Phytomedicine 19 (2012) 571–574

Contents lists available at SciVerse ScienceDirect

Phytomedicine

journal homepage: www.elsevier.de/phymed

Non-insulin dependent anti-diabetic activity of (2S, 3R, 4S) 4-hydroxyisoleucine of fenugreek (*Trigonella foenum graecum*) in streptozotocin-induced type I diabetic rats

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ARTICLE INFO

Keywords:
Fenugreek
Trigonella foenum graecum
4-Hydroxyisoleucine
Diabetes
Streptozotocin
Rat

ABSTRACT

The seeds of fenugreek, *Trigonella foenum graecum*, commonly used as a spice in Middle Eastern countries and widely used in south Asia and Europe, are known to have anti-diabetic properties. They contain an unusual amino acid (2S, 3R, 4S) 4-hydroxyisoleucine (4HO-Ile), so far found only in fenugreek, which has anti-diabetic properties of enhancing insulin secretion under hyperglycaemic conditions, and increasing insulin sensitivity. Here we describe for the first time the anti-diabetic activity of 4HO-Ile in a model of type I diabetes, streptozotocin-treated rats, where levels of insulin are much reduced, by 65%, compared to normal animals. Treatment of diabetic rats with daily doses of 4HO-Ile at 50 mg/kg/day for four weeks could reduce plasma glucose in the diabetic group. Moreover the high levels of lipids (cholesterol, HDL, LDL and triglycerides) and uric acid in the diabetic rats, could be restored to levels found in non-diabetic controls by the treatment with 4HO-Ile. These results demonstrate that 4HO-Ile has significant anti-diabetic activities that are independent of insulin and suggest the potential of 4HO-Ile as an adjunct to diabetes treatment and for type 1 as well as type 2 diabetes.

Introduction

Fenugreek, *Trigonella foenum graecum*, has long been used in traditional treatments of diabetes (Al-Habori and Raman 1998) and is widely cultivated in India, the Mediterranean and China. Its long history has prompted a number of small clinical trials to assess the efficacy and safety of fenugreek seed powder in the treatment of type 1 and type 2 diabetes (reviewed in Basch et al. 2003), with variable but promising results. Analysis of active, soluble components of fenugreek seed revealed an unusual amino acid, 4-hydroxyisoleucine (4HO-Ile), that had anti-diabetic potential through its ability to stimulate secretion of insulin from rat pancreatic islet cells (Sauvaire et al. 1998). 4HO-Ile comprises 80% of the free amino acid content, and 0.6% (w/w), of defatted fenugreek seeds (Sauvaire et al. 1984, 1998). The molecule has three chiral centres and 90% is found in the form with stereochemistry (2S, 3R, 4S) and 10% with stereochemistry (2R, 3R, 4S) (Sauvaire et al. 1984; Alcock et al. 1989). A comparison of the activity of the (2S, 3R, 4S) stereoisomer with the (2R, 3R, 4S) isomer and another 10 congeners revealed the (2S, 3R, 4S) isomer to be the most potent form tried, when measuring insulin release from isolated rat pancreatic islets (Broca et al. 2000). So far 4HO-Ile has only been reported to be found in fenugreek seed, and all subsequent discussion is limited to the active 2S, 3R, 4S stereoisomer that is the predominant form in this plant species.

In the rat islet insulin secretion assay treatment with 4HO-Ile was found to be 15 to 25 times more potent than the branched chain amino acids L-leucine and L-isoleucine (Broca et al. 2000). Under these conditions the stimulated release of insulin from isolated human islets and perfused rat pancreas was also noted (Sauvaire et al. 1998; Broca et al. 2000). Furthermore 4HO-Ile could also improve glucose control in oral glucose tolerance tests in normal rats and dogs which was attributed to the increase in circulating insulin after 4HO-Ile treatment (Broca et al. 1999). Other studies on normal, type 2 diabetic, or obese Zucker *fa/fa* rats indicated that 4HO-Ile could have another anti-diabetic mode of action, by improving insulin sensitivity (Broca et al. 1999, 2004). This mechanism could explain the improvement in glucose clearance and lipidemia in dyslipidemic hamsters (Narender et al. 2006), fructose-fed rats (Haeri et al. 2009), and in *db/db* mice (Singh et al. 2010), after treatment with 4HO-Ile.

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doi:10.1016/j.phymed.2012.01.004

To date there have been no studies examining the effect of 4HO-Ile on models of type I diabetes since all the studies cited above are on normal animals or tissue, or animal models of type 2 diabetes. To investigate the mode of action of 4HO-Ile further we used a rat model of type 1 diabetes induced by streptozotocin in which levels of insulin are much reduced, allowing an examination of the hypoglycaemic and lipid modulating properties of 4HO-Ile independent of insulin sensitization. We found that 4HO-Ile had significant hypoglycaemic activity in this model and could also induce a significant reduction of serum triglyceride, LDL and uric acid close to levels found in non-diabetic control rats. The data show that 4-OH-Ile can ameliorate metabolic syndrome conditions independently of insulin and strengthen the case for assessment of 4HO-Ile in clinical trials.

Materials and methods

Animals

Male Wistar rats were purchased from the Pharmacological Research Center of Tehran University of Medical Sciences. Eight week-old rats weighing between 220 and 250 g were used at the start of each treatment protocol. Rats were housed in separate cages in an animal room kept at constant temperature (25 °C) with a 12 h light–dark cycle. A standard rat chow pellet and water were provided *ad libitum* throughout the experimental period. The animals were maintained in accordance with the Animal Ethics Committee of the University of Medical Science, Qom, Iran, following.

Induction of diabetes

Rats were divided into three groups each of six, normal controls (NC), diabetic controls (D) and diabetic rats treated with 4HO-Ile (D4H). Rats were rendered type 1 diabetic by intraperitoneal injection of streptozotocin (60 mg/kg) dissolved in 0.1 M citrate buffer (pH 4.5) for five days consecutively (Motyl and McCabe 2009). After one week blood glucose concentration was measured with a Glucometer on a drop of blood from the tail. Rats were considered to be diabetic if blood glucose levels were greater than 300 mg/dl. The treatment group of diabetic rats were intubated daily with a solution of 4HO-Ile at a dose equivalent to 50 mg/kg/day for 4 weeks (Haeri et al. 2009). Normal control rats and diabetic control rats were intubated with saline alone.

Serum lipid profile, insulin, glucose and uric acid

At the end of the experiment rats were anaesthetized with ether and blood samples were collected through cardiac puncture in heparinized tubes and immediately centrifuged at 1000 × *g* for 15 min. Plasma was removed and stored at −20 °C. Glucose, triglycerides, cholesterol and LDL concentration were measured on an autoanalyser (Biosystem, Spain). Plasma insulin was measured by ELISA (DRG International, NJ, USA). Plasma uric acid was measured using a timed end point method on a Beckman Coulter Synchron LX20 (Rosolowsky et al. 2008).

Statistical analysis

Data were analysed using SPAW version 18.0 and are expressed as mean ± SD. ANOVA with Tukey and Bonferroni *post hoc* tests were used to determine significance of differences between groups. $p < 0.05$ was considered to be statistically significant.

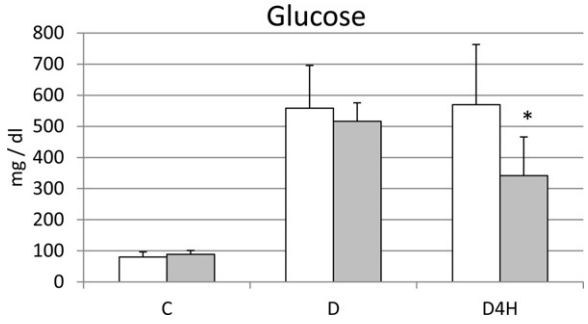

Fig. 1. Plasma glucose at the beginning and end of the 4HO-Ile treatment. Mean and SD plasma glucose of five or six rats per group. Control rats (C). Diabetic (D) and treatment (D4H) groups were made diabetic by repeated doses of STZ. Treatment with 4HO-Ile started one week after the STZ treatment and continued for four weeks. Glucose was measured at the start of the 4HO-Ile treatment (□) and at the end of four weeks treatment (■). 4HO-Ile induced a significant (*$p < 0.05$) decrease in plasma glucose within the treatment group after four weeks.

Results and discussion

Repeated dose STZ model of diabetes

STZ has been used for several decades to induce a type 1 diabetic state in rodents, usually administered as a single intravenous (i.v.) dose (Davidson and Kaplan 1977; Rees and Alcolado 2005). We compared three protocols for administering STZ through the intraperitoneal (i.p.) route as an alternative, to avoid difficulties and losses though i.v. injection. Single injections i.p. of STZ at doses of 60 mg/kg or 150 mg/kg were compared with the procedure of Motyl and McCabe (2009) of single injections i.p. of STZ at 60 mg/kg for five consecutive days. Insulin and glucose levels were monitored at two weeks after treatment and compared with control rats which received vehicle (0.1 M citrate pH 4.5) only. Single doses of STZ induced increases in glucose levels, but not to levels greater than 300 mg/dl, the threshold for diabetic phenotype (data not shown). Repeated doses of STZ induced glucose levels of 520 ± 13 mg/dl compared with 79 ± 3 mg/dl for controls ($n = 2$), and insulin levels were below the limit of detection (0.2 μg/l) compared with 1.79 μg/l in controls. On this basis the repeated doses of STZ were used to induce a type 1 diabetic state in rats.

After dividing the rats into three treatment groups the animals were treated with STZ or vehicle for five days, left for seven days and then intubated with 4HO-Ile (50 mg/kg/day) or saline vehicle daily for a further four weeks. Rats treated with STZ had a markedly elevated plasma glucose compared with controls one week after STZ administration (Fig. 1), that was sustained for a further four weeks (Fig. 1, groups D and D4H). Insulin levels in the diabetic groups were significantly lower than normal controls with 60–70% decreased levels (Fig. 2). The diabetic rats had a markedly higher intake of food and water compared with controls (Fig. 3), but the hyperphagia did not cause any increase in body weight in the diabetic rats compared with controls. Body weights (mean ± SD) at the end of the study were 289 ± 31 g (C), 269 ± 15 g (D) and 267 ± 5 (D4H). There was no significant difference between groups. The lipid profile of the diabetic rats was also consistent with a diabetic phenotype, with significantly elevated TG, cholesterol, HDL and LDL compared with the control group (Fig. 4). The changes in glucose and lipids coupled with the marked decrease in insulin indicate a type 1 diabetic phenotype is induced by the repeated i.p. doses of STZ.

Treatment of diabetic rats with 4HO-Ile

Generally the diabetic animals treated with 4HO-Ile had an improved appearance and it was noticeable that the heavy ocular