarray analysis. It should be noted that microarrays frequently

Table 6
Gene expression profiles that were reversed when cells that were treated with BV plus LPS were compared with those that were treated with LPS alone for 1 h.

Symbol	Gene name	Accession No	LPS (1 h), BV (1 h)	
			Up	Down
Irg1	Immunoresponsive gene 1	XM_127883.4	21.08	0.63
Ccl5	Chemokine ligand 5	NM_013653.1	9.84	0.63
Cdc42ep4	CDC42 effector protein 4	NM_020006.1	7.64	0.62
Oasl1	2–5 oligoadenylate synthetase-like 1	NM_145209.2	6.27	0.58
Mefv	Mediterranean fever	NM_019453.1	4.46	0.51
Slc7a11	Solute carrier family 7, member 11	AK037742	3.47	0.61
Cryba4	Crystallin, beta A4	NM_021351.1	3.41	0.62
Lpin2	Lipin 2	AK048657	3.37	0.61
Ehd1	EH-domain containing 1	NM_010119.3	2.82	0.61
Itga5	Integrin alpha 5	NM_010577.2	2.79	0.60
Il4i1	Interleukin 4 induced 1	NM_010215.1	1.91	0.60
Serpinb2	Serine proteinase inhibitor, clade B, member 2	NM_011111.2	1.82	0.64
Il13ra2	Interleukin 13 receptor, alpha 2	NM_008356.1	1.73	0.64

Table 7
Signaling pathway of the genes that exhibited reversed expression after BV treatment for 30 min.

Pathway	Symbol	Gene name	Accession No	Fold change
Toll-like receptor signaling	Tnf	Tumor necrosis factor	NM_013693	0.37
	Nfkbia	Nuclear factor of kappa light chain gene enhancer in B-cells inhibitor, alpha	NM_010907	0.52
	Jun	Jun oncogene	NM_010591.1	0.59
	Tnfaip3	Tumor necrosis factor, alpha-induced protein 3	NM_009397.2	0.38
	Traf1	Tnf receptor-associated factor 1	AK089281	0.59
MAPK signaling	Map3k8	Mitogen activated protein kinase kinase kinase 8	NM_007746.1	0.46
	Gadd45a	Growth arrest and DNA-damage inducible 45 alpha	NM_007836.1	0.38
Jak-STAT signaling	Crebbp	CREB binding protein	XM_148699.3	0.35
	Socs3	Suppressor of cytokine signaling 3	NM_007707.2	0.43
	Tnfaip2	Tumor necrosis factor, alpha-induced protein 2	NM_009396.1	0.45
Cytokine–cytokine receptor interaction	Tnfsf9	Tumor necrosis factor (ligand) superfamily, member 9	NM_009404.1	0.65
	Ptpn11	Protein tyrosine phosphatase, non-receptor type 11	NM_011202.2	0.65
Cell adhesion molecules	Icam1	Intercellular adhesion molecule	NM_010493.2	0.51

The 1.5-fold differentially expressed genes were mapped to their relevant pathways using GenPlex v2.4 software (ISTECH Inc., Korea).The pathway resources are provided by the KEGG database.

underestimate the fold changes in gene expression when compared to techniques that are relatively more sensitive, such as real-time PCR (Kane et al., 2000).

4. Discussion

In previous study, we showed that BV significantly inhibited the secretion of inflammatory cytokines that occurred when RAW 264.7 cells were stimulated with LPS (Jang et al., 2005). Subsequently, we performed a microarray assay to elucidate the molecular mechanism by which BV exerts its effect. The results of the microarray analysis showed that 282 genes were differentially expressed by RAW 264.7 cells following 30 min of BV treatment, and that of these cells, MAP3K8, MYD116, TNF, TNFAIP3, SOCS3, TRAF1, JUN, NFKBIA, TNF-α-inducible protein 3 (TNFAIP3), and CBP were known to regulate inflammation.

TNF and JUN were up-regulated following LPS stimulation, however, treatment with BV suppressed their expression. TNF is a multifunctional proinflammatory cytokine that is predominantly secreted by monocytes and macrophages and has effects on lipid metabolism, coagulation, insulin resistance, and endothelial function (Shirai et al., 1985), whereas JUN is a component of activator protein 1 (AP-1). The induction of AP-1 by pro-inflammatory cytokines and genotoxic stress is primarily mediated by the JNK and p38 MAPK cascades (Chang and Karin, 2001). Once activated, the JNKs translocate to the nucleus, where they phosphorylate c-Jun, thereby enhancing its transcriptional activity (Karin, 1995).

MAP3K8 was up-regulated following LPS stimulation, however, BV treatment suppressed its expression. MAP3K8 is known as tumor progression locus2 (TPL2) or cancer Osaka thyroid (COT). Dumitru et al. reported that TNF-alpha induction by LPS was regulated post-transcriptionally via a Tpl2/ERK-dependent pathway, and they showed that Tpl2 knockout mice produced low levels of TNF-alpha when exposed to lipopolysaccharide. However, they also found that LPS stimulation of peritoneal macrophages obtained from these mice did not activate MEK1, ERK1, or ERK2 but that they did activate JNK, p38 MAPK, and NF-kappaB (Dumitru et al., 2000).

Myd116, which is rapidly induced by IL-6 in M1 myeloblastic leukemia cells, is expressed in bone marrow cells. Blocking MyD116 expression with antisense oligos promotes hematopoietic cell survival, suggesting that MyD116 plays a major role in terminal differentiation associated apoptosis (Liebermann and Hoffman, 2003). In this study, MYD116 was increased 32 fold by LPS treatment for 30 min when compared to the control, however, its expression was decreased by BV treatment.

SOCS3 is induced by LPS or CpG-DNA stimulation in macrophages (Bode et al., 1999). It was recently reported that SOCS3 was significantly increased in peripheral blood mononuclear cells obtained from rheumatoid arthritis patients when compared with healthy volunteers, and that these differences occurred primarily as a result of up-regulation of SOCS3 in peripheral blood monocytes. In addition, it was reported that a significant portion of macrophages in the synovial tissues expressed SOCS3 protein (Isomaki et al., 2007). In this study, SOCS3 expression was highly increased by LPS treatment, but decreased by BV treatment.

CBP is involved in regulation of the NF-κB and of the AP-1 pathways (Zhong et al., 1998). Phosphorylation of p65 of the NF-κB heterodimer or of c-Jun, a component of the AP-1 complex, leads to interaction with CBP. This general coactivator protein bridges DNA-bound transcription factors to the basal transcription machinery, thereby enhancing the expression of target genes involved in the inflammatory response (Matt, 2002). In this study, CBP expression was increased 3.07-fold by LPS treatment, but decreased 0.35-fold by BV treatment.

TRAF1 is a strongly anti-apoptotic protein involved in signaling by the TNF-α receptor superfamily members (Wang et al., 1998;

Table 8
Signaling pathway of the genes that exhibited reversed expression after BV treatment for 1 h.

Pathway	Symbol	Gene name	Accession No	Fold change
Toll-like receptor signaling	Ifnb1	Interferon beta 1	NM_010510.1	0.57
	Pik3r2	Phosphatidylinositol 3-kinase, regulatory subunit, polypeptide 2	NM_008841.1	0.61
	Ccl5	Chemokine (C-C motif) ligand 5	NM_013653.1	0.63
Jak-STAT signaling	Il13ra2	Interleukin 13 receptor, alpha 2	NM_008356.1	0.64
Cytokinecytokine receptor interaction	Cxcl16	Chemokine (C-X-C motif) ligand 16	NM_023158	0.63
	Il17r	Interleukin 17 receptor	NM_008359	0.65

The 1.5-fold differentially expressed genes were mapped to their relevant pathways using GenPlex v2.4 software (ISTECH Inc., Korea).The pathway resources are provided by the KEGG database.

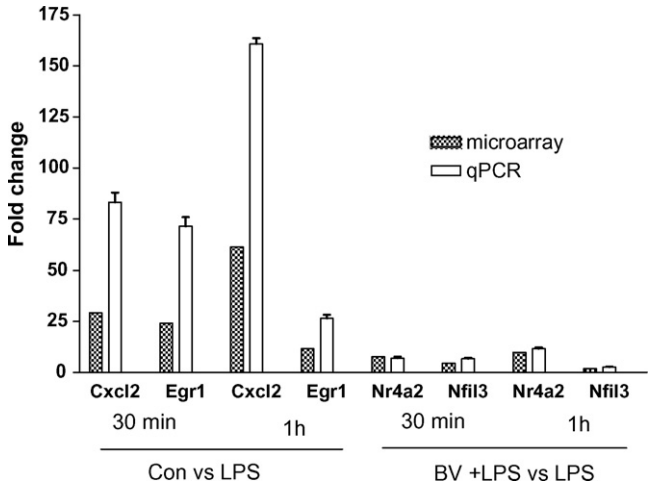

Fig. 3. Real-time PCR (qPCR) analysis of control (Con), LPS and BV plus LPS treated cDNA confirms gene transcript up-regulation for each time point. Transcripts for selected genes were assayed by Real-time PCR and expressed as the fold-increase relative to normalized GAPDH. Each bar represents the mean of three replicates ± S.E.M.

Durkop et al., 2003). TRAF1, when in heterotrimeric complexes with TRAF2, has been shown to interact with the cytosolic domains of CD40, CD30, TNF receptor-1 (TNFR1), and TNF receptor-2 (TNFR2) (Wang et al., 1998; Durkop et al., 2003). The outcome of these interactions, which includes enhanced gene expression, proliferation, or apoptosis, is in large part dependent upon the ratio of TRAF1 and TRAF2 in the complexes, with excess TRAF1 tending to promote cell survival. Lymphocytes, macrophages, and dendritic cells are known to produce TRAF1 (Bradley and Pober, 2001), and in this study, TRAF1 was also highly up-regulated by LPS, but down-regulated by BV treatment.

Tnfaip3 (A20) is a cytoplasmic protein required for the termination of Toll-like receptor-induced activity of the transcription factor NF-κB and proinflammatory gene expression in macrophages, and this function was shown to protect mice from endotoxic shock (Boone et al., 2004).

NF-κB is sequestered in the cytoplasm by IκB-α (NFKBIA gene product), however, LPS treatment results in the degradation of IκB-α via ubiquitination in an IκB kinase (IKK)-dependent manner (Pando and Verma, 2000; Ghosh and Karin, 2002). The removal of IκB-α allows the nuclear translocation of NF-κB, which subsequently leads to the transcription of downstream target genes, including IκB-α itself (Brown et al., 1993).

In our results, TNFAIP3 (A20) and NFKBIA were up-regulated by LPS, but down-regulated by BV treatment. These results were similar to those of Mookherjee et al. (2006), who reported that endogenous human host defense peptide caused inhibition of LPS-triggered pro-inflammatory genes but did not inhibit the LPS-induced expression of some of the known negative regulators of NF-κB, such as TNFAIP3 and NFKBIA (IκBα). A balanced response to signals of injury requires a transient cellular activation of a panel of genes together with inhibitory systems that temper the overwhelming inflammation. In this context, the activation of genes such as TNFAIP3 and NFKBIA appears to counter-balance the inflammatory response to LPS activation by limiting prolonged activation and increased cytokine production. We propose that BV plays a role in the delicate balancing of inflammatory responses in homeostasis, as well as in combating inflammation.

In conclusion, our results showed that BV decreased the expression of various genes related to the inflammatory effects which occur in LPS stimulated RAW 264.7 cells. In addition, we confirmed these microarray data using real time PCR. Previous studies have demonstrated that BV can induce an inflammatory response in several assay systems (Lariviere and Melzack, 1996; Cui et al., 2008). To examine whether repeat treatment with BV and then LPS can induce desensitization in certain signal pathways, we conducted microarray assay in BV only treated cells compared with un-treated cells (Table 2). Although BV could up-regulate inflammatory gene such as Nr4a2, Osm, IL1b, CXCL2 and CCL7, these genes were not same as the reversed expression genes by BV plus LPS (Tables 5 and 6), suggesting that tolerance effects by repeated stimulation by LPS and BV were insignificant. Taken together, these results indicate that BV may have anti-inflammatory effects in inflammatory diseases that are mediated by macrophages, such as rheumatoid arthritis and septic shock, and that these effects may occur as a result of the decreased expression of MAP3K8, MYD116, TNF, SOCS3, TRAF1, JUN, TNFAIP3, and CBP. However, the precise mechanism by which the anti-inflammatory effects occur, as well as the major bioactive component remains to be elucidated.

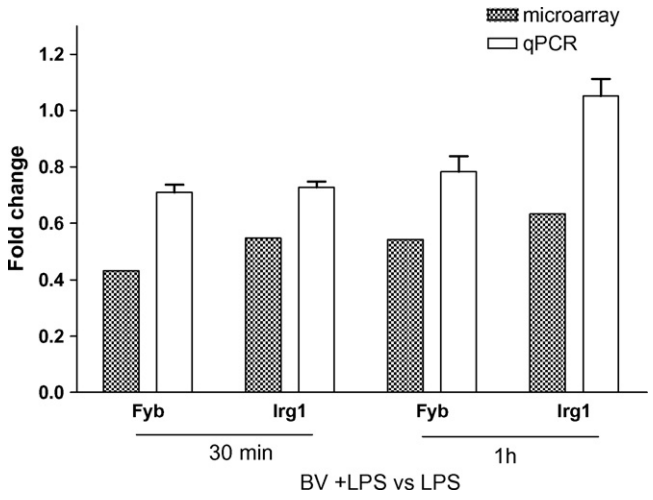

Fig. 4. Real-time PCR (qPCR) analysis of control (Con), LPS and BV plus LPS treated cDNA confirms gene transcript down-regulation for each time point. Transcripts for selected genes were assayed by Real-time PCR and are expressed as the fold-increase relative to normalized GAPDH. Each bar represents the mean of three replicates ± S.E.M.

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