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Nitric Oxide Dependency in Inflammatory Response-related Gene Transcripts Expressed in Lipopolysaccharide-treated RAW 264.7 Cells

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Accepted 23 November 2009

Abstract

Cytotoxic Nitric oxide (NO) overproduced by inducible NO Synthase (iNOS or NOS2), which was induced in inflammatory reactions and immune responses directly or indirectly affects the functions as host defense and can cause normal tissue damage. Microarray analysis was performed to identify gene profiles of both NO-dependent and -independent transcripts in RAW 264.7 macrophages that use selective NOS2 inhibitors aminoguanidine (100 μM) and L-canavanine (1 mM). A total of 3,297 genes were identified that were up- or down-regulated significantly over 2-fold in lipopolysaccharide (LPS)-treated macrophages. NO-dependency was determined in the expressed total gene profiles and also within inflammatory conditions-related functional categories. Out of all the gene profiles, 1711 genes affected NO-dependently and -independently in 567 genes. In the categories of inflammatory conditions, transcripts of 16 genes (Pomp, C8a, Ifih1, Irak1, Txnrd1, Ptafr, Scube1, Cd8a, Gpx4, Ltb, Fasl, Igk-V21-9, Vac14, Mbl1, C1r and Tlr6) and 29 geneas (IL-1beta, Mpa2l, IFN activated genes and Chemokine ligands) affected NO-dependently and -independently, respectively. This NO dependency can be applied to inflammatory reaction-related functional classifications, such as cell migration, chemotaxis, cytokine, Jak/STAT signaling pathway, and MAPK signaling pathway. Our results suggest that LPS-induced gene transcripts in inflammation or infection can be classified into physiological and toxic effects by their dependency on the NOS2-mediated NO release.

Keywords: Microarray, Nitric oxide, Lipopolysaccharide, Inflammation, NO-dependency, Macrophage

Cytotoxic nitric oxide (NO) is a reactive free radical modulator molecule that is overproduced from macrophage and other immune cells in several immune and inflammatory responses that have two major functions, such as host defense and immunomodulator[1]. The NO released during inflammatory and immune reactions is generally mediated by induction of an inducible NO Synthase isoform (iNOS or NOS2), and several cells, including macrophages[2-4]. Macrophages release inflammatory and cytotoxic cytokines after stimulation by agents, such as lipopolysaccharide (LPS). Generally, proinflammatory cytokines, like tumor necrosis factor-alpha (TNFα), interferon-gamma (IFNγ), interleukine-1beta (IL-1β), and IL-2 and a lipid mediator plate-activating factor (PAF) can trigger the gene expression of NOS2 in tissue macrophages[5,6]. Although the roles of cytotoxic NO in inflammatory and immune reactions have been studied previously, the use of it is controversial and uncertain because of the pro- or anti-inflammatory properties of NO and pro- or anti-apoptotic, as demonstrated in NOS2-deficient or selective inhibitors-treated cells and animal models[7-9].

In vivo endotoxic shock induced with bacterial LPS or *in vitro* LPS-treated macrophages are well known inflammatory conditions that can identify the overproduced NO-mediated mechanisms and their control. In these models, LPS treatment increases the production of autocrines like prostaglandin E2 and prostacycline PGI2, endothelin-1 as well as NO. The production of the autocrines is inhibited by pre-treatment of selective inhibitors of NOS2, aminoguanidine, and L-canavanine[10-12]. In addition to inducting NO release from inflammatory cells, the direct effect of several NO donors on the expression and stabilization of inflammation-related gene transcripts have been evaluated for NO-dependent activation or inhibition, as described in studies on an inflammatory transcription factor

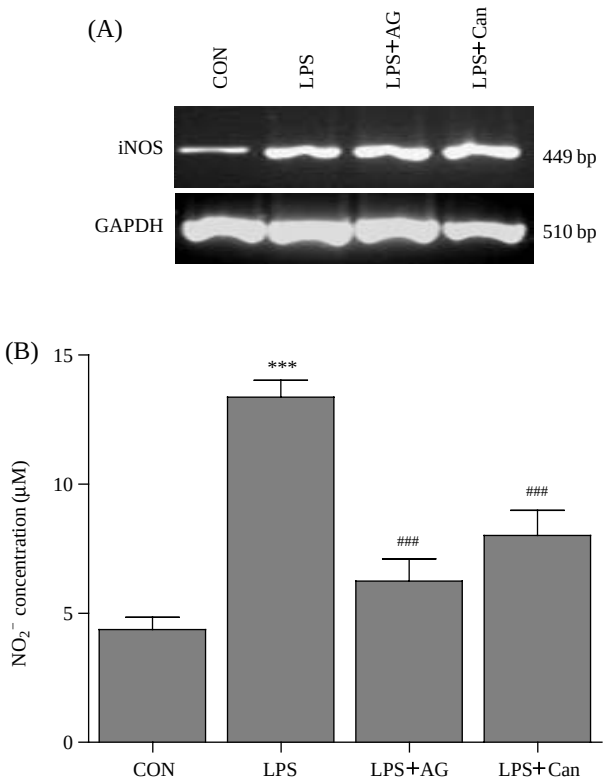

Figure 1. Changes in the expression level of NOS2 mRNA (A) and the concentration of NO metabolite nitrite (NO_2^-) (B). The levels of NOS2 mRNA expression determined by RT-PCR was increased by LPS treatment, but the increased levels were unchanged by pretreatment of NOS2-selective inhibitors aminoguanidine (AG) and L-canavanine (Can) in RAW 264.7 macrophages. The levels of nitrite concentration measured by Griess reaction method increased significantly compared to the untreated control (CON) ($P < 0.001$), but the increase was significantly prevented by pretreatment of AG ($P < 0.001$) or Can ($P < 0.001$).

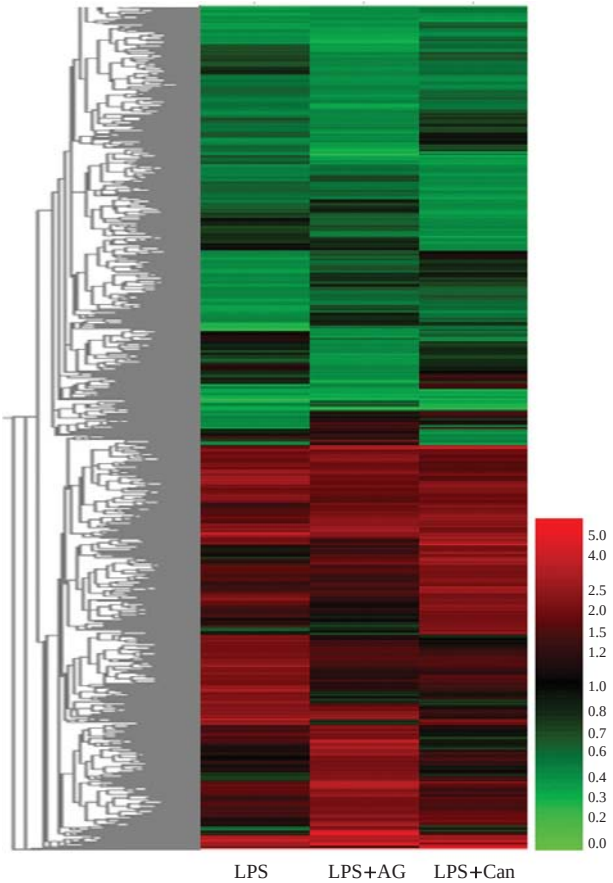

Figure 2. Hierarchical cluster image showing the total gene expression profiles in RAW 264.7 macrophages treated with LPS, LPS+AG and LPS+Can. Red and green colors represent up-regulation and down-regulation of the expression level of gene transcripts.

hypoxia-inducible factor-1 (HIF-1)[13-16]. However, there are several differences between the expressed genes and the effects of the treatment of NO donors and stimulation of *in vivo* NOS2-mediated NO release[17-19].

Thus, in the present study, we performed gene transcripts microarray analysis to determine the NO-dependency on the total and inflammation-related gene profiles in LPS-stimulated RAW 264.7 macrophages. These profiles were also compared with microarray data based on the crosstalk between NO and hypoxia that was published recently[20].

NOS2 mRNA Expression Confirmed by RT-PCR

As shown in Figure 1A, NOS2 mRNA expression was induced in RAW 264.7 cells treated with LPS for 6 h. Selective inhibition of NOS2 with aminoguanidine (AG) or L-canavanine (Can) had no effect on the increased NOS2 mRNA levels. This indicates that the control of NOS2-mediated NO production by using competitive substrate inhibitors was not related to elevated NOS2 mRNA levels, that is NO-independent expression of NOS2 mRNA.

Determination of Nitric Oxide Levels for Microarray

Using the general lipopolysaccharide (LPS) treatment to induce an inducible nitric oxide synthase (*iNOS* or *NOS2*) gene, a typical NO metabolite nitrite concentration was determined in culture media. In particular, LPS was treated for 6 hr because of the maximum level of the NOS2 mRNA expression (data not shown). As shown in Figure 1B, LPS treatment resulted in a significant increase in nitrite concentration (13.36±

Table 1. Representative list for the numbers of 2-fold changed gene transcripts.

	Significant over	Significant down	Total
LPS vs. control	1581	1716	3297
LPS+AG vs. control	1585	1587	3172
LPS+Can vs. control	1755	2096	3851

Table 2. Functional categories for gene transcripts 2-fold up- or down-regulated in microarray analysis.

Categories	LPS vs. control	LPS+AG vs. control	LPS+Can vs. control
Proliferation	64	73	76
Translation	97	115	125
Inflammatory response	28	27	33
Immune response	119	103	141
Cell adhesion	83	79	89
Lipid metabolism	85	71	93
Cell differentiation	150	140	176
Apoptosis	101	92	122
Cell growth	22	28	40
Transport	405	370	485
Cell cycle	101	115	132
Transcription	330	333	397
Signal transduction	437	431	538
Homeostasis	46	42	68
Response to stress	120	123	158
Total	3297	3172	3851

The numbers indicate the gene transcripts changed by the ratio above 2.0 or below 0.5 compared with untreated control cells.

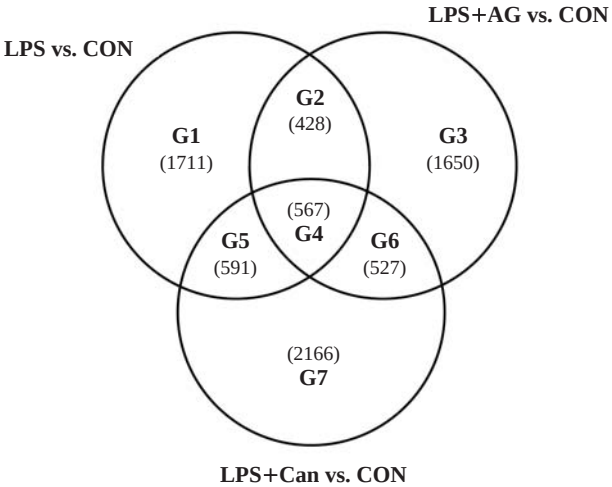

Figure 3. Venn diagram of gene transcripts divided by grouping. The up-regulated and down-regulated genes filtered from three groups (LPS, LPS+AG and LPS+Can) were compared to each other as well as those from untreated control group. The total number of genes (parentheses) represented in each pure group (G1-G7) indicates the overlapping and pure genes compared among seven groups NO. Particularly, the genes (1711) in group 1 (G1) are induced by LPS, but the induction is completely blocked by pretreatment of AG or Can, thus indicates NO-dependent expression pattern. In contrast, the genes in group 4 (G4) indicates NO-independent expression, which were changed by over 2-fold in all three groups.

0.66 μM compared to control level 4.37 ± 0.47 μM). The increase was inhibited by pretreatment of 100 μM AG (6.24 ± 0.86 μM) or 1 mM Can (8.01 ± 0.98 μM).

Total Gene Expression Profiles

A hierarchical analysis revealed that transcriptional alterations of 7,622 genes were assessed by using a Roche NimbleGen mouse whole genome 12-plex array and an Axon GenePix 4000B scanner with associated software in the RAW 264.7 cells treated LPS, LPS+AG or LPS+Can (Figure 2). In 3297 gene transcripts that changed by more than 2-fold in the LPS-treated cells, 1581 gene transcripts, including immune response-related genes, such as Tnf, Il1b, Ntrk1, Cd8a, Tgfbr2, C8a, Il6 and Ccl2-5 genes were upregulated while 1716, including RNA metabolism-related genes, such as Dars2, Pnpt1, Lcmt2, Prmt2, Fars2, Ints7, Adarb1, Mbnl1, Nol3, Sf4, Txnl4, Sart1, Creg2 were down-regulated (Table 1). In addition, 3172 (1585 up-regulated and 1587 downregulated) and 3851 (1755 upregulated and 2096 downregulated) gene transcripts were changed by more than 2-fold in LPS+AG- and LPS+Can-treated groups, respectively (Table 1). These genes were classified to functional properties as shown in Table 2.

NO-dependent and -independent Gene Expression Profiles in Total Gene Transcripts

The Venn diagram in Figure 3 shows the number of the regulated total gene transcripts of all groups (Groups 1-7). As shown in the Venn diagram of Figure 3, the NO released from the LPS-treated macrophage cells had 1711 up- or downregulated genes (833 upregulated and 878 downregulated gene transcripts) by more than 2-fold. The expression levels were partially or completely prevented by the pretreatment of both selective inhibitors AG (100 μM) and Can (1 mM) of the *NOS2* gene. This result shows the NO-dependent gene expression profile (Group 1). However, LPS-induced regulation of the other 567 genes (266 up-regulated and 301 down-regulated gene transcripts) that increased by more than 2-fold was not affected by the AG or Can that was pretreated to control *NOS2* gene-mediated NO release (Group 4). This gene expression profile indicates NO-independent gene expression.

Table 3. NO-dependent gene expression profiles in inflammatory reaction-, immune response- and response to stress-related genes.

GenBank ID	Gene title	Symbol	Fold change		
			LPS	LPS+AG	LPS+Can
XM_893473	Phosphatudylinositol-specific phospholipase C	Plcxd2	4.01	1.30	1.07
AK165073	Heat shock protein 12A	Hspa12a	3.60	1.59	1.13
NM_025624	proteasome maturation protein	Pomp	3.39	1.44	1.85
AB077295	complement component 8, alpha polypeptide	C8a	3.01	0.77	1.00
AK168646	interferon induced with helicase C domain 1	Ifih1	2.93	1.62	0.84
AY184362	interleukin-1 receptor-associated kinase 1	Irak1	2.58	1.04	1.46
BC037643	thioredoxin reductase 1	Txnrd1	2.25	1.29	1.44
XM_357441	platelet-activating factor receptor	Ptafr	2.40	0.79	0.62
BC066066	signal peptide, CUB domain, EGF-like 1	Scube1	2.22	1.19	1.23
AK088128	CD8 antigen, alpha chain	Cd8a	2.09	1.10	1.29
BC106147	glutathione peroxidase 4	Gpx4	2.06	1.43	1.18
NM_008518	lymphotoxin B	Ltb	2.04	1.20	1.11
AF288572	Fas ligand (TNF superfamily, member 6)	Fasl	0.49	1.42	0.83
XM_993285	immunoglobulin kappa chain variable 21 (V21)-9	Igk-V21-9	0.46	1.05	0.77
NM_146216	Vac14 homolog (S. cerevisiae)	Vac14	0.45	0.84	0.96
NM_010775	mannose binding lectin (A)	Mbl1	0.43	0.93	0.74
AF459018	complement component 1, r subcomponent	C1r	0.39	0.86	0.72
NM_011604	toll-like receptor 6	Tlr6	0.39	0.71	0.67
NM_021501	protein inhibitor of activated STAT4	Pias4	0.21	1.00	0.86

Table 4. NO-independent gene expression profiles in inflammatory reaction-, immune response- and response to stress-related genes.

GenBank ID	Gene title	Symbol	Fold change		
			LPS	LPS+AG	LPS+Can
BC108956	chemokine (C-X-C motif) ligand 2	Cxcl2	117.62	108.49	88.56
BC024930	chemokine (C-C motif) ligand 2	Ccl2	25.01	21.75	23.86
BC055070	chemokine (C-C motif) ligand 4	Ccl4	11.57	10.32	9.15
BC099532	C-type lectin domain family 4, member e	Clec4e	10.36	12.85	9.44
BC033508	chemokine (C-C motif) ligand 3	Ccl3	5.93	5.90	5.58
NM_008361	Fc receptor, IgG, low affinity IIb	Fcgr2b	5.16	7.04	5.84
NM_011940	interferon activated gene 204	Ifi204	4.10	5.80	5.51
NM_009263	guanylate nucleotide binding protein 1	Gbp1	4.07	5.46	4.54
NM_010259	interferon activated gene 202B	Ifi202b	3.55	2.46	6.74
NM_019948	interleukin 1 beta	Il1b	3.43	2.59	3.31
NM_011337	interferon activated gene 203	Ifi203	3.37	2.41	3.41
NM_011198	prostaglandin-endoperoxide synthase 2	Ptgs2	3.35	2.41	3.54
BC057858	secreted phosphoprotein 1	Spp1	3.36	2.99	4.09
NM_033622	leukocyte immunoglobulin-like receptor, subfamily B, member 4	Lilrb4	3.28	2.72	2.97
BC100398	Mitogen activated protein kinase 7	Mapk7	3.20	2.96	2.86
XM_001001561	macrophage activation 2 like	Mpa2l	3.22	3.38	4.89
NM_013652	interferon-induced protein 35	Ifi35	2.88	3.02	2.04
BC002113	2′-5′ oligoadenylate synthetase 1E	Oas1e	2.71	2.04	3.33
BC010548	superoxide dismutase 2, mitochondria	Sod2	2.40	2.75	2.41
BC002113	secreted phosphoprotein 1	Spp1	2.66	2.48	3.02
BC057969	chemokine (C-C motif) ligand 5	Ccl5	2.39	2.58	2.11
NM_010187	histocompatibility 28	H28	2.37	2.15	2.79
NM_008329	predicted gene, EG630499	EG630499	2.24	2.34	2.83
BC033508	2′-5′ oligoadenylate synthetase-like 2	Oasl2	2.16	2.67	2.94
AB093243	zinc finger protein 292	Zfp292	0.46	0.46	0.40
NM_009263	secreted phosphoprotein 1	Spp1	0.45	0.46	0.47
NM_011337	tumor necrosis factor (ligand) superfamily, member 13b	Tnfsf13b	0.45	0.41	0.39
NM_013652	tumor necrosis factor (ligand) superfamily, member 15	Tnfsf15	0.39	0.49	0.41
NM_027320	linker for activation of T cells family, member 2	Lat2	0.33	0.45	0.49

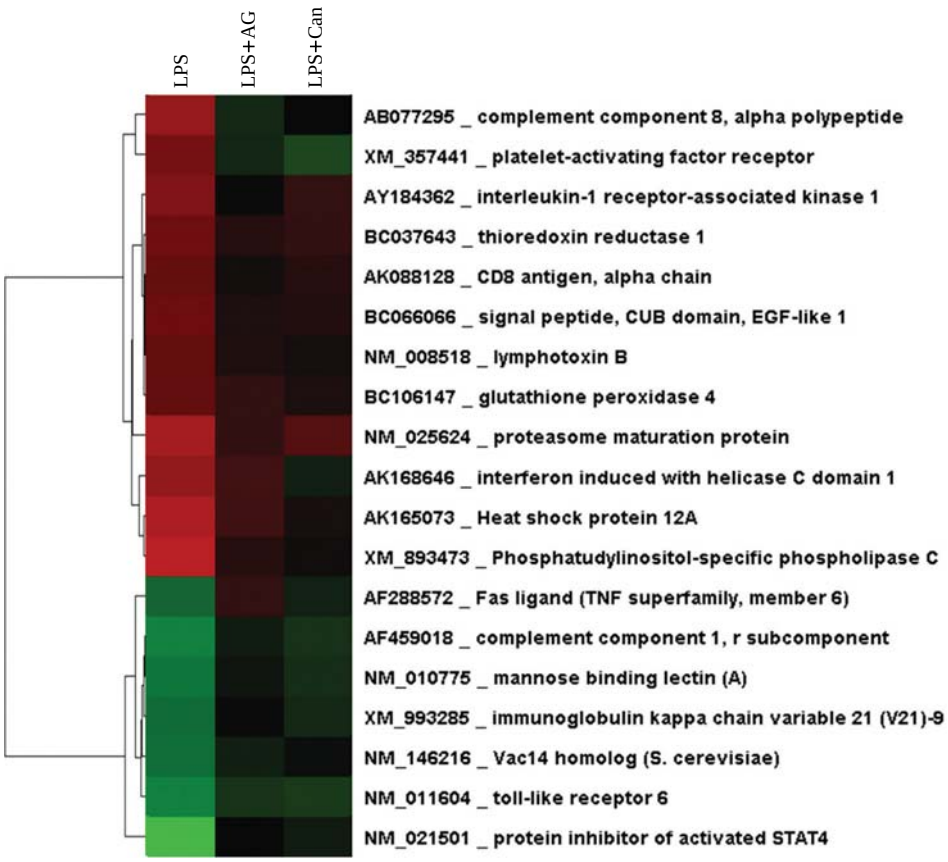

Figure 4. Clustergram of the NO-dependently expressed genes within inflammatory and immune responses-related gene profiles in RAW 264.7 macrophages treated with LPS.

NO-dependent and -independent Gene Expression Profiles of Inflammatory Reaction-, Immune Response- and Response to Stress-related Gene Transcripts

NO-dependently regulated 16 genes were involved in three related functional categories of inflammatory response-, immune response- and response to stress, while 29 genes were regulated NO-independently (Tables 3 and 4, Figures 4 and 5). In particular, up-regulated gene transcripts, like C8a (Complement component 8, alpha polypeptide), Ptafr (Platelet-activating factor receptor), Scube1 (Signal peptide, CUB domain, EGF-like 1), Cd8a (CD8 antigen, alpha chain) and Ltb (lymphotoxin B) were completely NO-dependent. The NO-independent gene expression profiles that were also in these categories are listed in Table 4. In particular, the gene transcripts of chemokine (C-C motif), ligands (Ccl2-5), and Cxcl2 were highly up-regulated and NO-independent (Table 4).

Principal Component Analysis (PCA)

PCA classification was performed to represent the differences between inflammatory or cytotoxic NO-mediated gene expression profiles with candidate transcripts (45 and 238 genes, respectively) selected from the inflammatory reaction- and immune response-related gene expression profiles (Figure 6).

Discussion

NO is a potent mediator that is involved in many biological functions, including macrophage cytotoxicity and non-specific immunity against parasites, bacteria, and viruses. The present study demonstrates that NO-dependent and -independent gene expression profiles can be characterized by using two selective inhibitors of iNOS aminoguanidine (AG) and L-canavanine (Can) in mouse macrophages (RAW 264.7) treated with LPS for 6 h. In particular, the study focused on the trigger point of gene expression early after LPS treatment. Thus, all of the RNAs were isolated at a maximum level of NOS2 mRNA expression (6 h). Although bacterial LPS have been known to be a potent inducer of *NOS2* gene *in vivo* and in macrophages *in vitro*, the roles of NO in the inflammatory responses to LPS have been controversial and poorly understood.

Generally, pure NO dependency should be evaluated at the early time of inflammation because the highly produced NO in a culture medium or serum by long-

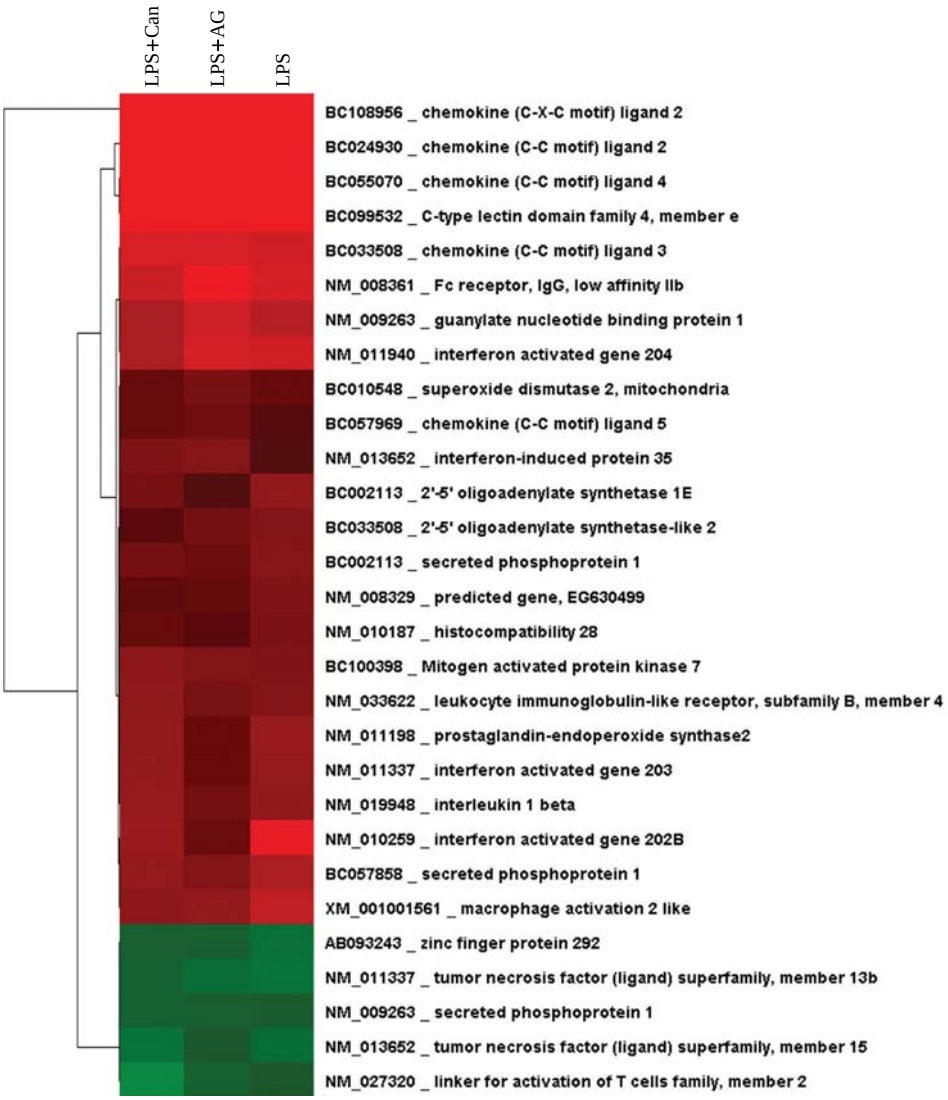

Figure 5. Clustergram of the NO-independently expressed genes within inflammatory and immune responses-related gene profiles in RAW 264.7 macrophages treated with LPS.

term treatment or chronic infection can react with superoxide anion (O_2^-) to generate a highly cytotoxic oxidant peroxynitrite ($ONOO^-$)[21,22]. Therefore, a low or moderate dose of NO early on is sufficient to cause a mild NO-mediated modulation of gene expression and toxicity during the period of LPS exposure. Its effect is completely controlled by the inhibition of NO production with AG or Can, as shown in Figure 1B. In addition, macrophages activated by inflammatory stimuli, such as LPS and Interferon-gamma (IFN-γ), release proinflammatory cytokines and chemokines, and can generate NO via expression of NOS2[23,24]. In particular, this NO affects directly or indirectly the expression of several gene transcripts, and the effects can be characterized as NO-dependency, as discussed in this study. In the current study, the common gene transcripts that were analyzed were classified as inflammatory reaction-, immune response-, and response to stress-related functional categories.

The phosphatidylinositol-specific phospholipase C (Plcxd2) gene transcript was up-regulated NO-dependently in this study. However, conversely, Plcxd2 gene expression is required for LPS-induced NO production in macrophages[25]. If the Plcxd2 gene expression depends on the inflammatory NO release, it is possible for there to be a reciprocal interaction between Plcxd2 and NOS2-mediated NO production. In addition, the platelet-activating factor receptor (Ptafr) gene transcript was also up-regulated NO-dependently, as shown in Table 3. Generally, the platelet-activating factor (PAF) induces NO overproduction from *Trypanosoma cruzi*-infected macropahges[26]. LPS-induced NOS2 expression and NO release also are suppressed by pretreating the PAF receptor antagonist *in vivo*, as well as *in*

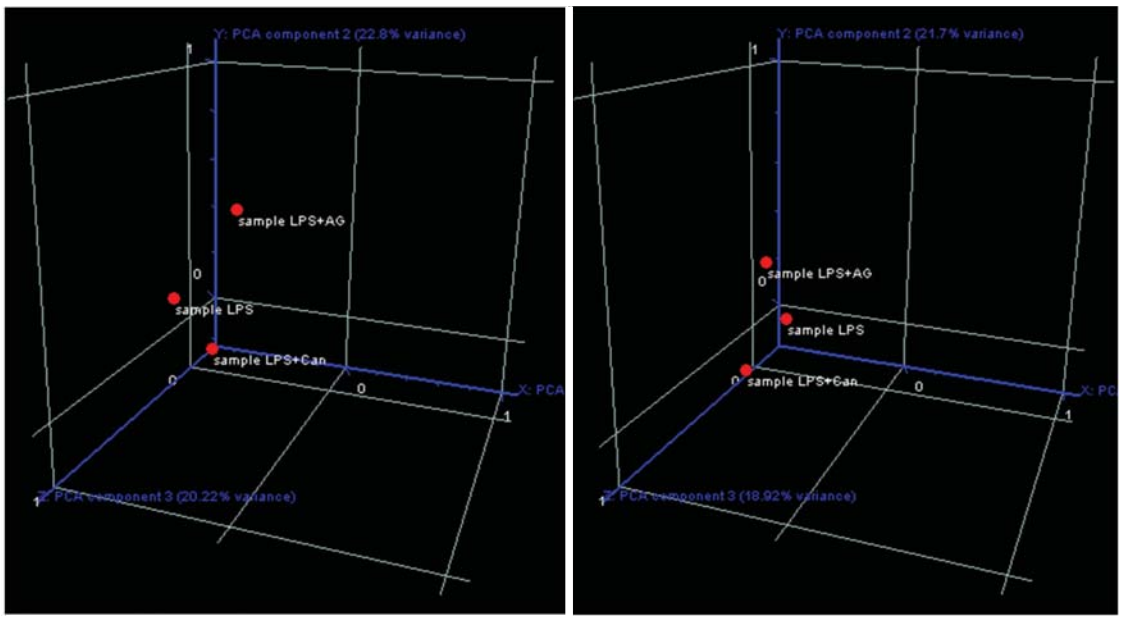

Figure 6. Principle component analysis (PCA) for immune responses- (A) and inflammatory response- (B) related gene expression profiles modified by over 2-fold.

vitro Kupffer cells[27]. Therefore, reciprocal interaction between Ptafr and *NOS2* gene expression can be suggested from our study.

Proteasome maturation protein (Pomp, a homolog of the yeast proteasome maturation factor Ump1) is known as an essential factor of mammalian proteasome biogenesis, which interacts directly with the ER membrane and can be increased by several stresses[28,29]. The proteasome is the major cellular protease that is responsible for the degradation of normal short-lived or oxidized proteins. In this study, the Pomp gene was upgraded by 3.4-folds, but its increase was inhibited by the blocking of inflammatory NO, such as the NO-dependent expressed genes (Table 3). The Ump1-over-expressed cells were observed to be highly enhanced proteasome-mediated protein degradation in responses to oxidative stress[30]. Thus, proteasome activity that can remove the oxidized or damaged proteins can be increased, especially in LPS-induced inflammatory conditions, and can thus be evaluated as a beneficial effect of NO. Furthermore, NO-dependent upgraded heat shock protein (Hspa12a), thioredoxin reductase 1 (Txnrd1), and glutathione peroxidase 4 (Gpx4) gene transcripts have been implicated in anti-inflammatory responses as antioxidant enzymes. Interestingly, these antioxidant enzymes are induced NO-dependently. They neutralize the concomitantly released reactive oxygen species (ROS). However, superoxide dismutase 2 (SOD2), a typical antioxidant enzyme that scavenges superoxide, was induced NO-independently, as shown in Table 4. The key point of our study for Pomp and several antioxidant enzymes, except for SOD2, is whether there is a NO-dependent induction pathway in LPS-treated macrophages or in the early time of innate immunity.

Recently, inflammatory reactions-, immunity-, and apoptosis-related gene expression profiles have been reported in macrophages activated by LPS treatment, advanced glycation endproducts (AGEs), and infections of *Francisella tularensis* LVS or *Streptococcus pyogenes*[31-35]. These studies demonstrated that pro-inflammatory cytokines and chemokines, like TNF-α, IL-1β, MCP-1 and Ccl2-5, were upregulated and downregulated in gene transcripts, such as Hps6, LstI, and Fxyd5, but little was known regarding the gene expression patterns for NO-dependency. According to our results, several chemokines that contain monocyte chemotactic protein 1 [MCP-1, Ccl2 or chemokine (C-C motif) ligand 2] were highly and NO-independently upregulated to 2.39-117.62-folds by LPS-treatment. In contrast, the treatment of NO donors was upregulated Ccl3 (MCP1α), but downregulated by hypoxia[35]. In addition, Bosco *et al*. reported that chemokine (C-C motif) ligands were downregulated by hypoxia[36]. Their results have important differences when they were compared with our NO-independent expression profile for the listed five chemokine (C-C motif) ligands 2-5 and chemokine (C-X-C) motif) ligand 2. The NO donor-direct treatment could not be evaluated as a NO-dependent pattern because NO di-

rectly, or indirectly, affects several signaling pathways. Furthermore, the gene transcripts [interleukin 1 beta (IL-1β), interferon activated genes (Ifi35, Ifi202B, Ifi203 and Ifi204), and macrophage activation 2 like (Mpa2l)] were also expressed highly in inflammatory conditions and upregulated NO-independently. Therefore, inflammatory activities for chemokines, ligands, and receptors, as well as interleukines and interferons, should be controlled independently with overproduced NO, which has been shown in many inflammatory diseases, such as active inflammatory bowel disease, psoriasis, and cancer[37-39]. We have compared the commonly expressed genes expression profiles between genes induced by NO-donors[20] and by controlling LPS-induced NO as this result. Of 46 genes up-regulated by NO-donors, expression for 10 gene transcripts (Sgpp1, Sfrs1, Adh7, Slc7a11, P2ry12, Slmap, Igf2bp1, Mpst, Prkcn and Ttk) were same with those of NO-dependently up-regulated genes in our total gene expression profiles.

As expected, prostaglandin-endoperoxide synthase 2 (Ptgs2, cyclooxygenase 2, or COX-2) was upregulated, which were implicated in the immediate early response gene products, like prostaglandin (PGs) and thromboxane (TXs) in inflammatory and immune cells. Unfortunately, however, the *NOS2* gene transcript co-induced with COX-2 in microarray was not detected, though it was clearly detectable in RT-PCR (Figure 1). This may have been caused by the quality of oligonucleotides in the microarray of the *NOS2* gene.

This NO dependency can be applied to inflammatory reaction-related functional classifications, like cell migration, chemotaxis, cytokine, Jak/STAT signaling pathway, and MAPK signaling pathway. These results suggest that LPS-induced gene transcripts in inflammation or infection can be classified to physiological (beneficial) and toxic (deleterious) effects by the inflammatory NO dependency.

Materials & Methods

RAW264.7 Cell Treatment and Preparation

The murine macrophage-like cell line RAW 264.7 cells (1×10^6 cells/cm^2) were grown in HEPES-Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with antibiotics (100 unit/mL penicillin and 100 μg/mL streptomycin) and 10% heat-inactivated fetal bovine serum (Invitrogen) at 37°C in an atmosphere of 5% CO_2. The washed macrophage cells were pre-treated with aminoguanidine (100 μM) or L-canavanine (1 mM) for 30 min as a selective or natural inhibitor of inducible nitric oxide synthase (iNOS or NOS2), respectively, and then incubated with 1 μg/mL lipopolysaccharide (LPS, Sigma) for 6 h.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

RT-PCR was carried out using the specific primers for the iNOS (sense: 5′-ATGTCCGAAGCAAACATCAC-3′; antisense: 5′-TAATGTCCAGGAAGTAGGTG-3′) and GAPDH (sense: 5′-TCGTGGAGTCTACTGGCGT-3′; antisense: 5′-GCCTGCTTCACCACCTTCT-3′) genes, as published by Kim *et al.*[40]. The PCRs were performed for 30 cycles with the following conditions: denaturation at 95°C for 1 min, annealing at 52°C for 1 min, and elongation at 65°C for 2 min.

Determination of NO Metabolite NO_2^- Concentration

The concentration of NO metabolite nitrite (NO_2^-) in the cell-culture media was determined by the Griess reaction[41] as a measure of iNOS activity. The culture medium (100 μL) was mixed with the same volume (100 μL) of Griess reagent (50 μL of 1% sulfanilamide prepared in 5% phosphoric acid and 50 μL 0.1% N-naphthylethylene-diamine prepared in milliQ water). The samples were then incubated at room temperature for 10 min and protected from light. Sodium nitrite (Sigma) was used as a standard. The optical absorbance was measured at 546 nm with a microplate reader (Power Wave 340, Bio-TEK Instruments, INC, Winooski, VT).

RNA Isolation, Preparation of Fluorescent DNA Probe and Hybridization

Total RNA was extracted from the RAW 264.7 macrophage cells by using the TRI REAGENT (MRC, OH) according to the manufacturer's instructions. Each total RNA sample (20 μg) was labeled with Cyanine-3 conjugated dCTP (Amersharm, Piscataway, NJ) by a reverse transcription reaction that uses reverse transcriptase, SuperScrip ll (Invitrogen, Carlsbad, California). The labeled cDNAs were then concentrated by using the ethanol precipitation method. The concentrated Cy3 labeled cDNAs were resuspended in 10 μL of the hybridization solution (GenoCheck, Korea). Afterwards, the labeled cDNAs were placed on Roche NimbleGen Mouse whole genome 12-plex array (Roche NimbleGen, Inc., WI) and covered by a NimbleGen X4 mixer (Roche NimbleGen, Inc., WI). The slides were hybridized for 12 hr at 42°C MAUI system (Biomicro systems, Inc. UT). The hybridized slides were washed in 2 X SSC, 0.1% SDS for 2 min, 1 X SSC for 3 min, and then 0.2 X SSC for 2 min at room temperature. The slides were centrifuged at 3,000 rpm for 20 sec to dry.

Microarray Data Analysis

The Roche NimbleGen mouse whole genome 12-plex array was submitted to Roche NimbleGen Inc. for microarray design and manufacture with maskless, digital micromirror technology. Twelve replicates of the genome were included per chip. An average of 3 different 60-base oligonucleotides (60-mer probes) represented each gene in the genome. 60-mer probes were selected so that each probe had at least three mismatches compared to all the other 60-mers in the target genome. A quality control check (hybridization) was performed for each array, which contained on-chip control oligonucleotides. The arrays were analyzed by using an Axon GenePix 4000B scanner with associated software (Molecular Devices Corp., Sunnyvale, CA). Gene expression levels were calculated with NimbeScan Version 2.4 (Roche NimbleGen, Inc., WI). Relative signal intensities for each gene were generated using the Robust Multi-Array Average algorithm. The data were processed based on the median polish normalization method that used the NimbeScan Version 2.4 (Roche NimbleGen, Inc., WI). This normalization method aimed to make the distribution of intensities for each array in a set of arrays the same. The normalized, and log transformed, intensity values were then analyzed using GeneSpring GX 10 (Agilent technologies, CA). The fold change filters included the requirement that the genes be present in at least 200% of controls for upregulated genes and fewer than 50% of controls for downregulated genes. Hierarchical clustering data were composed of clustered groups that behave similarly across experiments that use GeneSpring GX 10 (Agilent technologies, CA). The clustering algorithm was Euclidean distance, average linkage.

Statistical Analysis

Statistical analyses were performed by using GraphPad Prism 4 System Data were expressed as means ± S.D. Student's *t*-test was performed to determine the significant differences of nitrite concentrations between groups.

Acknowledgements

This study was supported by the sabbatical Leave Program of Anyang University.

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