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Journal of Nutritional Biochemistry 24 (2013) 222–230

Journal of Nutritional Biochemistry

# Positive and negative regulation of insulin action by genistein in the endothelium

Xuejiao Gao[a], Kang Liu[a], Fang Huang[a], Dongyan Zhang[a], Xudan Guo[b], Min Wang[b], Baolin Liu[a,*]

[a]Department of Pharmacology of Chinese Materia Medica, China Pharmaceutical University, Nanjing 211198, P.R. China
[b]College of Food Science and Engineering, Northwest A & F University, YangLing, Shaanxi 712100, P. R. China

Received 18 December 2011; received in revised form 3 May 2012; accepted 4 May 2012

**Abstract**

Genistein is an isoflavone phytoestrogen with biological activities in management of metabolic disorders. This study aims to evaluate the regulation of insulin action by genistein in the endothelium. Genistein inhibited insulin-stimulated tyrosine phosphorylation of insulin receptor substrate-1 (IRS-1) and attenuated downstream Akt and endothelial nitric oxide synthase (eNOS) phosphorylation, leading to a decreased nitric oxide (NO) production in endothelial cells. These results demonstrated its negative regulation of insulin action in the endothelium. Palmitate (PA) stimulation evoked inflammation and induced insulin resistance in endothelial cells. Genistein inhibited IKKβ and nuclear factor-κB (NF-κB) activation with down-regulation of tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) production and expression. Genistein inhibited inflammation-stimulated IRS-1 serine phosphorylation and restored insulin-mediated tyrosine phosphorylation. Genistein restored insulin-mediated Akt and eNOS phosphorylation, and then led to an increased NO production from endothelial cells, well demonstrating its positive regulation of insulin action under insulin-resistant conditions. Meanwhile, genistein effectively inhibited inflammation-enhanced mitogenic actions of insulin by down-regulation of endothelin-1 and vascular cell adhesion protein-1 overexpression. PA stimulation impaired insulin-mediated vessel dilation in rat aorta, while genistein effectively restored the lost vasodilation in a concentration-dependent manner (0.1, 1 and 10 μM). These results suggested that genistein inhibited inflammation and ameliorated endothelial dysfunction implicated in insulin resistance. Better understanding of genistein action in regulation of insulin sensitivity in the endothelium could be beneficial for its possible applications in controlling endothelial dysfunction associated with diabetes and insulin resistance.


*Keywords:* Genistein; Insulin resistance; Endothelial dysfunction; Inflammation

## 1. Introduction

Insulin plays a key role in the stimulation of glucose uptake in skeletal muscle and adipose tissue. Besides the well-known regulatory role in the maintenance of glucose homeostasis, it also exerts its action on the blood vessel to promote vasodilatation by stimulation of nitric oxide (NO) production from endothelium. The vasodilator action of insulin is mediated *via* phosphatidylinositol 3-kinase (PI3K)-dependent signaling pathway. This pathway requires activation of insulin receptor (IR) tyrosine kinase, which then phosphorylates insulin receptor substrate-1 (IRS-1), leading to binding and activation of PI3K; PI3K activation, in turn, phosphorylates and activates Akt, which directly activates endothelial nitric oxide synthase (eNOS) and then increases NO production from endothelial cells [1,2]. In the endothelium, specific impairment of insulin PI3K signaling results in the loss of insulin-mediated NO production, leading to endothelial dysfunction. Endothelial dysfunction has been demonstrated in type 2 diabetes as well as in obese and insulin-resistant patients [3,4], and it has been well established that endothelial dysfunction and insulin resistance are frequently comorbid states and responsible for the increased cardiovascular risks in diabetic patients [5,6].

Flavonoids are nearly ubiquitous in plants and rich in seeds, citrus fruits, olive oil, tea and red wine. Genistein, an isoflavone phytoestrogen, is found in a number of plants, especially in soybeans and soy products which are the primary food sources for flavonoids dietary intakes in Asia. Soybeans and soy products are often highlighted for their health-enhancing properties, and accumulating evidence demonstrates that genistein regulates many biological activities, among which appropriate management of metabolic disorders interests people very much [7]. Epidemiologic data demonstrated that genistein intakes lowered the risk of type 2 diabetes in overweight Japanese women [8]. Recently, several reports have showed that some flavonoids, including genistein, inhibited insulin-mediated glucose uptake in adipocytes [9–12]. Genistein inhibits glucose transporter 4 (GLUT4)-mediated glucose uptake in mouse adipocytes and counteracts the antilipolytic action of insulin in isolated rat adipocytes [9,13]. These results suggest that genistein might attenuate insulin sensitivity in regulating glucose homeostasis. In contrast, some studies reported the antidiabetic actions of genistein. Haneishi et al. reported that genistein stimulated the insulin-dependent signaling pathway in hepatocytes [14]. Genistein is shown to lower glucose and improve glucose

* Corresponding author.
*E-mail address:* zhongyao440@163.com (B. Liu).

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http://dx.doi.org/10.1016/j.jnutbio.2012.05.008

tolerance in diabetic rats [15]. Genistein also demonstrates some chemoprotection properties in islet B cell damage [16,17].

The evidence mentioned above reveals a somewhat paradoxical situation in the effect of genistein on insulin action. We noticed that the effect of genistein on insulin-mediated glucose uptake was observed in experiments carried out in a given situation, i.e., physiological situation, but its antidiabetic actions were driven mainly from pathological models. In fact, genistein is a multi-functional compound, and therefore, it may behave differently in regulation of insulin action under different situations. Endothelial insulin resistance is tightly associated with the cardiovascular complications of diabetes. Although many studies focused on genistein inhibition of insulin-mediated glucose uptake in adipocytes, up to now, little is known about its regulation of insulin action in the endothelium, especially in the insulin-resistant state. Insulin action in the endothelium is mainly to promote NO production, and therefore, in the present study, we investigated the effect of genistein on insulin-mediated NO production in endothelial cells and observed that genistein inhibited insulin-mediated NO production by inhibition of insulin-mediated IRS-1 tyrosine phosphorylation. Meanwhile, we stimulated endothelial cells with palmitate (PA) to induce insulin resistance and also observed that genistein inhibited inflammatory response and ameliorated insulin resistance by positive modulation of serine/tyrosine phosphorylation of IRS-1. These results well demonstrated its dual regulation of insulin action in the endothelium. Better understanding of genistein action in regulation of insulin sensitivity in the endothelium should be beneficial for its possible therapeutic applications in controlling endothelial dysfunction implicated in insulin resistance.

## 2. Materials and methods

### 2.1. Animals

Sprague–Dawley rats (200–250 g), supplied by the Laboratory Animal Center of Nanjing Qinglongshan, were used for aorta relaxation study. The care and treatment of these rats were performed in accordance with the Provisions and General Recommendation of Chinese Experimental Animals Administration Legislation.

### 2.2. Reagents

Genistein (98% in purity) was purchased from Nanjing Zelang Medical Technology Co., Ltd. (Nanjing, China), and dissolved in dimethyl sulfoxide (0.1% v/v). PA was obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China), and dissolved in absolute ethanol at 200 mM as stock solution and then was further diluted with medium containing 10% free fatty acid (FFA)-free and low-endotoxin bovine serum albumin to obtain a concentration of 5 mM before use. The following items were purchased from the cited commercial sources: RPMI Medium 1640 and fetal bovine serum (FBS) from Sigma (St. Louis, MO, USA); insulin from Wanbang Biochemical Pharmaceutical Company (Xuzhou, Jiangsu, China); sodium salicylate from Tianjin Kemiou Chemical Agent Center (Tianjin, China); all primers from Sangon Biotec Co., Ltd. (Shanghai, China); TRNzol Plus, BU-Taq DNA polymerase, DNA Markerland BU-SuperScript RT kit from Biouniquer Technology Co., Ltd. (Nanjing, China); anti-phospho-IRS-1 (Ser307) (BS4104), anti-IRS-1 (R301) (BS1408), anti-phospho-Akt (T308) (BS4008), anti-Akt (A444) (BS1810), anti-phospho-IKKβ (Y199) (BS4320) and anti-IKKβ (F182) (BS1407) from Bioworld Technology (St. Paul, MN, USA); horseradish-peroxidase (HRP)-conjugated anti-rabbit and anti-mouse IgG antibodies, phospho-NF-κB p65 (Ser536) antibody (#3031), NF-κB p65 antibody (#3034), phospho-eNOS (Ser 1177) antibody (#9571), eNOS (49G3) antibody (#9586) and PD98059 from Cell Signaling Technology, Inc. (Beverly, MA, USA); PY99 (sc-7020) from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA); wortmannin from Beyotime Institute of Biotechnology (Nanjing, China).

### 2.3. Cell culture

Human umbilical vein endothelial cells (HUVECs) were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China) and were examined morphologically and identified as endothelial cells by positive immunocytochemistry staining for factor VIII. HUVECs were maintained in RPMI 1640 supplemented with 10% (vol/vol) FBS, streptomycin (100 U/ml) and penicillin (100 U/ml) under an atmosphere of 5% $CO_2$ and 95% humidified air at 37°C. The medium was renewed every 2 days until the cells were grown to confluence.

### 2.4. Detection of NO production from HUVECs with fluorescence microscopy

HUVECs in the exponential phase were seeded at $2\times10^4$ cells per well in 24-well plates. Cells were grown to confluence and pretreated with genistein at concentrations of 0.1, 1, or 10 μM with or without control agents as follows: wortmannin (0.1 μM) and salicylate (5 mM) for 0.5 h. After incubation, cells were stimulated with or without PA (100 μM) for another 0.5 h and then washed twice with PBS. After loading with 5 mM of NO-specific fluorescent dye 3-amino,4-aminomethyl-2′,7′-difluoresceindiacetate (DAF-FM DA, Beyotime, Shanghai, China) at 37°C for 0.5 h and kept in the dark. Cells were rinsed with PBS and incubated with insulin (0.1 μM) for 5 min, then fixed in 2% paraformaldehyde (vol/vol) at 4°C for 5 min. Fixed cells were examined using an Olympus IX81 inverted microscope with attached charge-coupled device camera (Retiga Exi, Burnaby, BC, Canada) using appropriate filters. The dye was excited at 495 nm, and the emission was detected at 515 nm.

### 2.5. Determination of tumor necrosis factor (TNF)-α and interleukin (IL)-6 production in endothelial cells

Cells were subcultured into 24-well plates at $2\times10^4$ cells per well and grown to confluence. They were pretreated with genistein or salicylate for 0.5 h and then incubated with PA for an additional 12 h. The medium was collected on ice and centrifuged at 1000g for 10 min at 4°C. The levels of TNF-α and IL-6 in the supernatant were assayed with commercial enzyme-linked immunosorbent assay (ELISA) kits (R&D, USA).

### 2.6. Reverse transcription polymerase chain reaction (RT-PCR) assay

For the detection of TNF-α and IL-6 gene expression, cells were grown to confluence in six-well plates, pretreated with genistein or salicylate for 0.5 h and stimulated with PA for 12 h. For endothelin-1 (ET-1) and vascular cell adhesion molecule-1 (VCAM-1) gene determination, HUVECs were grown to confluence in six-well plates and then incubated with genistein, salicylate, wortmannin or PD98059 for 0.5 h, respectively, and then stimulated with PA for 2 h. After incubation, cells were treated with insulin for 20 min in the continued presence of the inhibitors or genistein. Total RNA was isolated from the cultures using TRNzol Plus, and its concentration was calculated from spectrophotometric measurements at 260 nm. Total RNA (500 ng) from each RNA sample was used with the RT kit according to manufacturer's protocol (Biouniquer Technology Co., Ltd.). An aliquot of cDNA (5 μl of RT mixture) was dissolved in 50 μl of the reaction mixture containing 10× PCR buffer (final $Mg^{2+}$ concentration: 1.5 mM), 10 mM of dNTP mixture, 10 μM of forward and reverse primers (TNF-α, IL-6, ET-1, VCAM-1 and β-actin) and 1 U of Taq polymerase. Primers were used as follows: β-actin [161 base pairs (bp)], forward primer: 5′-ACATCTGCTGGAAGGTGGAC-3′, reverse primer: 5′-GGTACCACCATGTACCCAGG-3′; IL-6 (408 bp), forward primer : 5′-TAGCGC CTCACA-CAGACAG-3′, reverse primer: 5′-GGCTGGCATATGTGCTTGGG-3′; TNF-α (519 bp), forward primer: 5′-CTCTGGCCGAGGCAGTCGA-3′, reverse primer: 5′-GGCTGG CAT-ATGTGCTTGGG-3′; ET-1 (436 bp), forward primer: 5′-CGTTGTTCCGTATGGACTT G-3′; reverse primer: 5′-AGGCTATGGCTTCAGACAGG-3′; VCAM-1 (540 bp), forward primer: 5′-AGCCACATCAGTATCCAAGAGGAG-3′; reverse primer: 5′-CAATAACCAAC TCTATGTTCCTTTTC-3′. Amplification products obtained in PCR were separated by electrophoresing in 1.5% agarose gel with 0.5 μg/ml ethidium bromide and visualized under UV light, and then the signal intensity was detected by Bio-Rad image system and analyzed with the computer program Quantity One (Bio-Rad). For semiquantitative analysis, the signals were normalized (TNF-α, IL-6, ET-1, VCAM-1 to the cDNA levels of the β-actin housekeeping gene) and expressed as a ratio.

### 2.7. Western blot analysis

Endothelial cells were pretreated with genistein or salicylate at given concentrations for 0.5 h, followed by addition of PA or insulin. Cells were washed two times with ice-cold PBS, collected and lysed in Western blot lysis buffer [Tris–HCl 50 mM, pH 7.2, containing 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1% NP-40, NaCl 0.15 mM, sodium orthovanadate 1 mM]. The cell lysates were then incubated at 4°C for 0.5 h and cleared by centrifugation at 13,000g for 15 min at 4°C. The protein concentration of each sample was determined using a Bicinchoninic Acid Protein Assay kit (Biosky Biotechnology Corporation, Nanjing, China). The proteins were separated by 10% SDS–polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride (PVDF) membranes (0.22 μm, Millipore Co., Ltd.) by semidry electrophoretic transfer. The PVDF membranes were blocked with 5% skim milk in TBST buffer (Tris–HCl 5 mM, pH 7.6, NaCl 136 mM, 0.05% Tween-20) for 2 h at room temperature or overnight at 4°C and then incubated with primary antibody (1:800 dilution in TBST) at 4°C overnight. The PVDF membrane was washed three times with TBST buffer and then incubated with the secondary antibody at room temperature for 1 h. HRP-labeled anti-rabbit or anti-mouse IgG was used as the secondary antibody (1:2000 dilution in TBST). The signals were detected by enhanced chemiluminescence (ECL) and quantized by densitometry with Image-Pro Plus 6.0 (IPP 6.0) software.

### 2.8. Preparation of aortic rings and assessment of endothelium-dependent relaxation

Rats were sacrificed by cervical dislocation. The thoracic aorta was immediately removed and placed in 4°C Krebs–Henseleit (K–H) solution (NaCl 118.3 mM, KCl

4.7 mM, $MgSO_4 \cdot 7H_2O$ 1.2 mM, $KH_2PO_4$ 1.2 mM, $CaCl_2$ 2.5 mM, $NaHCO_3$ 25 mM, edetate calcium disodium 0.026 mM, glucose 5.0 mM, pH 7.4) and gassed with 95% $O_2$–5% $CO_2$. The dissected aortas were cleaned of connective tissue and cut into rings (5 mm long). Care was taken to avoid abrading the intimal surface to maintain the integrity of the endothelial layer. For measurement of vascular responses, aortic rings were suspended in an organ bath containing 30 ml of K–H solution maintained at 37°C, pH 7.4, and continuously aerated with 95% $O_2$ and 5% $CO_2$. A resting tension of 1.0 g was applied to the aortic ring, and changes in tension were measured with a force-displacement transducer connected to a polygraph. After 1-h equilibration period, segments were exposed to 60 mM of KCl to assess their viability. The functionality of vascular endothelium was confirmed by the ability of relaxation after exposed to 10 μM acetylcholine to relax segments contracted with 1 μM phenylephrine (the relaxation was over 80%). After confirming the integrity of the endothelium, the aortic ring was precontracted with phenylephrine (1 μM), and then insulin (0.001–1 μM) was added cumulatively to evoke an endothelium-dependent relaxation. The relaxation induced by insulin was expressed as a percentage of the phenylephrine-induced contraction. After washout, the aortic ring was incubated with genistein for 0.5 h followed by the addition of PA for another 0.5 h. After the washout of genistein and PA, the aortic ring was precontracted with phenylephrine (1 μM). When the contraction was stable, increasing concentrations of insulin (0.001–1 μM) were added to produce endothelium-dependent relaxation.

### 2.9. Statistical analysis

Results were expressed as mean±S.D. Differences were analyzed by one-way analysis of variance followed by Student–Newman-Keuls test. A value of $P<.05$ was regarded as significant.

## 3. Results

### 3.1. Genistein regulated insulin-mediated NO production in endothelial cells

As insulin action in the endothelium is to induce NO production, we first determined the effect of genistein on regulation of insulin-mediated NO production in endothelial cells under normal and insulin-resistant conditions. There was a basal production of NO in the endothelial cell, and NO production was substantially increased by insulin stimulation. Treating cells with genistein at the concentration ranging from 0.1 to 10 μM inhibited insulin action on NO production, as we observed that NO production was significantly decreased (Fig. 1A). PA stimulation of endothelial cells blunted insulin action, leading to a decrease in NO production. Genistein treatment protected cells against PA insult and effectively restored the loss of insulin-mediated NO production (Fig. 1B). These results showed the dual effect of genistein on insulin action in endothelial cells. Wortmannin, a specific inhibitor of PI3K, inhibited insulin-stimulated NO production, but no significant change was observed when cells were treated with genistein in the presence of wortmannin (Fig. 1C).

### 3.2. Genistein inhibited insulin-mediated tyrosine phosphorylation in IRS-1

Insulin increased NO production in the endothelium through PI3K signaling. Insulin binds to IRs and then induces tyrosine phosphorylation of IRS-1, generating the major docking sites for PI3K signaling. As shown in Fig. 2, insulin stimulation induced tyrosine IRS-1 phosphorylation (detected by antibody of PY99). Genistein is a potent inhibitor of tyrosine kinase, and we observed that genistein inhibited insulin-mediated IRS-1 tyrosine phosphorylation in a concentration-dependent manner. This result suggested that the inhibition of tyrosine kinase was involved in its modulation of IRS-1 phosphorylation modification.

### 3.3. Genistein inhibited insulin-induced Akt activation and eNOS phosphorylation

PI3-kinase is responsible for downstream insulin signaling along Akt/eNOS pathways in endothelial cells. Insulin stimulation induced IRS-1 tyrosine phosphorylation and thereby led to PI3K signaling activation indicated by the enhanced downstream Akt and eNOS phosphorylation. Genistein treatment blunted insulin-mediated Akt phosphorylation and attenuated subsequent eNOS phosphorylation, demonstrating its inhibition of insulin PI3K signaling along Akt/eNOS pathways. The resultant inhibition of eNOS phosphorylation should be responsible for the loss of insulin-mediated NO production. Results were shown in Fig. 3A and B.

### 3.4. Genistein inhibited IKKβ/NF-κB activation evoked by PA stimulation

PA stimulation inhibited insulin-mediated NO production, and the inhibition was reversed by salicylate treatment, suggesting that inflammatory response was involved in PA insult. IKKβ/NF-κB signaling is a regulator of inflammation, and PA stimulation resulted in IKKβ and NF-κB phosphorylation activation. As shown in Fig. 4A and B, genistein attenuated enhanced IKKβ phosphorylation and effectively inhibited NF-κB activation by inhibition of p65 phosphorylation. Salicylate also showed a similar inhibitory tendency as genistein.

### 3.5. Genistein decreased TNF-α and IL-6 expression and production in endothelial cells

NF-κB activation up-regulates the expression of a wide range of proinflammatory cytokines, including TNF-α and IL-6. We observed that TNF-α and IL-6 mRNA expression and protein production were markedly increased when cells were exposed to PA stimulation. Genistein treatment effectively attenuated TNF-α and IL-6 mRNA expression. Meanwhile, the increased TNF-α and IL-6 production was also significantly decreased by genistein in a concentration-dependent manner. These results suggested that inflammatory response was inhibited by genistein in the endothelium. Results were shown in Fig. 5A and B.

### 3.6. Genistein modulated IRS-1 serine/tyrosine phosphorylation in the presence of PA

It has been well established that inflammation is tightly associated with insulin resistance. IRS-1 serine phosphorylation is a key event linking inflammation to the impairment of insulin signaling. PA stimulation induced IRS-1 serine phosphorylation, which disturbed insulin-mediated tyrosine phosphorylation of IRS-1. Genistein treatment reduced PA-induced serine phosphorylation of IRS-1 and subsequently restored insulin-mediated IRS-1 tyrosine phosphorylation at concentrations ranging from 0.1 μM to 10 μM, well demonstrating its beneficial modulation of IRS-1 serine/tyrosine phosphorylation (Fig. 6A and B). Salicylate also showed a similar modulation on IRS-1 serine and tyrosine phosphorylation as genistein.

### 3.7. Genistein restored insulin-stimulated Akt and eNOS phosphorylation in the presence of PA

PA stimulation inhibited insulin-mediated tyrosine phosphorylation of IRS-1 and thereby impaired downstream signaling along the PI3K/Akt/eNOS pathway. We observed that insulin-mediated Akt and eNOS phosphorylation was reduced when cells were exposed to PA stimulation. PA stimulation attenuated insulin-mediated Akt phosphorylation, but the inhibitory tendency was effectively reversed by genistein treatment. Akt activation can directly induce eNOS phosphorylation, which is responsible for NO production in the endothelium. As expected, genistein was shown to restore insulin-mediated eNOS phosphorylation. Salicylate also restored Akt and eNOS phosphorylation. Results were shown in Fig. 7A and B.

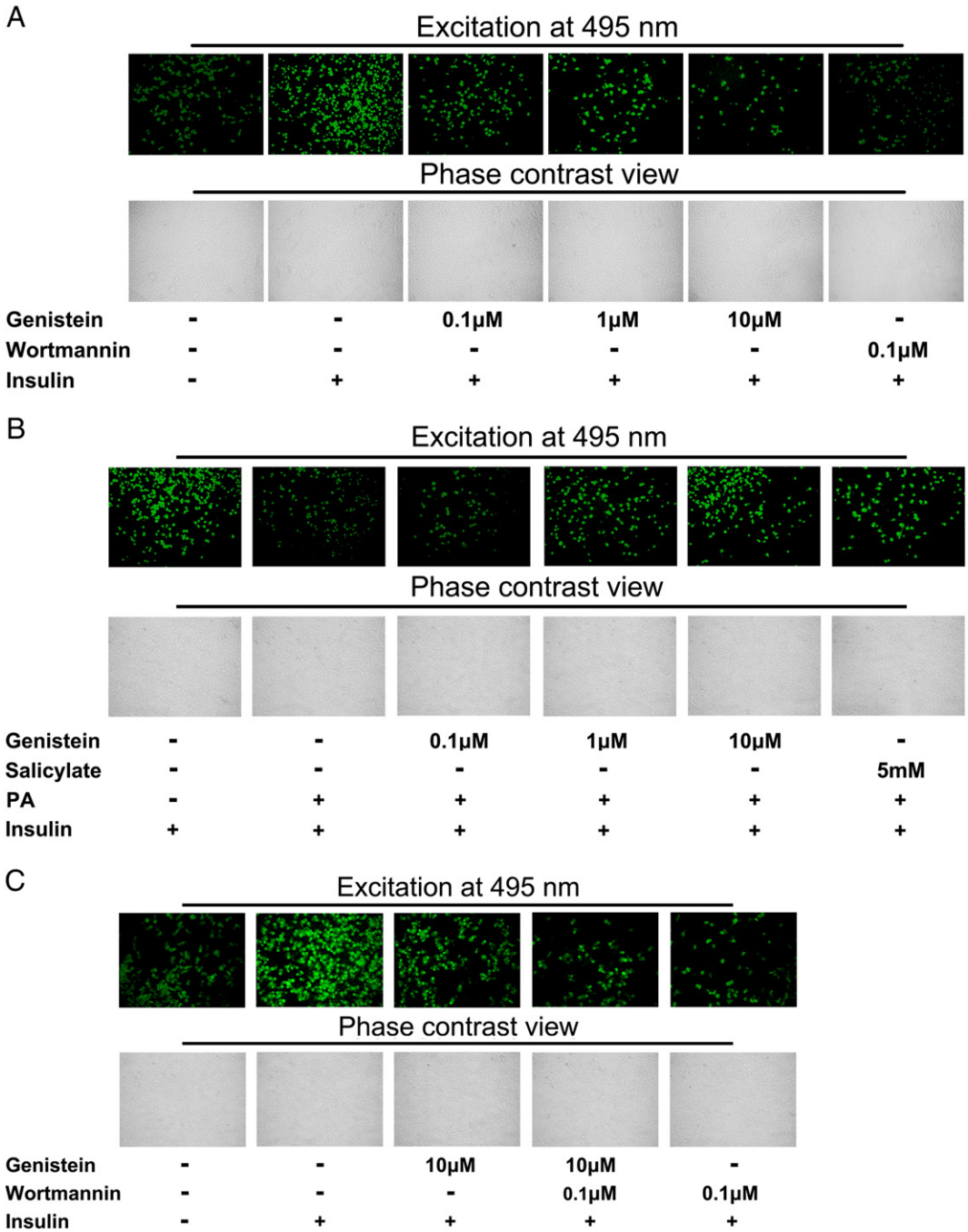


Fig. 1. Genistein regulated insulin-mediated NO production in endothelial cells. (A–B) Cells were pretreated with genistein, wortmannin or salicylate, respectively, for 0.5 h and then incubated with or without PA (100 μM) for another 0.5 h, followed by insulin (0.1 μM) stimulation for 5 min. Salicylate was taken as a positive control. (C) HUVECs were pretreated with genistein for 0.5 h and then incubated with wortmannin (0.1 μM) for another 0.5 h, followed by insulin (0.1 μM) stimulation for 5 min. Intracellular NO production was viewed with fluorescence microscopy. The results were for one representative of three independent experiments.

### *3.8. Genistein inhibited ET-1 and VCAM-1 expression in endothelial cells*

Insulin regulates ET-1 and cell adhesion molecules expression *via* MAPK pathways in the endothelium. We observed that insulin increased the basal expression of ET-1 and VCAM-1, and this action was enhanced by wortmannin treatment. PA itself induced ET-1 and VCAM-1 expression, and moreover, it further increased ET-1 and VCAM-1 expression in response to insulin. Genistein treatment effectively inhibited ET-1 and VCAM-1 overexpression in a concentration-dependent manner. PD98059, a specific inhibitor of ERK, significantly inhibited ET-1 and VCAM-1 expression, suggesting the involvement of ERK activation. Results were shown in Fig. 8A and B.

### *3.9. Genistein restored insulin-mediated vessel relaxation in rat aorta*

Insulin-resistant endothelial dysfunction is characterized by the loss of insulin-mediated vessel relaxation. As shown in Fig. 9, insulin induced vasodilation at concentrations ranging from 0.001 μM to 1 μM. Stimulation of rat aorta with PA impaired insulin action, leading to a significant decrease of vessel relaxation, as the percentages of relaxation induced by 0.1 μM and 1 μM insulin were reduced from

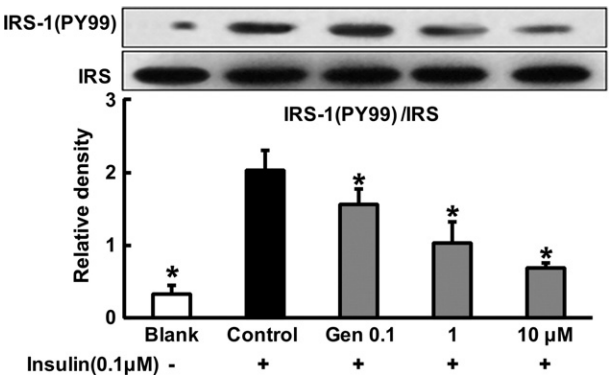


Fig. 2. Genistein inhibited insulin-mediated tyrosine phosphorylation of IRS-1 in endothelial cells. HUVECs were pretreated with genistein for 0.5 h and then incubated with insulin (0.1 μM) for 20 min. After incubated with insulin, tyrosine phosphorylation of IRS-1 was determined by Western blot. The results were expressed as the mean±S.D. of three independent experiments. *$P$<.05 vs. control.

80.1% to 42.9% (0.1 μM of insulin) and from 97.7% to 66.6% (1 μM of insulin), respectively. Genistein treatment effectively restored the loss of insulin-mediated vessel relaxation in a concentration-dependent manner, as largest relaxations (1 μM of insulin) were restored to 79%, 93% and 92% by genistein at concentrations of 0.1, 1 and 10 μM, respectively. Salicylate treatment effectively restored the loss of insulin-mediated vasodilation.

## 4. Discussion

Emerging evidence demonstrates that genistein influences insulin action in adipose and muscle tissue [12,18], and we wondered whether it affected insulin action in endothelial cells. In the present study, we showed that genistein inhibited insulin-stimulated NO production, and this action was consistent with its inhibition of insulin-mediated glucose uptake in adipocytes [12]. PA stimulation reduced NO production in response to insulin, but genistein effectively restored the loss of insulin-mediated NO production. These results well demonstrated a dual regulation of insulin action by genistein in endothelial cells.

Same as insulin signaling in skeletal muscle and adipose tissue, insulin promotes NO production in the endothelium through PI3K signaling. In the present study, wortmannin, an inhibitor of PI3K, reduced NO production in response to insulin, indicating that PI3K-dependent signaling was involved in insulin-mediated NO production. Insulin binds to IR and activates the receptor tyrosine kinases (RTKs), resulting in the initiation of signaling transduction. Genistein is proposed to be a specific inhibitor of tyrosine protein kinases, and this action is associated with its antitumor activity [19]. In the present study, genistein inhibited insulin-mediated NO production, and we wondered whether genistein regulated insulin action by its inhibition of tyrosine kinase. Because tyrosine phosphorylation of IRS-1 is a key control for downstream insulin PI3K signaling [20], we investigated its regulation of IRS-1 tyrosine phosphorylation and observed that genistein treatment attenuated insulin-stimulated IRS-1 tyrosine phosphorylation. This result suggested that tyrosine kinase inhibition was involved in the regulation of insulin action by genistein. But the evidence is not fully consistent with its action in adipocytes. Nomura and coworkers showed that genistein inhibited glucose uptake in adipocytes without affecting insulin-stimulated IR-β phosphorylation, which is a result derived from RTKs [12]. Different from tyrosine phosphorylation of IRS-1, IR-β phosphorylation is a direct result from insulin-stimulated autophosphorylation of the receptor itself. The selective inhibition of tyrosine kinase by genistein may be an acceptable explanation for its different actions as mentioned above.

Insulin-mediated IRS-1 tyrosine phosphorylation generates docking sites for PI3K activation [21]. PI3K activates downstream target Akt, which directly phosphorylates eNOS, leading to NO production. Genistein inhibited insulin-mediated IRS-1 tyrosine phosphorylation, and this action should affect downstream PI3K signaling. As Akt is a downstream effector of PI3K, we showed the effect on PI3K by determination of Akt phosphorylation. As expected, we observed that insulin-enhanced Akt and eNOS phosphorylation was attenuated by genistein treatment. This result suggested that genistein inhibited insulin PI3K signaling, and the resultant down-regulation of eNOS phosphorylation should be responsible for the decreased NO production from endothelial cells. The result is somewhat different from some studies which reported that genistein inhibited insulin-mediated glucose uptake in adipocytes by targeting GLUT4 without affecting insulin-mediated Akt activity [12,9]. In the present study, we demonstrated that the suppression of NO production by genistein was relative with attenuated Akt phosphorylation, but we did not think that this action is a result derived from its direct effect on Akt activity. In fact, the final down-regulation of Akt and eNOS phosphorylation should be a result derived from its upstream inhibition of IRS-1 tyrosine phosphorylation in response to insulin. In adipocytes, genistein targeted GLUT4 without affecting insulin-mediated Akt activity [12,9], and this action just suggested that genistein might affect GLUT4 translocation in an insulin-independent manner.

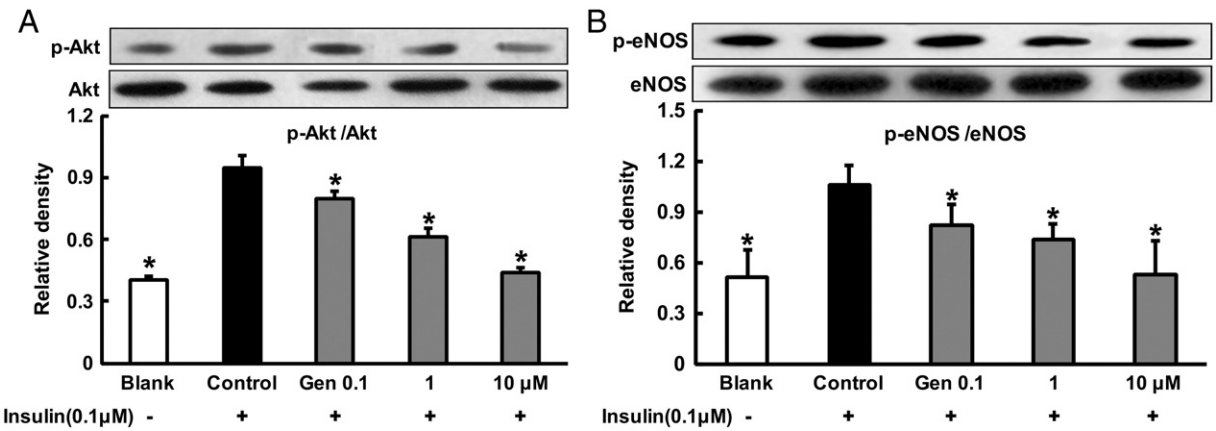


Fig. 3. Genistein inhibited insulin-induced Akt and eNOS phosphorylation in endothelial cells. (A–B) HUVECs were pretreated with genistein for 0.5 h followed by addition of insulin (0.1 μM) for 20 min, and then Akt (A) and eNOS (B) phosphorylation was detected by Western blot. Data were expressed as the mean±S.D. of three independent experiments. *$P$<.05 vs. control.

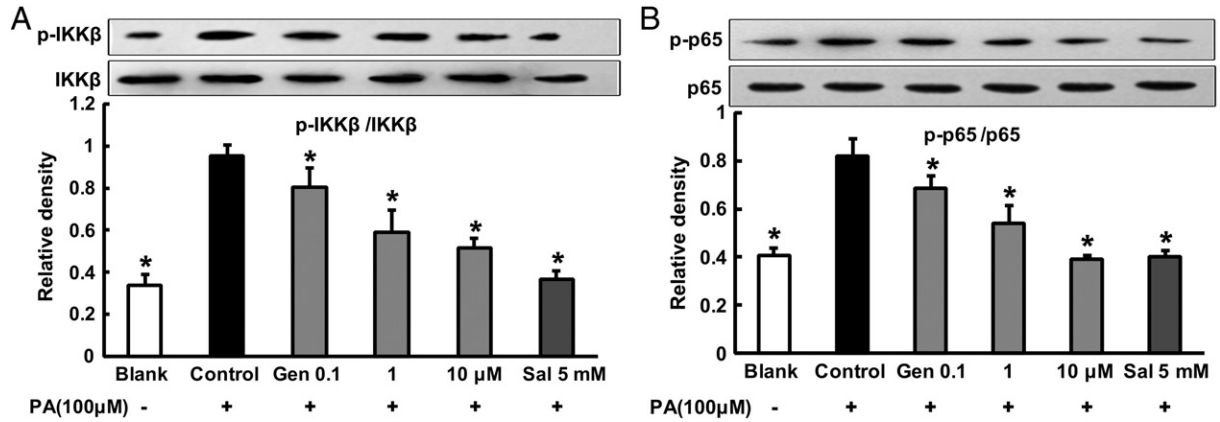


Fig. 4. Genistein inhibited PA-induced IKKβ and NF-κB p65 phosphorylation in endothelial cells. HUVECs were pretreated with genistein or salicylate for 0.5 h followed by addition of PA (100 μM) for another 0.5 h, and then IKKβ (A) and p65 (B) phosphorylation was determined by Western blot. Salicylate was taken as a positive control. Data obtained from three separate experiments were expressed as the mean±S.D. $^{*}P<.05$ vs. control.

Genistein inhibited the production of inflammatory mediators by tyrosine kinase inhibition [22], and we wondered whether its anti-inflammatory activity contributed to attenuation of insulin resistance in the endothelium. Elevation in circulating FFA is an important pathological factor for insulin resistance [23]. As PA is a major component of the total serum FFA, we used PA as a stimulus to induce inflammatory response in endothelial cells. Genistein inhibited TNF-α and IL-6 production with down-regulation of relative gene expression. Meanwhile, enhanced IKKβ and NF-κB p65 phosphorylation was also attenuated by genistein treatment. Because IKKβ can regulate the expression of inflammatory molecules, including TNF-α and IL-6, through subsequent NF-κB activation, these results suggested that genistein inhibited inflammatory response in an IKKβ/NF-κB-dependent manner. The result was also consistent with the published report which showed that genistein reduced the production of proinflammatory molecules with down-regulation of NF-κB activation in brain microvascular endothelial cells [24]. Serine phosphorylation of IRS-1 is a key event linking inflammation to insulin resistance, because more inflammatory molecules, including TNF-α, IL-6, IKKβ and FFA, can inhibit insulin-mediated IRS-1 tyrosine phosphorylation by inducing serine phosphorylation, and thereby impaired downstream insulin PI3K signaling [20]. PA stimulation induced IRS-1 phosphorylation accompanied with down-regulated expression of IRS-1 tyrosine phosphorylation, indicating that the functional interaction

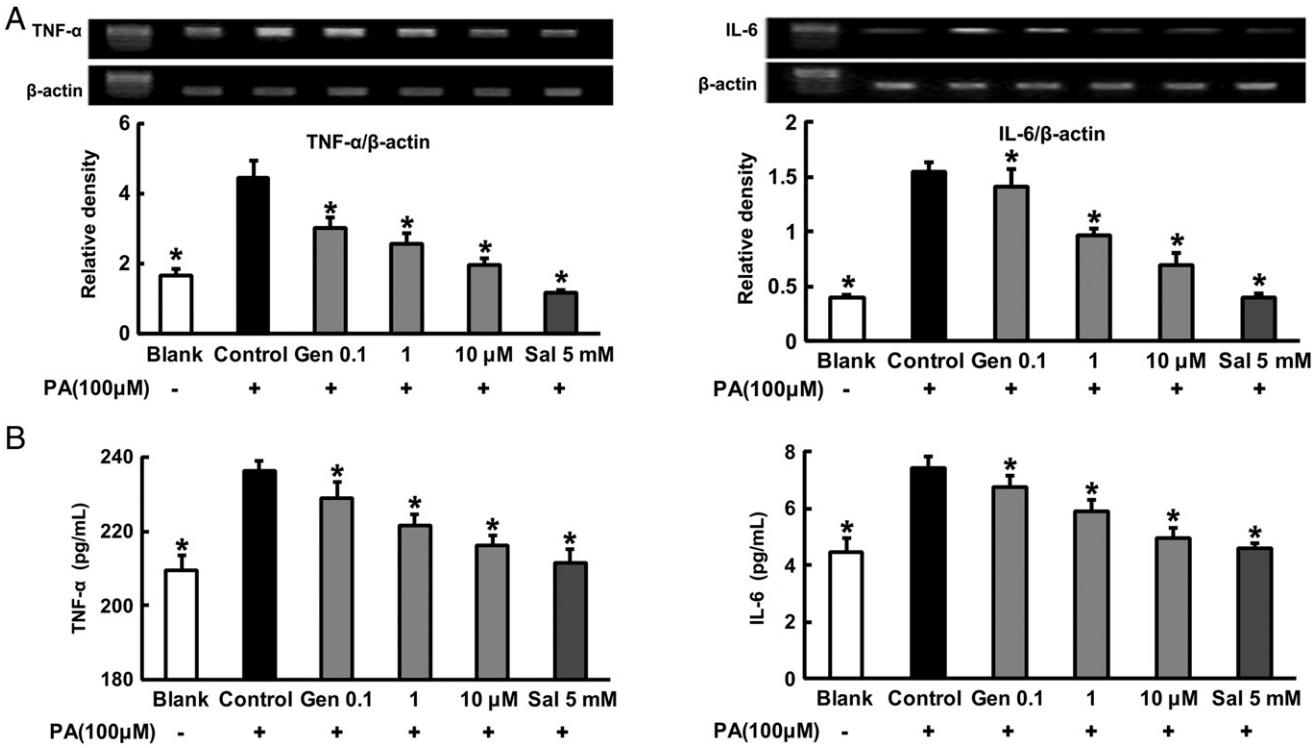


Fig. 5. Genistein decreased TNF-α and IL-6 mRNA expression and production in PA-stimulated endothelial cells. (A) HUVECs were pretreated with genistein or salicylate for 0.5 h and then incubated with PA (100 μM) for 12 h. TNF-α and IL-6 mRNA expression was determined by RT-PCR. Salicylate was taken as a positive control. The results were expressed as the mean±S.D. of three independent experiments. $^{*}P<.05$ vs. control. (B) HUVECs were pretreated with genistein or salicylate for 0.5 h and then incubated with PA (100 μM) for 12 h. The concentrations of TNF-α and IL-6 in the conditioned medium were measured with ELISA kits. Salicylate was taken as a positive control. The results were expressed as the mean±S.D. ($n=4$). $^{*}P<.05$ vs. control.

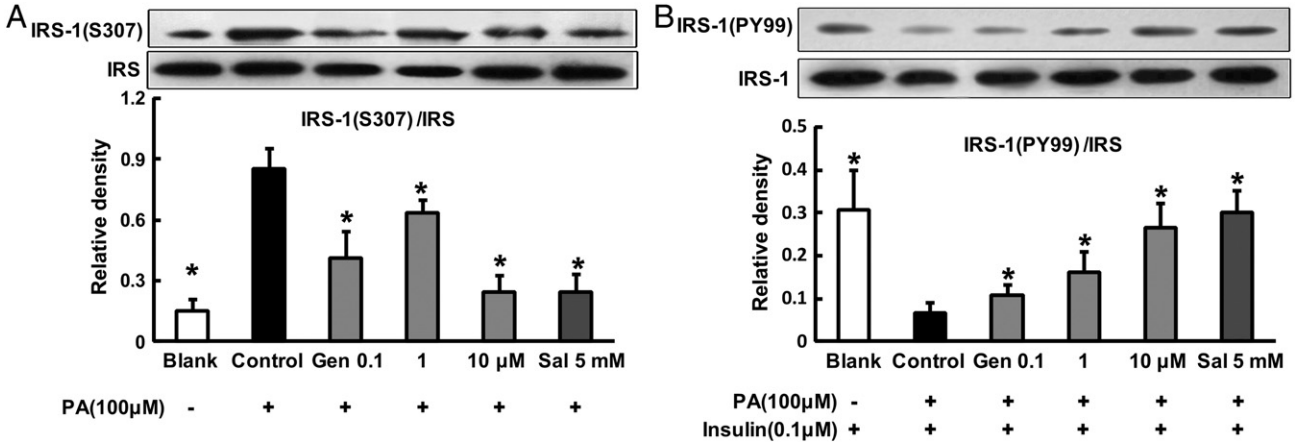


Fig. 6. Genistein modulated serine and tyrosine phosphorylation of (IRS-1 in PA-stimulated endothelial cells. HUVECs were pretreated with genistein or salicylate for 0.5 h and then incubated with PA (100 μM) for 0.5 h. (A) Serine phosphorylation of IRS-1 (S307) was determined by Western blot. (B) After stimulation with PA, HUVECs were treated with insulin (0.1 μM) for 20 min, and tyrosine phosphorylation of IRS-1 (PY99) was determined by Western blot. Salicylate was taken as a positive control. Data were expressed as the mean±S.D. of three independent experiments. $^{*}P<.05$ vs. control.

between IR and IRS-1 was blocked. Salicylate, a potent inhibitor of inflammation, can reverse the change, suggesting that inflammatory response was involved in the phosphorylation modification of IRS-1. Genistin inhibited PA-induced IRS-1 serine phosphorylation and effectively restored insulin-mediated tyrosine phosphorylation in the presence of PA, well demonstrating its beneficial effect on IRS-1 function by inhibition of inflammation.

Insulin resistance is characteristic of the impairment of PI3K signaling. PA stimulation attenuated insulin stimulated IRS-1 tyrosine phosphorylation and then impaired insulin PI3K signaling evidenced by attenuated Akt and eNOS phosphorylation. Genistein treatment restored insulin-mediated Akt and eNOS phosphorylation, demonstrating its improvement of insulin PI3K signaling. Because genistein is a multifunctional compound and some of its biomodulations are associated with PI3K or Akt activity [25,26], it is necessary to know whether it can regulate insulin-mediated NO production by its original influence on PI3K under insulin-resistant conditions. We pretreated endothelial cells with wortmannin, a specific inhibitor of PI3K, to inhibit insulin-mediated NO production and observed the effect of genistein on insulin-mediated NO production (Fig. 1C). Different from its action in PA stimulation, no significant change in NO production was observed. This result excluded the direct action of genistein on PI3K signaling when PI3K activation was blocked, and further supported the conclusion that the improvement of insulin PI3K signaling by genistein was derived from its positive modulation of IRS-1 phosphorylation against PA insult.

In addition to promoting NO production, insulin also stimulates ET-1 and adhesion molecule expression in endothelial cells, and the mitogenic action is mediated through MAPK (ERK) pathways. A balance between PI3K-dependent and MAPK-dependent functions of insulin is important for the maintenance of the endothelial homeostasis. Under insulin-resistant conditions in the endothelium, impaired PI3K signaling enhances mitogenic action of insulin and led to decreased NO production and increased ET-1 expression characteristic of endothelial dysfunction [27,28]. In the present study, treating cells with wortmannin increased insulin-stimulated ET-1 and VCAM-1 expression, suggesting that inhibition of PI3K enhanced the mitogenic action of insulin. We had shown that PA stimulation resulted in impairment of PI3K signaling, and this action should affect MAPK-dependent actions of insulin. As a result, insulin-stimulated ET-1 and VCAM-1 expressions were greatly enhanced when cells were exposed to PA. PD98059, a specific inhibitor of ERK, down-regulated ET-1 and VCAM-1 expression, further confirming the involvement of MAPK activation. Genistein

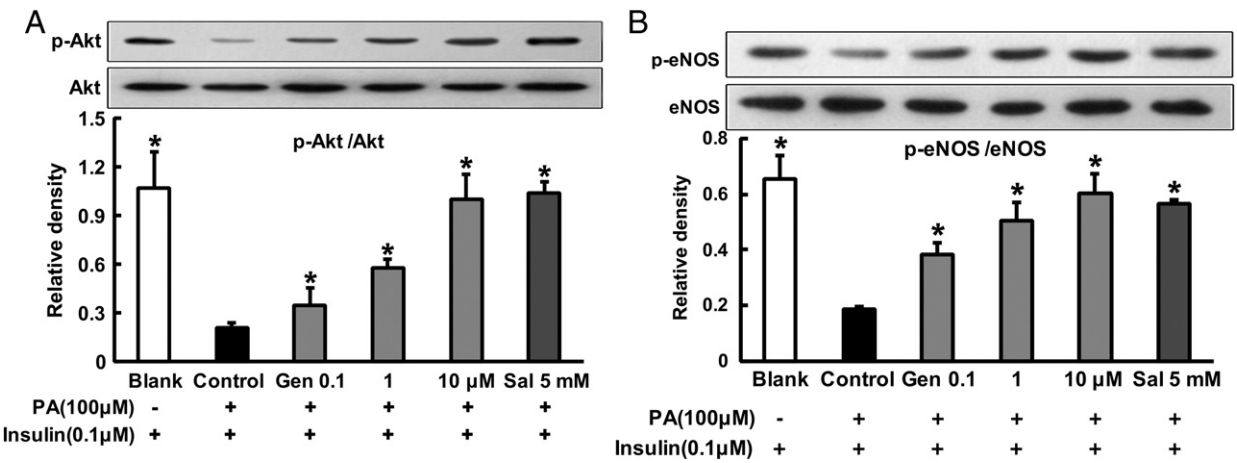


Fig. 7. Genistein restored insulin-stimulated Akt and eNOS phosphorylation in PA-stimulated endothelial cells. Cells were pretreated with genistein for 0.5 h and then incubated with PA (100 μM) for 0.5 h. After incubation, cells were treated with insulin (0.1 μM) for 20 min, and then Akt activation (A) and eNOS phosphorylation (B) were detected by Western blot. Salicylate was taken as a positive control. Data were expressed as the mean±S.D. of three independent experiments. $^{*}P<.05$ vs. control.

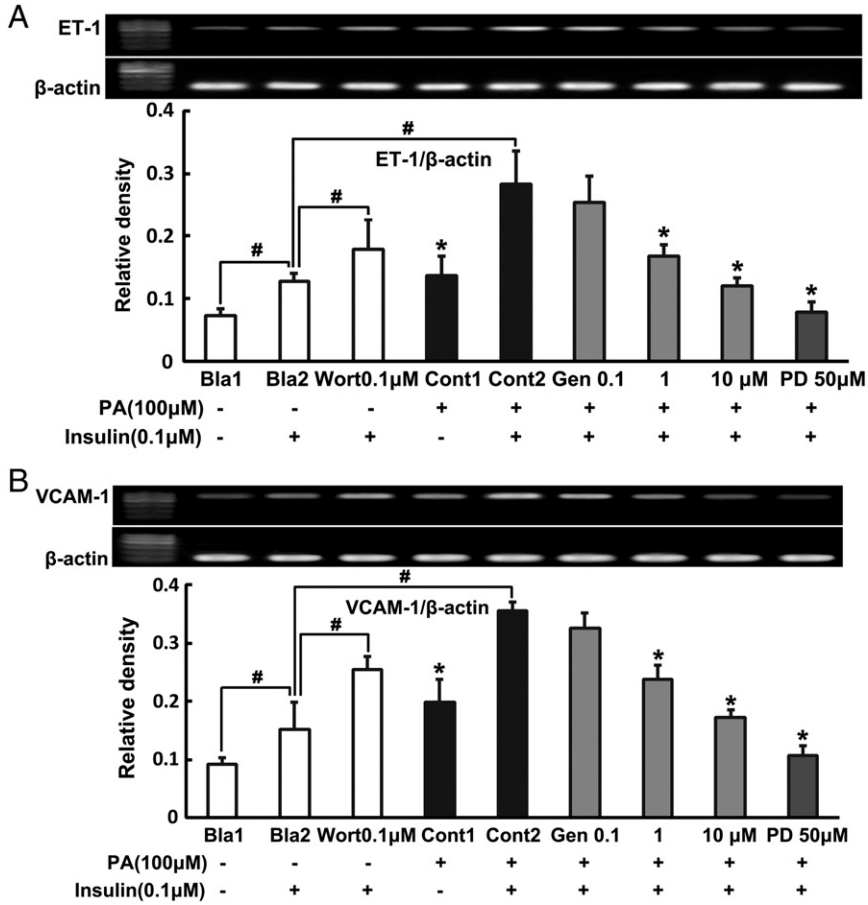


Fig. 8. Genistein reduced ET-1 and VCAM-1 mRNA expression in PA-treated endothelial cells. HUVECs were pretreated with genistein, PD98059 or wortmannin (Wort) for 0.5 h and incubated with PA (100 μM) for another 2 h. Then cells were stimulated with or without insulin (0.1 μM) for 20 min. ET-1 (A) and VCAM-1 (B) mRNA expression was detected by RT-PCR. Salicylate was taken as a positive control. The results were expressed as the mean±S.D. of three independent experiments. #$P$<.05 vs. blank 2 (Bla2); *$P$<.05 vs. control 2 (Cont2).

not only restored insulin-mediated NO production in a PI3K-dependent manner, but also inhibited ET-1 and VCAM-1 expression, and therefore, it was reasonable to believe that the improvement of IRS-1/PI3K signaling by genistein should be a key regulation for restoration of the balance between PI3K-dependent and MAPK-dependent functions of insulin.

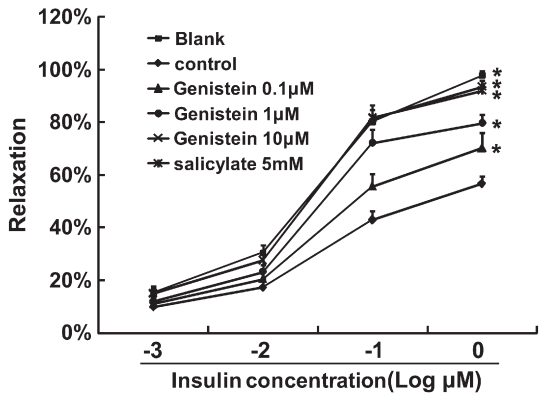


Fig. 9. Effects of genistein on insulin-mediated endothelium-dependent relaxation in PA-treated aortic rings. The aortic ring was pretreated with genistein or salicylate for 0.5 h and then stimulated with PA (100 μM) for 0.5 h. After washing out of genistein and PA, the aortic ring was precontracted with phenylephrine (1 μM), and then the relaxation was induced by insulin treatment. Salicylate was taken as a positive control. The results were expressed as the mean±S.D. ($n$=4). *$P$<.05 vs. control.

Insulin-resistant endothelial dysfunction is characterized by the loss of insulin-mediated vasodilation, and therefore, we investigated the effect of genistein on insulin-mediated vasodilation in rat aorta. PA stimulation induced the loss of insulin-mediated vasodilation, but the change was effectively reversed by genistein treatment. It has been reported that administration of genistein ameliorated endothelial dysfunction in hypertensive rats with the involvement of calmodulin [29]. Different from classical cholinergic vasodilators (e.g., acetylcholine), insulin-mediated eNOS activation is less relative with calcium influx and calmodulin [30]. To eliminate the possible influence, we pretreated aorta with genistein and then washed it out, so the positive result should be relative its chemoprotection of endothelium homeostasis against PA insult.

In fact, genistein is a multifunctional compound. In addition to inhibition of inflammation, its other bioactivities, such as antioxidation [31], estrogenic action [32] and regulation of AMPK [33,34], may take part in regulation of insulin sensitivity, although details remain to be determined. In the present study, we showed opposite effects of genistein on insulin action in the endothelium. Genistein inhibited insulin-mediated IRS-1 tyrosine phosphorylation and thereby attenuated insulin signaling along PI3K/Akt/eNOS pathways, leading to a decreased NO production in endothelial cells. PA stimulation evoked inflammation and induced insulin resistance in the endothelium. Genistein inhibited inflammatory response in an IKKβ/NF-κB-dependent manner and facilitated insulin PI3K signaling by beneficial modulation of IRS-1 function. Meanwhile, genistein inhibited enhanced mitogenic actions of insulin by down-regulation of ET-1 and VCAM-1 expression, demonstrating its beneficial regulation of PI3K-

dependent and MAPK-dependent actions of insulin. The resulting increase in insulin-mediated NO should be responsible for its amelioration of endothelial dysfunction.

## Acknowledgments

This work is supported by the National Natural Science Foundation of China (Grant No. 81072976) and Natural Science Foundation of Jiangsu Province of China (Grant No. BK2010430). We are grateful to Dr. Shuifei Zhuang (Stowers Institute for Medical Research, Kansas City, MO, USA) for her careful checking of the language of the manuscript.

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