Chronic Administration of Daidzein, a Soybean Isoflavone, Improves Endothelial Dysfunction and Attenuates Oxidative Stress in Streptozotocin-induced Diabetic Rats

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The effect of chronic daidzein, a soybean isoflavone, on aortic reactivity of streptozotocin-diabetic rats was studied. Male diabetic rats received daidzein for 7 weeks a week after diabetes induction. Contractile responses to KCl and phenylephrine (PE) and relaxation response to acetylcholine (ACh) were obtained from aortic rings. Maximum contractile response of endothelium-intact rings to PE was significantly lower in daidzein-treated diabetic rats relative to untreated diabetic rats, and endothelium removal abolished this difference. Endothelium-dependent relaxation to ACh was significantly higher in daidzein-treated diabetic rats as compared with diabetic rats and pretreatment of rings with nitric oxide synthase inhibitor N(G)-nitro-l-arginine methyl ester and/or indomethacin attenuated it. Two-month diabetes also resulted in an elevation of malondialdehyde (MDA) and decreased superoxide dismutase (SOD) activity, and daidzein treatment significantly reversed the increased MDA content and reduced activity of SOD. Therefore, chronic treatment of diabetic rats with daidzein could prevent some abnormal changes in vascular reactivity in diabetic rats through nitric oxide and prostaglandin-related pathways, and via attenuation of oxidative stress in aortic tissue and endothelium integrity seems essential for this effect. Copyright © 2012 John Wiley & Sons, Ltd.

Keywords: daidzein; diabetes mellitus; streptozotocin; aorta; oxidative stress.

INTRODUCTION

The prevalence of diabetes mellitus (DM) is increasing worldwide: it is a major health problem in the 21st century, and prevalence of DM is estimated to increase to 366 million by 2030 (Wild et al., 2004). Cardiovascular disorders continue to constitute major causes of morbidity and mortality in diabetic patients in spite of significant achievements in their diagnosis and treatment (Cocherl, 2007). Changes in vascular responsiveness to vasoconstrictors and vasodilators are mainly responsible for the development of some vascular complications in diabetes (Nasri et al., 2011). Most of these complications are due to increased serum glucose and augmented generation of reactive oxygen species, which finally lead to endothelium dysfunction (Naito et al., 2011).

Soybean (Glycine max L.) has long been known as one important source of protein. In addition to protein, soybean also contains various nutrients and functional components including isoflavones such as daidzein (Mateos-Aparicio et al., 2008). Epidemiological evidence suggests that consuming soy products in population lowers incidence of cardiovascular disease, and this has led to the suggestion that isoflavones may be beneficial for cardiovascular health because of their protective property (Erdman, 2000; Goodman-Gruen and Kritz-Silverstein, 2001; Moore, 2011). Studies in experimental animals in which cardiovascular responses to these isoflavones have been assessed are broadly supportive of their protective role. In this respect, daidzein has been shown to improve vascular function through nitric oxide (NO) pathway (Sobey et al., 2004; Woodman et al., 2004). Endothelium-dependent relaxation of rat aorta and main pulmonary artery by daidzein has also been reported (Mishra et al., 2000). Meanwhile, daidzein exerts protective effects on ventricular remodeling in rats with myocardial hypertrophy (Zhou et al., 2007). Soy isoflavonoes may improve glucose homeostasis and delay progression of type 2 diabetes as reviewed before (Kwon et al., 2010), and there is some evidence on antidiabetic potential of daidzein (Choi et al., 2008). Nevertheless, the in vivo protective effect of daidzein on vascular system in diabetes has not been documented yet. Therefore, this study was designed to assess for the first time the...
beneficial effect of chronic daidzein treatment on improvement of aortic reactivity dysfunction of streptozotocin (STZ)-diabetic rats and to investigate some underlying mechanisms.

MATERIALS AND METHODS

Animals

Male albino Wistar rats (Pasteur’s institute, Tehran, Iran) weighing 220–280 g were housed in an air-conditioned colony room at 21 ± 2 °C and were supplied with standard pellet diet and tap water ad libitum. Procedures involving animals and their care were conducted in conformity with National Institutes of Health guidelines for the care and use of laboratory animals.

Experimental protocol

The rats were rendered diabetic by a single intraperitoneal dose of 60 mg kg⁻¹ STZ freshly dissolved in ice-cold 0.1 M citrate buffer (pH 4.5). Age-matched normal animals that received an injection of an equivalent volume of buffer comprised a non-diabetic control group. One week after STZ injection, overnight fasting blood samples were collected, and serum glucose concentrations were measured using glucose oxidation method (Zistchimie, Tehran). Only those animals with a serum glucose level higher than 250 mg/dl were selected as diabetic. During the next weeks, diabetes was reconfirmed by the presence of polyphagia, polydipsia, polyuria and weight loss. Normal and hyperglycemic rats (a total of 48) were randomly allocated and similarly grouped into six groups (eight in each): normal vehicle (N), diabetic, diabetic treated with 0.1 M daidzein, diabetic treated with 1.5 g daidzein per 1.5 g body weight, diabetic treated with 1.5 g daidzein per 1.5 g body weight for 40 min, and diabetic treated with 1.5 g daidzein per 1.5 g body weight for 40 min and 30 min before the experiment with NO. Rats were allowed to recuperate for at least 30 min.

At the end of the equilibration period, dose–response curves with KCl (10-50 mM) and PE (10⁻³–10⁻⁵ M) in the presence and absence of endothelium were obtained in aortic rings in a cumulative manner. To evaluate ACh (10⁻⁴–10⁻⁵ M)-induced vasodilatation in rings with endothelium, they were preconstricted with a submaximal concentration of PE (10⁻⁶ M), which produced 70–80% of maximal response. The sensitivity to the agonists was evaluated as pD2, which is the negative logarithm of the concentration of the drug required to produce 50% of the maximum response.

To determine the participation of NO, rings were incubated 30 min before the experiment with N (omega)-L-arginine methyl ester (L-NAME) (100 µM, a non-selective NO synthase inhibitor). To determine the participation of endothelial vasodilator factors in response to ACh, segments were incubated with indomethacin (INDO) (10 µM, an inhibitor of cyclooxygenase-derived prostanoide synthesis) 30 min before the experiment with ACh.

After each vasoreactivity experiment, aortic rings were blotted and weighed, and the cross-sectional area (csa) was calculated using the following formula: cross-sectional area (mm²) = weight (mg) × [length (mm) × density (mg mm⁻²)]⁻¹. The density of the preparations was assumed to be 1.05 mg/mm² (Abebe et al., 1990).

Determination of MDA concentration in aortic rings

After removing aortic segments and cleansing them of extra tissues, they were blotted dry and weighed, then made into 5% tissue homogenate in ice-cold 0.9% saline solution. A supernatant was obtained from tissue homogenate by centrifugation (1000 × g, 4 °C, 5 min). The malondialdehyde (MDA) concentration (thiobarbituric acid reactive substances, TBARS) in the supernatant was measured as described before (Roghani and Baluchnejadmojarad, 2009). Briefly, trichloroacetic acid and TBARS reagent were added to supernatant, then mixed and incubated at 100 °C for 80 min. After cooling, the sample was centrifuged at 1000 × g for 20 min, and the absorbance of the supernatant was read at 532 nm. TBARS results were expressed as MDA equivalents using tetraethoxypropane as standard.

Measurement of SOD activity in aortic rings

The supernatant of tissue homogenate were obtained as described earlier (Baluchnejadmojarad and Roghani, 2008). Briefly, supernatant was incubated with xanthine and xanthine oxidase in potassium phosphate buffer (pH 7.8, 37 °C) for 40 min, and nitro-blue tetrazolium (NBT) was added to the bath in order to assess the endothelial integrity of the preparations. Endothelium was considered to be intact when this drug elicited a vasorelaxation ≥50% of the maximal contraction obtained in vascular rings precontracted with PE. The absence of acetylcholine relaxant action in the vessels indicated the total removal of endothelial cells. After assessing the integrity of the endothelium, vascular tissues were allowed to recuperate for at least 30 min.

Isometric contractions were induced by the addition of phenylephrine (PE, 1 µM), and once the contraction was stabilized, a single concentration of acetylcholine (ACh) (1 µM) was added to the bath in order to assess the endothelial integrity of the preparations. Endothelium was considered to be intact when this drug elicited a vasorelaxation ≥50% of the maximal contraction obtained in vascular rings precontracted with PE. The absence of acetylcholine relaxant action in the vessels indicated the total removal of endothelial cells. After assessing the integrity of the endothelium, vascular tissues were allowed to recuperate for at least 30 min.

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(NBT) was added. Blue formazan was then monitored spectrophotometrically at 550 nm. The amount of protein that inhibited NBT reduction to 50% maximum was defined as 1 nitrite unit of superoxide dismutase (SOD) activity.

Chemicals

Phenylephrine, daidzein, STZ, Cremophor, ACh, INDO and L-NAME were purchased from Sigma Chemical (St. Louis, Mo., USA). All other chemicals were purchased from Merck (Germany) and Darupakhsh Co. (Tehran, Iran). Indomethacin solution was prepared in ethanol in such a way that the maximal ethanol concentration of the medium was less than 0.001% (v/v).

Data and statistical analysis

All values were given as means±SEM. Contractile response to PE was expressed as grams of tension per cross-sectional area of tissue. Relaxation response for ACh was expressed as a percentage decrease of the maximum contractile response induced by PE. Statistical analysis was carried out using repeated measure ANOVA and one-way ANOVA followed by Tukey post-hoc test. A statistical p-value less than 0.05 was considered significant.

RESULTS

After 8 weeks, the weight of the vehicle-treated diabetic rats was found to be significantly decreased as compared with controls (p<0.01) and daidzein treatment at both doses, especially at a dose of 10 mg/kg, caused a non-significant lower reduction in weight in diabetic rats as compared with vehicle-treated diabetic rats. Untreated diabetic rats had also an elevated serum glucose level over those of control rats (p<0.0005), and treatment of diabetic rats with daidzein at a dose of 10 mg/kg caused a significant decrease in the serum glucose level relative to diabetic rats (p<0.05). In addition, daidzein treatment of control rats did not produce any significant change regarding serum glucose level (Fig.1).

Cumulative addition of KCl (10–50 mM) and PE (10^{-16}–10^{-5} M) resulted in concentration-dependent contractions in aortas of all groups (Fig. 2). The maximum contractile responses to KCl and PE in the aortas from vehicle-treated diabetic rats in the presence of endothelium were found to be significantly (p<0.01–0.005) greater than vehicle-treated control rats, and concentration–response curve of endothelium-intact aortas from daidzein-treated diabetic rats (at a dose of 10 mg/kg) to PE (and not to KCl) was significantly attenuated compared with vehicle-treated diabetic rats (p<0.05).

Although endothelium-denuded aortic rings in all groups showed a higher contractile response to KCl and PE, the observed changes between treated and untreated diabetic rats were attenuated after endothelium removal. This clearly indicates the necessity of endothelium presence for beneficial vascular effect of daidzein. In addition, aortic rings with endothelium from daidzein-treated control group showed a non-significant reduction in contractile response to KCl and PE as compared with vehicle-treated controls. There were also no significant differences among the groups in terms of the pD2 (data not shown), indicating that there has not been any significant change in the sensitivity of aortic rings from different groups.

Addition of ACh resulted in concentration-dependent relaxations in all aortic rings precontracted with PE (Fig. 3). As was expected, endothelium-dependent relaxation responses induced by ACh was significantly lower in vehicle-treated diabetic rats in relation to vehicle-treated controls (p<0.05–0.005). Meanwhile, the existing difference between daidzein-treated (at a dose of 10 mg/kg) and vehicle-treated diabetic rats was only significant (p<0.05) at concentrations higher than 10^{-5} M. Relaxation response of daidzein-treated control rats was non-significantly greater than control group. Regarding relaxation response to ACh, pre-incubation of aortic rings with L-NAME almost completely abolished the vasodilator response to ACh in segments from daidzein20-treated diabetic rats, indicating the important role of endothelium-derived NO in the vascular effect of daidzein (Fig. 4).

Pre-incubation of aortic segments from daidzein10-treated diabetic rats with INDO also moderately and significantly diminished the endothelial vasodilator response to ACh (p<0.05–0.01) (Fig. 4).

Regarding aortic lipid peroxidation markers (Table 1), STZ-induced diabetes resulted in an elevation of MDA content, and decreased SOD activity (p<0.005–0.001)
in aortic tissue and chronic treatment of diabetic group with daidzein at a dose of 10 mg/kg significantly reversed the increased MDA content \( (p < 0.05) \) and reduced activity of SOD \( (p < 0.005) \).

**DISCUSSION**

In this study, administration of daidzein for 7 weeks did have a mild hypoglycemic effect: it reduced the enhanced contractility of aortic rings to PE and increased ACh-induced relaxation, which was partly due to the involvement of NO and prostaglandins pathways because the relaxation was blocked in the presence of L-NAME and/or INDO. In addition, endothelium removal clearly affected PE-induced contractions in daidzein-treated diabetic rats. Regarding oxidative stress markers, daidzein treatment attenuated the increased MDA content and reduced activity of SOD.

Vascular dysfunction is one of the complicating features of diabetes in humans, and its experimental model and hyperglycemia is the primary cause of micro and macrovascular complications in diabetic condition (Madonna and De Caterina, 2011). Compared with the aortic rings from control animals, contraction of aortas to KCl and PE from diabetic rats significantly increased, which was consistent with the previous studies (Roghani and Baluchnejadmojarad, 2009), and chronic daidzein was capable to attenuate this change only for PE-induced contractions. Impaired endothelial function (Olukman et al., 2010), enhanced sensitivity of calcium channels (Chang et al., 1993), increased vasoconstrictor prostanoids due to increased superoxide anions and increased sensitivity to adrenergic agonists (Abebe, 2008) might all be responsible for increased contractile responses in diabetic rats, which may have been improved following daidzein treatment.

In endothelial cells of most vascular beds, ACh could stimulate production and release of endothelial-derived relaxing factors including nitric oxide, prostacyclin and endothelium-derived hyperpolarizing factor and, in this way leads, to relaxation of vascular smooth muscle in an endothelium-dependent manner (Flammer and Luscher, 2010; Takaki et al., 2008; Zhang et al., 2011). The ACh-induced relaxation response is endothelium-dependent and NO-mediated (Roghani and Baluchnejadmojarad, 2009). The results of this work revealed that the endothelium-dependent relaxant response was reduced in aortas from STZ-induced diabetic rats, and this reduced relaxation was partially recovered by daidzein treatment. Although some researchers asserted that the

**Figure 2.** Cumulative concentration–response curves for KCl and phenylephrine in aortic preparations 8 weeks after experiment in the presence (A) and absence (B) of endothelium (means ± SEM). \# \( p < 0.05 \) (as compared with diabetic).

**Figure 3.** Cumulative concentration–response curves for ACh in endothelium-intact aortic rings precontracted with phenylephrine 8 weeks after experiment. Relaxation responses are expressed as a percentage of the submaximal contraction induced by phenylephrine, which produced 70–80% of maximal response (means ± SEM). \# \( p < 0.05 \) (as compared with diabetic).
sensitivity to acetylcholine decreases in diabetes (Abebe, 2008), the results of this research, in accordance with those of many previous ones (Silan, 2008), reveals that diabetes condition in long term only decreases the maximum responses to ACh but not the sensitivity (pD2).

Impaired endothelium-dependent relaxation in STZ-induced diabetic rat might be due to increased blood glucose level and decreased blood insulin level. It has been shown that hyperglycaemia causes tissue damage with several mechanisms, including advanced glycation end product formation, increased polyol pathway flux, apoptosis and reactive oxygen species formation (Hartge et al., 2007). Our results showed that daidzein treatment could exert a mild hypoglycemic effect in STZ-induced diabetic rats; therefore, its beneficial effect on aortic tissue of diabetic rats should be partly due to its hypoglycemic effect. Some damaging effect of diabetes on vascular tissue of diabetic animals is also believed to be due to enhanced oxidative stress, as shown by enhanced MDA and decreased activity of defensive enzymes such as SOD (Baluchnejadmojarad and Roghani, 2008), and as was observed in this study. This could also lead to diabetes-induced functional changes in vascular endothelial cells and the development of altered endothelium-dependent vasoreactivity. The results of the present study showed that chronic treatment of daidzein significantly decreased MDA content and enhanced SOD activity in aortic tissue from diabetic rats, indicating that the improvement in vascular responsiveness from daidzein may be partly due to ameliorating lipid peroxidation and oxidative injury. These results clearly suggested that another cause of
the effect of daidzein on improving the endothelial dysfunction is due to its antioxidative activity.

In conclusion, to the best of our knowledge, this is the first study to report that in vivo chronic treatment of diabetic rats with daidzein dose-dependently could prevent the functional changes in vascular reactivity observed in diabetic rats through NO and prostaglandin-dependent pathways, and via attenuation of aortic lipid peroxidation. Our data may be helpful in the development of new natural drugs to improve endothelial function and to prevent cardiovascular diseases.

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REFERENCES


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