Title: Paeonol attenuates H_2O_2-induced NF-κB-associated amyloid precursor protein expression

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Running title: Paeonol attenuates H_2O_2-induced amyloid precursor protein

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Abbreviations:

AD: Alzheimer's disease; APP: amyloid precursor protein; CNTN2: contactin 2; EDTA: Ethylenediaminetetraacetic acid; GRM3: metabotropic glutamate receptor 3; GSEA: gene set enrichment analysis; GSH: glutathione; HIF1: hypoxia-inducible factor 1; IL16: interleukin 16; JNK: c-jun N-terminal kinase; KRT5: keratin 5; MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MX1: myxovirus resistance 1; NF-κB: nuclear factor-κB; NIK: NF-κB inducing kinase; OPRM1: opioid receptor, mu 1; REN: renin; RLU: relative luciferase unit; ROS: reactive oxygen species; SOD: superoxide dismutase; THBS1: thrombospondin 1; TLR9: toll-like receptor 9; TLR: toll-like receptor; TRIF: TIR domain containing adaptor.

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, we induced NF-κB activation by H_2O_2 in human neuroblastoma SH-SY5Y cells. Then we investigated whether paeonol, one of the phenolic phytochemicals isolated from Chinese herbs *Paeonia suffruticosa* Andrews (MC), attenuated the H_2O_2-induced NF-κB activity. By Western blot, we found that paeonol inhibited the phosphorylation of IκB and the translocation of NF-κB into the nucleus. By electrophoretic mobility shift assay and luciferase reporter assay, we confirmed that paeonol reduced DNA binding ability and suppressed the H_2O_2-induced NF-κB activation. Using microarray combined with gene set analysis, we found that the suppression of NF-κB was associated with mature T cell up-regulated genes, c-jun N-terminal kinase pathway, and two hypoxia-related gene sets, including hypoxia up-regulated gene set and hypoxia inducible factor 1 targets. Moreover, using 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, we also identified the gene expression profile accompanying the suppression of NF-κB by paeonol. The new gene set that can be targeted by paeonol provided a potential usage of this drug and a possible pharmacological mechanism for other phenolic compounds that protect against oxidative-related injury.

Key words: 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 causal of many neurodegenerative diseases, including as 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 damage to lipid, protein, and nucleic acid, thereby directly inducing 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 comprises five members, including c-Rel, p65, RelB, p50, and p52. These members associate in homo- or heterodimers (most commonly p50-p65 dimer) and control transcription by binding to NF-κB sites in the promoter or enhancer region of their target genes. NF-κB dimmers 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 development of ROS-associated age-associated diseases (Braun et al., 2009; Tan et al., 2008). Active NF-κB also has been shown to be overexpressed in brain of AD patients (Boissiere et al., 1997).

Because NF-κB plays an important role in oxidative stress-induced neuronal damage, antioxidants that can cause a reduction in NF-κB are considered to be candidates to prevent or treat ROS-associated neuronal diseases (Pratico, 2008). Phenolic compounds, a group of phytochemicals that exist in a variety of herbs, are well-known for their antioxidative abilities (Manach et al., 2005). Paeonol (2'-hydroxy-4'-methoxyacetophenone) is one of the phenolic compounds isolated from Chinese herbs *Paeonia suffruticosa* Andrews (MC) and *Paeonia lactiflora* Pall (PL). Both herbs are traditionally used as anti-inflammatory drugs and sedative drugs 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). 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). Besides, 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 then 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.

EXPERIMENTAL PROCEDURE

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) plus 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 treatment

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. Luciferase assay was perform 24 h after a 24 h 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 16-18 h, SH-SY5Y cells were pre-treated with various concentrations of paeonol 1 h before H_2O_2 (5 μM) exposure. For microarray, Western blotting for APP, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and luciferase reporter assays, the cells were harvested 24 h after H_2O_2 treatment. For electrophoretic mobility shift assay (EMSA), Western blotting for NF-κB, IκBα, and phospho-IκBα, the nuclear and cytoplasmic proteins were obtained 4 h 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 SuperFect® transfection reagent (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Twenty-four hours later, the cells were selected 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 MTT colorimetric assay. Cells were cultivated in 96-well culture plates. After a 16-18-h incubation at 37°C, cells were pre-treated with various amounts of paeonol 1 h before H_2O_2 treatment. After incubation for another 24 h, a one-tenth volume of 5 mg/mL MTT was then added to the culture medium. After a 4-h incubation at 37°C, an equal cell culture 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 12000×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 h 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 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, 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 2 independent plates using an RNeasy Mini kit (Qiagen, Valencia, CA, USA) and quantified 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). RNA samples with RNA integrity numbers greater than 8.0 were pooled together and accepted 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 expression 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).

"GeneSetTest" function implemented in the limma package of R program was used to test the significantly altered gene sets by 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 single dye was two and that for dual dye was two.

Statistical analysis

Data are expressed as mean ± SEM. Data of different groups were compared using one-way ANOVA followed by 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 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 concentration of H_2O_2 for 24 h. Each dose of H_2O_2 was used twice in two different plates at the same time, one for MTT assay and the other for luciferase assay. As shown in Fig.2, the luciferase activity increased in a dose-dependent manner without changing cell viability when exposed to low dose 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 level. When the concentration of H_2O_2 was greater than 10 μM, luciferase activity declined. Under the high dose of H_2O_2 exposure, cell viability decreased at H_2O_2 concentrations greater than 50 μM. The TC_{50} of H_2O_2 was approximately 68 μM. We chose 5 μM H_2O_2 to induce the maximum NF-κB activity in the following experiments.

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

Effect of paeonol on H_2O_2-induced NF-κB activation

The first method to measure NF-κB activity was the luciferase reporter assay.. SH-SY5Y/NF-κB cells were pre-treated with various amounts of paeonol for 1 h prior to 5 μM H_2O_2 exposure. As shown in Fig. 4A, paoenol reduced the H_2O_2-induced luciferase activity in a dose-dependent manner. Paeonol at doses up to 0.4 mM did not alter the viability of SH-SY5Y/NF-κB cells. These 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 NF-κB-binding consensus sequence. SH-SY5Y/NF-κB cells showed an increase in DNA binding activity by 1.68 fold 4 h 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, lane 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 h and then exposed to H_2O_2 for 24 h. The microarray data were 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. The 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

We used GSEA to identify and compare gene sets enriched in paeonol-treated SH-SY5Y cells with those enriched in cells exposed to H_2O_2 only. Twenty-one gene sets were identified ($p < 0.01$) (Supplementary information table S1) and the top ten gene sets that were highly altered by paeonol are listed in Table 1. Mature T cell-up-regulated gene set was the most significantly altered gene set by paeonol. Moreover, 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 above mentioned 2789 differentially expressed genes regulated by paeonol was analyzed by network analysis, which revealed that NF-κB was the primary center of the network (Supplementary information Figure 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. There were 49 genes that were directly linked to NF-κB in the network (Supplementary information table S2). The top ten 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, we confirmed the change of APP expression by examining its protein level using Western blotting. SH-SY5Y cells were pre-treated with various concentrations of paeonol 1 h prior to H_2O_2 exposure, and then incubated with 5 μM of H_2O_2 for 24 h. Total protein was extracted and an antibody that recognizes three isoforms of APP (immature, sAPP, and mature) was used. Western blotting showed that 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 h 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 in a narrow range of physiologically relevant doses (1-10 μM) without inducing cytotoxic effects (Droge, 2002; Gamaley and Klyubin, 1999). In SH-SY5Y cells, the NF-κB activation is observed both during an exposure of 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 low-dose 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. Lethal dose of H_2O_2 stimulates the protein expression of ERK1 (p42)/2 (p44), JNK, and PKB (Ruffels et al., 2004) , and mRNA expression 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 stress (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 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 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 that demonstrated 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 possible act through.

The DNA microarray data with gene set analysis revealed that among the top ten gene sets that were altered by paeonol, mature T cell-up-regulated gene set, comprising genes that are differentially expressed in mature T cells was the most significantly altered gene set by paeonol (Balduini et al., 2006). Moreover, hypoxia up-regulated and HIF1 target genes, those comprise 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). Moreover, genes that are related to JNK pathway were also regulated by paeonol. Among the above mention pathways, 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 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 NF-κB and JNK pathway (Aggarwal and Shishodia, 2006).

Using network analysis of the paeonol-regulated genes derived from the microarray data, 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 ten 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 regulated by paeonol can be roughly divided into 2 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 interferon and protects against viral infection (Haller et al., 2007). IL-16 is a microglial-produced chemoactive cytokine and it has been suggested that IL-16 might contribute to 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 second group of paeonol-regulated genes that were associated with NF-κB was the neuronal function-related genes, including OPRM1, CNTN2, and GRM3. THBS1 codes for thrombospondin. This protein functions in postnatal neuronal migration (Blake et al., 2008) and has been demonstrated to be necessary for synaptic plasticity and functional recovery after stoke (Liauw et al., 2008). OPRM1 is an opioid receptor that is correlated with mechanical allodynia after nerve injury (Back et al., 2006). CNTN2, which codes for 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). Base on the above mentioned findings and the results of 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 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 protein expression of APP and found that the drug 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 level and protein level (Lin et al., 2008; Tang and Chua, 2008).

Resveratrol also has been shown to reduce β-amyloid levels as well (Marambaud et al., 2005). The above studies reported curcumin and resveratrol attenuate APP and β-amyloid levels by promote its clearance. However, none of those studies proposed possible pathways through which these phenolics might participate in. In this study, we found that the suppression of APP by paeonol was coincident with the suppression of NF-κB, implying that paeonol suppressed APP level via NF-κB pathway. Actually, the induction of APP by NF-κB has been reported. No matter activated by mental ion, CD40, IL-1β, or glutamate, NF-κB has been shown to activate APP expression (Grilli et al., 1996; Ait-Ghezala et al., 2007; Walton and Wang, 2009). This might be because 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, the component of *Paeonia suffruticosa* Andrews and *Paeonia lactiflora* Pall. Both herbs have 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 the ability of suppressing inflammatory-related gene sets, including mature T cell-up-regulated, up-regulated during EMT induction, and LIF down-regulated gene sets. On the other hand, their sedative effect might be due to the regulation of neuronal function-related genes, including opioid receptor, thrombospondin, contactin 2, and glutamate receptor. In addition to the classical use of *Paeonia suffruticosa* Andrews and *Paeonia lactiflora* Pall, results of our study provided evidence that suggest 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). Base on our results, 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 has on the processing of APP and memory function in animal models.

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Figure legends

Fig. 1. The chemical structure of paeonol.

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

Fig. 3. Inhibition of H_2O_2-induced IκB phosphorylation and NF-κB nuclear translocation by paeonol. Data are shown as mean ± 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 with untreated cells; * $p < 0.05$ compared with H_2O_2-treated cells.

Fig.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 pre-treated with paeonol for 1 h and then exposed to 5 μM H_2O_2. The percentage

at the bottom represents the amount of shifted probes relative to untreated cells. Data are shown as mean ± SEM of three independent assays. # $p < 0.05$ compared with untreated cells. * $p < 0.05$, and *** $p < 0.001$ compared with H_2O_2-treated cells.

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

Fig. 6 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 untreated group. Values are mean ± SEM of three independent assays. # $p < 0.05$ compared with untreated cells; * $p < 0.05$ compared with H_2O_2-treated cells.

Fig. S1. Network analysis of paeonol-regulated genes. Differentially expressed genes were analyzed by BiblioShere 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; the right side represents the response to paeonol. Blue color represents down-regulated genes; yellow and red color represents up-regulated genes.

Fig. 1

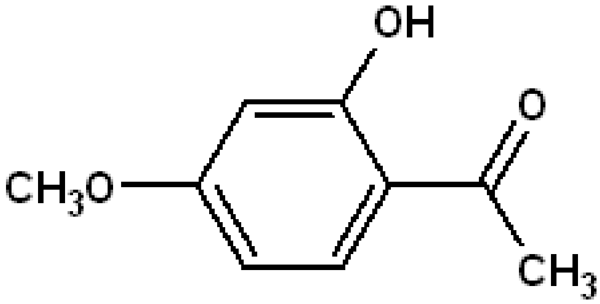
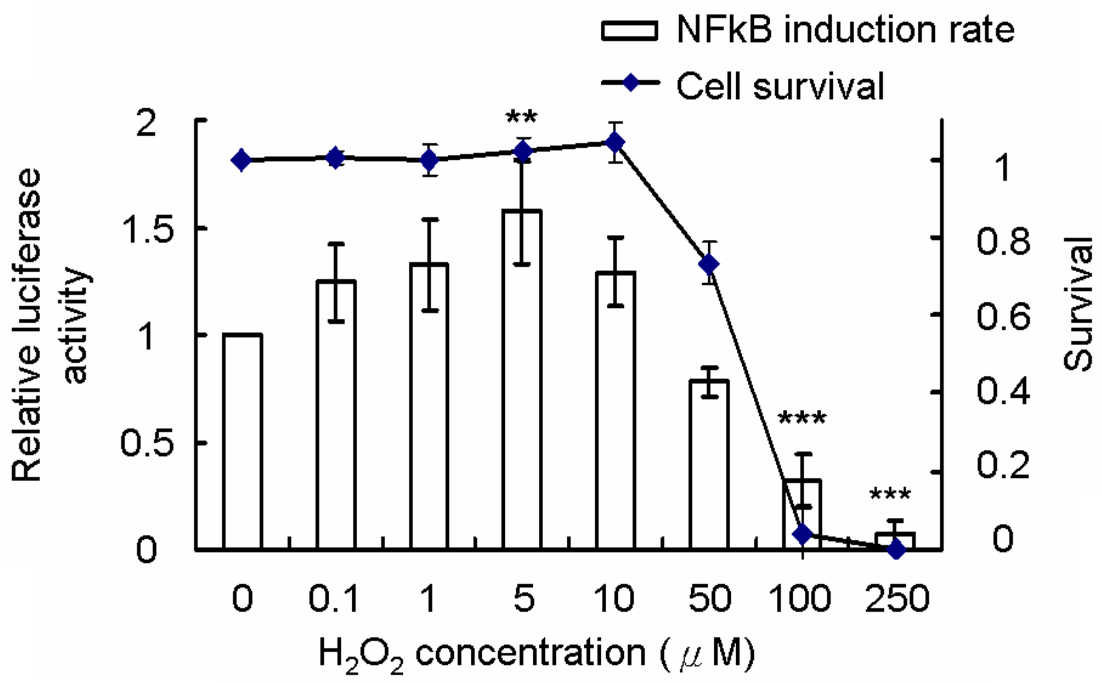


Fig. 2



NFkB induction rate
Cell survival
Relative luciferase activity
Survival
2
1.5
1
0.5
0
1
0.8
0.6
0.4
0.2
0
**
***
***
0
0.1
1
5
10
50
100
250
H_2O_2 concentration (μ M)

Fig. 3

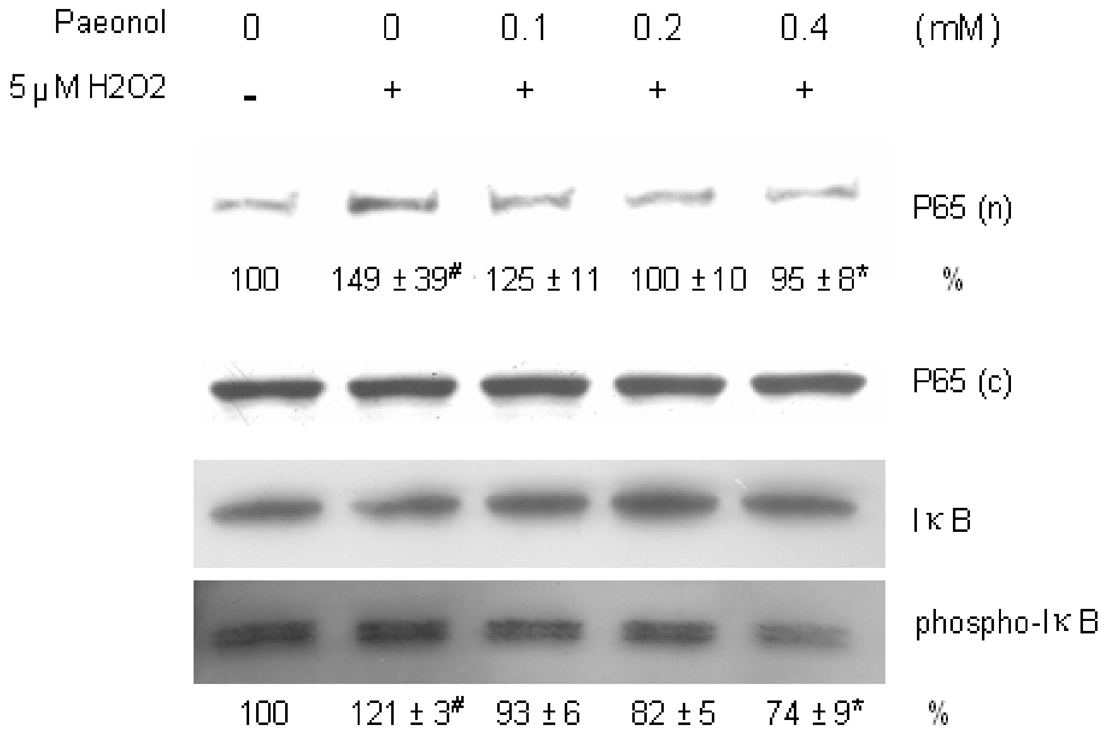
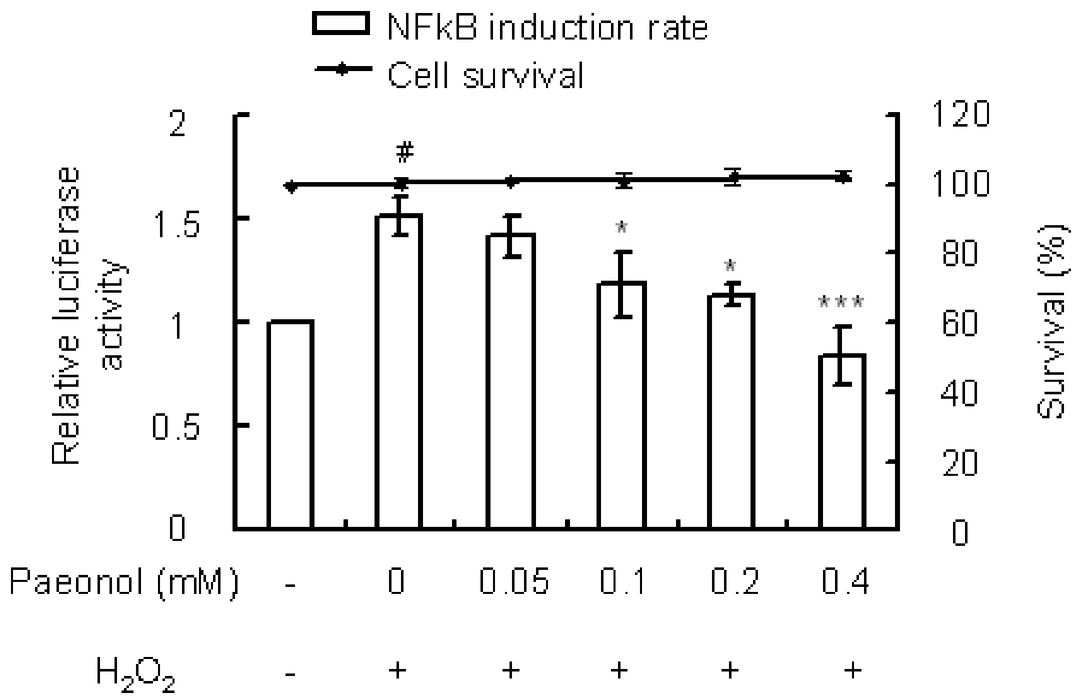

Paeonol 0 0 0.1 0.2 0.4 (mM)
5 μM H2O2 - + + + +
P65 (n)
100 149 ± 39# 125 ± 11 100 ± 10 95 ± 8* %
P65 (c)
Iκ B
phospho-Iκ B
100 121 ± 3# 93 ± 6 82 ± 5 74 ± 9* %

Fig. 4A



NFkB induction rate
Cell survival
Relative luciferase activity
Survival (%)
2
1.5
1
0.5
0
120
100
80
60
40
20
0
#
*
*
***
Paeonol (mM) - 0 0.05 0.1 0.2 0.4
H2O2 - + + + + +

Fig. 4B

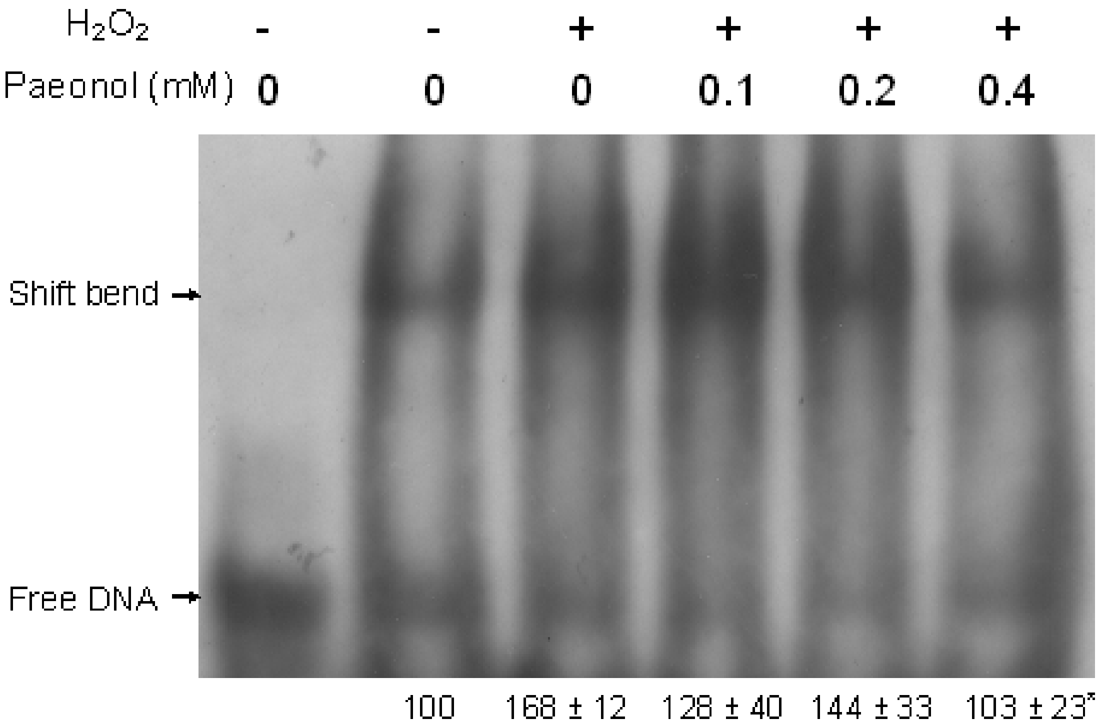
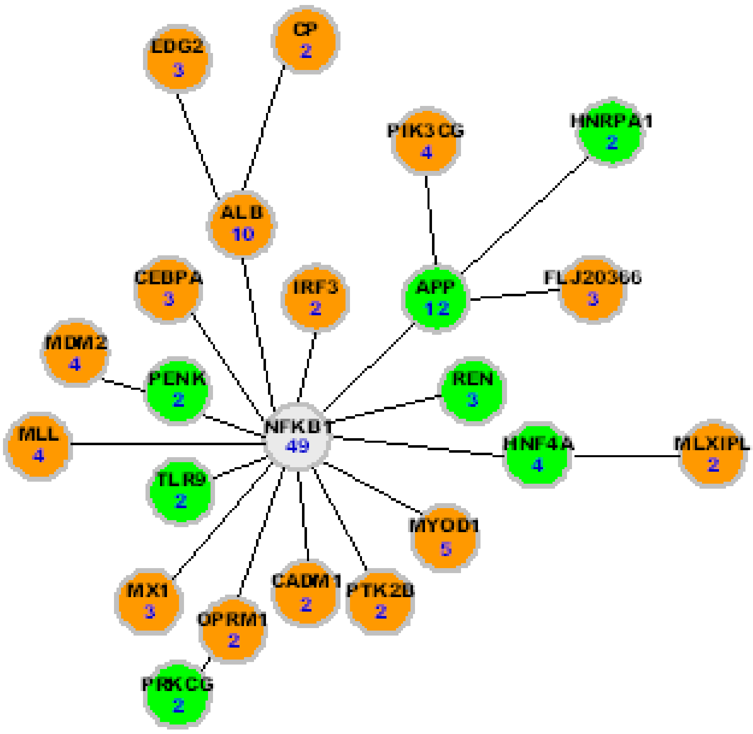

H2O2 - - + + + +
Paeonol (mM) 0 0 0 0.1 0.2 0.4
Shift bend
Free DNA
100
168 ± 12
128 ± 40
144 ± 33
103 ± 23*

Fig. 5


EDG2
3
CP
2
PIK3CG
4
HNRPA1
2
ALB
10
CEBPA
3
IRF3
2
APP
12
FLJ20366
3
MDM2
4
PENK
2
REN
3
MLL
4
NFKB1
49
HNF4A
4
MLXIPL
2
TLR9
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MYOD1
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MX1
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CADM1
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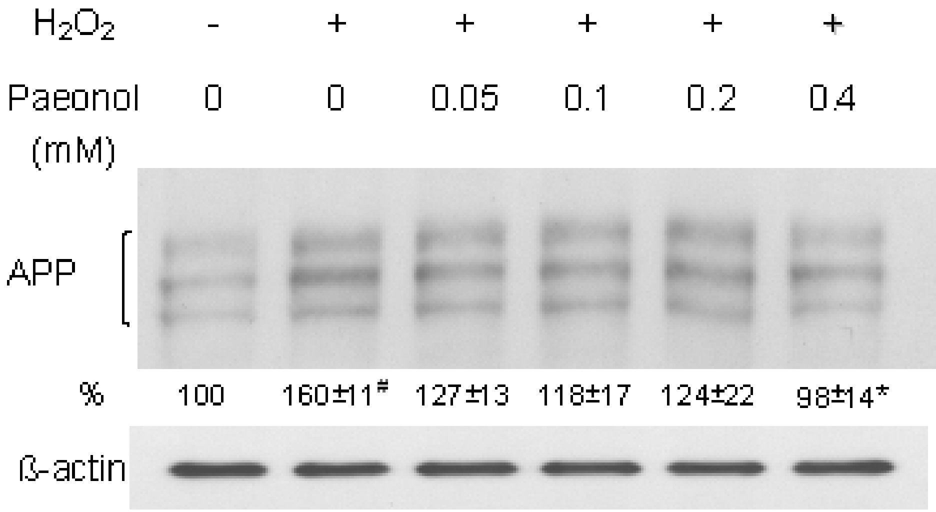
PTK2B
2
OPRM1
2
PRKCG
2

Fig. 6



H2O2 - + + + + +
Paeonol (mM) 0 0 0.05 0.1 0.2 0.4
APP
% 100 160±11# 127±13 118±17 124±22 98±14*
ß-actin

Table 1 List of the significantly altered gene sets 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.

Table 2 List of the top ten genes altered by paeonol that were primarily associated with NF-κB from network analysis.

Gene name	Gene description	Fold change	
		By H_2O_2	By paeonol
MX1	myxovirus resistance 1	-5.88	16.50
TICAM1	TIR domain 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

Supplementary information

Table S1 List of 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 List of genes that were primarily associated with NF-κB from 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
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