White bean amylase inhibitor administered orally reduces glycaemia in type 2 diabetic rats

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A purified pancreatic α-amylase inhibitor (α-AI) from white beans (Phaseolus vulgaris) was administered orally (100 mg/kg body weight dissolved in 9 g NaCl/l) for 22 d to non-diabetic (ND) and type 2 diabetic (neonatal diabetes models n0-STZ and n5-STZ) male Wistar rats. Mean glycaemia (mmol/l) declined from day 4 of the α-AI administration in ND rats (5.48 (SEM 0.08) v. 4.39 (SEM 0.13); P<0.05), n0-STZ diabetic rats (7.94 (SEM 0.42) v. 5.56 (SEM 0.32); P<0.01) and n5-STZ diabetic rats (17.34 (SEM 2.58) v. 11.93 (SEM 1.96)), until the end of treatment: ND (5.22 (SEM 0.21) v. 3.97 (SEM 0.06); P<0.01); n0-STZ (8.10 (SEM 0.19) v. 5.21 (SEM 0.30); P<0.01); and n5-STZ (16.36 (SEM 2.14) v. 7.69 (SEM 1.34); P<0.01). There was a decrease in water intake (ml/d) in the α-AI-treated diabetic rats: n0-STZ (30 (SEM 0.10) v. 22 (SEM 1.50); P<0.01) and n5-STZ (76 (SEM 5.04) v. 57 (SEM 4.85); P<0.01). Food intake (g/d) decreased in all three groups: ND (23 (SEM 0.31) v. 20 (SEM 0.03); P<0.05); n0-STZ (22 (SEM 0.55) v. 16 (SEM 0.98); P<0.01); and n5-STZ (31 (SEM 0.58) v. 23 (SEM 1.20); P<0.01). The enterocty surrace and malse rate activities (U/g proteins) were high (P<0.01) in the untreated diabetic rats, n0-STZ (45 (SEM 4) and 152 (SEM 10), respectively) and n5-STZ (67 (SEM 12) and 151 (SEM 10), respectively) with respect to the ND rats (24 (SEM 2) and 74 (SEM 10), respectively). After α-AI treatment, enzyme activities declined in both diabetic rats, n0-STZ (21 (SEM 2) and 85 (SEM 11); P<0.01) and n5-STZ (28 (SEM 7) and 75 (SEM 19); P<0.05), to values close to those in the ND rats. In conclusion, α-AI significantly reduced glycaemia in both the ND and diabetic animals and reduced the intake of food and water, and normalized the elevated disaccharidase levels of the diabetic rats.

Rats: α-Amylase inhibitor: Type 2 diabetes: Glycaemia: Disaccharidases

For many individuals affected with type 2 diabetes, postprandial hyperglycaemia may be the only manifestation of their diabetes (Lebovitz, 1999). There is increasing evidence that postprandial hyperglycaemia is an important contributing factor to the development of diabetic complications (Ceriello, 2005). In diabetic patients and diabetic rats, abnormal increases in the activities of sucrase and isomaltase are observed in the small intestine (Adachi et al. 1999). Postprandial hyperglycaemia can be partially controlled by delaying digestion and absorption of carbohydrates by pharmacological inhibition of α-glucosidase activity (acarbose, miglitol) or fibre ingestion (Jenkins et al. 2002; Chiasson et al. 2004). The other approach to control postprandial hyperglycaemia is based on the inhibitory action of pancreatic α-amylase. Inhibitors of pancreatic α-amylase have been detected in many cereals and some pulses (Bowman, 1945; Jaffé & Lette, 1968; Marshall & Lauda, 1975; Mulimani & Rudrappa, 1994). In particular, the white bean (Phaseolus vulgaris) contains a high level of such an inhibitor (Moreno et al. 1990). Using an inhibitor of α-amylase isolated and purified from white beans, it has been shown that the prolonged administration of the amylase inhibitor reduced blood glucose levels and body weight gain in non-diabetic (ND) Wistar rats (Tormo et al. 2004).

The objectives of the present work were to isolate and purify a pancreatic α-amylase inhibitor (α-AI) from white beans (Phaseolus vulgaris) and to study the effect of administering the α-AI orally for 22 d to ND and type 2 diabetic (neonatal diabetes models n0-STZ and n5-STZ) male Wistar rats (2-5 months old).

Materials and methods
Purification of the α-amylase inhibitor
The pancreatic α-AI was purified from white beans (Phaseolus vulgaris) by ion exchange chromatography following the method of Pusztaї et al. (1995) with minor modifications as previously described (Tormo et al. 2004). Basically, bean meal (1 kg) was stirred in 10 litres acid acetic (20 mmol/l) containing 0.2 g ascorbic acid/l for 30 min and, after adjusting to pH 5.0 with NaOH (1 mol/l), the slurry was stirred for another 2 h. After being left to stand in a cold room overnight, the extract was centrifuged (10000g for 15 min), 1.5 g CaCl2 was added to clear the supernatant and this was adjusted to pH 9.0 with NaOH (1 mol/l). The heavy precipitate, formed after being left to stand in a cold room overnight, was removed by centrifugation (3000g for 10 min) and the

Abbreviations: α-AI, α-amylase inhibitor; ND, non-diabetic.

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supernatant adjusted to pH 3.8 with 1 mol HCl/l. After another night in a cold room, the extract was cleared by centrifugation (10000g for 15 min) and diluted twofold with distilled water. The diluted supernatant was further purified by ion exchange chromatography on a Sulphopropyl Fast Flow Fast Flow column (5 cm × 7.5 cm, 150 ml bed volume) equilibrated with 25 mmol Na-formate buffer (25 mmol/l), pH 3.8. After the extract passed through, the column was washed with formate buffer until the extinction value at 280 nm fell below 0.01; then the α-AI was eluted with 0.15 mol NaCl/l in formate buffer. The α-AI fractions from several chromatograms were combined and rechromatographed on the Sulphopropyl Fast Flow column under the same conditions. To remove small molecular weight impurities, the concentrated eluates from the column were passed through a Sephacryl 100 column (Amersham Pharmacia Biotech), equilibrated with Na-phosphate buffer (50 mmol/l), pH 7.5, and the first peak containing α-AI was collected, dialysed against water and freeze-dried. The yield was about 1.5–2.4 g α-AI/kg bean meal.

Test of α-amylase inhibitor purity

The haemagglutination activity of the α-AI preparations was measured according to a previously reported method (Le Berre-Anton et al. 1997). Briefly, in U-bottomed microtitration plates, 25 μl twofold serial dilutions of 1 mg α-AI/ml in 100 mM Tris, 150 mM NaCl buffer (pH 7.4) were mixed at room temperature with an equal volume of a 1% (v/v) suspension of human O Rh-+ erythrocytes washed three times in the same buffer. Haemagglutination was read 2 h later at room temperature and (as a control) after being left to stand at 4°C for 12 h.

PAGE was carried out using the Miniprotein II System (Bio-Rad Laboratories, Alcobendas, Madrid, Spain) with 15% acrylamide gel (Pusztai et al. 1988).

Animal experiments

ND and type 2 diabetic (models n0-STZ and n5-STZ; Portha et al. 1974, 1989) adult (2.5 months) male Wistar rats were used. The n0-STZ model was obtained by a single dose of streptozotocin (Sigma-Aldrich Química S.A., Alcobendas, Madrid, Spain; 100 mg/kg body weight) dissolved in a citrate buffer (0.1 mol/l) at pH 4.5 administered intraperitoneally on the day of birth, and the n5-STZ model was induced by a single dose of streptozotocin (80 mg/kg body weight) on day 5 after birth. In adulthood, the n0-STZ rats showed mild basal hyperglycaemia, an approximately 50% reduction in pancreatic insulin content, and no insulin resistance and the n5-STZ rats showed frank basal hyperglycaemia and glucose intolerance, a marked reduction of pancreatic insulin stores, and insulin resistance (Portha et al. 1989; Tormo et al. 2004). They had been maintained on a standard diet (maintenance diet Letica, Panlab S.L., Barcelona, Spain; 61.4% (w/w) carbohydrate (100% starch), 30.4% fibre, 15.1% protein and 2.7% fat) with free access to food and water and housed in a room at 24°C with light from 08.00 to 20.00 hours. The animals were cared for in accordance with the principles of the Guide to the Care and Use of Experimental Animals (Real Decreto, 1988) and the protocol was approved by the Animal Ethics Committee of the Universidad de Extremadura. The α-AI at doses of 100 mg/kg body weight dissolved in NaCl (9 g/l) were administered orally for 22 d through a gastric cannula in a single dose at 20.30 hours.

Analytical methods

Every day at 09.00 hours (overnight rats fed ad libitum), the body weight was measured and the ingestion of food and water was recorded. Glucose concentration was measured in 2 μl blood extracted from the tail of the animal with reactive strips read in a Glucocard Memory (Menarini Diagnostics, Barcelona, Spain). At the beginning, halfway through (day 10) and at the end of the treatment (day 22), the plasma insulin levels were measured by RIA with a rat insulin kit which uses a specifically synthesized antibody against rat insulin (DRG’s Instrument GmbH, Marburg, Germany).

At the end of the treatment the rats were killed in the morning by pentobarbital overdose. The abdomen was cut open, and the small intestine, pancreas, liver and the large intestine were removed, rinsed with NaCl (9 g/l), blotted dry and weighed. The small intestine length was measured under 5g tension. Epithelial cells of the small intestine were isolated (Watford et al. 1979) and the sucrase and maltase activities were determined in isolated enterocytes following the method of Dahlqvist (1964) as described (Tormo et al. 2004). The protein concentration was determined by the micro-Lowry method (Sigma-Aldrich Química, Alcobendas, Madrid, Spain).

Expression of results and statistical analysis

Values are expressed as means and their standard errors. Statistical analyses were performed using the program InStat for Macintosh version 1.12. Repeated-measures ANOVA was used to assess changes in the level of glycaemia and immuno-reactive insulin in the same experimental group. When P < 0.05, the significance of the difference was estimated by the Bonferroni test. The Mann–Whitney U test was used to determine differences between groups. A value P < 0.05 was considered statistically significant.

Results

In control ND rats, who were administered daily NaCl (9 g/l) alone, blood glucose remained constant throughout the experimental period at about 5.0–5.5 mmol/l. The glycaemia declined slightly after the α-AI administration with respect to day 0. This reduction was statistically significant from day 4 (5.5 (SEM 0.4) vs. 4.4 (SEM 0.1); P < 0.05) until the end of treatment (day 22: 5.2 (SEM 0.2) vs. 3.9 (SEM 0.3); P < 0.001) (Fig. 1). In n0-STZ diabetic rats, in the absence of α-AI administration, glycaemia remained constant throughout the experimental period (7.7–8.1 mmol/l). In these rats the blood glucose levels were significantly reduced after the α-AI administration (7.9 (SEM 0.4) vs. 5.5 (SEM 0.3); P < 0.01) at day 4 and this decline in glycaemia was maintained until the end of the treatment (8.1 (SEM 0.2) vs. 5.2 (SEM 0.2); P < 0.001 at day 22). In n5-STZ diabetic rats under NaCl (9 g/l) administration, high blood glucose levels were
measured (15·9–17·3 mmol/l) throughout the experimental period. After the α-AI administration an abrupt reduction of glycaemia was observed (17·3 (SEM 2·6) vs 11·9 (SEM 1·9); *P < 0·01 at day 4) that continued until the end of the experimental period (16·4 (SEM 2·1) vs 7·7 (SEM 1·3); *P < 0·01 at day 22). As shown in Table 1, plasma insulin levels were significantly reduced at day 0 in diabetic rats with respect to that measured in the corresponding ND rats. There were no significant differences in the plasma insulin levels measured in ND and diabetic rats during the experimental period except for the plasma insulin values measured in n5-STZ diabetic rats at day 22 in the absence of α-AI administration.

In the absence of α-AI administration, water intake (Table 2) was significantly increased in n5-STZ diabetic rats versus that measured in ND rats. After α-AI administration there was no reduction in water intake in the ND rats. But there was a decrease in water intake in the α-AI-treated diabetic rats. In the absence of α-AI administration, food intake (Table 2) was significantly increased in n5-STZ diabetic rats with respect to ND rats. The administration of the amylase inhibitor (α-AI) produced a decrease in food intake in all three experimental groups. The anorexigenic effect of the α-AI administration was reflected in a smaller weight increase rate during the experimental period, that was statistically significant (*P < 0·05) in ND rats.

As shown in Table 3, in the absence of α-AI administration the length and weight of the small intestine was significantly increased in diabetic rats. The α-AI administration reduced significantly the weight of the liver and the pancreas in both diabetic and ND rats and the weight of large intestine in n5-STZ diabetic rats, without modification of the weight and length of small intestine.

The enterocyte sucrase and maltase activities (Table 4) were high (*P < 0·01) in the untreated diabetic rats, n0-STZ and n5-STZ, with respect to the ND rats. After α-AI administration, the enzyme activities declined in both diabetic rats to values close to those in the ND rats.

### Discussion

#### Method and purification yield

As reported previously (Tormo et al. 2004), the α-AI preparations contained four polypeptide bands of 32, 29, 17 and 16 kDa, similar to the results reported by other workers (Le Berre-Anton et al. 1997). The test for haemagglutination activity showed no evidence of contamination of the α-AI preparation with kidney bean lectin, again results that were...
and n5-STZ) after 22 d of daily administration of NaCl or α-amylase inhibitor (α-Al; 100 mg/kg body weight) from kidney beans suspended in NaCl (9 g/l).§

(Mean values with their standard errors for six determinations for each experimental group)

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<th>α-Al</th>
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<tr>
<td>124</td>
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<td>118</td>
</tr>
<tr>
<td>n0-STZ</td>
<td>132*</td>
<td>3</td>
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<tr>
<td>n5-STZ</td>
<td>139*</td>
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<td>n5-STZ</td>
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<th>Large intestine (g)</th>
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<tr>
<td>1.16</td>
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<td>n5-STZ</td>
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Mean values were significantly different from those of the ND NaCl rats: **P < 0.01.
**P < 0.001.

Mean values were significantly different from those of the n0-STZ NaCl rats:
††P < 0.001.
††P < 0.01.

††P < 0.5.
†‡‡P < 0.01.

§ For details of procedures, see pp. 539–540.

similar to previous reports (Maranesi et al. 1984; Pusztai et al. 1995). This preparation contained a high inhibitory activity as tested by measuring in vitro the inhibition of the amylase activity of the porcine amylase as described in Tormo et al. (2004).

Table 4. Values of sucrase and maltase measured in enterocytes isolated from the small intestine of non-diabetic (ND) and diabetic (n0-STZ and n5-STZ) after 22 d of daily administration of NaCl or α-amylase inhibitor (α-Al; 100 mg/kg body weight) from kidney beans suspended in NaCl (9 g/l).§

(Mean values with their standard errors for six determinations for each experimental group)

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<th>α-Al</th>
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<td>SEM</td>
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<tr>
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<td>2</td>
<td>29</td>
</tr>
<tr>
<td>n0-STZ</td>
<td>45**</td>
<td>4</td>
</tr>
<tr>
<td>n5-STZ</td>
<td>67***††</td>
<td>12</td>
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<table>
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<th>NaCl</th>
<th>α-Al</th>
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<tbody>
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<td>ND</td>
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</tr>
<tr>
<td>74</td>
<td>10</td>
<td>67</td>
</tr>
<tr>
<td>n0-STZ</td>
<td>152</td>
<td>10**</td>
</tr>
<tr>
<td>n5-STZ</td>
<td>151</td>
<td>75††</td>
</tr>
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</table>

Mean values were significantly different from those of the ND NaCl rats: **P < 0.01.
**P < 0.001.

Mean values were significantly different from those of the n0-STZ NaCl rats:
††P < 0.001.
†‡‡P < 0.01.

††P < 0.5.
†‡‡P < 0.01.

§ For details of procedures, see pp. 539–540.

Hypoglycaemic effect

The n0-STZ diabetic rats presented a slight increase in basal glycaemia. The hyperglycaemia was clearly seen in the n5-STZ diabetic rats. The present results show that the α-Al isolated and purified from white kidney beans significantly reduces glycaemia levels in rats following chronic administration in both ND and diabetic rats. Similar results have been described by other workers in growing (120 g) ND Wistar rats (Kotaru et al. 1989), and for healthy and type 2 diabetes subjects (Layer et al. 1986; Boivin et al. 1987; Jain et al. 1991). These previously reported studies were all carried out under acute conditions, while in the present work the effect of prolonged daily administration of the α-amylase inhibitor in two models of type 2 diabetic rats was investigated, and the present results provide support for its therapeutic potential in treating postprandial hyperglycaemia in diabetic rats.

Diabetic rats presented a slight decrease in insulinemia with respect to ND rats. These data are similar to those reported by Portha et al. (1974, 1989). The results showed no significant changes in plasma insulin levels after α-Al treatment and suggest that α-Al is a potent inhibitor of rat pancreatic α-amylase pancreatic. Other workers (Kotaru et al. 1989) report a decline in plasma insulin levels after the administration of α-Al purified from the cranberry bean variety of Phaseolus vulgaris together with an experimental diet in growing male Wistar rats. Healthy and diabetic subjects (Layer et al. 1986), who were administered 50 g starch together with 10 g inhibitor, presented reduced levels of postprandial plasma insulin and C-peptide during the time that glucose levels were greater than the fasting levels.

Intake of water and food, and body weight

Food and water intake were significantly increased in n5-STZ diabetic rats, while the weight increase rate was similar in the three experimental groups. The present results demonstrated that the chronic administration of α-Al reduced food intake in all three experimental groups, water intake was reduced in the diabetic rats and there was a significant reduction in the weight increase rate in ND rats. As the α-Al was administered by a gastric cannula the anorexigenic effect observed could not be attributed to a lack of palatability of the product reducing the energy intake. A similar anorexigenic effect has been known for many years (Jaffé & Lette, 1968; Puls & Kneup, 1973; Pusztai et al. 1995). It has also been difficult to explain how the chronic administration of α-Al reduces food intake. Studies on human subjects have shown that the inhibition of pancreatic amylase is associated with a delay in gastric emptying, and that the arrival of a greater amount of undigested carbohydrates in the ileum also slows gastric emptying (Jain et al. 1991). As by those previous workers, in the present study too no signs of malabsorption were observed, such as diarrhoea or increase in stools (data not shown). This seems to be an interesting finding, since α-glucosidases often cause diarrhoea and other collateral effects. Adequate amylase inhibition, however, could delay intestinal absorption and reduce body weight by diminishing food intake without malabsorption (Kataoka & DiMagno, 1999).
Intestinal tissue morphology

In man and in experimental animals, diabetes produces changes in the function and structure of the intestinal tract (Eittar & Carr, 1997; Zhao et al. 2003). In the present study the length and weight of small intestine was significantly increased in diabetic rats and the chronic administration of α-AI did not modify small intestine length and weight, but it led to weight changes in the liver and pancreas of ND and diabetic rats and in the large intestine of n5-STZ rats. On the contrary, other workers (Pusztaï et al. 1995), administering different doses of α-AI, also purified from white beans (10, 20, 40 g/d) to 19-d-old Wistar rats, observed a slight but significant increase in the weight of the small intestine, with an even more pronounced increase in weight of the caecum. According to those authors, this is clearly the consequence of poor breakdown of the dietary starch in the small intestine and its accumulation in the caecum. With respect to the liver and the pancreas, their absolute weight was less in the α-AI-treated rats, and similar to the values reported by other workers (Pusztaï et al. 1995), although in that study the differences were only significant in the case of the liver and with the highest doses of the inhibitor.

Disaccharidase activity

The activities of the disaccharidases maltase and sucrase are increased in the mucosa of the small intestine in the n0-STZ and n5-STZ diabetic rats, as has been described in diabetic patients and other diabetic animal models (Caspari et al. 1972; Tormo et al. 2002; Martínez et al. 2003). The increase in disaccharidase activity in diabetes can contribute to the appearance of postprandial hyperglycaemia peaks and consequently to the development of the chronic complications of diabetes, and justifies the pharmacological use of intestinal α-glucosidase inhibitors in the treatment of type 2 diabetes. It has been reported in normal rats made hyperglycaemic by an intravenous administration of dextrose monohydrate, that hyperglycaemia directly increased the activities of the intestinal disaccharidases maltase and sucrase and that hyperglycaemia was partly responsible for the increased activities of disaccharidases in diabetic rats (Murakami & Ikeda, 1998). The present results agree with those previously reported. The reduction of hyperglycaemia after α-AI treatment produced a significant reduction in the increased maltase and sucrase activities in diabetic rats to values close to those in the ND rats. These changes, together with the effect of the inhibitor itself, could cause a delay in glucose entering the bloodstream from the intestine without there being the symptoms of malabsorption that are observed in some patients with the administration of α-glucosidase inhibitors.

In conclusion, the results of the present study have shown that a pancreatic α-AI purified from white beans and administered orally for 22 d to Wistar rats significantly reduced glycaemia levels without significantly altering insulinaemia levels in both the ND and diabetic (n0-STZ and n5-STZ) animals. It also reduced the intake of food and body weight gain in all animals and reduced the intake of water in diabetic rats. The administration of the amylase inhibitor normalized the elevated sucrase and maltase activities measured in enterocytes from diabetic rats. The present results show that chronic administration of α-AI from white beans improved postprandial hyperglycaemia in type 2 diabetic rats and could provide support for its therapeutic potential in treatment or prevention of the complications of type 2 diabetes and obesity.

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