THE ROLE OF L-TYPE CALCIUM CHANNELS IN THE VASCULAR EFFECT OF *TRIGONELLA FOENUM-GRAECUM L.* IN DIABETIC RATS

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ABSTRACT

Some ion channels like voltage-operated calcium channels (VOCC) within the plasma membrane of vascular muscle cells from the walls of resistance arteries and arterioles play a central role in the regulation of vascular tone. On the basis of reports about the beneficial attenuating effect of fenugreek (*Trigonella foenum-graecum L.;* TFG) on the contractile reactivity of aortic rings of diabetic rats, this study was carried out to evaluate the possible involvement of L-type voltage-operated calcium channels in the vascular effect of this medicinal plant. For this purpose, male Wistar rats were made diabetic using streptozotocin (STZ, 60 mg/Kg, i.p). The extract-treated control and diabetic rats received aqueous leaf extract of TFG (200 mg/Kg, i.p.) every other day for two months. At the end of the study, contractile response of isolated aortic rings to KCl and noreadrenaline (NA) was determined in the absence and presence of the calcium channel blocker nifedipine. The results showed that aortic rings from diabetic rats are more responsive to the effect of KCl and NA than those of controls, TFG extract treatment could attenuate the enhanced contractile response of aortic rings of diabetic rats, and nifedipine pretreatment could partially neutralize the beneficial effect of this extract. It is concluded that TFG extract attenuates the enhanced vascular reactivity in chronic diabetic rats and voltage-operated calcium channels are in part responsible for this effect of TFG extract.

Keywords: Fenugreek, Aorta, Contractility, Calcium channel, Rat

INTRODUCTION

Ion channels in the plasma membrane of vascular muscle cells that form the walls of resistance arteries and arterioles play a central role in the regulation of vascular tone (1). Current evidence indicates that vascular smooth muscle cells express different types of voltage-gated Ca^{2+} channels, which may be involved in the regulation of vascular tone. Calcium influx through these channels provides a major source of activator Ca^{2+} used by resistance arteries and arterioles (2). Fenugreek (*Trigonella foenum-graecum L.*) is generally a plant with traditional medicinal use in diabetes and its beneficial effects have been demonstrated in diabetic animals and both insulin-dependent and non-insulin-dependent diabetic animals (3-5). In the previous studies, endothelium-dependent attenuating effect of *Trigonella foenum-graecum L.* on the contractile vascular reactivity of aortic rings of diabetic rats were shown (6) and this experimental study was carried out to evaluate the role of voltage-gated calcium channels in the vascular effect of aqueous leaf extract of this medicinal plant in male diabetic rats.

MATERIALS AND METHODS

Drugs and Chemicals

Noradrenaline bitartrate, nifedipine, and acetylcholine-HCl were purchased from Sigma Chemical (St. Louis, Mo., USA). Streptozotocin was obtained from Upjohn Chemical company (France). Prednisolone, imipramine, and timolol were obtained from Darupakhsh and Sinadarou pharmaceutical companies (Tehran, Iran) as a gift. All other chemicals were purchased from Merck (Germany). Nifedipine as a calcium channel blocker was dissolved in dimethylsulfoxide (DMSO). Further dilutions of the drugs were made in Physiological Saline Solution (PSS).

TFG Extract Preparation

Fresh fenugreek was obtained from local grocery (Tehran) in April 2002 and was systemically identified by the botanists in Department of Biology (Shaheed Beheshti University, Tehran, Iran). Then, green leaves were separated, cleaned, and shade dried at room temperature. Thereafter, 125 g of dried leaves was grounded and the obtained powder was mixed with 1000 ml of

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boiling distilled water for a period of 15 min under continuous stirring. The obtained mixture was filtered twice through a mesh and the resulting liquid was dried under continuous stirring (30-34 ºC), until a concentrated residue (67% w/w) was obtained (29 g). This stock extract was maintained at –20 ºC until being used. Fenugreek extract of lower concentrations was prepared by dilution of the stock with cold sterile 0.9% saline solution.

Animals

Male albino Wistar rats (Pasteur's institute, Tehran, Iran) weighing 225-265 g (8-10 weeks old) were housed in an air-conditioned colony room on a light/dark cycle (20-22 °C with a humidity of 30-40%) and supplied with standard pellet diet and tap water ad libitum. Procedures involving animals and their care were conducted in accordance with the NIH guidelines for the care and use of laboratory animals.

Experimental Procedure

The animals were randomly divided into four experimental groups: vehicle-treated control (VC, n = 8), extract-treated control (EC, n = 8), vehicle-treated diabetic (VD, n = 10), and extract-treated diabetic (ED, n = 10). Diabetes was induced by a single intraperitoneal injection of streptozotocin (STZ, 60 mg/Kg) dissolved in cold 0.9% saline immediately before use. Control and extract-treated animals received every other day normal sterile saline solution and aqueous extract of fenugreek extract (200 mg/Kg, i.p.) respectively. This extract was also administered every other day to the extract-treated diabetic animals from third day of the study. Serum glucose levels and body weight were measured one week before and four and eight weeks after the experiment was started. Diabetes was verified by a serum glucose level higher than 250 mg/dl using glucose oxidation method (glucose oxidase kit, Zistchimie, Tehran). All treatments continued for two months.

After two months, the rats were anesthetized with diethyl ether, decapitated, and through opening the abdomen, descending thoracic aorta was carefully excised and placed in cold physiological saline solution (PSS) containing (mM): NaCl (118), KCl (4.6), $MgSO_4$ (1.2), KH_2PO_4 (1.2), glucose (11.1), $NaHCO_3$ (27.2), and $CaCl_2$ (1.8). Thereafter, the aorta was cleaned from excess connective tissues and fat and cut into rings of approximately 4 (3-5) mm in length. Aortic rings were suspended between two triangular-shaped wires. One wire was attached to a fixed tissue support in a 50 ml isolated tissue bath containing PSS (pH 7.4) which was maintained at 37°C and continuously aerated with a mixture of 5% CO_2 and 95% O_2. The other end of each wire was attached through a cotton thread to Narco Biosystems F60 isometric force transducer coupled to signal amplifier and connected to a Pentium-III (IBM-compatible) computer via an A/D interface. Recording and analysis of data were performed using Behineh Arman Physiograph I software. In all experiments, special care was taken to avoid damaging the luminal surface of endothelium. The rings were allowed to equilibrate for 90 min under a resting tension of 2 g before starting of experiments which in preliminary experiments was shown to be the optimal resting tension. During equilibration period, the rings were washed every 30 min. For examination of the endothelial integrity, pre-constricted rings with noradrenaline (NA, 1 μM) were exposed to 10 μM acetylcholine (ACh). All experiments for NA were done in the presence of 1 μM timolol, 1 μM imipramine, and 1 μM prednisolone to eliminate the effects of β-adrenoceptors, neuronal uptake, and extraneuronal uptake respectively. Then, concentration-response curves were obtained with KCl and thereafter with NA in aortic rings with or without endothelium. In this respect, KCl (10-50 mM) and NA (10^{-9}-10^{-4} M) were added in a cumulative manner until a maximum response was achieved. In all experiments, after addition of each dose, a plateau response was obtained before addition of a subsequent dose. Another alternate series of rings were pretreated with nifedipine as a calcium channel blocker 5 min before addition of KCl and/or NA as a vasoconstrictor.

After each experiment, aortic rings were dried at 45°C for 5 min, weighed, and cross-sectional area (CSA) was calculated using the following formula: Cross-sectional area (mm^2) = weight (mg) × [length (mm) × density (mg/mm^3)]$^{-1}$. The density of the preparations was assumed to be 1.05 mg/mm^3 (7)

Data and statistical analysis

All values were given as means ± S.E.M. and were analyzed by repeated measure ANOVA (body weight and serum glucose level) and one way ANOVA (contractile responses) with a significant level of $P<0.05$.

RESULTS

Body weight, Serum glucose, and Cross-sectional area

Body weight and serum glucose level were measured before and at different weeks after the experiment was started (Table 1). At the end of 8th week, the body weight of the vehicle-treated diabetic rats was found to be significantly lower as compared to data that were obtained one week before starting of experiment ($p<0.01$). Vehicle-treated diabetic rats also had elevated serum

Table 1. Body weight and serum glucose level of control, diabetic, and extract-treated diabetic rats at different weeks

	Body weight (g)			Serum glucose (mg/dl)		
	Week 0	Week 4	Week 8	Week 0	Week 4	Week 8
Control	238.1 ± 4.2	267.5 ± 5.9	294.3 ± 4.9	102.7 ± 4.1	96.8 ± 3.5	107.4 ± 4.1
Control + TFG	237.7 ± 5.8	248.4 ± 4.3	258.3 ± 5.4	99.8 ± 5.6	91.7 ± 4.2	87.6 ± 4.8
Diabetic	241.7 ± 5.4	190.7 ± 6.7[b]	176.6 ± 6.5[c]	104.7 ± 4.7	379.8 ± 15.7[c]	391.5 ± 18.9[c]
Diabetic + TFG	227.3 ± 5.2	214.9 ± 7.1[a]	211.7 ± 6.7[b]	96.5 ± 5.8	281.7 ± 12.9[b]	241.9 ± 13.1[b]

a: P<0.05, b: P<0.01, c: P<0.001 (In comparison with control group)

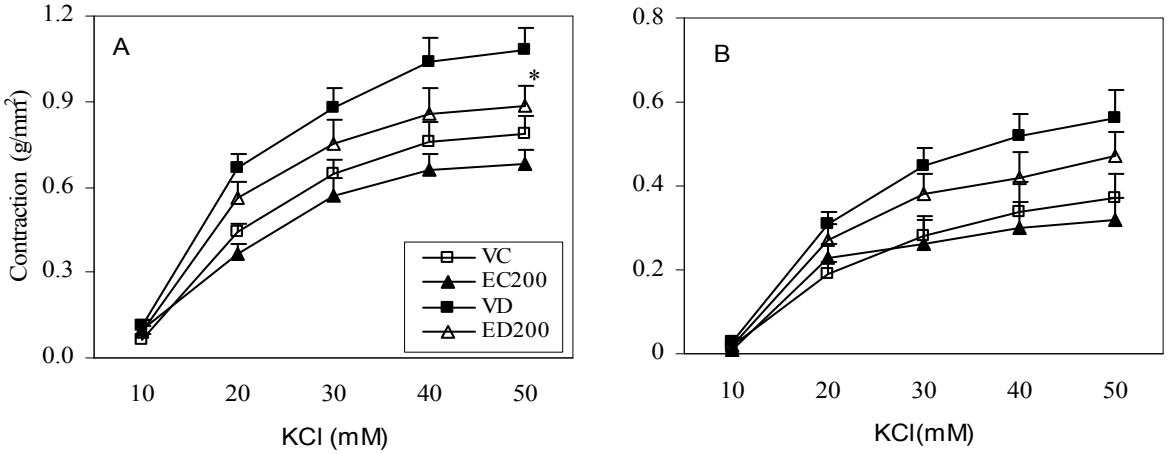

Figure. 1. Cumulative concentration-response curves for contractile reactivity of aortic rings to KCl (10-50 mM) from vehicle-treated control (VC), extract-treated control (EC200), vehicle-treated diabetic (VD), and extract-treated diabetic group (ED200) in the absence (A) and presence (B) of nifedipine pretreatment. The TFG extract was administered at a dose of 200 mg/Kg (i.p.) for two months. * *P*<0.05 (as compared to VD)

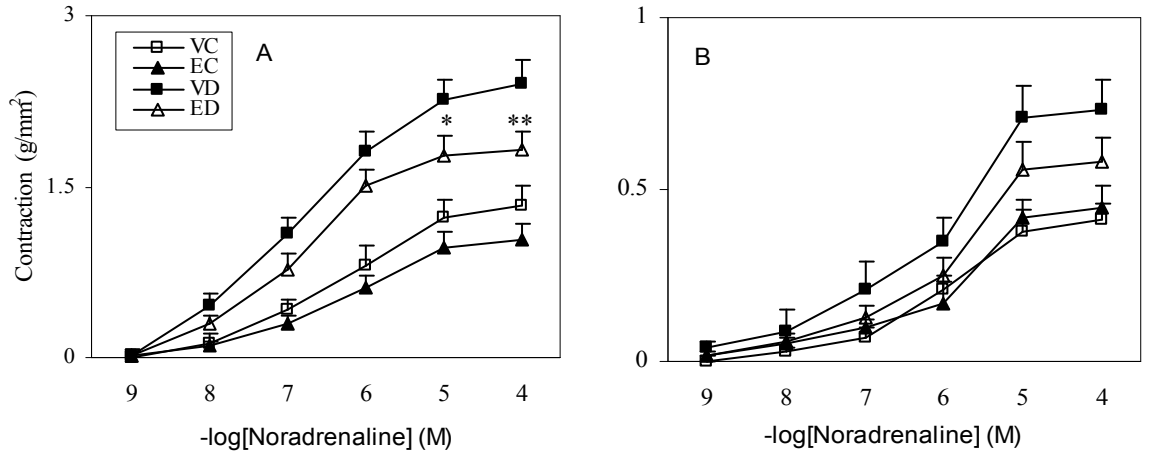

Figure 2. Cumulative concentration-response curves for contractile reactivity of aortic rings to noreadrenaline (10^{-9}-19^{-4} M) from vehicle-treated control (VC), extract-treated control (EC), vehicle-treated diabetic (VD), and extract-treated diabetic group (ED) in the absence (A) and presence (B) of nifedipine pretreatment. The TFG extract was administered at a dose of 200 mg/Kg (i.p.) for two months. * P<0.05, ** P<0.01 (as compared to VD)

glucose level compared with those of control rats. Treatment of diabetic rats with fenugreek extract at a dose of 200 mg/kg for a period of two months caused a significant reduction in serum glucose level and an increase in body weight in extract-treated diabetic rats as compared to vehicle-treated diabetics ($p<0.05$). Furthermore, there was a significant reduction in cross-sectional area of aortic rings in vehicle-treated-diabetic group which was not improved by administration of the extract.

Vascular Contractility

Addition of KCl in a cumulative manner (10-50 mM) resulted in concentration-dependent contraction in aortic rings of all groups (Fig. 1). The contractile response to this non-specific agonist at concentrations greater than 20 mM in vehicle-treated diabetic rats was significantly higher than control rats, and treatment of diabetic rats with TFG at a dose of 200 mg/Kg for two months caused a significant reduction ($p<0.05$) in contractile response of aortic rings to NA at a concentration of 50 mM (Fig. 1A). Furthermore, treatment of control rats with TFG leaf extract did not produce any significant changes in contractile responsiveness of aortic rings. On the other hand, nifedipine pretreatment partially and significantly reduced, but not abolished the observed difference between extract-treated diabetic and vehicle-treated diabetic groups (Fig. 1B).

Cumulative addition of NA (10^{-9}-10^{-4} M) to the organ bath resulted in concentration-dependent contraction in aortic rings in all of the groups (Fig. 2). The contractile response to NA at concentrations higher than 10^{-7} in vehicle-treated diabetic rats was found to be significantly higher than vehicle-treated control rats, and treatment of diabetic rats with TFG at a dose of 200 mg/Kg for a period of two months caused a significant reduction in contractile response of aortic rings to NA (at concentrations equal or higher than 10^{-5}) (Fig. 2A). Furthermore, treatment of control rats with TFG leaf extract did not produce any significant changes in contractile responsiveness of aortic rings. Meanwhile, pretreatment of rings with nifedipine as a calcium channel blocker partially and significantly reduced the observed difference between TFG-treated diabetic and diabetic groups (Fig. 2B).

DISCUSSION

The results of the present study demonstrated that aortas from 2-months STZ-diabetic rats are more responsive to the effect of KCl and NA than those of the corresponding controls, TFG extract treatment could attenuate the enhanced contractile response of aortic rings from diabetic rats, and nifedipine pretreatment could partially neutralize the beneficial effects of this extract.

Similar results showing increased vascular responsiveness to contractile agents in STZ-diabetic rats has also been reported previously (7-8). In this respect, some possible factors that could be involved in the increased vascular smooth muscle responsiveness to α_1-adrenoceptor agonists in diabetic rats are a) deficient endothelial activity, b) enhanced phosphoinositide (PI) metabolism (9), c) enhanced sensitivity of calcium channels (7), and d) increased sensitivity to adrenergic agonists (10). Furthermore, oxidative stress is increased due to excessive production of oxygen-free radicals and decreased antioxidant defense systems (11) and this phenomenon could be responsible for augmented contractility together with deficient endothelial activity in diabetic state (12).

Our results clearly demonstrated that fenugreek extract at a dose of 200 mg/kg could partially counteract the increased contractile response of endothelium-intact aortic rings of diabetic rats following application of KCl and NA. The beneficial effects of fenugreek extract on KCl- and NA-induced contractions was specific for aortas of diabetic rats, because the extract treatment did not produce any significant changes in control preparations. The results (unpublished data for pD_2) also directly indicate that TFG extract treatment did not change the sensitivity of vascular smooth muscle of diabetic rats to NA. The protective effect of aqueous leaf extract of TFG could be attributed to its glycosidic compounds with hypoglycemic and anti-hyperglycemic properties (3). In this study, pretreatment of aortic rings from TFG extract-treated diabetic rats caused an attenuation (not abolishment) of the beneficial effects of the extract. This clearly indicate that other mechanisms and pathways including endothelial-derived relaxing factor (nitric oxide), vasorelaxant prostaglandins, and/or reduced mobilization of intracellular calcium may also be involved in the reduction of the the effects of TFG extract (13-14). Further studies are warranted to evaluate the possible related mechanisms.

In conclusion, the findings of this study clearly indicated that treatment of diabetic rats with aqueous leaf extract of fenugreek could partially attenuate the increased reactivity of the thoracic aorta and this beneficial effect is mediated in part through voltage-operated calcium channels.

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