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Genistein inhibits TNF- α -induced endothelial inflammation through the protein kinase pathway A and improves vascular inflammation in C57BL/6 mice

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Abstract

Genistein, a soy isoflavone, has received wide attention for its potential to improve vascular function, but the mechanism of this effect is unclear. Here, we report that genistein at physiological concentrations (0.1 μ M–5 μ M) significantly inhibited TNF- α -induced adhesion of monocytes to human umbilical vein endothelial cells (HUVECs), a key event in the pathogenesis of atherosclerosis. Genistein also significantly suppressed TNF- α -induced production of adhesion molecules and chemokines such as sICAM-1, sVCAM-1, sE-selectin, MCP-1 and IL-8, which play key role in the firm adhesion of monocytes to activated endothelial cells (ECs). Genistein at physiologically relevant concentrations didn't significantly induce antioxidant enzyme activities or scavenge free radicals. Further, blocking the estrogen receptors (ERs) in ECs didn't alter the preventive effect of genistein on endothelial inflammation. However, inhibition of protein kinase A (PKA) significantly attenuated the inhibitory effects of genistein on TNF- α -induced monocyte adhesion to ECs as well as the production of MCP-1 and IL-8. In animal study, dietary genistein (0.1% genistein in the diet) significantly suppressed TNF- α -induced increase in circulating chemokines and adhesion molecules in C57BL/6 mice. Genistein treatment also reduced VCAM-1 and monocytes-derived F4/80-positive macrophages in the aorta of TNF- α treated mice. In conclusion, genistein protects against TNF- α induced vascular endothelial inflammation both *in vitro* and *in vivo* models. This anti-inflammatory effect of genistein is independent of the ER-mediated signaling machinery or antioxidant activity, but mediated via the PKA signaling pathway.

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Disclosure Statements

The authors have nothing to disclose.

Keywords

genistein; vascular inflammation; TNF- α ; protein kinase A; endothelial cells

INTRODUCTION

Atherosclerotic vascular disease is a major cause of morbidity and mortality in the industrial world and claims the lives of over 40 percent of the nearly 2.4 million Americans who die each year [1]. Although the pathogenesis of atherosclerotic vascular disease involves multifactorial processes, accumulating evidence demonstrates that inflammation and its subsequent endothelial dysfunction play a fundamental role in the initiation and progression of atherosclerosis [2]. The recruitment of monocytes by the activated endothelial cells (ECs) mediates the vascular inflammation and leads to the development of atherosclerosis [3]. Inflammation was reported to involve in the important stages of atherosclerosis such as adhesion and migration of monocytes into subendothelial space followed by the formation and rupture of atherosclerotic plaque [3]. It is now recognized that atherosclerosis is strongly modulated by pro-inflammatory mediators such as thrombin, tumor necrosis factor- α (TNF- α), and cell adhesion molecules secreted by injured ECs including monocyte chemoattractant protein-1 (MCP-1), vascular cell adhesion molecule-1 (VCAM-1), and intracellular adhesion molecule-1 (ICAM-1) [2, 4–6].

TNF- α is a major monocytes/macrophage-derived cytokine which possesses proatherogenic activity. Previous studies have shown that TNF- α increases EC permeability and induces the expression of chemokines and adhesion molecules [7]. TNF- α is remarkably elevated in the plasma and arteries in animals and humans with vascular complications [8–10] and is believed to be critically involved in the pathogenesis of atherosclerosis. High levels of TNF- α can induce endothelial cell apoptosis [9] and disrupt endothelial integrity leading to cardiovascular disease [11]. These results indicate that TNF- α may play an important role in endothelial dysfunction and the subsequent development of vascular disease. Since inflammation-induced endothelial dysfunction is important in the development of atherosclerosis, agents that can suppress the inflammatory pathway in vascular endothelial cells are candidate therapies to prevent vascular endothelial dysfunction.

Genistein, a major isoflavone in soy and red clover, has drawn wide attention due to its potential beneficial effects and various biological actions. Genistein has a weak estrogenic effect [12] by binding to estrogen receptors (ERs) [13] and at pharmacological dosage inhibits protein tyrosine kinase (PTK) [14]. Recent human intervention studies using soy phytoestrogens suggest a beneficial effect on atherosclerosis [15], markers of cardiovascular risk [16, 17], and vascular endothelial function [16, 17]. Data from animal studies also suggest a protective role of genistein in the vasculature [18, 19]. Data from in vitro studies demonstrate that genistein may exerts anti-atherogenic effects by inhibiting proliferation of vascular endothelial [20] and smooth muscle cells [21]. However, the concentrations (>30 μ M) used in most of these studies are far greater than the physiological relevant concentrations of genistein (< 5 μ M) that can be achieved following the consumption of genistein [22, 23]. Recently we have reported that genistein at physiological relevant concentrations reduces hyperglycemia-induced vascular inflammation in ECs [24]. Vascular inflammation is not only mediated by hyperglycemia but also by many other important factors such as dyslipidemia and various proinflammatory mediators such as TNF- α [3]. The effect of genistein on proinflammatory mediator-induced vascular inflammation in ECs and the molecular mechanisms involved are largely unknown. We hypothesize that genistein prevents TNF- α -induced vascular inflammation. Hence we carried out this study to evaluate the role of genistein at physiologically achievable concentrations in the prevention

of TNF- α -induced endothelial inflammation in human umbilical vein endothelial cells (HUVECs), and the effect of dietary intake of genistein on TNF- α -induced vascular inflammation in C57BL/6 mice. We also analyzed various inflammatory components and examined the possible mechanisms involved.

MATERIALS AND METHODS

Materials

Primary HUVECs and endothelial growth factors were purchased from Lonza (Walkersville, MD). M199 media; FBS, cell culture supplements, and calcein-AM were from Invitrogen (Carlsbad, CA); protein assay kits were from Bio-Rad (Hercules, CA); Human and mouse soluble adhesion molecules ICAM-1 (sICAM-1), VCAM-1 (sVCAM-1) and E-selectin (sE-Selectin), mouse chemokines MCP-1/JE and KC, and ELISA kits for the determination of human IL-8 and MCP-1 were from R&D Systems (Minneapolis, MN). cAMP enzyme immunoassay (EIA) kit was from Assay Design Inc. (Ann Arbor, MI); antibody for VCAM-1 was from Santa Cruz Biotechnology (Santa Cruz, CA) and antibody for F4/80 was from Bachem Peninsula Laboratories, LLC (San Carlos, California). ICI 182,780 (ICI) was purchased from Tocris Cookson (Balwin, MO); human monocytic U937 cells were from ATCC (Manassas, VA), genistein H89, and other general chemicals were obtained from Sigma-Aldrich (St. Louis, MO) and stock solution of 20 mM genistein in dimethyl sulfoxide was stored at -80°C before use.

Cell culture

HUVEC were cultured in M199 medium containing 2% FBS and endothelial growth supplements EGM2 at 37°C in a 5% CO_2 /95% air environment. Human monocytic U937 cells were cultured in DMEM medium with 10% FBS.

Monocyte adhesion assay

The determination of monocyte adhesion to ECs was conducted using U937 cells as described by us previously [24]. In brief, HUVECs were grown to confluence in 96-well plates and treated with various concentrations of genistein (0.1 – 10 μM) for 30 min before addition of TNF- α (2 ng/ml) for 24 h. In some experiments, HUVECs were pretreated with ER blocker ICI 182,780 (1 μM) or PKA specific inhibitor H89 (10 μM) for 30 min before addition of genistein (5 μM). Cells were then incubated with medium containing TNF- α (2 ng/ml) in the continued presence or absence of genistein for 24 h. To examine the post-treatment effect of genistein on TNF- α induced monocyte adhesion to ECs, HUVECs were incubated with TNF- α (2 ng/ml) for 24 h followed by addition of with various concentrations of genistein for another 24 h. HUVECs were gently washed with serum free medium and calcein-AM labeled U937 cells (5×10^4 /ml DMEM medium containing 1% FBS) were then added to HUVECs. After 1 h incubation, HUVEC monolayer was gently washed with phosphate buffered saline (PBS) to remove unbound monocytes. The fluorescence was measured to determine the bound monocytes using a FLX800 multi-detection microplate reader (Bio-Tek Instruments) at excitation and emission wavelengths of 496 and 520 nm, respectively.

Measurements of MCP-1 and IL-8 in cell culture supernatants

HUVECs were pretreated with or without genistein (0.1–10 μM) for 30 min before addition of TNF- α (2 ng/ml) for 24 h. The cell culture supernatants were collected and the production of MCP-1 and IL-8 by HUVECS were measured by using ELISA kits.

Intracellular cAMP assay

The accumulation of cAMP in HUVECs was determined by a specific EIA assay kit. HUVECs were pre-incubated with 5 μ M genistein for 30 min at 37°C. Cells were then treated with TNF- α (2 ng/ml) in the continued presence or absence of genistein for 24 h. After the treatment period, the supernatant was rapidly aspirated and the intracellular cAMP content was measured as we previously described [25].

Electron paramagnetic resonance spin-trapping assay for ROS-scavenging activity of genistein

Spin trap 5,5-dimethylpyrroline-*N*-oxide (DMPO)-spin was used to measure hydroxyl radicals generated by the Fenton reaction ($\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \cdot\text{OH} + \text{OH}^-$) in the presence or absence of various concentrations of genistein. Reactants were mixed in test tubes in a final volume of 0.1 ml containing 50 μ M of Fe^{2+} , 50 μ M of H_2O_2 and 80 mM of DMPO and the reaction mixture was then transferred to a capillary tube for EPR spectral analysis. Xanthine/xanthine oxidase system was used to generate superoxide to measure the superoxide-scavenging activity of genistein as described by us [26]. Briefly, 0.1 ml mixture solution contained 50 mM PBS, pH 7.4, 0.1 mM DTPA, 10 mM DEPMPO, 360 μ M xanthine and 32 mU/ml xanthine oxidase in the absence or presence of genistein. After incubation at 37 °C for 10 min, the reaction mixture was then transferred to a capillary cell for EPR spectral studies. Spectra were recorded at room temperature with a spectrometer (Bruker D-200 ER, IBM-Bruker), operating at X-band with a TM cavity and capillary cell, as described previously [27]. The EPR spectrometer settings were: modulation frequency, 100 KHz; X band microwave frequency, 9.5 GHz; microwave power, 20 mW; modulation amplitude, 1.0 G (gauss); time constant, 160 s; scan time, 200 s; and receiver gain, 1×10^5 . Spectral simulations were performed on the EPR data by matching directly to the spectra as described previously [28].

GSH and antioxidant enzymes assays

HUVECs (1×10^7) were incubated with 0.1 – 50 μ M genistein for 24 h. After 24 h, cells were pelleted by centrifugation at 200 *g* at 4°C for 5 min. Cells were then washed once with PBS and resuspended in ice-cold 50 mM potassium phosphate buffer, pH 7.4, containing 2 mM EDTA and 0.1% Triton X-100. The cells were sonicated, followed by centrifugation at 13,000 *g* for 10 min at 4°C to remove cell debris. The supernatant was then collected for the enzyme assays. The protein concentrations were measured using a Bio-Rad protein assay kit with bovine serum albumin (BSA) as the standard. The measurements of the cellular superoxide dismutase (SOD), glutathione (GSH), glutathione reductase (GR), glutathione peroxidase (GPx), glutathione transferase (GST), NAD(P)H:quinone oxidoreductase 1 (NQO-1) and catalase (CAT) were performed according to previously described methods [29, 30].

Animals and genistein treatment

Ten-week-old male C57BL/6 mice were obtained from Jackson Laboratory. Mice were housed in micro-isolator cages in a pathogen-free facility. After an initial acclimation period, mice were provided free access to a genistein-free rodent diet (modified AIN 93G diet; Dyet, Inc., PA) for one week to minimize any possible circulating genistein from previous dietary intake. Then the mice were randomly divided into 3 groups with 12 mice per group (control, TNF- α , TNF- α + genistein). Mice were fed a diet containing either 0 or 0.1% genistein with corn oil substituted for soybean oil [31]. This genistein dosage is close to those which humans can realistically consume (approximately a human intake of 75–100 mg/day) [32–35]. Previously we have reported the bioavailability of genistein and plasma genistein levels reached 0, 1.20 ± 0.03 , 1.90 ± 0.20 , 5.05 ± 0.49 μ M, in rats fed a diet

containing 0, 0.2, 0.5, and 2.0 g/kg diet of genistein respectively [36]. The dosage of genistein used in our *in vitro* and animal studies may overlap the reported achievable plasma genistein levels (0.74 – 6 μ M) in humans following consumption of a soy meal [37]. After one week, the mice were induced with intraperitoneal injection (i.p.) of TNF- α (Sigma Chemical, St. Louis, MO) at 25 μ g/kg daily for 7 consecutive days. A number of previous studies have shown that administration of the TNF- α to rodents at such dosage regimen significantly increased intercellular adhesion molecule expression, arteriolar leukocyte adhesion and vascular barrier dysfunction [8, 38–41]. Control mice received i.p. PBS. During the TNF- α administration, mice were continually treated with the control or genistein diet. Body weight and feed intake were recorded weekly throughout the study. The mice were euthanized after 2 h of the last TNF- α injection after being deprived of food for overnight, and serum samples were frozen at -80°C for the analysis. All experimental protocols were approved by the Institutional Animal Care and Use Committee at Virginia Tech and it conforms to the Guide for the Care and Use of Laboratory Animals published by US National Institutes of Health.

Measurements of serum chemokines and adhesion molecules

MCP-1/JE, KC, and soluble forms of ICAM-1 (sICAM-1) and VCAM-1 (sVCAM-1) in the serum were measured by ELISA kits according to the manufacturer's instructions.

Analysis of VCAM-1 and F3/80 expressions in mouse aorta

The aorta was cleaned of adherent fat, placed in 10% buffered formalin overnight, and then processed and embedded in paraffin. A series of tissue sections (5 μ m thickness) were prepared; Immunohistochemical sections were deparaffinized in xylene and rehydrated through a graded series of alcohol washes. Endogenous peroxidase activity was blocked by incubating in 0.3% H_2O_2 for 30 min. Antigen retrieval was carried out by boiling sections for 10 min in 0.01 M citrate buffer pH 6.0, cooled at room temperature for 30 min and then with 1.5% normal horse serum (Vector Laboratories) in PBS for at least 20 min at room temperature. Immunohistochemistry for VCAM-1 was performed with a rabbit anti-VCAM-1 primary antibody diluted 1:500 (Santa Cruz Biotechnology) using the Vectastain Elite Rabbit IgG kit (Vector Laboratories). Immunohistochemistry for F4/80 was performed with a rat monoclonal anti-F4/80 primary antibody diluted 1:200 (Bachem) using the Vectastain Elite Rat IgG kit (Vector Laboratories). Primary antibody incubation steps were carried out over-night at 4°C . The appropriate secondary antibodies from the rabbit or rat Vectastain ABC-AP kit (Vector Laboratories) were used according to manufacturer's instructions. Visualization was performed using 3,3'-diaminobenzidine (Dako). Nuclei were counterstained with Harris hematoxylin for 3 min. Photographs of immuno-stained mouse aorta ($40\times$ magnification) were digitized and captured using a AMG EVOS XL digital inverted bright field and phase contrast microscope (Bothell, WA). Quantitative analysis of VCAM-1 and F4/80 expressions in aorta was performed with an image-analysis program (Image J 1.46, National Institutes of Health Image, Bethesda, MD, USA) as previously described [42, 43].

Statistical analysis

All data were subjected to analysis of variance (ANOVA) using GraphPad Prism[®] software and are expressed as mean $\pm$ SEM. Data from *in vitro* studies were derived from at least three independent experiments performed in duplicate, and data from animal studies were obtained from at least 8 mice in each group. Significant treatment differences were subjected to Tukey's multiple comparison tests. $P < 0.05$ was considered different.

RESULTS

Genistein inhibits TNF- α -induced binding of U937 monocytes to ECs and reduces the production of chemokines and adhesion molecules in ECs

Exposure of HUVECs to TNF- α significantly induced the adhesion of U937 monocytes to HUVECs (Fig. 1A). However pretreatment with genistein as low as 0.1 μ M significantly inhibited TNF- α -induced binding of U937 monocytes to HUVECs and 10 μ M genistein suppressed the adhesion by 50% (Fig. 1A). MCP-1 and IL-8 are essential for the firm adhesion of monocyte to ECs [44, 45] and exposure of ECs to TNF- α significantly induced the production of MCP-1 and IL-8 (Figs. 1B and C). Pretreatment with genistein as low as 0.1 μ M concentration also significantly inhibited TNF- α -induced MCP-1 and IL-8 production in ECs (Figs 1 B–C). Genistein pretreatment at 0.1 μ M, 1 μ M, and 10 μ M concentrations also significantly suppressed TNF- α -induced production of adhesion molecules sICAM-1 (Fig. 1D), sVCAM-1 (Fig. 1E), and sE-selectin (Fig. 1F), and this inhibitory effect of genistein is consistent with its effect on monocyte adhesion (Fig. 1A). While pretreatment of genistein reduced endothelial inflammation, post-treatment of genistein failed to suppress TNF- α -induced adhesion of monocytes to HUVECs (data not shown). These results indicate that genistein post-treatment is not efficient to afford protection against TNF- α -induced endothelial inflammation.

Physiologically relevant concentrations of genistein have no significant antioxidant effects

TNF- α plays a central role in inflammation by the release of both superoxide and hydrogen peroxide-derived hydroxyl radical [46]. We investigated whether the anti-inflammatory effect of genistein on TNF- α might due to the ability of genistein in scavenging superoxide and hydroxyl radical. Using electron paramagnetic resonance spectroscopy (EPR) in combination with DEPMPO or DMPO-spin trapping technique, we determined the ability of genistein in scavenging superoxide and hydroxyl radicals that were generated from xanthine oxidase/xanthine system and the Fenton Reaction, respectively. However genistein at concentrations <10 μ M had no significant free radical scavenging activity (Figs. 2 A–D).

Further, we investigated whether the anti-inflammatory effect of genistein is mediated through other redox mechanisms such as induction of endogenous antioxidants and phase 2 enzymes in ECs. In that regard, we determined whether genistein treatment could increase the activities of GR, GPx, GST, NQO1, SOD and CAT in cultured HUVECs. There were no significant differences in the induction of these enzymes by genistein at concentrations <10 μ M (Fig. 2E). However, genistein at pharmacological concentration (50 μ M) significantly induced the activities of these enzymes indicating that higher concentration of genistein is necessary for the effective induction of these endogenous antioxidant enzymes.

The inhibitory effect of genistein on TNF- α -induced vascular inflammation in ECs is independent of ERs

Genistein has weak estrogenic effects in some tissues by binding to ERs [12] and we examined whether the genistein effect is mediated through ERs. The ER antagonist ICI 162,780, which successfully inhibited the estrogen effect of 17 β -estradiol in ECs in our previous study [47], did not inhibit the effect of genistein on TNF- α -induced EC-monocyte interaction (Fig. 3) indicating that genistein activity is not mediated through the ER-mediated mechanisms.

The inhibitory effect of genistein on TNF- α -induced vascular inflammation in ECs is mediated via PKA

Cyclic AMP/PKA signaling reportedly plays a role in inhibiting vascular endothelium from pro-inflammatory cytokine-induced damage [48] and depressing leukocyte adhesion to ECs

[49]. We have recently demonstrated that genistein is an activator of cAMP signaling system in ECs [25]. We thus investigated whether this pathway is involved in the protective action of genistein against TNF- α -induced inflammation in ECs. Inhibition of PKA by H89, a selective PKA inhibitor, significantly attenuated the inhibitory effect of genistein on TNF- α -induced monocyte adhesion to ECs (Fig. 4A). Further, the addition of PKA inhibitor significantly suppressed the protective effect of genistein against TNF- α -induced MCP-1 and IL-8 production in ECs (Figs. 4 B and C). TNF- α also significantly decreased the levels of intracellular cAMP in the ECs (Fig. 4D). However, co-incubation with genistein significantly improved this detrimental effect of TNF- α on the ECs (Fig. 4D). These results suggest that the anti-inflammatory effect of genistein may be mediated at least partially through the PKA-mediated mechanism.

Dietary supplementation of genistein reduces TNF- α -induced vascular inflammation in C57BL/6 mice

We further assessed whether genistein has the potential to prevent TNF- α -induced vascular inflammation *in vivo*. Genistein treatment to the mice had no effect on animal body weight and food intake (data not shown). The serum concentrations of MCP-1/JE, KC (the mouse homolog of human MCP-1 and IL-8 respectively), sICAM-1, sVCAM-1, and sE-Selectin were significantly greater in TNF- α -treated mice than those in control mice (Fig. 5A–E). Dietary genistein significantly suppressed the TNF- α -induced increase in circulating MCP-1/JE, KC, sICAM-1, sVCAM-1 and sE-Selectin (Figs. 5A–E), suggesting that genistein indeed has an anti-inflammatory effect *in vivo*, given that the secretion of these chemokines and adhesion molecules plays a key role in the firm adhesion of monocytes to activated ECs and subsequent monocyte recruitment into sub-endothelial dysfunction [2, 4–6].

To further verify the anti-inflammatory effect of genistein *in vivo*, we employed immunohistochemistry to identify F4/80, a commonly used marker of mouse vascular monocyte-derived macrophages and VCAM-1 expression in mouse aorta. Monocytes are known to recruit into the vessel wall and subsequently differentiate into macrophages to form lipid-rich foam cells during inflammation [50–52]. As shown in Fig. 6A, an abundance of F4/80-positive macrophages was present in the mouse aorta in TNF- α -treated group, indicating the vessels are activated and inflammatory. Dietary genistein significantly suppressed F4/80-positive monocytes-derived macrophages in mouse aorta (Figs. 6A and C). Consistently, strong VCAM-1 staining in TNF- α -treated group was seen on the surface of mouse aorta compared to control group (Fig. 6B). In contrast, dietary genistein reduced the intensity of VCAM-1 staining in the TNF- α -treated animals (Fig. 6B and D).

DISCUSSION

Inflammation-induced enhanced monocytes binding to ECs followed by their transmigration into the vessel wall play a major role in the progression of atherosclerotic lesions. Upon accumulation, monocytes transform into macrophages and lipid-rich foam cells, which ultimately lead to the development of atherosclerosis [53]. Thus compounds that suppress the ECs-monocytes interaction can prevent the morbidity and mortality associated with atherosclerosis.

In the present study, genistein at physiologically relevant concentrations (0.1 – 5 μ M) suppresses TNF- α -triggered EC-monocyte interaction. TNF- α was shown to induce the expression and release of a series of adhesion molecules and chemokines which are involved in the inflammatory response in ECs [54]. Chemokines such as MCP-1 and IL-8 are the key mediators in the regulation of enhanced EC-monocyte interaction and subsequent monocyte recruitment into vascular tissue [55]. Both MCP-1 and IL-8 are reported to highly express in

human atherosclerotic lesions, and mice lacking receptors for MCP-1 and IL-8 are less susceptible to atherosclerosis [55]. IL-8 is not expressed in mice and KC is the mouse homolog of human IL-8 [45], a primary mediator of monocyte binding to atherosclerotic endothelium in mice [56]. In this study, TNF- α significantly increases the secretion of these chemokines in HUVECs and in TNF- α -treated mice indicating the critical role of these chemokines in the TNF- α -induced vascular inflammation [55, 57, 58]. Mice treated with genistein abolished TNF- α -induced increases in circulating MCP-1/JE and KC which are consistent with the suppressive effect of genistein on chemokine production in HUVECs.

Cytokines such as TNF- α induces the expression of adhesion molecules on the cell surface of ECs resulting in the adhesion and migration of monocytes to the subendothelial space [3]. The adhesion molecules such as ICAM-1, VCAM-1, and E-selectin have been suggested to be atherosclerotic inflammatory markers [59, 60]. Indeed, previous studies have demonstrated increased expression of these endothelium-derived adhesion molecules in advanced human coronary atherosclerotic plaques as well as in experimental models of atherosclerosis [59, 60]. In this study, genistein suppressed the TNF- α -induced endothelial production of sICAM-1, sVCAM-1, and sE-selectin in HUVECs. Consistent to our in vitro results, genistein treatment suppressed the circulating levels of these adhesion molecules in the serum of TNF- α -treated mice. These results suggest that the anti-inflammatory effect of genistein on vascular inflammation in vivo may be partially mediated by inhibition of chemokines and adhesion molecules.

F4/80 is an approximately 125 kDa transmembrane protein that is a specific cell surface marker of murine macrophages. Previous studies have demonstrated highly presence of monocyte-derived F4/80-positive macrophages in mouse aorta during the inflammation [50–52], suggesting the recruitment of monocytes to the aortic endothelium. Our immunohistochemical analyses have further shown the increased expression of VCAM-1 and abundance of F4/80-positive macrophages in the mouse aorta of TNF- α -treated group suggesting that vascular wall in TNF- α -treated mice are inflammatory. However, genistein treatment reduced the expression of VCAM-1 and F4/80-positive macrophages, suggesting that genistein may primarily target vascular wall for exerting this anti-inflammatory action. This genistein effect could be partially due to its action on modulation of IL-8 and MCP-1 expressions, which are consistent with our in vitro finding that genistein suppresses TNF- α -induced inflammation of ECs.

We further investigated signaling responses to genistein at cellular level to determine the possible molecular mechanisms underlying the vascular effects of genistein. TNF- α -induced reactive oxygen species (ROS) production in ECs and its subsequent induction of oxidative stress are involved in the modulation of the TNF- α -induced-vascular inflammation [46]. However, genistein at concentrations used in the present study has no significant effect on various antioxidant enzyme activities in ECs and it does not scavenge free radicals. These results indicate that the activity of genistein on TNF- α -induced endothelial dysfunction is not mediated through an antioxidant mechanism.

Previous studies have shown that endogenous estrogen has the vasculoprotective effect via the ER-dependent mechanisms. Due to the similarity with estrogen in structure, genistein has been found to have both weak estrogenic and anti-estrogenic effects in vitro and in vivo [12]. Our data indicate that genistein action on TNF- α -triggered inflammation was independent of the classical ERs, because the specific ER antagonist ICI 162,780, which can effectively block both ER α - and ER β -mediated estrogen action by inhibiting receptor dimerization and inducing their degradation [61–63], did not inhibit the effect of genistein. Genistein is a well-known inhibitor of PTK being frequently used in studies involving PTK-mediated cellular events. However, we have recently showed that genistein can inhibit PTK

only at pharmacological concentration (100 μ M) [47], which is as high as 1,000-fold of the effective concentration of genistein used in the present study. Thus, the anti-inflammatory effect of genistein in ECs is likely independent of PTK.

PKA is also known as cAMP-dependent protein kinase, whose activity is dependent on cellular levels of cAMP. Cyclic AMP/PKA signaling are recognized components of pathways responsible for maintaining normal vascular health by repressing gene transcription of vascular cell adhesion molecules [64–67] and maintaining normal endothelial barrier function [68, 69]. TNF- α -triggered vascular dysfunction has also been linked to cAMP/PKA signaling and TNF- α reduces intracellular cAMP levels via activation of endothelial cGMP-stimulated phosphodiesterase [70]. We have recently demonstrated that genistein at physiologically achievable doses directly acts on endothelial cells leading to accumulation of intracellular cAMP and subsequent activation of PKA [25]. PKA is the downstream signal of cAMP whose activity is known to be dependent on the level of cAMP in the cells. We also showed that the activation of the cAMP-signaling system is not related to any known action of genistein, such as inhibition of PTK or binding to ERs [25, 47], suggesting a novel effect of genistein on vasculature. The possible mechanism of genistein on cAMP may be through the activation of adenylate cyclase (AC). Indeed, we have already demonstrated that genistein can directly activate cAMP signaling by stimulating AC activity in ECs [25, 47, 71]. Further, there is possibility that genistein may activate AC/cAMP cascade by modulating the plasma membrane associated G-protein, preferably G α s that activates AC/cAMP dependent pathway by stimulating the production of cAMP from ATP, an aspect that is currently under investigation in our laboratory. In the present study, TNF- α greatly reduced intracellular cAMP levels in ECs, which was improved by genistein treatment, and inhibition of PKA significantly abolished the inhibitory effects of genistein on TNF- α -induced monocyte adhesion to ECs as well as MCP-1 and IL-8 production. These data suggest that the anti-inflammatory effect of genistein in ECs is at least partially mediated via the PKA-mediated mechanism.

In summary, genistein at physiological relevant concentrations significantly inhibited TNF- α -mediated adhesion of monocytes to ECs and suppressed TNF- α -induced production of chemokines and adhesion molecules in ECs. In addition, dietary supplementation of genistein reduced circulating chemokines and adhesion molecules in plasma, and suppressed the expression of VCAM-1 and F4/80 in the aorta of TNF- α treated C57BL/6 mice. The protective effect of genistein on vascular inflammation is independent of ERs or its potential antioxidant effect, but largely depends on a PKA-mediated mechanism. These findings provide the evidence suggesting that genistein may be a novel agent to protect vasculature against TNF- α -caused inflammation and dysfunction.

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Abbreviations

CAT	catalase
CXCL1/KC	Chemokine (C-X-C motif) ligand 1
DMPO	5,5-dimethylpyrroline-N-oxide
EPR	electron paramagnetic resonance

ER	estrogen receptor
FBS	fetal bovine serum
GPx	glutathione peroxidase
GR	glutathione reductase
GSH	glutathione
GST	glutathione transferase
HUVECs	human umbilical vein endothelial cells
ICAM-1	intercellular adhesion molecule-1
IL-8	interleukin-8
MCP-1/JE	mouse /monocyte chemotactic protein-1/JE
MCP-1	monocyte chemotactic protein-1
ROS	reactive oxygen species
PKA	protein kinase A
PTK	protein tyrosin kinase
sE-Selectin	soluble E-Selectin
sICAM-1	soluble intercellular adhesion molecule-1
SOD	superoxide dismutase
sVCAM-1	soluble vascular adhesion molecule-1
TNF-	Tumor necrosis factor-
VCAM-1	vascular adhesion molecule-1

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Fig. 1. Genistein inhibits TNF- α -induced monocyte adhesion (A) and suppressed the production of IL-8 (B), MCP-1 (C), sICAM-1 (D), sVCAM-1 (E), and sE-Selectin (F) in HUVECs
 HUVECs were pre-treated with various concentrations of genistein (G) for 30 min before addition of TNF- α (T 2 ng/ml) in the presence or absence of genistein for 24 h. U937 cells were labeled with the fluorescent probe and the adhesion was determined. IL-8, MCP-1, sICAM-1, sVCAM-1, and sE-Selectin were measured by ELISA. Data are expressed as mean $\pm$ SEM from three experiments. *, $p < 0.05$ vs. control; #, $p < 0.05$ vs. TNF- α alone-treated cells. IL-8, interleukin-8; MCP-1, monocyte chemoattractant protein-1; sICAM-1, soluble intercellular adhesion molecule-1; sVCAM-1, soluble vascular adhesion molecule-1; sE-Selectin, soluble E-Selectin.

Fig. 2. Genistein at physiological relevant concentrations has no significant scavenging activities on superoxide (A–B) and hydroxyl radical (C–D) or effects on antioxidant enzyme activities (E) in ECs

A: Electron paramagnetic resonance (EPR) spectra of DEPMPO-superoxide spin adduct in the absence and presence of genistein or SOD. B: Effects of genistein on the DEPMPO-superoxide spin adduct generation by xanthine/xanthine oxidase system. C: EPR spectra of DMPO-hydroxyl spin adduct in the absence and presence of genistein. D: Effects of genistein on the DMPO-hydroxyl radical spin adduct generation by $\text{Fe}^{2+}/\text{H}_2\text{O}_2$ system. The scavenging ratio (Q) was defined as $Q = (1 - H_x/H_0) \times 100\%$, where H_0 denotes the relative signal intensity at 3480 G in the EPR spectrum of the control and H_x is in the control system, but in the presence of indicated concentrations of genistein. E: HUVECs were incubated with the indicated concentrations of genistein for 24 h, followed by measurement of cellular glutathione reductase (GR), glutathione peroxidase (GPx), glutathione S-transferase (GST), NAD(P)H:quinone oxidoreductase 1 (NQO1), superoxide dismutase

(SOD) and catalase (CAT). Data are expressed as $\pm$ SEM from three experiments. * <0.05 vs. control.

Fig. 3. Effect of genistein on TNF- α -induced monocyte adhesion to ECs is independent of ERs
 HUVECs were pre-incubated for 30 min with or without ICI 182,780 (I), then with genistein for 30 min, followed by addition of 2 ng/ml TNF- α in the presence or absence of genistein for 24 h. G, Genistein (5 μ M); T, TNF- α (2 ng/ml); I, ICI 182,780 (1 μ M). Data are expressed as mean $\pm$ SEM from three experiments. *, $p < 0.05$ vs. control; #, $p < 0.05$ vs. TNF- α -alone-treated cells.

Fig. 4. Genistein inhibition of TNF- α -mediated inflammation in ECs is mediated via PKA

Panels A–C, HUEVCs were pre-incubated with 10 μ M H89 (H) or vehicle, followed by addition of 5 μ M genistein (G) prior to stimulation with 2 ng/ml TNF- α in the presence or absence of genistein for 24 h. Cell adhesion was measured using fluorescent labeled U937 monocytes (Fig. 4A). IL-8 (Fig. 4B) and MCP-1 (Fig. 4C) were measured by ELISA. Panel D, HUVECs were pre-incubated with 5 μ M genistein (G) or vehicle for 30 min followed by the addition of 2 ng/ml TNF- α (T) for 24 h. Intracellular cAMP was extracted and measured by EIA. Data are expressed as mean $\pm$ SEM from three experiments. *, $p < 0.05$ vs. control; #, $p < 0.05$ vs. TNF- α alone-treated cells.

Fig. 5. Dietary genistein reduces chemokines (A–B), and adhesion molecules (C–E) in the serum of TNF- α treated mice

Values are mean $\pm$ SEM, n= 8–10. *, p<0.05 vs. control; #, p<0.05 vs. TNF- α alone-treated mice. sICAM-1, soluble intercellular adhesion molecule-1; sVCAM-1, soluble vascular adhesion molecule-1; sE-Selectin, soluble E-Selectin; **MCP-1**/JE, mouse monocyte chemotactic protein 1/JE; TNF- α , Tumor necrosis factor- α ; CXCL1/KC, Chemokine (C-X-C motif) ligand 1.

Fig. 6. Immunohistochemical staining for F4/80-positive monocytes-derived macrophages and adhesion molecule VCAM-1 in aortic cross-sections

Representative photomicrographs of immunohistochemical staining for F4/80-positive monocytes-derived macrophages (Fig. 6A) and VCAM-1 (Fig. 6B). Quantitative analysis of F4/80 (Fig. 6C) and VCAM-1 (Fig. 6D). Arrows indicate typical positive stained regions and original magnification is 40 $\times$. T, TNF- α ; T+G, TNF- α + genistein; Data are expressed as mean $\pm$ SEM, n=5, *, p<0.05 vs. control; #, p<0.05 vs. TNF- α alone-treated mice.