Mechanisms of soy isoflavones in the regulation of vascular function

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ABSTRACT

Cardiovascular diseases (CVD) are the leading cause of morbidity and mortality in the United States. It is also well recognized that the incidence of CVD is substantially increased in postmenopausal women due to the loss of estrogen. Experimental and clinical data support vascular protective effects of estrogen by various mechanisms. However, administration of estrogen is also associated with an increased incidence of heart disease which limits its therapeutic potential. Given the demonstrated risks of conventional estrogen therapy, a search for novel, cost-effective, alternative vasoactive agents for prevention of CVD is of major importance in the effort to decrease the burden of CVD morbidity. Genistein, a major soy isoflavone, may be one of those alternative agents because of its selective affinity to estrogen receptor-beta and various beneficial effects on CVD. However, the mechanism of the cardioprotective effects of genistein is still unclear. The objectives of this study were (1) to investigate the effect of genistein on the expression of endothelial nitric oxide synthase (eNOS) both *in vitro* and *in vivo*; (2) to define the mechanism by which genistein regulates eNOS expression; and, (3) to examine whether genistein protects against tumor necrosis factor-alpha (TNF-α)-induced apoptosis in human aortic endothelial cells (HAECs). The results demonstrated that genistein, at physiologically achievable concentrations (1-10 μM) in individuals consuming soy products, enhanced the expression of eNOS protein and

subsequently elevated nitric oxie (NO) synthesis in both HAECs and human umbilical vein endothelial cells, concomitant with the increased eNOS mRNA expression (2.6-fold of control) and eNOS promoter activity, suggesting that genistein activates eNOS transcription. Furthermore, dietary supplementation of genistein to spontaneously hypertensive rats restored aortic eNOS levels, improved aortic wall thickness, and alleviated hypertension, confirming the biological relevance of the *in vitro* findings. However, the effects of genistein on eNOS and NO were not mediated by activation of estrogen signaling, mitogen-activated protein kinase, phosphatidylinositol 3-kinase/Akt kinase, protein kinase C or inhibition of typrosine kinases, but possibly through activating the cAMP/protein kinase A/cAMP responsive elemant binding protein pathway. These data suggest that genistein has direct genomic effects on the vascular wall that are unrelated to its known actions, leading to increase in eNOS expression and NO synthesis, thereby improving vascular homeostasis.

We also found that genistein (5-10 μM) significantly inhibited TNF-α-induced apoptosis in HAECs as determined by caspase-3 activation, apoptotic cell detection and DNA laddering. The anti-apoptotic effect of genistein was associated with an enhanced expression of anti-apoptotic Bcl-2 protein and its promoter activity that was ablated by TNF-α. Moreover, this anti-apoptotic effect of genistein was not mediated by extracellular signal-regulated kinase 1/2**,** protein kinase A, or estrogen receptor. However, inhibition of p38 mitogen-activated protein kinase (p38) by SB203580 completely abolished the cytoprotective effect of genistein, suggesting that genistein acted through the p38-dependent pathway. Accordingly, stimulation

of HAECs with genistein resulted in rapid and dose-dependent activation of p38. Unlike TNF-α which specifically activated p38α, genistein selectively induced phosphorylation of p38β, suggesting that p38β, but not p38α, is essential for the cytoprotective effect of genistein. These findings provide the evidence that genistein acts as a survival factor for vascular ECs to protect cells against apoptosis via activation of p38β.

Taken together, the resuls of the present study suggest that genistein can act directly on vascular ECs, improves endothelium homeostasis by promoting eNOS expression and endothelial-derived NO synthesis through activating the cAMP/PKA/CREB cascade, and protects against TNF-α-induced apoptosis via activation of p38 β. These data potentially provide a basic mechanism underlying the physiological effects of genistein in the vasculature.

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LIST OF ABBREVIATIONS

A

7-AAD: 7-amino actinomycin D

AC: Adenylate cyclase

ANOVA: Analysis of variance

AdPKI: Adenovirus encoding protein kinase A inhibitor

B

BAECs: Bovine aortic endothelial cells

C

CaM: Calmodulin

cAMP: Cyclic adenosine monophosphate

CRE: cAMP-responsive element

CREB: cAMP-responsive element binding protein

CVD: Cardiovascular diseases

D

DMSO: Dimethyl sulfoxide

E

E2: 17β-estradiol

ER: Estrogen receptor

ECs: Endothelial cells

ERK1/2:Extracellular signal-regulated kinase 1/2

ERK/MAPK, ERK-mitogen activated protein kinase

ERRα1: estrogen-related receptor α1

eNOS: Endothelial nitric oxide synthase

ERs: Estrogen receptors

ET-1: Endothelin-1

F

FBS: Fetal bovine serum

H

HAECs: Human aortic endothelial cells

HBSS: Hank's balanced salts solution;

Hsp90: Heat shock protein 90

HUVECs: Human umbilical vein endothelial cells

I

ICAM-1: Intercellular adhesion molecule-1

L

LBD: Ligand binding domain

LDL: Low density lipoprotein

M

MCP-1: Macrophage chemoattractant protein-1

MMPs: Matrix metalloproteinases

MOI: Multiplicities of infection

N

nNOS: neuronal nitric oxide synthase

NO: Nitric oxide

P

P38: p38 mitogen-activated protein kinase

P38α: p38 mitogen-activated protein kinase alpha

P38β: p38 mitogen-activated protein kinase belta

PBS: Phosphate-buffered saline

PI3K: Phosphatidylinositol 3-kinase

PI3K/AKT, phosphoinositol-3 kinase/protein kinase B (AKT)

PKA: Protein kinase A

PKC: Protein kinase C

PPARs: Peroxisome proliferator-activated receptors

PTK: protein tyrosine kinase

R

ROS: Reactive oxygen species

S

siRNA: Small interfering RNA

SHR, spontaneously hypertensive rats

T

TNF-α: Tumor necrosis factor-alpha

TUNEL: Terminal deoxynucleotidyltransferase dUTP nick-end labeling

V

VCAM-1: Vascular cell adhesion molecule-1

VSMCs: Vascular smooth muscle cells

W

WKY, Wistar-Kyoto rats

CHAPER 1

Introduction

Background

Cardiovascular diseases (CVD), including coronary heart disease, heart failure, stroke and hypertension, are the leading cause of illness and death in the United States, which accounted for 36.3% of all deaths in 2004, or 1 of every 2.8 deaths in the United States (1). Indeed, CVD claims more lives each year than cancer, chronic lower respiratory diseases, accidents and diabetes mellitus combined, and the estimated direct and indirect cost of CVD for 2007 is $431.8 billion (1).

Previous studies show that women within their reproductive age have lower rate of CVD than that of age-matched men, but these reduced rate of CVD diminish with the onset of menopause and even higher in postmenopausal women than in age-matched men (1), suggesting that estrogen plays a key role in preventing CVD in premenopausal women. Indeed, experimental studies have shown that natural estrogens, such as 17β-estradiol, can lower plasma levels of low-density lipoprotein cholesterol and lipoprotein Lp(a), raise plasma levels of high-density lipoprotein cholesterol (2, 3), and protect blood vessels from atherosclerotic lesion formation (4). 17 β-estradiol also accelerates endothelial cell recovery after balloon injury (5) and inhibits vascular smooth muscle cell (VSMCs) proliferation (6). Therefore, estrogen replacement therapy was used to reduce the high rate of CVD in postmenopausal women (7). However, the effect of administration of estrogen for cardioprotection remains controversial (8, 9), and estrogen replacement therapy is further

limited by carcinogenic effects in women and feminizing effects in men (10). Therefore, a search for novel, cost-effective, alternative vasoactive agents for prevention of CVD is of major importance in the effort to decrease the burden of CVD morbidity.

Isoflavones have drawn wide attention due to their potentially beneficial effects on some human degenerative diseases in the last decade (11). Genistein, the most abundant isoflavone in soy, has a number of biological actions. It is a well-known tyrosine kinase inhibitor at pharmacological dose and is a selective ligand of the estrogen receptor (ER)-β (12). A wealth of literature shows that isoflavones have beneficial effects on obesity (13, 14), hormone-dependent cancer (15), osteoporosis (16) and cardiovascular diseases (17). Epidemiological studies demonstrate that soy isoflavones intake in American postmenopausal women is inversely associated with cardiovascular disease risk factors (18, 19). Some human intervention studies suggest a beneficial effect on atherosclerosis (20), markers of cardiovascular risk (21) vasomotor tone (22), systemic arterial compliance (23), and vascular endothelial function (24). Data from animals and *in vitro* studies suggest a protective role of isoflavones in cardiovascular events (25-33). Furthermore, genistein reduces the size of infarction and experimental myocardial ischemia-reperfusion injury (34), and improves endothelial dysfunction induced by oophorectomy in rats (35). Genistein consistently caused vascular relaxation of aorta, pulmonary and coronary arteries in animals (31, 32, 36-39). However, the mechanisms of the action of soy isoflavones are still unclear despite efforts to elucidate them. Therefore, my dissertation research is focused on elucidating the fundamental role for genistein in the regulation of vascular function.

Vascular endothelium, a single layer of endothelial cells (ECs) lining the lumenal side of the vessels, is not only a biological barrier separating circulating blood and peripheral tissues, but also a form of sensory organ having the ability to monitor, integrate and transduce blood born signals. Endothelium can speak outward to platelets and leukocytes or inward to VSMCs, and transduce environmental stimuli such as hormones, cytokines and bacterial products as well as mechanical forces like fluid shear stress, wall tension and intraluminal pressure. Endothelium also secrets critical vasoactive factors such as nitric oxide (NO) to modulate vascular function. Thus, endothelium plays a pivotal role in maintaining normal vascular function.

Endothelium is a dynamic and interactive element which maintains vascular homeostasis. Any impairment of the integrity of this continuous single-cell formed layer causes endothelial dysfunction, which leads to acute and chronic inflammatory process, immunologic reactions, apoptosis, hyperpermeability and eventually various cardiovascular events. Various stimuli such as viral infection, bacterial toxins and tumor necrosis factor-alpha (TNF-α) impair the integrity of endothelium anatomically (eg, surface blebbing, intracellular gaps, death) and functionally, leading to increased platelet adherence, intrinsic coagulation and decreased NO synthesis.

Endothelium-derived NO, synthesized by endothelial NO synthase (eNOS) from amino acid L-arginine and molecular oxygen, plays a key role in maintaining vascular tone and the integrity of endothelium. NO suppresses endothelial release of inflammatory cytokines and expression of adhesion molecules, thereby preventing vascular infiltration of leukocytes. In addition, NO prevents atherogenesis by inhibition of the proliferation and migration of

VSMCs, therefore inhibiting intimal fibrosis and atherosclerosis. Dysregulation of NO has been proposed as both cause and consequence of the endothelial dysfunction that leads to CVD. Indeed, many CVD risk factors such as being male (1), advancing age (40), cigarette smoking (41), high blood pressure (42, 43), diabetes (44), are associated with the reduction of NO release via decreasing the activity and/or expression of eNOS in human or animals.

Hypotheses

Previous studies have established a role for estrogen in the vascular ECs to enhance NO synthesis through genomic stimulation of eNOS expression (45), and by ERs-mediated, non-genomic eNOS activation (46). We recently demonstrated that genistein acutely stimulates NO production by phosphorylation of eNOS in ECs (47, 48). However, it is unknown whether genistein has a similar genomic effect on eNOS. Studies have reported that administration of soy protein improves eNOS expression and subsequently reduces blood pressure in rats (49). However, other studies demonstrated that the beneficial effect of genistein on endothelial function is not through enhancing eNOS expression (50). Although genistein has been shown to enhance eNOS promoter activity in a transformed human ECs (51), it is not clear whether genistein directly up-regulates eNOS expression in primary ECs and thereby reduces blood pressure *in vivo*. In the first project of my study, I **tested the hypothesis that genistein improves eNOS expression and subsequently increases NO synthesis in primary HAECs and in spontaneously hypertensive rats (SHR), and this improved NO synthesis is associated with a blood pressure-lowering effect of genistein in SHR.**

Recently, we demonstrated that genistein targets the cAMP signaling pathway and regulates cAMP-regulated gene expression that is not related to its estrogenic effect or inhibition of protein tyrosine kinase (PTK) in vascular ECs (48). Genistein also has been shown to increase intracellular accumulation of cAMP in other cells such as pancreatic beta-cells (52), airway epithelial cells (53) and cardiomyocytes (54), suggesting that genistein possibly influences a wide spectrum of cAMP-mediated biological activities. Cyclic AMP is a central signaling molecule in a variety of cellular systems and plays an important role in maintaining normal vascular function. Activation of PKA by cAMP stimulates the phosphorylation of cAMP-responsive element binding protein (CREB) at Ser-133 which subsequently interacts with cAMP-responsive element (CRE, TGACGTCA) or CRE-like sequences of target genes and therefore regulates gene expression in response to elevated cAMP (55). Interestingly, recent studies show that activation of PKA improves eNOS expression *in vivo* (56), suggesting that eNOS could be regulated by cAMP signaling. Indeed, recent studies showed that a CRE site is located within eNOS promoter that is involved in the regulation of eNOS expression (57), suggesting that the eNOS expression may be directly regulated by CREB. Based on these data, my second project tested the **hypothesis that genistein may regulate the expression of eNOS through the PKA-dependent activation of CREB.**

Endothelial cells apoptosis may be an important factor that initiates the pathogenesis of aging-associated vascular disease such as atherosclerosis (58) and acute coronary syndrome (59). Many classic risk factors of CVD such as oxidized LDL (60), aging (61), high concentration of reactive oxygen species (62) and cytokines such as TNF-alpha (63)

stimulate EC apoptosis. Furthermore, TNF-α, a potent inducer of EC apoptosis (64), is remarkably elevated in diabetic animals or humans with vascular complications (65-67), and high levels of TNF-α in the blood were associated with a high prevalence of atherosclerosis in old humans (68), suggesting that this inflammatory cytokine may play an important role in the process of atherosclerosis. Moreover, increased TNF-α down-regulates the expression of Bcl-2, one of the major anti-apoptotic proteins in vascular ECs (69). Thus, reducing TNF-α-induced ECs apoptosis may provide an important strategy to prevent CVD. Interestingly, isoflavones can augments production of NO (47, 48), a well known inhibitor of apoptosis at low levels. Recent studies demonstrated that genistein protects against TNF-α-induced apoptosis in osteoblastic cells (70) and homocysteine-induced apoptosis in clonal ECs (71). Therefore**, for the third project of my dissertation, I examined whether genistein protects against TNF-α-induced apoptosis in HAECs and then further determined the underlying mechanism.**

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CHAPER 2

Literature Review [1]

Abstract

Genistein, a natural bioactive compound derived from legumes, has drawn wide attention during the last decade because of its potentially beneficial effects on some human degenerative diseases. It has a weak estrogenic effect and is a well-known non-specific tyrosine kinase inhibitor at pharmacological doses. Epidemiological studies show that genistein intake is inversely associated with the risk of cardiovascular diseases. Data from animal and *in vitro* studies suggest a protective role of genistein in cardiovascular events. However, the mechanisms of the genistein action on vascular protective effects are unclear. Past extensive studies exploring its hypolipidemic effect resulted in contradictory data. Genistein also is a relatively poor antioxidant. However, genistein protects against pro-inflammatory factor-induced vascular endothelial barrier dysfunction and inhibits leukocyte-endothelium interaction, thereby modulating vascular inflammation, a major event in the pathogenesis of atherosclerosis. Recent studies found that genistein exerts a novel non-genomic action by targeting on important signaling molecules in vascular endothelial cells (ECs). Genistein rapidly activates endothelial nitric oxide synthase and production of nitric oxide in ECs. This genistein effect is novel since it is independent of its known effects, but

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mediated by the cyclic adenosine monophosphate/protein kinase A (cAMP/PKA) cascade. Further studies demonstrated that genistein directly stimulates the plasma membrane-associated adenylate cyclases, leading to activation of the cAMP signaling pathway. In addition, genistein activates peroxisome proliferator-activated receptors, ligand-activated nuclear receptors important to normal vascular function. Furthermore, genistein reduces reactive oxygen species (ROS) by attenuating the expression of ROS-producing enzymes. These new findings reveal the novel roles for genistein in the regulation of vascular function and provide a basis for further investigating its therapeutic potential for inflammatory-related vascular disease.

Keywords: genistein, endothelial cells, inflammation, cAMP, nitric oxide, peroxisome proliferator-activated receptor, antioxidant, atherosclerosis.

INTRODUCTION

The prevalence of cardiac and other vascular diseases rises in the aging population. It is also well recognized that the incidence of cardiovascular disease is substantially increased in postmenopausal women due to the loss of estrogen. Clinical studies have demonstrated that hormone replacement therapy prevents cardiovascular disease in early menopausal women [1, 2], but in postmenopausal women, this vasculoprotective effect of hormone replacement therapy is not consistent in recent clinical trials [3-7]. Moreover, the use of estrogen as a cardioprotective agent is further limited by its carcinogenic effects in women and feminizing effects in men [8]. Thus, there is interest in finding novel alternative agents that may have beneficial effects on the vasculature without some of the side effects of estrogen.

Isoflavones have received widespread attention over the past decade because of their preventive potential against some highly prevalent chronic diseases, such as cardiovascular disease [9], osteoporosis [10, 11] and hormone related cancers [12, 13]. Epidemiological studies show that soy isoflavone intake in American postmenopausal women is inversely associated with cardiovascular disease risk factors [14, 15]. Data from animals and *in vitro* studies suggest a protective role of isoflavones in cardiovascular events [16-23]. Genistein, a major isoflavone abundant in soy, has various biological actions including a weak estrogenic effect by binding to estrogen receptors (ERs) [24] , and inhibition of protein tyrosine kinases (PTK) [25]. Recent studies demonstrate that genistein has anti-atherogenic effects by inhibiting the proliferation of vascular smooth muscle cells (VSMCs) [26]. Some human intervention studies suggest the beneficial effects of genistein on atherosclerosis [27],

markers of cardiovascular risk [28, 29], vascular motor tone [30, 31], vascular endothelial function [32], and systemic arterial compliance [33]. In addition, it has been shown that genistein reduces the size of infarction and experimental myocardial ischemia-reperfusion injury [34], and improves endothelial dysfunction induced by oophorectomy in rats [35], thus providing additional evidence for a cardioprotective effect of genistein. In studies with animal models, genistein consistently caused vascular relaxation of aorta, pulmonary and coronary arteries [22, 36, 37]. However, the mechanism of genistein action in vasculature is still not clear. Past studies have extensively explored its hypolipidemic [38], anti-oxidative [39-44] and the estrogenic effects [45]. While genistein may have both ER-dependent and independent actions in vasculature, its average effect on plasma lipid profile is neutral [16, 31, 33, 46-49]. The effects of genistein on these aspects related to vascular health have been reviewed elsewhere [7, 42, 50-55]. This review focuses on the recent studies exploring the molecular targets for genistein in vasculature which may provide better understanding of the fundamental roles of genistein in vascular health. Furthermore, the possible relationship of chemical structure of genistein with its function as a ligand for important molecules in vascular cells is also discussed.

GENISTEIN IS A SELECTIVE ER-β LIGAND

Genistein consists of two aromatic rings (A and B) linked through a heterocyclic pyrane ring (C) [56]. The chemical structure of genistein is similar to that of 17β-estradiol (E2), the endogenous estrogen primarily acting through the ERs in humans. Typically, ER

ligand comprises two hydroxyl groups separated by a rigid hydrophobic linker region. In addition, an effective ligand possesses a phenolic hydroxyl group since ligand recognition is achieved through a combination of specific hydrogen bonds between ligand and the ER. As shown in Fig. **1**, the A and C rings of genistein are similar to the A and B rings of E2, and the actual distance between the two hydroxyl groups on both molecules is nearly identical. This structural similarity indicates that genistein could potentially bind to the ERs. Actually, genistein has long been known to exert estrogenic effect. Indeed, although it is not completely clear how genistein interacts with the ERs, it is believed that genistein binds to the ERs through its phenolic hydroxyl group at C4' which interacts with the Glu-Arg-water triad in the ERs and also through flavone hydroxyl group at C7 which interacts with the distal histidine residue at the end of the ERs cavity [57]. Unlike E2 however, which binds to both ERα and ERβ with nearly equal affinity, genistein shows much higher affinity to ERβ (87% of E2) than to ERα (4% of E2) [24]. This binding specificity may be due to slight difference in steroid binding sites of two receptors. In the binding site of ERβ, amino acid residue Met336/Ile373 is substituted for Leu384/Met421 in ERα [57]. While Met421 (ERα) and Ile373 (ERβ) are important in determining the ligand binding orientation, residues Leu384 (ERα) and Met336 (ERβ) may be the major determinants for the ligand binding to the ERs. As observed in the X-ray binding mode, the Met336 residue in the ERβ interferes with the methyl group of E2 and consequently decreases the affinity of E2 to ERβ [58]. In addition, the overall pocket size of ERβ-ligand is slightly smaller than that of ERα (390 Å for ERβ-Genistein vs. 490 Å for ERα-E2). This reduction in size is mainly due to the substitution of Met336 in ERβ for Leu384 in ERα [59]. These structural and conformational differences

between two receptors may contribute primarily to the observed differential affinity of genistein to ERα and ERβ. Furthermore, the hydroxyl group at C5 of genistein significantly increases its binding selectivity for ERβ though it has no specific interaction with the protein [59]. This is substantially supported by a recent study demonstrating that daidzein, a genistein analog and another isoflavone which is only lack of the hydroxyl group at C5 compared to genistein (Fig. **1**), essentially has no binding activity to either ERα or ERβ (0.1% and 0.5% of E2, respectively) [24]. Therefore, genistein is a plant-derived novel selective ERβ agonist. The structural similarity and difference between genistein and E2 may largely contribute to the ER-dependent and ER-independent functions of genistein [60-62].

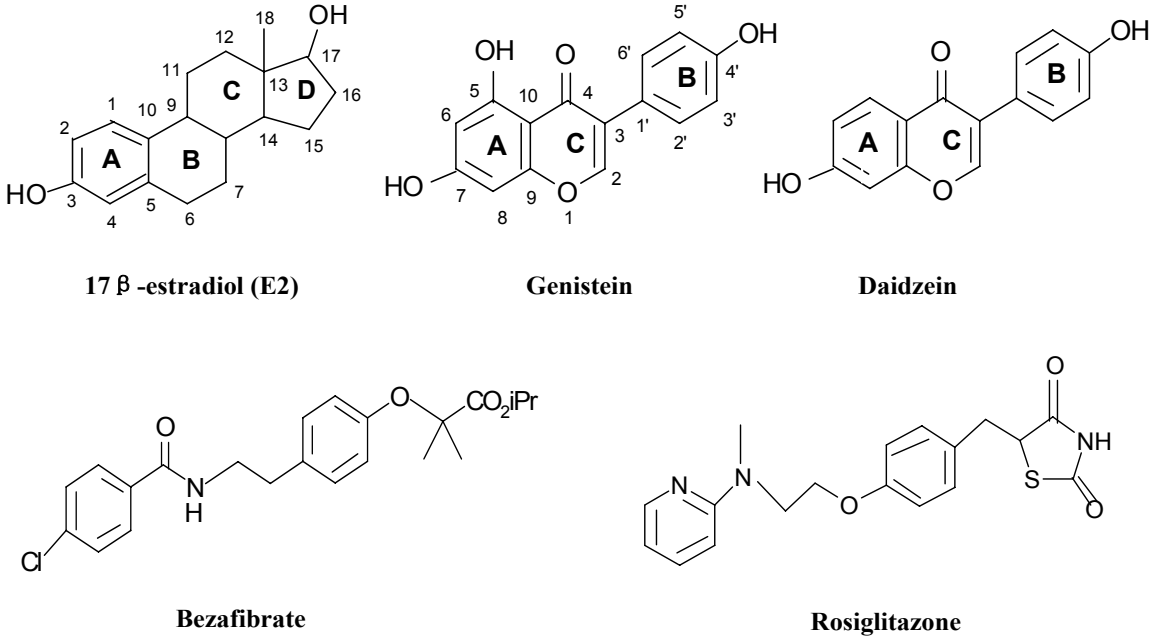

Fig. (1). Chemical structures of genistein, daidzein, estradiol and ligands of PPARα and PPARγ.

As genistein has weak estrogenic effect, there is concern about its potential adverse effects in various estrogen-dependent organs although research evidence is not established

and the relationship between doses, duration, beneficial or harmful effects are unclear. Genistein may has minimal uterotrophic effect by preferentially binding to ERβ since the main ER subtype in the uterus is ERα [63], which may primarily mediate the uterotrophic effect of estrogen. Indeed, although genistein has been shown to affect development of the reproductive system and immune functions in experimental animals [64], but no such an effect has been reported in infants fed infant formula containing this compound or in various mature animal species fed pharmacological doses of genistein [16, 65-69]. Indeed, the dose of genistein required to produce an effect on uterus of mice is 10,000-fold higher than that of E2 [69]. A recent study also showed that genistein provides similar vasculoprotective effect as E2 but has no adverse effect on the reproductive system in peripubertal moneys [16] and rats [65]. Both ERs are expressed in vascular endothelial and smooth muscle cells [70-72]. While the role of ERα in estrogen regulation of vascular function has been well established [73, 74], increasing evidence suggest that ERβ is also important in mediating various vascular protective effects of estrogen [65, 75-82]. Therefore, genistein may be a novel candidate as an alternative for estrogen based vasculoprotective drug that can protect vascular systems but devoid of uterotrophic side effects.

GENISTEIN AND VASCULAR INFLAMMATION

Atherosclerosis is the hardening and thickening of artery walls characterized by the deposition of atheromatous plaques. Atherosclerotic vascular disease is a major cause of morbidity and mortality in the industrial world [83]. In experimental studies, dietary

supplementation of genistein significantly reduced atherosclerosis in various animal models [84, 85]. It has been demonstrated that genistein also markedly inhibits diet-induced coronary artery atherosclerosis in nonhuman primates [8]. Further, administration of genistein significantly prevents thrombotic vessel occlusion [86]. However, the potential mechanisms of the beneficial effects of genistein on atherosclerosis are still unclear. During the last decade, extensive studies have focused largely on elucidating the effect of genistein on lipid profiles because hyperlipidemia contributes to atherosclerosis [87]. However, the results show that the effect of genistein on plasma lipid profiles, such as low-density lipoprotein and triglycerol, is essentially neutral [16, 31, 33, 46-49, 88, 89], suggesting that the anti-atherogenic effect of genistein is not due to a change in the plasma lipids.

Atherosclerosis is now recognized as a chronic inflammatory process since inflammation is involved in all stages of atherosclerosis from initiation through progression and, ultimately, the thrombotic complications of atherosclerosis [38, 90-92]. Vascular endothelium, which not only serves as a biological barrier separating circulating blood and peripheral tissues, but also secretes various vasoactive substances, plays a pivotal role in maintaining normal vascular function. Dysfunction of endothelium, impaired nitric oxide (NO) production, recruitment of immune cells to activated ECs, increase in endothelial permeability and subsequent transmigration of immune cells into the vessel wall are key early events in the development of atherosclerosis [93]. These important pathophysiological components are mediated by various pro-inflammatory mediators and cell adhesion molecules secreted by injured ECs such as thrombin, tumor necrosis factor-α (TNF-α), macrophage chemoattractant protein-1 (MCP-1), vascular cell adhesion molecule-1 (VCAM-

1), and intercellular adhesion molecule-1 (ICAM-1) [94-97]. Emerging evidence indicates that genistein exerts multifaceted anti-inflammatory effects in vasculature [98-100], suggesting that genistein may prevent atherosclerosis by suppressing vascular inflammation. Recent studies have demonstrated that genistein inhibits hyperpermeability of cultured vascular ECs induced by thrombin [101], an inflammatory mediator produced on the surface of injured endothelium, causing disturbance of its barrier function [102]. While the biological relevance of this *in vitro* study needs to be determined, another study showed that oral administration of genistein significantly reduced retinal vascular leakage of diabetic rats in a dose-response fashion [103], suggesting that genistein may prevent inflammation-induced vascular barrier dysfunction and thereby related vascular disease such as atherosclerosis. It was also demonstrated that genistein inhibits lipopolysaccharide-stimulated TNF-α production in a macrophage cell line [104] and TNF-α levels *in vivo* [105]. Genistein also dose-dependently inhibits TNF-α-induced MCP-1, ICAM-1 ,VCAM-1 [106-108] and matrix metalloproteinases (MMPs) [109] secretion both *in vitro* and *in vivo*, and the proliferation of VSMCs from spontaneously hypertensive rats [110].

The underlying mechanisms by which genistein affects TNF-α and adhesion molecules secretion have yet to be elucidated. However, some recent reports suggest that the inhibitory effect of genistein on some of these pro-inflammatory molecules may be mediated by NO since genistein increases NO bioactivity in ECs [101, 111]. As an anti-atherosclerotic molecule, EC-derived NO protects against atherosclerotic lesion formation by inhibition of MCP-1 and VCAM-1 expression [112], VSMC proliferation [113], and MMPs activity [114]. In fact, it was recently found that the inhibitory effect of genistein on VCAM-1 is dependent

on NO production [115]. In obese and diabetic patients [116] and in animals [117] with pre-atherosclerosis complications, NO bioactivity is impaired in association with elevated TNF-α. Our unpublished observations demonstrated that genistein dose-dependently restores the TNF-α-down-regulated endothelial NO synthase (eNOS) expression both in mRNA and protein levels in human aortic ECs (HAECs). In consistent with these observations, genistein also has been found to inhibit the interaction of leukocytes and vascular ECs *in vitro* [118, 119]. However, the biological relevance of this *in vitro* observation remains to be determined.

The specific chemical structure responsible for the inflammation-suppressing effect of genistein is not clear. One study showed that the structurally closely related isoflavone daidzein which differs with genistein only in the substitution of the 5C-hydroxyl group with a hydrogen (41), failed to inhibit TNF-α production [120], suggesting that the 5 position of the A ring may be an important component for an anti-inflammatory effect of genistein. However, other studies demonstrated that daidzein has similar effects as genistein on TNF-α generation [106, 120, 121]. It has been shown that replacement of 7C-hydroxyl group with a glucose molecule reverses the inhibitory effect of genistein on TNF-α production [120]. In addition, substitution of a sulfate for the 4'C- and/or 7C-hydroxyl group similarly ablates the inhibitory effects of genistein on MCP-1, VCAM-1 and ICAM-1 [122]. These results suggest that these two hydroxyl groups may be essential for the anti-inflammatory effects of genistein.

GENISTEIN, NO AND THE cAMP SIGNALING

Endothelial NOS-derived NO plays a critical role in the protection of a variety of vascular functions including vasorelaxation [123, 124], anti-inflammation [125, 126], anti-atherogenesis [127] and inhibition of platelet adhesiveness [128, 129]. Previous studies establish a role for estrogen in the regulation of vascular function. Estrogen can act directly on the vascular ECs to enhance NO production through genomic stimulation of eNOS expression [130] as well as via non-genomic, receptor-mediated elevation of the enzymatic activity [70]. Similarly, it has been demonstrated that genistein intake can increase circulating nitrate/nitrite [31] and endothelium-dependent vasodilatation in humans [30, 31]. Previous studies also indicate that daidzein [131-133] and its metabolite equol [134] augment NO bioavailability without ER involvement. However, it is still unclear whether increased nitrate/nitrite by genistein reflects increased NO production or its bioavailability. A more recent study demonstrated that genistein may regulate vascular function by directly modulating eNOS, because genistein dose-dependently elevates NO release (Fig. **2A**) by directly phosphorylation of Ser1179-eNOS (Fig. **2B**) in bovine aortic endothelial cells (BAECs) [61]. The rapid activation of eNOS by genistein (10-120 min) is not mediated by binding to the ERs, but dependent on the protein kinase A (PKA) pathway. Consistent with the PKA-dependent action of eNOS by genistein, a further study demonstrated that genistein targets the cAMP signaling pathway in both BAECs and human umbilical vein endothelial cell (HUVECs) by primarily activating plasma membrane-associated adenylate cyclase through the non-genomic mechanisms that are not related to its estrogenic effect or inhibition of PTK (Fig. **3A**) [101]. The elevation of cAMP by genistein stimulates PKA activity, which

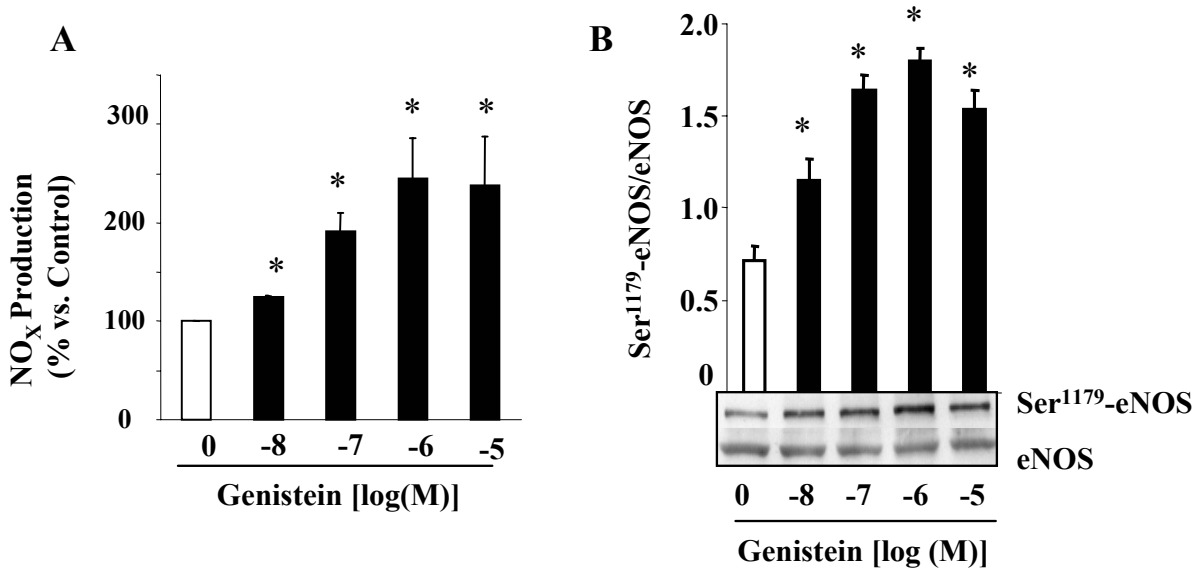

Fig. (2). Genistein rapidly stimulates NO production by direct phosphorylation of Ser1179-eNOS in ECs. Bovine aortic endothelial cells were incubated with various concentrations of genistein or vehicle for 10 min. NO production (**A**) and eNOS phosphorylation (**B**) were measured with specific kits and Western blot respectively (from reference 61). The bar graphs represent three independent experiments. *, $p<0.05$ *vs.* vehicle alone-treated control.

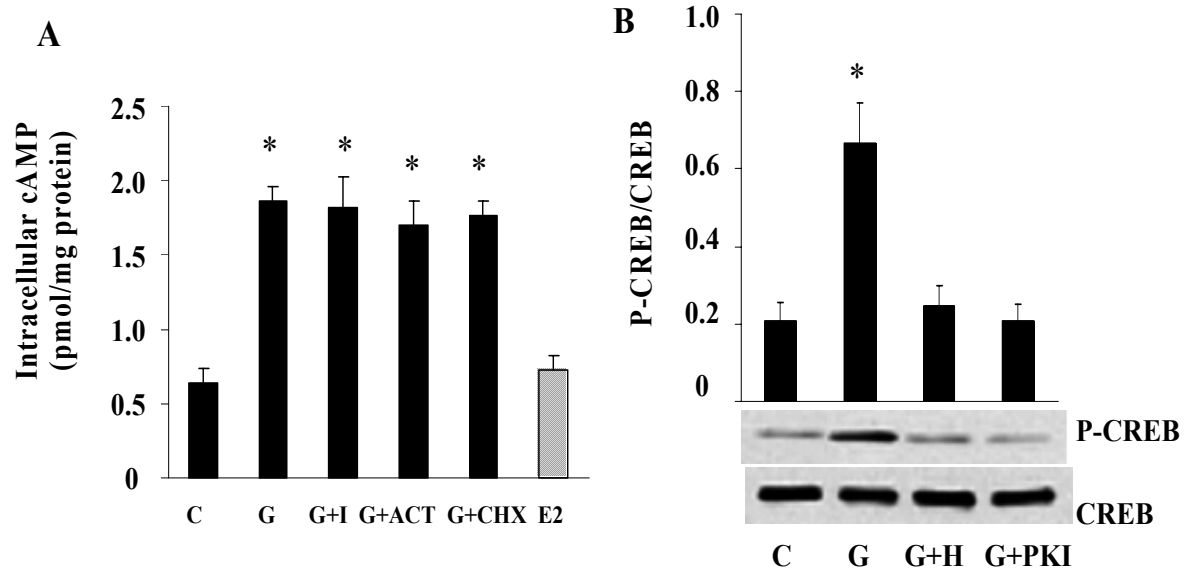

Fig. (3). Genistein activates the cAMP/PKA/CREB cascade in the ER-and transcription-independent mechanisms in ECs. **A:** Intracellular cAMP accumulation in ECs stimulated with

genistein (G, 5 μM), E2 (E2, 10 nM) or vehicle (C) in the presence or absence of ICI 182,780 (I, 10 μM), actinomycin D (AT, 10 μM), or cycloheximide (CX, 10 μM) was measured by an EIA kit; **B:** CREB phosphorylation (P-CREB) and total protein expression in ECs incubated with genistein (G, 5 μM) or vehicle (C) in the presence or absence of H89 (H, 10 μM),or PKI (PKI, 2 μM) were determined by Western blot (from reference 101). The bar graphs represent three independent experiments. *, $p<0.05$ *vs.* vehicle alone-treated control.

subsequently activates cAMP-responsive element-binding protein (CREB) (Fig. **3B**) and regulates gene expression in vascular ECs [101]. Genistein also has been shown to increase intracellular accumulation of cAMP in other tissues including pancreatic beta-cells [135], airway epithelial cells [136] and cardiomyocytes [137], suggesting that genistein possibly influences a wide spectrum of cAMP-mediated biological activities.

Cyclic AMP is a central signaling molecule in a variety of cellular systems and plays an important role in maintaining normal vascular function. Activation of the cAMP/PKA pathway directly phosphorylates multiple residues of eNOS, leading to the rapid activation of eNOS and NO production in ECs [138, 139]. In addition, the presence of functional cAMP-responsive element sites within the human eNOS promoter [140] suggests that the eNOS expression may be directly regulated by CREB. Indeed, a recent study demonstrated that genistein increased the eNOS gene expression in vasculature in rats [141]. This genistein effect on eNOS may be at the transcriptional level since it promotes the eNOS promoter activity in a human EC line (EA.hy926) [60]. Our unpublished studies further demonstrated that genistein increases the eNOS protein and mRNA expression as well as the eNOS

promoter activity in primary HAECs, confirming above results from transformed vascular cells and further suggesting a human relevant effect of genistein. However, it is still unknown whether genistein can act via the cAMP cascade to regulate eNOS expression in vasculature.

In addition, activation of the cAMP/PKA signaling inhibits vascular inflammation by depressing the adhesion of leukocytes to ECs [142] possibly through PKA-mediated CREB phosphorylation [143]. Furthermore, elevation of intracellular cAMP concentration in ECs improves barrier function by decreasing intercellular gap formation and endothelial permeability that result from various inflammatory mediators [144-151]. All these events are implicated in various pathological conditions such as the development of arteriosclerosis, suggesting that the cAMP elevating agent genistein may retard the process of some chronic vascular diseases by targeting the cellular cAMP/PKA pathway. A recent study also suggests that cAMP-dependent mechanisms may be involved in genistein-induced vascular relaxation [152]. Collectively, many of these genistein effects are either mediated through the cAMP signaling or are compatible with the declared action of cAMP, suggesting that the effect of genistein on the cellular cAMP/PKA cascade may represent a central mechanism and play a key role in a wide range of vascular protective effects. These findings thus may provide an explanation for these previously reported versatile actions of genistein observed in animal and human studies.

In contrast to above observations, a recent study reported that acute exposure of HUVECs to genistein (0.1 μM, 30 sec-2 min) stimulates activation of eNOS that is independent of the ERs but mediated by the extracellular signal-regulated kinase1/2

(ERK1/2) and phosphatidylinositol 3-kinase/Akt pathways, whereas intracellular cAMP production is unaffected [134]. This ER-independent, non-genomic activation of eNOS by genistein is consistent with above results. The discrepancies between these findings are not clear, which may be due to differences in cell type (HUVEC *versus* BAEC), species (human *versus* bovine) and the duration of genistein treatment ($\leqq$2 min *versus* $\geqq$10 min).

Genistein may also have indirect effect on eNOS activation by modulating those proteins that regulate eNOS activity. It is documented that eNOS activation is inhibited by caveolin-1 [153] but stimulated by calmodulin (CaM) [154] and heat shock protein 90 (Hsp90) [155]. Hsp90, eNOS, and caveolin-1 could form a heterotrimeric complex in ECs, and CaM displaces eNOS from caveolin-1 which facilitates Hsp90 binding to eNOS, thereby reducing the inhibitory action of caveolin-1 on eNOS [156]. Interestingly, genistein [157, 158] and daidzein [132, 159] increases CaM expression and reduces caveolin-1 levels in rats, which are associated with elevated plasma NO [157]. However, these genistein actions may not be relevant to its acute effect on eNOS activation.

GENISTEIN AND PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR (PPAR) ACTIVITY

The PPARs are ligand-activated transcription factors and are one of the major members of nuclear receptor family. Three PPAR isoforms, namely α, β/δ, and γ, have been identified. While each PPAR has distinct tissue expression pattern [160, 161], both PPARα and PPARγ are expressed in vascular ECs [162, 163] and VSMCs [164, 165]. The crucial

roles of PPARs in vascular homeostasis have been reviewed elsewhere [161, 166]. PPARγ and PPARα are recognized as integral members of pathways that control inflammation [167-169]. In the endothelium, ligand-activated PPARs result in an inhibition of cytokine-dependent induction of adhesion molecules and subsequent recruitment of immune cells to ECs [168, 170]. Recent studies showed that genistein inhibits the expression of vascular adhesion molecules [122] and pro-inflammatory cytokines [171] in ECs and immune cells, resulting in the inhibition of platelet aggregation and monocyte migration. These results suggest that genistein may be a potential anti-inflammatory agent. While the anti-inflammatory mechanism for genistein is not clear, several lines of evidence show that this genistein effect may be mediated through PPARγ [172]. A study reported that genistein acts as a ligand of PPARγ [62] and was later confirmed by several studies showing that genistein induces both PPARα and PPARγ activity by binding to the ligand-binding domains (LBD) within the transcriptional factors [173, 174]. Consistently, genistein has been shown to induce PPARγ-driven reporter gene activity in murine RAW 264.7 cells [175]. Our study also demonstrated that genistein stimulates the PPARγ promoter activity in BAECs, while its protein expression is not altered (unpublished observation). In addition, genistein has been shown to induce gene and protein expression of PPARα and subsequently increase expression of genes involved in lipid metabolism in hepatocyte cells [175, 176].

It is unclear how genistein interacts with PPARs. Like other nuclear receptors, PPARs have N-terminal transactivation domains, highly conserved DNA-binding domains, and LBD. When ligand enters into a pocket in the LBD and subsequently activates the receptor and forms obligate heterodimers with the 9-cis retinoic acid receptor, the heterodimers then bind

to the specific PPAR responsive elements within the target gene promoter and, subsequently regulate the specific gene expression or other signaling pathways [177, 178]. As shown in Fig.**1**, the typical ligand of PPARs is composed of a polar head and a hydrophobic tail. The A ring with its hydroxyl group of genistein mimics the polar head of rosiglitazone, while genistein's B ring with its hydroxyl group is similar to the hydrophobic tail of these PPAR ligands. However, it is largely unknown whether genistein activates PPARs because of these structural similarities of genistein with typical PPAR ligands. Nevertheless, genistein, as a ligand of PPARs, may exert multiple protective effects on vasculature.

Reduced NO biosynthesis and elevated endothelin-1 (ET-1) production are the major characteristics of endothelial dysfunction [179], which is a hallmark of the initiation of aging-associated vascular diseases. Recent studies show that activation of PPARα and PPARγ significantly increases the production of NO from ECs by stimulation of eNOS activity and protein expression [180-183]. In addition, both ligands of PPARα and PPARγ inhibit thrombin-induced ET-1 secretion in ECs [184]. Concisely, numerous studies have indicated that genistein stimulates the activity and expression of eNOS [61, 131, 141, 185, 186] and inhibits ET-1 expression [187, 188] in both *in vitr*o and in animals. Consistently, a recent human study also reported that dietary supplementation of genistein decreased plasma levels of ET-1 while circulating NO was elevated in postmenopausal women [189]. The effects of genistein on NO and ET-1 are unlikely mediated through the inhibition of PTK since the genistein levels used in these studies are much lower than the minimal dose of genistein (≥10 μM) required for inhibiting these enzymes [190, 191]. As described above, genistein could activate both PPARα and PPARγ [173, 174] and improves PPARα expression [175, 176].

Taken the regulatory roles of PPARα/γ in NO and ET-1 production as aforementioned, it is tempting to speculate that genistein may modulate the expression of eNOS and ET-1 and NO production partially via activation of these transcriptional factors, and subsequently prevents vascular inflammation. In addition, ample evidence demonstrates that PPARα and PPARγ modulate vascular inflammation possibly by reducing expression of inflammatory cytokines, adhesion molecules and extracellular matrix proteases [169] [192] [193]. Therefore, it is conceivable that the anti-inflammatory effect of genistein in ECs could be partially dependent on activation of PPARs. Indeed, a recent study showed that genistein inhibits leukocyte-EC interactions by the PPARγ–mediated pathway in an *in vitro* stimulated blood flow environment [98], revealing an additional anti-inflammatory mechanism for genistein in vasculature.

There is accumulating evidence that reactive oxygen species (ROS)-induced oxidative stress contributes to vascular inflammation and dysfunction [194-196] through multiple mechanisms [197-199]. Thus, reduction of ROS becomes one of important strategies to prevent cardiovascular diseases. While it has been shown that genistein is a relatively poor ROS scavenger [98, 200, 201], it may reduce superoxide production by suppressing protein expression of endothelial NAD(P)H oxidase [131, 157, 172, 202], a major source of superoxide in blood vessels which is implicated in oxidative stress-related vascular diseases [203-206]. Interestingly, genistein exerts this effect through activation of PPARγ [172], suggesting that PPARγ is a key upstream molecules that may mediate various vascular protective effects of genistein.

CONCLUSIONS

Genistein has been shown to exert beneficial effect on cardiovascular system, although it only has a limited effect on plasma lipids. As a highly selective agonist of ERβ, genistein may act on vasculature partially through the ER-dependent mechanisms, given the role for estrogen in the regulation of vascular function. However, it appears that genistein has ER-independent stimulatory effects on multiple cellular signaling pathways and transcriptional factors including eNOS, cAMP, ERK1/2, Akt, and PPARα and PPARγ which potentially offer a wide spectrum of beneficial effects on vasculature and therefore are attractive molecular targets by which to prevent cardiovascular disease (Fig. **4**). These findings from substantial recent studies provide new insights into the fundamental role of genistein in vascular health and have initiated important new areas for investigation. Meanwhile, it should be pointed out that genistein exists primarily as glucuronide and sulfate conjugates in blood circulation and only a small percentage of genistein (1.59% to 8.42% of total genistein)

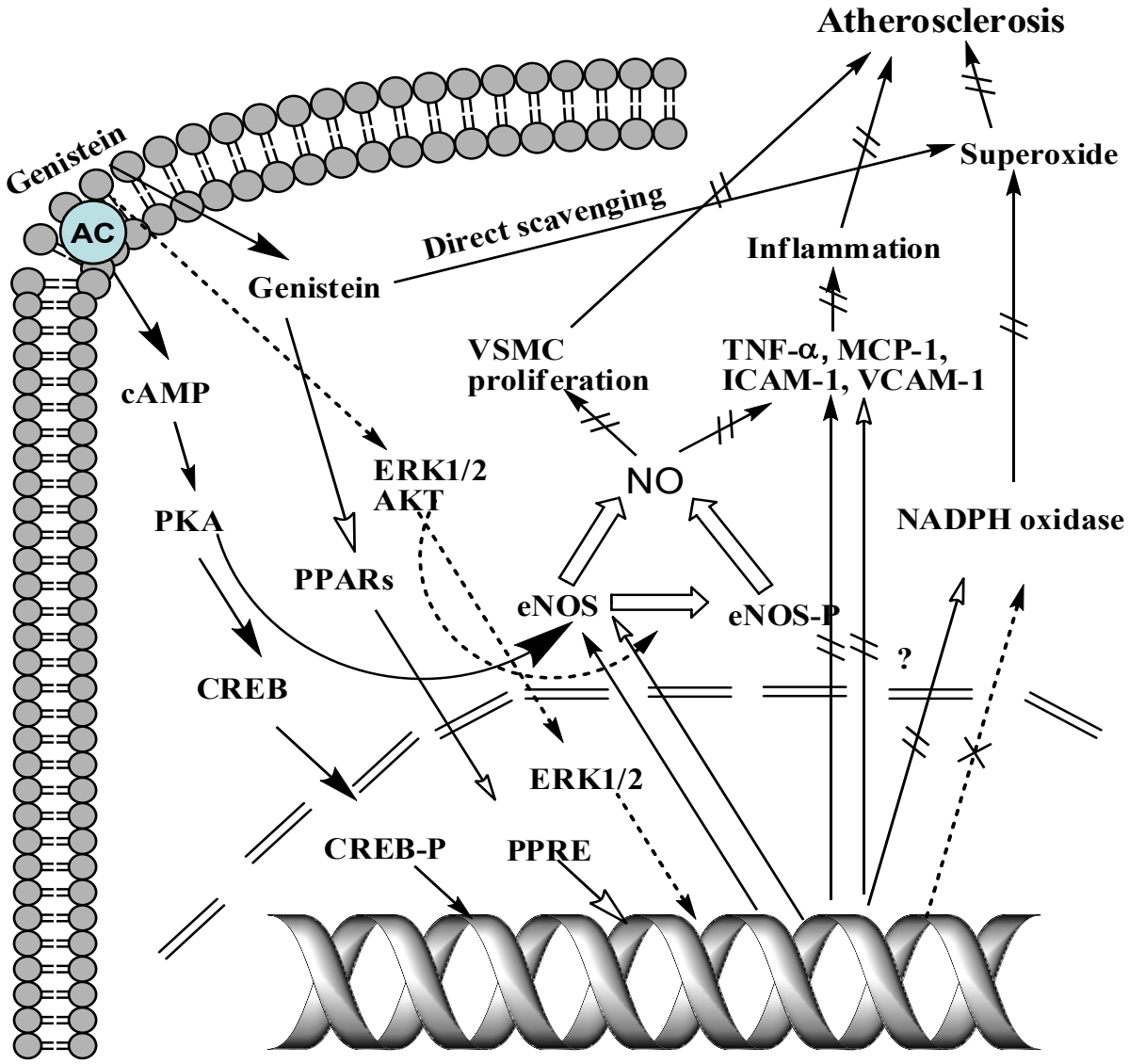

Fig. (4). Scheme summarizing molecular targets for genistein in vasculature and consequent vasculoprotective effect. Genistein activates the cAMP/PKA cascades, ERK1/2 and Akt and PPARs, which subsequently stimulates NO production by direct activation of eNOS and/or stimulation of eNOS expression in ECs. Elevated NO inhibits TNF-α, VCAM-1 and MCP-1 expression and VSMCs proliferation. Activation of PPARγ by genistein also inhibits the expression of NAPDH oxidase, thereby reducing superoxide production. Consequently, modulation of these molecule events by genistein prevents endothelial inflammation and atherosclerosis.

present in free aglycone form in both humans and rodents [207, 208]. Given that achievable plasma total genistein concentrations in both rodents and humans consuming soy products are less than 5 μM [209-211]. Results from many *in vitro* studies were obtained with genistein aglycone concentrations that are likely beyond those that might be achieved through dietary genistein intake. Thus, the physiological relevance of these *in vitro* findings is unclear. While genistein conjugates in the serum are reported to be biologically active with less potency than free genistein [212], their vascular effects are unknown. Studies in this area are needed to identify primary component that is responsible for cardiovascular health benefit of genistein supplement. Regardless, as a molecule capable of activating multiple intracellular molecules or related pathways essential for normal vascular function, genistein may offer an unique structural model and perspective for the design of new analog compounds that can simultaneously and adequately activate these molecular targets, thereby providing novel therapeutic approaches to cardiovascular disorders.

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ABBREVIATIONS

BAECs = Bovine aortic endothelial cells

CaM = Calmodulin

CREB = cAMP-responsive element binding protein

E2 = 17β-estradiol

ECs = Endothelial cells

ERK1/2 = Extracellular signal-regulated kinase 1/2

eNOS = Endothelial nitric oxide synthase

ERs = Estrogen receptors

ET-1 = Endothelin-1

HAECs = Human aortic endothelial cells

Hsp90 = Heat shock protein 90

HUVECs = Human umbilical vein endothelial cells

ICAM-1 = Intercellular adhesion molecule-1

LBD = Ligand binding domain

MCP-1 = Macrophage chemoattractant protein-1

MMPs = Matrix metalloproteinases

NO = Nitric oxide

PKA = Protein kinase A

PPARs = Peroxisome proliferator-activated receptors

PTK = Protein tyrosine kinase

ROS = Reactive oxygen species

TNF-α = Tumor necrosis factor-alpha

VCAM-1 = Vascular cell adhesion molecule-1

VSMCs = Vascular smooth muscle cells

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CHAPTER 3

Genistein, a soy phytoestrogen, up-regulates the expression of human endothelial nitric oxide synthase and lowers blood pressure in spontaneously hypertensive rats [2]

Abstract

Genistein, a soy phytoestrogen, may improve vascular function but the mechanism of this effect is unclear. Endothelial-derived nitric oxide (NO) is a key regulator of vascular tone and atherogenesis. Previous studies have established that estrogen can act directly on vascular endothelial cells to enhance NO synthesis through genomic stimulation of endothelial nitric oxide synthase (eNOS) expression. However, it is unknown whether genistein has a similar effect. We therefore investigated whether genistein directly regulates NO synthesis in primary human aortic endothelial cells (HAEC) and human umbilical vein endothelial cells (HUVEC). Genistein, at physiologically achievable concentrations in individuals consuming soy products, enhanced the expression of eNOS and subsequently elevated NO synthesis in both HAEC and HUVEC, with 1-10 μmol/L genistein inducing the maximal effects. However, the effects of genistein on eNOS and NO were not mediated by activation of estrogen signaling or inhibition of typrosine kinases, two known biological actions of genistein. Genistein (1-10 μmol/L) increased eNOS gene expression (1.8-2.6-fold of control)

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and significantly increased eNOS promoter activity of the human eNOS gene in HAEC and HUVEC, suggesting that genistein activates eNOS transcription. Dietary supplementation of genistein to spontaneously hypertensive rats restored aortic eNOS levels, improved aortic wall thickness, and alleviated hypertension, confirming the biological relevance of the *in vitro* findings. Our data suggest that genistein has direct genomic effects on the vascular wall that are unrelated to its known actions, leading to increase in eNOS expression and NO synthesis, thereby improving hypertension.

KEY WORDS: genistein; nitric oxide synthase; nitric oxide; endothelial cells; blood pressure, spontaneously hypertensive rats.

Introduction

The prevalence of cardiac and other vascular diseases rises in aging population. It is also well recognized that the incidence of cardiovascular disease (CVD)[3] is substantially increased in postmenopausal women due to the loss of estrogen. Experimental and clinical data support vascular protective effects of estrogen by various mechanisms (1). However, administration of estrogen is also associated with an increased incidence of heart disease which limits its therapeutic potential (2). In addition, the use of estrogen as a cardioprotective agent is further limited by carcinogenic effects in women and feminizing effects in men (3). Given the demonstrated risks of conventional estrogen therapy, a search for novel, cost-effective, alternative vasoactive agents for prevention of CVD is of major importance in the effort to decrease the burden of CVD morbidity.

The soy phytoestrogen genistein has drawn wide attention due to its potential healthy benefits in preventing chronic diseases such as CVD (4, 5), obesity (6, 7) and osteoporosis (8). Epidemiological studies show that genistein intake in American postmenopausal women is inversely associated with CVD risk factors (9, 10), supporting a beneficial role for genistein administration to aging individuals. Some human intervention studies suggest the beneficial effects of genistein on atherosclerosis (11), markers of cardiovascular risk (12, 13), vascular motor tone (14), vascular endothelial function (15), and systemic arterial compliance (16). Data from animals and *in vitro* studies also suggest a protective role of genistein in cardiovascular events (17, 18). However, the mechanism of genistein action in vasculature is

still not clear, which hinders our further determining the physiological and pharmacological role of this nutraceutical compound in vascular function. Past studies have extensively explored its hypolipidemic (19), anti-oxidative (20, 21) and the estrogenic effects (22). While genistein may have both estrogen receptors (ER)- dependent and independent actions in vasculature, its average effect on plasma lipid profile is neutral (23). Interestingly, recent studies have shown that the beneficial effects of genistein on endothelial function in postmenopausal women can be blocked by N^G-monomethyl-L-arginine, the inhibitor of endothelial nitric oxide synthase (eNOS) (24, 25). Moreover, genistein restores the nitric oxide (NO)-mediated vascular relaxation in ovariectomized (26) or chronically hypoxic (27) rats. Furthermore, long-term dietary supplementation of genistein elevates the plasma NO concentrations and reduces the plasma endothelin-1 levels in healthy postmenopausal women (15). Given the importance of NO in modulating vascular homeostasis, it is tempting to propose that genistein exerts vasculoprotective effects by regulating NO levels.

Previous studies have established a role for estrogen in the vascular endothelial cells (EC) to enhance NO synthesis through genomic stimulation of eNOS expression (28), and by ERs-mediated, non-genomic eNOS activation (29). We recently demonstrated that genistein acutely stimulates NO production by phosphorylation of eNOS via the cAMP/protein kinase A (PKA) cascade in EC (30, 31). However, it is unknown whether genistein has a similar genomic effect on eNOS. Studies have reported that administration of soy protein improves eNOS expression and subsequently reduces blood pressure in rats (32). However, other studies demonstrated that the beneficial effect of genistein on endothelial function is not through enhancing eNOS expression (33). Although genistein has been shown to enhance

eNOS promoter activity in a transformed human EC (34), it is not clear whether genistein directly up-regulates eNOS expression in primary EC and thereby reduces blood pressure *in vivo*. In the present study, we tested whether genistein improves eNOS expression and subsequently increases NO synthesis in primary human aortic EC (HAEC) and in spontaneously hypertensive rats (SHR), and whether this is associated with a blood pressure-lowering effect of genistein.

Materials and Methods

Materials. Primary HAEC and endothelial growth factors were purchased from Cambrex Bioscience (Rockland, ME); primary human umbilical vein endothelial cells (HUVEC) were obtained from the Cardiovascular Research Cell Culture Core at the University of Iowa; competent cells for plasmid multiplication, M199 media, fetal bovine serum (FBS) and other cell culture supplements were obtained from Invitrogen (Carlsbad, CA); eNOS and β-actin monoclonal antibodies were purchased from Cell Signaling Technology (Beverly, MA); the superSignal chemiluminescence detection system was obtained from Pierce (Rockford, IL); nitrocellulose membranes, SYBR green supermix, cDNA synthesis and protein assay kits were from Bio-Rad (Hercules, CA); human eNOS promoter (-1193/+109) linked to a firefly luciferase reporter gene was kindly provided by Dr. William Sessa at Yale University; plasmid purification and RNeasy Mini kits were from Qiagen (Valencia, CA); primers were synthesized by Integrated DNA Technologies (Coralville, IA); transfection reagents were obtained from Targeting system (Santee, CA); dual luciferase reporter assay kits were obtained from Promega (Madison, WI); nitrite/nitrate fluorometric assay reagents were purchased from Cayman Chemical (Ann Arbor, MI); ICI182,780 was from Tocris (St. Louis, MO); genistein and daidzein were purchased from LC Laboratories (Woburn, MA) and Sigma (St. Louis, MO); 17β-estradiol (E2), protease and phosphatase inhibitor cocktails and other general chemicals were obtained from Sigma (St. Louis, MO). Stock solutions of

genistein, daidzein or E2, at 20 mmol/L in dimethylsulfoxide (DMSO), were stored at -80^{o}C before use.

Cell culture. HAEC were cultured in M199 medium containing 2% FBS and endothelial growth supplements-EGM2 and HUVEC were cultured in 20% FBS M199 medium at 37^{o}C in a 5% CO2/95% air environment. The medium was changed every other day until the cells became confluent. HAEC and HUVEC were passaged by using 0.05% trypsin and passages 4–6 were used in all experiments.

Animals and Diets. 4-wk old male, spontaneously hypertensive rats (SHR) and Wistar-Kyoto rats (WKY) were purchased from Harlan Inc.(Indianapolis, IN). Rats were housed in a room maintained on a 12h light/dark cycle under constant temperature (22–25°C) with free access to food and water. The protocol of this study was reviewed and approved by the Institutional Animal Care and Use Committee At Virginia Polytechnic Institute and State University. After an initial acclimation period, SHR were randomly divided into 6 groups and were fed a basal soy-free AIN-76A diet (35) containing genistein at 0, 0.2, 0.5, or 2.0 g /kg diet for 19 wk. WKY were fed the basal AIN-76A diet for the same period. To determine whether genistein can improve established hypertension, adult SHR with overt hypertension (20 wk old) were randomly divided into 2 groups and fed either 0 or 2.0 g genistein /kg diet until their blood pressure was significantly lowered. Then, both groups of rats were fed the same basal diets for 6 wk.

Plasma genistein measurements. On the last day of the study, blood samples were drawn 30 min after food intake from the retrobulbar plexus through heparinized capillary tubes. Plasma

was collected by centrifugation at 16,000 x g for 15 min. An aliquot of 250 μL serum per sample was used for extraction of genistein using a previously described method (36). Genistein in the extracted samples was determined by using the HPLC system (Waters2695) with a Luna Phenyl-hexyl column (5 μ C_{18} 100 R) (36).

Blood pressure, heart rate, body weight, and food intake measurements. Every other week, rat blood pressure and heart rate were determined after a warming period using the Kent CODA 2 series computerized non-invasive blood pressure system (Kent Scientific, Litchfield, CT) as described (37). During these measurements, rats were under 0.8% isoflurane anesthesia, which had no effect on blood pressure as determined in our preliminary study. The digital values for the systolic, diastolic blood pressure and heart rate were recorded. Readings were taken for 20 cycles from each rat with the highest and the lowest values excluded. To minimize stress-induced variations in blood pressure, all measurements were taken by the same person in the same peaceful environment. Body weight and feed intake were recorded weekly throughout the study to determine whether genistein has any effect on these parameters.

Measurement of aortic wall thickness. The rats were killed using CO_2 and segments of thoracic aorta were fixed in 10% neutral buffered formalin solution for 24 h. Aorta segments were then embedded in frozen embedding media, cut into 5μm section, and stained with Verhoeff's Van Gieson, which specifically stains elastic tissue fibers. Stained sections were photographed by a computer-operated Olympus BH-2 photomicroscope. The wall thicknesses

of aorta were measured using Image-pro plus system (Media Cyberretic, Inc.). Ten measurements were performed for each sample, and the average value was used as the thickness of the sample.

NO Measurement. To investigate the effect of genistein on NO release *in vitro*, confluent cells grown in 12-well plates were treated with genistein, vehicle (DMSO) or other agents in complete medium, over a range of concentrations and time points, as indicated in the figure legends. For assays focused on the effect of prolonged incubation with genistein, culture media were renewed in the third day from the initial treatment. In some experiments, cells were pretreated with ICI 182,780 (1 μmol/L), a highly specific inhibitor of ERs, for 30 min before addition of agonists. Following treatment, cells were adapted into Hank's balanced salts solution (HBSS; 135 mmol/L NaCl, 1.2 mmol/L $CaCl_2$, 1.2 mmol/L $MgCl_2$ 1.2, 5 mmol/L KOH, 10 mmol/L HEPES, 10 mmol/L glucose, pH 7.4) supplemented with L-arginine (0.1 mmol/L) for 30 min, followed by stimulation with 10 μmol/L A23187 for 30 min. Culture supernatants were then collected for NO assay as determined by measuring the sum concentration of NO_2^- and NO_3^- using a fluorometric assay kit following the manufacturer's instructions. Briefly, cell supernatants were treated with NO_3^- reductase for 30 min at room temperature to reduce NO_3^- to NO_2^-, which then reacted with 2,3-diaminonaphthalene for 10 min to yield the fluorescent product 1(H)-naphthotriazole. Fluorescence was measured in a fluorescence microplate reader (Bio-Tek, Winooski, VT) with excitation and emission wavelengths of 365 and 450 nm, respectively. Fluorescence data were converted into concentrations based on standard curves constructed with $NaNO_3$,

normalized to protein concentration of the samples, and then expressed as folds of vehicle-treated controls.

Immunoblot analysis. Following experimental treatments, EC or aortic vessels from rats were harvested in lysis buffer and performed immunoblot analysis as previous described (30, 31) . The tissues were sonicated (EC) or homogenized with a Rotor–stator homogenizer (aorta) and then centrifuged at 10,000 × g for 5 min. Protein levels of the extracts were measured using a Bio-Rad assay kit. Equal amounts of protein from cell extracts were subjected to immunoblot. Membranes were probed with antibody against eNOS. The immunoreactive proteins were detected by chemiluminescence. Nitrocellulose membranes were stripped and re-probed with β-actin. The protein bands were digitally imaged for densitometric quantitation with a software program (Gene tools, Synoptics Ltd. UK). eNOS protein level was normalized to β-actin expression from the same sample.

Quantitative real-time PCR analysis. Total RNA from genistein- or vehicle-treated HAEC was isolated using the RNeasy Mini Kit following the manufacturer's protocol. Then, 0.5 μg of total RNA from each sample was reverse transcribed to cDNA using the iScript cDNA synthesis kit. eNOS was amplified on an iCycler IQ real-time quantitative PCR system using iQ SYBR Green supermix with β-actin as an internal control. A melting curve analysis was performed on each sample to verify that no non-specific products were synthesized. The reaction mixtures contained 100 nmol/L primers, 50 ng cDNA, and 12.5μL iQ SYBR Green supermix (0.2 mmol/L of each dNTP, 25 units/mL iTaq DNA polymerase, SYBR Green I, 10 nmol/L fluorescein, 3 mmol/L $MgCl_2$, 50 mmol/L KCl, and 20 mmol/L Tris-HCl) as

described previously (38). The primers used in quantitative real-time RT-PCR were eNOS (forward: 5'-GACATTGAGAG CAAAGGGCTGC-3'; reverse: 5'-CGGCTTGTCACCTCCTGG-3'), and β-actin (forward: 5'-CATGCCATCCTGCGTCTGGA-3', reverse: 3'-CCGTGGCCATCTCTTGCTCG-5') (39). The eNOS mRNA level was normalized to that of β-actin, and expressed as folds of control.

eNOS promoter activity assay. A reporter plasmid containing a human eNOS promoter (-1193/+109) linked to a firefly luciferase reporter gene (eNOS-Luc) was amplified with competent cells and purified using Qiagen's Maxi kit according to the manufacturer's instructions. For transient transfection of the plasmids, EC were grown in 24-well plates until 50-70% confluence. The cells were then co-transfected with 1.2 μg of eNOS-Luc and 0.5 ng of pRL reporter 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 phenol-red free M199 medium containing 2% FBS for 24 h. Treated 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 as described (40).

Statistical analysis. Data was analyzed with one-way, or two-way ANOVA where designated, using the SAS® program. Data are expressed as the mean±SE. For the time course study, initial values (d1) from vehicle-treated cells were set as the control. Treatment and time point

differences, as well as interaction between genistein and other agents if significant, were subjected to Tukey's multiple comparison tests, where $p < 0.05$ was considered significant.

Results

Genistein enhances NO synthesis in HAEC. We first examined whether long-term exposure of genistein stimulates NO synthesis in HAEC. Genistein significantly stimulated NO synthesis following 5 d of incubation (Fig. 1*A*). The effect of genistein was concentration-dependent, with genistein concentrations of ≥ 1 μmol/L inducing significant NO production. The time-course study showed that genistein (5 μmol/L)-stimulated NO production was significantly increased after 3 d of exposure to genistein, with about 1.1 fold increase at 5 d compared to that at 1 d of incubation with genistein (Fig. 1*B*).

Genistein-induced NO production is independent of ER and protein tyrosine kinase (PTK). Genistein has weak estrogenic effects in some tissues by binding to ER (41). In addition, previous studies have shown that E2 also can stimulate NO production in human EC (28). However, incubation of the cells with excess amounts of the ER antagonist ICI 182,780 did not block genistein-induced NO release (Table 1). The activity of ICI 182,780 used in this study was validated through blocking the cytoprotective effect of E2 in our recent study (40). In addition, while genistein enhanced NO synthesis as expected, chronic exposure of EC to E2 (10 nmol/L) did not stimulate NO production in HAEC (Table 1). These results suggest that the effect of genistein on NO production in EC is independent of the estrogen signaling mechanism.

To evaluate whether genistein enhances NO production through inhibition of PTK, we compared the effect of genistein with that of daidzein, an analogue of genistein that is

inactive for PTK inhibition, on NO production. Daidzein was as potent as genistein in stimulation of NO production (Table 1). However, there was no additive effect between genistein and daidzein, suggesting that two molecules may act through the same mechanisms in stimulation of NO production.

Genistein enhances eNOS protein through up-regulating mRNA transcription in HAEC. Genistein increased eNOS protein levels, with 1 μmol/L genistein inducing a significant effect, although the maximal effect of genistein on eNOS protein expression was achieved at 10 μmol/L concentration (1.5 fold of control) (Fig.2*A*). These results are consistent with the effect of genistein on NO production (Fig. 1*A*), suggesting that the elevated NO production by genistein may be attributable to an increase in eNOS protein expression. To investigate whether genistein elevates eNOS protein level via a transcriptional mechanism, we first tested whether genistein had an effect on eNOS mRNA expression in HAEC by using quantitative real-time PCR. Exposure of HAEC to various concentrations of genistein for 5 d, the same duration used to study genistein-induced eNOS protein expression and NO production, increased eNOS mRNA levels to 2.6 fold of control at 10 μmol/L genistein (Fig. 2*B*), consistent with its effect on eNOS protein expression and NO production. This result suggests that genistein may regulate eNOS expression at the transcriptional level. To confirm this, HAEC were transfected with a human eNOS promoter-driven reporter gene, followed by stimulation with genistein. Genistein significantly elevated human eNOS promoter activity to about 1.8-fold of control at 10 μmol/L (Fig. 2*C*), consistent with its effect on eNOS

expression and NO synthesis. However, E2 (10 nmol/L), which failed to enhance NO production, also had no effect on the eNOS promoter activity in HAEC (data not shown).

Genistein increases NO production, eNOS protein expression and promoter activity in HUVEC. To determine whether genistein has a similar effect on another type of EC, we performed this study with HUVEC. The results demonstrated that genistein as low as 10 nmol/L induced NO production (Fig. 3*A*) and eNOS expression (Fig. 3*B*) in HUVEC, with a maximal effect at 1-10 μmol/L genistein. We further transfected the eNOS promoter-driven luciferase gene constructs in HUVEC. Genistein stimulated the eNOS promoter activity with a maximal effect at 1-10 μmol/L in HUVEC (Fig. 3*C*), confirming a transcriptional effect of genistein in HAEC.

***In vivo* effects of genistein.** To confirm *in vivo* the importance of the genomic effects of genistein on eNOS, we tested whether dietary supplementation of genistein can improve eNOS expression and reduce blood pressure in SHR, a widely used hypertension animal model, given that the eNOS/NO signaling is critical for maintaining vascular tone. As expected, dietary supplementation of genistein significantly elevated plasma genistein levels. Under our experimental conditions, plasma genistein levels in rats fed 0, 0.2, 0.5, 2.0 g/kg diet of genistein were 0, 1.20±0.03, 1.90±0.20, 5.05±0.49 μmol/L, respectively, which overlap the concentrations used in our *in vitro* studies and attainable plasma levels in humans (0.74-6.0 μmol/L) following consumption of soy products or isoflavones as dietary

supplements (42, 43). Genistein treatment significantly reduced both the elevated systolic and diastolic blood pressures in SHR (Table 2), whereas heart rate was not altered by dietary supplementation of genistein (data not shown). In addition, we found that dietary supplementation of genistein for 6 wk lowered blood pressure in adult SHR after the onset of hypertension. Impressively, this blood pressure-lowering effect of genistein was still significant at 6 wk after genistein withdrawal from the diet (Fig. 4 *A*). Genistein had no effect on body weight and food intake throughout the experimental period (data no shown). Furthermore, we found that aortic wall thickness was significantly greater in SHR than in WKY (Fig. 4 *B*), confirming previous study showing that the higher blood pressure is associated with the increased aortic wall thickness (44). However, genistein administration significantly decreased aortic wall thickness in SHR (Fig. 4 *B*). Previous studies have reported that eNOS protein expression was significantly reduced in SHR which led to hypertension in these animals (45, 46). To examine whether genistein has an effect on eNOS in these animals, as a possible explanation of its blood pressure-lowering effect, we measured the eNOS protein expression in aortic vessels by Western blotting. Our results showed that dietary intake of genistein restored eNOS protein content in the vasculature of SHR, with doses of 0.5-2.0 g/kg diet inducing eNOS expression similar to that in WKY (Fig.4 *C*), suggesting that genistein administration likely reduces hypertension via a modulation of eNOS expression.

Discussion

Vascular EC, which not only serve as a biological barrier separating circulating blood and peripheral tissues, but also secrete various vasoactive substances, play a pivotal role in maintaining normal vascular function. Therefore, a major goal of our study was to determine whether genistein has a direct effect on vascular EC and thereby provide the molecular mechanisms by which genistein exerts some beneficial effects on the vasculature. We have demonstrated that, genistein, at physiologically achievable concentrations, activates eNOS transcription, leading to eNOS synthesis and NO production in human primary vascular EC. We further showed that this genistein effect on eNOS is present *in vivo*, confirming the biological relevance of the *in vitro* findings. Endothelium-derived NO is not only a potent vasodilator but also possesses anti-inflammatory (47), anti-atherogenic (48), anti-thrombotic (49), and anti-apoptotic (50) properties. Consistent with the key role of NO in vascular function, dietary administration of genistein lowered blood pressure in hypertensive rats. Recent studies reported that postmenopausal women taking genistein for 6 months have increased plasma levels of nitrate and nitrite, the stable metabolites of NO, and enhanced flow-mediated vasodilation in the forearm (51). Our finding that genistein directly targets EC to regulate eNOS is therefore important, since it may provide a molecular explanation for some vascular protective effects observed in animal and human studies (32, 51).

Genistein is considered as a specific ERß agonist since it binds to ERß with an affinity comparable to that of E2 but has a considerably lower affinity for ERα (52). Studies showed that E2 may regulate the transcription of eNOS in an ER-dependent manner in these cells (53,

54). However, our data indicate that genistein regulation of eNOS and NO was independent of ERs. First, the specific ER antagonist ICI 182,780 did not inhibit the effect of genistein on eNOS activation. Second, while E2 potentiated the effect of genistein on NO production, it had no effect on NO and eNOS promoter activity in HAEC. Third, daidzein, an analogue of genistein that is essentially lack of affinity for ERs (52), also induced an increase in NO production similar to that caused by genistein in HAEC. Thus, the transcriptional effect of genistein on eNOS is independent of this classical estrogen signaling mechanism. In line with our finding, a recent study showed that the effect of genistein on eNOS promoter activity is not mediated through ERs in transformed vascular cells (34). In addition, accumulating evidence indicates that genistein exerts various vascular effects that are ER-independent (31, 55). While both ERs are present in vascular EC, the role for ERβ in vascular function remains to be investigated. Some studies indicated that the effect of E2 on NO is mediated through ERα but not ERβ (56), providing a possible explanation for an ER-independent effect of genistein on NO, given that genistein only has about 4% affinity to ERα compared with E2 (52). Recently, an estrogen-related receptor α1 (ERRα1), a member of the steroid/thyroid hormone receptor superfamily expressed in EC, was reported to up-regulate eNOS promoter and protein expression in EC that was not related to ERs (57). Interestingly, this ERRα1-mediated eNOS expression pattern is similar to that observed in genistein-treated EC. It is therefore compelling to investigate whether genistein regulates eNOS through this estrogen-related signaling pathway.

Previous studies established that phosphoinositol-3-kinase/Akt (PI3K/Akt) and ERK-mitogen activated protein kinases (ERK/MPAK)-mediated pathways are two important

signaling cascades mediating eNOS activation by many stimuli in vascular EC (58, 59). However, activation of these signaling pathways only leads to acute eNOS activation without an increase in protein expression, suggesting that genistein-induced eNOS expression is unlikely related to PI3K/Akt or ERK/MAPK activity. Indeed, pharmacological inhibition of these pathways had no effect on genistein-stimulated eNOS and NO (data not shown). Cyclic AMP responsive element (CRE) sites are present within neuronal NO synthase (nNOS), which regulate nNOS gene expression through binding with CRE binding protein (CREB) (60). A recent study reported that eNOS also contains CRE sites through which the cAMP signaling regulates eNOS transcription (61). We recently found that genistein directly activates the cAMP signaling system and regulates CRE-mediated gene expression in primary vascular EC (31). Our unpublished results showed that genistein dose-dependently increased CREB phosphorylation in HAEC, which is required to activate transcription of target genes, and this effect was abolished by H89, an inhibitor of PKA. Thus, it is conceivable that genistein may, at least in part, up-regulate eNOS expression via activation of cAMP signaling, which is an ongoing area of investigation in this laboratory.

We have shown that dietary administration of genistein reduced the thickness of the wall of the aorta and improved arterial blood pressure in SHR, a widely used animal model for the study of human hypertension, as these rats spontaneously develop the metabolic features similar to the pathogenesis of human hypertension (62). Our study also showed that genistein had no effect on heart rate, food intake and body weight, suggesting that the beneficial effect on blood pressure is not due to alteration of these parameters. Our further animal studies demonstrated that genistein also can improve blood pressure in adult SHR with well-

developed hypertension, suggesting a possibly therapeutic potential of genistein for hypertension. Remarkably, after 6 weeks of genistein withdrawal, the blood pressure in genistein-fed SHR was still significantly lower than that in control SHR. Previous studies demonstrated that eNOS expression is reduced in SHR compared to that of normal rats (45, 63) which was further confirmed in this study. However, dietary supplementation of genistein restored eNOS levels in aortic vessels isolated from these rats, suggesting that the reduced eNOS expression contributes to the increased blood pressure in SHR, given the important role of eNOS in regulating vascular homeostasis. These outcomes are consistent with previous studies showing that genistein increases eNOS in rat aorta, liver (32) and heart (64). While it is presently unknown how genistein affects *in vivo* eNOS expression, the evidence from our *in vitro* study suggests that genistein may induce eNOS protein expression by directly targeting the vascular wall.

Progressive arterial hypertrophy is an important component of vasculature adaptation to the elevated arterial pressure. It has been found in the present study that the thickness of arterial wall is significantly greater in SHR than in WKY, consistent with previous observations (44). However, genistein administration significantly decreased aortic wall thickness in SHR. Recent studies showed that genistein inhibits the proliferation of vascular smooth muscle cells (VSMC) isolated from SHR, suggesting that genistein may have a direct effect on VSMC in vessel wall, though this effect was obtained only at pharmacological doses of genistein (65). It has been established that eNOS-derived NO inhibits VSMC cell growth (66), and our *in vitro* and *in vivo* data indicated that genistein has a direct genomic effect on eNOS expression, it is therefore intriguing to speculate that a secondary action

whereby genistein enhances eNOS may contribute to the overall inhibitory effect of genistein on VSMC growth, and thereby improves blood pressure. This aspect however, needs further investigation.

In summary, this study demonstrates for the first time to our knowledge, that genistein can enhance eNOS gene transcription and protein synthesis in primary human vascular EC, leading to NO production. Dietary genistein administration stimulated eNOS expression, improved vessel wall thickening, and alleviated hypertension in SHR, confirming the biological relevance of the *in vitro* findings. These findings potentially provide a basic mechanism underlying the physiological effects of genistein in the vasculature.

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ABBREVIATIONS

CRE, cAMP-responsive element; CREB, cAMP-responsive element binding protein; CVD, cardiovascular disease; DMSO, dimethylsulfoxide; E2, 17β-estradiol; EC, endothelial cells; eNOS, endothelial nitric oxide synthase; ERK/MAPK, ERK-mitogen activated protein kinase; ERRα1, estrogen-related receptor α1; ER, estrogen receptors; FBS, fetal bovine serum; HAEC, human aortic endothelial cells; HBSS, Hank's balanced salts solution; HUVEC, human umbilical vein endothelial cells; nNOS, neuronal nitric oxide synthase; NO, nitric

oxide; PI3K/AKT, phosphoinositol-3 kinase/AKT; PKA, protein kinase A; PTK, protein tyrosine kinase; SHR, spontaneously hypertensive rats; VSMC, vascular smooth muscle cells; WKY, Wistar-Kyoto rats.

Tables

TABLE 1 Genistein-stimulated NO production is independent of ER and PTK in HAEC [1]

	Treatments				2-Way ANOVA, P values		
	C	G	X[2]	G+X	G	X	G+X
I	1±0.0[b]	1.80±0.11[a]	1.14±0.03[b]	1.72±0.14[a]	0.0003	0.5673	0.0004
E2	1±0.0[b]	1.77±0.17[a]	0.95±0.08[b]	2.31±0.21[a]	0.0021	0.995	0.0001
D	1±0.0[b]	1.73±0.16[a]	1.67±0.18[a]	1.74±0.08[a]	0.0016	0.0009	0.0009

[1] NO production in confluent HAEC stimulated with vehicle (C) or genistein (G, 5 μmol/L) in the presence or absence of ICI 182,780 (I, 1 μmol/L), 17β-estrodial (E2, 10 nmol/L), or daidzein (D, 5 μmol/L) for 5 d. Values are mean±SE from four separate experiments and expressed as fold of the control. Means without a common letter differ, $P<0.05$.

[2] X=I, E2 or D.

TABLE 2 Dietary supplementation of genistein lowered blood pressure in SHR[1]

	WKY	SHR			
Genistein, g/kg	0	0	0.2	0.5	2
Systolic, mmHg	146.4±4.7[c]	217.1±3.9[a]	200.8±3.2[b]	196.0±5.6[b]	188.1±3.9[b]
Diastolic, mmHg	97.6±2.6[d]	155.1±3.3[a]	149.8±2.4[a,b]	142.4±2.7[b,c]	140.4±4.0[c]

[1] Blood pressure in rats fed a basal or genistein diet for 19 wk. Values are means ± SE, n=8 rats. Means without a common letter differ. P<0.05.

Figures

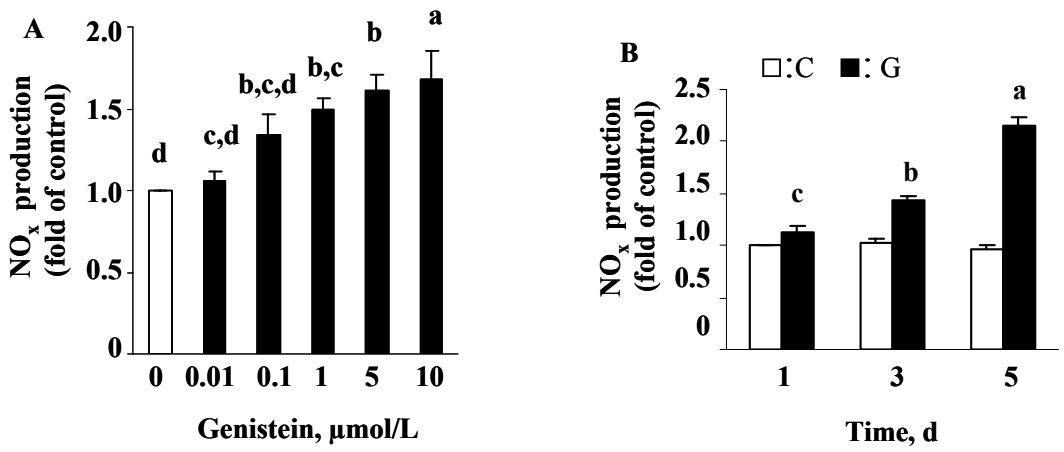

FIGURE 1 NO production in confluent HAEC incubated with various concentrations (0.01-10 µmol/L) of genistein or vehicle (DMSO) for 5 d (*A*), or with 5 µmol/L genistein for different times (*B*). Nitrite/nitrate (NO_x) secreted was measured and at baseline was 0.074±0.003 µmol/L. Values are mean±SE from four separate experiments and expressed as fold of the control. Bars without a common letter differ, *P*<0.05.

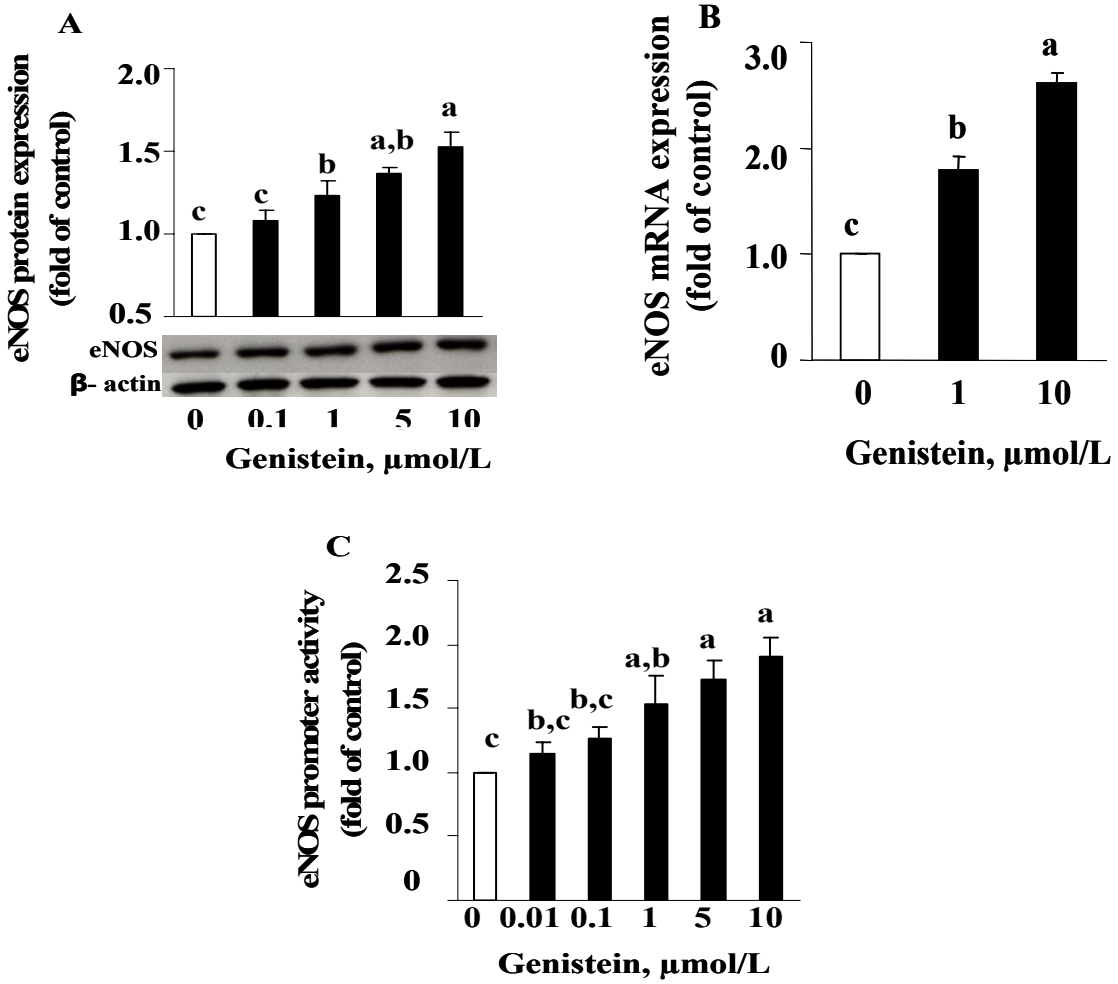

FIGURE 2 *A*. eNOS protein (*A*) or mRNA (*B*) expression normalized to β-actin content in HAEC treated with various concentrations of genistein or vehicle for 5 d; *C*. eNOS promoter activity in transfected HAEC stimulated with genistein or vehicle for 24 h. Values are mean±SE from three separate experiments and expressed as fold of the control. Means without a common letter differ, $P<0.05$.

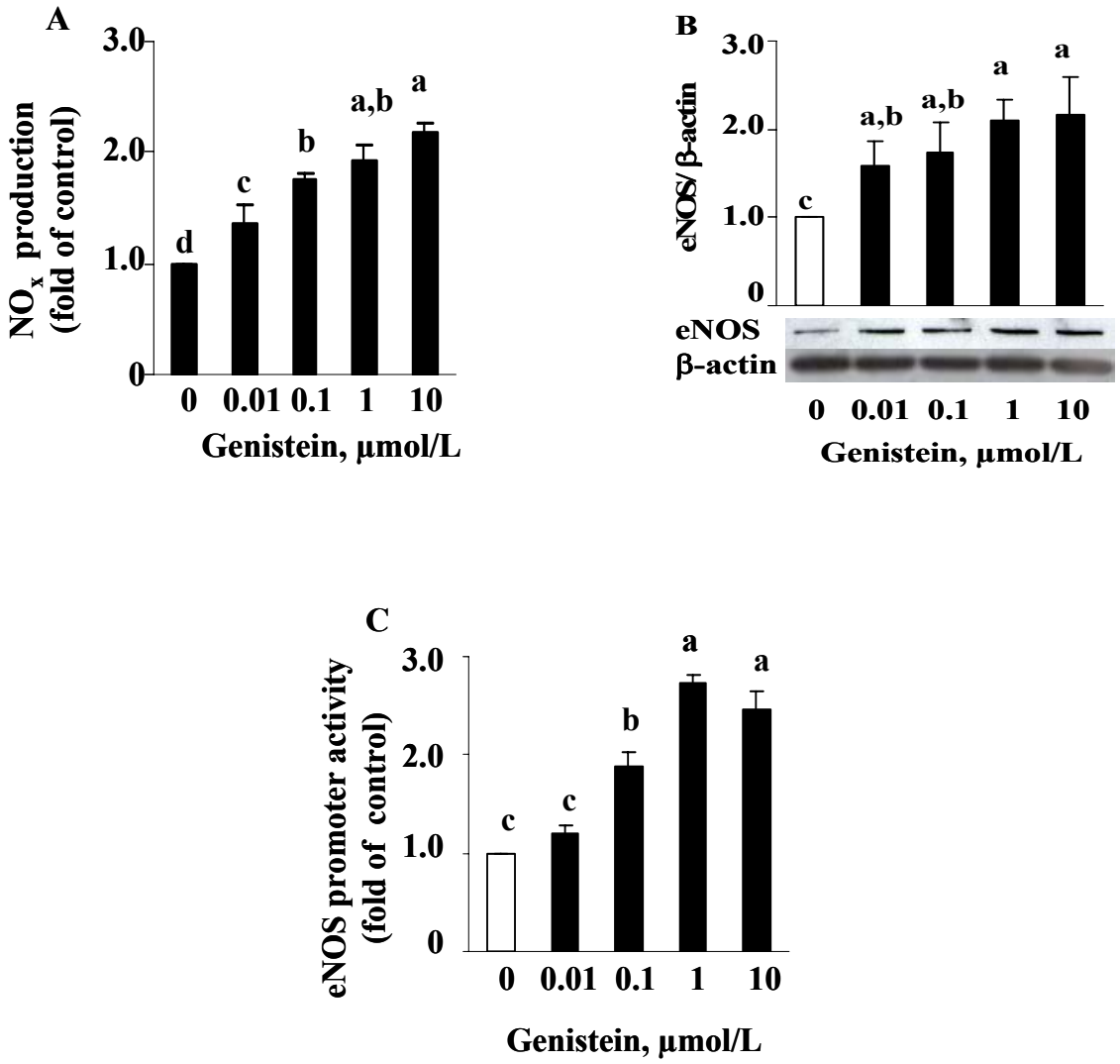

FIGURE 3 NO production in the supernatants (*A*) and eNOS protein expression normalized to β-actin content (*B*) in HUVEC treated with genistein or vehicle for 48 h. *C*. eNOS promoter activity in transfected HUVEC stimulated with genistein or vehicle for 24h. Values are mean±SE from four separate experiments and expressed as fold of the control. Means without a common letter differ, $P<0.05$.

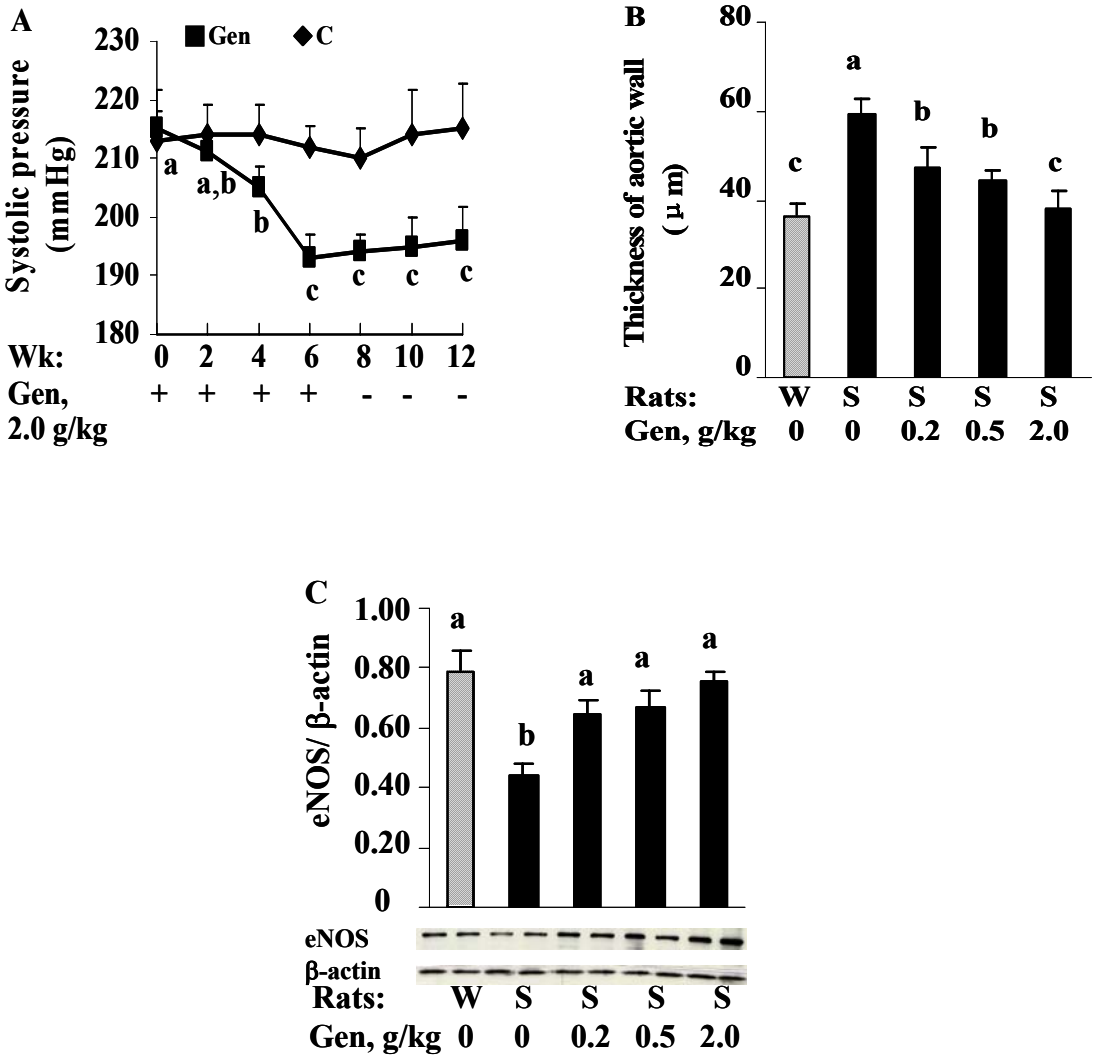

FIGURE 4 *A*. Systolic blood pressure in adult SHR fed a basal or genistein (Gen) diet for 6 wk followed by a genistein-free diet for additional 6 wk. Aortic wall thickness (*B*) and eNOS protein normalized to β-actin content (*C*) in WKY (W) and SHR (S) fed a basal or genistein (Gen) diet for 19 wk. Data are mean±SE (n=8 rats). Values without a common letter differ, $P<0.05$.

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CHAPTER 4

Phytoestrogen genistein up-regulates human endothelial nitric oxide synthase expression through activation of protein kinase A

Short Title: Genistein and eNOS regulation

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Abstract

We previously reported that genistein, a phytoestrogen, up-regulates endothelial nitric oxide synthase (eNOS) expression and consequently improves nitric oxide (NO) release in human vascular endothelial cells (ECs), an effect that is not related to its potential estrogenic action. In the present study, we further investigated the underlying mechanism by which genistein modulates eNOS expression in vascular ECs. Genistein enhanced protein expression of eNOS and subsequently elevated NO synthesis in human aortic ECs (HAEC). Inhibition of the mitogen-activated protein kinase, phosphatidylinositol 3-OH kinase/Akt kinase or protein kinase C had no effect on the enhanced NO synthesis by genistein. However, adenoviral transfer of the specific endogenous PKA inhibitor gene completely abolished PKA activity and the genistein-stimulated eNOS expression and NO production, suggesting that genistein acts through a PKA-dependent pathway. Furthermore, genistein dose-dependently augmented PKA activity and subsequently activated the phosphorylation of CREB at ser-133. These findings provide the first evidence that genistein induces eNOS protein expression specifically via the activation of the PKA/CREB-mediated mechanism that is not dependent on its potential estrogenic effect in vascular ECs. These data, along with our previous findings, reveal an important signaling pathway in vascular ECs that is activated by genistein and suggest that this plant-derived compound may play a beneficial role in vascular function through targeting the cAMP/PKA/CREB/eNOS/NO signaling.

Key Words: genistein; eNOS; cAMP; protein kinase A; protein kinase inhibitor; endothelial cells; CREB.

Introduction

Endothelial-derived nitric oxide (NO), synthesized by endothelial NO synthase (eNOS) from amino acid L-arginine and molecular oxygen, plays a pivotal role in maintaining vascular homeostasis. Attenuation of the activity and/or expression of eNOS are directly associated with various cardiovascular events, including hypertension (1, 2), atherosclerosis (3) and stroke (4).

Genistein, a major soy isoflavone, has received wide attention because of its potential beneficial effects on various human degenerative diseases such as cardiovascular disease. Data from human intervention studies suggest the beneficial effects of genistein on vascular motor tone (5, 6), systemic arterial compliance (7) atherosclerosis (8) and markers of cardiovascular risk (9, 10). Accumulating studies show that genistein increases circulating NO levels in healthy postmenopausal women (11) and animals (12, 13), although the primary source of this increased NO release in these in vivo studies is not clear. We (14, 15) and others (16) recently demonstrated that genistein may act directly on vascular endothelial cells (ECs) to enhance eNOS activity and expression which consequently induces NO synthesis and release. Further, data from our animal studies showed that genistein enhanced eNOS expression in spontaneously hypertensive rats, consistent with findings in a previous study (17). While estrogen has been shown to regulate eNOS expression both in cultured ECs and in vivo (18, 19) and genistein has weak estrogenic effect which was presumed in many previous studies as a mechanism that mediate various genistein effect, our recent studies provided evidence that the genistein effect on human eNOS expression is not dependent on

the estrogen-related signaling mechanism (15). Therefore, how genistein regulates eNOS and NO is unknown.

Recently, we demonstrated that genistein targets the cAMP signaling pathway and regulates cAMP-regulated gene expression that is not related to its estrogenic effect or inhibition of protein tyrosine kinase (PTK) in vascular ECs (20). Genistein also has been shown to increase intracellular accumulation of cAMP in other tissues including pancreatic beta-cells (21), airway epithelial cells (22) and cardiomyocytes (23), suggesting that genistein possibly influences a wide spectrum of cAMP-mediated biological activities. Cyclic AMP is a central signaling molecule in a variety of cellular systems and plays an important role in maintaining normal vascular function. Various important biological events elicited by the cAMP/PKA signaling is mediated through activation of cAMP-responsive element binding protein (CREB), a transcriptional factor primarily mediating cAMP-regulated gene transcription by binding to cAMP responsive element (CRE) within the genes. Interestingly, recent studies showed that eNOS gene contains CRE sites within its promoter region, suggesting that eNOS expression may be directly regulated by CREB (24). Actually, it has been found that activation of PKA improved eNOS expression in vivo (25). In the present study, we tested the hypothesis that genistein may regulate eNOS expression through the PKA-dependent activation of CREB in ECs**.** We found that genistein improves eNOS expression through a mechanism that is not related to protein kinase C (PKC), phosphatidylionsitol-3 kinase (PI3K) or extracellular signal-regulated kinases in human aortic endothelial cells (HAECs). However, genistein stimulates PKA activity and subsequently activates the phosphorylation of CREB in HAECs. We further provided evidence through

molecular intervention studies that induction of eNOS expression by genistein is dependent on activation of PKA.

Material and Methods

Materials Primary human aortic endothelial cells (HAEC) and endothelial growth supplements (EGM2) were purchased from Cambrex Bioscience (Rockland, ME); M199 media was obtained from Invitrogen (Carlsbad, CA); PepTag® assay for non-radioactive detection of cAMP-dependent protein kinase A was from Promega (Madison, WI); antibodies for eNOS, phospho-CREB, CREB and β-actin were from Cell Signaling Technology (Beverly, MA); nitrocellulose membranes and protein assay kits were from Bio-Rad (Hercules, CA); genistein, protease and phosphatase inhibitor cocktails, H89, P3115, PD98059, LY294002 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.

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Cell culture Primary HAECs were cultured in M199 medium containing 2% FBS and endothelial growth supplements-EGM2 at 37◦C in a 5% CO2/95% air environment. The medium was changed every other day until the cells became confluent. HAECs were passaged after 0.05% trypsin treatment and passages 4–6 were used in all experiments.

Western blot analysis Equal amounts of protein from cell extracts were subjected to Western blot analysis as described previously (20, 26). Membranes were probed with antibody against phospho-CREB or eNOS. The immunoreactive proteins were detected by chemiluminescence. Nitrocellulose membranes were stripped and reprobed with CREB or β-actin in the case of phospho-CREB or eNOS. The protein bands were digitally imaged for densitometric

quantitation with a software program (Gene tools, Synoptics Ltd. UK). eNOS and phospho-CREB expression was normalized to that of β-actin and CREB respectively from the same sample, and expressed as folds of vehicle-treated controls.

NO Measurement To investigate the effect of genistein on NO release *in vitro*, confluent cells grown in 12-well plates were treated with genistein, vehicle (DMSO) or other agents in complete medium, over a range of concentrations and time points, as indicated in the figure legends. For assays focused on the effect of prolonged incubation with genistein, culture media were renewed in the third day from the initial treatment. Following treatment, cells were adapted into Hank's balanced salts solution (HBSS; 135 mM NaCl, 1.2 mM $CaCl_2$, 1.2 mM $MgCl_2$ 1.2, 5 mM KOH, 10 mM HEPES, 10 mM glucose, pH 7.4) supplemented with L-arginine (0.1 mM) for 30 min, followed by stimulation with 10 μM A23187 for 30 min. Culture supernatants were then collected for NO assay as determined by measuring the sum concentration of NO_2^- and NO_3^- as we previously described (14). Fluorescence data were converted into concentrations based on standard curves constructed with $NaNO_3$, normalized to protein concentration of the samples, and then expressed as folds of vehicle-treated controls.

Adenoviral PKA inhibitor gene construct and infection Replication-deficient adenovirus containing the complete sequence of endogenous PKA inhibitor cDNA (AdPKI) was constructed as originally described by Lum et al. (27). For determining infection efficiency,

HAECs were exposed to adenovirus at 100-1000 multiplicities of infection (MOI) per cell in 0.15 ml of serum-free M199 medium for 1h at 37 ^{o}C and then cultured in complete medium for 24 h at 37 ^{o}C. Heat-inactivated AdPKI (65°C overnight) served as the control. After infection, the cells were collected for PKA activity assay as described below. For eNOS and NO analysis, HAECs were infected with AdPKI or heat-inactivated AdPKI at 1000 MOI/cells for 24 h, and then treated with 1-10 μM genistein or vehicle for 5 d, followed by eNOS and NO assays.

PKA activity assay HAECs or AdPKI-infected HAECs treated with genistein or vehicle (DMSO) were collected in PKA extraction buffer [25mM Tris-HCl, 0.5mM EDTA, 0.5mM EGTA, 10mM β-mercaptoethanol, 1μg/ml leupeptin,1μg/ml aprotinin and 5 mM PMSF, pH 7.4]. Cytoplasmic proteins were harvested by sonication and centrifugation. The enzymatic activity of PKA in cell extracts was determined by measuring the phosphorylation of kemptide as previously described (20). Phosphorylated kemptide was separated from unphosphorylated substrate on a 0.8% agarose gel by electrophoresis and visualized under UV light using an AlphaImager imaging system (Alpha Innotech Co., San Leandro, CA). The images of the fluorescent gels were photographed, and the amount of substrate phosphorylation was determined by quantifying the fluorescence intensity of the peptide bands.

Statistical analysis Data was analyzed with one-way ANOVA using SAS® program and expressed as mean±standard error (SE). Treatment differences were subjected to Tukey's multiple comparison tests, where $p < 0.05$ was considered significant.

Results

Genistein improves eNOS protein expression and NO production To initially determine the effects of chronic exposure of HAECs to genistein on eNOS expression and NO synthesis, confluent HAECs were incubated with various concentrations of genistein (1-10 μM) for 5 d with culture medium refreshed in third d. As shown in Fig. 1 A, genistein dose-dependently increased eNOS protein expression, with 10 μM genistein inducing about 60% increase over the control. To confirm the biological importance of this increased eNOS expression by genistein, we evaluated A23187-induced NO production in HAECs treated with genistein or vehicle. Consistent with the eNOS expression pattern, genistein stimulated NO release in a concentration-dependent manner, reaching a maximal level at 10 μM genistein (Fig. 1B).

Genistein-stimulated NO production is independent of PKC, PI3K or ERK1/2 Previous studies have determined that inhibition of PKC up-regulates eNOS transcription (28) and pharmacological doses of genistein could inhibit PKC activity in human chronic myeloid leukemia cells (29). We therefore tested whether PKC mediates the effect of genistein on eNOS. Co-incubation of the cells with p3115, a specific PKC inhibitor, had no effect on eNOS-derived NO production induced by chronic exposure of HAECs to genistein (Fig. 2 C). It have been established that PI3K/Akt and ERK/MAPK-mediated pathways are two important signaling cascades mediating eNOS activation by various stimuli in ECs (30-33). To elucidate the intracellular signaling involved in the genomic regulation of eNOS by genistein, we then examined whether the PI3K/Akt or ERK1/2 pathways were involved in genistein-induced NO synthesis. Pre-incubation of HAECs with the PI3K inhibitor,

LY294002, or the ERK/MAPK blocker, PD098059, had no effect on either basal or genistein-induced NO production (Fig. 2A & 2B).

Genistein-enhanced eNOS expression and NO production are mediated by PKA There is evidence that the eNOS promoter contains CRE sites, and activation of CREB phosphorylation can stimulate eNOS expression in ECs (34). Our recent study showed that genistein activates cAMP signaling in ECs (20). We thus hypothesized that genistein may augments eNOS expression via activation of the PKA/CREB pathway. To that end, HAECs were infected with AdPKI, an adenovirus construct containing the specific endogenous PKA inhibitor gene. As shown in Fig. 3 A, treatment of cultured HAECs with 500-1000 MOI of AdPKI resulted in an up to 95 % reduction in PKA activity compared with that of untreated ECs, whereas infection with heat-inactivated AdPKI had no significant effect on PKA activation. Accordingly, infection of HAECs with AdPKI significantly attenuated genistein-induced eNOS expression and NO production (Fig. 3 B& C), whereas heat-inactivated AdPKI was inactive (data not shown). Taken together, these results indicate that activation of the cAMP/PKA cascade is necessary and sufficient for genistein-induced eNOS expression in HAECs, suggesting a central role for the cAMP/PKA signaling in mediating the genomic effect of genistein on eNOS expression.

Genistein activates the PKA/CREB cascade We further demonstrated that genistein significantly augmented PKA activity and increased CREB phosphorylation at ser-133 in HAECs (Fig. 4A & B) in a dose-dependent manner, a pattern that is consistent with the

increased eNOS protein expression by genistein. To further confirm that activation of CREB by genistein is PKA-dependent, the cells were pre-incubated with PKA inhibitor H89 (10 μM) for 30 min, followed by addition of genistein. We found that genistein-stimulated CREB phosphorylation was completely abolished by H89 (Fig. 4C), suggesting that CREB lies downstream of PKA in genistein-induced signaling which may ultimately mediate genistein-stimulated eNOS expression in ECs.

Discussion

Cardiovascular diseases (CVD) are the leading cause of morbidity and mortality in the United States (35). Many CVD risk factors such as being male (35), aging (36), cigarette smoking (37), high blood pressure (38, 39), and diabetes (40), are associated with reduced NO release due to decreased activity and/or expression of eNOS in both humans and animals, suggesting that eNOS plays a central role in the pathogenesis of CVD. In addition, eNOS expression was significantly decreased in ovariectomized and fertile rats (41), consistent with the finding that pre-menopausal women have lower incidence rate of CVD and hypertension than that of age-matched men, but this reduced CVD rate diminishes with the onset of menopause and even higher in postmenopausal women than in men (35), suggesting a vascular protective effect of estrogen. Indeed, estrogen replacement therapy appears to reduce the risk of cardiovascular disease (42) and increase the circulating NO (43) in postmenopausal women, confirming that estrogen is cardioprotective at least in part, through promoting endothelium-derived NO synthesis. Therefore, maintenance of functional eNOS is of importance in the prevention and therapy for CVD. However, administration of estrogen is associated with an increased incidence of heart disease (44). In addition, estrogen has potential carcinogenic effects in women and feminizing effects in men (45). These side effects limit its use as a cardio-protective agent. Therefore, a search for safe, alternative eNOS-promotive agents for prevention of CVD is of major importance in the effort to decrease the burden of CVD morbidity.

While eNOS is a constitutive enzyme, its expression can be regulated by a variety of factors. For instance, fluid flow (46), vascular endothelial growth factor (47), insulin (48) and

hydrogen peroxide (49) up-regulate eNOS expression, whereas tumor necrosis factor-alpha (50), hypoxia (51), endotoxins (52) and other CVD risk factors down-regulate eNOS transcription. We therefore hypothesized that genistein up-regulates eNOS expression through increasing gene transcription in ECs. We found that genistein, at low concentrations (1-10 μM), enhanced the expression of eNOS protein and subsequently elevated NO synthesis in HAECs, consistent with our recent findings that genistein enhanced eNOS promoter activity (15, 16) and mRNA expression (15), suggesting that genistein enhances NO synthesis via transcriptional up-regulation of eNOS in ECs.

Previous studies showed that estrogen can act directly on vascular ECs to enhance NO production through both genomic stimulation of eNOS expression (53) and membrane receptor-mediated, non-genomic stimulation of the enzymatic activity (54). However, we have determined that genistein up-regulates eNOS via the ER- and PTK-independent mechanisms (15), which promoted us to investigate other potential pathways that mediate this genistein effect. Previous study showed that PKC phosphorylates eNOS at Thr497 which subsequently suppresses eNOS activity in bovine aortic ECs (BAECs) (55). In addition, inhibition of PKC increases eNOS mRNA and protein expression and subsequently elevates NO synthesis in BAECs (28), although the mechanism of this action is not clear. However, inhibition of PKC had no effect on basal or genistein-stimulated NO release, suggesting that this genistein effect is not related to PKC in HAECs. Indeed, as to our knowledge, there is no published data so far showing an effect of genistein on PKC activity in ECs. Genistein was reported to affect Akt and ERK1/2 MAP kinase activities which can modulate eNOS activity and NO production (31). In our present study however, genistein-induced eNOS expression

and NO release were not related to PI3K/Akt or ERK1/2 activity. Actually, these kinases may primarily regulate the acute eNOS activation in response to extracellular stimuli such as steroids (56), growth factors (57) and shear stress (58).

We found that adenoviral transfer of endogenous PKA inhibitor gene abrogated genistein-stimulated eNOS protein expression and subsequent NO production. AdPKI is highly specific and efficient because it could completely block the PKA activity in HAECs, whereas heat-inactivated AdPKI had no significant effect on PKA activity, suggesting that genistein enhances eNOS expression and NO production through activation of PKA. Indeed, we recently indicated that genistein activates the cAMP signaling system and subsequently regulates cAMP-mediated gene expression in ECs (14). In our present study, we demonstrated that genistein enhanced PKA activity in HAECs, further confirming that genistein activates the classic cAMP/PKA pathway in ECs. However, it is still unknown how activation of PKA by genistein regulates eNOS.

Endothelial NOS promoter contains several regulatory elements including shear stress-responsive element, estrogen-responsive element and activator protein-1 binding site which modulate eNOS gene transcription in response to respective stimuli (50, 59, 60). In addition to these mechanisms that regulates eNOS expression, a recent study found that eNOS gene contains functional cAMP responsive elements (CRE) that also positively regulates eNOS gene trasncription (24), consistent with other recent findings that activation of cAMP signaling stimulated eNOS expression in ECs (61, 62). These results suggest that the cAMP/PKA/CREB cascade plays a role in regulating eNOS expression. Our further studies demonstrated that genistein rapidly activated PKA-dependent phosphorylation of

CREB at ser-133 in HAECs. While not determined, it is reasonably speculated that genistein may regulates eNOS through activation of PKA-dependent CREB, given that the phosphorylation of CREB at ser-133 is necessary for its binding to CRE to regulate gene transcription (63), and that eNOS promoter contains CRE sites. However, studies showed that the catalytic subunit of PKA can activate CREB-binding protein (CREB-BP), a CREB coactivator that is required for CREB to regulate gene transcription (64). In addition, there is literature providing evidence that eNOS and the catalytic subunit of PKA are colocalized in the restricted intracellular locations in ECs (65), suggesting a direct interaction between PKA and eNOS. Therefore, it is possible that genistein may regulate eNOS by simultaneously acting on multiple targets which include PKA, CREB and CREB-BP. Therefore, further studies are needed to determine whether CREB indeed is the distal signal molecule that primarily mediates the genistein effect on eNOS.

In summary, genistein has various biological actions. We have demonstrated here for the first time to our knowledge that genistein enhances the expression of eNOS and subsequently increases NO synthesis via the cAMP/PKA/CREB pathway in primary human ECs. These findings add new information to the functional repertoire of this food-derived small molecule and form the basis for further evaluating its potential in preventing or treating cardiovascular disease. Future studies therefore will be aimed at determining if PKA/CREB/eNOS/NO signaling elicited by genistein *in vitro* is physiologically relevant *in vivo*.

Acknowledgments

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Abbreviations

AdPKI, adenovirus encoding protein kinase A inhibitor; BAECs, bovine aortic endothelial cells; CRE, cAMP-responsive element; CREB, cAMP-responsive element binding protein; CVD, cardiovascular disease; DMSO, dimethylsulfoxide; E2, 17β-estradiol; ECs, endothelial cells; eNOS, endothelial nitric oxide synthase; ERK1/2, extracellular signal-regulated kinase 1/2; ERK/MAPK, ERK-mitogen activated protein kinase; ERs, estrogen receptors; HAEC, human aortic endothelial cells; HBSS, Hank's balanced salts solution; NO, nitric oxide; PI3K, phosphoinositol-3 kinase; PI3K/AKT, phosphoinositol-3 kinase/ protein kinase B (AKT); PKA, protein kinase A; PKC, protein kinase C; PTK, protein tyrosine kinase; MOI, multiplicities of infection.

Figures

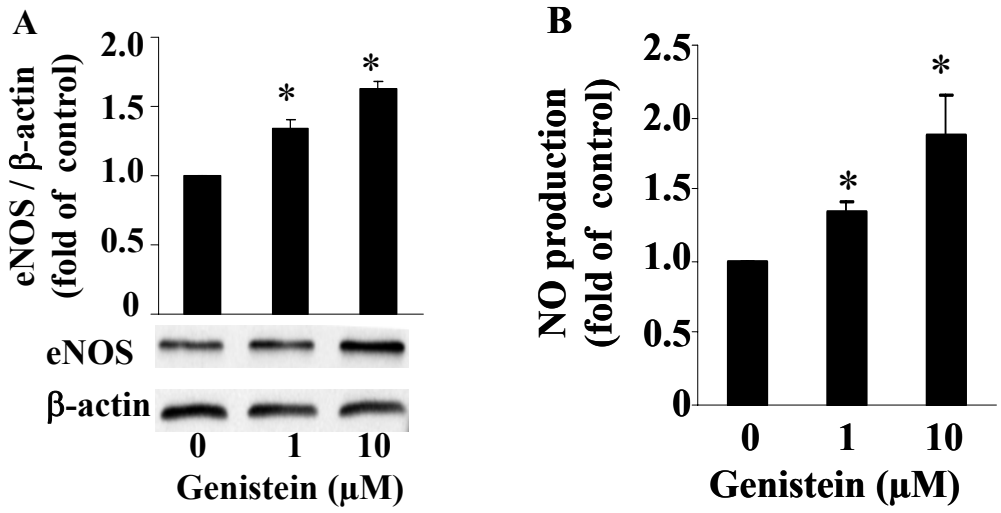

FIG.1 Genistein enhances eNOS protein expression and NO production in HAECs. Confluent HAECs were incubated with various concentrations (1-10 μM) of genistein or vehicle (DMSO) for 5 d. **A.** eNOS protein level in cell extracts was analyzed with Western blotting and normalized to β-actin content. Values (mean±SE) were expressed as fold increase over control derived from four separate experiments, a set of representative graphs and bar graph (mean±SE) were shown. *, $P<0.05$ *vs.* vehicle-alone treated control. **B.** Nitrite/nitrate (NO_x) production stimulated by ionophore 23187 was measured using a fluorometric assay kit and normalized to protein content. Values (mean±SE) were expressed as fold of the control derived from four separate experiments. *, $P<0.05$ *vs.* vehicle-alone treated cells.

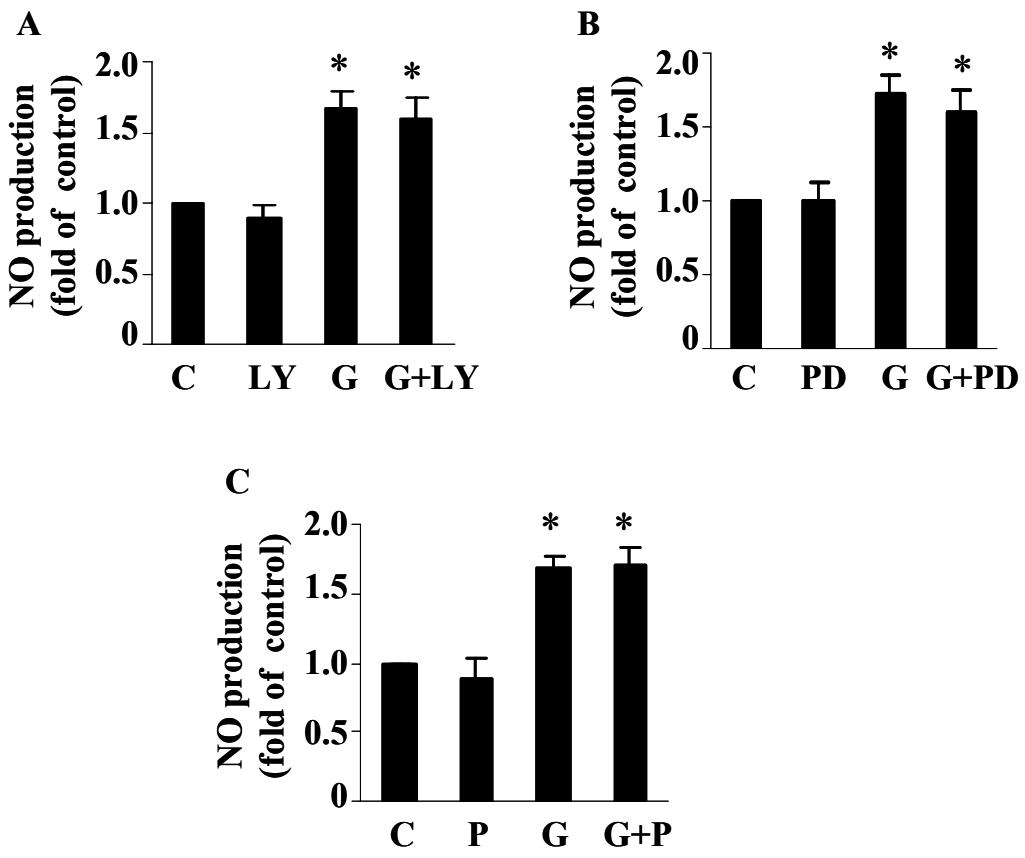

FIG.2 The promotive effect of genistein on NO production is not dependent on PI3K, ERK1/2 and PKC pathways in HAECs. Confluent HAECs were pre-incubated with either LY294002 (LY; 10 μM), the PI3K inhibitor (**A**), PD 98059 (PD; 10 μM), a ERK inhibitor (**B**) or P3115 (P; 20 μM), a PKC inhibitor (**C**) for 30 min followed by addition of genistein (G; 10 μM) for 5 d. Nitrite/nitrate (NO_x) production stimulated with ionophore 23187 was measured using a fluorometric assay kit and normalized to protein content. Values (mean±SE) were expressed as fold of the control derived from four separate experiments, *, $P<0.05$ *vs.* vehicle-alone treated control.

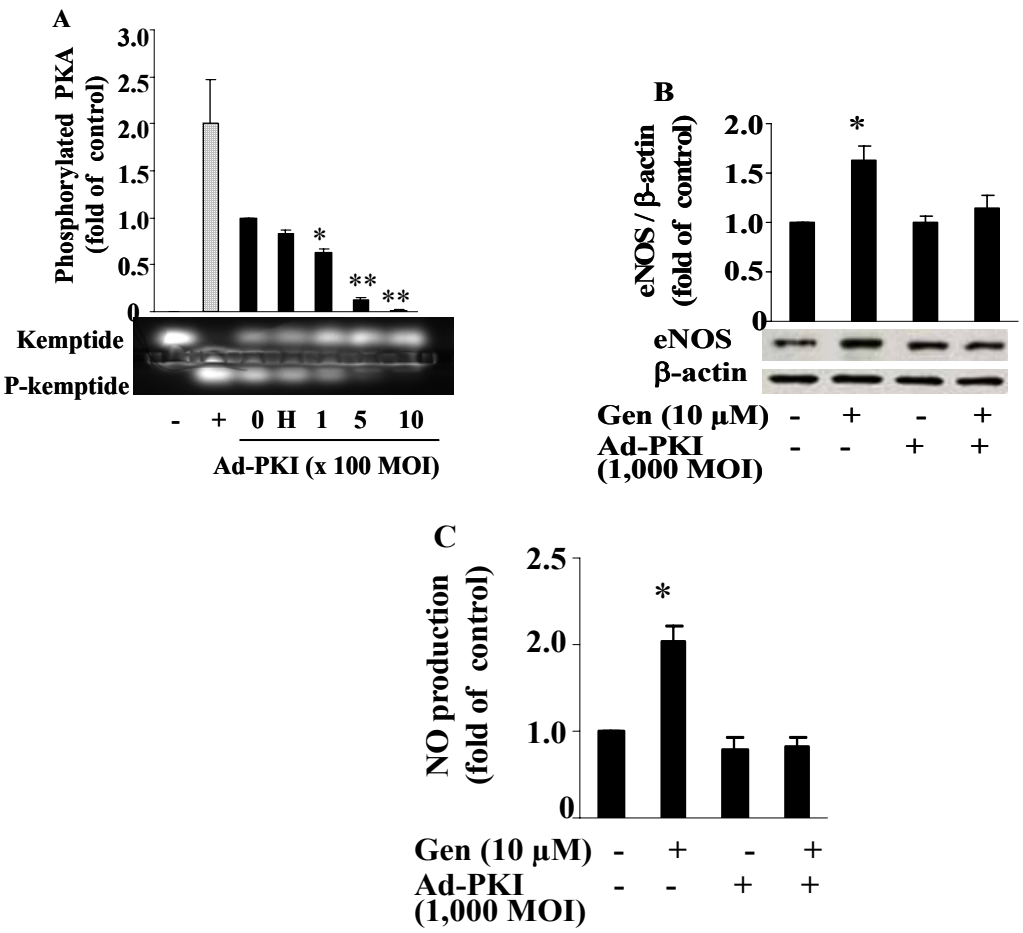

FIG.3 PKA mediates genistein-enhanced eNOS protein expression and NO production in HAECs. **A.** Different concentration and heat-inactivated AdPKI virus were transfected into HAEC for 24 h. Cell lysate were used to measure PKA activity using a non-adioactive cAMP-dependent protein kinase A PepTag® assay. **B**& **C.** AdPKI transfected HAECs were treated in the presence or absence of genistein (G; 10 μM) for 5 d. eNOS protein in cell extracts was analyzed with Western blotting and normalized to β-actin content. Values (mean±SE) were expressed as fold of control derived from four separate experiments, a set of representative graphs and bar graph (mean±SE) were shown. *, $P<0.05$ *vs.* vehicle-alone treated control (**B**). Nitrite/nitrate (NO_x) production stimulated with ionophore 23187 was measured using a fluorometric assay kit and normalized to protein content. Values (mean±SE)

were expressed as fold of control derived from four separate experiments, *, $P<0.05$ *vs.* vehicle-alone treated control (**C**).

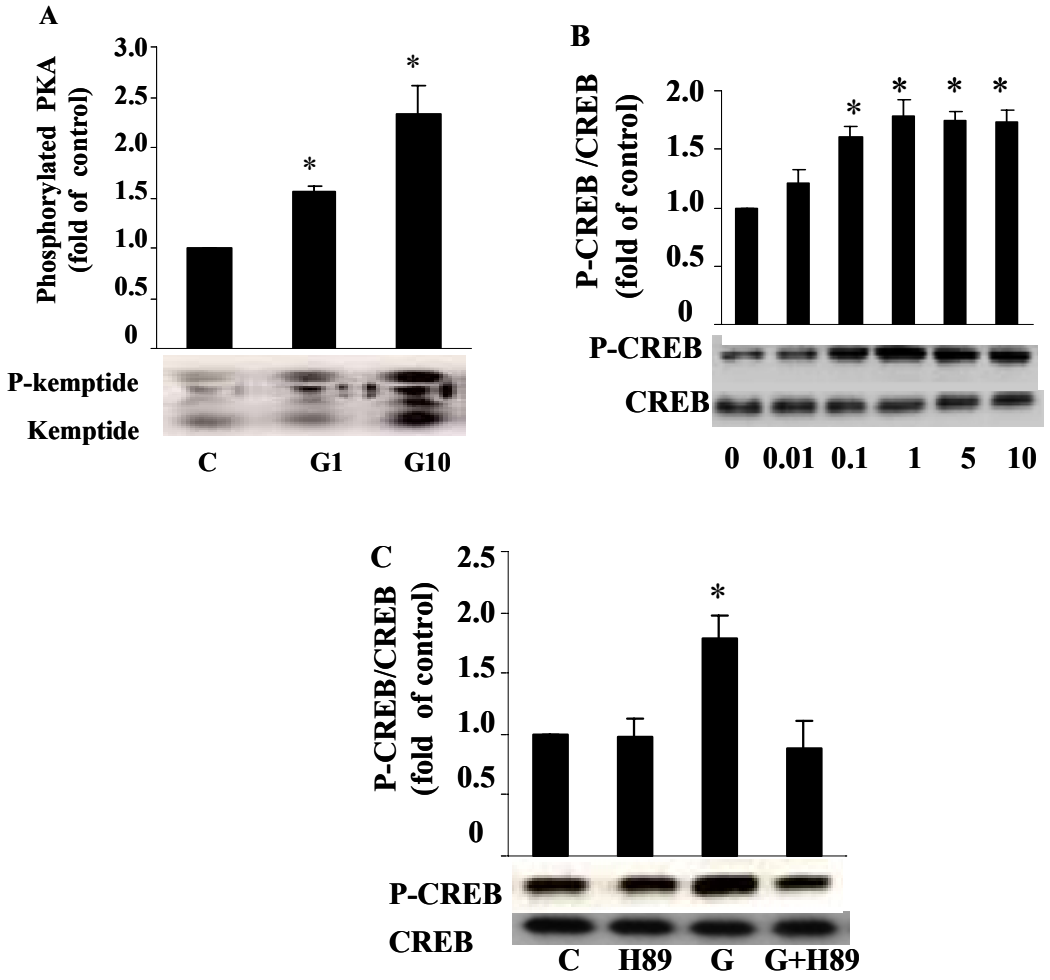

FIG.4 Genistein activates PKA/CREB cascade. Confluent HAECs were serum-starved in HBSS buffer for 30min and followed by stimulation of various concentration of genistein (G; 0.01-10 μM) for 15 min. PKA activity (**A**) and CREB phosphorylation (**B** & **C**) were measured using a non-radioactive cAMP-dependent protein kinase A PepTag® assay and Western blot respectively. **C.** H89, a specific inhibitor of PKA was pre-incubated for 30 min before genistein stimulation. After normalizing with total PKA or CREB, values (mean±SE) were expressed as fold of control derived from three separate experiments, *, $P<0.05$ *vs.* vehicle-alone treated control.

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CHAPTER 5

Isoflavone genistein protects human vascular endothelial cells against tumor necrosis factor-α-induced apoptosis through the p38β mitogen-activated protein kinase

Short Title: genistein and endothelial apoptosis

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Abstract

Isoflavone genistein may have beneficial effects on vascular function, but the mechanism is unclear. In the present study, we investigated whether genistein protects vascular endothelial cells (ECs) against apoptosis induced by tumor necrosis factor-α (TNF-α), a pro-inflammatory cytokine associated with the pathogenesis of atherosclerosis. 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. This effect was dose-dependent and maximal at 5-10 μM concentrations. The anti-apoptotic effect of genistein was associated with an enhanced expression of anti-apoptotic Bcl-2 protein and its promoter activity that was ablated by TNF-α. Inhibition of extracellular signal-regulated kinase 1/2**,** protein kinase A, or estrogen receptor had no effect on the cytoprotective effect of genistein. However, inhibition of p38 mitogen activated protein kinase (p38) by SB203580 completely abolished the cytoprotective effect of genistein, suggesting that genistein acted through the p38-dependent pathway. Accordingly, stimulation of HAECs with genistein resulted in rapid and dose-dependent activation of p38. Unlike TNF-α which specifically activated p38α, genistein selectively induced phosphorylation of p38β, suggesting that p38β, but not p38α, is essential for the cytoprotective effect of genistein. These findings provide the evidence that genistein acts as a survival factor for vascular EC to protect cells against apoptosis via activation of p38β. Preservation of the functional integrity of the endothelial monolayer may represent an important mechanism by which genistein exerts its vasculoprotective effect.

Key Words: genistein; apoptosis; caspase-3; Bcl-2; endothelial cells; 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 (4-9), recent human studies have shown that dietary supplementation of genistein alone has a significantly beneficial effect on atherosclerosis (10), markers of cardiovascular risk (11, 12), vascular motor tone (13, 14), vascular endothelial function (15), and systemic arterial compliance (16). Data from animal and *in vitro* studies also consistently suggest a protective role of genistein in cardiovascular events (17-23). It also has been shown that genistein reduces the size of infarction and experimental myocardial ischemia-reperfusion injury (24), and improves endothelial dysfunction induced by oophorectomy in rats (25), providing consistent evidence for a cardioprotective effect of genistein. However, the mechanism of genistein action in vasculature is still not clear.

Past studies have extensively explored its hypolipidemic (26), anti-oxidative (27, 28) and the estrogenic effects (29), which all play a potential role in initiation of atherosclerosis (30-32). While genistein may have both ER-dependent and independent actions in vasculature, its average effect on plasma lipid profile is neutral (16, 33). In addition, genistein is not a physiologically effective antioxidant or scavenger of reactive oxygen species (34, 35), although it has been reported to exhibit antioxidant activity in aqueous phase systems (36, 37)

and prevent LDL oxidation at pharmacological doses (38, 39). Recently, we (40, 41) and others (42) 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 (43) and acute coronary syndrome (44), which is enhanced by circulating inflammatory cells and other risk factors such as tumor necrosis factor-α (TNF-α). A recent study demonstrated that genistein protects against TNF-α-induced apoptosis in osteoblastic cells (45). However, it is unknown if genistein exerts a similar effect on ECs. 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 p38β, which may underline the ability of genistein to rescue ECs from TNF-α-induced apoptosis.

Material and Methods

Materials

Primary human aortic endothelial cells (HAECs) and endothelial growth supplements (EGM2) were purchased from Cambrex Bioscience (Rockland, ME); M199 media, caspase-3 assay kits and competent cells for plasmid trasnformation were from Invitrogen (Carlsbad, CA); antibodies against p38 , phospho-p38 (Thr180/Tyr182), p38α, p38β, 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, H89, PD098059, LY294002, 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 μM) for 48 h. Apoptotic cells were counted using flow cytometry as described (46) 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 ×g for 4 min at 4°C. The cells were then incubated in PBS containing 20 μg/ml of 7-AAD at 4°C for 20 min. After washed once with PBS, cells were suspended in 200 μl 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 (47). 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 $Na_4P_2O_7$, 1 mM glycerolphosphate, and 1Mm Na_3VO_4, pH 7.4) supplemented with protease (1:500) and phosphatase (1:100) inhibitor cocktails, and cell extracts were collected by centrifugation at 12,000 ×g for 10 min at 4°C. An aliquot of supernatant (100 μg protein) was incubated with p38α or p38β monoclonal antibody (1:200) with gentle mixing at 4°C. 12 h later, 80 μl 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 ×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 μg 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.

Statistical analysis

Data was analyzed with one-way ANOVA using SAS® program and expressed as mean±standard error (SE). Treatment differences were subjected to a Tukey's multiple comparison test, where $p < 0.05$ was considered significantly different.

Results

The effect of genistein on EC 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 (48). As shown in Fig. 1 A & 1B, genistein dose-dependently attenuated TNF-α-induced apoptosis, with 10 μM inducing the maximal effect. 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 μM) reduced the percentage of TNF-α-induced apoptotic cells from 33.0% to 15.7% (Fig. 1C, 1D). 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 (49) and caspase-3 is one of the key proteases involved in the convergence of disparate apoptotic signaling pathways. Paralleling 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 μM) 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 (29), and 17β-estradiol has been shown to protect ECs from apoptosis through an ER-dependent mechanism (50, 51), we examined whether ERs are involved in the cytoprotective effect of genistein. Our results demonstrated that ICI 182,780 (10 μM), the highly specific inhibitor of ER, did not ablate the inhibitive effect of genistein on caspase-3 activity (Fig. 4A). Both PKA (52, 53) and ERK1/2 (54) 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

(41, 55). Therefore, we investigated whether the anti-apoptotic effect of genistein is mediated through these pathways. The results showed that incubation of HAECs with the PKA inhibitor, H89 (Fig.4B), or the ERK1/2 pathway blocker, PD98059 (10 μM) (Fig. 4C), had no effect on the inhibitory effect of genistein on caspase-3 activity. Both H89 and PD098059 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 p38β

Previous studies showed that p38 mediates the anti-apoptotic effect of heme oxygenase-1 in ECs (56) and genistein can activate p38 in mammary epithelial cells (57). We therefore examined whether p38 is involved in the anti-apoptotic effect of genistein. As shown in Fig. 5, SB203580 (40 μM), a specific inhibitor of p38, abolished the inhibitory effect of genistein on caspase-3 activity in HAECs. 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 p38α, one of four p38 isoforms (58), is a pre-apoptotic molecule in ECs (56), whereas activation of p38β exerts an anti-apoptotic effect (59, 60). Data from immunoprecipitatation assay showed that genistein stimulated the phosphorylation of p38β but simultaneously inhibited p38α activation. On the contrary, TNF-α remarkably activated p38α but had no significant effect on the phosphorylation of p38β (Fig.6 C, 6D), suggesting that genistein and TNF-α have a

differential effect on p38α and p38β. Therefore, it is likely that that genistein protects EC against TNF-α-stimulated apoptosis by selective activation of p38β.

Discussion

Vascular endothelium, a single layer of ECs lining the lumenal 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 (61, 62). TNF-α, a proinflammatory cytokine, is remarkably elevated in the plasma and artery both in animals and humans with vascular complications (63-65). It is believed that TNF-α is critically involved in the pathogenesis of atherosclerosis. Indeed, high levels of TNF-α can induce EC apoptosis (64), which disrupts endothelial integrity and leads to cardiovascular disease (66). Isoflavone genistein may exert beneficial effects on vasculature which are always explained by its presumably hypolipidemic, weak estrogenic and antioxidantive 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. The 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 μM) (67, 68). However,

our current data shows that genistein is survival factor for human ECs when used at relatively lower concentrations (5-10 μM). This result is in line with recent studies demonstrating that genistein (2.5 μM) can inhibit homocysteine- and oxidized LDL-induced apoptosis in transformed ECs (69, 70). 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 100 μM (71, 72). It was reported that total circulating genistein levels in humans and animals consuming soy products or isoflavone supplements are between 0.74-6.0 μM, and that the genistein concentrations in tissues may be even higher (73-75). Therefore, the genistein concentrations that produced biological effects observed in this study (5-10 μM) 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 (76), while genistein conjugates in the serum are reported to be lesser biologically active than free genistein (77), 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 EC survival could be realized in hostile environment such as diabetes where plasma TNF-α level is dramatically elevated (64). It would probably not be necessary to achieve concentrations that high in plasma in order to observe a beneficial effect because the anti-apoptotic effect of genistein on ECs is chronic and could be cumulative. In deed, administration of genistein has been found

to reduce apoptosis of myocytes and attenuate myocardial ischemia/reperfusion injury in rabbits (78).

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 (79). Therefore, overexpression of Bcl-2 has been shown to protect ECs against TNF-α-induced apoptosis (80). In consistent with the protective effect of genistein on TNF-α-induced apoptosis, our studies showed 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 17β-estradiol has been reported to protect ECs against stimuli-induced apoptosis that is mediated by the ER-dependent mechanisms (50), 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, did not block the inhibitory effect of genistein on capsase-3 activity in HAECs. It is unlikely that the inability of this agent to block the effect of genistein on apoptosis is due to a lack of efficacy, because we previously reported that, at the same concentration used, ICI 182, 780 completely abolished the 17β-estradiol-induced endothelial nitric oxide synthase activity in ECs (40). Second, daidzein, a genistein analogue that is essentially lack of affinity to the ERs, also protected ECs against TNF-α-induced apoptosis as observed in osteoblastic cells (45). In addition, previous studies demonstrated

that genistein activates ERK1/2 and PKA in ECs (41, 55), which play important roles in promoting ECs survival (54, 55). However, the cytoprotective effect of genistein was not related to ERK1/2 or PKA. While neither ERK1/2 nor PKA mediate the genistein effects on cell apoptosis, their potential role in other genistein-induced vascular effects deserves further study.

In the present study, we showed that the cytopreventive effect of genistein on TNF-α-induced apoptosis was abolished by SB203580, a specific inhibitor of p38α and p38β that has been widely used in investigation of the biological functions involving the p38 kinase signaling pathway. While p38α is the most widely expressed isoform of p38 family, both p38α and p38β are equally expressed in vascular ECs (58). We further demonstrated that genistein activated the phosphorylation of p38 over the same concentration range as its effect on apoptosis. Interestedly, 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 (81, 82). Studies show that p38α and p38β exerts different biological actions. Activation of p38α has been shown to induce apoptosis in ECs (56), L929 fibroblasts (83), myocytes (84) and HeLa cells (59), whereas p38β actually promotes survival of these cells (56, 59, 83, 84). In addition, cardiomyocytes and fibroblasts derived from p38α deficient mice are less susceptible to undergo apoptosis (85), suggesting that p38α is a pro-apoptotic molecule both in vitro and in vivo. These data suggest that p38α and p38β have antagonistic effects in controlling cellular apoptosis and therefore the balance between these two kinases may decide the cell survival or apoptosis. In an effort to define the roles of p38 isoforms in genistein effect, we initially determined

whether genistein and TNF-α may have differential effects on p38α and p38β activation in ECs. We found that genistein selectively activated p38β whereas TNF-α predominantly induced p38α activity in HAECs, suggesting that p38β 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 p38β 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 demonstrated that the cytoprotective effects of genistein were ER-, ERK1/2- and PKA-independent but were mediated through the p38β 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; E2, 17β-estradiol; EC, endothelial cells; ER, estrogen receptors; ERK1/2, extracellular signal-regulated kinase ½; 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 belta; 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.

Figures

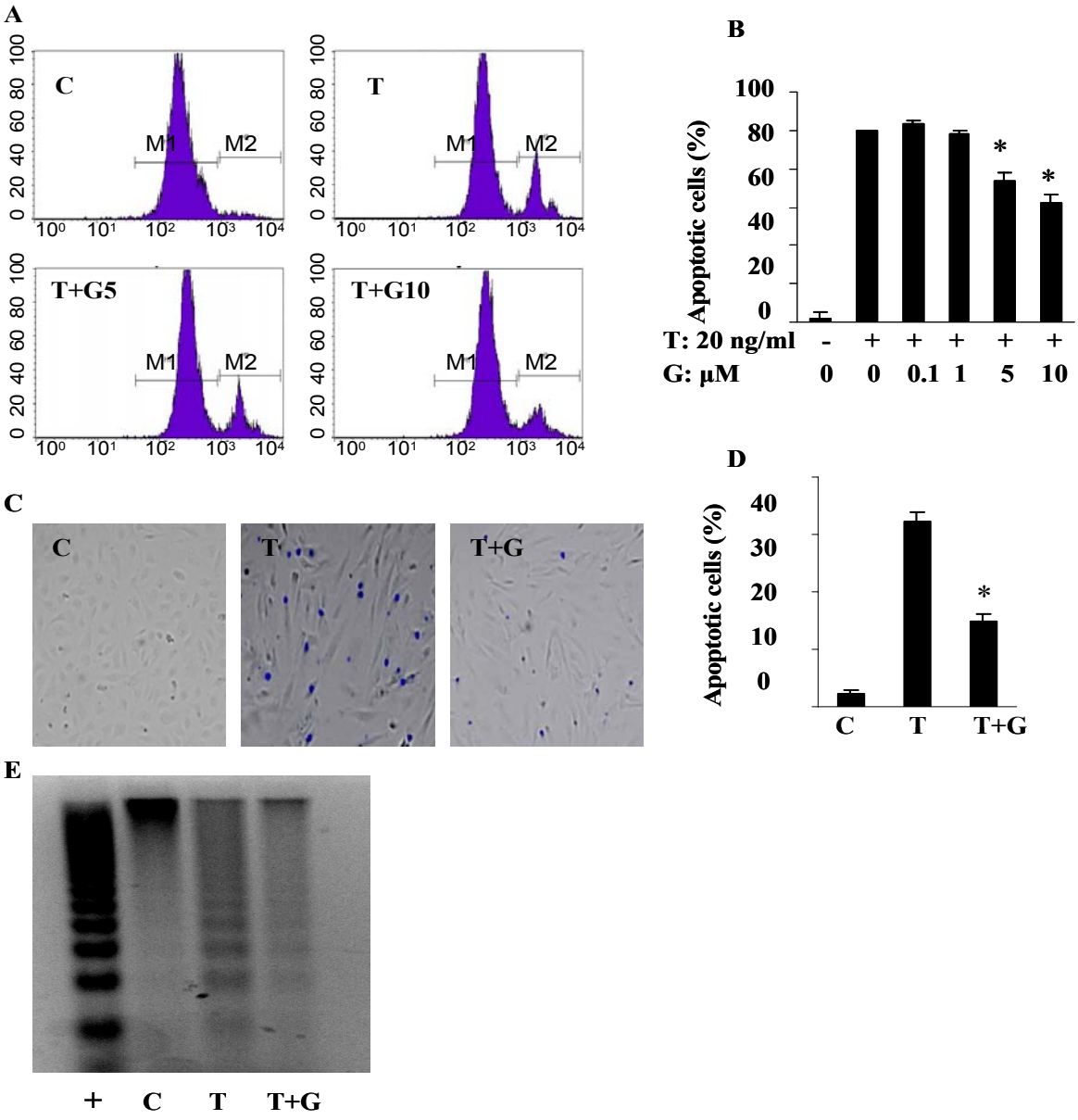

FIG.1 Genistein protects against TNF-α-induced apoptosis in HAECs. **A, B**. Confluent HAECs were treated with or without TNF-α (T, 20 ng/ml) in the presence or absence of and various concentrations of genistein (G, 0-10 μM) for 48 h, and apoptotic cells were stained with 7-ADD and determined using flow cytometry. A representative image and bar graph (mean±SE) of four independent experiments were shown. *, $P < 0.05$ *vs*. TNF-α-alone treated cells. **C, D.** HAECs cultured on chamber slideswere incubated with or without TNF-α (T, 20

ng/ml) in the presence or absence of genistein (G, 10 μM) for 48 h. Apoptotic cells were detected using TUNEL method. A representative image and bar graph (mean±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 was shown.

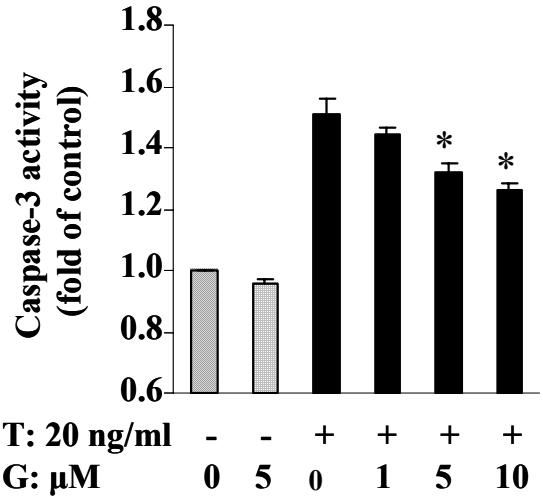

FIG.2 Genistein inhibits TNF-α-induced caspase-3 activity in HAECs. Confluent HAECs were exposed to TNF-α (T, 20 ng/ml) withor without various concentrations of genistein (G, 0-10 μM) for 9 h, and cells were collected to measure caspase-3 activity using an assay kit. Data are means±SE derived from four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

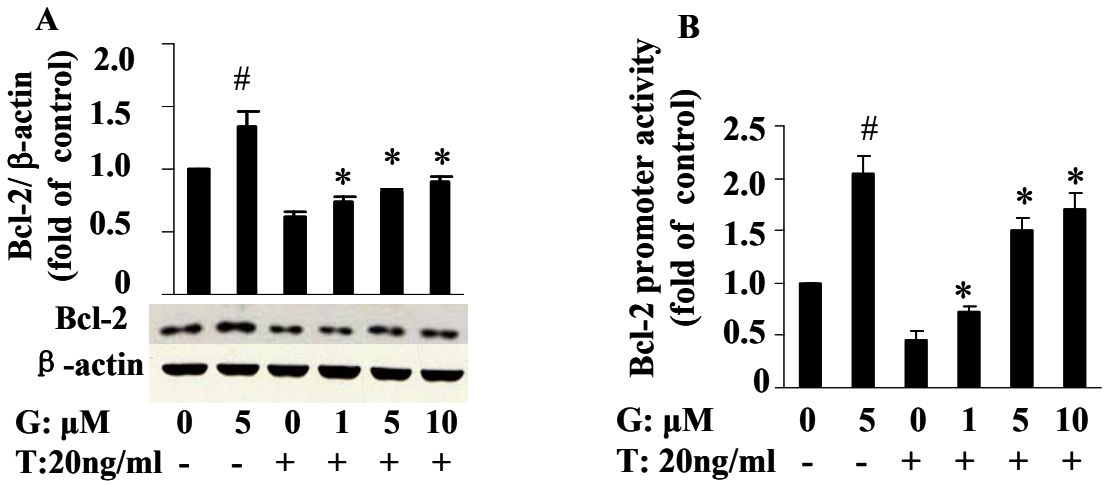

FIG.3 Genistein restores TNF-α-inhibited Bcl-2 protein expression and promoter activity in ECs. **A**. Confluent HAECs were treated with or without TNF-α (T, 20 ng/ml) in the presence or absence of various concentrations of genistein (G, 1-10 μM) for 48 h. The Bcl-2 level in the cell extracts were measured 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 μM) for 24 h. Bcl-2 promoter activity in the cell lysates was measured using a dual-luciferase kit. Values are mean±SE obtained from three separate experiments and expressed as folds of control. *, $P<0.05$ vs. TNF-α-alone treated cells.

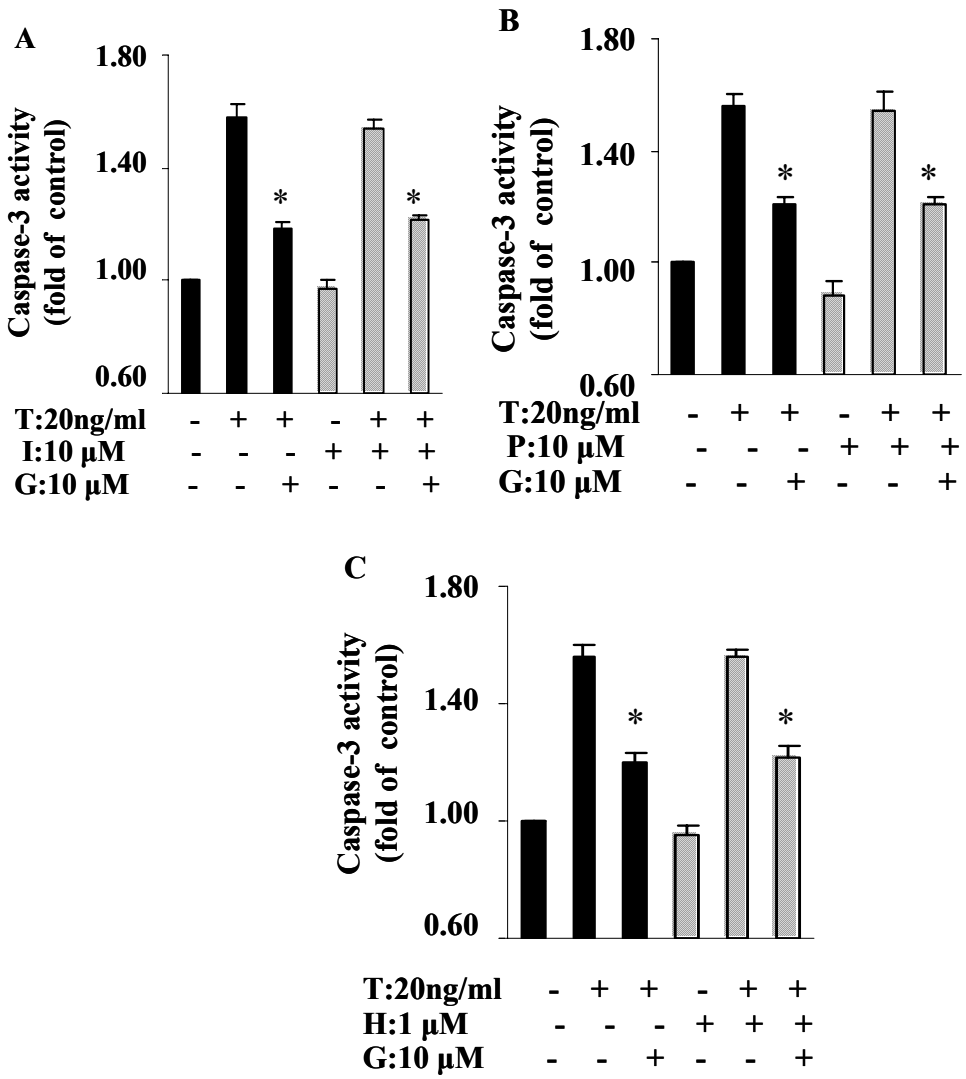

FIG.4 The inhibitory effect of genistein on TNF-α-induced caspase-3 activity is not dependent on ER, PKA and ERK1/2 pathways. HAECs were pre-incubated with ICI 182780 (ICI; 10 μM), PD 98059 (P; 10 μM), or H89 (H; 1 μM) for 30 min followed by addition of TNF-α (T; 20 ng/ml) with or without genistein (G; 5 μM) 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±SE) were expressed as folds of control. *, $P<0.05$ vs. TNF-α alone-treated cells.

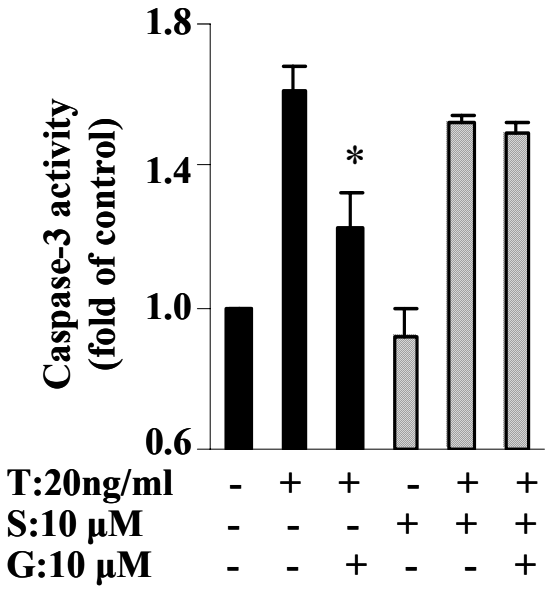

FIG.5 The inhibitory effect of genistein on TNF-α-induced caspase-3 activity is mediated by p38 pathway. HAECs were pre-incubated with SB203580 (S; 40 μM), a P38 inhibitor for 30 min followed by addition of TNF-α (T; 20 ng/ml) with or without genistein (G; 5 μM) for 9 h, caspase-3 activity in the cell lysates was measured. The experiment was repeated four times and data (means±SE) were expressed as folds of control. *, $P<0.05$ vs. TNF-α alone-treated cells.

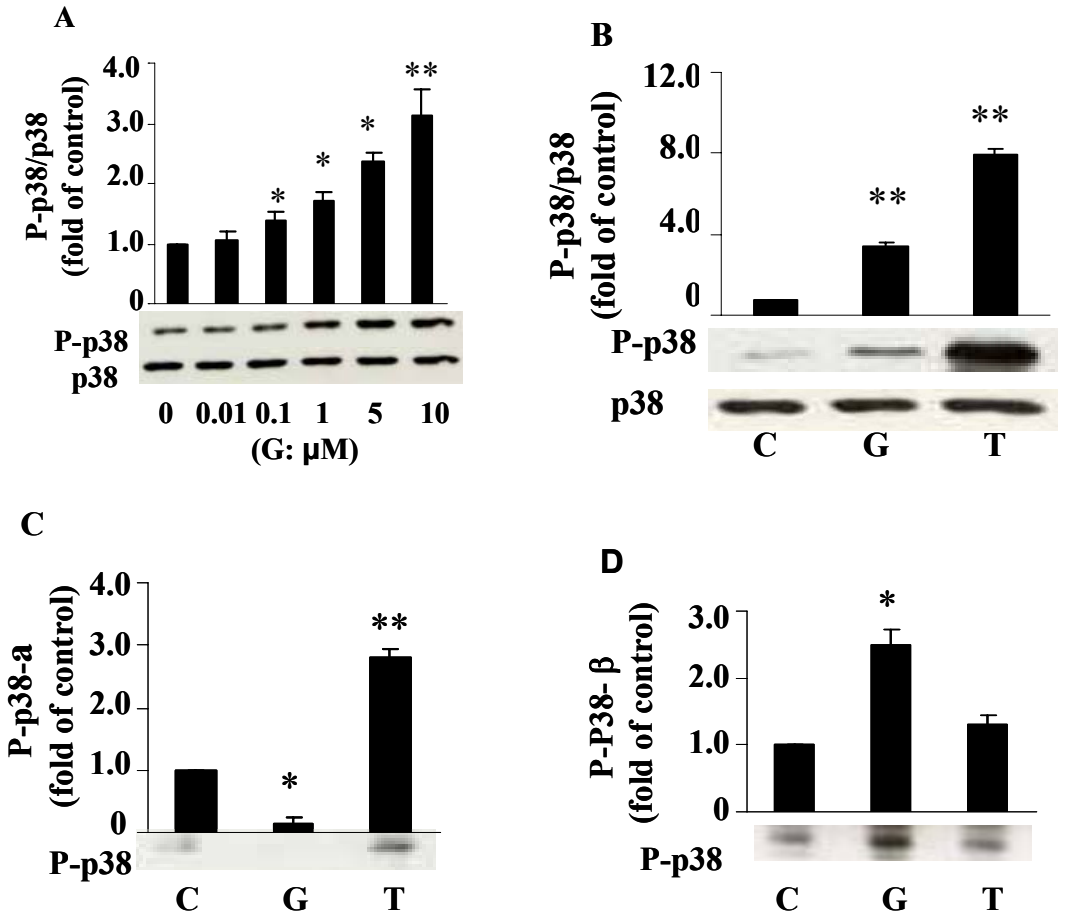

FIG.6 Genistein activates p38β phosphorylation while inhibits p38α phosphorylation in HAECs **A.** HAECs were incubated with various concentrations of genistein (G; 0.01-10 μM) for 15 min. **B.** HAECs were incubated with , with either genistein (G; 5 μM), TNF-α (T; 20 ng/ml) or vehicle (C) for 15 min. The phosphorylation of p38 was detected by Western blot using a phospho-specific p38 antibody, normalized to total p38. **C, D.** HAECs treatd with genistein (G; 5μM), TNF-α (T; 20 ng/ml) or vehicle (C) were lysed and immunoprecipitated with p38α or p38β antibody, followed by measuring the phosphorylation of p38 using Western blot. The experiment was repeated three times and data (means±SE) were expressed as folds of control. *, $P<0.05$, and ** $p<0.01$ vs. vehicle alone-treated control.

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CHAPTER 6

Conlusions and recommendations for future research

Conclusions

Genistein has been shown to exert beneficial effect on cardiovascular system, although it only has a limited effect on plasma lipids. As a highly selective agonist of ERβ, genistein may act on vasculature partially through the ER-dependent mechanisms, given the role for estrogen in the regulation of vascular function. However, it appears that genistein has ER-independent stimulatory effects on multiple cellular signaling pathways and transcriptional factors including eNOS, cAMP, ERK1/2, Akt and PPARs, which potentially offer a wide spectrum of beneficial effects on vasculature and therefore are attractive molecular targets by which to prevent cardiovascular disease. However, the mechanism of genistein action in vasculature is still not clear. In the present study, we demonstrated for the first time to our knowledge, that genistein can enhance eNOS gene transcription and protein synthesis in primary human vascular EC, leading to NO production. Dietary genistein administration stimulated eNOS expression, improved vessel wall thickening, and alleviated hypertension in SHR, confirming the biological relevance of the *in vitro* findings. Our results further indicated that genistein-enhanced eNOS expression and NO synthesis in primary human ECs are mediated by cAMP/PKA/CREB pathway. We also 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 demonstrated that the cytoprotective effects of genistein were ER-, ERK1/2- and PKA-independent but were mediated through the p38β signaling pathway, thereby defining a novel mechanism of this genistein action in vascular ECs. These findings add new information to the functional repertoire of this food-derived small molecule and form the basis for further evaluating its potential in preventing or treating cardiovascular disease.

Future research recommendations

1 Determine the role that CREB plays in genistein-enhanced eNOS expression. Although the highly specific PKA inhibitor blocked the genistein-stimulated eNOS expression and NO synthesis in our current study, and CREB is located downstream of PKA signaling, further studies are still needed to determine whether CREB plays a role in genistein signaling to eNOS. Regarding this, pharmacological or molecular intervention studies such as transfection of siRNA of CREB or dominant-negative CREB constructs can be employed to address this question.

2 Investigate whether genistein can reverse impaired eNOS expression by TNF-α in ECs. TNF-α negatively regulates eNOS expression by inhibiting eNOS promoter activity (1) and lowering its mRNA stability (2). It is interesting to test whether genistein could restore TNF-α-reduced eNOS expression in ECs.

3 Examine whether genistein protects against TNF-α-induced apoptosis *in vivo.* Endothelial integrity is modulated by a variety of factors and EC apoptosis is a complex process *in vivo*. Although genistein inhibits TNF-α-induced ECs apoptosis *in vitro*, it is not clear whether administration of genistein offers the same protective effect on ECs *in vivo*, which is an ongoing project in this laboratory.

4 Determine the mechanism by which genistein protects against EC apoptosis. Low level NO is well recognized as an antiapoptotic molecule, and genistein can enhance both eNOS-derived NO synthesis and p38 activity in ECs as aforementioned, combining that p38 mediates eNOS-derived NO synthesis regulation (3), it is conceivable to test whether the protective effect of genistein on TNF-α-induced apoptosis is directly mediated by promoting p38/eNOS/NO cascade.

5 Investigate the antiapoptotic effects of other polyphenols such as resveratrol and catechins. Both resveratrol and catechins have been demonstrated to activate p38 β and induces eNOS/NO signaling (3-6). Based on the results from my research which show that genistein protects against EC apoptosis through activation of p38β, it is very interesting to evaluate whether these polyphenols provide similar protective effect on ECs.

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VITA

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After graduating with his Master of Science in Microbiology and Immunology in 1998, Hongwei moved Qingdao, a great city in the northeast of China, where he took a research scientist position in the Qingdao Animal Husbandry and Veterinary Institute. He remained there for six years. It was there in Qingdao that two major things happened in his life. First, he met the woman who would later become his wife, Sinqin, and later have their lovely son Haijun. Second, he decided that he was ready for a new challenge in life. He enjoyed his work, but was ready to return to school to pursue his Ph.D.

In 2004, Hongwei enrolled at Virginia Tech, to work under Dr. Dongmin Liu on the phytochemicals functions and mechanisms on chronic diseases. In fall 2007, Hongwei will graduate from Virginia Tech with a Ph.D. in human nutrition, food and exercise. He has decided continue working with Dr. Dongmin Liu as a postdoctoral researcher.