

Activation of nuclear factor erythroid 2-related factor 2 and PPAR γ plays a role in the genistein-mediated attenuation of oxidative stress-induced endothelial cell injury

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Abstract

We investigate the cytoprotective effects and the molecular mechanism of genistein in oxidative stress-induced injury using an endothelial cell line (EA.hy926). An oxidative stress model was established by incubating endothelial cells with H₂O₂. According to the present results, genistein pretreatment protected endothelial cells against H₂O₂-induced decreases in cell viability and increases in apoptosis. Genistein also prevented the inhibition of B-cell lymphoma 2 and the activation of caspase-3 induced by H₂O₂. Genistein increased superoxide dismutase (SOD), catalase (CAT) and glutathione (GSH) levels and attenuated the decrease in these antioxidants during oxidative stress. We also found that genistein induced the promoter activity of both nuclear factor erythroid 2-related factor 2 (Nrf2) and PPAR γ . Additionally, genistein induced the nuclear translocation of Nrf2 and PPAR γ . While genistein caused the up-regulation of both Nrf2 and PPAR γ , it also activated and up-regulated the protein expression and transcription of a downstream protein, haem oxygenase-1 (HO-1). Moreover, the use of Nrf2 small interfering RNA transfection and HO-1- or PPAR γ -specific antagonists (Znpp and GW9662, respectively) blocked the protective effects of genistein on endothelial cell viability during oxidative stress. Therefore, we conclude that oxidative stress-induced endothelial cell injury can be attenuated by treatment with genistein, which functions via the regulation of the Nrf2 and PPAR γ signalling pathway. Additionally, the endogenous antioxidants SOD, CAT and GSH appear to play a role in the antioxidant activity of genistein. The present findings suggest that the beneficial effects of genistein involving the activation of cytoprotective antioxidant genes may represent a novel strategy in the prevention and treatment of cardiovascular endothelial damage.

Key words: Genistein: Oxidative stress: Endothelial cells: Nuclear factor erythroid 2-related factor 2

Oxidative stress due to abnormal exposure to reactive oxygen species (ROS) plays a key role in the pathogenesis of atherosclerosis and CVD⁽¹⁾. Endothelial cells lining the vasculature are particularly sensitive to oxidative stress as high concentrations of ROS in the circulating blood directly keep in contact with the endothelium. Overproduction of ROS can damage vascular endothelial cells, and numerous *in vivo* and *in vitro* studies have implicated that endothelial cell damage and cell death may play a role in the development of atherogenesis⁽²⁾. However, the underlying mechanism by which oxidative stress induces endothelial cell damage and cell death has not been fully established. Therefore, further

insight into this mechanism is required before a new preventive strategy against oxidative stress-induced endothelial cell injury can be developed for the treatment of atherosclerosis.

Epidemiological evidence suggests that populations consuming soya products rich in isoflavones have a lower incidence of CVD. The benefits of soya isoflavones for the prevention of atherosclerosis and CVD have been examined in many clinical trials and animal models^(3,4). Genistein, the primary isoflavone found in soybeans, has been highlighted for its antioxidant effects and ability to activate the transcription of various genes. Due to its antioxidative properties, genistein effectively scavenges ROS and inhibits lipid

Abbreviations: ARE, antioxidant responsive element; Bcl-2, B-cell lymphoma 2; CAT, catalase; DMEM, Dulbecco's modified Eagle's medium; ER, oestrogen receptor; FITC, fluorescein isothiocyanate; GSH, glutathione; HO-1, haem oxygenase-1; HUVEC, human umbilical vein endothelial cells; Nrf1, nuclear factor erythroid 2-related factor 1; Nrf2, nuclear factor erythroid 2-related factor 2; ROS, reactive oxygen species; SOD, superoxide dismutase; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labelling.

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peroxidation⁽⁵⁾. In addition, genistein reverses alterations in the protein expression profile induced by cell stress⁽⁶⁾ and protects endothelial cells from oxidative stress-induced damage by maintaining or activating several survival signalling pathways⁽⁷⁾. However, there is only limited experimental evidence addressing the impact of genistein on antioxidant gene expression in the vasculature^(8,9). Various other phytochemicals, such as (–)-epigallocatechin-3-gallate⁽¹⁰⁾, quercetin⁽¹¹⁾, resveratrol⁽¹²⁾ and puerarin⁽¹³⁾, have been demonstrated to activate antioxidant genes as well as their downstream cytoprotective enzymes. However, the effects of genistein and the underlying mechanisms regarding oxidative stress-induced endothelial cell injury remain unclear.

The most important cellular defence mechanism against ROS involves nuclear factor erythroid 2-related factor 2 (Nrf2), which mediates antioxidant responsive element (ARE) sequences in the promoter regions of phase II and antioxidant genes⁽¹⁴⁾. Among the various antioxidant enzymes regulated by Nrf2/ARE, haem oxygenase-1 (HO-1) has been highlighted for its cytoprotective involvement in the vascular system, including vascular tone regulation, inhibition of smooth muscle proliferation, inhibition of endothelial apoptosis and promotion of angiogenesis⁽¹⁵⁾. PPAR γ also plays an important role in vascular regulation. Recent studies have indicated that there is a mechanistic link between PPAR γ and oxidative stress in various cell types^(16,17), and that the activation of PPAR γ exerts beneficial effects in endothelial cells^(18,19). The present study aims to examine the cytoprotective effects of genistein on oxidative stress-induced loss of cell viability and increased apoptosis in EA.hy926 endothelial cells. Moreover, the present study assesses whether Nrf2, PPAR γ , HO-1 and the endogenous antioxidants superoxide dismutase (SOD), catalase (CAT) and glutathione (GSH) are involved in the mechanism of cellular protection.

Experimental methods

Reagents

Genistein, H₂O₂, Znpp, GW9662, hemin and NADPH were purchased from Sigma. Genistein was prepared as a 0.1 M stock solution in dimethyl sulfoxide; an equal volume of dimethyl sulfoxide was used as a control in each experiment. Polyclonal rabbit anti-human Nrf2 (sc-722) and monoclonal mouse anti-human PPAR γ (sc-7273) antibodies were purchased from Santa Cruz Biotechnology. Polyclonal rabbit anti-human HO-1 (SPA-895) antibody was obtained from Stressgen. Rabbit anti-human activated-caspase-3 p17 (BS7004), B-cell lymphoma 2 (Bcl-2, BS1511), β -tubulin (BS1482) and lamin B1 (BS3547) antibodies were purchased from Bioworld Technology. Goat anti-rabbit and goat anti-rabbit IgG peroxidase conjugate were obtained from Pierce Biotechnology (Thermo Scientific). Nrf2 small interfering RNA (siRNA) (sc-37030), control siRNA (sc-37007), control siRNA (fluorescein conjugate) (sc-36869) and siRNA transfection reagent (sc-29528) were purchased from Santa Cruz Biotechnology. The Cell Counting Kit-8 was purchased from Dojindo Laboratories. The Annexin V-FITC Apoptosis

Detection Kit was obtained from BestBio. The *In Situ* Cell Death Detection Kit was purchased from Roche Diagnostics. The reagent kit used for the measurement of SOD (A001-3), CAT (A007) and GSH (A006) was purchased from Nanjing Jiancheng Bioengineering Institute. The BioEasy SYBR Green I Real Time PCR Kit was obtained from Bioer Technology. The ARE-luciferase reporter gene was kindly provided by Dr Donna D. Zhang (University of Arizona). The PPAR γ -luciferase reporter gene was kindly provided by Dr Ronald M. Evans (the Salk Institute for Biological Studies).

Cell culture and treatment

EA.hy926 cells were obtained from the American Type Culture Collection. Cells were maintained at 37°C in an incubator with a humidified atmosphere of 5% CO₂ and were cultured in Dulbecco's modified Eagle's medium (DMEM), which was supplemented with 10% heat-inactivated fetal bovine serum, 2 mM-L-glutamine, penicillin (60 μ g/ml) and streptomycin (100 μ g/ml), until they reached 90–95% confluence. The culture medium was replaced every 3 d.

Measurement of cell viability

Cell viability was assessed using a Cell Counting Kit-8 assay. WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2H-tetrazolium, monosodium salt) is reduced by dehydrogenases in cells to produce a water-soluble formazan dye (orange in colour). The relative amount of formazan dye in cells is directly proportional to the number of living cells. For this assay, cells were seeded at a density of 5000 cells/well in ninety-six-well plates with six replicate wells for each concentration in the same plate. Before detection, the Cell Counting Kit-8 reagent was diluted 10-fold with DMEM, and 100 μ l of the Cell Counting Kit-8 solution were then added to each well. After 2.5 h incubation, the optical density (OD) value was read at 450 nm using a multimode microplate reader (Infinite M200; Tecan). The OD₄₅₀ value is proportional to the degree of cell viability. The results shown are derived from at least three independent experiments.

Flow cytometry assay

Cells grown in six-well plates were harvested, washed and double-stained with annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (Annexin V-FITC Apoptosis Detection Kit, Bestbio), incubated for 15 min at room temperature in the dark, and analysed by flow cytometry.

Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labelling assay

Apoptosis was detected via terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labelling (TUNEL) analysis using the *In Situ* Cell Death Detection Kit (Roche Diagnostics) according to the manufacturer's instructions. EA.hy926 cells were seeded on sterile glass coverslips in twelve-well plates overnight and pre-incubated with genistein

for 10 h before being exposed to H₂O₂ for 24 h. Cells were then washed twice with PBS, fixed with 4% methanol-free paraformaldehyde for 10 min, washed twice with PBS and permeabilised with 0.2% Triton X-100 for 5 min. After two more washes, each glass coverslip was covered with equilibration buffer for 10 min. The buffer was then aspirated, and the glass coverslips were incubated with terminal deoxynucleotidyl transferase buffer at 37°C for 1 h. Chromosomal DNA was stained with 4',6-diamidino-2-phenylindole, the cells were mounted on glass slides and staining was analysed using confocal laser scanning microscopy.

Assessment of superoxide dismutase, catalase and glutathione levels

Cells were pre-incubated with genistein (0, 20, 100 and 500 nM) for 10 h, while H₂O₂-treated cells were incubated either with or without 500 nM-genistein for 10 h, followed by additional incubation with H₂O₂ for 24 h. The medium was then removed, cells were washed twice with cold PBS and cells were removed from the plates while kept on ice. Cell suspensions were sonicated three times for 3–4 s each on ice and then centrifuged at 14 000 rpm (21 913 g) for 25 min at 4°C. Cell supernatants were used for the assessment of antioxidant enzyme activity and GSH levels. The analysis of SOD, CAT and GSH levels was performed according to the manufacturers' instructions. Protein concentration was determined via the Bradford method; bovine serum albumin was used as a reference standard.

Luciferase reporter gene assay

Cells in twenty-four-well plates were transfected using LipofectAMINE™ 2000 reagent (Invitrogen) with either an ARE-luciferase plasmid or PPAR γ -luciferase plasmid together with a renilla luciferase expression plasmid (used as an internal reporter to normalise for variations in transfection efficiency) and pGL 4.74 (hRluc/TK) (Promega). The cells were then treated with genistein for 10 h, and the activity of both firefly and renilla luciferases was measured using the dual luciferase reporter assay system from Promega.

Western blot analysis

After the treatment, cells were lysed in lysis buffer (50 mM-Tris, pH 8.0, 150 mM-NaCl, 0.1% SDS, 1% Triton X-100, 0.5% deoxycholate and protease inhibitors), and the protein concentrations of the cell lysates were determined by the Bradford assay. Lysate aliquots (40 μ g/well) were separated by 10–12% SDS-PAGE and then transferred onto polyvinylidene fluoride membranes. Membranes were subsequently incubated with primary antibodies overnight at 4°C, washed with PBS and incubated with horseradish peroxidase-conjugated secondary antibodies (1:5000 dilution). Visualisation was performed using electro chemiluminescence reagents and developed on radiographic film.

Real-time PCR

Total RNA was extracted using Trizol reagent (Bioer), and genomic DNA was digested with DNase. mRNA was reverse-transcribed into complementary DNA using Moloney murine leukaemia virus reverse transcriptase (Bioer). Gene expression was determined by quantitative real-time PCR using a SYBR Green PCR kit. The primer sequences used for expression analysis are as follows: *Nrf2*, 5'-ATTGCCTGTAAGTCCTGTCA-3' (forward) and 5'-ACTGCTCTTTGGACATCATTTCG-3' (reverse); *HO-1*, 5'-CGATGGGTCCTTACTC-3' (forward) and 5'-GGCTCCTTCCTCCTT-3' (reverse); *PPAR γ* , 5'-AGGTTTGGGCGGATGCCACA-3' (forward) and 5'-AGATCGCCCTGCCTTTGCT-3' (reverse); glyceraldehyde-3-phosphate dehydrogenase, 5'-TGACCACTGCTTAG-3' (forward) and 5'-GATGCAGGATGATGTTTC-3' (reverse); *18S rRNA*, 5'-GTAACCCGTTGAACCCATT-3' (forward) and 5'-CCAT CCAATCGTAGTAGCG-3' (reverse); β -actin, 5'-ACCAACTGGGACGATATGGAGAAGA-3' (forward) and 5'-ACGACCAGAGGCATACAGGGACAA-3' (reverse). The real-time PCR conditions were as follows: 94°C for 2 min followed by forty-five cycles of 94°C for 10 s and 72°C for 45 s. Data are presented as fold change in gene expression compared with the control group.

Assessment of haem oxygenase-1 activity

After the treatment, cells were washed twice with cold PBS, harvested by gently scraping the cells off the dish (on ice), and centrifuged (1500 g for 15 min at 4°C). Cell pellets were resuspended in cold KH₂PO₄ buffer (pH 7.4), frozen at –70°C, thawed three times and sonicated on ice before centrifugation at 14 000 rpm (21 913 g) for 15 min at 4°C. The supernatant (20 μ l) was added to a reaction mixture containing 4.5 mM-NADPH, 3 mg protein of rat liver cytosol prepared from the one 5000 g supernatant fraction as a source of biliverdin reductase, 0.1 M-cold KH₂PO₄ buffer (pH 7.4) and 2 mM-hemin (1.8 ml). The reaction was performed at 37°C for 30 min in the dark and terminated by putting the samples on ice. A reaction without NADPH served as the control. The extracted bilirubin was calculated by determining the difference in absorption between 464 and 530 nm.

Small interfering RNA transfection

Nrf2 siRNA transfection was performed according to the manufacturer's instructions. Cells were seeded in a six-well tissue culture plate (2 \times 10⁵ cells/well) in 2 ml antibiotic-free normal growth medium supplemented with fetal bovine serum. Cells were incubated at 37°C in a CO₂ incubator overnight. A mixture of 6 μ l Nrf2 siRNA and 6 μ l transfection reagent was incubated for 45 min at room temperature. The mixture was then added to the cells with 800 μ l antibiotic/serum-free medium. The final concentration of Nrf2 siRNA was 60 nM. The culture cells transfected with control siRNA were treated in parallel. Additionally, FITC-labelled siRNA was used as a control.

Statistical analysis

Data are expressed as means and standard deviations. Statistical significance was analysed via ANOVA, and differences among the groups were assessed via Tukey's test using SPSS version 13.0 software (SPSS, Inc.). Student's *t* test was also used when comparing the means of the two groups. Differences were considered statistically significant at $P < 0.05$.

Results

Genistein preserves the viability of endothelial cells during oxidative stress

To assess the potential protective effect of genistein at physiological concentrations, cells were pretreated with genistein (1–500 nM) before exposure to 650 μM -H₂O₂ for an additional 24 h. As shown in Fig. 1, genistein prevented the loss of cell viability in EA.hy926 cells during oxidative stress. Cells treated with genistein (10–500 nM) displayed concentration-dependent protective effects (56.9–67.9% viable cells) compared with H₂O₂-treated cells (26.1% viable cells). These results are consistent with Xu's study⁽⁷⁾.

Genistein protects against cell apoptosis induced by oxidative stress

To further demonstrate the protective effects of genistein on endothelial cells during oxidative stress, we evaluated the effects of genistein on apoptosis induced by H₂O₂ in EA.hy926 cells. Using annexin V-FITC and propidium iodide flow cytometric analysis, we observed that apoptosis induced by H₂O₂ was significantly inhibited when cells were pretreated with genistein (Fig. 2(a)). In addition, cells exposed to H₂O₂ demonstrated increased TUNEL staining, whereas the genistein treatment decreased the number of TUNEL-stained positive cells (Fig. 2(b)).

Caspase-3 plays a pivotal role in the terminal execution phase of apoptosis induced by a wide range of stimuli. Under normal conditions, caspase-3 is undetectable in EA.hy926 cells; however, we observed that H₂O₂ activated caspase-3

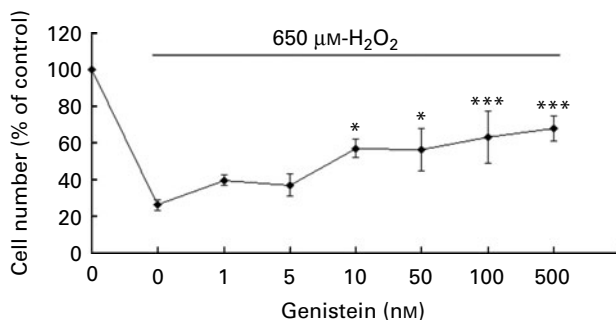


Fig. 1. Genistein attenuates the loss of cell viability during oxidative stress. EA.hy926 cells were pretreated with genistein (1–500 nM) for 10 h and subsequently incubated with H₂O₂ (650 μM) for an additional 24 h, and cell viability was then assessed by the Cell Counting Kit-8 assay. Values are means ($n = 6$ for each group), with standard deviations represented by vertical bars. Mean values were significantly different compared with the H₂O₂-treated group: * $P < 0.05$, *** $P < 0.001$.

in a time-dependent manner (Fig. 2(c)), while genistein pretreatment significantly inhibited caspase-3 expression induced by H₂O₂ (Fig. 2(d)). The Bcl-2 family proteins play a critical role in the regulation of cell proliferation and apoptosis under both normal and oxidative stress conditions. Incubation with H₂O₂ significantly inhibited the expression of Bcl-2 protein. Genistein pretreatment promoted a significant increase in Bcl-2 protein expression levels (Fig. 2(e)) compared with cells treated with H₂O₂ alone. These data confirm that genistein inhibits H₂O₂-induced apoptosis in EA.hy926 cells.

Genistein treatment promotes an increase in superoxide dismutase, catalase and glutathione levels

A basic estimation of endogenous antioxidants, including SOD, CAT and GSH, was assessed in genistein-treated EA.hy926 cells in both the presence and absence of oxidative stress. As shown in Fig. 3, genistein pretreatment resulted in a significant increase in SOD (Fig. 3(a)) and CAT (Fig. 3(b)) activity in a dose-dependent manner. H₂O₂ stimulation led to significant decreases in antioxidant enzyme (SOD and CAT) activity, while pre-incubation with genistein (500 nM) significantly attenuated the decreases in SOD and CAT activity, thereby returning enzyme activity to normal levels. Exposing EA.hy926 cells to genistein caused an increase in GSH levels (Fig. 3(c)). A slight induction in GSH levels was observed after the treatment with 20 and 100 nM-genistein, while treatment with 500 nM-genistein induced significant increases in GSH levels. Although cellular GSH levels decreased during oxidative stress, GSH levels were elevated after the pretreatment with 500 nM-genistein. The present results that display the elevation of antioxidant enzyme activity and GSH levels following genistein treatment alone and the ability of genistein to maintain cellular levels of SOD, CAT and GSH during oxidative stress are consistent with the reports of Choi *et al.*⁽²⁰⁾ and Liu *et al.*⁽²¹⁾.

Genistein activates nuclear factor erythroid 2-related factor 2

Nrf2 signalling plays a major role in the protection of cells against chemical and radiation stress, while it also promotes cell survival. To examine whether genistein activates Nrf2, a plasmid containing an ARE-dependent firefly luciferase reporter gene was utilised as described previously⁽²²⁾. Genistein treatment induced ARE-dependent luciferase activity in a dose-dependent manner (Fig. 4(a)). A slight induction (1.5-fold) in ARE-dependent luciferase activity was observed at concentrations as low as 20 nM and reached a maximum induction (4.0-fold) at 500 nM. Cells were treated with 500 nM-genistein for the indicated times and proteins were extracted for Western blot analysis. As shown in Fig. 4(c), treatment with 500 nM-genistein led to increased Nrf2 expression for at least 10 h as observed in whole cell extracts. The same phenomenon was observed in the nuclear extract (Fig. 4(d)); the highest level of Nrf2 expression was observed at 6 h. Moreover, treatment with 20–500 nM-genistein for 6 h also induced Nrf2 nuclear translocation (Fig. 4(e)). Of note,

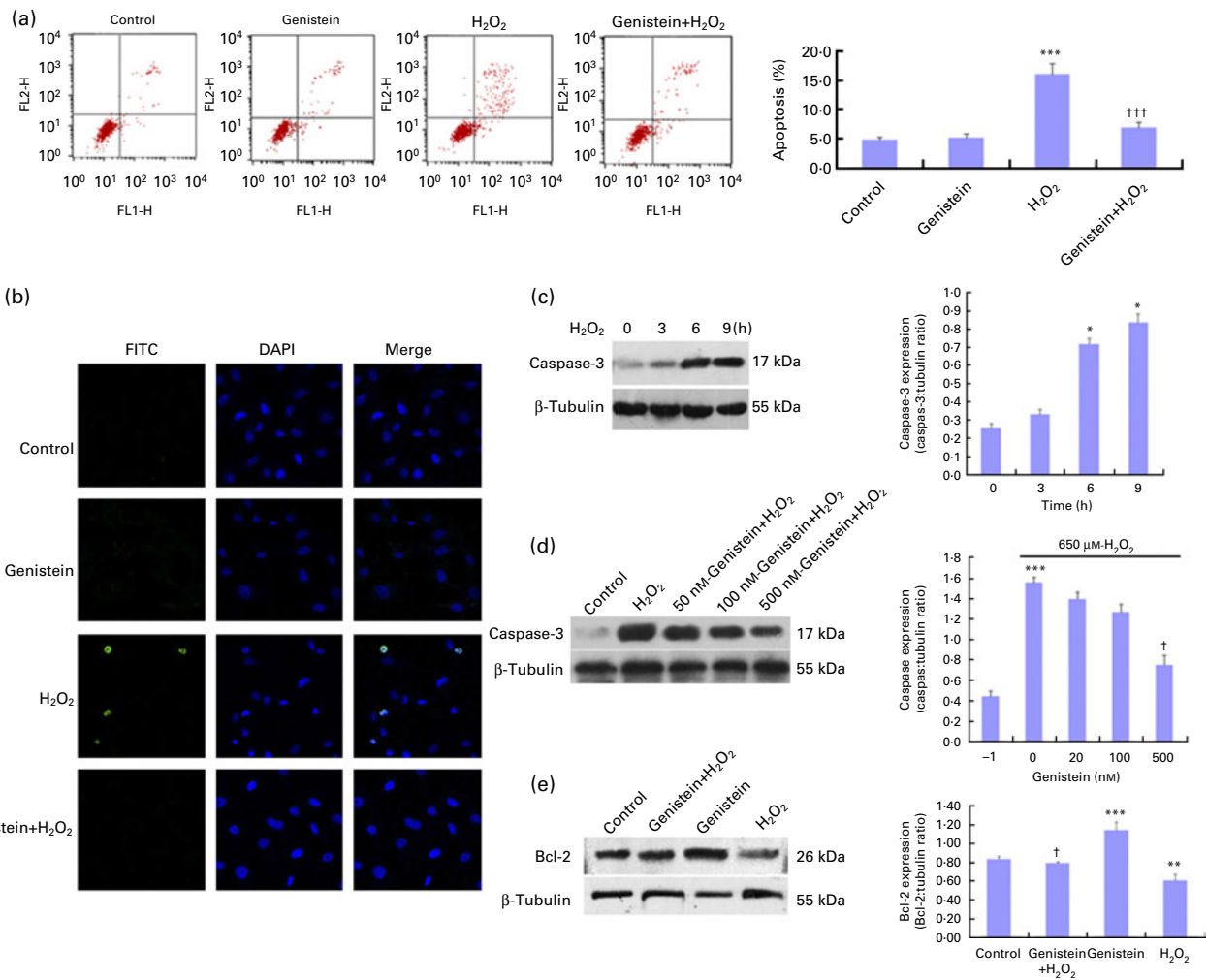


Fig. 2. Genistein suppresses oxidative stress-induced cell apoptosis. (a) EA.hy926 cells were pretreated with genistein (500 nM) for 10 h and then incubated with 650 μM -H₂O₂ for 24 h. Cells were then harvested and labelled with annexin V-fluorescein isothiocyanate (FITC) and propidium iodide, and apoptosis was subsequently analysed via flow cytometry. Representative flow cytometric histograms of the different groups are shown. Values are means (*n* 3 for each group) for the level of apoptosis for each treatment group, with standard deviations represented by vertical bars. Mean values were significantly different compared with the negative control (no oxidative stress) group (*** *P* < 0.001) and the non-genistein group (††† *P* < 0.001). (b) Cells were seeded on sterile glass coverslips in twelve-well plates overnight and pretreated with genistein for 10 h before stimulation with 650 μM -H₂O₂ for 24 h. A terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labelling assay was then performed according to the manufacturers' instructions. (c) The cells were exposed to 650 μM -H₂O₂ for the indicated times and then evaluated for activated caspase-3 p17 expression. *Mean value was significantly different from that at 0 h (*P* < 0.05). (d) Cells were pretreated with various concentrations of genistein for 10 h (the positive control was left untreated) before stimulation with 650 μM -H₂O₂ for 9 h, and the activated caspase-3 p17 levels were then assessed in cell extracts via Western blot analysis. The expression levels were normalised to β -tubulin expression levels. ***Mean value was significantly different from that of the untreated control (*P* < 0.001). † Mean value was significantly different from that for 0 μM -genistein (*P* < 0.005). (e) Cells were pre-incubated either with or without genistein (500 nM) for 10 h and then exposed to 650 μM -H₂O₂ for 24 h. Bcl-2 levels in cell extracts were measured by Western blot analysis and were normalised to β -tubulin expression levels. Mean value was significantly different from that of the control: ***P* < 0.01, ****P* < 0.001. † Mean value was significantly different from that of H₂O₂ alone (*P* < 0.05). DAPI, 4',6-diamidino-2-phenylindole; FL1-H, fluorescence 1-height-FITC(log); FL2-H, fluorescence 2-height-propidium iodide (log).

treatment with 500 nM-genistein resulted in significant increases in *Nrf2* mRNA expression levels (Fig. 4(b)). These data suggest that genistein is a potential inducer of Nrf2 in endothelial cells.

Genistein activates PPAR γ

PPAR γ plays an important role in vascular regulation. PPAR γ activation inhibits endothelial inflammation by suppressing inflammatory gene expression, thereby hindering endothelial damage. To assess whether genistein activates PPAR γ , a

plasmid containing the PPAR response element X3-luciferase reporter gene was transfected in EA.hy926 cells, and gene activation was examined using a dual luciferase reporter gene assay. As shown in Fig. 5(a), activation of the PPAR γ promoter was enhanced (1.7-fold in the 500 nM-treated group). Additionally, PPAR γ protein expression was analysed using Western blotting in EA.hy926 cells after the treatment with genistein (500 nM) for the indicated times. The genistein treatment induced PPAR γ expression in a time-dependent manner (Fig. 5(c)). According to the present results, the ratio of PPAR γ :tubulin in non-genistein-treated cells was 0.7, cells

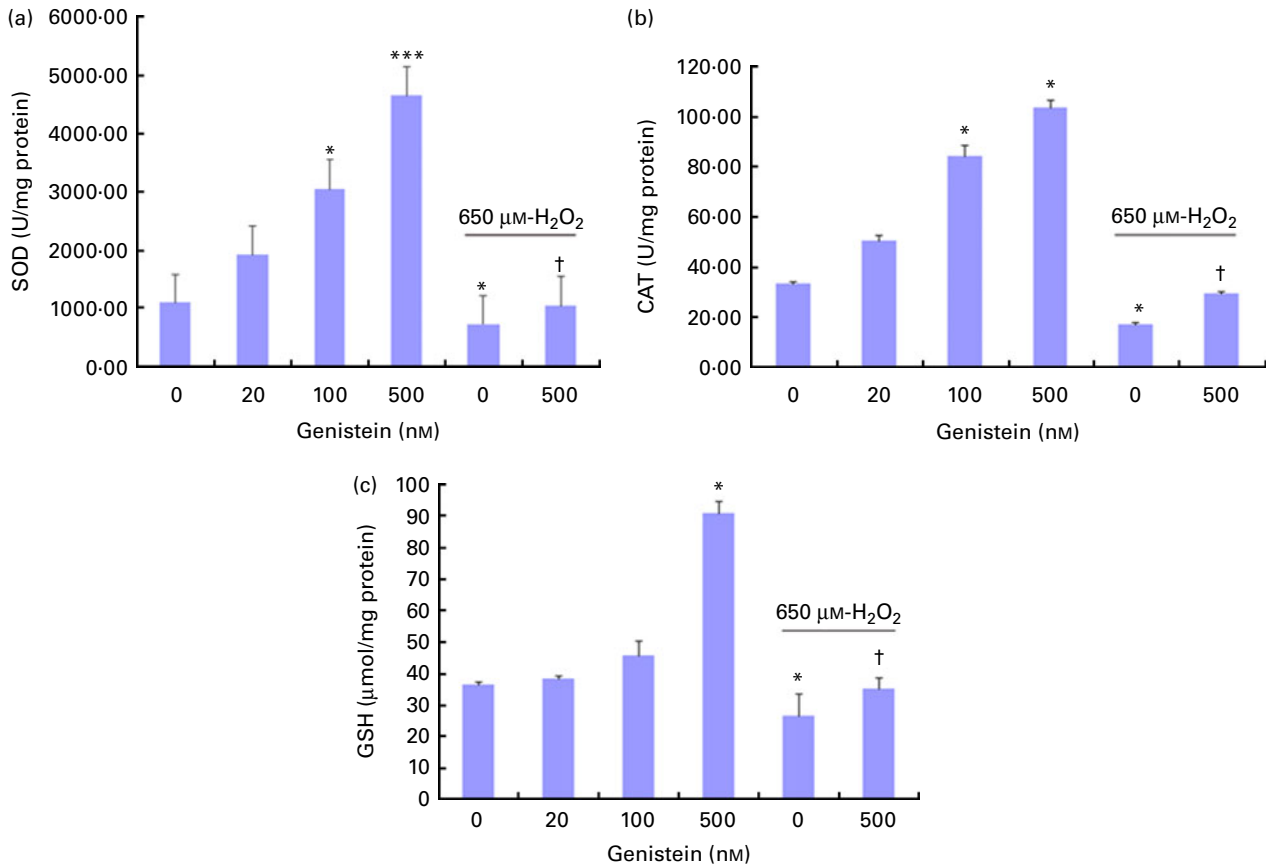


Fig. 3. Genistein up-regulates superoxide dismutase (SOD), catalase (CAT) and glutathione (GSH) levels. EA.hy926 cells were treated with various concentrations of genistein (0, 20, 100 and 500 nm) for 10 h, while H_2O_2 -treated cells were pretreated with or without 500 nm-genistein, followed by incubation with 650 $\mu\text{M-H}_2\text{O}_2$ for 24 h. Cell extracts were then prepared for the assessment of the SOD, CAT and GSH levels. The effects of genistein on (a) SOD activity, (b) CAT activity and (c) GSH levels were detected using a commercial kit. One unit of SOD activity is defined as a 50% decrease in the rate of cytochrome C reduction. One unit of CAT will decompose 1.0 $\mu\text{mol H}_2\text{O}_2$ to O_2 and water per min at pH 7.0 at 25°C at a substrate concentration of 50 $\text{m}\mu\text{-H}_2\text{O}_2$. Values are means (n 3 for each group), with standard deviations represented by vertical bars. Mean values were significantly different compared with both the non-genistein group (* $P < 0.05$, *** $P < 0.001$) and the H_2O_2 control group († $P < 0.05$).

treated with genistein for 1 h displayed a slight induction (ratio 1.3), and the expression induction ratio reached 2.0 after a 4–10 h treatment with genistein. As expected, genistein led to significant increases in $\text{PPAR}\gamma$ accumulation within the nucleus of EA.hy926 cells (Fig. 5(d)). Moreover, the genistein treatment also significantly increased $\text{PPAR}\gamma$ mRNA expression levels (3.3-fold) compared with the control cells (Fig. 5(b)). Above all, these data demonstrate that genistein is able to activate $\text{PPAR}\gamma$ in endothelial cells. However, the effect that genistein displayed on $\text{PPAR}\gamma$ activation was less pronounced than its effect on Nrf2 activation, which suggests that genistein is able to induce the gene expression of various antioxidant genes, albeit at varying degrees.

Genistein up-regulates haem oxygenase-1 expression and activity

As HO-1 is an important component of cellular defence against oxidative stress and is regulated by the Nrf2/ARE-mediated defensive pathway or $\text{PPAR}\gamma$, we also assessed whether genistein could induce HO-1 gene expression. Cells were again exposed to 500 nm-genistein for the indicated

times, and time-dependent (Fig. 6(a)) and dose-dependent (Fig. 6(b)) increases in HO-1 protein expression levels were observed. The genistein treatment for 5–10 h led to increases in HO-1 mRNA expression levels compared with the control cells (Fig. 6(c)). In addition, HO-1 activity was also tested, and as shown in Fig. 6(d), a dose-dependent induction of HO-1 activity was observed after the treatment with genistein for 10 h.

Nuclear factor erythroid 2-related factor 2, $\text{PPAR}\gamma$ and haem oxygenase-1 mediate the protective effect of genistein

To further investigate whether Nrf2 plays a role in genistein-induced protection, EA.hy926 cells were transfected with Nrf2 siRNA, while FITC-labelled control siRNA-transfected cells served as a control (Fig. 7(a)). Nrf2 protein expression levels were significantly reduced by Nrf2 siRNA transfection, and the up-regulation of Nrf2 by genistein (500 nm) was lost after siRNA transfection (Fig. 7(b)). Moreover, the protective effect against H_2O_2 -induced cell death was reduced after the transfection with Nrf2 siRNA (Fig. 7(c)), thereby suggesting that the

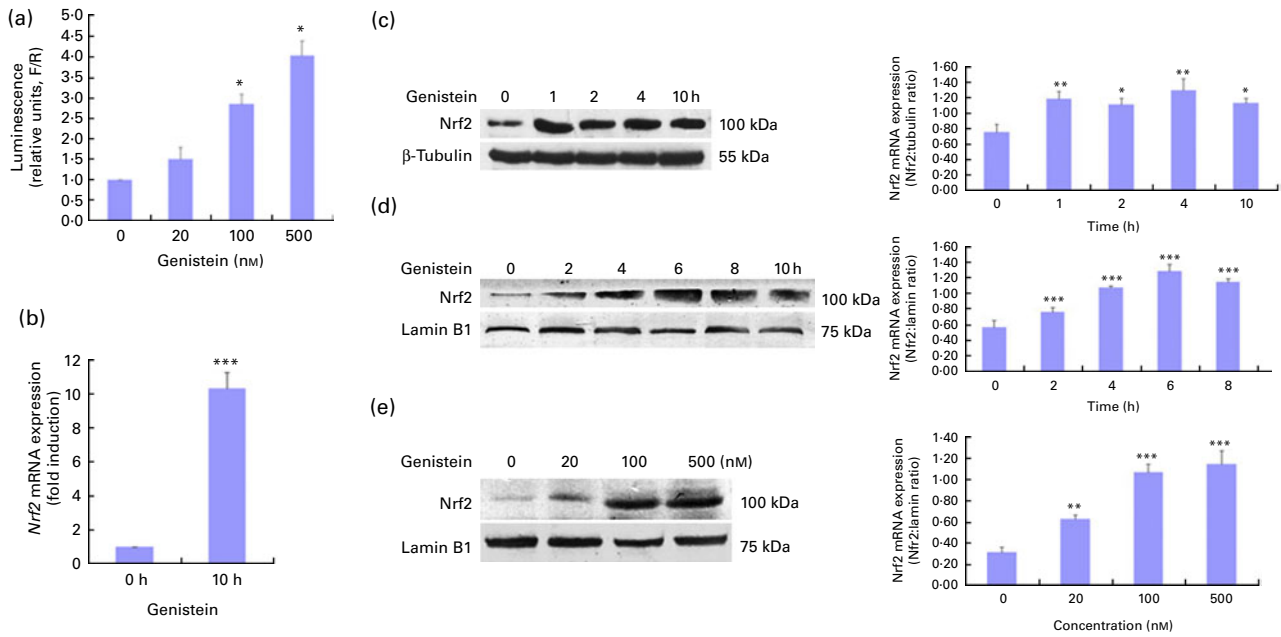


Fig. 4. Genistein activates nuclear factor erythroid 2-related factor 2 (Nrf2). (a) Effect of genistein on Nrf2 promoter activity. EA.hy926 cells were transfected with the antioxidant responsive element-reporter plasmid and treated with genistein (20–500 nm) for 10 h. The cells were then harvested, and luciferase activity was determined. (b) Effects of genistein on *Nrf2* mRNA expression levels. Cells were pretreated with genistein (500 nm) for 10 h, and total RNA was extracted. *Nrf2* mRNA expression levels were assessed by real-time PCR. (c) Effects of genistein on Nrf2 protein expression levels. Cells were pretreated with genistein (500 nm) for the indicated times, and cell extracts were then prepared for Western blot analysis. (d, e) Effects of genistein on the nuclear translocation of Nrf2 protein. Cells were either pretreated with genistein (500 nm) for the indicated times (d) or treated with the indicated concentrations for 6 h (e), and nuclear extracts were then prepared for Western blot analysis. Nuclear extracts were prepared using the Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime) according to the manufacturer's instructions. Values are means (*n* 3 for each group), with standard deviations represented by vertical bars. Mean values were significantly different compared with the non-genistein control group: * *P* < 0.05, ** *P* < 0.01, *** *P* < 0.001.

activation of Nrf2 plays a role in the genistein-mediated protection against oxidative stress-induced cell damage.

To investigate the involvement of PPAR γ and HO-1 in the protective mechanism of genistein, we applied a PPAR γ -specific antagonist (GW9662) and an HO-1-specific antagonist (Znpp) to determine whether they would block the protective effects of genistein. Both GW9662 (10 μ M) and Znpp (10 μ M) partially blocked the protective effects of genistein (Fig. 7(d)), indicating that the activation of PPAR γ and HO-1 is at least partly involved in genistein-mediated protection. The involvement of HO-1 further suggests that *Nrf2* and PPAR γ , two antioxidant genes, play a fundamental role in genistein-induced protection.

Discussion

In the present study, we have shown that treatment with low (physiological) concentrations of the soya isoflavone genistein significantly protects vascular endothelial cells against oxidative stress-induced loss of cell viability and apoptosis. This protective effect was mediated by both the gene regulation of *Nrf2* and PPAR γ , and their downstream gene *HO-1*, as well as cellular antioxidants, including SOD, CAT and GSH. These findings shed light on the mechanism by which genistein protects vascular endothelial cells against oxidative stress.

Endothelial cell damage and subsequent apoptosis are key events in the pathogenesis of various vascular diseases, such as oxidative stress-induced atherosclerosis⁽²³⁾. Genistein

(μ M, pharmacological concentrations) has previously been demonstrated to hinder oxidative stress-induced endothelial damage, as displayed by the inhibition of cell proliferation and secretion⁽²⁴⁾ as well as a decrease in cell apoptosis^(7,25–27) and permeability⁽²⁸⁾. Here, the present results show that genistein displayed cytoprotective effects at physiological concentrations (nm) and significantly inhibited oxidative stress-induced cell apoptosis. Furthermore, the 100–500 nm concentration of genistein found to exert the potent cytoprotective effects is within the concentration range found in the plasma of Asian populations with a diet rich in soya protein (300–500 nm)^(8,29), which suggests that a soya-rich diet may be beneficial for atherosclerosis patients.

However, controversy exists regarding the effect of genistein-mediated activity (antioxidant or pro-oxidant) on apoptosis. Genistein has been shown to induce apoptosis in many cancer cell types, including breast cancer cells, prostate cancer cells, non-small cell lung cancer cells, head and neck squamous carcinoma cells, and pancreatic cancer cells⁽³⁰⁾. This genistein-induced pro-apoptotic effect may be due to its regulation of apoptosis-associated genes, such as *Bcl-2*, *Bax*, *Bcl-xL*, *PARP* (poly-ADP-ribose polymerase) and *caspase-3*⁽³⁰⁾. There is limited direct evidence that genistein's pro-apoptotic effect is attributed to its pro-oxidant activity. Ullah *et al.*⁽³¹⁾ have found that genistein induced breast cancer cell death through the mobilisation of endogenous Cu ions and generation of ROS. However, genistein exerts an antioxidant effect via the inhibition of ROS generation,

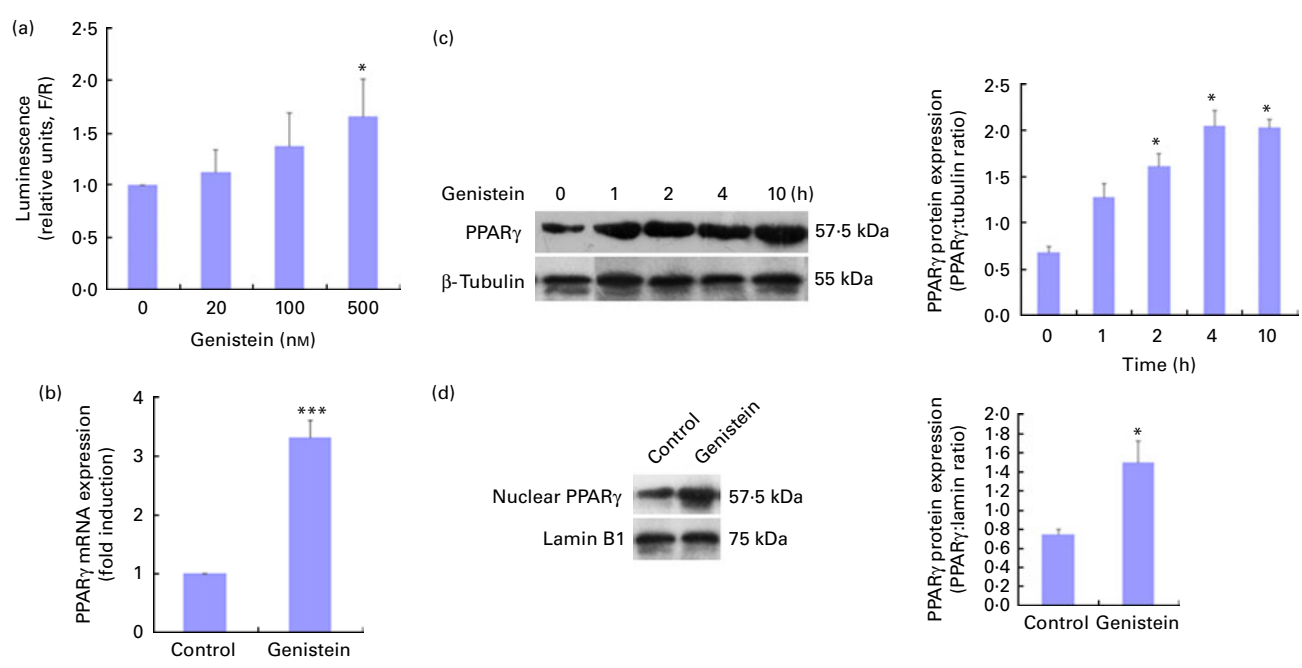


Fig. 5. Genistein activates PPAR γ activity. (a) Effect of genistein on PPAR γ promoter activity. EA.hy926 cells were transfected with a PPAR γ -reporter plasmid and treated with genistein (20–500 nM) for 16 h. Cells were subsequently harvested, and then luciferase activity was determined. F/R, firefly/renilla (b) Effect of genistein on PPAR γ mRNA expression levels. Cells were pretreated with genistein (500 nM) for 10 h, and total RNA was extracted. The PPAR γ mRNA expression levels were assessed by real-time PCR. (c) The effects of genistein on PPAR γ protein expression levels. Cells were pretreated with genistein (500 nM) for the indicated times, and cell extracts were prepared for the analysis of PPAR γ expression via Western blot. (d) Effects of genistein on the nuclear translocalisation of PPAR γ protein. Cells were pretreated with genistein (500 nM) for 4 h, and nuclear extracts were prepared for Western blot analysis. Values are means (n 3 for each group), with standard deviations represented by vertical bars. Mean values were significantly different compared with the non-genistein control group: * $P < 0.05$, *** $P < 0.001$.

thereby resulting in the inhibition of NF- κ B activation, which plays a role in angiogenesis and metastasis. Genistein also acts as a chemopreventive agent against CVD, osteoporosis and Alzheimer's disease. It has been reported that genistein reduces the levels of apoptosis by enhancing the expression of anti-apoptotic proteins in endothelial cells, human retinal pigment epithelial cells and neuronal PC12 cells. In addition to the direct scavenging of ROS and the inhibition of the oxidation of LDL, the ability of genistein to activate cell signalling pathway molecules, including endothelial No synthase, nuclear factor erythroid 2-related factor 1 (Nrf1) and Nrf2, makes it an optimal natural antioxidant with potential clinical use. The observation that genistein exerts a pro-apoptotic effect on cancer cell while normal cells remain unaffected may be due to differences in cell type, pathophysiology and behavioural gene expression in cancer and CVD. Unlike normal cells, cancer cell types are characterised by deregulated cell growth and cell proliferation, invasive and metastatic potential, and constitutively active NF- κ B signalling, resulting in high levels of oxidative stress. It has previously been reported that genistein concentrations that induce apoptosis range from approximately 30 to 200 μ M, based on studies in various cancer cell lines. Interestingly, these high genistein concentrations are non-toxic to normal cells. According to the present observations, genistein (50 μ M) has little influence on endothelial cell proliferation and apoptosis. Moreover, curcumin has been shown to induce apoptosis and enhance the activity of oxaliplatin on colon cancer cells, while

immortalised normal colon cells remain unaffected⁽³²⁾. Additionally, (–) epigallocatechin gallate causes apoptosis in epidermoid carcinoma cells, but not in normal keratinocytes⁽³³⁾. Therefore, we could not simply analyse genistein as an antioxidant or a pro-oxidant while studying its anti-apoptotic or pro-apoptotic effect under different pathophysiology. This is especially true for mechanistic studies, which require careful consideration and realistic approaches with respect to dose, choice of cell types and growth conditions.

Bcl-2 is an anti-apoptotic protein that plays an important role in sustaining cell viability and function⁽³⁴⁾. Both Bcl-2 and Bax, another Bcl-2 family member, have been shown to be regulated by oxidative stress⁽³⁵⁾. However, Bcl-2 regulation by phyto-oestrogens during oxidative stress conditions has only been reported in a few studies^(7,35). Here, we verified that Bcl-2 protein expression was up-regulated by genistein in EA.hy926 cells, thereby suggesting that this effect may play a role in the mechanism by which genistein protects against oxidative stress-induced endothelial cell injury.

Genistein is the most prominent soya isoflavone and has been shown to inhibit the growth of cancer cells via the regulation of genes associated with the homeostatic control of cell-cycle progression and apoptosis. At high concentrations (10–50 μ M), genistein has also been shown to act as an inhibitor of protein-tyrosine kinases⁽³⁶⁾. Although we used a relatively low genistein concentration (100–500 nM) in the present study, we observed a significant cytoprotective effect against oxidative stress in endothelial cells, which correlates

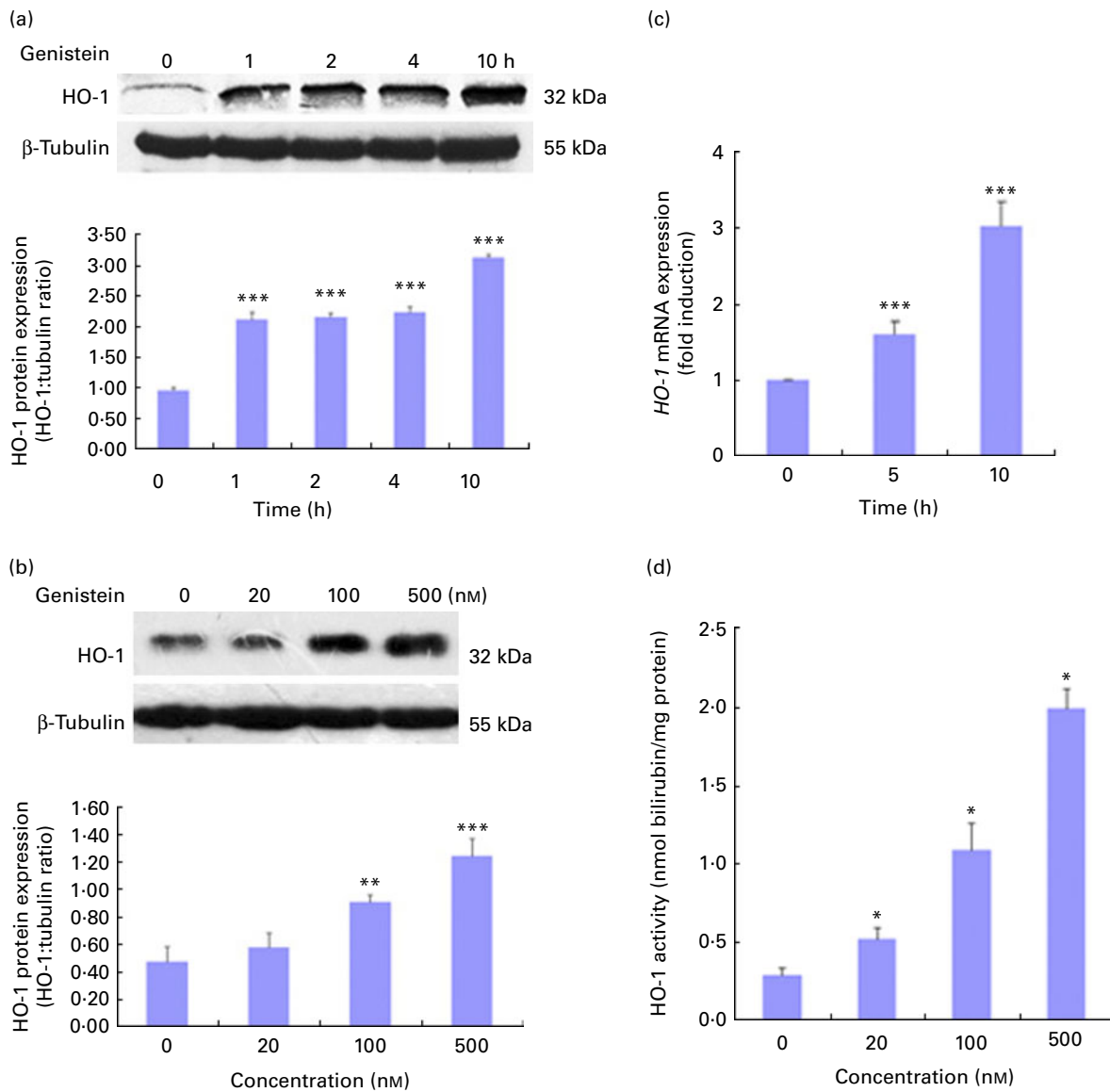


Fig. 6. Genistein up-regulates haem oxygenase-1 (HO-1) expression. (a, b) Effect of genistein on HO-1 protein expression levels. EA.hy926 cells were either pretreated with genistein (500 nM) for the indicated times (a) or treated with various concentrations of genistein for 10 h (b). Cell extracts were then prepared, and HO-1 expression levels were analysed using Western blotting. (c) Effect of genistein on HO-1 mRNA expression levels. Cells were pretreated with genistein (500 nM) for the indicated times, and total RNA was extracted. The HO-1 mRNA expression levels were assessed by real-time PCR. (d) HO-1 activity was measured 10 h after exposure to various concentrations of genistein. Values are means (*n* 3), with standard deviations represented by vertical bars. Mean values were significantly different compared with the non-genistein control group: * *P* < 0.05, ** *P* < 0.01, *** *P* < 0.001.

with the results described by Xu *et al.*⁽⁷⁾. Interestingly, plasma genistein levels of patients receiving genistein or dietary soya supplementation were previously found to be 18 (SD 20) nM⁽³⁷⁾, while plasma genistein concentrations ranged from 50 to 800 ng/ml (equal to 185 nM–3 μM) in Japanese subjects with traditional soya-rich diets^(29,38). Therefore, the beneficial effects of genistein occur at dietary concentrations. In addition, previous studies have indicated that a 10–50 μM-genistein concentration is required for the protein-tyrosine kinases-inhibitory effects of genistein, thereby suggesting that the protective effects observed in the present study may be a result of the antioxidative properties of genistein rather than its inhibitory effects on protein-tyrosine kinases. Moreover, as it is difficult to take in

such high levels (μM) simply through dietary soya supplementation and may also be difficult to achieve via drug intervention, physiologically relevant concentrations (nM) of genistein that can be obtained with either a reasonable diet or pharmacologically relevant supplementation are more realistic for the clinical setting. Therefore, a change in diet or the use of supplements may help to improve vascular dysfunction in patients by decreasing oxidative stress.

Nrf2 has been implicated in the regulation of genes involved in response to oxidative stress⁽³⁹⁾. Nrf2-null mice exhibit an increased susceptibility to oxidants, colitis, lupus-like immune nephritis, emphysema, carcinogenesis and vascular damage⁽⁴⁰⁾. Based on the involvement of Nrf2 in chemoprevention, a

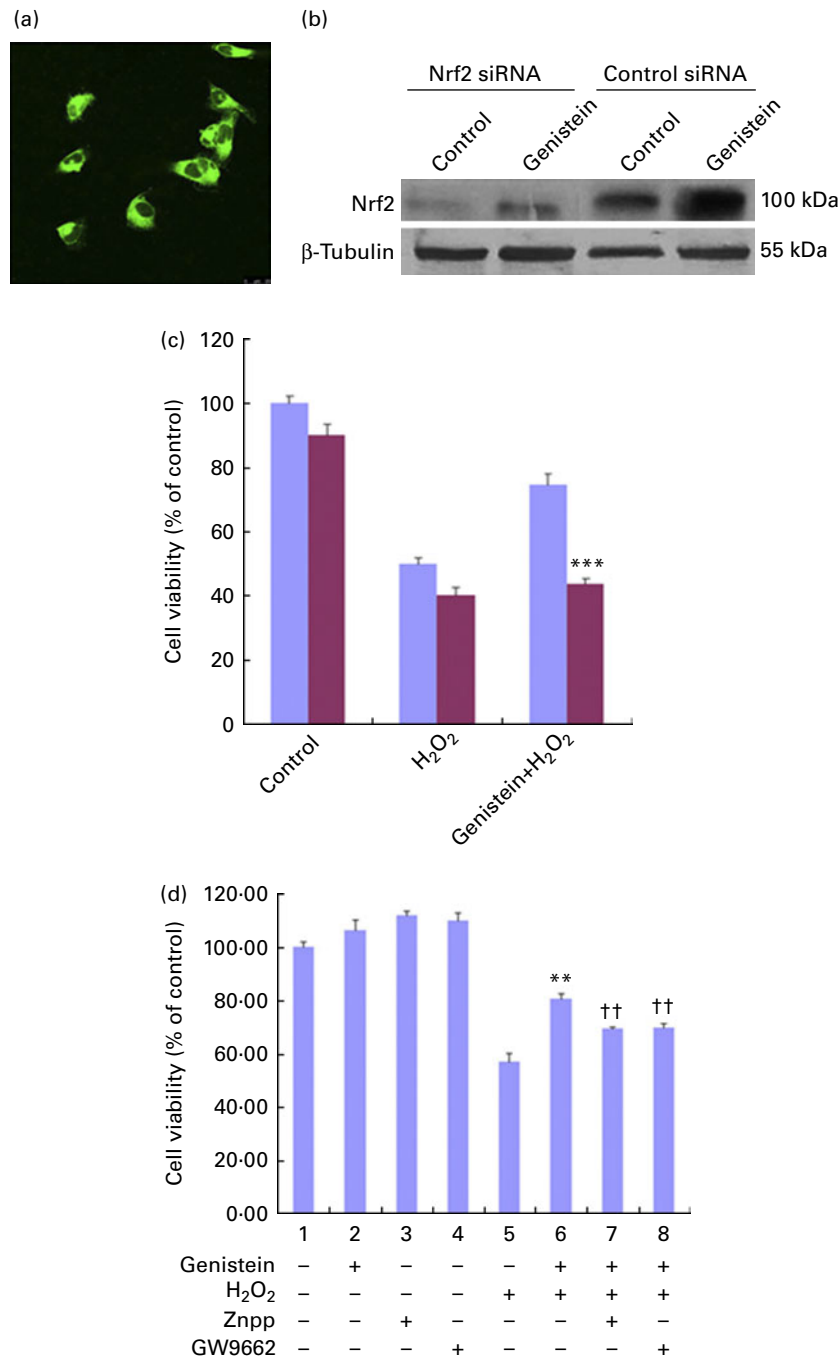


Fig. 7. Inhibition of nuclear factor erythroid 2-related factor 2 (Nrf2), PPAR γ and haem oxygenase-1 attenuates the cytoprotective effect of genistein. EA.hy926 cells were seeded on sterile glass coverslips in twenty-four-well plates overnight, and small interfering RNA (siRNA) transfection was then performed according to the manufacturers' instructions. (a) A control siRNA (fluorescein-conjugated) transfection was used as a control. (b) Cells were transfected with either Nrf2 siRNA or control siRNA, treated with genistein (500 nm) for 10 h, and harvested. Nrf2 protein levels were assessed by Western blot analysis. (c) After transfection with Nrf2 siRNA (■) or control siRNA (□), cells were pretreated with genistein (500 nm) for 10 h and then incubated with H_2O_2 (650 μ M) for 24 h. Cell viability was determined by the Cell Counting Kit-8 (CCK-8) assay. Values are means ($n=6$), with standard deviations represented by vertical bars. *** Mean values were significantly different compared with the control siRNA transfection (genistein plus H_2O_2) group ($P < 0.001$). (d) Cells were treated with genistein (500 nm) for 10 h and then incubated with H_2O_2 (650 μ M) with the addition of either Znpp (10 μ M) for 24 h or GW9662 (10 μ M) for 30 min. Next, cells were treated with genistein (500 nm) for 10 h and then incubated with H_2O_2 (650 μ M) for 24 h. Cell viability was assessed using the CCK-8 assay. Values are means ($n=6$ for each group), with standard deviations represented by vertical bars. Mean values were significantly different compared with both the H_2O_2 -only control group (** $P < 0.01$, *** $P < 0.001$) and the genistein plus H_2O_2 control group (†† $P < 0.01$, ††† $P < 0.001$).



high-throughput screening for small-molecule Nrf2 activators represents an innovative strategy to enhance resistance to environmental insults. HO-1 plays a key role in the maintenance of antioxidant homeostasis during cellular stress⁽⁴¹⁾. Human atherosclerotic lesions have been shown to exhibit enhanced gene expression of *HO-1*, while the overexpression of *HO-1* in vascular tissues has been shown to protect against atherogenesis and restenosis in rodent models of hypercholesterolaemia and vascular injury⁽⁴²⁾. Until now, evidence displaying that genistein may regulate the expression of genes encoding for phase II and antioxidant enzymes was limited and controversial. *In vivo* studies have shown that Nrf2 protein levels were not altered by dietary genistein in the liver of feeding rats⁽⁴³⁾. A pharmacological dose of genistein (50 μM) was previously found to activate Nrf1, but not Nrf2, in EA.hy926 cells⁽⁴⁴⁾. Genistein treatment has been shown to lead to increased quinone reductase activity, although daidzein and equol displayed a greater impact on Nrf2 activity at physiological concentrations (1 and 5 μM) compared with genistein⁽⁴⁵⁾. Borrás *et al.*⁽⁴⁶⁾ have reported that genistein (0.5 μM) up-regulated antioxidant and longevity-related genes as well as MnSOD levels in MCF-7 cells. However, we herein provide the first demonstration that a low, physiologically relevant concentration of genistein (20–500 nM) can activate Nrf2 and a downstream gene, *HO-1*, in endothelial cells. Moreover, we demonstrated that the effect of genistein on H₂O₂-induced loss of cell viability was blocked by Nrf2 siRNA transfection and the HO-1 antagonist Znpp, which suggests that low concentrations of genistein may protect cells via the activation of the Nrf2/HO-1 pathway. Furthermore, the present findings indicate that physiologically relevant concentrations of genistein, commonly found in the plasma of Eastern populations who regularly consume soya-based foods, may improve cellular redox imbalance via the activation of an antioxidant gene. It remains to be determined whether these plasma levels can also be achieved in Western populations, as these populations generally do not consume soya products, and thus there may be variations in the disposition of genistein in different populations.

Both human genetic analyses and studies in transgenic mice have demonstrated the importance of PPAR γ in vascular disorders⁽⁴⁷⁾. Angiotensin II has been implicated in both hypertension and atherosclerosis in mice, and studies on endothelial cell dysfunction display decreases in PPAR γ mRNA and protein expression⁽⁴⁸⁾. However, the impact of oxidative stress on PPAR γ activity is not clear. Recent studies have shown that oxidative stress attenuates PPAR γ expression and activity in endothelial cells through the suppression of PPAR γ transcription at least partly as a result of the activation of inhibitory redox-regulated transcription factors⁽⁴⁹⁾. PPAR γ activation in endothelial cells inhibits inflammation by suppressing the expression of inflammation-associated genes, thereby reducing endothelial damage^(18,19). Genistein has been shown to act as an agonist of PPAR γ in an *in vitro* reporter gene assay experiment⁽⁵⁰⁾, while the oestrogen-induced production of a PPAR ligand has also been reported⁽⁵¹⁾. In our studies, we observed that genistein (80 μM), a natural PPAR γ ligand, could induce HT29 cell apoptosis via the

activation of PPAR γ (R Rong unpublished results). In the present study, we demonstrated that low concentrations of genistein also induced PPAR γ promoter activity and led to increases in PPAR γ protein expression and transcription levels in EA.hy926 cells, while the effects of genistein during cellular oxidative stress were attenuated by a PPAR γ -specific antagonist, GW9662. These results are consistent with studies that displayed that genistein (0.5 μM) activates PPAR γ in cultured astrocytes⁽⁵²⁾. In addition, it should be noted that PPAR γ ligands are also able to up-regulate HO-1⁽⁵³⁾. The up-regulation of HO-1 by genistein further suggests the involvement of the PPAR γ signalling pathway in genistein-induced endothelial cell protection. Several clinical trials have demonstrated the apparent cardiovascular benefits of thiazolidinediones (especially rosiglitazone), a class of drugs used to treat type 2 diabetes mellitus that functions by binding to PPAR γ ⁽⁴⁷⁾. Genistein may also exert cardiovascular benefits through PPAR proteins. Therefore, the present results provide a basis for new approaches in the treatment of oxidative stress-induced atherosclerosis.

Low concentrations of genistein have been shown to activate the oestrogen receptor (ER). At high concentrations, genistein inhibits the growth of both ER α - and ER β -positive MCF-7 cells as well as ER α -negative and ER β -positive (MDA-MB-231) breast cancer cells via an ER-independent mechanism; however, the stimulation of MCF-7 cell growth by treatment with low concentrations of genistein functions via the ER⁽⁵⁴⁾. Similar reports of ER α and ER β immunoreactivity were first identified in human umbilical vein endothelial cells (HUVEC) by Joy *et al.*⁽⁵⁵⁾. Genistein has also been reported to up-regulate ER β expression in HUVEC⁽⁷⁾. Moreover, studies have suggested that oestrogenic activity is involved in genistein-mediated antioxidant effects (quinone reductase and MnSOD)^(45,46) and that endothelial cells also represent an important oestrogen target. However, the present data display conflicting results regarding the interactions between oestrogen and the antioxidant genes mediated by soya isoflavones. Nutritionally relevant plasma concentrations of equol stimulated NO synthesis in an ER-independent manner⁽⁵⁵⁾. This mounting evidence suggests that the mechanism of genistein activity is more complex than simple receptor interactions or antioxidant activation. Therefore, further analysis should be focused on the possible involvement of ER-mediated antioxidant genes (*Nrf2* and *PPAR γ*) in endothelial cells.

In conclusion, genistein can protect against oxidative stress-induced endothelial cell injury by altering the expression of antioxidant genes and elevating antioxidant enzyme and GSH levels. The present results provide the basis for further evaluation of the effects of soya isoflavones with regard to their potential in the treatment of CVD. Moreover, the present findings suggest that the lower incidence of CVD in Asian populations compared with Western populations may be at least partially due to the higher plasma concentration of genistein in Asian individuals.

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