Microarray Analysis of Gene Expression by Rhei Rhizoma Water Extracts in a Hypoxia Model of Cultured Neurons

HyunSook Lee, Jinyoung Song and Il Soo Moon*

Department of Anatomy, College of Medicine, Dongguk University, Gyeongju 780-714, Korea

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In this study, we investigated the effect of Rhei Rhizoma (RR; 大黃) water extract on gene expression in a hypoxia model of cultured rat hippocampal neurons. RR water extract (2.5 μg/ml) was added to the culture media on day 10 *in vitro* (DIV10), and a hypoxic shock (2% O_2/5% CO_2 , 37°C, 3 h) was given on DIV13. After maintaining the cultures in normoxia for 24 hr, total RNA was isolated and used for microarray analysis. The MA-plot indicated that most genes were up- or downregulated within 2-fold. There were more downregulated genes (725 ea) than upregulated ones (472 ea) when larger than Global M value 0.2 (i.e., >15% increase) or smaller than Global M value -0.2 (i.e., >15% decrease) were considered. Antiapoptosis genes such as Tegt (2.4-fold), Nfkb1 (2.4-fold) Veg (1.8-fold), Ngfr (1.6-fold) were upregulated, while pro-apoptosis genes such as Bad (-64%), Cstb (-66%) were downregulated. Genes for combating environmental stress (stress response genes) such as Defb3 (2.7-fold), Cygb (2.2-fold), Ahsg (2.18-fold), Alox5 (2-fold) were upregulated. Genes for cell proliferation (cell cycle-related genes) such as Erbb2 (1.84-fold), Mapk12 gene (1.8-fold) was upregulated. Therefore, RR water extracts upregulate many pro-survival genes while downregulating many pro-death genes. It is interpreted that these genes, in combination with other regulated genes, can promote neuronal survival in a stress such as hypoxia.

Key words : Cell culture, hippocampal neuron, hypoxia, microarray, Rhei Rhizoma

Introduction

Rhei Rhizoma (RR; 大黃), a widely used folk medicine in Southeast Asian, consists of the underground parts (rhizome and root) of *Rheum officinale* Baill. and *Rheum palmatum* L. (Polygonaceae). Major constituents of RR are anthranoids, rheinosides, rhein, and stilben such as rhaponticin and rhapontigenin [48]. Beneficiary effects of RR on human health have been reported. For example, RR extracts lower serum cholesterol and improve diabetic nephropathy [40,46]. Effects of RR on lowering serum cholesterol are attributed to rhein, which are transformed from anthranoids sennoside A and B by bacterial enzymes in large intestine [55]. Rhaponticin in the rhizome of RR has extensive anti-allergic and anti-thrombotic properties [46]. RR extracts decreased serum creatinine levels in diabetic patients with neuropathy and retinopathy [18].

Studies on the nervous system are relatively scarce. It has been reported that RR improves memory ability. By comparing the effects of the Compound Tong Jiang Oral Liquid with Da Huang added (TJ) and Qi Yin Oral Liquid (QY) without Da Huang on senile persons' memory ability, Tian *et al.* [55] discovered that TJ improves senile persons' memory ability, in addition to shortened interval and duration of defecation. However, effects of RR on the nervous system are not studied at the cellular level. Previously, our laboratory reported that RR suppresses production of reactive oxygen species (ROS) and loss of mitochondrial membrane potential (MMP) in a hypoxia model of rat hippocampal neurons in culture [34]. Here, we report results of a microarray analysis on the expression of genes in hypoxia.

***Corresponding author**
Tel : +82-54-770-2414, Fax : +82-54-770-2434
E-mail : moonis@dongguk.ac.kr

Materials and Methods

Preparation of water-extracts of RR

Rhei Rhizoma (RR; 大黃) was selected according to the Korean Pharmacopoeia and obtained from Dongguk University Oriental Hospital (Gyeongju, Korea). Distilled water was added to the RR powder and it was agitated for 4 hr at room temperature (RT) followed by overnight at 4°C. After centrifugation (15,000 rpm, 15 min, RT), the supernatant was filter-sterilized (pore size 0.2 μm) and stored at -20°C in small aliquots.

Neuronal culture

Cortices from time-pregnant rats (Sprague-Dawley) at embryonic day 18 (E_{18}) were dissected, dissociated by trypsin treatment and mechanical trituration, and plated onto poly-lysine-coated 60 mm dishes at a density of ~500 cells/mm^2 as described [2,17]. Cells were plated initially in Neurobasal medium supplemented with B27 (Invitrogen, Carlsbad, CA, USA), 25 μM glutamate, and 500 μM glutamine, and fed 5 days after plating and weekly thereafter with the same media (without glutamate).

Hypoxia

Hypoxic shock was given to cells by transferring culture plates to a humidified CO_2 Water Jacketed Incubator (Forma Scientific Inc., Maretta, OH, USA) which was equilibrated at 2% O_2/5% CO_2 (37°C). After 3 hr incubation, plates were returned to normoxic incubator (5% CO_2, 37°C).

Total RNA preparation

On day 10 *in vitro* (DIV10) RR water extract (2.5 μg/ml) was added directly into culture media, given a hypoxic shock on DIV13, and further incubated for 24 hr in normoxia. The culture was briefly washed with 5~10 ml of ice-cold PBS, and cells were lysed by adding 1.0 ml of solution D [4.0 M guanidinium thiocyanate, 25 mM sodium citrate-2H_2O, 0.5% (w/v) sodium lauryl sarcosinate, 0.1 M β-mercaptoethanol]. Cell lysates were transferred to a microfuge tube and homogenized using a tissue homogenizer for 15~30 sec. Per 1.0 ml of the homogenate, 0.1 ml of 2.0 M sodium acetate (pH 4.0), 1.0 ml of phenol (4°C), 0.2 ml of chloroform-isoamyl alcohol was added, mixed well, and incubated on ice for 15 min. After centrifugation (10,000× *g*, 20 min, 4°C), supernatants were mixed with same volumes of isopropanol. After incubation for 2 hr at -20°C, RNA was precipitated by centrifugation (10,000× *g*, 30 min, 4°C) and dissolved in solution D. RNA was precipitated once more by isopropanol and washed twice with 75% alcohol, dissolved in diethyl pyrocarbonate-treated water, and stored at -70°C.

Microarray

Microarray analysis was performed on Rat 44K 4-Plex Gene Expression platforms (Agilent) in Digital Genomics (Seoul, Korea) using total RNAs prepared through 9 independent experiments.

Indirect labeling of probes using aminoallyl-dUTP

To 20-50 μg of total RNA, 2 μl of oligo(dT) primer (0.5 μg/μl) were added and the final volume was adjusted to 31 μl with RNase-free water. The mixture was incubated at 70°C for 10 min, then, chilled on ice for 1 min. The first cDNA strand was synthesized using SuperScript II (200 U/μl; Invitrogen, Carlsbad, CA) at 42°C for 1 hr. To hydrolyze RNA, 16.5 μl of 1.0 M NaOH and 16.5 μl of 0.5 M EDTA were added, and incubate at 65°C for 15 min. The pH was neutralized by adding 16.5 μl of 1.0 M HCl, and cleaned the cDNA reaction with Microcon YM-30. The sample was dried in a speed vac, and resuspend in 9.0 μl of 0.1 M sodium carbonate buffer. NHS-ester Cy dye (2.0 μl in DMSO) was added and incubated for 1 hr in the dark at RT. The coupling reaction was cleaned up with QIAquick PCR purification kit. The elution step was repeated twice with 30 μl elution buffer to get 60 μl of elution volume. The Cy3 and Cy5 sample were dried in a speedvac and proceeded to the hybridization step.

Hybridization

The dried and labeled samples were dissolved in a reasonable volume of hybridization buffer depending on the area to be covered (typically, a 22×22 mm coverslip requires 25 μl of hybridization buffer, and a 22×60 mm coverslip requires 60 μl). The labeled sample preparation was heated for 5 min at 95°C, spun down for 30 sec, and the slides were placed in the hybridization chamber. 20 μl of 3× SSC was put to the chamber at both ends of the slide, the labeled targets were added onto the slide surface, and coverslips were carefully placed on top of the slide. The hybridization chamber was sealed and incubated in 42°C waterbath or hybridization oven for more than 16 hr. The coverslips were removed by immersing the slide in 2× SSC/0.1% SDS at 42°C, placed in 2× SSC/0.1% SDS for 5 min at 42°C, 0.1× SSC/0.1% SDS for 10 min at RT, then 4 times in 0.1× SSC for 1 min at RT. The slides were dried by centrifuge at 650 rpm for 5 min.

Image scanning

Images were captured on the ScanArray Lite using the ScanArray 3.0 software (Perkin Elmer Life Sciences, Boston, MA) at the scanning resolution of 50 μm. The laser power and PMT voltage were adjusted to get comparable intensity in Cy3 and Cy5 images. The scanning resolution was set to 10 μm.

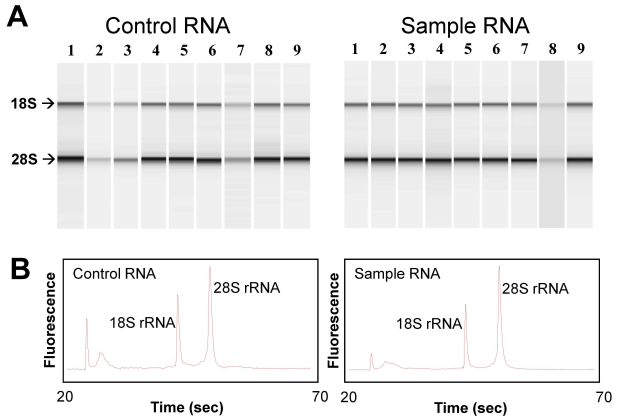
Results and Discussion

RNA quality

Preparation of undegradated total RNA in high purity is essential for a successful microarray. The quality of total RNA preparations were monitored by the RNA 6000 Pico Assay Solution using 2100 Bioanalyzer (Agilent Inc.). No degradation was evident in the gel-like images of 9 control and sample preparations (Fig. 1A), and 28S and 18S ribosomal RNA peaks of the electrophoretic data of each preparation no. 1 (Fig. 1B) were sharp without tails. The rRNA ratios (28S/18S) of each preparation were close to 2.0 (Fig. 1C). These data verifies a good quality of RNA.

MA-plot

An MA-plot of microarray data, where M and A represent $\log_2$(Cy5 intensity/Cy3 intensity) and $1/2\log_2$(Cy5 intensity×Cy3 intensity), respectively, is a plot of log-ratio of two expression intensities versus the mean log-expression of the two [9]. As shown in Fig. 2, most M values are between ±1.0, meaning that up- or downregulation scales are mostly less than 2-fold.



C

sample	rRNA ratio	sample	rRNA ratio*
C1	1.6	D1	2.05
C2	1.64	D2	1.79
C3	1.98	D3	1.98
C4	1.87	D4	1.68
C5	1.82	D5	1.89
C6	1.9	D6	1.99
C7	1.72	D7	1.99
C8	1.63	D8	2.12
C9	1.97	D9	1.91

* rRNA Ratio; 28S/18S ribosomal RNA

Fig. 1. RNA quality control. The quality of RNA preparations were monitored by the RNA 6000 Pico Assay Solution using 2100 Bioanalyzer (Agilent Inc.). Gel-like images of 9 control and sample preparations (*A*) as well as one electrophoretic profile (*B*) were shown. rRNA Ratios (28S/18S ribosomal RNA) were shown in *C*. Data *B* is from one of nine independent experiments.

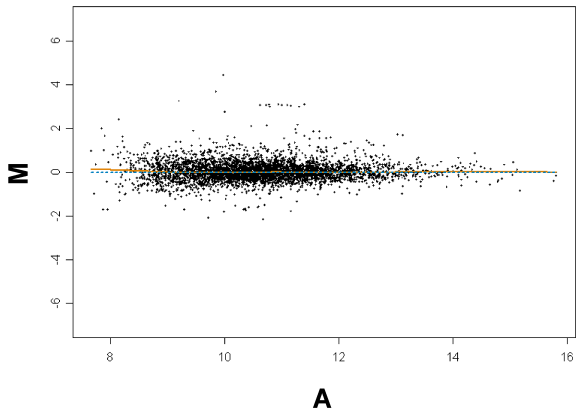


Fig. 2. An MA-plot of microarray data.

Functional distribution of regulated genes

Genes were classified into nine functional groups (apoptosis, cell cycle, immune response, physiological process, signal transduction, stress response, transcription). The overall distribution of the genes in each groups that were up- or downregulated by larger than Global M value 0.2 (i.e., >15% increase or >15% decrease) by RR water extracts

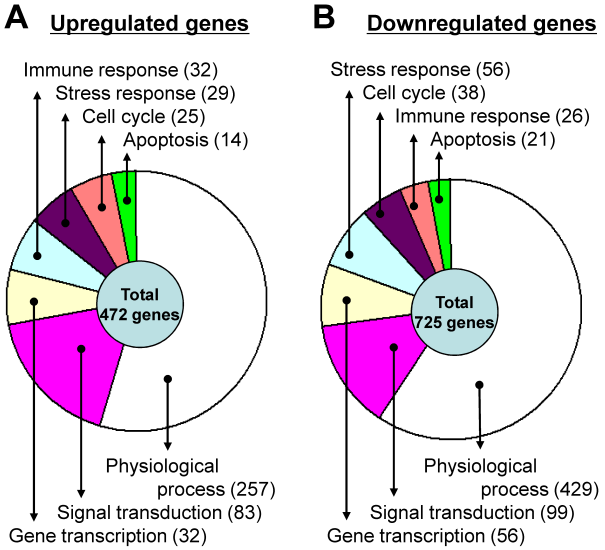


Fig. 3. Functional distribution of regulated genes. The overall distribution of the genes in each groups that were up- or downregulated (*A* and *B*, respectively) by larger than Global M value 0.2 (i.e., >15% increase or <15% decrease) by RR water extracts at 1 day after hypoxic shock was shown.

Table 1. Cell death-related genes

Gene symbol	GenBank. ACC	Global.M	Title
Tegt	AA819031	1.28322	Testis enhanced gene transcript
Nfkb1	AA858801	1.242249	Nuclear factor of kappa light chain gene enhancer in B-cells 1, p105
Mgmt	AI044880	1.22528	O-6-methylguanine-DNA methyltransferase
Vegf	AA924335	0.845627	Vascular endothelial growth factor
Ngfr	AI500767	0.67439	Nerve growth factor receptor (TNFR superfamily, member 16)
Bat3	AA900362	0.591977	HLA-B-associated transcript 3
Rtn4	AA901032	0.465308	Reticulon 4
Notch2	AA999021	-0.41489	Notch gene homolog 2, (Drosophila)
G6pdx	AA899102	-0.41632	Glucose-6-phosphate dehydrogenase
Lcn2	AI137617	-0.43974	Lipocalin 2
Txnl1	AA875390	-0.44684	Thioredoxin-like (32 kD)
Inha	AA955146	-0.44989	Inhibin alpha
Dnajb9	AI136414	-0.45228	DnaJ (Hsp40) homolog, subfamily B, member 9
P2rx1	AI070938	-0.47793	Purinergic receptor P2X, ligand-gated ion channel, 1
Casp2	AA875622	-0.55095	Caspase 2
Igf1	AA963258	-0.58785	Insulin-like growth factor 1
Bad	AA964163	-0.7099	Bcl2-associated death promoter
Cstb	AA859814	-0.7308	Cystatin B
Tpt1	AA900176	-0.84745	Tumor protein, translationally-controlled 1
Gpx1	AA964788	-1.01287	Glutathione peroxidase 1

at 1 day after hypoxic shock was shown in Fig. 3. There were more downregulated genes (725 ea) than upregulated ones (472 ea). Lists of genes in each groups are shown in Table 1-7. In the following sections, meanings for only apoptosis-, stress response-, and cell cycle-related genes are investigated, because these genes are most important in cell survival.

Upregulated apoptosis-related genes

Genes that were up- or downregulated by larger than Global M value 0.2 (i.e., >15% increase or >15% decrease) by RR water extracts was shown in Table 1. Genes upregulated more than 2-fold are testis enhanced gene transcript (Tegt; Global M=1.28322), nuclear factor of kappa light chain gene enhancer in B-cells 1, p105 (Nfkb1; Global M=1.242249), and O-6-methylguanine-DNA methyltransferase (Mgmt; Global M=1.22528).

Testis enhanced gene transcript (Tegt)

Tegt was upregulated by 2.4-fold. Tegt, also known as Bax inhibitor-1 (BI-1), is conserved in both animal and plant species [5,58]. Tegt can inhibit the endoplasmic reticulum (ER) stress proteins as well as the accumulation of ROS, thereby protecting the cells [4,33,61]. Tegt is a suppressor of apoptosis [61], and overexpression of Tegt in rat nigral CSM14.1 and human SH-SY5Y neuroblastoma cells markedly protected cell death induced by thapsigargine, a stress agent blocking the Ca^{2+}-ATPase of the ER [8]. Moreover, Tegt was neuroprotective in oxygen-glucose as well as serum deprivation [8]. Since Tegt functions as a suppressor of apoptosis, its upregulation by RR water extracts is expected to be beneficial for cell survival in hypoxia.

Nuclear factor of kappa light chain gene enhancer in B-cells 1, p105 (Nfkb1)

Nfkb1 was upregulated by 2.4-fold. NF-κB is an evolutionarily conserved signaling module that plays a critical role in the immune system among many biological processes [1]. In many cell types, the most abundant form of NF-κB is the p50/p65 heterodimer, which forms a ternary complex with the inhibitor of NF-κB, IκBα, and remains inactive in the cytoplasm. Upon stimulation, IκBα is rapidly degraded, allowing translocation of p50/p65 heterodimers into the nucleus [16,24,29,32] and activation of genes related to inflammation and proliferation. p105 is the precursor of p50 component of NF-κB [24]. Increase in NFKB1 is expected to provide a beneficial environment for neuronal survival in hypoxia.

O-6-methylguanine-DNA methyltransferase (Mgmt)

Mgmt was upregulated by 2.3-fold. O^6-methylguanine (O^6MeG) is the most critical DNA lesion. Another pre-mutagenic methylation lesion is O^4-methylthymine (O^4MeT). O^6MeG and O^4MeT are repaired by MGMT (also referred to as ATase, AGT, AGAT; E.C. 2.1.1.63) [15,37]. If not repaired, O^6MeG can cause cell death, chromosomal aberrations, mutations and cancer. One MGMT molecule can repair only one alkyl adduct. Therefore, the cell's capacity for removing DNA O^6-alkylguanine adducts depends on the total

number of MGMT molecules per cell. Upregulation of Mgmt would be highly beneficial to neuronal survival in hypoxia.

Vascular endothelial growth factor (Vegf)

Vegf was upregulated by 1.8-fold. Vegf was also significantly upregulated (Global M=0.8456266). VEGF can activate anti-apoptotic kinase and maintain survival signals in endothelial cells [19,36]. It has been shown that VEGF can prevent cell death in various conditions. For example, VEGF prevented apoptosis of human umbilical vein endothelial cells (HUVEC) from high glucose exposure [63]. In high glucose exposure, pretreatment of VEGF lowered ROS generation, calcium overload, Bax/Bcl-2 ratio, caspase-3 activation in HUVEC. *In vitro*, VEGF-A stimulates axonal outgrowth, improves neuronal survival, and prevents hippocampal cells from apoptosis induced by serum withdrawal [26,51,52]. Recently, VEGF-A was proved to be a survival

Table. 2. Stress-related genes

Gene symbol	GenBank. ACC	Global.M	Title
Defb3	AA819022	1.458768	Defensin beta 3
Nfkb1	AA858801	1.242249	Nuclear factor of kappa light chain gene enhancer in B-cells 1, p105
Mgmt	AI044880	1.22528	O-6-methylguanine-DNA methyltransferase
Cygb	AA866399	1.167176	Cytoglobin
Ahsg	AA955349	1.125401	Alpha-2-HS-glycoprotein
Alox5	AI044102	1.049389	Arachidonate 5-lipoxygenase
Sod3	AA963644	0.860088	Superoxide dismutase 3, extracellular
Crp	AA926359	0.835698	C-reactive protein, petaxin related
Thbd	AA818521	0.826701	Thrombomodulin
Ptafr	AA924637	0.748363	Platelet-activating factor receptor
Fen1	AA819793	0.705112	Flap structure-specific endonuclease 1
Insig2	AA818627	0.656895	Insulin induced gene 2
Gng7	AA925506	0.633971	Guanine nucleotide binding protein, gamma 7
Xpo1	AA818465	0.618395	Exportin 1, CRM1 homolog (yeast)
Clu	AA818413	0.602256	Clusterin
A2m	AA817954	0.591385	Alpha-2-macroglobulin
Ptprc	AA924685	0.513707	Protein tyrosine phosphatase, receptor type, C
Tsc2	AA899998	0.474074	Tuberous sclerosis 2
Pros1	AA925039	0.428995	Protein S (alpha)
Pparg	AI111890	0.401628	Peroxisome proliferator activated receptor, gamma
Apex1	AA900301	-0.40687	Apurinic/apyrimidinic endonuclease 1
Ogg1	AA859654	-0.41122	8-oxoguanine-DNA-glycosylase
Gm1960	AI045017	-0.41831	Gene model 1960, (NCBI)
Tlr4	AI044119	-0.42067	Toll-like receptor 4
LOC81816	AA859229	-0.42814	Ubiquitin conjugating enzyme
Slc2a1	AA875020	-0.44564	Solute carrier family 2 (facilitated glucose transporter), member 1
Pros1	AA875352	-0.44855	Protein S (alpha)
Inha	AA955146	-0.44989	Inhibin alpha
Dnajb9	AI136414	-0.45228	DnaJ (Hsp40) homolog, subfamily B, member 9
Fga	AA925421	-0.46384	Fibrinogen, alpha polypeptide
Defb1	AA998504	-0.46674	Defensin beta 1
Txnrd2	AA925357	-0.46952	Thioredoxin reductase 2
Ninj1	AI044670	-0.47247	Ninjurin 1
Ddt	AA900788	-0.47251	D-dopachrome tautomerase
Evl	AA997968	-0.504	RNB6
Erbb3	AA924236	-0.50868	V-erb-b2 erythroblastic leukemia viral oncogene homolog 3 (avian)
Cd81	AA964328	-0.54156	CD 81 antigen
Camk2g	AA866334	-0.58588	Calcium/calmodulin-dependent protein kinase II gamma
Trip10	AI059990	-0.59428	Thyroid hormone receptor interactor 10
Prkab1	AA924247	-0.63369	Protein kinase, AMP-activated, beta 1 non-catalytic subunit
Itga1	AA964090	-0.6439	Integrin alpha 1
Pla2g1b	AA955059	-0.71966	Phospholipase A2, group IB
Crry	AA925136	-0.83107	Complement receptor related protein
C1qb	AA925356	-0.88625	Complement component 1, q subcomponent, beta polypeptide
Gpx1	AA964788	-1.01287	Glutathione peroxidase 1

Table 3. Cell cycle-related genes

Gene symbol	GenBank. ACC	global.M	Title
Erbb2	AA858862	0.877964	V-erb-b2 erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma derived oncogene homolog (avian)
Vegf	AA924335	0.845627	Vascular endothelial growth factor
Mapk12	AA924917	0.843205	Mitogen-activated protein kinase 12
Fen1	AA819793	0.705112	Flap structure-specific endonuclease 1
Bmyc	AA998830	0.550368	Brain expressed myelocytomatosis oncogene
Tsc2	AA899998	0.474074	Tuberous sclerosis 2
Ccng1	AA956549	0.470117	Cyclin G1
Cdc25b	AA924021	0.456182	Cell division cycle 25B
Vav1	AA925725	0.418808	Vav 1 oncogene
Ccnd1	AA875417	0.417766	Cyclin D1
Wt1	AA965119	-0.40316	Wilms tumor 1
Ran	AA858998	-0.41427	RAN, member RAS oncogene family
Notch2	AA999021	-0.41489	Notch gene homolog 2, (Drosophila)
Ccng1	AA925280	-0.41908	Cyclin G1
Inha	AA955146	-0.44989	Inhibin alpha
Raf1	AA900883	-0.45641	Murine leukemia viral (v-raf-1) oncogene homolog 1 (3611-MSV)
Pcm1	AI072209	-0.47699	Pericentriolar material 1
Erbb3	AA924236	-0.50868	V-erb-b2 erythroblastic leukemia viral oncogene homolog 3 (avian)
Cdc25a	AI060206	-0.56259	Cell division cycle 25A
Camk2g	AA866334	-0.58588	Calcium/calmodulin-dependent protein kinase II gamma
Tgfb2	AA899488	-0.66711	Transforming growth factor, beta 2
Bin1	AA859289	-0.66941	Myc box dependent interacting protein 1
Fgr	AA901385	-0.74246	FGR
Cdk4	AA819907	-0.78598	Cyclin-dependent kinase 4
Mas1	AI071498	-0.87495	MAS1 oncogene

Table 4. Immune response genes

Gene symbol	GenBank. ACC	Global.M	Title
Nfkb1	AA858801	1.2422	Nuclear factor of kappa light chain gene enhancer in B-cells 1, p105
Ahsg	AA955349	1.1254	Alpha-2-HS-glycoprotein
Alox5	AI044102	1.0494	Arachidonate 5-lipoxygenase
Crp	AA926359	0.8357	C-reactive protein, petaxin related
Gbp2	AA819701	0.7649	Guanylate binding protein 2, interferon-inducible
Ptafr	AA924637	0.7484	Platelet-activating factor receptor
Znf179	AA997188	0.716	Zinc finger protein 179
A2m	AA817954	0.5914	Alpha-2-macroglobulin
Igbp1	AA997141	0.5478	Immunoglobulin (CD79A) binding protein 1
Cd5	AA925584	0.5264	CD5 antigen
Ptprc	AA924685	0.5137	Protein tyrosine phosphatase, receptor type, C
Tsc2	AA899998	0.4741	Tuberous sclerosis 2
Vav1	AA925725	0.4188	Vav 1 oncogene
Gbp2	AA923928	0.4061	Guanylate binding protein 2, interferon-inducible
Pparg	AI111890	0.4016	Peroxisome proliferator activated receptor, gamma
Fth1	AA818441	-0.4014	Ferritin, heavy polypeptide 1
Gm1960	AI045017	-0.4183	Gene model 1960, (NCBI)
Tlr4	AI044119	-0.4207	Toll-like receptor 4
Inha	AA955146	-0.4499	Inhibin alpha
Ddt	AA900788	-0.4725	D-dopachrome tautomerase
Hla-dmb	AI146187	-0.4949	Major histocompatibility complex, class II, DM beta
Cd81	AA964328	-0.5416	CD 81 antigen
Itga1	AA964090	-0.6439	Integrin alpha 1
Mx2	AI030615	-0.6496	Myxovirus (influenza virus) resistance 2
Bad	AA964163	-0.7099	Bcl2-associated death promoter
Crry	AA925136	-0.8311	Complement receptor related protein
C1qb	AA925356	-0.8862	Complement component 1, q subcomponent, beta polypeptide

Table 5. Physiological process-related genes

Gene symbol	GenBank. ACC	Global.M	Title
Timm44	AI045558	1.713614	Translocator of inner mitochondrial membrane 44
Ass	AA924544	1.613404	Arginosuccinate synthetase
Nr3c2	AI029599	1.495669	Nuclear receptor subfamily 3, group C, member 2
Nt5	AA858866	1.49235	5 nucleotidase
Defb3	AA819022	1.458768	Defensin beta 3
Pfkp	AA819266	1.342494	Phosphofructokinase, platelet
Tegt	AA819031	1.28322	Testis enhanced gene transcript
Cabp1	AI111726	1.281876	Calcium binding protein 1
Sdha	AA875474	1.25314	Succinate dehydrogenase complex, subunit A, flavoprotein (Fp)
Nfkb1	AA858801	1.242249	Nuclear factor of kappa light chain gene enhancer in B-cells 1, p105
Faah	AI136093	1.232485	Fatty acid amide hydrolase
Mgmt	AI044880	1.22528	O-6-methylguanine-DNA methyltransferase
Cygb	AA866399	1.167176	Cytoglobin
Tceb3	AA818727	1.160721	Transcription elongation factor B (SIII), polypeptide 3
Folh1	AI059788	1.145236	Folate hydrolase
Ahsg	AA955349	1.125401	Alpha-2-HS-glycoprotein
Deaf1	AA997917	1.084914	Deformed epidermal autoregulatory factor 1 (Drosophila)
Fxyd1	AA818939	1.07493	FXYD domain-containing ion transport regulator 1
Mte1	AA899721	1.061956	Mitochondrial acyl-CoA thioesterase 1
Alox5	AI044102	1.049389	Arachidonate 5-lipoxygenase
Nr1d2	AA858968	1.048974	Nuclear receptor subfamily 1, group D, member 2
Arf2	AA875379	1.031227	ADP-ribosylation factor 2
Col2a1	AA899303	1.010234	Procollagen, type II, alpha 1
Ppp1cb	AA901261	1.010125	Protein phosphatase 1, catalytic subunit, beta isoform
Rpo1-2	AA964945	-1.01213	RNA polymerase 1-2
Gpx1	AA964788	-1.01287	Glutathione peroxidase 1
Lss	AA997956	-1.05279	2,3-oxidosqualene: lanosterol cyclase
Hibadh	AA859729	-1.07892	3-hydroxyisobutyrate dehydrogenase
Kcnj16	AI111944	-1.09891	Potassium inwardly-rectifying channel, subfamily J, member 16
Mmp23	AA900609	-1.10199	Matrix metalloproteinase 23
Copb2	AA964331	-1.12599	Coatomer protein complex, subunit beta 2 (beta prime)
Slc10a1	AA999182	-1.27549	Solute carrier family 10 (sodium/bile acid cotransporter family), member 1
Mybbp1a	AA899306	-1.28789	MYB binding protein (P160) 1a
Ppp2cb	AA866273	-1.45478	Protein phosphatase 2a, catalytic subunit, beta isoform
Enpp3	AA859670	-1.58063	Ectonucleotide pyrophosphatase/phosphodiesterase 3
Ctsh	AA819336	-1.87779	Cathepsin H

factor for retinal neurons to ischemic injury [41]. Therefore, upregulation of Vegf would provide a beneficial environment for neuronal survival in hypoxia.

Nerve growth factor receptor (Ngfr)

Ngfr was upregulated by 1.6-fold (Global M=0.6743903). The receptors for neurotrophins are members of a family of highly similar transmembrane tyrosine kinases (TrkA, TrkB, and TrkC). Nerve growth factor (NGF) binds mainly TrkA. Binding of NGF to TrkA initiates activation of phosphatidylinositol 3-kinase/Akt, MAPK, and phospholipase C-γ1 signaling pathways [28], leading to the prevention of apoptotic cell death and promotion of cellular differentiation. Therefore, upregulation of Ngfr would be highly beneficial to neuronal survival in hypoxia.

Downregulated apoptosis-related genes

Bcl2-associated death promoter (Bad). Bad was downregulated by -64% (Global M=-0.7098992). The Bcl-2 family proteins play an essential role in cell death regulation. BAD is a pro-apoptotic member of the Bcl-2 family and a regulatory target of survival signaling [65]. Among pro-apoptotic members, BAD belongs to BH3-only proteins that sense stress signals and initiate the death program [25]. Therefore, downregulation of Bad would prevent apoptosis and lead to cell survival.

Cystatin B (Cstb)

Cstb gene was downregulated by -66% (Global M= -0.7308042). Cystatin-B-deficient homozygous mice show behavioral defects such as poor balance while moving and a

Table 6. signal transduction-related genes

Gene symbol	GenBank.ACC	Global.M	Title
Gnrhr	AI146077	1.268131	Gonadotropin releasing hormone receptor
Nfkb1	AA858801	1.242249	Nuclear factor of kappa light chain gene enhancer in B-cells 1, p105
Ahsg	AA955349	1.125401	Alpha-2-HS-glycoprotein
Arf2	AA875379	1.031227	ADP-ribosylation factor 2
Plcd4	AA955840	0.904427	Phospholipase C, delta 4
Erbb2	AA858862	0.877964	V-erb-b2 erythroblastic leukemia viral oncogene homolog 2, neuro/ glioblastoma derived oncogene homolog (avian)
Vegf	AA924335	0.845627	Vascular endothelial growth factor
Mapk12	AA924917	0.843205	Mitogen-activated protein kinase 12
Plcg1	AA899848	0.836405	Phospholipase C, gamma 1
Ptafr	AA924637	0.748363	Platelet-activating factor receptor
Bmpr1a	AA924837	0.739263	Bone morphogenetic protein receptor, type 1A
Rhoa	AA819093	0.706041	Plysia ras-related homolog A2
Pthr1	AI059504	0.69255	Parathyroid hormone receptor 1
Ngfr	AI500767	0.67439	Nerve growth factor receptor (TNFR superfamily, member 16)
Sgne1	AA998689	0.672918	Secretory granule neuroendocrine protein 1
Tle4	AA859009	0.656743	Transducin-like enhancer of split 4, E(spl) homolog (Drosophila)
Avpr1a	AI045872	0.64867	Arginine vasopressin receptor 1A
Dtr	AA818658	0.636677	Diphtheria toxin receptor
Gng7	AA925506	0.633971	Guanine nucleotide binding protein, gamma 7
Arrb2	AI136065	0.632451	Arrestin, beta 2
Git1	AA997450	0.631166	G protein-coupled receptor kinase interactor 1
Gng8	AA899129	0.626027	Guanine nucleotide binding protein (G protein), gamma 8 subunit
Madh4	AA997371	-0.61697	MAD homolog 4 (Drosophila)
Itga1	AA964090	-0.6439	Integrin alpha 1
Rab11a	AA963374	-0.65162	RAB11a, member RAS oncogene family
Plau	AA923863	-0.68778	Plasminogen activator, urokinase
Gprk2l	AA963235	-0.69101	G protein-coupled receptor kinase 2, groucho gene related (Drosophila)
Bmp6	AI071095	-0.70819	Bone morphogenetic protein 6
Fgr	AA901385	-0.74246	FGR
Ghrh	AI058642	-0.7589	Growth hormone releasing hormone
Olr59	AA997671	-0.76061	Olfactory receptor gene Olr59
Cdk4	AA819907	-0.78598	Cyclin-dependent kinase 4
Ptpro	AI136174	-0.78835	Protein tyrosine phosphatase, receptor type, O
Stat5b	AA955730	-0.85212	Signal transducer and activator of transcription 5B
Mas1	AI071498	-0.87495	MAS1 oncogene
Gnb2l1	AA859083	-1.21224	Guanine nucleotide binding protein, beta polypeptide 2-like 1
Ppp2cb	AA866273	-1.45478	Protein phosphatase 2a, catalytic subunit, beta isoform
Gcgr	AA962949	-1.78369	Glucagon receptor

lack of motor coordination (Pennacchio et al., 1998). Previously, these mice exhibited neuronal loss in various brain regions including cerebellar granule cell layer, entorhinal cortex, and hippocampus [43,50]. This phenotype is similar to the progressive myoclonus epilepsy of the Unverricht-Lundborg type (locus symbol EPM1) which is related to the increased activity of cysteine proteins cathepsins [47]. Since Cystatin B is a cysteine protease inhibitor, cytoplasmic cathepsins are free to activate the apoptotic pathways in this pathogenesis [64]. However, the meaning of Cstb downregulation in hypoxia is not clear.

Tumor protein, translationally-controlled 1 (Tpt1)

Tpt1 gene was downregulated by 80% (Global M= -0.8474458). TPT1 is widely expressed and conserved throughout vertebrates. The tpt1 is the strongest differentially expressed gene between tumor and tumor-reversed states in human leukemia and breast-cancer cells [57]. The meaning of Cstb downregulation in hypoxia is not clear.

Glutathione peroxidase 1 (Gpx1)

Gpx1 gene was downregulated by 2-fold (Global M= -1.012869). GPX1 is an anti-oxidant enzyme. Over-expression of Gpx1 or Cu/Zn-superoxide dismutase (SOD1) protects neuronal apoptosis after focal cerebral ischemia [11,30]. Knockout of SOD1 or Gpx1 increases neuronal cell damage after focal cerebral ischemia [7,31]. The reason why Gpx1, an anti-oxidant gene, is reduced is not clear.

Table 7. Transcription-related genes

Gene symbol	GenBank. ACC	Global.M	Title
Timm44	AI045558	1.713614	Translocator of inner mitochondrial membrane 44
Nr3c2	AI029599	1.495669	Nuclear receptor subfamily 3, group C, member 2
Nfkb1	AA858801	1.242249	Nuclear factor of kappa light chain gene enhancer in B-cells 1, p105
Tceb3	AA818727	1.160721	Transcription elongation factor B (SIII), polypeptide 3
Deaf1	AA997917	1.084914	Deformed epidermal autoregulatory factor 1 (Drosophila)
Nr1d2	AA858968	1.048974	Nuclear receptor subfamily 1, group D, member 2
Tle4	AA859009	0.656743	Transducin-like enhancer of split 4, E(spl) homolog (Drosophila)
Rbm16	AA963906	0.646814	RNA binding motif protein 16
Pou3f4	AA996525	0.546103	POU domain, class 3, transcription factor 4
Neurod3	AI028971	0.530241	Neurogenic differentiation 3
Adnp	AA900236	0.515365	Activity-dependent neuroprotective protein
Zhx1	AA817888	0.495373	Zinc-fingers and homeoboxes 1
Nptxr	AI045501	0.47642	Neuronal pentraxin receptor
Myod1	AA955902	0.459998	Myogenic differentiation 1
Dlx5	AA998469	0.424502	Distal-less homeobox 5
Adnp	AA818064	0.414277	Activity-dependent neuroprotective protein
Aes	AA875427	0.41274	Amino-terminal enhancer of split
Ilf3	AA899489	0.412524	Interleukin enhancer binding factor 3
Pparg	AI111890	0.401628	Peroxisome proliferator activated receptor, gamma
Notch3	AA875382	-0.40184	Notch 3
Wt1	AA965119	-0.40316	Wilms tumor 1
Taf9l	AI059951	-0.41251	TAF9-like RNA polymerase II, TATA box binding protein (TBP)-associated factor, 31kDa
Notch2	AA999021	-0.41489	Notch gene homolog 2, (Drosophila)
Madh2	AA858489	-0.41862	MAD homolog 2 (Drosophila)
Foxq1	AI030728	-0.42124	HNF-3/forkhead homolog-1
Stat5b	AA875320	-0.43957	Signal transducer and activator of transcription 5B
Gtf2a2	AA924402	-0.46704	General transcription factor Iia 2
Sfrp4	AI058284	-0.52688	Secreted frizzled-related protein 4
Rlzfy	AA925476	-0.52961	Zinc finger protein Y1 (RLZF-Y)
Myog	AI030924	-0.53956	Myogenin
Rab2	AI070618	-0.5426	RAB2, member RAS oncogene family
Mta1	AA899830	-0.55242	Metastasis associated 1
Ssrp1	AA996926	-0.5661	Structure specific recognition protein 1
Unr	AI070521	-0.59953	Unr protein
TCEB2	AA817828	-0.60517	Transcription elongation factor B (SIII), polypeptide 2
Madh4	AA997371	-0.61697	MAD homolog 4 (Drosophila)
Msx1	AI029934	-0.64503	Homeo box, msh-like 1
Rab11a	AA963374	-0.65162	RAB11a, member RAS oncogene family
Ppard	AI058392	-0.659	Peroxisome proliferator activated receptor delta
Mtpn	AI044524	-0.70996	Myotrophin
Stat5b	AA955730	-0.85212	Signal transducer and activator of transcription 5B
Yy1	AA901302	-0.86165	YY1 transcription factor
Rpo1-2	AA964945	-1.01213	RNA polymerase 1-2
Mybbp1a	AA899306	-1.28789	MYB binding protein (P160) 1a

Stress response-related genes

Genes that were up- or downregulated larger than Global M value 0.2 (i.e., >15% increase or >15% decrease) by RR water extracts were shown in Table 2. Genes upregulated more than 2-fold are defensin beta 3 (Defb3; Global M= 1.458768), Nfkb1, Mgmt, cytoglobin (Cygb; Global M= 1.167176), alpha-2-HS-glycoprotein (Ahsg; Global M= 1.125401), and arachidonate 5-lipoxygenase (Alox5; Global M=1.049389).

Defensin beta 3 (Defb3)

The Defb3 gene was upregulated by 2.7-fold (Global M=1.458768). Mammalian cells express a number of peptide antibiotics as a innate host defense system [27]. Defensins and cathelicidin are the two major classes of antimicrobial peptides in humans [14,42]. Defensins are divided into α- and β-defensins [42]. α-Defensins are expressed in small intestine (neutrophils and Paneth cells), whereas human β-defensins (hBDs) are mainly found in epithelial tissues [42].

hBD-1 is constitutively expressed in various epithelial tissues [12,66], while hBD-2 is mainly found in skin, respiratory and gastrointestinal tracts [21,36]. hBD-3, the third type β-defensin, is inducible upon stimulation with bacteria and cytokines in both epithelial and non-epithelial tissues [21,13]. Therefore, upregulation of Defb3 would protect neurons in hypoxia.

Cytoglobin (Cygb)

The Cygb gene was upregulated by 2.2-fold (Global M=1.167176). In vertebrates, four types of globins have been reported: hemoglobin (Hb) in red blood cell [23], myoglobin (Mb) in muscle [59,60], neuroglobin (Ngb) and cytoglobin (Cygb). Cygb is ubiquitously expressed [3,20], and is upregulated in rat heart and liver following hypoxia [49] or in brain by hypoxia [10]. When Cygb mRNA was knocked down by siRNA, cell death was increased upon hydrogen peroxide-treatment [35], suggesting that Cygb protects cells against oxidative stress. In addition, Ngb expression was up-regulated following hypoxic challenges [54] and in ischemic-hypoxia brain injury [53]. Thus, Cygb that binds an oxygen (O_2) molecule may be induced under oxidative stress to protect cells from death.

Alpha-2-Heremans-Schmid (HS)-glycoprotein (Ahsg)

Ahsg was upregulated by 2.18-fold (Global M=1.125401). AHSG is a serum glycoprotein. ahsg-KO mice demonstrated impaired tolerance to ischemia suggesting that AHSG exerts a protective effect against ischemia in the cardiomyocyte [39,45]. Thus, upregulation of Ahsg is expected to protect cells from death under hypoxic stress.

Arachidonate 5-lipoxygenase (Alox5)

Alox5 was upregulated by 2-fold (Global M=1.049389). ALOX5 is a dual-function protein that converts arachidonate to 5-hydroperoxyeicosatetrenoic and subsequently to leukotriene A4 [44]. Tong *et al.* [56] showed that 5-LOX inhibitor Rev-5901 blocked cell proliferation in pacreactic cancer and induced apoptosis *in vivo* and *in vitro*. This results indicate that downstream products of ALOX5 stimulate cell proliferation and promote cell survival. Therefore, upregulation of ALox5 would help cells to survive in hypoxia.

Cell cycle-related genes

The up- or downregulated cell cycle-related genes are shown in Table 3. The erythroblastic leukemia viral oncogene homolog 2 (Erbb2), Vascular endothelial growth factor (Vegf) and Mitogen-activated protein kinase 12 (Mapk12) were most highly increased.

Erbb2

The Erbb2 gene was upregulated by 1.84-fold (Global M=0.8779643). The ErbB-2 · ErbB-3 dimer signals through the mitogen-activated protein kinase (MAPK) pathway, which stimulates cell proliferation. This dimer also signals through the PI3K/Akt pathway. This pathway promotes cellular survival and antiapoptotic signals [6]. Recently, it has been reported that ErbB2 expression increased TNF-induced apoptosis [62]. These results indicate that Erbb2 is involved in cell proliferation and survival. Therefore, upregulation of Erbb2 is beneficial for cellular survival.

Mapk12

The Mapk12 gene (also called Erk6 or SAPK3) was upregulated by 1.8-fold (Global M=0.8432047). Mapk12 responds to environmental stress and pro-inflammatory cytokines and phosphorylate downstream targets. c-jun is one of the downstream targets of Mapk12 [38]. Activation of c-jun stimulates cell proliferation. A stimulation to proliferate is expected to promote cell survival.

Summary

As indicated by MA-plot, most genes were up- or downregulated within 2-fold by RR water extracts. Antiapoptosis genes such as Tegt (2.4-fold), Nfkb1 (2.4-fold) Veg (1.8-fold), Ngfr (1.6-fold) were upregulated, while pro-apoptosis genes such as Bad (-64%), Cstb (-66%) were downregulated. Genes for combating environmental stress (stress response genes) such as Defb3 (2.7-fold), Cygb (2.2-fold), Ahsg (2.18-fold), Alox5 (2-fold) were upregulated. Genes for cell proliferation (cell cycle-related genes) such as Erbb2 (1.84-fold), Mapk12 gene (1.8-fold) was upregulated. Therefore, RR water extracts upregulate many pro-survival genes while downregulating many pro-death genes. It is interpreted that these genes, in combination with other regulated genes, can promote neuronal survival in a stress such as hypoxia.

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초록 : 배양신경세포의 저산소증모델에서 대황 물추출물에 의한 유전자 표현 변화의 microarray 분석

이현숙 · 송진영 · 문일수*
(동국대학교 의과대학 해부학교실)

대황(Rhei Rhizoma; RR, 大黃)은 *Rheum officinale* Baill.와 *Rheum palmatum* L. (Polygonaceae)의 땅속부분으로 남아시아의 민속의학에서 간 및 신장의 손상을 치료하는데 널리 이용되고 있다. 본 연구에서는 배양한 흰쥐 해마신경세포의 저산소증모델을 이용하여 대황의 물추출물이 유전자 표현에 미치는 영향을 microarray 방법을 이용하여 조사하였다. 배양 후 10일(DIV10)에 추출물을 배지에 2.5 μg/ml 농도로 첨가하고, DIV13에 저산소증(2% O_2/5% CO_2, 37°C, 3 h)을 유발한 후 24시간 후에 total RNA를 분리하여 microarray에 사용하였다. MA-plot에 의하면 표현이 변화된 대부분의 유전자는 ±2배 이내로 증감되었다. 이 가운데 Global M 값이 0.2(즉, 15%)보다 더 증가한 유전자는 472종, Global M 값이 -0.2(즉, -15%)보다 더 감소한 유전자는 725종이였다. 세포의 생존과 관련된 유전자 가운데 세포자연사 억제유전자인 Tegt (2.4배), Nfkb1 (2.4배), Veg (1.8배), Ngfr (1.6배) 등이 크게 증가하였으며, 반면에 자연사 촉진유전자인 Bad (-64%), Cstb (-66%)는 감소하였다. 스트레스를 극복하는데 필요한 유전자인 Defb3 (2.7배), Cygb (2.2배), Ahsg (2.18배), Alox5 (2배) 등도 크게 증가하였다. 그리고 세포 성장을 촉진하는 유전자인 Erbb2 (1.84배), Mapk12 (1.8배)도 크게 증가하였다. 따라서 대황의 물추출물은 세포생존에 필요한 유전자를 증가시키고, 세포사를 유도하는 유전자는 감소시킴으로서 저산소증 스트레스에서 신경세포의 사망을 억제하는 것으로 해석된다.