OPEM

Oriental Pharmacy and Experimental Medicine 2003 **3(3)**, 129-132

Fenugreek seed polyphenols inhibit RBC membrane Na^+/K^+-ATPase activity

Carani V Anuradha*, Subramanian Kaviarasan and Kodali Vijayalakshmi

Department of Biochemistry, Annamalai University, Annamalai Nagar 608 002, Tamil Nadu, India

SUMMARY

The hypoglycemic and hypolipidaemic effects of fenugreek seeds (*Trigonella foenum graecum*) are well established. Owing to the wide spread use of the seeds by healthy individuals and by diabetic patients we wanted to test whether the seeds can affect biological systems such as membrane transport function. In the present study fenugreek seed polyphenols were extracted and their effect on erythrocyte membrane-bound sodium-potassium adenosine triphosphatase (Na^+/K^+-ATPase) activity was studied *in vitro*. Fenugreek seed polyphenols inhibited Na^+/K^+-ATPase in erythrocyte membrane of diabetic and normal subjects. Maximum inhibition was observed at 100 µl of extract containing 0.75 mM gallic acid equivalents. The uncoupling of membrane ATPases *in vitro* suggest that polyphenols from fenugreek seeds may possess a positive inotropic effect.

Key words: *Trigonella foenum graecum*; RBC membrane; Na^+/K^+-ATPase; Diabetes

INTRODUCTION

Polyphenols constitute a large group of naturally occurring substances in the plant kingdom, which include the flavonoids. Flavonoids are recognized to possess anti-inflammatory, antioxidant, antiallergic, antithrombotic, hepatoprotective, antiviral and anticarcinogenic properties (Middelton *et al.*, 1992). Flavonoids exhibit a wide range of favorable biological effects as a consequence of their antioxidant properties. Further the flavonoids have been demonstrated to influence the activity of many mammalian enzyme systems (Jinsart *et al.*, 1992).

Fenugreek (*Trigonella foenum graecum*), an annual herb of the leguminosae family, is cultivated in several Asian and African countries. Seeds of fenugreek are commonly used as condiment in Indian homes. The seeds are reported to posses anti-hyperglycemic (Sharma and Raghuram, 1990) and antioxidant properties (Anuradha and Ravikumar, 1999) and are potentially useful as antidiabetic agent. The lipid lowering effect of the seeds are also demonstrated in the diabetic rat (Khosla *et al.*, 1995).

*Correspondence: Dr CV Anuradha, Reader, Department of Biochemistry, Annamalai University, Annamalai Nagar 608 002 Tamil Nadu, India. Tel: +91-4144-238343; Fax: +91-4144-238080; E-mail: cvaradha@hotmail.com

Whole fenugreek seeds contain 48% total fibre, which includes 20% gum and 28% neutral detergent fibre (NDF), 26% protein comparable to soybean about 4% of saponins. Fenugreek seeds are reported to be rich in flavonoids (>100 mg/g) (Gupta and Nair, 1999). About 5 different flavonoids namely vitexin, trixin, naringenin, quercetin and tricin-7-0-beta-Dglucopyranoside have been identified in the seeds (Shang *et al.*, 1998).

In view of the beneficial properties and widespread use of the seeds by the general population, it is necessary to generate data to ascertain its effects on the membrane function. The erythrocyte membrane has long served as a convenient model system for testing new concepts and methodology in membrane biochemistry.

All cells depend on membrane transport systems for uptake or release of solutes and metabolites and maintenance and regulation of ion gradients. Maintenance of the cation gradient by Na^+/K^+-ATPase is essential in the control of hydration, volume, nutrient uptake and fluidity of cells. This ATP-dependent cation pump is uncoupled in a number of common dysfunctional states including diabetes (Raccah *et al.*, 1996).

In this paper we report the *in vitro* effect of fenugreek seed polyphenols on the erythrocyte

membrane-bound enzyme, Na^+/K^+-ATPase in diabetic and normal subjects.

MATERIALS AND METHODS

Blood samples were collected from age- and sex-matched control (n=30) and diabetic (n=30) subjects (type1) after an overnight fast with informed consent. The subjects were non-obese, non-smokers and nonconsumers of alcohol, normotensives and were free of nutrient supplementation. The patients did not have diabetic complications and were treated with oral antidiabetic drug, glibenclamide (sulfonylurea). The age of the subjects ranged from 22 to 55 years and their mean duration of diabetes was 4.0.±1.1 years. BMI ranged from 20.91-26.82 kg/m^2. The study protocol was approved by the institutional human ethical committee, Rajah Muthiah Medical College and Hospital, Annamalai University, Annamalai Nagar.

Glucose in blood was determined by the method of Sasaki *et al.* 1927. The blood samples were centrifuged at 4°C for 10 min at 1000 g to remove plasma and buffy coat. The red blood cells (RBC) were washed thrice with 0.89% sodium chloride. RBC membranes were prepared by the method of Quist (1980).

Assay of Na^+/K^+-ATPase activity

Na^+/K^+-ATPase activity was assayed in RBC membrane according to the procedure of Bonting (1970). The final assay mixture contained 1ml of 184 mM Tris-Hcl buffer, pH7.5, 0.2 ml of 50 mM magnesium sulphate, 0.2 ml of 50 mM potassium chloride, 0.2 ml of 600 mM sodium chloride, 0.2 ml of 1mM EDTA and 0.2 ml of 40 mM ATP. After equilibration at 37°C for 10 minutes the reaction was initiated by adding 0.2 ml of enzyme. After incubation for 15 minutes at 37°C the reaction was stopped by adding 10% TCA. The liberated phosphorus was estimated in the supernatant. RBC membrane protein content was measured by the method of Lowry *et al.* (1951).

Extraction of fenugreek seed polyphenols

Polyphenols were extracted from fenugreek seeds by the method of Xia *et al.* (1998). For this, 100 g of the seed powder was mixed with 2 volumes of 80% methanol and kept for 5 days. This was filtered and evaporated. The residue obtained was dissolved in water and washed with petroleum ether several times. Polyphenols were extracted with ethyl acetate containing 10 ml/L glacial acetic acid. Extraction was carried out at room temperature for 36 hours and the combined ethyl acetate layer was concentrated by evaporation under the hood. The content of total polyphenols was determined by the method of Singleton and Rossi (1965) using gallic acid as standard and used for *in vitro* studies. The total phenolic. content was expressed as mM gallic acid equivalents (GAE).

In vitro treatment with fenugreek seed polyphenols

Aliquots (0.2 ml) of RBC membrane were treated with polyphenol extract at final concentrations ranging from 0.18 to 0.72 mM GAE in phosphate buffered saline, pH 7.4 and incubated at 37°C for 30 min prior to enzyme assay. Control tubes without the extract were incubated simultaneously.

Statistical analysis

The results obtained were expressed as means±SD. The students t-test was used to assess the statistical significance. A value of $P<0.05$ was considered statistically significant.

RESULTS

Fasting plasma glucose was 5.37±1.4 mmol/L in control subjects and 10.61±2.4 mmol/L in patients with type 1 diabetes.

The activity of Na^+/K^+-ATPase was significantly lower in diabetic patients ($P<0.001$) as compared to control subjects. (Control - 0.52 ± 0.08, diabetic-0.35 ± 0.06 mmoles Pi liberated/min/mg protein).

The total polyphenol content of the seeds was determined to be 0.48 gm% in our study. An aqueous solution prepared from the polyphenol inhibited Na^+/K^+-ATPase activity.

Figure 1 gives the dose-response effect of fenugreek polyphenol extract on the inhibition of erythrocyte membrane Na^+/K^+-ATPase activity in control and type 1 diabetic subjects. Significant decreases in activity occurred at 0.55 mM GAE and 0.74 mM GAE of extract. Maximum decrease (37%) was observed at 0.74 mM (GAE) in diabetic RBC membrane

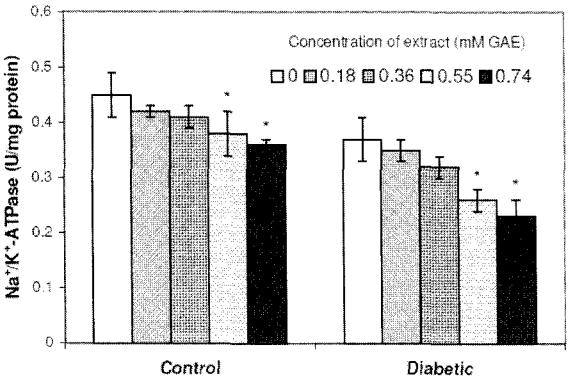

Fig. 1. Effect of fenugreek seed polyphenol extract on the inhibition of erythrocyte membrane Na^+/K^+-ATPase activity in control and diabetic subjects. Values are average of six independent determinations. *$P<0.05$ as compared with the level without extract.

while the decrease was 20% at 0.74 mMGAE in RBC membrane from control subjects.

DISCUSSION

The ion pump Na^+/K^+-ATPase is an ubiquitous membrane protein and is an active transporter of ions Na^+ and K^+ across the cell. It belongs to the P_2 type pumps with 10 passes through the membrane. It is essential for maintaining and regulating the ionic cellular milieu and by virtue of this it is thought to influence functions such as growth, differentiation and contractability of excitable and non-excitable cells.

Alterations in Na^+/K^+-ATPase activity have been described in human diabetes. The reduction is attributed to high glucose- induced peroxidation, glycosylation of proteins and alterations in the structure and function of membranes (Rajeswari *et al.*, 1991). Na^+/K^+-ATPase activity in peripheral nerves, heart and skeletal muscle is significantly reduced in diabetic animals (Kjeldsen *et al.*, 1987). An inhibitory effect of diabetes on Na^+/K^+-ATPase activity in aorta was observed in alloxan-treated rabbits (Gupta *et al.*, 1992). Diabetes-induced Na^+/K^+-ATPase activity dysfunction is related to the electrophysiological abnormalities and hence contributes to neuropathy. A link between this modification and the chronic complications of the disease has been hypothesized (Raccah *et al.*, 1996).

We found an inhibitory effect of fenugreek seed polyphenols on erythrocyte membrane Na^+/K^+-ATPase activity. The inhibition was dose-dependent and was observed in both control and diabetic samples. While going through the mechanism of action of the extract, it can be suggested that decreased activity of ATPase may be due to the conformational changes in the structure of the enzyme brought about by the extract. Flavones and flavonols containing hydroxy groups inside the phenyl radical in ortho- and vicinal positions exhibit high inhibitory effects on the membrane enzymes. The presence of dimethyl allyl groups in flavanones leads to the inhibitory activity (Umarova *et al.*, 1998). The polyphenolic structures of flavonoids similar to cholesterol partition into hydrophobic core of the membrane and cause modulation in lipid fluidity (Arti *et al.*, 2000). This could sterically hinder diffusion of ions and other transport processes.

Quercetin, a flavon-3-ol, a well-known inhibitor of ATPase activity of purified reconstituted P-glycoprotein is richly present in fenugreek seeds. Inhibition by quercetin is not related to the ouabain-binding site of the enzyme Na^+/K^+-ATPase (Hirano, 1989). Quercetin produces inotropism in the frog and rabbit heart and pig kidney medulla (Bhansali *et al.*, 1987) by inhibiting Na^+/K^+-ATPase pump and by stimulating β-adreno receptors involving adenylate cyclase-cAMP system ultimately increasing availability of calcium from intracellular sites. Clinical data however is not available for quercetin.

The inhibitory effect was significant at concentrations of polyphenol ranging from 0.55 to 0.75 mM GAE. The findings suggest fenugreek seeds may possess positive inotropic effect and may be potentially useful as cardiotonic agent. This preliminary study opens up an area for further research. Evaluation of similar effect in myocardial tissue by the polyphenol extract could be useful before they are used in pharmacological therapy.

REFERENCES

Anuradha CV, Ravikumar P. (1999) Effect of fenugreek seeds on blood lipid peroxidation and antioxidants in diabetic rats. *Phyto. Res.* **13**, 197-201.

Arti A, Byren TM, Nair MG, Strasburg GM. (2000) Modulation of liposomal membrane fluidity by flavonoids and isoflavonoids. *Arch. Biochem. Biophys.* **373**, 102-109.

Bhansali BB, Vyes S, Goyal RK (1989) Cardiac effects of quercetin on isolated rabbit and frog heart preparations. *Ind. J. Pharmacol.* **19**, 100-107.

Bonting SL. (1970) Sodium-potassium activated adenosine triphosphatase and cation transport In: *Membrane and ion transport*, edited by Bittar EE, p. 257-260. Interscience, London.

Gupta R, Nair S. (1999) Antioxidant flavonoids in common Indian diet. *South Asian J. Prev. Cardiol.* **3**, 83-94.

Gupta S, Sussman I, Mc Arthur CS, Tomheim K, Cohen RA, Ruderman NB. (1992) Endothelium dependent inhibition of Na^+/K^+-ATPase activity in rabbit aorta by hyperglycemia. Possible role of endothelium- derived nitric oxide. *J. Clin. Invest.* **90**, 727-732.

Hirano T, Oka K, Akiba M. (1989) Effects of synthetic and naturally occurring flavonoids on Na^+/K^+-ATPase: aspects of the structure activity relationship and action mechanism. *Life sci.* **45**, 1111-1117.

Jinsart W, Ternai B, Polya GM. (1992) Inhibition of rat liver cyclic AMP dependent protein kinase by flavonoids. *Biol.Chem. Hoppe- seyler* **373**, 205-211.

Kjeldsen K, Braendgaard H, Sidenius P, Larsen JS, Norgaard A. (1987) Diabetes decreases Na^+/K^+- pump concentration in skeletal muscles, heart ventricular muscle, and peripheral nerves of rat. *Diabetes* **36**, 842-848.

Khosla P, Gupta DD and Nagpal RK. (1995) Effect of *Trigonella foenum graecum* (fenugreek) on serum lipids in normal and diabetic rats. *Ind. J. Pharmacol.* **27**, 89-93.

Lowry OH, Rose brough NJ, Farr AL, Randall RJ. (1951) Protein measurement with the Folin phenol reagent. *J. Biol.Chem.* **193**, 265-275.

Middleton E, Kandaswami C, Theoharides TC. (2000) The effect of plant flavonoids on mammalian cells: Implication for inflammation, heart disease and cancer. *Pharmacol. Rev.* **52**, 673-751.

Quist EM. (1980) Regulation of erythrocyte membrane shape by calcium ions. *Biochem. Biophys. Res. Commun.* **92**, 631-637.

Raccah D, Fabreguetts C, Azulay JP, Vague P. (1996) Erythrocyte Na^+/K^+-ATPase activity, metabolic control and neuropathy in IDDM patients. *Diabetes care.* **19**, 564-568.

Rajeswari P, Natrajan R, Nadler JL, Kumar D, Kalra VK. (1991) Glucose induces lipid peroxidation and inactivation of membrane-associated ion transport enzymes in human erythrocytes *in vivo* and *in vitro*. *J. Cell. Physiol.* **49**, 100-109.

Sasaki T, Matsui S, Sonae A. (1972) Effect of acetic acid concentration on the colour reaction in the *O*-toludine boric acid method for blood glucose estimation. *Rinsho kagaku.* **1**, 346-353.

Shang M, Cai S, Han J, Li J, Zhao Y, Zheng J, Namba T, Kadota S, Tezuka Y, Fan W. (1998) Studies on flavonoids from fenugreek (*Trigonella foenum graecum L*). *Zhongguo Zhong Yao Za Zhi* **23**, 614-616.

Sharma RD, Raghuram TC. (1990) Hypoglycemic effect of fenugreek seeds in non-insulin dependent diabetic subjects. *Nutr. Res.* **10**, 731-739.

Singleton SL, Rossi JA. (1965) Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagent. *Am. J. Enol. Vitic.* **16**, 144-158.

Umarova FT, Khushbactova ZA, Batirov EH, Mekler VM. (1998) Inhibition of Na^+/K^+-ATPase by flavonoids and their inotropic effect. Investigation of the structure-activity relationship. *Cell. Biol.* **12**, 27-40.

Xia J, Allenbrand B, Sun GV. (1998) Dietary supplementation of grape polyphenols and chronic ethanol administration on LDL oxidation and platelet function in rats. *Life Sci.* **63**, 383-390.