Microarray Study of Genes Differentially Modulated in Response to Nitric Oxide in Macrophages

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Abstract: Nitric oxide (NO) has been known to play important roles in numerous physiologic processes including neurotransmission, vasorelaxation, and cellular apoptosis. Using a mouse cDNA gene chip, we examined expression patterns and time course of NO-dependent genes in mouse macrophage RAW264.7 cells. Genes shown to be up-regulated more than two fold or at least at two serial time points were further selected and validated by RT-PCR. Finally, 81 selected genes were classified by function as signaling, apoptosis, inflammation, transcription, translation, ionic homeostasis and metabolism. Among those, genes related with signaling, apoptosis and inflammation, such as guanylate cyclase 1, soluble, alpha3 (*Gucy1a3*); protein kinase C, alpha (*Pkcα*); lymphocyte protein tyrosine kinase (*Lck*); BCL2/adenovirus E1B 19 kDa-interacting protein (*Bnip3*); apoptotic protease activating factor 1 (*Apaf1*); X-linked inhibitor of apoptosis (*Xiap*); cyclin G1 (*Ccng1*); chemokine (C-C motif) ligand 4 (*Ccl4*); B cell translocation gene 2, anti-proliferative (*Btg2*); lysozyme 2 (*Lyz2*); secreted phosphoprotein 1 (*Spp1*); heme oxygenase (decycling) 1 (*Hmox1*); CD14 antigen (*Cd14*); and granulin (*Grn*) may play important roles in NO-dependent responses in murine macrophages.

Key words: nitric oxide; microarray; mouse macrophage RAW264.7 cells; apoptosis

Macrophages play an indispensable role in protecting host organisms from invading pathogens. They kill bacterial pathogens, clear dead cells and present antigens to other immune cells. When activated by pathogenic microorganisms, they produce various immune mediators, IL-1, IL-6, TNF-α, complement proteins, reactive oxygen species (ROS), and nitric oxide (NO) (MacMicking et al., 1997). NO is a diffusible gas endogenously produced by inducible NO synthase (iNOS). iNOS can be expressed at high levels when stimulated with bacterial components plus a T-cell derived cytokine, IFN-γ (Kim et al., 1997a). The released NO molecules have strong cytotoxic effects and are essential for killing bacteria; blocking the production of NO by disrupting NOS in mice leads to reduced resistance to bacterial infection (MacMicking et al., 1995). Although not all cellular systems are equally affected, diffusive NO is, in general, toxic not only to microbial pathogens but also to host cells, including macrophages themselves (Albina et al., 1993; Messmer et al., 1996). Henceforth, macrophages have been used as a model for NO-induced apoptosis.

Conversely, NO is an important molecule involved in various physiological processes and in many different organs, including the regulation of blood vessel dilatation, myocardial depression, neurotransmitter function, innate immune responses, and pathologic effects by inducing apoptosis as well as protecting cells against apoptosis (Toda et al., 1996; Balligand and Cannon, 1997; Kim et al., 1997b; Zhao et al., 1999; Bogdan et al., 2000; An et al., 2006). These effects of NO can originate from nitration or nitrosylation of tyrosine or cysteine residues of functionally critical proteins and its interaction with transition metals that regulate activities of enzymes, receptors, RNA-binding proteins and transcription factors (Bogdan, 2001). NO has been known to directly or indirectly activate various transcription factors including NF-κB, AP-1, Sp1, Egr-1, and HIF-1 (Kim et al., 1997a; Marshall et al., 2000).

In this study, we tried to identify NO-responsive genes in mouse macrophages using cDNA microarray. RAW264.7 macrophages were treated with SNAP (a nitric oxide donor) for various time periods. About 250-350 genes were shown to be up-regulated for each time point up to 15 hr after the treatment. Genes increased in more than two fold or at least two serial time points were further selected and validated by RT-PCR. Finally, the selected genes were

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functionally classified as apoptosis, inflammation, signaling, transcription and translation, ionic homeostasis and metabolism -related factors.

MATERIALS AND METHODS

Cell culture

RAW264.7 (mouse macrophage cell line) cells were obtained from American Type Culture Collection (Manassas, VA, USA). They were maintained at 37°C in a humidified atmosphere of 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (Hyclone, South Logan, UT, USA) and antibiotic-antimycotics (Gibco-BRL, Gaithersburg, MD, USA).

RNA isolation and treatment

RAW264.7 cells were plated at 1×10^7 cells/100 mm dish and grown for 12 h before the addition of the NO donor, S-nitroso-N-acetylpenicillamine (SNAP) (Sigma chemical, St. Louis, MO, USA). They were treated with 100 μM SNAP for 0.5, 1.5, 3, 6, 9, 12 and 15 h. Cells were harvested and total RNA was isolated using RNeasy Midi Kit (Qiagen, Hilden, Germany) for cDNA microarray and TRIzol® Reagent (Invitrogen, Carlsbad, CA, USA) for RT-PCR analysis, according to the manufacturers' instructions.

cDNA Microarray analysis

Mouse cDNA chips (25 K) were prepared at Genome Research Center, Korea Research Institute of Bioscience and Biotechnology (Daejeon, Korea). Microarray analysis was performed as previously described (Kim et al., 2007). Briefly, 20 μg of total RNA for each time point of SNAP treatment was reverse transcribed using Superscript Reverse transcriptase (Invitrogen). The cDNA samples were fluorescently labeled with Cy5 (tester) or Cy3 (driver), purified with a Microcon YM-30 (Millipore, USA) to exclude unincorporated dNTPs, and hybridized to the microarray chips using Genisphere cDNA Array kit (Genisphere, Hatfield, PA, USA). The hybridized chips were washed twice with 2X SSC/0.2% SDS and once with 95% ethanol at room temperature and scanned at 10 μm resolution on ScanArray 5000 scanner (Packard, Billerica, MA, USA). The microarray data were normalized using SAM package (http://www.r-project.org, http://braju.com/R/). The intensity of each hybridization signal was photometrically calculated using the GenePix Pro v4.0 program (Axon Instruments). The microarray clustering data were analyzed using the software "Cluster and Tree View" (http://rana.lbl.gov/EisenSoftware.htm). Functional annotation was performed by searching NCBI Database.

RT-PCR analysis

Total RNA was reverse transcribed using M-MLV Reverse Transcriptase (Promega, Madison, WI, USA). Semi-quantitative PCR was performed using primer pairs listed in Table 1. PCR cycling conditions were as follows: 19~28 cycles at 95°C for 30 s, 55~65°C for 30 s and 72°C for 1 min. The PCR products were resolved by electrophoresis

Table 1. Primers used for RT-PCR. Sense sequences are listed first that is followed by the antisense sequences

Gene	Primer sequences		PCRproduct(bp)
Adam8	5'-GCT TGG CCT CTG GCT GCT CAG -3'	5'-TCT TTG ACC CCA CAG GTC CCA G-3'	501
Apaf1	5'-CCG CAA TTG TTT GCT TCA ACA TAG -3'	5'-TGT ACA CCC CCT GAA AAG CAA CC-3'	501
Bnip3	5'-ATG TCG CAG AGC GGG GAG GAG -3'	5'-TCA GAA GGT GCT AGT GGA AGT TG-3'	564
Btg2	5'-ATG AGC CAC GGG AAG AGA ACC G-3'	5'-CTA GCT GGA GAC GGC CAT CAC -3'	477
Ccl4	5'-CAT ACC CCG AGC AAC ACC ATG -3'	5'-CAA TGG TGG ACC ATC TCC ATG -3'	521
Ccng1	5'-ATG ATA GAA GTA CTG ACA ACT GAC-3'	5'-GGT GTC GTG AAC GAG TGA ATA AT-3'	501
Ccrn4l	5'-GCT CGC CCC GCC GGT GCT CG-3'	5'-AAG TAG TGG TCC ACT TCC TGG A-3'	501
Cd14	5'-CGT GTG CTT GGC TTG TTG CTG T-3'	5'-CTT GAG TCC AGG CTT TAG CCA C -3'	501
Cdkn1a	5'-ATG TCC AAT CCT GGT GAT GTC CG-3'	5'-GGC GGG GCT CCC GTG GGC AC -3'	501
Higd1a	5'-GGG CCT GCT GGC TTC TAG ATT G-3'	5'-ATT GTA GAT ACA AAC ACG TAA CCT-3'	500
Hmox1	5'-TGG AGC GTC CAC AGC CCG ACA-3'	5'-TAA AAA AAG CCA GGC CCT CCC C-3'	501
Id2	5'-ATG AAA GCC TTC AGT CCG GTG A-3'	5'-TTA GCC ACA GAG TAC TTT GCT AT-3'	405
Igtp	5'-ATG GAT TTA GTC ACA AAG TTG CCA-3'	5'-TGT TTC TAA GCT CTG GGA AGT CG-3'	766
Lyz2	5'-CTC TCC TGA CTC TGG GAC TCC-3'	5'-TGC TCG AAT GCC TTG GGG ATC T-3'	371
Pmm1	5'-GCA TCC TCT GCC TGT TTG ACG-3'	5'-CGG ATC TTC TCC TTC TTG TCC A -3'	451
Sat1	5'-ATG GCT AAA TTT AAG ATC CGT CC-3'	5'-TCA CTC CTC TGC TGC CAT TTT TA-3'	516
Spp1	5'-CTG TTT GGC ATT GCC TCC TCC C -3'	5'-AGA GGT GAG GTC CTC ATC TGT G-3'	501
Stfa2l1	5'-GAT TTC TTC TCA GCA CCC TGC C-3'	5'-GGG GGA ATC AGG TCA AGT TGG A-3'	370
Tex261	5'-ATG TGG TTC ATG TAC GTG CTG AG-3'	5'-TCA GTA TAT CTT CTG CCG ACT GG-3'	591

on a 1% agarose gel with ethidium bromide. All reactions were performed in duplicate.

RESULTS

Time course analysis of genes differentially expressed in response to NO

We previously determined the concentration of SNAP that was equivalent to the concentration of endogenous NO released by activated RAW264.7 macrophages (Yook et al., 2004). We used LPS/IFN-γ as an endogenous NO inducer because LPS/IFN-γ increased the expression of iNOS and caused the release of endogenous NO; about 20 mM nitrite

accumulated in the culture medium after exposure to 500 ng/ml LPS for 24 h, and a similar amount of nitrite was generated by 100 μM SNAP under the same conditions.

In this study, 100 μM SNAP was used to treat RAW264.7 cells. Cells were treated with SNAP for 0.5, 1.5, 3, 6, 9, 12 and 15 h, and harvested for RNA preparation. cDNA prepared from each of the RNA samples was subjected to microarray analysis using gene chips containing 25392 mouse cDNA probes. After duplicate experiments of each time point, 250-350 genes were selected to be induced or suppressed. They were first analyzed by hierarchical clustering in order to see a rough expression pattern (Fig. 1A). Various sets of genes were shown to be up-regulated
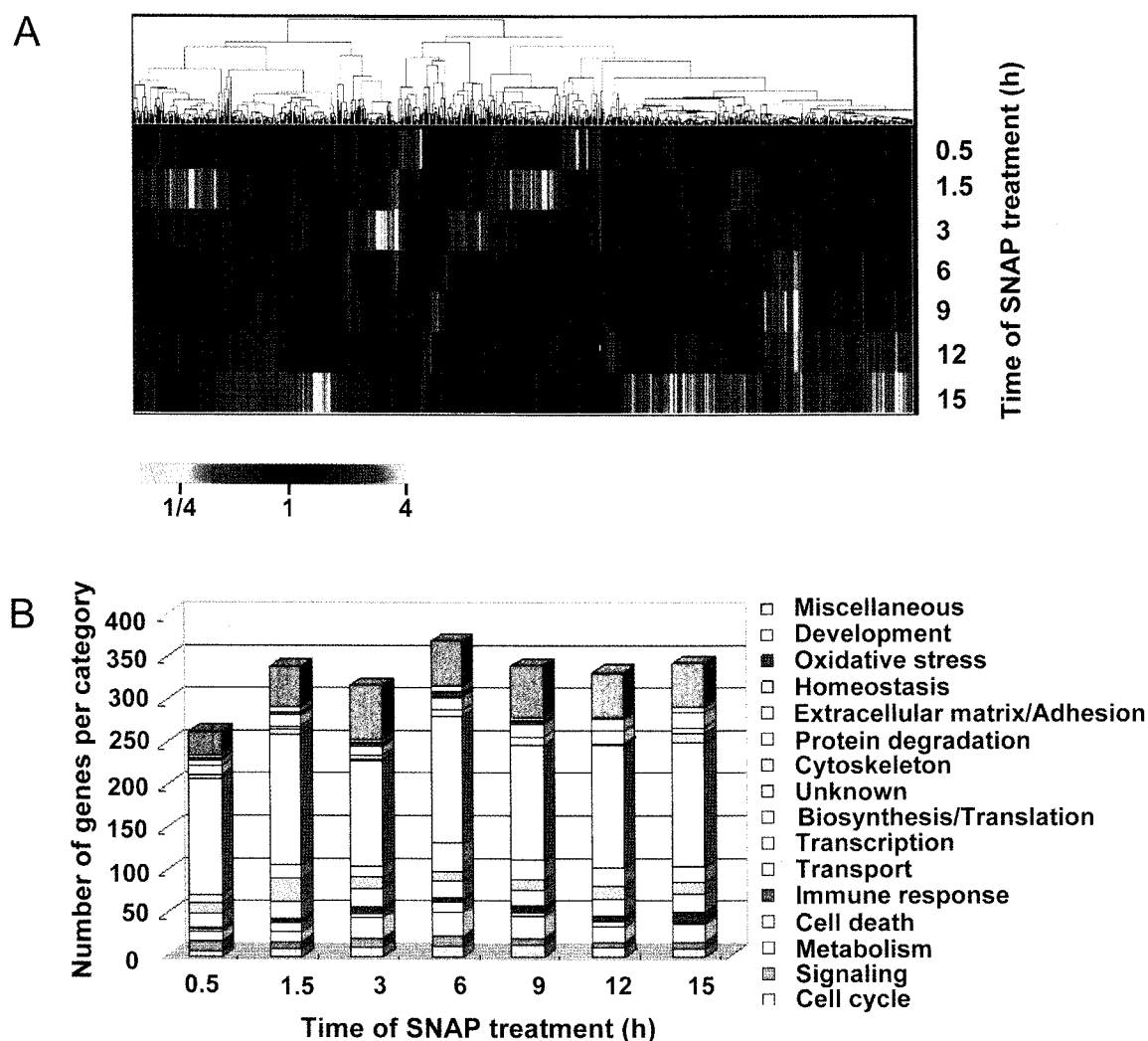

Fig. 1. Analysis of hierarchical clustering and functional categorization of microarray data demonstrating genes differentially expressed by NO. A: Cluster diagrams representing up- and down-regulation of genes by SNAP. Mouse macrophage RAW 264.7 cells were incubated with 100mM SNAP for the indicated time periods and total RNA was prepared. cDNA was synthesized by reverse transcription using Cy3 and Cy5 for mouse chip hybridization. Four different chips were used for each time point in duplicate. The results were analyzed with MultiExperiment Viewer Version 4.0beta program. The NO-dependent up-regulated and down-regulated genes are shown in red and green, respectively, and fold indicated by color bar at the bottom of the figure. The Y-axis is the time of SNAP treatment, and X-axis shows selected genes. B: Functional classification of up-regulated genes by NO treatment. Fourteen different categories were adopted to classify the selected genes by function according to NCBI protein and PubMed database. Genes for each functional group were counted, presented as different colors, and stacked on square column. The genes that could not be included in the 14 categories were classified into miscellaneous or unknown.

(red) or down-regulated (green) following the NO treatment. Next, we categorized the selected genes into functional groups according to the NCBI protein database and Source (Fig. 1B). Fourteen different functional categories were used for analyzing the up-regulated genes. As expected from the large number of probes arrayed in the gene chips (25392 cDNA), significant portions of the selected genes were assorted as undetermined. The number of genes induced by NO showed distinct peaks in categories of transcription, extracellular matrix/adhesion, biosynthesis/ translation, and metabolism. A number of genes involved in transcription reached a maximum within 1.5 h of SNAP treatment. On the other hand, the genes involved in extracellular matrix/adhesion, biosynthesis/translation and metabolism reached a maximum at a relatively later time point, around 6 h. Other genes categorized to oxidative stress, transport, immune response, cell death and signaling were also significantly up-regulated throughout the time points.

Hierarchy of up-regulated gene expression in response to NO

To further determine the hierarchy of gene expression induced by NO treatment, we tried to select genes that showed a significant increase from early to late time points. For each time point, we selected genes induced more than 2 fold or up-regulated for at least two time points. The selected genes were sorted into early (0.5-1.5 h), middle (3-6 h) and late (9-15 h) time points and depicted in color square profiles (Fig. 2).

Within 1.5 h of NO treatment, genes such as heme oxygenase (decycling) 1 (*Hmox1*), cyclin G1 (*Ccng1*) and guanylate cyclase 1, soluble, alpha3 (*Gucy1a3*) were shown to be up-regulated. These genes have already been reported to be NO-responsive in various conditions and several different cell lines (Brockhaus and Brüne, 1999; Chen, 2002; Chen et al., 2002). The results demonstrate the reliability of our microarray experiments.

Within 6 h, genes such as HIG domain family, member 1A (*Higd1a*) similar to hypoxia induced gene 1, BCL2/ adenovirus E1B 19 kDa-interacting protein (*Bnip3*), protein kinase C, alpha (*Pkca*), cyclin-dependent kinase inhibitor 1A (P21) (*Cdkn1a*), lymphocyte protein tyrosine kinase (*Lck*), B cell translocation gene 2, anti-proliferative (*Btg2*), MAP kinase-interacting serine/threonine kinase 2 (*Mknk2*), apoptotic protease activating factor 1 (*Apaf1*), calmodulin 1 (*Calm 1*) and IFN-γ induced GTPase (*Igtp*) were significantly up-regulated, suggesting activation of intracellular signaling or apoptosis during this time.
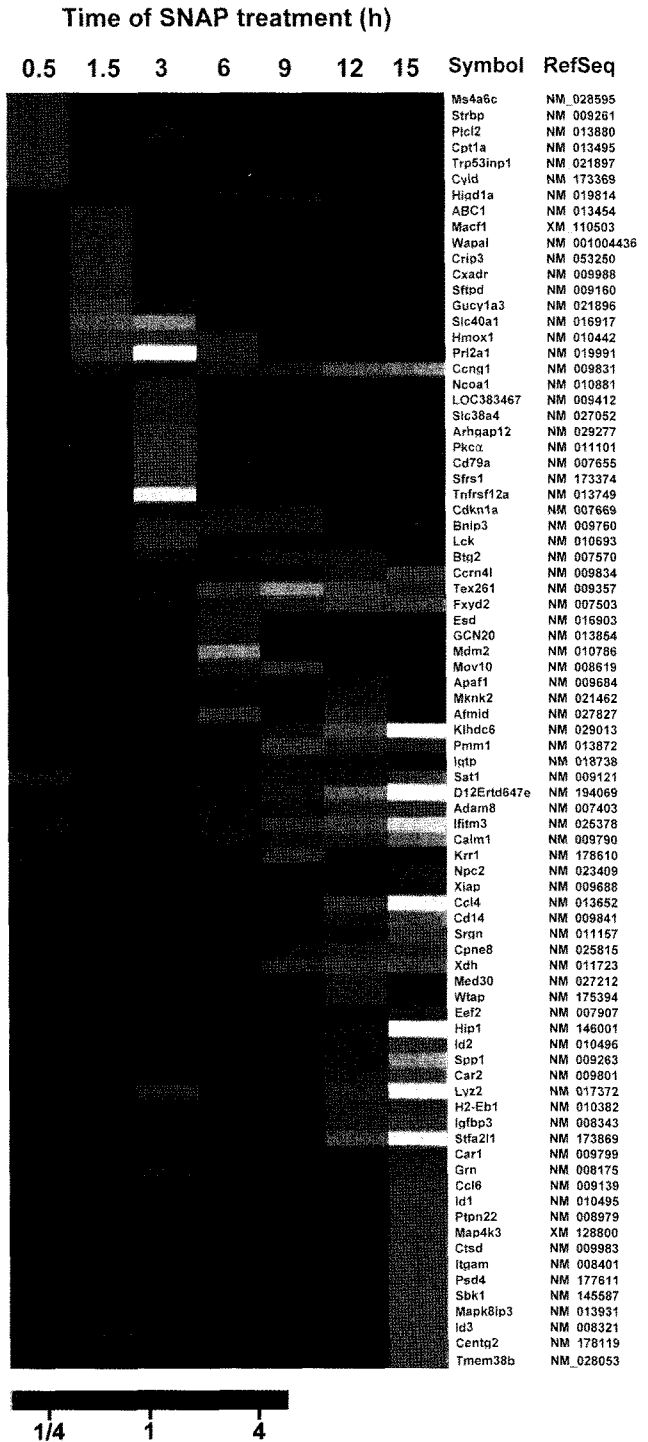
Time of SNAP treatment (h)

Fig. 2. Hierarchy of up-regulated genes in response to NO. Genes that were induced by more than 2 fold or at least at 2 time points were selected. The 81 genes were analyzed using MultiExperiment Viewer Version 4.0beta program. The NO-dependent up-regulation and down-regulation were shown in red and green, respectively, and the fold indicated on color bar in the bottom of the figure. The X-axis is the time of SNAP treatment, and Y-axis shows selected genes.

Symbol	RefSeq
Ms4a6c	NM_028595
Strbp	NM 009261
Plcl2	NM 013880
Cpt1a	NM 013495
Trp53inp1	NM 021897
Cyld	NM 173369
Higd1a	NM 019814
ABC1	NM 013454
Macf1	XM 110503
Wapal	NM 001004436
Crip3	NM 053250
Cxadr	NM 009988
Sftpd	NM 009160
Gucy1a3	NM 021896
Slc40a1	NM 016917
Hmox1	NM 010442
Prl2a1	NM 019991
Ccng1	NM 009831
Ncoa1	NM 010881
LOC383467	NM 009412
Slc38a4	NM 027052
Arhgap12	NM 029277
Pkcα	NM 011101
Cd79a	NM 007655
Sfrs1	NM 173374
Tnfrsf12a	NM 013749
Cdkn1a	NM 007669
Bnip3	NM 009760
Lck	NM 010693
Btg2	NM 007570
Ccrn4l	NM 009834
Tex261	NM 009357
Fxyd2	NM 007503
Esd	NM 016903
GCN20	NM 013854
Mdm2	NM 010786
Mov10	NM 008619
Apaf1	NM 009684
Mknk2	NM 021462
Afmid	NM 027827
Klhdc6	NM 029013
Pmm1	NM 013872
Igtp	NM 018738
Sat1	NM 009121
D12Ertd647e	NM 194069
Adam8	NM 007403
Ifitm3	NM 025378
Calm1	NM 009790
Krr1	NM 178610
Npc2	NM 023409
Xiap	NM 009688
Ccl4	NM 013652
Cd14	NM 009841
Srgn	NM 011157
Cpne8	NM 025815
Xdh	NM 011723
Med30	NM 027212
Wtap	NM 175394
Eef2	NM 007907
Hip1	NM 146001
Id2	NM 010496
Spp1	NM 009263
Car2	NM 009801
Lyz2	NM 017372
H2-Eb1	NM 010382
Igfbp3	NM 008343
Stfa2l1	NM 173869
Car1	NM 009799
Grn	NM 008175
Ccl6	NM 009139
Id1	NM 010495
Ptpn22	NM 008979
Map4k3	XM 128800
Ctsd	NM 009983
Itgam	NM 008401
Psd4	NM 177611
Sbk1	NM 145587
Mapk8ip3	NM 013931
Id3	NM 008321
Centg2	NM 178119
Tmem38b	NM_028053

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Table 2. Functional Classification of NO-induced genes selected by microarray

Functions Accession No.	Full name	Symbol
Phosphorylation and signaling		
NM_021896	guanylate cyclase 1, soluble, alpha 3	Gucy1a3
NM_009790	calmodulin 1	Calm1
NM_018738	interferon gamma induced GTPase	Igtp
NM_010693	lymphocyte protein tyrosine kinase	Lck
NM_021462	MAP kinase-interacting serine/threonine kinase 2	Mknk2
NM_011101	protein kinase C, alpha	Pkcα
NM_013872	phosphomannomutase 1	Pmm1
Cell death and survival		
NM_009684	apoptotic protease activating factor 1	Apaf1
NM_009760	BCL2/adenovirus E1B 19kDa-interacting protein 1, NIP3	Bnip3
NM_007570	B-cell translocation gene 2, anti-proliferative	Btg2
NM_007669	cyclin-dependent kinase inhibitor 1A (P21)	Cdkn1a
NM_019814	HIG1 domain family, member 1A	Higd1a
NM_009688	X-linked inhibitor of apoptosis	Xiap
Inflammation		
NM_010442	heme oxygenase (decycling) 1	Hmox1
NM_009160	surfactant associated protein D	Sftpd
NM_013652	chemokine (C-C motif) ligand 4	Ccl4
NM_009841	CD14 antigen	Cd14
NM_008175	granulin	Grn
NM_009263	secreted phosphoprotein 1, osteopontin	Spp1
Other functions		
NM_009831	cyclin G1	Ccng1
NM_019991	prolactin family 2, subfamily a, member 1	Prl2a1
NM_007503	FXYD domain-containing ion transport regulator 2	Fxyd2
NM_007907	eukaryotic translation elongation factor 2	Eef2
NM_146001	huntingtin interacting protein 1	Hip1
NM_010496	inhibitor of DNA binding 2	Id2
NM_017372	lysozyme 2	Lyz2
NM_009121	spermidine/spermine N1-acetyl transferase 1	Sat1
NM_173869	stefin A2 like 1	Stfa2l1

After 9 h, genes such as chemokine (C-C motif) ligand 4 (*Ccl4*) which is also called macrophage inflammatory protein-1 beta (*MIP-1β*), secreted phosphoprotein 1 (*Spp1*) which is other designated osteopontin (*Opn*), CD14 antigen (*Cd14*), granulin (*Grn*), lysozyme 2 (*Lyz2*), X-linked inhibitor of apoptosis (*Xiap*), inhibitor of DNA binding 2 (*Id2*) were induced by NO treatment. Most of these genes function in inflammation or cell death. The functional classification of these induced genes is summarized in Table 2. Undoubtedly other functional genes related with transcription, translation, ionic homeostasis, metabolism and so on were also induced by NO treatment in macrophages (data not shown).

Correlation of data between microarray and RT-PCR analysis

We further tried to confirm the increased expression of selected genes by semi-quantitative RT-PCR. RAW264.7 cells were treated with SNAP for the indicated time points and total RNA were subjected to RT-PCR using primers designed to produce 500-700 bp PCR products (Table 1 and Fig. 3). *Hmox1* showed peak expression between 1-3 hr. *Adam8, Ccrn41, Pmm1, Higd1a, Bnip3, Cdkn1a*, and *Btg2* peaked at 6-12 h. The RNA expression of *Ccl4, Ccng1, Sat1, Stfa2l1, Tex261, Id2, Spp1* and *Lyz2* started to increase at early time points, and peaked at 12-18 h. These results evidently demonstrated good correlation between
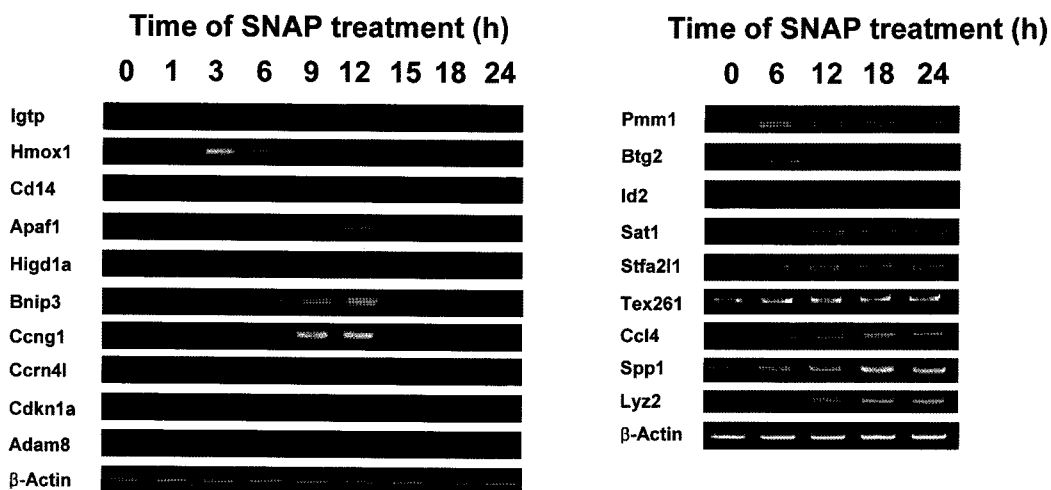
Fig. 3. RT-PCR analysis of the genes selected from microarray analysis. RAW264.7 macrophages were treated with 100 μM SNAP for the indicated time periods. Total RNA was reverse-transcribed and analyzed by semi-quantitative RT-PCR, using primers indicated in Table 1. β-actin was used as an internal standard.

microarray and RT-PCR data.

RT-PCR also showed interesting patterns that had not been revealed by microarray; *Apaf1*, *Cd14* and *Igtp* showed biphasic patterns of RNA expression at middle (3-6 h) and late (12-18 h) time points.

DISCUSSION

Microarray analysis has been used for screening a large pool of genes in various cells using many different stimuli. Once the cDNA or oligo gene chips are developed at maximal efficiency level, the microarray analysis will become a powerful technique for testing induction or suppression of gene expression.

Until now, NO-induced genes or their down-stream signaling pathway have been searched using microarray analysis in hepatocytes, NIH3T3 fibroblasts, lung endothelial cells and human monocytes (Zamora et al., 2002; Hemish et al., 2003; Li et al., 2004; Turpaev et al,. 2005). Since the induction of genes by NO is known to be largely concentration- and cell type-dependent, each of the microarray experiments about the NO-responsive genes revealed different results and conclusions. Hemish et al. (2003) used NIH3T3 fibroblasts and several inhibitors of signaling pathways to define NO-responsive signaling pathways. PI3-kinase, PKC, NF-κB, and p53 signaling were found to be activated to induce genes such as Bcl-2-associated X protein (*Bax*), Bcl2-like 1 (*Bcl2l1*, = *Bcl-xL*), *p21* (equal to *Cdkn1a*), *mdm2*, 14-3-3, etc. They also found particular kinetic waves of total number of genes following NO treatment.

Using hepatocytes infected with adenovirus expressing iNOS, proinflammatory transcription factors, cytokines, cytokine receptors and proteins involved in proliferation,

energetics and apoptosis were shown to be modulated (Zamora et al., 2002). Furthermore, *HO-1* (NM_010442, *Hmox1*) were shown to be up-regulated by NO and found to be involved in reduction of liver injury following ischemia/reperfusion. In lung endothelial cells, 17 genes in 13 signal pathways were increased or decreased in cells treated with NOC-18, a NO donor (Li et al., 2004). In another recent report, human monocytic cells treated with DTPA-NO were used for microarray analysis to verify NO-dependent induction of IL-8, p21 and MKP-1 (Turpaev et al., 2005).

The purpose of the present study is to investigate the down-stream effects of NO in macrophages and also to obtain information about genes responsible for functional regulation. Macrophages are typical myeloid cells used to study NO-dependent physiologic regulation by iNOS induction through treatment with LPS and IFN-γ. Although apoptosis seems to be the major consequence of NO-treatment in the cells, other functional activity also remains to be uncovered. In the present study, we showed that when 14 different functional categories were used for analyzing the up-regulated genes, a number of genes categorized into transcription, biosynthesis/translation, extracellular matrix/adhesion, metabolism, oxidative stress, transport, immune response, cell death and signaling were significantly up-regulated throughout the whole time points (Fig. 1B). Among them, the genes involved in transcription, biosynthesis/translation, extracellular matrix/adhesion, and metabolism showed a wave of peaks at 1.5-6 h following SNAP treatment. However, when we further selected genes induced more than 2 fold or up-regulated for at least two time points, genes such as *higd1a*, *Bnip3*, *Pkcα*, *Cdkn1a*, *Lck*, *Btg2*, *Mknk2*, *Apaf1* and *Igtp* were significantly up-regulated within 6 h (Fig. 2 and Table 2). These are genes

involved in intracellular signaling or apoptosis. After 9 h, genes such as *Ccl4, Spp1, Cd14, Grn* and *Xiap* were induced. These genes are involved in inflammation or cell death. Collectively, the genes involved in signaling, apoptosis and inflammation may play important roles in NO-dependent response, at least in part, in macrophages.

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