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Research Paper

Phytoestrogen genistein supplementation increases eNOS and decreases caveolin-1 expression in ovariectomized rat hearts

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Abstract: This study examined whether genistein influences the production of nitric oxide (NO) and expression of endothelial nitric oxide synthase (eNOS) and the modulators of eNOS activity in ovariectomized (OVX) rat hearts. Female mature Sprague-Dawley rats were subjected to bilateral ovariectomy, OVX rats were randomly divided into four groups: 17β-estradiol (0.1 mg/kg, s.c. daily) was used as the positive control; low dose of genistein (0.5 mg/kg, s.c. daily); high dose of genistein(5.0 mg/kg, s.c. daily) and model. Sham operations as controls, the treatment lasted 6 weeks. Blood pressure, heart rate, plasma estradiol, heart and uterine weights were measured. Nitrite production in the myocardium was determined by nitrate reductase method. Protein level of eNOS, caveolin-1 and calmodulin was determined by Western blot. The results showed that nitrite production and eNOS protein in homogenized ventricular tissue was attenuated by approximately 53% and 67% in OVX rats compared with those in sham rats, respectively. Genistein increased nitrite production in rat heart in a dose-dependent manner, genistein at the dose of 5 mg /kg·d^{-1} resumed nitrite production to a level similar to that in sham operated rats. Administration of genistein also increased eNOS protein expression in OVX rats myocardium with a concomitant decrease in the expression of caveolin-1, an endogenous eNOS inhibitory protein. Another eNOS stimulatory protein, calmodulin, was unchanged in these treatments. These effects were also observed in rats treated with 17β-estradiol. Genistein at the dose of 5.0 mg/kg·d^{-1} augmented uterine weight but this side effect in reproductive system was less than that of 17β-estradiol. These results suggest that genistein supplementation and estrogen replacement therapy directly increase eNOS functional activity and NO production in the hearts of the OVX rats, but genistein has less side effects on the reproductive system than 17β-estradiol.

Key words: genistein; nitric oxide; endothelial nitric oxide synthase; caveolins

金雀异黄酮通过减少卵巢切除大鼠心肌小凹蛋白 -1 的表达而增加一氧化氮的生成

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摘 要: 观察金雀异黄酮(genistein)替代治疗对卵巢切除大鼠心肌中一氧化氮(nitric oxide, NO)和内皮型一氧化氮合酶(endothelial nitric oxide synthase, eNOS)的影响。成年雌性 Sprague-Dawley 大鼠经双侧卵巢切除术, 假手术组作为对照，术后三周将行卵巢切除术的大鼠随机分为低剂量 genistein (0.5 mg/kg·d^{-1})、高剂量 genistein (5.0 mg/kg·d^{-1})、17-β 雌二醇 (0.1 mg/kg·d^{-1})和模型组(100 μl /d 芝麻油)，各组均皮下注射给药并给予不含大豆的饲料喂养 6 周, 测定大鼠尾动脉血压、心率，麻醉后放血处死大鼠称量子宫重量；放免法检测血浆中总雌二醇，亚硝酸还原酶法检测心肌匀浆中 NO, Western blot 检测心肌中 eNOS 的表达以及 eNOS 的调节蛋白小凹蛋白 -1 (caveolin-1)和钙调素(calmodulin)的表达情况。结果显示各组间大鼠血压无显著性差异, 同 17-β 雌二醇一样, genistein 能呈剂量依赖性地增加心肌组织中 eNOS 表达量和 NO 生成, 同时 genistein 能明显降低内源性 eNOS 活性抑制物 caveolin-1 的表达, 而不影响 eNOS 活性正性调节蛋白钙调素的表达。与溶媒对照组比较，0.5 mg/kg·d^{-1} 的 genistein 不增加子宫重量, 5.0 mg/kg·d^{-1} 的 genistein 增加子宫重量 3 倍, 但较 17-β 雌二醇(增加 6 倍)的作用小(P<0.01)。上述结果提示，植物雌激素

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genistein 剂量依赖性地上调心肌组织 eNOS 的活性并增加 NO 的生成，减少抑制 eNOS 活性的小凹蛋白 -1 表达。

关键词: 金雀异黄酮；一氧化氮；内皮型一氧化氮合酶；小凹蛋白
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Population-based observational studies revealed that the incidence of cardiovascular events is higher in postmenopausal women than that in premenopausal women, but the increased incidence was cut in half after taking estrogen or phytoestrogen replacement therapy in postmenopausal women [1,2]. Estrogen and phytoestrogen have important protective effects on the cardiovascular system that are mediated, to a large extent, by an enhancement in nitric oxide (NO) production by the endothelial isoform of NO synthase (eNOS) due to increases in both eNOS expression and level of activation [3,4].

eNOS, originally identified in large vessel endothelium, is also expressed in cardiac myocytes. eNOS is quantitatively associated with caveolin, the structural protein of caveolae, which serves to inhibit eNOS. Cell stimulation with Ca^{2+} mobilizing agonists promotes calmodulin (CaM) binding to eNOS and caveolin dissociation from the enzyme, rendering the enzyme active [5]. The isoform caveolin (caveolin-1, Cav-1) expressed in myocytes, therefore, modulates the catalytic activity of cardiac eNOS and hence regulates NO production and its biological effects [6].

Estrogen is able to increase NO production through up-regulation of the activity of eNOS. Estrogen replacement therapy, however, has adverse effects on the reproductive system and a risk of venous thromosis that limit their therapeutic use. Genistein, a dietary-derived isoflavonoid bearing an isoflavonoid structure, has many protective effects of 17β-estradiol (E_2) on the cardiovascular system, such as reversing endothelial dysfunction in ovariectomied rats, improving the activity of eNOS, reducing infarct size in an experimental model of myocardial ischaemia-reperfusion injury [7,8]. However, it is unknown whether genistein influences the production of NO and expression of eNOS in hearts. In this study, we used genistein and E_2 supplementation to the ovariectomized rats to investigate their effects on NO production and the eNOS expression, and their effects on the posttranslational allosteric modulators of eNOS, caveolin-1 and calmodulin, in hearts.

1 MATERALS AND METHODS

1.1 *Animals preparation and experimental protocol*

Female mature Sprague-Dawley rats (200~220 g, Laboratory Animal Center, Central South University, China) were subjected to bilateral ovariectomy (OVX). Sham operated animals (sham) were used as controls. All rats were housed in standard conditions, light controlled cycle (06:00~18:00) and were given free access to soybean-free chow and drinking water. Experiments were consistent with the Guide for the Care and Use of Laboratory Animals (NIH Publication NO85-23, revised 1996).

OVX was performed as described in other studies [9]. Briefly, the rats were anesthetized using pentobarbital sodium (35 mg/kg, i.p.). The lower part of the back was shaved and a single 1.5 to 2 cm incision was made in the skin to expose the back muscles. A small 1 to 2 cm incision was made in the muscles overlying the ovaries on both sides, and the ovaries were isolated, tied off with sterile suture, and removed. The muscles and the skin were sutured separately, and the rats were allowed to recover for 3 weeks before the time of the experiment. Sham operation rats were performed by exposing the ovaries without isolation.

Three weeks after surgery, the OVX rats were randomly assigned to four treatment groups, 12 rats each group. The first group received the vehicle as model (OVX, 100 μl sesame oil, s.c. daily); the second group was given with a low dose of genistein (L-GEN, 0.5 mg/kg in 100 μl sesame oil, s.c. daily); the third group received a high dose of genistein (H-GEN, 5.0 mg/kg in 100 μl sesame oil, s.c. daily); the forth group received 17β-estradiol (E_2, 0.1 mg/kg in 100 μl sesame oil, s.c. daily) was used as the positive control; sham operation rats were treated with vehicle as control (sham, 100 μl sesame oil, s.c. daily). The treatment lasted for 6 weeks and all rats were given soybean-free diet during the treatment.

1.2 *Arteria systolic pressure, heart rate, body weight and uterine assay*

Systolic arterial blood pressure and heart rate (HR) were measured by the tail cuff method at baseline conditions. Body weight was also monitored at the same time points every three days. At the end of experiment uterus and the hearts were removed immediately and were subsequently weighed.

1.3 *Blood E_2 concentration and nitrite production in*

the myocardium

At the end of experiment, blood samples were collected from the carotid artery, plasma was obtained by centrifuging at 3 000×*g*, plasma E_2 concentration was measured by using the radioimmunoassay kit (Jiuding Biological Engineering Company, Tianjin). Nitrite production in the myocardium was determined by nitrate reductase method. Briefly, at the end of experiment, the rats were anesthetized using pentobarbital sodium (35 mg/kg, i.p.), the right carotid artery was cannulated and a PE-50 tube was inserted into the left ventricle. The heart was perfused with physiological saline and was taken out, the great vessel, atria and right ventricular free wall were removed, ventricular tissue samples were homogenized in a lysis buffer comprised of 25 mmol/L Hepes (pH=7.2), 140 mmol/L NaCl, 5.4 mmol/L KCl at 0 ºC. After centrifugation at 10 000×*g* for 5 min, reduction of nitrate to nitrite with the method of nitrate reductase kits (Jiancheng Biological Engineering, Nanjing), the amount of nitrite was corrected by protein amount which was measured by the Bradford method (Bio-Rad).

1.4 *Western blot analysis*

Western blot analysis was conducted as described elsewhere[6]. Briefly, ventricular tissue samples were homogenized in a lysis buffer (0.5 ml/100 mg tissue) comprised of 50 mmol/L Tris (pH 7.5), 0.1 mmol/L EGTA, 0.1 mmol/L EDTA, 2 μmol/L leupeptin, 1 mmol/L phenylmethylsulfonylfluoride, 1% (*V/V*) Nonidet P-40, 0.1% SDS, and 0.1% deoxycholate at 0 ºC, after centrifugation (12 000×*g* for 5 min) protein was quantified in the supernatant using Bradford assay. For Western blot analysis of eNOS, caveolin-1 and calmodulin, protein was separated through 8%, 12% SDS polyacrylamide gel and electrotransferred to PVDF membranes, unbound sites were blocked 2 h at room temperature with 5% (*W/V*) nonfat milk in Tris-buffered saline containing 20 mmol/L Tris-HCl (pH 7.6), 140 mmol/L NaCl, and 0.1% (*W/V*) Tween-20. The membranes were incubated with the specified primary antibody [anti-eNOS, anti-caveolin dilution at 1:1 000, and anti-calmodulin dilution at 1:800 (Santa Cruz Biotechnology)] in TBS buffer containing 5% nonfat dry milk overnight at 4 ºC. After 4 washes, the blots were incubated with secondary antibodies linked to horseradish-peroxidase labeled anti-rabbit IgG (Santa Cruz Biotechnology) for 1 h at room temperature. The blots were developed in a chemiluminescence system (Santa Cruz Biotechnology) and then visualized by exposure to Kodak X-ray film. The accuracy of protein loading on the gel was verified by re-probing with mouse monoclonal β-actin antibody (Neomaker Company). Densitometry was analyzed using the AlphaImager 2200 (Alpha Innotech).

1.5 *Statistical analysis*

Data are presented as mean ± SEM. Statistical analysis was performed with SPSS for Windows. Statistical comparison was determined by analysis of variance. $P<0.05$ was considered as being significantly different.

2 RESULTS

2.1 Effects of genistein and 17β-estradiol supplementation on ovariectomy

The effects of genistein treatment on body weight, heart weight, heart-to-body ratio, uterine weight, blood pressure (BP), heart rate (HR) and plasma concentrations of estradiol are presented in Table 1. Genistein treatment did not alter the decrease in plasma estradiol levels (< 3.5 pmol/L) produced by bilateral ovariectomy. BP and HR values in OVX rats treated with genistein were similar to those in vehicle-treated controls. OVX animals gained an average

Table 1. Effects of genistein and E_2 treatment on body weight, uterine weight, heart weight, heart-to-body ratio (HW/BW), blood pressure (BP), heart rate (HR) and serum concentrations of estradiol

	OVX	L-GEN	H-GEN	E_2	Sham
Δ Body weight (g)	105±11	99±8	102±6	67±9 *	71±7 *
Uterus (mg)	108±6	112±10	252±9*	637±35$^{*,**,\#}$	494±97$^{*,\#}$
Heart weight (mg)	995±48	976±59	985±43	933±32 *	925±45 *
HW/BW (mg/g)	3.16±0.09	3.17±0.06	3.18±0.08	3.33±0.09 *	3.31±0.07 *
BP (systolic mmHg)	132±8	124±7	121±6	125±10	122±9
HR (beats/min)	371±12	365±13	369±9	361±16	364±10
Estradiol (pmol/ L)	<3.5	<3.5	<3.5	132±18*,*	36±11*

mean ± SEM. Δ Body weight, the increase value of body weight during the whole experiment; OVX, ovariectomized treated with vehicle; L-GEN, treated with 0.5 mg/kg·d^{-1} genistein. H-GEN, treated with 5 mg/kg·d^{-1} genistein; Sham, sham operation rats treated with vehicle. $^{*}P<0.05$ *vs* OVX, $^{**}P<0.05$ *vs* sham rats, $^{\#}P<0.05$ *vs* H-GEN. $n=12$.

of (105±11) g, which was greater than that in sham-operated controls [(71±7) g, $P<0.05$]. The total heart weight in 17β-estradiol treated and sham groups was lower than that in OVX group, 17β-estradiol treatment resulted in a higher heart-to-body weight ratio than that in vehicle controls. Genistein did not change the gain of body weight and total heart weight compared with OVX group. In addition, genistein at dose of 0.5 mg/kg·d^{-1} did not augment uterus weight, compared with OVX treated with vehicle, dose of 5 mg/kg·d^{-1} augmented significantly uterus weight [(252±9) mg, $P<0.05$]. Rats treated with 17β-estradiol had greater uterus weight than that of the OVX treated with high dose of genistein [(637±35) mg, $P<0.01$].

2.2 Nitrite production in the myocardium

Nitrite production in the homogenized ventricular tissue was studied at the end of experiment. The effects of genistein and estradiol supplementation were shown in Fig. 1. Ovariectomy markedly reduced nitrite production in homogenized ventricular tissue. Genistein supplementation increased nitrite production in a dose-dependent manner. Genistein at the dose of 5 mg/kg·d^{-1} and E_2 treatment could restore the reduced nitrite production caused by OVX to the level similar to sham operated rats. Nitrite production in the myocardium showed no significant difference between the sham operated rats and E_2, H-GEN treated rats.

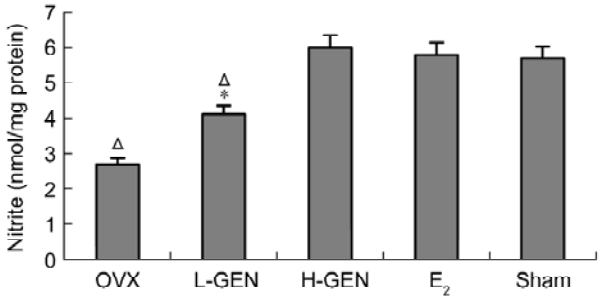

Fig. 1. Effects of genistein and E_2 supplementation on nitrite production in the homogenized ventricular tissue. The amount of nitrite was corrected by protein amount which was measured by the Bradford method. mean ± SEM. $^{*}P<0.05$ *vs* H-GEN, $^{\triangle}P< 0.05$ *vs* sham, n=12.

2.3 eNOS, caveolin-1 and calmodulin protein level in each group

As shown in a representative Western blot by densitometry analysis (Fig.2*A*), a significant reduction in the ventricular eNOS protein was detected in OVX rats compared with that in sham operated rats. Genistein supplementation increased the eNOS protein expression compared with that of the OVX group and displayed a dose-dependent effect. 5 mg/kg·d^{-1} genistein could restore the reduced eNOS protein expression caused by ovariectomy to the level similar to that in the sham operated controls. On the other hand, when compared with caveolin-1 expression in the drug treatment groups (Fig.2*B*), the result was reversed. Genistein decreased caveolin-1 expression and also displayed a dose-dependent effect. As shown in Fig.2*B*, calmodulin expression in heart tissues was not different among the five groups of rats.

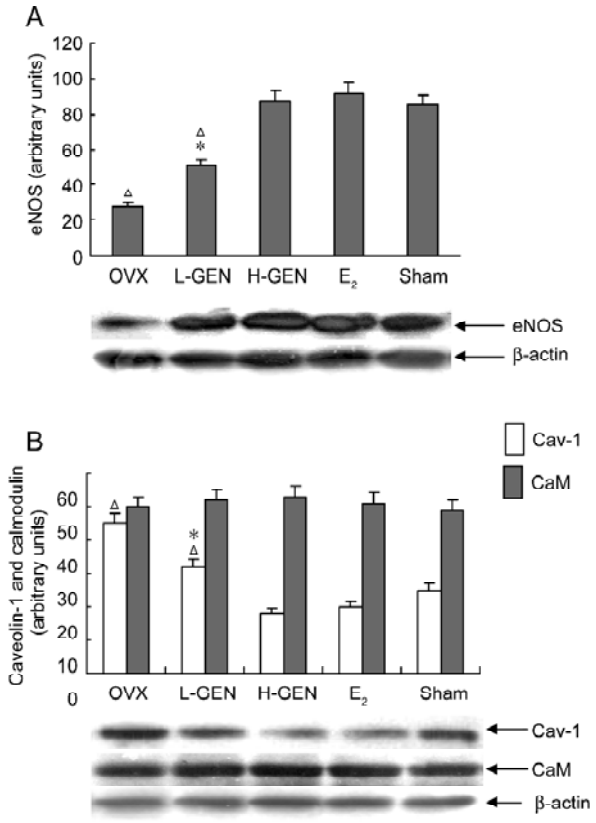

Fig. 2. *A*: Western blot analysis of effects of genistein and E_2 supplementation on eNOS protein expression in the rat hearts in each group. β-actin was used as internal control. *B*: Densitometric value of caveolin-1 (Cav-1) and calmodulin (CaM) protein expression in the rat hearts. $^{*}P<0.05$ *vs* H-GEN, $^{\triangle}P<0.05$ *vs* sham. n=12.

3 DISCUSSION

The present study demonstrated that phytoestrogen genistein supplementation increased eNOS protein expression and NO production in the hearts of OVX rats. The study was extended to investigate the two counterbalancing allosteric modulators, caveolin-1 and calmodulin in the regulation of eNOS activity by genistein treatment. Our data show: (1) both phytoestrogen genistein treatment and E_2 are able to increase the eNOS expression and NO production in the hearts of OVX rats in dose-dependent manners. (2) Caveolin-1, a negative modulator of eNOS, is increased by OVX and restores to normal level of ex-

pression after genistein and E_2 replacement, however, the positive modulator calmodulin is not changed by genistein and E_2 treatment. (3) Genistein and E_2 show overlapping effects on the regulation of NO, however, the side effects of genistein on the reproductive system was obviously less than that of E_2.

Genistein is a naturally occurring plant-derived estrogen-like compound and has been shown to mimic many of the biological activities of 17β-estradiol. Previous reports have demonstrated that genistein has, either *in vitro* or in experimental animals, a positive effect on cardiovascular protection, thus, raising the possibility that it may have the potential to be a cardiovascular-protective agent, with the benefit of no increased risk of cancer or less side effects in the reproductive system. More specifically, it has been reported that the protective effects of genistein supplementation on the cardiovascular apparatus may be mediated by an increased production of NO from the vasculature [10,11]. NO has several actions that are vasoprotective such as vasodilatation, inhibition of platelet adhesion and aggregation, and inhibition of smooth muscle cell proliferation and migration, therefore, an increase in NO release may improve the protective effect on the cardiovascular system [12]. In our study, a low dose of genistein (0.5 mg/kg·d^{-1}) induced NO metabolic production in OVX rats and high dose of genistein (5 mg/kg·d^{-1}) increased NO production similar to normal level in the myocytes. In addition, we found that low dose of genistein did not augment uterus weight, which was found when high genistein 5 mg/kg·d^{-1} was administered to the OVX rats. But the side effect on reproductive system is significantly less when compared with that of E_2. This implies that the dose of genistein is critical in the induction of tissue-dependent biological actions.

It was important to investigate the mechanism by which genistein enhances NO production in cardiac tissue. This is regulated not only by the amount of eNOS protein in cardiac tissue but also by its catalytic activity [13]. We demonstrated that the expression of cardiac eNOS protein was up-regulated by genistein and E_2 replacement to OVX rats. Therefore, it is reasonable to conclude that the increase in NO production in OVX rats resulted, at least partly, from an increased cardiac eNOS protein expression. In agreement with our findings, other researchers also have shown that genistein up-regulates the expression of eNOS level, increases the activity in endothelial cells and enhances NO release [14].

The precise molecular mechanisms of genistein and E_2 supplementation in the regulation of eNOS activity still remain unknown. eNOS activity is under a posttranslational regulation, recent reports have shown that the eNOS activity is influenced by a microdomain in cell membrane named caveolae [15]. A characterized mechanism for regulating eNOS activity is its binding to the caveolar protein, caveolin-1, that association represses eNOS activity. Stimulation of eNOS occurs when Ca^{2+}-activated calmodulin displaces caveolin-1 from its binding site on the eNOS molecule [16]. Therefore, the abundance of caveolin-1 is involved in the eNOS activity and NO production. It has recently been reported that the protein expression of caveolin-1 in median eminence of rats was markedly increased by estrogen depletion, the increased caveolin-1 inhibited the activity of eNOS and reduced eNOS-dependent NO production [17]. In cerebral blood vessels, caveolin-1 and eNOS expressions altered in opposite directions with chronic estrogen depletion and repletion in female rats [18,19]. These results imply that estrogen can, either through a genomic or non-genomic effect on modulation caveolin-1, influence the activity of eNOS. In the present study, we found that not only E_2 but also genistein, in addition to theirs association with higher eNOS expression in the rat cardiac myocyte, was also associated with lower ventricular caveolin-1 expression. These findings, therefore, point to the possibility that the genistein-related potentiation of eNOS-dependent NO production is due not only to an up-regulation of eNOS protein expression, but also to a combination of increased eNOS and diminished caveolin-1 expression in the heart.

As a cautionary note, Western blot evaluations do not reveal whether the proteins are interacting in any way. Nevertheless, a higher ventricular expression of eNOS relative to caveolin-1 can be viewed as suggestive of a less eNOS/caveolin-1 association and a greater functional eNOS activity. Furthermore, we demonstrated that NO metabolic production, nitrite, was increased after genistein treatment. This suggests that genistein can increase the functional activity of cardiac eNOS.

In summary, we report that genistein, in a dose with similar potency to E_2, markedly increases eNOS protein expression and functional activity that can lead to the production of NO, a vasoactive molecule, in the hearts of OVX rats. These findings implicate that genistein has a potential protection to resist the diseases in which the release of NO is reduced in the heart. Phytoestrogen genistein supplementation increases eNOS and decreases caveolin-1 expression in ovariectomized rat hearts.

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