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Isoflavone genistein protects human vascular endothelial cells against tumor necrosis factor- α -induced apoptosis through the p38 β mitogen-activated protein kinase

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Abstract

Isoflavone genistein may have beneficial effects on vascular function, but the mechanism is unclear. Here, we investigated whether genistein protects vascular endothelial cells (ECs) against apoptosis induced by tumor necrosis factor- α (TNF- α). We show that genistein significantly inhibited TNF- α -induced apoptosis in human aortic endothelial cells (HAECs) as determined by caspase-3 activation, 7-amino actinomycin D staining, *in situ* apoptotic cell detection and DNA laddering. The anti-apoptotic effect of genistein was associated with an enhanced expression of Bcl-2 protein and its promoter activity. Inhibition of extracellular signal-regulated kinase 1/2, protein kinase A, or estrogen receptors had no effect on the cytoprotective effect of genistein. However, inhibition of p38 mitogen activated protein kinase (p38) completely abolished this genistein effect. Accordingly, stimulation of HAECs with genistein resulted in rapid activation of p38 β , but not p38 α . These findings provide the evidence that genistein acts as a survival factor for vascular ECs to protect cells against apoptosis via activation of p38 β . Preservation of the functional integrity of the endothelial monolayer may represent an important mechanism by which genistein exerts its vasculoprotective effect.

Keywords

genistein; endothelial cells; apoptosis; p38

Introduction

Bioactive compound genistein, one of the major isoflavones in soy and red clover, has various biological actions, including a weak estrogenic effect [1] by binding to estrogen receptors (ERs) [2], and inhibition of protein tyrosine kinases (PTKs) at pharmacological doses [3]. The potential effects of isoflavones on human vascular health have been extensively investigated during the past ten years. While the effects of an isoflavone mixture on human vascular health may be controversial [4–8], recent human studies have shown that dietary supplementation of genistein alone has a significantly beneficial effect on atherosclerosis [9], markers of cardiovascular risk [10,11], vascular motor tone [12,13], vascular endothelial function [14], and systemic arterial compliance [15]. Data from animal and *in vitro* studies also consistently suggest a protective role of genistein in cardiovascular events [16–22]. It also has been shown that genistein reduces the size of infarction and experimental myocardial ischemia-reperfusion injury [23], and improves endothelial dysfunction induced by oophorectomy in rats [24],

providing consistent evidence for a cardioprotective effect of genistein. However, the mechanism of genistein action in vasculature is still not clear.

Past studies have explored its hypolipidemic [25], anti-oxidative [26,27] and the estrogenic effects [28], which all play potential roles in the initiation of atherosclerosis [29–31]. While genistein may have both ER-dependent and independent actions in vasculature, its average effect on plasma lipid profile is neutral [15,32]. In addition, genistein is not a physiologically effective antioxidant or scavenger of reactive oxygen species [33,34], although it has been reported to exhibit antioxidant activity in aqueous phase systems [35] and prevent LDL oxidation at pharmacological doses [36,37]. Recently, we [38,39] and others [40] demonstrated a direct action of genistein on vascular endothelial cells (ECs) *in vitro* and *in vivo* to modulate vascular function through a mechanism independent of ERs.

The vascular endothelial monolayer, which separates circulating blood and peripheral tissues, plays a pivotal role in maintaining normal vascular function. Endothelial injury or loss of ECs due to aging-apoptosis contributes to the development of aging-associated vascular diseases such as arteriosclerosis [41] and acute coronary syndrome [42], which is enhanced by circulating inflammatory cells and other risk factors such as tumor necrosis factor- α (TNF- α). In the present study, we first examined whether genistein protects human aortic ECs (HAECs) from TNF- α -induced apoptosis. We then defined the cellular mechanism underlying this genistein action. We report the novel observation that genistein protects HAECs from TNF- α -induced apoptosis. This cytoprotective effect of genistein is reversed by inhibition of p38 mitogen-activated protein kinase (p38). Specifically, genistein induces the activation of p38 β , but not p38 α , which may underline the ability of genistein to rescue ECs from TNF- α -induced apoptosis.

Material and Methods

Materials

Primary HAECs, fetal bovine serum (FBS) and endothelial growth supplements (EGM2) were purchased from Lonza Walkersville (Walkersville, MD); M199 media, caspase-3 assay kits and competent cells for plasmid transformation were from Invitrogen (Carlsbad, CA); antibodies against p38, phospho-p38 (Thr180/Tyr182), p38 α , p38 β , Bcl-2 and β -actin were from Cell Signaling Technology (Beverly, MA); supersignal chemiluminescence detection system and Protein A beads were from Pierce (Rockford, IL); nitrocellulose membranes and protein assay kits were from Bio-Rad (Hercules, CA); Bcl-2 promoter-driven luciferase (Bcl-2-Luc) reporter construct was a kind gift from Dr. Linda M. Boxer, Stanford University, Stanford, CA; plasmid purification kit was from Qiagen (Valencia, CA); transfection reagents were from Targeting System (Santee, CA); dual luciferase reporter assay kits were from Promega (Madison, WI); terminal deoxynucleotidyltransferase dUTP nick-end labeling (TUNEL) and apoptotic DNA ladder kits were from Roche Applied Science (Indianapolis, IN); ICI182,780 was from Tocris (St. Louis, MO); genistein, TNF- α , SB203580, SB203474, H89, PD098059, protease and phosphatase inhibitor cocktails, 7-amino actinomycin D (7-AAD) and other general chemicals were from Sigma (St. Louis, MO); stock solutions of genistein, at 20 mM in dimethylsulfoxide (DMSO), were stored at -80°C before use.

Cell culture

Primary HAECs were cultured in M199 medium containing 2% FBS and endothelial growth supplements-EGM2 at 37°C in a 5% CO_2 /95% air environment. The medium was changed every other day until the cells became confluent. HAECs were passaged by using 0.05% trypsin treatment and passages 4–6 were used in all experiments.

Cell apoptosis assay

Confluent HAECs cultured in 12-well plates or on chamber slides were treated with TNF- α (20 ng/ml) in the presence or absence of genistein (0.1–10 μ M) for 48 h. Apoptotic cells were counted using flow cytometry as described [43] with minor modification. Briefly, treated HAECs were suspended using 0.05% trypsin-EDTA and washed by centrifugation using phosphate buffered saline (PBS) at 2,000 $\times$ g for 4 min at 4°C. The cells were then incubated in PBS containing 20 μ g/ml of 7-AAD at 4°C for 20 min. After washed once with PBS, cells were suspended in 200 μ l of PBS and applied to a FACS Calibur flow cytometer (BD, CA) to detect apoptotic cells based on the loss of membrane permeability. Data were analyzed using a CellQuest software (BD, CA). For *in situ* detection of apoptotic cells, cells were fixed with 4% (wt/vol) paraformaldehyde in PBS (pH 7.4) at room temperature for 1 h, and then permeabilized with 0.1 % Triton X-100 in 0.1% sodium citrate solution on ice for 5 min. The apoptotic cells were detected using TUNEL techniques as described [44]. For DNA laddering assay, treated HAECs were harvested into lysis buffer. DNA was isolated using an apoptotic DNA ladder kit following the manufacturer's protocol. DNA fragmentation was detected by standard agarose gel electrophoresis.

Caspase-3 activity assay

Cytosolic enzymatic activity of caspase-3 was measured essentially as described in the manufacturer's protocol. The caspase-3 activity in the cell lysates was normalized to the cellular protein concentration and expressed as fold of the control.

Immunoprecipitation

HAECs were harvested into lysis buffer (20 mM Tris/HCl, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM Na₄P₂O₇, 1 mM glycerolphosphate, and IMm Na₃VO₄, pH 7.4) supplemented with protease (1:500) and phosphatase (1:100) inhibitor cocktails, and cell extracts were collected by centrifugation at 12,000 $\times$ g for 10 min at 4°C. An aliquot of supernatant (100 μ g protein) was incubated with p38 α or p38 β monoclonal antibody (1:200) with gentle mixing at 4°C. 12 h later, 80 μ l of washed protein A beads was added to each sample and the mixture was incubated at 4°C for 2 h. The immunoprecipitates were collected following centrifugation at 12,000 $\times$ g for 3 min and sequentially washed three times in lysis buffer and once in water. The final pellets were resuspended and boiled in SDS-PAGE sample buffer at 95°C for Western blotting as described below.

Western blot analysis

Equal amounts of proteins from cell extracts were subjected to Western blot analysis as described previously (28, 29). Membranes were probed with antibody against phospho-p38 or Bcl-2. The immunoreactive proteins were detected by chemiluminescence. Nitrocellulose membranes were then stripped and reprobed with p38 or β -actin. The protein bands were digitally imaged for densitometric quantitation with a software program (Gene tools, Synoptics Ltd. UK). Phospho-p38 and Bcl-2 expression was normalized to that of p38 and β -actin, respectively, from the same sample.

Promoter activity assay

Bcl-2-Luc reporter plasmids were amplified with competent cells and purified using Qiagen's Maxi kit. For transient transfection of the plasmids, HAECs were grown in 24-well plates until 70% confluence. The cells were then co-transfected with 1.2 μ g of Bcl-2-Luc and 0.5 ng of pRL control plasmid per well using F-1 transfection reagent for 24 h according to the manufacturer's protocol. The transfected cells were then treated with various concentrations of genistein or vehicle in the presence or absence of TNF- α (20 ng/ml) in phenol-red free M199 medium containing 2% FBS for 24 h. Cells were harvested in reporter lysis reagent. Luciferase

activity, normalized to pRL activity in the cell extracts, was determined by using the dual luciferase reporter assay system [44].

Statistical analysis

Data was analyzed with one-way ANOVA using SAS[®] program and expressed as mean $\pm$ standard error (SE) derived from 3–4 independent experiments. Treatment differences were subjected to a Tukey's multiple comparison tests, where $p < 0.05$ was considered significantly different.

Results

The effect of genistein on ECs apoptosis

To determine whether genistein is a ECs survival factor, we first incubated HAECs with TNF- α (20 ng/ml) in the presence or absence of different concentrations of genistein for 48 h, and then detected apoptotic cells with flow cytometry using the fluorescent DNA-binding agent 7-AAD, which monitors the loss of membrane integrity during apoptosis [45]. As shown in Fig. 1, genistein dose-dependently attenuated TNF- α -induced apoptosis, with 10 μ M inducing the maximal effect (Fig. 1a, b). We further evaluated the anti-apoptotic effect of genistein by directly assessing the percentage of apoptotic cells using TUNEL assay. Consistent with the result observed by 7-AAD staining, addition of genistein (10 μ M) reduced the percentage of TNF- α -induced apoptotic cells from 33.0% to 15.7% (Fig. 1c, d). To further confirm the anti-apoptotic effect of genistein, we performed electrophoretic analysis of DNA fragmentation, a key feature of cell undergoing apoptosis. As shown in Fig. 1e, genistein ameliorated TNF- α -induced DNA fragmentation in HAECs.

Genistein reduces TNF- α -increased caspase-3 activity

The caspase proteins are critical components responsible for apoptosis [46] and caspase-3 is one of the key proteases involved in the convergence of disparate apoptotic signaling pathways. In parallel with the increased cell apoptosis, exposure of HAECs to TNF- α for 9 h increased the cellular caspase-3 activity to 1.5 fold of the control in HAECs (Fig. 2). However, co-incubation of HAECs with genistein (5–10 μ M) significantly reduced TNF- α -induced caspase-3 activity (Fig. 2).

Genistein reverses TNF- α -impaired Bcl-2 expression

It is well recognized that Bcl-2 plays an important protective role in cell viability. To elucidate the mechanism underlying cytoprotective effect of genistein on ECs, we first determined whether genistein could enhance the expression of the anti-apoptotic protein Bcl-2. As shown in Fig. 3a, exposure of HAECs to TNF- α for 48 h suppressed Bcl-2 protein level by 38% compared to that of the control. However, addition of genistein reversed the TNF- α -impaired Bcl-2 protein expression to a level similar to that of the control. Furthermore, genistein directly increased Bcl-2 promoter activity, as determined by a Bcl-2 promoter-driven luciferase reporter assay (Fig. 3b), indicating that genistein may directly regulate Bcl-2 expression at the transcriptional level.

The anti-apoptotic effect of genistein is independent of ERs, the extracellular signal-regulated kinase (ERK1/2) or protein kinase A (PKA) pathways

As genistein has weak estrogenic effects in some tissues by binding to ERs [28], and 17 β -estradiol has been shown to protect ECs from apoptosis through an ER-dependent mechanism [47,48], we examined whether ERs are involved in the cytoprotective effect of genistein. Our results demonstrated that ICI 182,780 (10 μ M), the highly specific inhibitor of ERs, did not ablate the inhibitory effect of genistein on caspase-3 activity (Fig. 4a), but blocked the anti-

apoptotic effect of 17 β -estradiol (Fig. 4b), suggesting that the anti-apoptotic effects of genistein and 17 β -estradiol are mediated by different mechanisms. To further confirm that ERs are not involved in the anti-apoptotic effect of genistein, we tested the cytoprotective effect of daidzein, an analogue of genistein with very low affinity to ERs. The result showed that daidzein also inhibited TNF- α -induced caspase-3 activity that was not abolished by ICI 182,780 (10 μ M) (Fig. 4c). Both PKA [49,50] and ERK1/2 [51] are reported to be involved in preventing EC apoptosis, and previous studies have shown that genistein stimulates the activity of PKA and ERK1/2 in ECs [39,52]. Therefore, we investigated whether the anti-apoptotic effect of genistein is mediated through these pathways. The results show that incubation of HAECs with the ERK1/2 pathway blocker, PD098059 (10 μ M) (Fig. 4d), or the PKA inhibitor, H89 (Fig. 4e), had no effect on the inhibitory effect of genistein on caspase-3 activity. Both PD098059 and H89 were active, since H89 completely inhibited PKA activity and subsequent CREB activation by genistein, and PD098059 blocked genistein-induced ERK1/2 phosphorylation (data not shown), using the same inhibitor concentration as in our experimental studies.

The anti-apoptotic effect of genistein on HAECs is mediated by p38 β

Previous studies showed that p38 mediates the anti-apoptotic effect of heme oxygenase-1 in ECs [53] and genistein can activate p38 in mammary epithelial cells [54]. We therefore examined whether p38 is involved in the anti-apoptotic effect of genistein. As shown in Fig. 5a, SB203580 (40 μ M), a specific inhibitor of p38, abolished the inhibitory effect of genistein on TNF- α -induced caspase-3 activity in HAECs, whereas SB203474, an inactive analogue of SB203580, had no such an effect (Fig. 5b), suggesting that the cytoprotective effect of genistein is mediated by the p38 signaling pathway. Incubation of HAECs with genistein induced a rapid increase in p38 phosphorylation (Fig. 6a), a magnitude that was about 42% of that evoked by TNF- α (Fig. 6b). Previous studies demonstrated that p38 α , one of four p38 isoforms [55], is a pre-apoptotic molecule in ECs [53], whereas activation of p38 β exerts an anti-apoptotic effect [56,57]. Data from immunoprecipitation assay shows that genistein stimulated the phosphorylation of p38 β but simultaneously inhibited p38 α activation. On the contrary, TNF- α remarkably activated p38 α (Fig. 6c), but it had no significant effect on the phosphorylation of p38 β , (Fig. 6d), suggesting that genistein and TNF- α have a differential effect on p38 α and p38 β . Therefore, genistein may protect EC against TNF- α -stimulated apoptosis by selective activation of p38 β .

Discussion

Vascular endothelium, a single layer of ECs lining the luminal side of the vessels, is not only a selective permeable barrier providing a continuous nonthrombogenic lining for the vascular system, but also a form of sensory organ having the ability to monitor, integrate and transduce blood born signals. ECs injury and subsequent apoptosis is a key event in the pathogenesis of various vascular diseases such as diabetes-caused atherosclerosis [58,59]. TNF- α , a proinflammatory cytokine, is remarkably elevated in the plasma and artery both in animals and humans with vascular complications [60–62]. It is believed that TNF- α is critically involved in the pathogenesis of atherosclerosis. Indeed, elevated TNF- α can induce EC dysfunction [60] and apoptosis [61], which disrupts endothelial integrity and leads to cardiovascular disease [63]. Genistein may exert beneficial effects on vasculature which are always explained by its presumably hypolipidemic, weak estrogenic and antioxidative effects, although the results are controversial. Therefore, the cellular and molecular mechanisms underlying the vascular effects of genistein are still unclear. In the present study, we found an important cellular effect for genistein and defined a novel signaling pathway mediating this genistein action, which may explain some of its beneficial vascular effects. We show for the first time to our knowledge that genistein protects against TNF- α -induced apoptosis in HAECs by selective activation of

p38 β . This activity of genistein was independent of the ER-mediated signaling and was not inhibited by PKA and ERK1/2 blockade.

Genistein has been studied for its possible beneficial effects on cancer prevention as it can induce tumor cell apoptosis at pharmacological doses (30–100 μ M) [64,65]. However, our current data shows that genistein is a survival factor for human ECs when used at relatively lower concentrations (5–10 μ M). This result is in line with recent studies demonstrating that genistein (2.5 μ M) can inhibit homocysteine- and oxidized LDL-induced apoptosis in transformed ECs [66,67]. Genistein is a well-known inhibitor of PTK, and is often used to study PTK-mediated signaling events. However, this cytoprotective effect of genistein on ECs is unlikely related to its effect on PTK because the concentrations of genistein required for effective inhibition of PTK are no less than 10 μ M [68,69]. It was reported that total circulating genistein levels in humans and animals consuming soy products or isoflavone supplements are between 0.74–6.0 μ M, and that the genistein concentrations in tissues may be even higher [70–72]. Therefore, the genistein concentrations that produced biological effects observed in this study (5–10 μ M) overlap those attainable in the plasma and tissues in humans following dietary supplementation. However, it must be noted that genistein primarily exists as glucuronide conjugates with reportedly free genistein accounting for only 5–26% of total genistein present in plasma in humans [73]. While genistein conjugates in the serum are reported to be lesser biologically active than free genistein [74], they may serve as excellent sources of biologically active genistein in circulation and within target tissues. Regardless, it is intriguing to speculate as to whether beneficial effects of genistein on ECs survival could be realized in hostile environment such as diabetes where plasma TNF- α level is dramatically elevated [61]. It would probably not be necessary to achieve concentrations that high in plasma to observe a beneficial effect because the anti-apoptotic effect of genistein on ECs is chronic and may be cumulative. In deed, administration of genistein has been found to reduce apoptosis of myocytes and attenuate myocardial ischemia/reperfusion injury in rabbits [75].

Bcl-2 is a well-known anti-apoptotic protein and studies showed that the expression of Bcl-2 is down-regulated by TNF- α in ECs, which is concomitant with the TNF- α -induced apoptosis [76]. Therefore, overexpression of Bcl-2 has been shown to protect ECs against TNF- α -induced apoptosis [77]. In consistent with the protective effect of genistein on TNF- α -induced apoptosis, our studies show that genistein could restore Bcl-2 protein expression ablated by exposure to TNF- α in HAECs. While it is unclear how genistein regulates Bcl-2 expression, it is clear from our data that genistein enhanced Bcl-2 promoter activity, suggesting that genistein may have a direct effect on Bcl-2 transcription, an effect that need further investigation.

While genistein has well-known weak estrogenic effect by binding to the ERs and 17 β -estradiol has been reported to protect ECs against stimuli-induced apoptosis that is mediated by the ER-dependent mechanisms [47], the novel protective effect of genistein on TNF- α -induced apoptosis in ECs is not dependent on the ER-mediated pathway. First, ICI 182,780, a highly specific ER inhibitor [38], did not block the inhibitory effect of genistein on caspase-3 activity, but negated such an effect of 17 β -estradiol in HAECs. In addition, daidzein, a genistein analogue that is essentially lack of affinity to the ERs, also protected ECs against TNF- α -induced caspase 3 activity that was not blocked by ER inhibition (Fig. 4c).

Previous studies demonstrated that genistein activates ERK1/2 and PKA in ECs [39,52], which may play a role in promoting ECs survival [51,52]. However, the cytoprotective effect of genistein on human ECs in the present study was not related to ERK1/2 or PKA. We show that the cytopreventive effect of genistein on TNF- α -induced apoptosis was abolished by SB203580, a specific inhibitor of p38 α and p38 β that has been widely used in investigation of the biological functions involving the p38 kinase signaling pathway. While there is concern

that pharmacological inhibitor may have nonspecific cellular action, which could lead to blockage of genistein effect that is not related to inhibition of p38 kinase, we found that this effect of SB203580 on genistein action was not shared by its inactive analog or pharmacological inhibitors of ERK1/2 and PKA, suggesting that p38 specifically mediates cytoprotective effect of genistein in ECs. While p38 α is the most widely expressed isoform of p38 family, both p38 α and p38 β are equally expressed in vascular ECs [55]. We further demonstrate that genistein activated the phosphorylation of p38 over the same concentration range as its effect on apoptosis. Of interest, exposure of HAECs to TNF- α elicited much more pronounced increase in p38 activity. A wealth of literature exists showing both pro-apoptotic and anti-apoptotic effect of p38 depending on cell-types and stimuli [78,79]. Studies showed that p38 α and p38 β exerts different biological actions. Activation of p38 α has been shown to induce apoptosis in ECs [53], L929 fibroblasts [80], myocytes [81] and HeLa cells [56], whereas p38 β actually promotes survival of these cells [53,56,80,81]. In addition, cardiomyocytes and fibroblasts derived from p38 α deficient mice are less susceptible to undergo apoptosis [82], suggesting that p38 α is a pro-apoptotic molecule both in vitro and in vivo. These data suggest that p38 α and p38 β have antagonistic effects in controlling cellular apoptosis and the balance between these two kinases may therefore decide the cell survival or apoptosis. To define the roles of p38 isoforms in genistein effect, we initially determined whether genistein and TNF- α may have differential effects on p38 α and p38 β activation in ECs. We found that genistein augmented p38 β activity but concomitantly inhibited p38 α activation, whereas TNF- α predominantly induced p38 α activity in HAECs, suggesting that p38 β may mediate the cytoprotective effect of genistein in ECs. Taken these results together, it is reasonable to suggest that genistein protects against TNF- α -induced apoptosis via promoting p38 β activity in ECs.

In conclusion, we provide data showing that genistein can inhibit apoptosis in human vascular ECs exposed to TNF- α , an inflammatory cytokine involved in the pathogenesis of various vascular diseases, suggesting that genistein may act as a survival factor in an inflammatory environment for these cells. We further demonstrate that the cytoprotective effect of genistein was ER-, ERK1/2- and PKA-independent but was mediated through the p38 β signaling pathway, thereby defining a novel mechanism of this genistein action in vascular ECs. These findings potentially provide a basic mechanism underlying some of the vasculoprotective effects of genistein.

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Abbreviations

7-AAD	7-amino actinomycin D
CREB	cAMP-responsive element binding protein
DMSO	dimethylsulfoxide
ECs	endothelial cells
ERs	

	estrogen receptors
ERK1/2	extracellular signal-regulated kinase 1/2
FBS	fetal bovine serum
HAECs	human aortic endothelial cells
LDL	low density lipoprotein
p38	p38 mitogen-activated protein kinase
p38α	p38 mitogen-activated protein kinase alpha
p38β	p38 mitogen-activated protein kinase beta
PBS	phosphate-buffered saline
PKA	protein kinase A
PTK	protein tyrosine kinase
TNF-α	tumor necrosis factor-alpha
TUNEL	terminal deoxynucleotidyltransferase dUTP nick-end labeling

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Fig. 1.

Genistein protects against TNF- α -induced apoptosis in HAECs. (**a**, **b**) Confluent HAECs were treated with or without TNF- α (T, 20 ng/ml) in the presence or absence of various concentrations of genistein (G, 0–10 μ M) for 48 h, and apoptotic cells were stained with 7-ADD and determined using flow cytometry. A representative image and bar graph (mean $\pm$ SE) of four independent experiments were shown. *, $P < 0.05$ vs. TNF- α -alone treated cells. (**c**, **d**) HAECs cultured on chamber slides were incubated with or without TNF- α (T, 20 ng/ml) in the presence or absence of genistein (G, 10 μ M) for 48 h. Apoptotic cells were detected using TUNEL method. A representative image and bar graph (mean $\pm$ SE) from three independent experiments were shown. *, $P < 0.05$ vs. TNF- α -alone treated cells. (**e**) HAECs grown in 6-

well plates were treated as stated in c and d. Genomic DNA was extracted and DNA fragmentation was detected by gel electrophoresis. A representative image from three experiments was shown.

Fig. 2. Genistein inhibits TNF- α -induced caspase-3 activity in HAECs. Confluent HAECs were exposed to TNF- α (T, 20 ng/ml) with or without various concentrations of genistein (G, 0–10 μ M) for 9 h. Cells were collected to measure caspase-3 activity using an assay kit. Data are means $\pm$ SE derived from four separate experiments and expressed as folds of control. *, $P < 0.05$ vs. TNF- α alone-treated cells.

Fig. 3.

Genistein restores TNF- α -inhibited Bcl-2 protein expression and promoter activity in ECs. (a) Confluent HAECs were treated with or without TNF- α (T, 20 ng/ml) in the presence or absence of various concentrations of genistein (G, 1–10 μ M) for 48 h. The Bcl-2 level in the cell extracts were measured by Western blot and normalized to β -actin content. (b) HAECs were co-transfected with Bcl-2 promoter-driven reporter constructs and pRL-CMV plasmids. Cells were then treated with or without TNF- α (T, 20 ng/ml) in the presence or absence of various concentrations of genistein (G, 1–10 μ M) for 24 h. Bcl-2 promoter activity in the cell lysates was measured using a dual-luciferase kit. Values are mean $\pm$ SE obtained from three separate experiments and expressed as folds of control. *, $P < 0.05$ vs. TNF- α -alone treated cells.

Fig. 4.

The inhibitory effect of genistein on TNF- α -induced caspase-3 activity is not dependent on the ER, ERK1/2, or PKA pathway. HAECs were pre-incubated with ICI 182780 (ICI, 10 μ M, **a**, **b**, **c**), PD 098059 (P, 10 μ M, **d**), or H89 (H, 1 μ M, **e**) in the presence or absence of genistein (G, 10 μ M, **a**, **d**, **e**), 17 β -estradiol (E, 10 μ M, **b**) or daidzein (D, 10 μ M, **c**) for 30 min, followed by addition of TNF- α (T, 20 ng/ml) for 9 h. Caspase-3 activity in the cell lysates was measured using an assay kit. The experiment was repeated four times and data (means $\pm$ SE) were expressed as folds of control. *, P<0.05 vs. TNF- α alone-treated cells.

Fig. 5.

The inhibitory effect of genistein on TNF- α -induced caspase-3 activity is mediated by p38 pathway. HAECs were pre-incubated with SB203580 (S0, 40 μ M, **a**), a p38 inhibitor, or SB203474 (S4, 40 μ M, **b**), a SB203580 inactive analog, for 30 min, followed by addition of TNF- α (T, 20 ng/ml) with or without genistein (G, 10 μ M) for 9 h. Caspase-3 activity in the cell lysates was measured. The experiment was repeated four times and data (means $\pm$ SE) were expressed as folds of control. *, P<0.05 vs. TNF- α alone-treated cells.

Fig. 6.

Genistein differentially modulates phosphorylation of p38 α and p38 β in HAECs. **(a)** HAECs were incubated with various concentrations of genistein (G, 0.01–10 μ M) for 15 min. **(b)** HAECs were incubated with either genistein (G; 10 μ M), TNF- α (T; 20 ng/ml) or vehicle (C) for 15 min. The phosphorylation of p38 was detected by Western blot using a phospho-specific p38 antibody, normalized to total p38. **(c, d)** HAECs treated with genistein (G, 10 μ M), TNF- α (T, 20 ng/ml) or vehicle (C) were lysed and immunoprecipitated with p38 α or p38 β antibody, followed by measuring the phosphorylation of p38 using Western blot. The experiment was repeated three times and data (means $\pm$ SE) were expressed as folds of control. *, $P < 0.05$ vs. vehicle alone-treated control.