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Paeonol Attenuates H_2O_2-Induced NF-κB-Associated Amyloid Precursor Protein Expression

Shan-Yu Su,[*,§] Chin-Yi Cheng,[*] Tung-Hu Tsai,[//] Chien-Yun Hsiang,[†] Tin-Yun Ho[*,¶] and Ching-Liang Hsieh[‡,§]

[*]*School of Chinese Medicine*
[†]*Department of Microbiology*
[‡]*Graduate Institute of Acupuncture Science*
China Medical University
Taichung 40402, Taiwan

[§]*Department of Chinese Medicine*
[¶]*Nuclear Medicine and PET Center*
China Medical University Hospital
Taichung 40447, Taiwan

[//]*Institute of Traditional Medicine*
National Yang-Ming University, Taipei, Taiwan

Abstract: Hydrogen peroxide (H_2O_2) has been shown to promote neurodegeneration by inducing the activation of nuclear factor-κB (NF-κB). In this study, NF-κB activation was induced by H_2O_2 in human neuroblastoma SH-SY5Y cells. Whether paeonol, one of the phenolic phytochemicals isolated from the Chinese herb *Paeonia suffruticosa* Andrews (MC), would attenuate the H_2O_2-induced NF-κB activity was investigated. Western blot results showed that paeonol inhibited the phosphorylation of IκB and the translocation of NF-κB into the nucleus. The ability of paeonol to reduce DNA binding ability and suppress the H_2O_2-induced NF-κB activation was confirmed by an electrophoretic mobility shift assay and a luciferase reporter assay. Using a microarray combined with gene set analysis, we found that the suppression of NF-κB was associated with mature T cell up-regulated genes, the c-jun N-terminal kinase pathway, and two hypoxia-related gene sets, including the hypoxia up-regulated gene set and hypoxia inducible factor 1 targets. Moreover, using

Correspondence to: Dr. Ching-Liang Hsieh, Graduate Institute of Acupuncture Science, China Medical University, 91 Hsueh-Shih Road, Taichung 40402, Taiwan. Tel: (+886) 4-2206-3366 (ext. 3600), Fax: (+886) 4-2203-5191; E-mail: clhsieh@mail.cmuh.org.tw
Dr. Chien-Yun Hsiang, Department of Microbiology, China Medical University, 91 Hsueh-Shih Road, Taichung 40402, Taiwan. Tel: (+886) 4-2205-3366 (ext. 2163), Fax: (+886) 4-2205-3764, E-mail: cyhsiang@mail.cmu.edu.tw
Dr. Tin-Yun Ho, Graduate Institute of Chinese Medical Science, China Medical University, 91 Hsueh-Shih Road, Taichung 40402, Taiwan. Tel: (+886) 4-2205-3366 (ext. 3302), Fax: (+886) 4-2203-2295, E-mail: tyh@mail.cmu.edu.tw

network analysis to investigate genes that were altered by H_2O_2 and reversely regulated by paeonol, we found that NF-κB was the primary center of the network and amyloid precursor protein (APP) was the secondary center. Western blotting showed that paeonol inhibited APP at the protein level. In conclusion, our work suggests that paeonol down-regulates H_2O_2-induced NF-κB activity, as well as NF-κB-associated APP expression. Furthermore, the gene expression profile accompanying the suppression of NF-κB by paeonol was identified. The new gene set that can be targeted by paeonol provided a potential use for this drug and a possible pharmacological mechanism for other phenolic compounds that protect against oxidative-related injury.

Keywords: Paeonol; Hydrogen Peroxide; Amyloid Precursor Protein; Microarray; Nuclear Factor-κb.

Introduction

The accumulation of reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), is suspected to be a cause of many neurodegenerative diseases, including Alzheimer's disease (AD), Parkinson's disease, Huntington's disease and amyotrophic lateral sclerosis (Bogdanov *et al.*, 1998; Cookson and Shaw, 1999; Behl, 1999). High doses of H_2O_2 cause severe damages to lipids, proteins, and nucleic acids, which directly induces cell death. However, subacute doses of H_2O_2 act as a signaling molecule modulating the activity of redoxsensitive transcription factors such as nuclear factor-κB (NF-κB) (Brown and Griendling, 2009; Bonello *et al.*, 2007). NF-κB is a transcription factor that is regulated by cellular stimulants, including pro-inflammatory cytokines, UV irradiation, and oxidants (Gao *et al.*, 2002). In vertebrates, the NF-κB family is comprised of five members, c-Rel, p65, RelB, p50, and p52. These members associate in homo- or heterodimers (most commonly the p50–p65 dimer) and control transcription by binding to NF-κB sites in the promoter or enhancer regions of their target genes. NF-κB dimers are expressed in a latent form bound to an inhibitory protein of the IκB family. This inactive form prevents their binding to DNA and facilitates their retention in the cytoplasm (Oliver *et al.*, 2009). NF-κB becomes activated after the phosphorylated IκB is degraded. The NF-κB protein is then translocated into the nucleus where it binds to a NF-κB specific DNA sequence (Schoonbroodt *et al.*, 2000). NF-κB regulates a variety of down-stream genes that code for cytokines, receptors, metabolism enzymes, detoxification enzymes, energetic enzymes, apoptotic enzymes, and other transcription factors (Gosselin and Abbadie, 2003). In the nervous system, NF-κB mediates neurodegeneration caused either by neurotoxins or during the development of ROS-associated and age-associated diseases (Braun *et al.*, 2009; Tan *et al.*, 2008). Activated NF-κB also has been shown to be overexpressed in the brains of AD patients (Boissiere *et al.*, 1997).

Since NF-κB plays an important role in oxidative stress-induced neuronal damage, antioxidants that can cause a reduction in NF-κB are considered as candidates to prevent or treat ROS-associated neuronal diseases (Pratico *et al.*, 2008). Phenolic compounds, a group of phytochemicals that exist in a variety of herbs, are well-known for their antioxidative

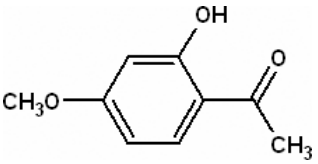


Figure 1. The chemical structure of paeonol.

abilities (Manach *et al.*, 2005; Cheng *et al.*, 2008). Paeonol (2′-hydroxy-4′-methoxyacetophenone) (Fig. 1) is one of the phenolic compounds isolated from the Chinese herbs *Paeonia suffruticosa* Andrews (MC) and *Paeonia lactiflora* Pall (PL). Both herbs are traditionally used as anti-inflammatory and sedative medicines to treat inflammation-associated allergic rhinitis, otitis and appendicitis, as well as to relieve hypertensive nerve-related insomnia, pain, and vexation (Chang and But, 1986; Huang *et al.*, 2009). Paeonol has been shown to increase superoxide dismutase (SOD) and glutathione (GSH) levels (Zhong *et al.*, 2009). Paoenol possesses a neuro-protective effect in cerebral artery occlusion and in ischemia-reperfusion models (Chen *et al.*, 2001; Mi *et al.*, 2005). Furthermore, paeonol attenuates neurotoxicity and ameliorates cognitive impairment induced by D-galactose, which generates superoxide anion and oxygen-derived free radicals (Zhong *et al.*, 2009). Paeonol also protects neuronal cells from inflammation, which is commonly caused by ROS (Nizamutdinova *et al.*, 2007; Hsieh *et al.*, 2006).

In this study, we aimed to investigate the effect of paeonol on H_2O_2-induced activation of NF-κB in human neuroblastoma SH-SY5Y cells, and to find the genes that were altered by paeonol in H_2O_2-treated cells by analyzing gene expression profiles using oligonucleotide DNA microarrays. The enriched genes were further analyzed to identify the possible pathway and network that paeonol might act through. We found that NF-κB is the primary center and amyloid precursor protein (APP) is the secondary center in the network of paeonol-regulated genes. Therefore, Western blot analysis was further used to demonstrate the regulation of APP protein by paeonol.

Methods and Materials

Cell Culture

SH-SY5Y neuroblastoma cells were kindly provided by Dr. Ching-Ju Lin (China Medical University, Taichung). Cells were maintained in RPMI-1640 medium (HyClone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS) (HyClone, Logan, UT, USA) and penicillin-streptomycin and incubated at 37°C in a humidified atmosphere with 5% CO_2. The medium was routinely changed every 2–3 days and the cells were subcultured using trypsin-EDTA solution when the cells reached a 90% confluence.

Drug Treatments

Paeonol was isolated from *Paeonia suffruticosa* as previously described (Hsieh *et al.*, 2006). For the induction of NF-κB activation, H_2O_2 was diluted with serum-free medium to reach

final concentrations ranging from 0.1 to 250 μM. A luciferase assay was performed 24 hours after a 24-hour H_2O_2 treatment. Paeonol was dissolved in dimethyl sulfoxide to a concentration of 0.4 M and stored at -30°C. SH-SY5Y cells were seeded in 25-cm^2 tissue culture flasks. After incubation for 16 –18 hours, SH-SY5Y cells were treated with various concentrations of paeonol 1 hour before H_2O_2 (5 μM) exposure. For the microarray, Western blotting for APP, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and the luciferase reporter assays, the cells were harvested 24 hours after H_2O_2 treatment. For the electrophoretic mobility shift assay (EMSA), Western blotting for NF-κB, IκBα, and phospho-IκBα, the nuclear and cytoplasmic proteins were obtained 4 hours after H_2O_2 treatment (Ha *et al.*, 2006). The preparations of nuclear protein extracts and cytoplasmic protein extracts have been described previously (Hsiang *et al.*, 2002).

Construction of SH-SY5Y/NF-κB Recombinant Cells

Plasmid pNF-κB-Luc, containing a nuclear factor-κB (NF-κB)-responsive element-driven luciferase reporter gene, was purchased from Stratagene (La Jolla, CA, USA). SH-SY5Y cells were transfected with 2.5 μg of *Alw*NI-linearized pNF-κB-Luc DNA and *Eco*RI-linearized pSV3-neo DNA by a SuperFect® transfection reagent (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Twenty-four hours later, the cells were selected by using 600 μg/ml G-418. The clones showing the highest luciferase activity were selected and designated as SH-SY5Y/NF-κB. The cell line was maintained in RPMI-1640 supplemented with 10% FBS and 600 μg/ml G-418.

MTT Assay

The viability of SH-SY5Y/NF-κB cells was monitored by a MTT colorimetric assay. Cells were cultivated in 96-well culture plates. After a 16–18-hour incubation at 37°C, cells were treated with various concentrations of paeonol 1 hour before H_2O_2 treatment. After incubation for another 24 hours, a one-tenth volume of 5 mg/ml MTT was added to the culture medium. After a 4-hour incubation, an equal volume of 0.04 N HCl in isopropanol was added to dissolve the MTT formazan. The absorbance value was measured at 570 nm using a microplate reader. Cell survival was calculated by (optical density of paeonol-treated cells/optical density of solvent-treated cells). The 50% toxicity concentration (TC_{50}) was defined as the H_2O_2 concentration that resulted in a 50% reduction in survival relative to the untreated cells and calculated using an interpolation method.

Luciferase Reporter Assay

Cells were washed with ice-cold phosphate-buffered saline (137 mM NaCl, 1.4 mM KH_2PO_4, 4.3 mM Na_2HPO_4, 2.7 mM KCl, pH 7.2), lysed with Triton lysis buffer (50 mM Tris-HCl, 1% Triton X-100, 1 mM dithiothreitol, pH 7.8), and centrifuged at $12,000 \times g$ for 2 min at 4°C. The luciferase activity was measured as described previously (Cheng *et al.*, 2009). Relative luciferase activity was calculated by dividing the relative luciferase unit (RLU) of treated cells by the RLU of untreated cells.

Western Blotting Analysis

SH-SY5Y cells were pre-treated with paeonol for 1 hour followed by H_2O_2 exposure. The protein extracts (10 μg) were then separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, probed with antibodies, and detected with the peroxidase-conjugated anti-rabbit antibody followed by chemiluminescence, as described previously (Hsiang *et al.*, 2005). Antibodies against APP and β-actin were purchased from Chemicon (Billerica, MA, USA); antibodies against p65, IκB, and phospho-IκB were purchased from Cell Signaling (Beverly, MA, USA). The intensities of bands on the gel were calculated by Gel-Pro® Analyzer (Media Cybernetics Inc., Bethesda, MD, USA).

Biotinylated EMSA

The biotin-labeled complementary oligonucleotides corresponding to the NF-κB-binding consensus sequence (5′-AGT TGA GGG G AC TTTC CCA GGC-3′) were annealed by heating to 90°C for 3 min and cooling slowly to 45°C. EMSA was performed as described previously (Hsiang *et al.*, 2005). Briefly, the nuclear extract (10 μg) was incubated in binding buffer at 25°C for 30 min with double-stranded oligonucleotide probes. After electrophoresis, protein bands were transferred to nylon membranes. Membranes were blocked in blocking solution and detected with alkaline phosphatase-conjugated streptavidin (Chemicon, Australia) followed by chemiluminescence (Roche, Germany).

Total RNA Isolation

Total RNA was extracted from two independent plates using an RNeasy Mini kit (Qiagen, Valencia, CA, USA) and quantified by using a Beckman DU800 spectrophotometer (Beckman Coulter, Fullerton, CA, USA). Samples with A260/A280 ratios greater than 1.8 were further evaluated using an Agilent 2100 bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Samples with RNA integrity numbers greater than 8.0 were pooled together and used for microarray analysis.

Microarray Analysis

Microarray analysis was performed as described previously (Hsiang *et al.*, 2009). Briefly, fluorescent cDNA targets were prepared from 20 μg of total RNA samples using the Amino Allyl cDNA Labeling Kit (Ambion, Austin, TX, USA) and Cy3/Cy5 dyes (Amersham Pharmacia, Piscataway, NJ, USA). The signal intensity of spots that passed these criteria were normalized and tested for differential expressions by the limma package of the R program (Smyth, 2005). For pathway analysis, the enriched genes were analyzed by the R program according to database C2 in the Gene Set Enrichment Analysis (GSEA) (http://www.broad.mit.edu/gsea/). (Mootha *et al.*, 2003). The "GeneSetTest" function

implemented in the limma package of the R program was used to test the significantly altered gene sets by the Wilcoxon test (Hsiang *et al.*, 2009). Furthermore, we constructed interaction networks of differentially expressed genes using BiblioSphere Pathway Edition software (Genomatix Applications, http://www.genomatix.de/index.html), that integrates literature mining and annotation analysis with promoter sequence analysis (Seifert *et al.*, 2005). The number of replicates for both single dye and dual dye was two.

Statistical Analysis

Data are expressed as mean ± SEM. Data in different groups were compared using one-way ANOVA followed by a post-hoc Duncan's test. A probability value less than 0.05 was considered statistically significant.

Results

Induction of NF-κB Activity by H_2O_2 in SH-SY5Y Cells

The induction of NF-κB activity by H_2O_2 in SH-SY5Y/NF-κB cells was assessed by a luciferase assay. In order to find the concentrations of H_2O_2 that lead to the maximum induction rate of NF-κB, cells were exposed to various concentrations of H_2O_2 for 24 hours. Each dose of H_2O_2 was plated in two plates, one for MTT assay and the other for luciferase assay. As shown in Fig. 2, the luciferase activity was increased in a dose-dependent manner without changing cell viability when exposed to lower doses of H_2O_2 (0.1 μM–5 μM). The luciferase activity in cells treated with 5 μM H_2O_2 was 1.5 fold higher than the control. When the concentration of H_2O_2 was greater than 10 μM, luciferase activity declined. Under the high doses of H_2O_2 exposure, cell viability was decreased

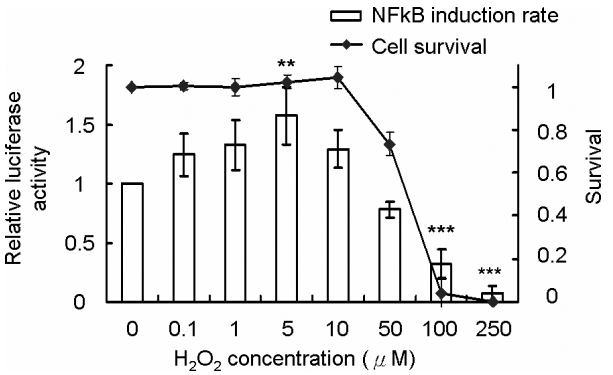


Figure 2. Activation of NF-κB activity by H_2O_2 in SH-SY5Y/NF-κB cells. SH-SY5Y/NF-κB cells were treated with various concentrations of H_2O_2 for 24 hours. The NF-κB activity was measured by a luciferase assay and the cell viability was accessed by MTT assay. **$p < 0.01$ and ***$p < 0.001$ compared to 0 μM of H_2O_2 for luciferase activity. Values were mean ± SD of three independent assays.

when H_2O_2 was greater than 50 μM. The TC_{50} of H_2O_2 was approximately 68 μM. Five μM H_2O_2 was chosen to induce the maximum NF-κB activity in the following experiments.

The Effect of Paeonol on H_2O_2-Induced IκB Phosphorylation and NF-κB Translocation

The effects of paeonol on IκB phosphorylation and the translocation of NF-κB protein were determined by Western blotting. Nuclear and cytoplasmic protein extracts were prepared after a 1-hour pre-treatment with paeonol and a 4-hour exposure to H_2O_2. The protein levels of NF-κB in the nucleus and cytoplasm were examined by anti-p65 antibody. The level of phospho-IκB in the cytoplasm was measured by anti-phospho-IκB antibody. In cells treated with H_2O_2, there was an increase of phosphorylated IκB in the cytoplasm and an increase of NF-κB protein in the nucleus (Fig. 3, lane 2). When cells were pre-treated with paeonol, both the phospho-IκB and the protein level of NF-κB in nucleus were decreased, implying that paeonol inhibited the H_2O_2-induced phosphorylation of IκB and the nuclear translocation of NF-κB (Fig. 3, lanes 3–5).

The Effect of Paeonol on H_2O_2-Induced NF-κB Activation

NF-κB activity was first measured by the luciferase reporter assay. SH-SY5Y/NF-κB cells were treated with various amounts of paeonol for 1 hour prior to 5 μM H_2O_2 exposure. As shown in Fig. 4A, paoenol reduced the H_2O_2-induced luciferase activity in a

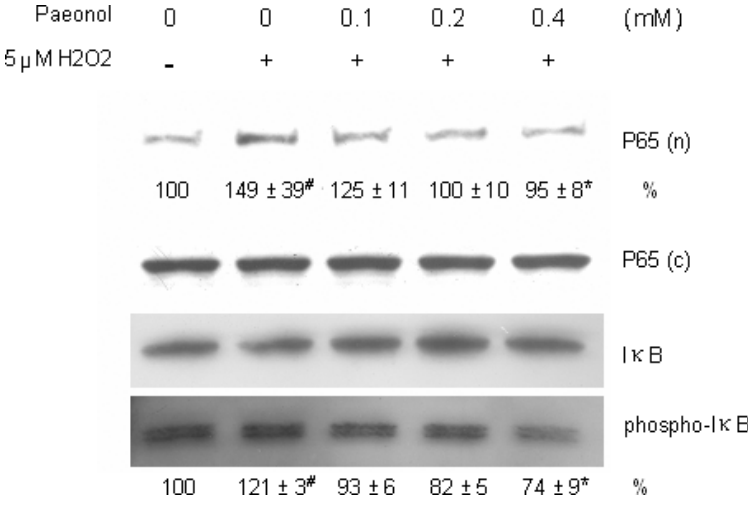
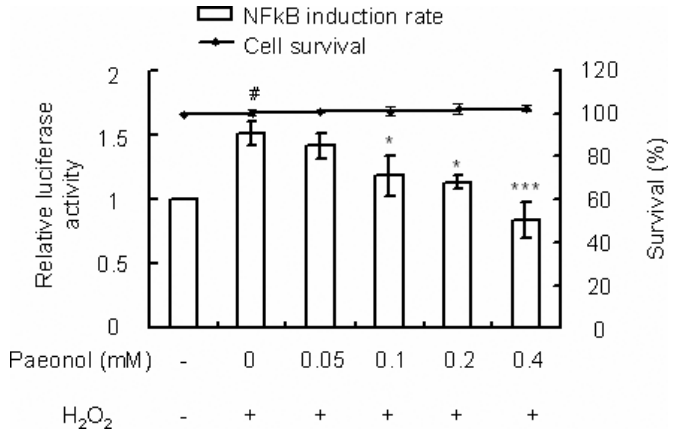
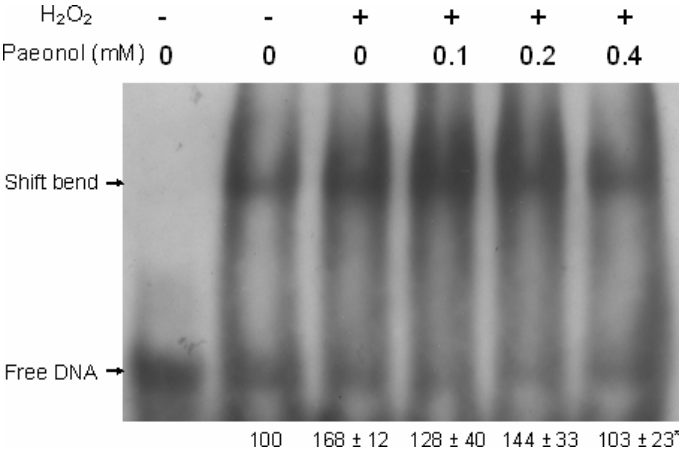


Figure 3. Inhibition of H_2O_2-induced IκB phosphorylation and NF-κB nuclear translocation by paeonol. Data are shown as mean $\pm$ SEM of three independent assays. The percentage below each lane of p65 (n) represents the amount of p65 protein in the nucleus, and the percentage below each lane of phospho-IκB represents the amount of phospho-IκB relative to unexposed cells. #$p < 0.05$ compared to the untreated cells; *$p < 0.05$ compared to H_2O_2-treated cells.



(A)



(B)

Figure 4. Inhibition of H_2O_2-induced NF-κB activity by paeonol in SH-SY5Y/NF-κB cells. (A) Luciferase reporter assay. (B) Biotinylated EMSA. SH-SY5Y/NF-κB cells were treated with paeonol for 1 hour and then exposed to 5 μM H_2O_2. The percentage at the bottom represents the amount of shifted probes relative to the untreated cells. Data are shown as mean $\pm$ SEM of three independent assays. #$p < 0.05$ compared to the untreated cells. *$p < 0.05$, and ***$p < 0.001$ compared to H_2O_2-treated cells.

dose-dependent manner. Paeonol, at doses up to 0.4 mM, did not alter the viability of SH-SY5Y/NF-κB cells. This data suggested that paeonol inhibited H_2O_2-induced NF-κB activation in neuroblastoma cells.

The effect of paeonol on the DNA binding activity of NF-κB was measured by EMSA. The nuclear extract was incubated with biotin-labeled oligonucleotides containing the NF-κB-binding consensus sequence. SH-SY5Y/NF-κB cells showed an increase in DNA binding activity by 1.68 fold 4 hours after H_2O_2 treatment (Fig. 4B, lane 3). Pre-treatment of paeonol decreased the H_2O_2-induced NF-κB binding activity (Fig. 4B, lanes 4–6).

Identification of Differentially Expressed Genes by Oligonucleotide DNA Microarrays

In order to profile the effects of paeonol on the gene expression accompanying NF-κB suppression under H_2O_2 exposure, SH-SY5Y cells were pre-treated with paeonol (0.4 mM) for 1 hour and then exposed to H_2O_2 for 24 hours. The microarray data was analyzed by the limma package within the bio-conductor program to examine the differentially expressed genes in cells treated with H_2O_2 and in cells pre-treated with paeonol before H_2O_2 exposure. Fold change greater than 1.8 was used as the cut-off point to choose the represented number of genes. In a total of 30,968 transcripts, 2789 transcripts, with fold changes greater than 1.8 in SH-SY5Y cells, were altered by H_2O_2. These genes include 1193 up-regulated and 1596 down-regulated transcripts. Among these 2789 transcripts, 778 transcripts were reversely regulated by paeonol, with fold changes greater than 1.8.

Gene Set Analysis and Gene Interaction Network Analysis of Paeonol-Regulated Genes

GSEA was used to identify and compare gene sets in paeonol-treated SH-SY5Y cells with those in cells exposed to H_2O_2 only. Twenty-one gene sets were identified ($p < 0.01$) (Supplementary information Table S1) and the top 10 gene sets highly altered by paeonol are listed in Table 1. The mature T cell-up-regulated gene set was the most significantly altered gene set by paeonol. Moreover, the hypoxia up-regulated gene set and HIF1 (hypoxia-inducible factor 1) targets were associated with hypoxia (Semenza, 2001; Leonard *et al.*, 2003), and the c-jun N-terminal kinase (JNK) pathway was highly related to environmental stress, including oxidation, ischemia, and ultraviolet radiation (Forman and Torres, 2002).

The relationship between the 2789 differentially expressed genes regulated by paeonol was analyzed by network analysis, which revealed that NF-κB was the primary center of

Table 1. Gene Sets Significantly Altered by Paeonol Treatment

Gene Set	Gene Number	p Value
Mature T cell up-regulated	106	0.0014
Fbw7 pathway	9	0.0019
Up-regulated during EMT induction	62	0.0022
Brain highest variance	45	0.0028
LIF down-regulated	29	0.0037
Hypoxia up-regulated	38	0.0037
Skp2 E2F pathway	10	0.0049
HIF1 targets	36	0.0056
Linoleic acid metabolism	31	0.0059
JNK down-regulated	33	0.0060

EMT: Epithelial-to-mesenchymal transition; LIF: leukemia inhibitory factor; HIF1: hypoxia-inducible factor 1; JNK: c-jun N-terminal kinase.

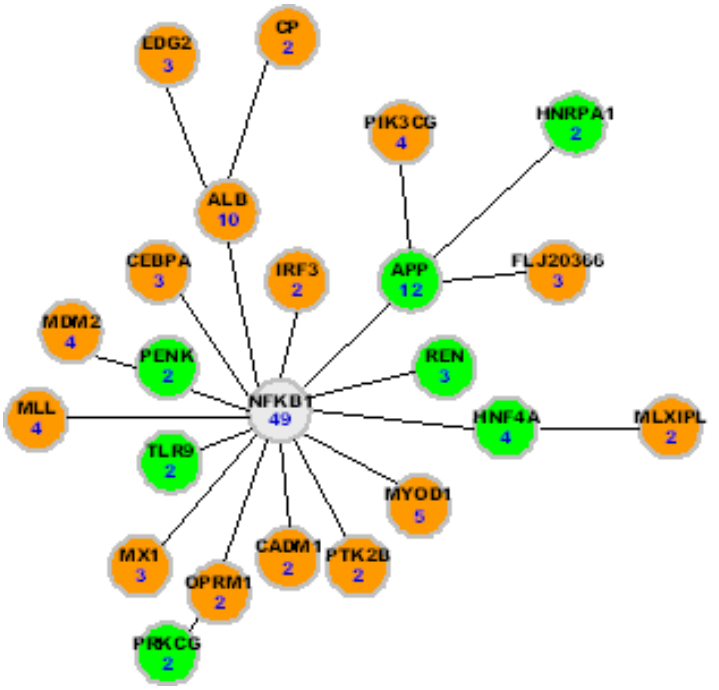


Figure 5. Network skeleton of genes altered by H_2O_2 and reversely regulated by paeonol. Differentially expressed genes were analyzed by BiblioSphere Pathway Edition software. Genes up-regulated by paeonol are colored orange and down-regulated genes are colored green. The number below a gene name represents the number of genes connected to it.

the network (Supplementary information Fig. S1 and Fig. 5). β-amyloid precursor protein (APP), which was up-regulated by H_2O_2 by 1.90-fold and down-regulated by paeonol by 1.94-fold, formed the secondary center of the network. Forty nine genes were directly linked to NF-κB in the network (Supplementary information Table S2). The top 10 genes that were differentially altered by paeonol included five down-regulated genes (myxovirus resistance 1 (MX1), TIR domain containing adaptor (TICAM1), thrombospondin 1 (THBS1), interleukin 16 (IL16), and opioid receptor, mu 1 (OPRM1)), and five up-regulated genes (toll-like receptor 9 (TLR9), renin (REN), contactin 2 (CNTN2), metabotropic glutamate receptor 3 (GRM3), and keratin 5 (KRT5)) (Table 2).

Confirmation of Paeonol-Reduced APP Protein Expression

APP, which formed the secondary center in the network analysis, is a precursor protein of β-amyloid, which is considered an important hallmark protein in Alzheimer's disease (Chong *et al.*, 2005). Therefore, the change of APP protein expression was examined using Western blotting. SH-SY5Y cells were treated with various concentrations of paeonol for 1 hour, and then incubated with 5 μM of H_2O_2 for 24 hours. Western blotting showed that

Table 2. Top Ten Genes Altered by Paeonol and Associated with NF-κB by Network Analysis

Gene Name	Gene Description	Fold Change	
		By H_2O_2	By Paeonol
MX1	Myxovirus resistance 1	–5.88	16.50
TICAM1	TIR containing adaptor inducing interferon-β	–2.00	5.88
THBS1	Thrombospondin 1	–2.67	5.67
IL16	Interleukin 16	–3.67	5.00
OPRM1	Opioid receptor Mu 1	–2.67	5.00
KRT5	Keratin 5	1.93	–3.73
GRM3	Glutamate receptor, metabotropic 3	2.37	–3.80
CNTN2	Contactin 2	2.07	–5.09
REN	Renin	10.60	–10.60
TLR9	Toll-like receptor 9	8.91	–12.25

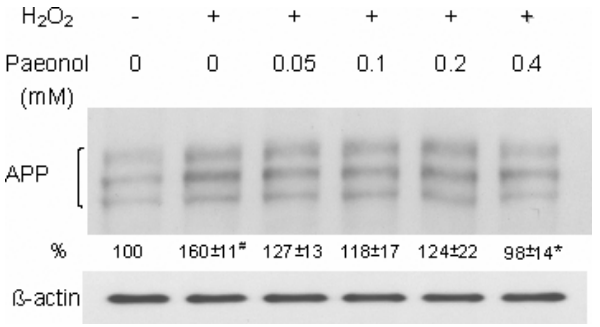


Figure 6. The effect of paeonol on the expression of H_2O_2-induced APP in SH-SY5Y. The percentage below each lane represents the relative APP level compared to the untreated group. Values are mean ± SEM of three independent assays. #$p < 0.05$ compared to the untreated cells; *$p < 0.05$ compared to H_2O_2-treated cells.

H_2O_2 treatment increased the expression of APP by 1.6-fold (Fig. 6, lanes 1 and 2). The induction of APP by H_2O_2 was diminished when paeonol was added 1 hour before H_2O_2 exposure (Fig. 6, lanes 2–5). The levels of APP protein were reduced to baseline after pre-treatment with 0.4 mM paeonol.

Discussion

H_2O_2 is a general and important ROS in the nervous system and it has been shown that H_2O_2 promotes NF-κB activation (Marshall *et al.*, 2000). However, the activation of NF-κB by H_2O_2 is cell-specific and concentration-specific (Bowie and O'Neill, 2000). In our study, H_2O_2 activated NF-κB activity in SH-SY5Y cells in a narrow range of physiologically relevant doses (1–10 μM) without inducing cytotoxic effects. In SH-SY5Y cells, the NF-κB activation is observed both during an exposure of a nearly-lethal dose of

H_2O_2 (100 μM) (Larouche *et al.*, 2008) and at doses less than 10 μM (Zhen *et al.*, 2008). The effect of a low-dose of H_2O_2 on the NF-κB pathway has also been reported in an *in vitro* reconstitution experiment, which demonstrates that NF-κB-inducing kinase (NIK) is activated by a narrow range of H_2O_2 (1–10 μM) (Li and Engelhardt, 2006). Lethal and non-lethal doses of H_2O_2 appear to induce different signals in cells. A lethal dose of H_2O_2 stimulates the protein expressions of ERK1 (p42)/2 (p44), JNK, and PKB (Ruffels *et al.*, 2004), and mRNA expressions of c-jun, c-fos, MAPK phosphatase-1 (Guyton *et al.*, 1996), as well as activates NFκB (Marshall *et al.*, 2000). On the other hand, non-lethal levels of H_2O_2 have been shown to activate signaling cascades that mediate the responses to cytokines, hormones, and physical and chemical stresses (Bonello *et al.*, 2007). Non-lethal levels of H_2O_2 also induce intracellular calcium concentration (Hong *et al.*, 2006) and NF-κB-dependent activation of Akt (Sen *et al.*, 2006), hyposiainducible factor-1 (Bonello *et al.*, 2007), and endothelial nitric oxide (Zhen *et al.*, 2008).

In this study, we demonstrate that paeonol could reduce the expression of NFκB-target genes by a luciferase reporter assay, and could suppress the binding of NFκB to its DNA binding sequence by EMSA. Moreover, our Western blotting suggested that paeonol inhibits NFκB activity even from the earlier process, because paeonol attenuates the level of IκB phosphorylation and the amount of NFκB protein that translocates into the nucleus. Previous studies also reported that paeonol suppress NFκB activity induced by tumor necrosis factor-α, trinitrobenzene sulfonic acid, and a high fat diet (Ishiguro *et al.*, 2006; Li *et al.*, 2009; Tsai *et al.*, 2008; Nizamutdinova *et al.*, 2007). Our study is the first time it was demonstrated that paeonol suppressed NFκB induced by an oxidative condition. We also first measured the paeonol-regulated transcription profile accompanying NFκB suppression, and designated possible pathways and networks that paeonol could possibly act through.

The DNA microarray data with gene set analysis revealed that among the top 10 gene sets altered by paeonol, the mature T cell-up-regulated gene set, comprised of genes that are differentially expressed in mature T cells, was the most significantly altered (Balduini *et al.*, 2006). Moreover, hypoxia up-regulated and HIF1 target genes, those comprised of genes that mediate adaptive response to reduced oxygen availability, were also altered by paeonol (Leonard *et al.*, 2003; Semenza, 2001). HIF1 is a transcription factor activated by low oxygen tension and also modulated by oxidative stress, such as H_2O_2 (Millonig *et al.*, 2009). Furthermore, genes that are related to JNK pathway were also regulated by paeonol. The activities of JNK and HIF1 have been shown to be related to NF-κB. JNK is one of the upstream modulators of NF-κB, and has been shown to be activated almost simultaneously with NF-κB by several environmental stressors (Zhang *et al.*, 2004). Moreover, some of the hypoxia-related genes are also regulated by NF-κB indirectly because the HIF1 promoter is responsive to NF-κB subunits (van Uden *et al.*, 2008). HIF1, the transcription factor activated by hypoxia, binds to the hypoxia-response elements in the promoter region and regulates the expression these hypoxia-related genes (Leonard *et al.*, 2003). Our data suggested that paeonol regulated JNK-related NF-κB activation and modulated NF-κB-associated hypoxia-response and inflammation-related genes. Previous studies have shown that many phenolic compounds from natural products, including curcumin, resveratrol,

anethole and flavonoids, exert their biological functions via suppressing both the NF-κB and JNK pathways (Aggarwal and Shishodia, 2006).

Using network analysis, we found that NF-κB formed the primary center of the network, even though NF-κB itself was not one of the input genes. This implies that paeonol does not alter the expression of the NF-κB gene, but regulates NF-κB activity. Among the top 10 genes that were primarily associated with NF-κB in the network, MX1, TICAM1 (TRIF), THBS1, IL16, and OPRM1 were down-regulatd by paeonol, and TLR9, REN, CNTN2, GRM3, and KRT5 were up-regulated. These 10 NF-κB-associated genes can be roughly divided into two groups. The first one is inflammatory-related genes, including MTX, TICAM1, IL-16, and TLR9. MTX codes for a myxovirus resistance protein that is induced by an interferon and protects against viral infection (Haller *et al.*, 2007). IL-16, a microglial-produced chemoactive cytokine, might contribute to the chemoattraction of pathogenic CD4 lymphocytes across the blood-brain barrier (Schluesener *et al.*, 1996). TLR9 is one of the toll-like receptors responsible for innate immunity. Extracellular microbial pathogens activate NF-κB via TLR signals (Letiembre *et al.*, 2007). TICAM1 is a toll-like receptor adaptor molecular recruited by toll-like receptor 3 to induce interferon beta in defense against microbial pathogens (Seya *et al.*, 2009). The other group was the neuronal function-related genes, including OPRM1, CNTN2, and GRM3. THBS1, thrombospondin, functions in postnatal neuronal migration (Blake *et al.*, 2008) and has been demonstrated to be necessary for synaptic plasticity and functional recovery after stroke (Liauw *et al.*, 2008). OPRM1 is an opioid receptor that is correlated with mechanical allodynia after nerve injury (Back *et al.*, 2006). CNTN2, the immunoglobulin contactin 2, functions in the maintenance of the myelinated neurons (Shimoda and Watanabe, 2009). GRM3 codes for a glutamate receptor, whose ligand is the major excitatory neuro-transmitter in the brain (Reis *et al.*, 2009). Based on the results of the gene set analysis, we speculate that paeonol regulated inflammatory-, hypoxia-, and neuronal function-related genes. The NF-κB-associated genes altered by paeonol in our study might form a new gene set, which could possibly provide a database of phenolic compound-regulated genes under an oxidative environment.

According to the network analysis, APP formed the secondary center of the network, implying that paeonol might participate in the expression of regulation of APP and APP-associated genes. Therefore, we further analyzed whether paeonol alters the protein expression of APP and found that it not only down-regulated APP at the transcription level, but also at the translation level. Resveratrol and curcumin are two other phenolic compounds that have been shown to reduce APP at the mRNA and protein levels (Lin *et al.*, 2008; Tang and Chua, 2008). Resveratrol has been shown to reduce β-amyloid levels as well (Marambaud *et al.*, 2005). Curcumin and resveratrol attenuate APP and β-amyloid levels by promoting its clearance. However, none of those studies proposed possible pathways through which these phenolics might participate. In this study, we found that the suppression of APP by paeonol was coincident with the suppression of NF-κB, implying that paeonol might suppress APP level via the NF-κB pathway. Actually, the induction of APP by NF-κB has been reported. It has been shown metal ion, CD40, IL-1β, or glutamate activated NF-κB can activate APP expression (Grilli *et al.*, 1996; Ait-Ghezala *et al.*, 2007;

Walton and Wang, 2009). This might be because the APP gene contains the NF-κB binding sequence in its promoter region and 5′-regulatory region (Grilli *et al.*, 1996; Song and Lahiri, 1998).

Our findings also provided an overall view for the effects of paeonol, a component of *Paeonia suffruticosa* Andrews and *Paeonia lactiflora* Pall. Both herbs have been shown to exhibit anti-inflammatory effects for the treatment of various symptoms with "blood heat" and sedative effects to either "relieve pain" or "eliminate vexation" (Bansky *et al.*, 2004). Results of this study showed that the anti-inflammatory effects of both herbs might be attributed to their ability to suppress inflammatory-related gene sets, including the mature T cell-up-regulated, up-regulated during EMT induction, and the LIF down-regulated gene sets. On the other hand, their sedative effect might be due to the regulation of neuronal function-related genes, including an opioid receptor, thrombospondin, contactin 2, and glutamate receptor. In addition to the classical use of *Paeonia suffruticosa* Andrews and *Paeonia lactiflora* Pall, our study provided evidence that suggests both herbs could be used to treat oxidative stress-associated inflammatory neuronal diseases, including Alzheimer's disease, Parkinson's disease, and Huntington's disease (Glass *et al.*, 2010). Paeonol down-regulated APP expression, implying that *Paeonia suffruticosa* Andrews and *Paeonia lactiflora* Pall might reduce the production of β-amyloid and, therefore, might prevent and treat Alzheimer's disease.

In conclusion, our work demonstrates that paeonol inhibited the activity of NF-κB induced by H_2O_2 in SH-SY5Y cells, as well as the expression of NF-κB-related genes. Furthermore, a group of differentially expressed genes that were regulated by paeonol were identified. These genes might comprise a potent new anti-oxidative gene set with which to test the protective effects of phenolic compounds against oxidation-related injury. Moreover, our study suggests that paeonol reduces the production of APP via suppressing NF-κB. Further study is needed to investigate the effects paeonol on the processing of APP and memory function in animal models.

Acknowledgments

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Supplementary Information

Table S1. The Significantly Altered Gene Sets by Paeonol Treatment

Index	Gene Set Name in GSEA	Gene Set Description	Gene Number	p Value
941	LEE_TCELLS3_UP	Mature T cell up-regulated	106	0.0014
431	FBW7PATHWAY	Fbw7 pathway	9	0.0019
1344	EMT_UP	Genes up-regulated during EMT induction	62	0.0022
353	CHESLER_BRAIN_HIGHEST_VARIANCE_GENES	Brain highest variance	45	0.0028
791	ABBUD_LIF_DN	LIF down-regulation	29	0.0037
1268	HYPOXIA_REG_UP	Hypoxia up-regulation	38	0.0037
60	SKP2E2FPATHWAY	Skp2 E2F pathway	10	0.0049
1539	HIF1_TARGETS	HIF1 targets	36	0.0056
1759	HSA00591_LINOLEIC_ACID_METABOLISM	Linoleic acid metabolism	31	0.0059
1611	JNK_DN	JNK down-regulation	33	0.0060
1419	SERUM_FIBROBLAST_CELLCYCLE	Cell-cycle dependent	138	0.0062
493	ACETAMINOPHENPATHWAY	Acetaminophen pathway	6	0.0068
1313	GN_CAMP_GRANULOSA_DN	Gonadotropins down-regulated	61	0.0078
77	PASSERINI_APOPTOSIS	Cell adhesion-related	43	0.0085
1086	CIS_RESIST_LUNG_UP	Cisplatin resistant down-regulated	11	0.0091
978	XU_CBP_DN	Down-regulated in CBP null B cells	55	0.0091
938	LINDSTEDT_DEND_UP	Up-regulated in dendritic cells	52	0.0092
790	SCHRAETS_MLL_UP	Up-regulated in mixed lineage leukemia	40	0.0092
1258	HDACI_COLON_CUR24HRS_DN	Down-regulated by curcumin	25	0.0095
24	KENNY_WNT_UP	Up-regulated by Wnt	51	0.0099
194	JECHLINGER_EMT_UP	Up-regulated for epithelial plasticity in tumor progression	57	0.0099

EMT: Epithelial-to-mesenchymal transition; LIF: leukemia inhibitory factor; HIF1: hypoxia-inducible factor 1; JNK: c-jun N-terminal kinase; CBP: crebbp-binding protein.

Table S2. Genes Primarily Associated with NF-κB by Network Analysis

GenBank Accession Number	Gene Name	Gene Description	Fold Change	
			By H_2O_2	By Paeonol
NM_002462.2	MX1	Myxovirus resistance 1	–5.88	16.50
NM_014261.1	TICAM1	TIR domain containing adaptor inducing interferon-beta	–2.00	5.88
NM_003246.2	THBS1	Thrombospondin 1	–2.67	5.67
NM_004513.3	IL16	Interleukin 16	–3.67	5.00
NM_000914.1	OPRM1	Opioid receptor Mu 1	–2.67	5.00
NM_152787.2	MAP3K7IP3	TAK1-binding protein 3	–5.34	3.67
NM_006044.2	HDAC6	Histone deacetylase 6	–2.67	3.67
BC042835	CXCL14	Chemokine (C-X-C motif) ligand 14	–3.67	3.67
AK091563	PENK	Proenkephalin	–1.64	3.09
NM_020639.2	RIPK4	Receptor-interacting serine-threonine kinase 4	–2.57	2.79
NM_002392.2	MDM2	Mdm2 transformed 3T3 cell double minute 2	–4.67	2.67
NM_153477.1	UXT	Ubiquitously-expressed transcript	–3.67	2.67
BF509092	NPPB	Natriuretic peptide precursor B	–2.67	2.67
AF130077	ALB	Albumin	–1.83	2.50
CA311890	SCGB1A1	Secretoglobin, family 1A, member 1	–1.83	2.50
NM_006691.2	LYVE1	Extracellular link domain containing 1	–2.33	2.50
NM_000765.2	CYP3A7	Cytochrome P450, family 3, subfamily A, polypeptide 7	–2.33	2.50
NM_004103.3	PTK2B	Protein tyrosine kinase 2 beta	–2.00	2.37
NM_001192.2	TNFRSF17	Tumor necrosis factor receptor superfamily, member 17	–2.14	2.27
NM_014333.2	CADM1	Immunoglobulin superfamily, member 4	–2.24	2.24
NM_004364.2	CEBPA	CCAAT/enhancer binding protein alpha	–2.75	2.13
NM_138454.1	TXNL6	Thioredoxin-like 6	–2.00	2.12
NM_002478.3	MYOD1	Myogenic factor 3	–1.89	2.05
NM_002720.1	PPP4C	Protein phosphatase 4 catalytic subunit	–2.6	2.0
NM_001977.2	ENPEP	Glutamyl aminopeptidase	–3.37	1.87
NM_001571.2	IRF3	Interferon regulatory factor 3	–1.84	1.94
NM_013351.1	TBX21	T-box 21	–2.33	1.83
NM_006247.2	PPP5C	Protein phosphatase 5 catalytic subunit	–1.83	1.83

Table S2. (*Continued*)

GenBank Accession Number	Gene Name	Gene Description	Fold Change	
			By H_2O_2	By Paeonol
NM_006183.3	NTS	Neurotensin	–1.83	1.83
NM_000638.2	VTN	Vitronectin	–1.83	1.83
NM_001228.2	CASP8	Caspase 8	–2.00	1.87
NM_001178.3	ARNTL	Aryl hydrocarbon receptor nuclear translocator-like	–3.33	1.83
NM_000715.2	C4BPA	Complement component 4 binding protein alpha	–1.83	1.83
NM_006167.2	NKX3-1	NK3 transcription factor related, locus 1	2.45	–1.80
NM_000484.2	APP	Amyloid beta (A4) precursor protein	1.90	–1.94
BM994488	FST	Follistatin	2.00	–2.00
NM_001003679.1	LEPR	Leptin receptor	2.00	–2.00
NM_018965.1	TREM2	Triggering receptor expressed on myeloid cells 2	1.95	–2.05
NM_033035.3	TSLP	Thymic stromal lymphopoietin	2.44	–2.60
NM_001259.5	CDK6	Cyclin-dependent kinase 6	2.17	–2.60
NM_012092.2	ICOS	Inducible T-cell co-stimulator	2.07	–2.64
NM_000457.3	HNF4A	Hepatocyte nuclear factor 4, alpha	2.00	–2.75
NM_000959.2	PTGFR	Prostaglandin F receptor	2.56	–2.73
NM_006144.2	GZMA	Granzyme A	2.17	–3.55
NM_000424.2	KRT5	Keratin 5	1.93	–3.73
NM_000840.2	GRM3	Glutamate receptor, metabotropic 3	2.37	–3.80
NM_005076.2	CNTN2	Contactin 2	2.07	–5.09
NM_000537.2	REN	Renin	10.60	–10.60
NM_017442.2	TLR9	Toll-like receptor 9	8.91	–12.25

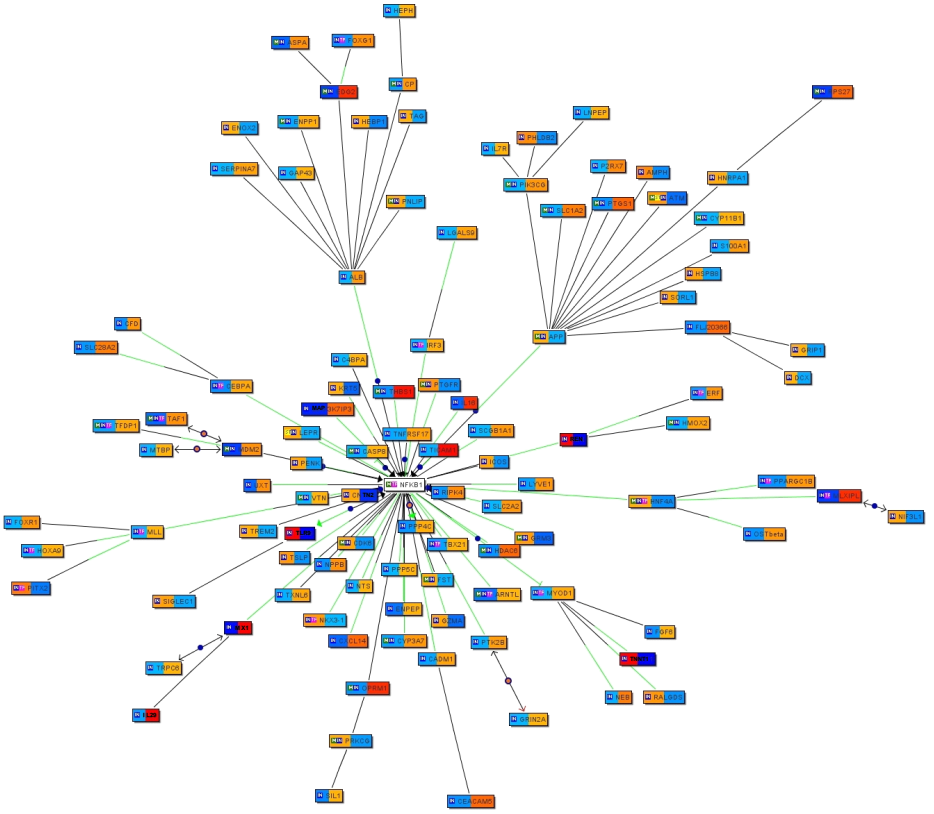

Figure S1. Network analysis of paeonol-regulated genes. Differentially expressed genes were analyzed by BiblioSphere Pathway Edition software. Input genes are marked as IN. Genes that code for a transcription factor are marked as TF. The product of the gene marked as ST is part of a Cenomatix signal transduction pathway. For each box, the left side represents the response to H_2O_2 and the right side represents the response to paeonol. A blue color represents down-regulated genes; a yellow and red color represent up-regulated genes.