4-Hydroxyisoleucine: experimental evidence of its insulinotropic and antidiabetic properties

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WE HAVE RECENTLY REPORTED that 4-hydroxyisoleucine (4-OH-Ile), an amino acid extracted and purified from fenugreek seeds (Trigonella foenum-graecum L. leguminosae), displays in vitro an insulinotropic activity of its insulinotropic and antidiabetic properties. Am. J. Physiol. 277 (Endocrinol. Metab. 40): E617–E623, 1999.—We have recently shown in vitro that 4-hydroxyisoleucine (4-OH-Ile), an amino acid extracted from fenugreek seeds, potentiates insulin secretion in a glucose-dependent manner. The present study was designed to investigate whether 4-OH-Ile could exert in vivo insulinotropic and antidiabetic properties. For this purpose, intravenous or oral glucose tolerance tests (IVGTTs and OGTTs, respectively) were performed not only in normal animals but also in a type II diabetes rat model. During IVGTT in normal rats or OGTT in normal dogs, 4-OH-Ile (18 mg/kg) improved glucose tolerance. The lactonic form of 4-OH-Ile was ineffective in normal rats. In non-insulin-dependent diabetic (NIDDM) rats, a single intravenous administration of 4-OH-Ile (50 mg/kg) partially restored glucose-induced insulin response without affecting glucose tolerance; a 6-day subchronic administration of 4-OH-Ile (50 mg/kg, daily) reduced basal hyperglycemia, decreased basal insulinemia, and slightly, but significantly, improved glucose tolerance. In vitro, 4-OH-Ile (200 µM) potentiated glucose (16.7 mM)-induced insulin release from NIDDM rat-isolated islets. So, the antidiabetic effects of 4-OH-Ile on NIDDM rats result at least in part, from a direct pancreatic B cell stimulation.

RESEARCH DESIGN AND METHODS

The experiments were performed either in male Wistar rats weighing 220–350 g or in male mongrel dogs weighing 13–16 kg. Rats were maintained on a 12:12-h light-dark schedule and were deprived of food 2 h before the experiments.

Intravenous Glucose Tolerance Tests

Normal rats. Normal rats were anesthetized with an intraperitoneal injection of pentobarbital (60 mg/kg) and maintained on a heated operating surface. After a cervical median incision and dissection, a catheter, filled with heparinized saline to prevent blood clotting, was inserted into each jugular vein. In all experiments, after a 30-min resting period, blood samples were taken through one catheter to determine plasma insulin and glucose levels. The other catheter was used for the injection of glucose in the absence or in the presence of 4-OH-Ile (at the dose of 18 mg/kg). Glucose alone (0.5 g/kg) or the mixture of glucose plus 4-OH-Ile was dissolved in 0.5 ml saline and injected within 5 s. Samples were collected before and 3, 5, 10, 15, 30, and 60 min after intravenous injection.

NIDDM rats. Ten-week-old rats weighing 220–230 g were made diabetic by an injection of NA (230 mg/kg ip) 15 min before an intravenous administration of STZ (65 mg/kg), according to the protocol recently described (13). The choice of this NIDDM model is justified by its advantages, namely a partial responsiveness to glucose and a well-preserved sensitivity to tolbutamide, the insulinotropic agent of reference.
Three weeks after diabetes induction, experiments were performed in three groups of rats; group 1: placebo-treated NIDD rats; group 2: 4-OH-Ile-treated NIDD rats; and group 3: placebo-treated normal rats. The effect of 4-OH-Ile on glucose tolerance was evaluated in both acute and subchronic experiments.

In acute experiments, each animal of the three groups was submitted to an intravenous glucose tolerance test (IVGTT). Group 2 received an injection of glucose (0.5 g/kg as a 30% solution) plus 4-OH-Ile (50 mg/kg dissolved in 0.1 ml saline), whereas groups 1 and 3 received an injection of glucose alone.

In subchronic experiments, before the beginning of treatment, each animal of the three groups was submitted to an IVGTT (0.5 g/kg glucose as a 30% solution) referred to as the "before treatment" test. Then, due to the scarceness of the compound, each group underwent a treatment restricted to 6 days; group 2 received for 5 days a daily administration of 4-OH-Ile (50 mg/kg in 1 ml saline ip) just before the dark period, whereas groups 1 and 3 received at the same time the saline placebo. Finally, on day 6, each group was submitted to a second IVGTT, referred to as the "under treatment" test; group 2 received an injection of glucose (0.5 g/kg) plus 4-OH-Ile (50 mg/kg), whereas groups 1 and 3 were injected with glucose alone.

For each IVGTT in both acute and subchronic experiments, glucose or the mixture of glucose plus 4-OH-Ile was injected through the tail vein of conscious rats. Blood samples were collected sequentially from the tail vessels before and 2, 6, 15, 30, and 60 min after the injection.

**Oral Glucose Tolerance Tests**

Normal dogs. In normal conscious dogs fasted for 16 h, the oral effectiveness of 4-OH-Ile was tested by performing oral glucose tolerance tests (OGTTs) induced by intragastric intubation. Glucose alone (1 g/kg) or the mixture of glucose plus 4-OH-Ile (18 mg/kg) was dissolved in 100 ml saline and administered within 1 min. In these experiments, blood samples were taken from a jugular vein to evaluate blood glucose and plasma insulin levels before and 3, 5, 10, 15, 30, 45, 60, 90, 120, 150, and 180 min after glucose administration.

Normal rats. In normal conscious rats, the effects of the lactonic form of 4-OH-Ile were evaluated during glucose tolerance tests. OGTTs were carried out by esophageal intubation. Glucose alone (2 g/kg) or the mixture of glucose plus the lactonic form of 4-OH-Ile (at the doses of 18 and 36 mg/kg) dissolved in 0.8 ml saline was administered within 1 min. Blood samples were taken from the tail vessels before and after intragastric glucose administration.

**Experiments on Isolated NIDD Rat Islets**

Diabetic rats were killed by decapitation and islets were isolated after collagenase digestion of the pancreas, according to a technique derived from that of Lacy and Kostianovsky (11). Isolated islets were preincubated for 60 min at 37.5°C in a Krebs-Ringer bicarbonate buffer (pH 7.4) containing 1 g/l BSA and 8.3 mM glucose. Thereafter, batches of three size-matched islets were incubated in the presence of different glucose concentrations (3.0, 8.3, or 16.7 mM) for 60 min in 1 ml of medium with or without 4-OH-Ile at 200 μM. At the end of the incubation period, an aliquot of the medium was frozen for further insulin RIA.

**Assays**

In all in vivo experiments, blood samples were collected in chilled tubes containing EDTA and rapidly centrifuged at 4°C. Plasma were removed and stored at −20°C until subsequent glucose and insulin determinations.

Insulin concentrations in plasma and incubation media were determined by RIA with charcoal separation (6), with an anti-porcine insulin antibody (ICN Biomedicals, Orsay, France) and pure rat or dog insulin (Novo, Copenhagen, Denmark) as the reference standard (the biological activities were 22.3 and 14.2 μU/ng, respectively).

Rat plasma glucose was determined by the glucose oxidase method (19), and dog blood glucose was measured with a Technicon autoanalyzer with the potassium ferricyanide procedure (1).

**Drugs**

STZ (Zanosar) was a gift from Upjohn (Paris, France); NA was purchased from Sigma (St. Louis, MO); 4-OH-Ile (linear form, pilot plant extraction) and its lactonic form were kindly provided by Legras Laboratory (Paris, France).

Aware of the greater efficiency of 4-OH-Ile vs. leucine to stimulate insulin release in vitro at physiological glucose concentration (18), we selected the dose of 18 mg/kg of 4-OH-Ile, i.e., approximately a 10-fold lower dose than the one previously used for leucine and found to increase plasma insulin in subjects (5).

**Data Analysis**

Data are expressed as means ± SE and have been statistically submitted to ANOVA followed by the multiple comparison test (22). For glucose tolerance tests in normal animals, variations in glycemia are expressed as area under the curve (AUC). During IVGTTs, glucose disappearance rates (coefficients K) were calculated from the slope of the logarithm of the postload plasma glucose concentration between 2 and 60 min and were expressed as percentages per minute. In diabetic rat experiments, insulin responses to glucose were expressed as increments (Δ, ng/ml) or integrated increments for AUC (ΔAUC, ng/ml) of plasma insulin, because absolute basal values were different due to the treatment. In addition, IVGTT data were also analyzed by calculating individual changes in glycemia-to-insulin (G/I) ratios vs. time of glucose injection.

**RESULTS**

**Effects of 4-OH-Ile in Normal Animals**

Normal rats. In normal anesthetized rats (Fig. 1), we studied the effects of 4-OH-Ile on intravenous glucose tolerance. After the intravenous injection of glucose alone (0.5 g/kg), plasma glucose concentration increased from 133 ± 7 to 282 ± 14 mg/dl (+112%) at 3 min and then progressively decreased. As expected, this rise in plasma glucose was associated with a simultaneous increase in insulin secretion. The addition of 4-OH-Ile at the dose of 18 mg/kg to glucose induced a two- to threefold greater insulin response, thereby reducing the increment of plasma glucose (from 127 ± 8 mg/dl in the basal state to only 204 ± 25 mg/dl at 3 min, +60%). The 30-min AUC for blood glucose was 5,421 ± 125 mg/dl in 4-OH-Ile-treated rats vs. 6,459 ± 67 mg/dl in controls (P < 0.01).

Normal dogs. In normal conscious dogs (Fig. 2), the effects of orally administered 4-OH-Ile were investigated on oral glucose tolerance. The intragastric administration of glucose alone (1 g/kg) induced an increase in glycemia, which reached 165 ± 4 mg/dl at 30 min (+75
mg/dl). This hyperglycemia provoked a stimulation of insulin secretion (+0.57 ng/ml at minute 15). When 4-OH-Ile (18 mg/kg) was added to glucose, the increments of plasma insulin levels were much higher (maximum at minute 15: +2.35 ng/ml, P < 0.05), and, consequently, the elevated hyperglycemia observed in controls was significantly blunted. The 45-min AUC for blood glucose was 5,027 ± 107 mg/dl in 4-OH-Ile-treated dogs vs. 6,290 ± 140 mg/dl in controls (P < 0.01).

Lactonic form of 4-OH-Ile in normal rats. The ability of 4-OH-Ile to cyclize into a lactonic form under acidic conditions prompted us to test the effects of the latter on insulin secretion. In normal conscious rats, the oral ingestion of 2 g/kg glucose resulted in an increase of plasma glucose and insulin levels. No change could be observed on simultaneous administration of the lactonic form of 4-OH-Ile at either 18 or 36 mg/kg. Thus at the higher dose, the peaks of hyperglycemia (minute 20) recorded were 179 ± 13 vs. 178 ± 10 mg/dl in controls. Likewise, insulin levels reached similar maximal values (minute 10): 6.6 ± 0.8 vs. 6.2 ± 1.4 ng/ml, respectively, in treated and control rats. Similar data were obtained in experiments in which the lactonic form of 4-OH-Ile was administered intravenously (data not shown).

Effects of 4-OH-Ile in NIDD Rats

Establishment and characteristics of the NIDDM model in adult rats. Ten-week-old rats were made diabetic by the combined administration of the B-cytotoxic agent STZ and a suitable partially protective dose of NA. Three weeks after diabetes induction, animals exhibited a moderate and stable hyperglycemia (163 ± 4 vs. 132 ± 3 mg/dl in normal rats, P < 0.01) and a slight nonsignificant reduction in insulinemia (1.96 ± 0.17 vs. 2.19 ± 0.20 ng/ml in controls). Accordingly, IVGTTs revealed a marked glucose intolerance attributable to a relative deficiency in the insulin response to glucose (see Figs. 3 and 5).

Effects of a single administration of 4-OH-Ile. In this NIDDM model raised in adult rat, 4-OH-Ile was first tested at 18 mg/kg during an IVGTT, but no significant effect was observed (Fig. 3). So, we progressively increased the dosage. When 4-OH-Ile was added at the dose of 50 mg/kg to the intravenous glucose load, we observed a greater increase in plasma insulin levels vs. placebo-treated NIDD rats. Thus insulinemia was clearly enhanced during the first 15 min (maximum at minute 2: 2.42 ± 0.48 ng/ml in 4-OH-Ile-treated NIDD rats vs. 1.38 ± 0.35 ng/ml in placebo-treated NIDD rats, P < 0.05, and vs. 3.88 ± 0.28 ng/ml in placebo-treated normal rats; Fig. 3A). However, no significant effect on glucose tolerance was observed (Fig. 3B).

Effects of a subchronic treatment with 4-OH-Ile. The 6-day subchronic treatment with 4-OH-Ile (50 mg/kg...
induced a significant reduction in basal hyperglycemia of diabetic rats (Fig. 4A). Thus after 6 days, the glycemia of 4-OH-Ile-treated NIDD animals (group 2) was 143.6 ± 4.4 vs. 163.5 ± 4.4 mg/dl before treatment (P < 0.01) and vs. 164.3 ± 6.4 mg/dl for placebo-treated NIDD rat (P < 0.01). In the same conditions, glycemia of placebo-treated normal rats was 132.6 ± 3 mg/dl. Moreover, a slight but significant reduction of basal insulinemia (Fig. 4B) occurred in 4-OH-Ile-treated NIDD rats (1.52 ± 0.12 ng/ml under treatment vs. 1.96 ± 0.11 ng/ml before treatment, P < 0.01), whereas in placebo-treated NIDD rats, a slight but not significant increase in basal insulinemia was observed (2.15 ± 0.24 under treatment vs. 1.75 ± 0.16 ng/ml before treatment). Additionally, there was no difference in body weight nor in food intake between 4-OH-Ile-treated NIDD rats and placebo-treated NIDD rats (weight gain was similar, reaching about +14 g/wk).

IVGTTs performed before treatment showed that the glucose intolerance was similar for both groups 1 and 2 (NIDD rats) with a deficiency in the insulin response, compared with group 3 (normal rats; Fig. 5, A and B). The subchronic administration of 4-OH-Ile clearly improved the small insulin response (Fig. 5C): 60-min \( \Delta AUC \) for insulinemia was 28.8 ± 8.0 ng/ml with 4-OH-Ile vs. 8.9 ± 3.9 ng/ml without 4-OH-Ile (P < 0.05). Consequently, a substantial improvement of glucose tolerance occurred with 4-OH-Ile, and glycemia returned closer to basal values (Fig. 5D). The postload glucose disappearance rate between 2 and 60 min increased in 4-OH-Ile-treated NIDD rats (K = 1.16 ± 0.04 vs. 0.89 ± 0.08% in placebo-treated NIDDM rats, P < 0.01). Thus, at minute 60, glycemia was 172 ± 5 mg/dl for 4-OH-Ile-treated NIDD rats vs. 221 ± 11 mg/dl for placebo-treated NIDD rat (P < 0.01). Comparatively, plasma glucose levels for placebo-treated normal rats were 150 ± 3 mg/dl at the same time.

We compared these beneficial effects of 4-OH-Ile treatment in diabetic rats by calculating the individual changes in the G/I during IVGTT. The decrease of G/I occurring in normal rats was drastically reversed into an increase in diabetic animals; subchronic treatment with 4-OH-Ile partly corrected this abnormality and resulted, at minute 15, in G/I values significantly lower than in placebo-treated diabetic rats (114 ± 6 vs. 150 ± 15, respectively, P < 0.05, and vs. 52 ± 4 for normal control rats). In vitro effects of 4-OH-Ile in NIDD rat islets. The effect of 4-OH-Ile at the same concentration used on normal islets (200 µM) was tested directly on isolated, incubated islets from NIDD rats (Fig. 6). On a per islet basis, insulin release from normal rat islets reached 1.24 ± 0.17, 3.47 ± 0.26, and 4.89 ± 0.28 ng/h for 3, 8.3, and 16.7 mM glucose, respectively.
Discussions

This study shows that the previously observed insulinotropic effect of 4-OH-Ile in vitro is maintained in vivo and accounts for the improvement of glucose tolerance occurring in two animal species (rats and dogs). This effect is restricted to the linear form of the amino acid. More importantly, we also demonstrate for the first time that this amino acid is able to reduce basal hyperglycemia and to moderately, but significantly, improve glucose tolerance in NIDD rats, pointing out that 4-OH-Ile may be considered as a novel potential antidiabetic drug.

In normal anesthetized rats, a single dose of 4-OH-Ile (18 mg/kg) strongly potentiated the insulin response to an intravenous glucose load and this effect was sufficient to improve glucose tolerance. However, in these experiments glycemia did not return to basal values, which could be due to our experimental conditions, i.e., surgical procedure in the cervical region and barbiturate-induced anesthesia. Indeed, large doses of barbiturates have been reported to cause abnormal glucose tolerance curves (7, 14). Even with this bias, the results nevertheless show a clear effect of 4-OH-Ile.

Actually, the ability of the amino acid to operate in vivo is strongly reinforced by our experiments in conscious normal dogs. These experiments further show that 4-OH-Ile (18 mg/kg) remains effective on insulin secretion and glucose tolerance after oral administration, suggesting that 4-OH-Ile is readily absorbed during gastrointestinal transit. In this respect, it is worthy to mention that the insulinotropic effect of 4-OH-Ile is only observed in the presence of the linear form of this amino acid, whereas its lactonic form is ineffective, even at a higher dosage (36 mg/kg), on both OGTT and IVGTT. These results are consistent with those in vitro showing that the lactonic form has no effect on insulin secretion by the isolated perfused

Fig. 5. Effects of a 6-day subchronic treatment with 4-OH-Ile (50 mg/kg ip daily) on plasma insulin (A and C) and glucose (B and D) levels in response to a glucose load (IVGTT, 0.5 g/kg) in NIDD rats. IVGTTs were performed before (A and B) and under (C and D) treatment (6th day) in 4-OH-Ile-treated NIDD rats (○) but also in placebo-treated normal rats (●) and placebo-treated NIDD rats (□). Basal insulinemia was 1.78 ± 0.09 and 1.53 ± 0.11 ng/ml, 1.72 ± 0.08 and 2.14 ± 0.20 ng/ml, and 2.15 ± 0.18 and 2.12 ± 0.20 ng/ml, respectively, for 2 successive IVGTTs and for each group of animals. Values are means ± SE of 8–9 animals.

Fig. 6. Effects of 4-OH-Ile (200 µM) on insulin release from NIDD rat isolated islets (D+) incubated in presence of different glucose concentrations (3, 8.3 and 16.7 mM). Normal rat islet (N) and NIDD rat islets (D) were incubated with glucose alone at same concentrations. Values are means ± of 7–10 experiments. *P < 0.05; **P < 0.01.
pancreas, in marked contrast with the linear form (data not shown). This excludes the possibility of an insulinitropic effect of the cyclic form of 4-OH-Ile, an alternative we had to consider because reversible lactonization of the amino acid is likely to occur due to gastric acidity.

Major information arises from the data obtained in NA plus STZ-induced diabetic rats, which constitute a new experimental diabetic model that mimics some features of human type II diabetes not shared by other established animal models of the disease, essentially a partial responsiveness to glucose and a well-preserved sensitivity to sulfonylureas (13). The NA plus STZ rat is a mildly diabetic animal without associated obesity, primarily characterized by reduced pancreatic insulin stores (~40% of normal) and defective insulin secretion, which presents a good stability of its diabetic state during at least 9 wk. So, this model appears particularly suitable to investigate the potential antidiabetic properties of new insulinitropic agents, such as 4-OH-Ile.

First, in this NIDD rat model, a single intravenous administration of 4-OH-Ile at the dose of 50 mg/kg during IVGTT partially restored the impaired insulin response to glucose. Thus the increment of plasma insulin levels induced by intravenous glucose injection in 4-OH-Ile-treated NIDD rats, albeit less important than in placebo-treated normal rats, was approximately twice that observed in placebo-treated NIDD rats. This single administration of 4-OH-Ile however appeared insufficient to significantly improve glucose tolerance in diabetic rats after 3–4 wk of established NIDDM.

Therefore, in an attempt to obtain a more pronounced effect, subchronic treatment with 4-OH-Ile was initiated. Our results show that daily administration of this amino acid during 6 days at the same dose (50 mg/kg) resulted in a beneficial impact on both basal plasma glucose and glucose tolerance even though complete correction could not be achieved. Indeed, the subchronic treatment of NIDDM rats with 4-OH-Ile resulted in a 65% reduction of the increase in basal glycemia and a substantial amelioration of the glucose intolerance during IVGTT. This antihyperglycemic effect can be attributed, at least partly, to the partial restoration of the insulin response to glucose in diabetic rats. The persistence of 4-OH-Ile insulinitropic activity in diabetic animals was confirmed by the data obtained in vitro on islets isolated from these NIDDM rats. Indeed 4-OH-Ile, at the same concentration (200 µM) effective in normal islets, potentiated insulin release of incubated diabetic rat islets in the presence of high glucose concentration (16.7 mM). In contrast, 4-OH-Ile was ineffective in the presence of low glucose concentration (3 mM). These data are in agreement with those recently reported in normal islets from rats and humans (18). However, at intermediate glucose concentration (8.3 mM), no significant potentiating effect of 4-OH-Ile (200 µM) was observed in islets isolated from NIDDM rats, unlike results obtained in normal islets (18). A possible explanation could be that this defect in the insulin response of diabetic rat islets results from the adverse influence of a hyperglycemic environment, a phenomenon known as glucose toxicity (12, 21). Chronic hyperglycemia could indeed alter the set point of the B cell, i.e., the dose-response relationships between glucose concentration and insulin secretion rate. If this is the case in these diabetic rat islets, the shift of this set point might preferentially result in a potentiation of insulin release by 4-OH-Ile at a higher glucose (16.7 mM) rather than at an intermediate glucose concentration (8.3 mM), which corresponds to basal glycemia of diabetic rats.

Taken together, the present data suggest that improvement of the diabetic state of NA plus STZ rats results, at least partly, from a direct stimulating effect of 4-OH-Ile on B cell function. In this respect, the slight decrease in basal insulinemia that occurs simultaneously with the reduction of basal hyperglycemia is surprising. This unexpected observation, the reason for which remains to be determined, might however be of great interest, all the more as chronic fasting hyperglycemia of type II diabetes is well known to be often associated with hyperinsulinism. In any case, reduction of basal hyperglycemia with a concomitant decrease in basal plasma insulin levels argues once more that the latter provide little information about insulin secretory capacity but rather are a better indicator of the degree of insulin resistance (20). As a consequence, additional beneficial effects of 4-OH-Ile on peripheral insulin sensitivity cannot be excluded and deserve further investigations. In this respect, even though in alloxan-induced insulin-dependent diabetic dogs, no such favorable effect of a fraction of fenugreek seeds could be observed (17; possibly due to the severity of this diabetic state and/or a low content of 4-OH-Ile in the seed extract), in agreement with a possible effect are the data of Raghuaram et al. (16); these authors suggested that fenugreek can improve peripheral glucose utilization, which contributes to an improvement in glucose tolerance in type II diabetic patients after daily ingestion for 15 days of high amounts (25 g) of fenugreek seed powder.

In summary, we have now demonstrated that 4-OH-Ile is able to stimulate insulin secretion in vivo and to improve glucose tolerance in normal rats and dogs. Another major finding is that these properties are also observed in a rat model of NIDDM previously shown to mimic major features of human type II diabetes. Indeed, in the NA plus STZ rat, subchronic administration of 4-OH-Ile reduced basal hyperglycemia and improved glucose tolerance thereby meeting, albeit modestly, two major aims in type II diabetes treatment. Therefore, the new insulinitropic agent 4-OH-Ile could now be considered of some interest for the treatment of NIDDM.

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