4-Hydroxyisoleucine

A Novel Amino Acid Potentiator of Insulin Secretion

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We report the characterization of a new insulinotropic compound, 4-hydroxyisoleucine. This amino acid has been extracted and purified from fenugreek seeds, which are known in traditional medicine for their antidiabetic properties. 4-Hydroxyisoleucine increases glucose-induced insulin release, in the concentration range of 100 µmol/l to 1 mmol/l, through a direct effect on isolated islets of Langerhans from both rats and humans. The stimulating effect of 4-hydroxyisoleucine was strictly glucose dependent; indeed, ineffective at low (3 mmol/l) or basal (5 mmol/l) glucose concentrations, the amino acid potentiated the insulin secretion induced by supranormal (6.6–16.7 mmol/l) concentrations of glucose. In addition, in the isolated perfused rat pancreas, we could show *1*) that the pattern of insulin secretion induced by 4-hydroxyisoleucine was biphasic, *2*) that this effect occurred in the absence of any change in pancreatic α- and δ-cell activity, and *3*) that the more glucose concentration was increased, the more insulin response was amplified. Moreover, 4-hydroxyisoleucine did not interact with other agonists of insulin secretion (leucine, arginine, tolbutamide, glyceraldehyde). Therefore, we conclude that 4-hydroxyisoleucine insulinotropic activity might, at least in part, account for fenugreek seeds' antidiabetic properties. This secretagogue may be considered as a novel drug with potential interest for the treatment of NIDDM. *Diabetes* 47:206–210, 1998

β-cell defect and insulin resistance are essential features of NIDDM (1–4), and both features are the focus of intensive investigations. In this context, plants are a source of many biochemical substances that present interesting therapeutic properties (5). Thus from traditional medicine, the antidiabetic properties of fenugreek (*Trigonella fœnum-grœcum* L. leguminosae) seeds have been known for a long time. These properties have been evaluated in animals (6–9) and humans (10) and are generally attributed to the high-fiber content of the seeds and the subsequent reduction of intestinal glucose absorption (11). We showed that the fiber-rich extract of fenugreek seeds from testa + endosperm was able to decrease hyperglycemia and glycosuria in insulin-dependent alloxan diabetic dogs (9). However, more recently, we reported (12) that chronic administration of a second fenugreek seed extract obtained from cotyledons + axes increased plasma insulin levels in normal rats. From this second extract, which was found to contain many water-soluble substances, including steroid saponins, trigonelline, flavonoids, and free amino acids, we have isolated and purified 4-hydroxyisoleucine, which is the most typical amino acid in this genus. This substance appeared to be a novel insulinotropic compound when tested in three different preparations, particularly human pancreatic islets.

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TLC, thin-layer chromatography; HPLC, high-performance liquid chromatography; $[\alpha]^{20}_{D}$, optical rotation.

RESEARCH DESIGN AND METHODS

Isolation and identification of 4-hydroxyisoleucine. Fenugreek seeds (cultivar Gouka) were obtained from our experimental field at the Montpellier II University, Montpellier, France. Mature seeds were ground and defatted with hexane using a Soxhlet apparatus, and the powder (100 g) was extracted with CH_3CH_2OH/H_2O (20/80) at room temperature. After a vacuum concentration, the basic compounds were fixed on an Amberlite IR 120, H^+ form, and eluted with 2 mol/l NH_4OH. The ammoniacal solution was concentrated and lyophilized (2.9 g), and then fractionated twice successively on a chromatography column filled with silica gel. Fractions containing 4-hydroxyisoleucine (determination by thin layer chromatography [TLC] and high-performance liquid chromatography [HPLC]) were pooled). Purification and repeated crystallizations gave 0.6 g pure 4-hydroxyisoleucine (overall yield from dried plant material, 0.56% wt/wt). HPLC analysis of 4-hydroxyisoleucine was carried out on a Shimadzu HPLC (LC. 6A) apparatus equipped with a Shimadzu fluorimeter (RF 530, Kyoto, Japan). We used a highly sensitive method based on precolumn formation of a derivative with *O*-phtaldialdehyde (13). Separation was performed on a reverse phase column (Adsorbosphere OPA HS 100 × 4.6 mm, 5 µm) with an elution gradient composed of CH_3COONa 65 mmol/l, 5% tetrahydrofurane (pH 5.7), and methanol. Detection was carried out by fluorescence analysis (λ excitation, 355 nm; λ emission, 410 nm). The chromatograph results revealed one well-separated peak (retention time 8 min, 14 s). All the physicochemical analyses ($[\alpha]^{20}_{D}$, IR spectra, mass spectra, fast atom bombardment [FAB] mode, ^1H and ^{13}C nuclear magnetic resonance [NMR] spectra) indicate that the substance isolated from fenugreek seeds is 4-hydroxyisoleucine (molecular formula: $C_6H_{13}O_3N$ with 2S, 3R, 4S configuration).

Pharmacological procedures

Rat pancreas isolation. Adult male Wistar rats weighing between 330 and 350 g were used in this study. The surgical procedure was described previously (14). Rats were anesthetized with sodium pentobarbitone (60 mg/kg i.p.). The pancreas was totally isolated and perfused through its own arterial system with a Krebs-Ringer bicarbonate buffer containing 2 g/l bovine serum albumin and glucose at appropriate concentrations. The solution had the following ionic composition (in millimoles per liter): NaCl 108, KH_2PO_4 1.19, KCl 4.74, $CaCl_2$ 2.54, $MgSO_4 \cdot 7H_2O$ 1.19, and $NaHCO_2$ 18. An O_2/CO_2 (95/5) mixture was continuously bubbled through this medium, while maintaining a pH level of 7.4. The preparation was kept at 37.5°C

and perfused at a constant pressure, selected to give a flow rate of 2.5 ml/min at the end of the stabilization period. Any change in pancreatic vascular resistance was thus detected by measuring the flow rate. In all experiments, there was a 30-min adaptation period before taking the first sample. Two more samples were taken at 40 and 45 min, with the 45-min time representing the reference value. 4-Hydroxyisoleucine was then perfused for 30 min or 10 min. Indeed, in some experiments, the duration of 4-hydroxyisoleucine perfusion was reduced to 10 min because of the scarceness of the drug. The effluent was collected for 1 min for each sample, which was then immediately frozen for pancreatic hormone (insulin, glucagon, somatostatin) radioimmunoassays.

Rat islet isolation. Islets were isolated after collagenase digestion of the pancreas from adult Wistar rats (15). Immediately after isolation, the islets were preincubated for 90 min at 37.5°C in a Krebs-Ringer bicarbonate buffer (pH 7.4) containing 1 g/l bovine serum albumin and 8.3 or 3 mmol/l glucose. Thereafter, batches of three islets were incubated in the presence of the appropriate glucose concentration (8.3 or 3 mmol/l) for 60 min in 1 ml medium. In these conditions, two experimental sets were performed; first, in the presence of 8.3 mmol/l glucose, the effects of different concentrations of 4-hydroxyisoleucine alone on insulin release were tested; second, a possible interaction of 4-hydroxyisoleucine on the insulin secretion induced by different other secretagogues (L-leucine, L-arginine, tolbutamide, D-glyceraldehyde) was also investigated in the presence of 3 mmol/l glucose, a concentration chosen to avoid interaction of glucose with 4-hydroxyisoleucine and other secretagogues. At the end of each incubation period, an aliquot of the medium was frozen for insulin radioimmunoassay.

Human organ donors and procurement of pancreatic tissue. Human pancreases were obtained from brain-dead organ donors in the context of a human islet transplantation protocol, essentially as reported previously (16). The protocol was approved by the hospital ethics committee, and in all cases informed consent was obtained from family members. After perfusion and extraction, organs were maintained at 4°C for 2–6 h in a solution developed by the University of Wisconsin. Human pancreatic islets were isolated after a modification of Ricordi's automatic digestion technique. For experiments described in this study, purification of pancreatic islets was accomplished by hand-picking under a stereomicroscope to ensure a comparable degree of purity in the different samples. Positive identification of islets was confirmed by staining aliquots with dithizone (17). For measuring insulin release, groups of eight islets were incubated for 90 min at 37°C in 1.0 ml bicarbonate-buffered medium, as described elsewhere (17). After removal of the incubation medium, the same islets were sonicated at 4°C in 0.5 ml acid-alcohol (ethanol 75%, H_2O 23.5%, HCl 10N 1.5%) for the measurement of their insulin content.

Assays. Insulin concentrations were measured by the method of Herbert et al. (18) using an antibody supplied by Miles Laboratories (Paris). ^{125}I-labelled insulin was obtained from International CIS (Gif-sur-Yvette, France); the standard used was rat insulin (Novo, Copenhagen, Denmark), whose biological activity was 22.3 μU/ng. The intra- and interassay coefficients of variation were 9 and 13.5%, respectively. The sensitivity was 0.1 ng/ml.

For glucagon and somatostatin determinations, samples were collected in chilled tubes containing 100 μl of a mixture of EDTA (32 mmol/l) and aprotinin (Antagosan, Hoechst Laboratories, Puteaux, France; 10,000 U/ml kallikrein inhibitor). Glucagon concentrations were measured by the method of Unger et al. (19) using BR124 glucagon antiserum from the Institut de Biochimie Clinique (Centre Médical Universitaire, Geneva, Switzerland); the standard used was Novo porcine glucagon. The intra- and interassay coefficients of variation were 10 and 15%, respectively. The sensitivity was 15 pg/ml. The results are expressed as picograms per milliliter equivalents of porcine glucagon.

Plasma somatostatin-like immunoreactivity (SLI) was assayed according to the previously described technique (20) using the 80°C antiserum from Dr. R. Unger (Health Science Center, Dallas, Texas). The intra- and interassay coefficients of variation were 10 and 14%, respectively. The sensitivity was 10 pg/ml.

Statistical analysis. Results were submitted to analysis of variance followed by the multiple comparison test of Newman-Keuls or to Student's *t* test.

Drugs. L-leucine, L-arginine, D-glyceraldehyde, and tolbutamide were purchased from Sigma (St. Louis, MO). Collagenase for pancreatic digestion was also obtained from Sigma. Aprotinin (Antagosan) was kindly supplied by Hoechst Laboratories (Puteaux, France).

RESULTS

Insulin release from isolated rat islets

Effects of 4-hydroxyisoleucine in the presence of glucose. In the presence of 8.3 mmol/l glucose, 4-hydroxyisoleucine (200 μmol/l) induced a significant increase in

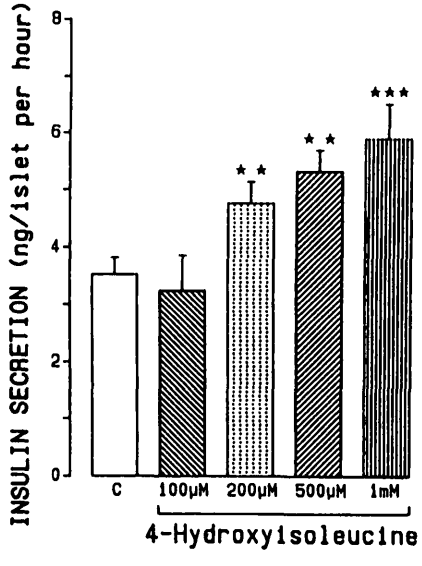

FIG. 1. Effect of 4-hydroxyisoleucine on insulin release from isolated rat islets in the presence of 8.3 mmol/l. Values are means ± SE from at least 10 different experiments. **P < 0.01, ***P < 0.001.

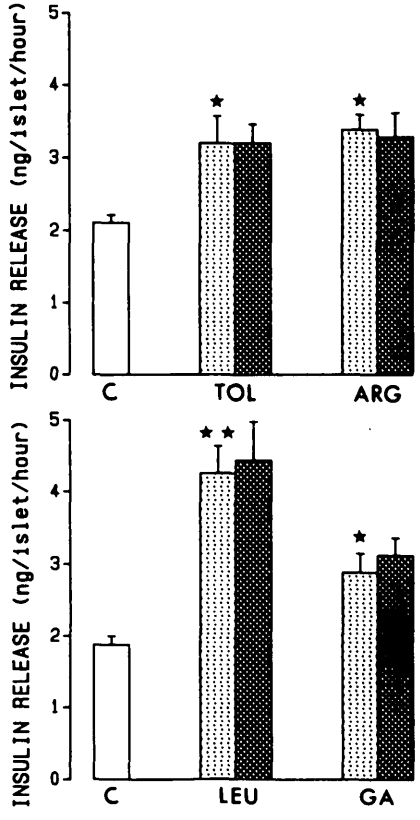

FIG. 2. Effect of 4-hydroxyisoleucine (200 μmol/l) on insulin release induced by other secretagogues in isolated rat islets incubated in the presence of 3 mmol/l glucose. Effects of each secretagogue alone (□): tolbutamide (200 μmol/l), L-arginine (20 mmol/l), L-leucine (10 mmol/l), and D-glyceraldehyde (5 mmol/l). Effects of each secretagogue plus 4-hydroxyisoleucine (▦). Values are means ± SE from at least seven different experiments. Control experiments (C) with glucose alone; *P < 0.05; **P < 0.01.

insulin release (4.8 ± 0.3 vs. 3.5 ± 0.2 ng · islet^{-1} · h^{-1} in controls, $P < 0.01$). This effect was concentration dependent in the range of 200–1,000 µmol/l (Fig. 1). These concentrations were much lower than those required for the stimulating effect of structural amino acid analogs such as leucine or isoleucine. A significant ($P < 0.05$) effect was obtained with L-leucine and L-isoleucine at concentrations of 5 and 3 mmol/l, respectively (6.3 ± 1.0 and 5.6 ± 0.7 vs. 3.4 ± 0.3 ng · islet^{-1} · h^{-1} in controls). Homoserine was completely ineffective in the range of 200 µmol/l to 20 mmol/l (4.1 ± 0.4 to 4.3 ± 0.5 vs. 3.7 ± 0.5 ng · islet^{-1} · h^{-1} in controls).

Effect of 4-hydroxyisoleucine in the presence of other secretagogues. In the presence of 3 mmol/l glucose, 4-hydroxyisoleucine at the concentration of 200 µmol/l was ineffective on insulin release (1.9 ± 0.2 vs. 1.7 ± 0.1 ng · islet^{-1} · h^{-1}). In the same experimental conditions, tolbutamide (200 µmol/l), L-arginine (20 mmol/l), L-leucine (10 mmol/l), and D-glyceraldehyde (5 mmol/l) elicited a significant ($P < 0.05$) increase in insulin release (Fig. 2). 4-Hydroxyisoleucine (200 µmol/l) did not modify the insulin-stimulating effect of these secretagogues.

Pancreatic hormones secretion from isolated perfused rat pancreas. In isolated pancreas, perfused in the presence of a slightly stimulating glucose concentration (8.3 mmol/l), 4-hydroxyisoleucine at a concentration of 200 µmol/l elicited an immediate insulin response, which persisted during the 30 min of administration (Fig. 3). The pattern of this response was clearly biphasic: the first phase peaked at +187 ± 39% (2 min, $P < 0.01$) of basal values and was followed by a second phase throughout the amino acid perfusion. At the end of the drug administration, insulin output progressively returned to basal values. In the same experimental conditions, the drug did not modify either the pancreatic flow rate or the glucagon and somatostatin pancreatic secretions (Table 1).

Interestingly, the stimulating effect of 4-hydroxyisoleucine was clearly related to the glucose concentration in the perfusion medium (Fig. 4*A*). Indeed, 4-hydroxyisoleucine (200 µmol/l) was ineffective in the presence of 5 mmol/l glucose (Fig. 4*B*). In the presence of 6.6 mmol/l glucose, the amino acid elicited a weak and transient insulin response (+3.7 ± 1.2 ng/min at 2 min); the stimulating effect of 4-hydroxyisoleucine was more pronounced in the presence of 8.3 mmol/l glucose (+18.3 ± 5.8 ng/min at 2 min). Finally, the drug induced a biphasic and long-lasting stimulation of insulin secretion in the presence of 10 mmol/l glucose (+47.6 ± 5.2 ng/min at 2 min).

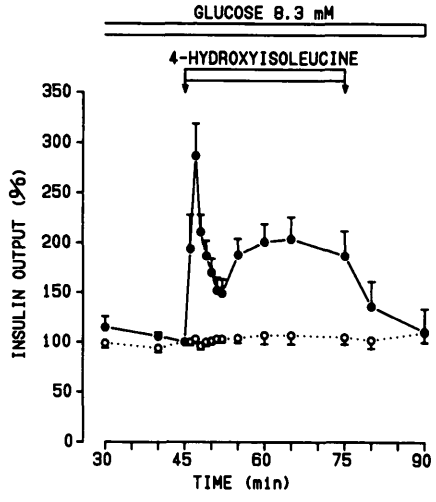

FIG. 3. Effect of 4-hydroxyisoleucine on insulin secretion in isolated perfused rat pancreas in the presence of 8.3 mmol/l glucose. 4-Hydroxyisoleucine was perfused at 200 µmol/l during 30 min (●), and ○ represents control experiments. The results are expressed as changes in percentage of the value at time 45 min, taken as reference (100%). Values are means ± SE of seven to nine experiments. The insulin output rate (ng/min) at 45 min was 12.6 ± 1.8 and 15.0 ± 2.1 for each set of experiments, respectively.

Insulin release from human pancreatic islets. Measurements of insulin release and content in the presence of 8.3 mmol/l of glucose and 4-hydroxyisoleucine at different concentrations (100–1,000 µmol/l) revealed that this amino

TABLE 1
Effects of 4-hydroxyisoleucine on insulin, glucagon, and somatostatin secretions and pancreatic flow rate in isolated perfused normal rat pancreas

	Insulin secretion (ng/min^{-1})	Glucagon secretion (pg/min^{-1})	Somatostatin secretion (pg/min^{-1})	Flow rate (ml/min^{-1})
Time (min)				
−15	12.86 ± 1.20	106 ± 27	36 ± 4	2.49 ± 0.02
−5	12.92 ± 1.01	105 ± 29	34 ± 4	2.52 ± 0.02
−1	11.75 ± 1.28	106 ± 27	44 ± 2	2.51 ± 0.01
4-Hydroxyisoleucine perfusion				
1	15.87 ± 2.36*	111 ± 39	36 ± 4	2.49 ± 0.02
2	29.57 ± 3.57†	117 ± 37	39 ± 4	2.48 ± 0.03
3	21.06 ± 2.45†	126 ± 36	45 ± 4	2.49 ± 0.02
4	17.90 ± 1.71*	137 ± 39	41 ± 7	2.49 ± 0.02
5	15.79 ± 1.78*	129 ± 31	51 ± 7	2.50 ± 0.02
6	13.14 ± 1.37*	140 ± 39	52 ± 14	2.49 ± 0.02
7	13.44 ± 1.81*	110 ± 34	53 ± 17	2.48 ± 0.02
10	16.11 ± 1.81*	137 ± 31	57 ± 20	2.47 ± 0.03
15	10.88 ± 1.29	128 ± 20	53 ± 15	2.48 ± 0.03

Data are means ± SE of five to six experiments. 4-Hydroxyisoleucine was perfused for 10 min at 200 µmol/l in the presence of 8.3 mmol/l glucose. *$P < 0.05$; †$P < 0.01$.

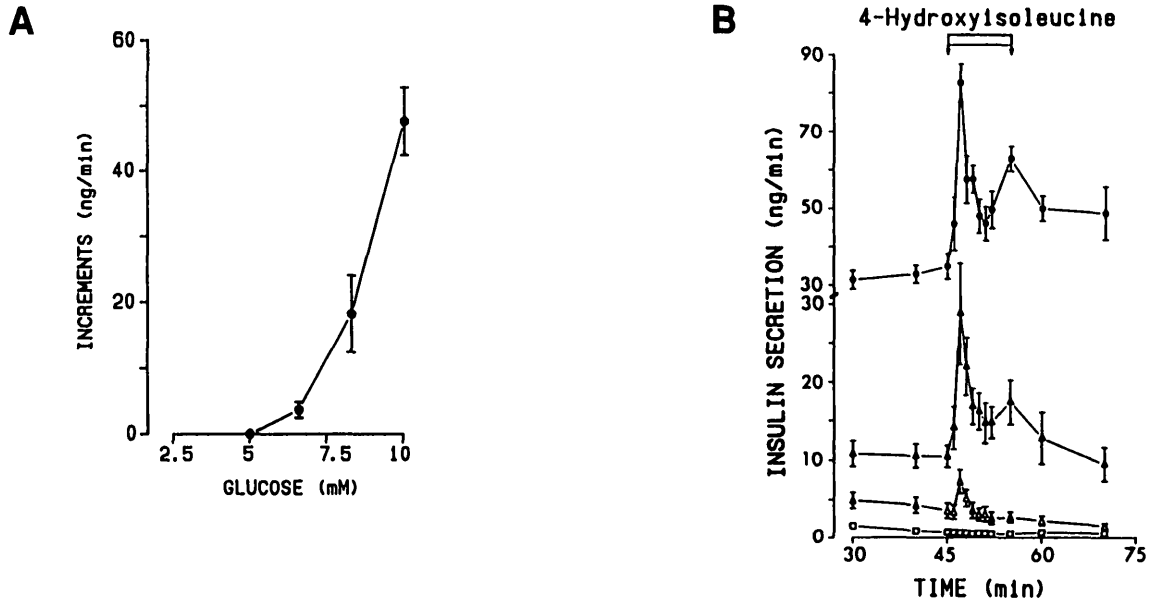

FIG. 4. The insulin-stimulating effect of 4-hydroxyisoleucine (200 µmol/l) is related to the concentration of glucose in the medium. *A*: Relationship between the maximal increment of insulin secretion and the glucose concentration in the medium. These increments express the difference between secretion at 47 min and basal secretion at 45 min. *B*: Kinetics of insulin secretion in the presence of 5 mmol/l (□), 6.6 mmol/l (△), 8.3 mmol/l (▲), and 10 mmol/l (●) glucose. Values are means ± SE of five to nine experiments.

acid stimulates insulin release without affecting insulin content. The amino acid induced a significant increase ($P < 0.05$) from a concentration of 100 µmol/l (Table 2).

Moreover, the stimulatory effect of 4-hydroxyisoleucine at the concentration of 200 µmol/l was studied in the presence of different glucose concentrations (3, 8.3, and 16.7 mmol/l). Ineffective in the presence of 3 mmol/l glucose, 4-hydroxyisoleucine significantly potentiated the insulin release induced by 8.3 mmol/l ($P < 0.05$) or 16.7 mmol/l ($P < 0.05$) glucose (Fig. 5).

DISCUSSION

The results of the present study demonstrate that 4-hydroxyisoleucine, an amino acid extracted and purified from fenugreek seeds, is a novel potentiator of insulin secretion.

It is worthy to mention that this amino acid is not present in mammalian tissues but only found in plants, especially in *Trigonella* species. Isolated and purified according to the procedures we developed, it is mainly distributed in fenugreek seeds in which it accounts for 80% of the total content in free amino acids (21). Consequently, it may be considered as a novel pharmacological substance.

In a previous report, Madar et al. (10) have shown that the daily administration of ground fenugreek seeds are able to lower blood glucose levels in NIDDM subjects. Since fenugreek seeds contain a high percentage of dietary fiber (60%), these authors proposed this latter fraction to be the major contributor for reducing the plasma glucose level. However, owing to *1*) the high content of 4-hydroxyisoleucine in the seeds (0.5% wt/wt) and to *2*) the low threshold concentration for this amino acid to stimulate insulin release in human islets, we can suggest that 4-hydroxyisoleucine might, at least in part, account for fenugreek antidiabetic effects in NIDDM.

The present data show that 4-hydroxyisoleucine stimulates insulin secretion through a direct action on pancreatic β-cells. Indeed, the amino acid is effective both in the isolated perfused pancreas and isolated islets from rats. The target of 4-hydrox-

TABLE 2
Effects of 4-hydroxyisoleucine on insulin release and insulin content of human pancreatic islets

Experimental conditions	Insulin release (pmol/8 islets)	Insulin content (pmol/8 islets)
Glucose 8.3 mmol/l	1.3 ± 0.3 (15)	46.2 ± 4.6 (15)
Glucose 16.7 mmol/l	2.6 ± 0.6 (16)	54.3 ± 5.1 (16)
Glucose 8.3 mmol/l + 0.1 mmol/l	2.6 ± 0.5 (16)*	53.8 ± 5.9 (16)
Glucose 8.3 mmol/l + 0.2 mmol/l	2.8 ± 0.5 (16)*	59.1 ± 5.5 (16)
Glucose 8.3 mmol/l + 0.5 mmol/l	2.9 ± 0.4 (16)†	51.4 ± 5.4 (16)
Glucose 8.3 mmol/l + 1 mmol/l	2.2 ± 0.3 (15)*	47.8 ± 5.4 (16)

Data are means ± SD (n). *$P < 0.05$; †$P < 0.005$ vs. control 8.3 mmol/l glucose.

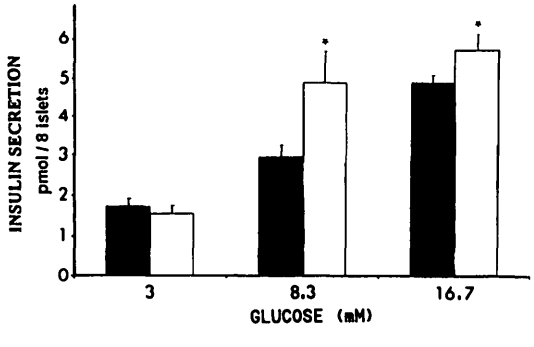

FIG. 5. The stimulating effect of 4-hydroxyisoleucine on insulin release from human islets is related to the concentration of glucose in the medium (□) control experiments (■). Values (mean ± SE of 17–22 determinations) are expressed as picomoles per eight islets per 90 min. *$P < 0.05$ vs. control islets.

yisoleucine in endocrine pancreas seems to be exclusively β-cells, since no change in pancreatic glucagon, somatostatin secretions, or in pancreatic vascular resistance was observed. Thus the activity of this amino acid contrasts with the effects of other insulin secretory amino acids such as arginine (22,23). Moreover, our results show that 4-hydroxyisoleucine is effective on insulin release in a much lower concentration range than its structural amino acids congeners. In our experimental conditions, the drug is 25 and 15 times more effective than leucine or isoleucine, respectively.

The originality of this amino acid is strengthened by our studies concerning the effects of the drug on the insulin response to other secretagogues. Indeed, we investigated the effects of four insulinotropic substances well known to stimulate insulin release, in basal conditions, by different mechanisms (β-cell membrane depolarization related or not to metabolic events). Neither the stimulatory effects of tolbutamide and L-arginine nor those of L-leucine and D-glyceraldehyde were modified by the addition of 4-hydroxyisoleucine (200 μmol/l). Thus it appears that, unlike as for glucose, this drug does not interact with these secretagogues.

It must be emphasized that 4-hydroxyisoleucine is devoid of any secretory effect under normal concentrations of glucose (5 mmol/l) mimicking normoglycemia. In contrast, the insulinotropic activity appeared and strongly developed upon increasing glucose concentrations to supranormal levels. To counteract the deficiency of insulin secretion present in NIDDM (24–26), sulfonylureas are currently the only therapeutic tool available. However, a common drawback of sulfonylureas is the risk of severe hypoglycemia (27,28). It seems worthwhile to search for other insulin-stimulatory agents, which may provide an alternative strategy for the treatment of the disease. From this point of view, it has to be emphasized that the glucose dependency of the response to 4-hydroxyisoleucine may be of interest in vivo in avoiding the risk of hypoglycemia.

In summary, because of *1*) the insulinotropic activity of this amino acid, only in the presence of supranormal levels of glucose, *2*) the great sensitivity of human β-cells to this amino acid, and *3*) the absence of acute toxicity (1 g/kg i.p. in mice, J.J. Serrano, personal communication), we conclude that 4-hydroxyisoleucine may be considered as a novel pharmacological insulinotropic compound potentially useful for the development of an alternative strategy in the treatment of NIDDM. The results of this study have led to a patent (Y.S., G.R., inventors; Jouvenet, assignee. Composition capable of stimulating insulin secretion intended for the treatment of non insulin-dependent diabetes. Fr Demande FR 2,695,317; 10 Mar 1995. U.S. patent 5,470,879; 28 Nov 1995. Eur. pat. appl. EP0,587,476).

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