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4-Hydroxyisoleucine: effects of synthetic and natural analogues on insulin secretion

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Abstract

4-Hydroxyisoleucine, a peculiar amino acid extracted from fenugreek seeds and never found in mammalian tissues, exhibits interesting insulinotropic activity. To investigate the structural requirements for this stimulating effect, the insulinotropic activity of the major isomer (2*S*,3*R*,4*S*) of 4-hydroxyisoleucine, in the presence of 8.3 mM glucose, was compared to that of (1) its minor isomer (2*R*,3*R*,4*S*) (2) its lactone form, (3) classical structurally related amino acids, and (4) synthetic monomethylated analogues. In the isolated, ex vivo, perfused rat pancreas, only the major isomer of 4-hydroxyisoleucine (200 μM) potentiated insulin release. On incubated isolated rat islets, the threshold concentration for a significant increase ($P < 0.05$) in insulin release was 200 μM for (2*S*,3*R*,4*S*) 4-hydroxyisoleucine, 500 μM for (2*S*,4*R*) and (2*S*,4*S*) γ-hydroxynorvalines as well as (2*S*,3*S*) and (2*S*,3*R*) γ-hydroxyvalines, and 1 mM or more for other congeners. In conclusion, the insulinotropic properties of 4-hydroxyisoleucine, in the micromolar range, are seen only in the presence of the linear major isoform; they also require carbon α in S-configuration, full methylation and carbon γ-hydroxylation.

Keywords: 4-Hydroxyisoleucine; Insulin release; Structural analogue; Fenugreek seed; Pancreas; (Rat)

1. Introduction

4-Hydroxyisoleucine (4-OH-Ile) is an original amino acid, not found in mammalian tissues, which is only present in some very specific plants, especially Trigonella species. This amino acid is mainly distributed in fenugreek (*Trigonella foenum graecum* L.) seeds in which it accounts for about 80% of the total content of free amino acids (Fowden et al., 1973, Sauvaire et al., 1984). In their attempt to investigate the stereochemistry of this amino acid, Sauvaire et al. (1984) could show that 4-OH-Ile, a molecule with 3 chiral centers, is present in fenugreek seeds in the form of two diastereoisomers: the major one with (2*S*,3*R*,4*S*) configuration, representing about 90% of the total content of 4-OH-Ile in the seeds, and a minor one with (2*R*,3*R*,4*S*) configuration. These results were confirmed by Hasan (1986) and Alcock et al. (1989). Moreover, other diastereoisomers of 4-OH-Ile seem to be seldom present in plants.

Recently, we discovered that the major isomer of 4-OH-Ile stimulates glucose-induced insulin secretion in the micromolar concentration range through a direct effect on pancreatic B cells in rats and humans (Sauvaire et al., 1998). This effect is glucose-dependent and occurs only in the presence of moderate (8.3 mM) to high (16.7 mM) glucose concentrations. Furthermore, in a new model of type 2 diabetes in rats (Masiello et al., 1998), (2*S*,3*R*,4*S*) 4-OH-Ile is still active and partly corrects hyperglycemia and glucose intolerance (Broca et al., 1999). Thus, 4-OH-Ile, a polar non-charged amino acid structurally related to branched-chain amino acids, may be considered as a novel

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drug, which could be a potential antidiabetic agent of interest in type 2 diabetes.

The present study was designed to get a first insight into structure-activity relationships between 4-hydroxyisoleucine and closely related substances in order to define the structural features required for this amino acid to be considered as the leader of a new class of insulin secretagogues. Therefore, using two different preparations, the isolated, ex vivo, perfused rat pancreas and incubated rat islets, we compared the insulinotropic effectiveness of the major isomer of 4-OH-Ile (2*S*,3*R*,4*S*; 90% in fenugreek seeds) with that of (1) its minor isomer (2*R*,3*R*,4*S*; 10% in fenugreek seeds), (2) its lactone form which occurs under specific conditions, (3) synthetic monomethylated derivatives, and (4) its classical structurally related amino acids such as leucine, isoleucine or homoserine.

2. Materials and methods

2.1. Obtention of 4-OH-Ile and related compounds (Fig. 1)

2.1.1. 4-OH-Ile major and minor isomers

Fenugreek seeds (cultivar Gouka) were obtained from our experimental field at the University Montpellier II in Montpellier, France. Mature seeds were ground and defatted with hexane using a Soxhlet apparatus; the powder (100 g) was extracted with CH_3CH_2OH/H_2O (20/80) at room temperature. After vacuum concentration, the total free amino acids were fixed on an Amberlite IR 120, H^+ form and eluted with 2 M NH_4OH. The ammonia 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 4-hydroxyisoleucine (overall yield from dried plant material, 0.56% wt./wt.).

The two isomers of 4-hydroxyisoleucine present in fenugreek seeds were separated on a HPLC semi-preparative column (Ultrabase C18, 150 × 10 mm). The mobile phase was pure water at a flow rate of 2 ml/min. Detection was carried out by U.V. absorption (200 nm) or refractometry (retention time was 9.6 min for the minor isomer (2*R*,3*R*,4*S*) and 10.2 min for the major isomer (2*S*,3*R*,4*S*)). This separation allowed the isolation of sufficient quantities of both isomers for pharmacological testing.

HPLC analysis of each isomer of 4-hydroxyisoleucine was carried out on a Shimadzu HPLC (LC. 6A) apparatus equipped with a Shimadzu fluorimeter (RF 530). We used a highly sensitive method based on precolumn formation of a derivative with *O*-phtaldialdehyde (Lindroth and Mopper, 1979). Separation was performed on a reverse-phase column (Adsorbosphere OPA HS 100 × 4.6 mm, 5

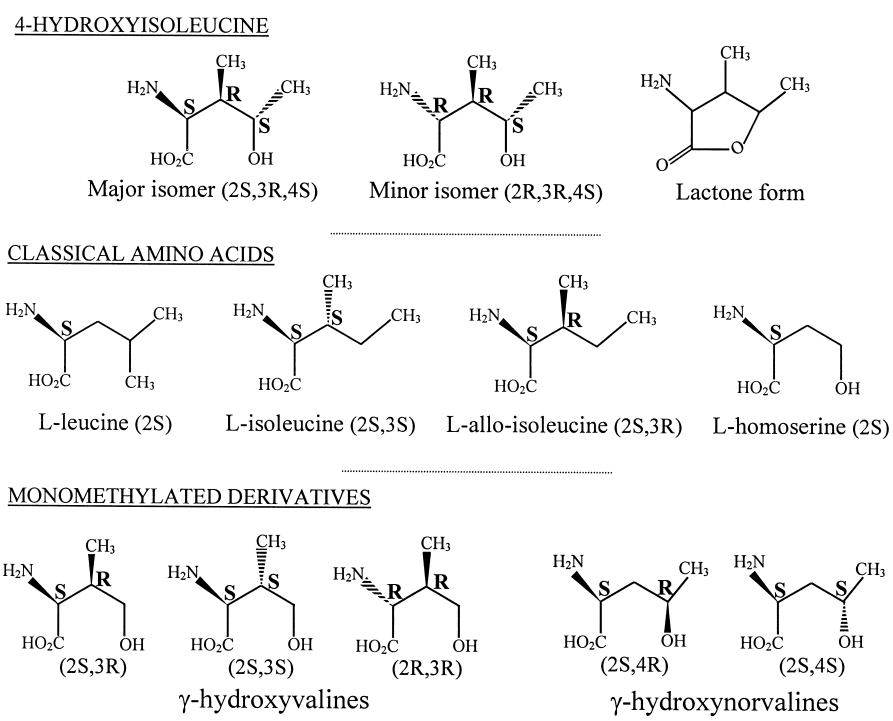

Fig. 1. Structures of 4-hydroxyisoleucine major and minor isomers, 4-hydroxyisoleucine lactone form, classical structurally related amino acids and synthetic monomethylated analogues.

μm) with an elution gradient composed of CH_3COONa 65 mM, 5% tetrahydrofurane (pH 5.7) and methanol. Detection and quantification were carried out by fluorescence analysis (λ excitation: 355 nm, λ emission: 410 nm).

Identity with 4-hydroxyisoleucine was confirmed by comparing the physicochemical data with those for an authentic sample provided by Sir Leslie Fowden, Rothamsted Experimental Station (Harpenden, UK).

2.1.2. Lactone form

The lactone form of 4-OH-Ile was kindly provided by the Société Jouvenet (Paris, France).

2.1.3. Synthetized related compounds

Monomethylated derivatives were synthetized by the Laboratoire des Acides Aminés Peptides et Protéines (UMR 5810 CNRS, Montpellier, France).

2.1.4. Classical structurally related amino acids

Amino acid analogues (L-leucine, L-isoleucine, L-homoserine, L-allo-isoleucine, D-isoleucine) were purchased from Sigma (St. Louis, MO, USA).

2.2. Pharmacological methods

Experiments were performed on male Wistar rats (Iffa Credo, Lyon, France). They received a standard pellet diet (UAR, Epinay Sur Orge, France) and had free access to tap water. Experiments were carried out in accordance with the ethical rules of animal experimentation recommended by French legislation. Drugs were tested ex vivo and in vitro using two preparations, the isolated perfused pancreas and isolated incubated islets, to ascertain whether drugs have a direct stimulatory effect on insulin secretion.

2.2.1. Experiments on the isolated, ex vivo, perfused pancreas

The rats weighed 330 to 360 g on the day of the experiments. After sodium pentobarbitone anaesthesia (60 mg/kg i.p.), the pancreas was totally isolated from all neighbouring tissues according to the technique of Loubatières et al. (1969). Then the organ was transferred to a thermostated (37 C) plastic chamber and perfused through its own arterial system with a Krebs Ringer Bicarbonate (KRB) buffer containing 2 g/l pure bovine serum albumin (fraction V, Sigma, St. Louis, MO, USA). The KRB buffer, administered with a peristaltic pump via an open circuit, had the following ionic composition (mM): NaCl 108, KH_2PO_4 1.19, KCl 4.74, $CaCl_2$ 2.54, $MgSO_4$ $7H_2O$ 1.19, and $NaHCO_2$ 18. A mixture of O_2/CO_2 (95/5) was continuously bubbled through this buffer to provide an adequate oxygen supply and to keep the pH close to 7.3. Each pancreas was perfused in the presence of 8.3 mM glucose, at a constant pressure (35–45 cm H_2O) selected to produce a pancreatic flow rate of 2.5 ml/min at the start of the experiments. In all experiments, a 30-min adaptation period was allowed before the first sampling. All samples were immediately frozen until insulin assay.

2.2.2. Experiments on isolated islets of Langerhans

Rats weighed 300 to 330 g at the time of the experiments. After they were killed by decapitation, the pancreas was dissected out and pancreatic islets were isolated by the collagenase method (Lacy and Kostianovsky, 1967). In all experiments, islets were preincubated for 60 min at 37 C in a KRB buffer containing 2 g/l bovine serum albumin and 8.3 mM glucose. Batches of 3 islets were then incubated for 60 min in tubes containing 1 ml medium plus drugs at different appropriate concentrations. At the end of the incubations, 200-μl aliquots were immediately frozen until insulin assay.

2.2.3. Insulin assay

Insulin concentrations were determined by a radioimmunological method (Herbert et al., 1965) using a guinea pig anti porcine insulin antibody (ICN, Paris, France). The standard used was rat insulin (Novo, Copenhagen, Denmark). The sensitivity of the assay was 0.1 ng/ml. Insulin output from isolated pancreas was calculated by multiplying the hormone concentration (ng/ml) in the effluent by the flow rate (ml/min).

2.2.4. Statistics

Insulin kinetics data are expressed as changes in percent of the value at time 45 min taken as reference for isolated perfused pancreas; insulin release from incubated islets is expressed in ng/islet per 60 min. They appear on figures as means ± S.E.M. for the indicated number of experiments. The statistical significance of the differences between means was determined using an analysis of variance in conjunction with the multiple comparison test of Newman–Keuls.

3. Results

The structure and spatial conformation of the various 4-OH-Ile isomers and related analogues are given in Fig. 1.

3.1. 4-OH-Ile: major, minor, lactone isomers

3.1.1. Major isomer of 4-OH-Ile

In the isolated rat pancreas, the stimulating effect on insulin secretion induced by the major isomer (2*S*,3*R*,4*S*) of 4-OH-Ile perfused at the concentration of 200 μM for 10 min was clearly dependent on the concentration of glucose present in the medium. Not observed in the presence of 5 mM glucose, the stimulating effect developed progressively when the glucose concentration rose. Thus, the areas under the curve corresponding to insulin secre-

tion during the 10-min period of administration were 1.8 ± 1.7 ng for 5 mM glucose, 4.4 ± 4.3 ng for 6.6 mM glucose, 70.4 ± 18.8 ng for 8.3 mM glucose ($P < 0.01$) and 206.7 ± 10.8 ng for 10 mM glucose ($P < 0.001$). For further investigations, we selected the slightly stimulating 8.3 mM glucose concentration.

In isolated rat islets incubated in the presence of 8.3 mM glucose, 4-OH-Ile (2*S*,3*R*,4*S*) increased insulin release in the 200 μM to 5 mM concentration range ($P < 0.01$) (Fig. 2). Thus, 200 μM appears as the threshold concentration for the major isomer of 4-OH-Ile to significantly potentiate glucose-induced insulin release.

3.1.2. Minor isomer of 4-OH-Ile

In the isolated rat pancreas perfused with 8.3 mM glucose, the major or minor isomer was added at the concentration of 200 μM for 10 min. Under these conditions, the major isomer of 4-OH-Ile (2*S*,3*R*,4*S*) elicited a rapid and significant biphasic insulin response (peak at min 2 + 210 ± 20%, $P < 0.01$) while the minor isomer (2R,3R,4S) was completely ineffective (Fig. 3A). In order to define the threshold concentration required to induce a significant potentiation of 8.3 mM glucose-induced insulin release, we tested both enantiomers at higher concentrations using incubated isolated islets. The concentration range tested was 5×10^{-5} to 2×10^{-3} M. The threshold concentration was always 200 μM for the major isomer (+55 ± 5% vs. controls, $P < 0.01$) but reached 1 mM for the minor isomer (+37 ± 10% vs. controls, $P < 0.05$) (Fig. 3B).

3.1.3. Lactone form of 4-OH-Ile

The ability of 4-OH-Ile to cyclicise into a lactone form under specific conditions, such as strong acidity, prompted us to test the effect of this latter form on insulin secretion.

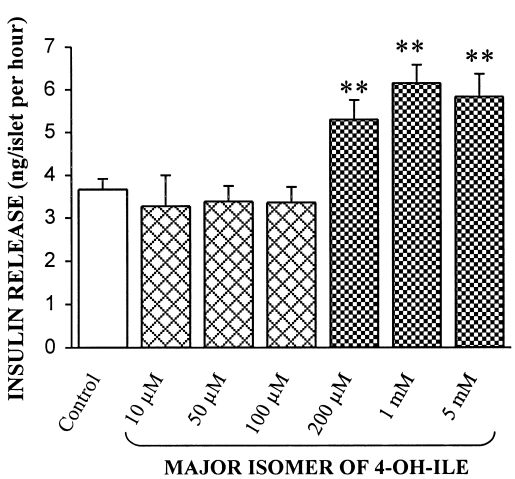

Fig. 2. Effect of the major isomer (2*S*,3*R*,4*S*) of 4-hydroxyisoleucine at increasing concentrations on insulin release from isolated rat islets incubated in the presence of 8.3 mM glucose. $^{**}P < 0.01$. Data are means ± S.E.M. of 15 to 23 determinations.

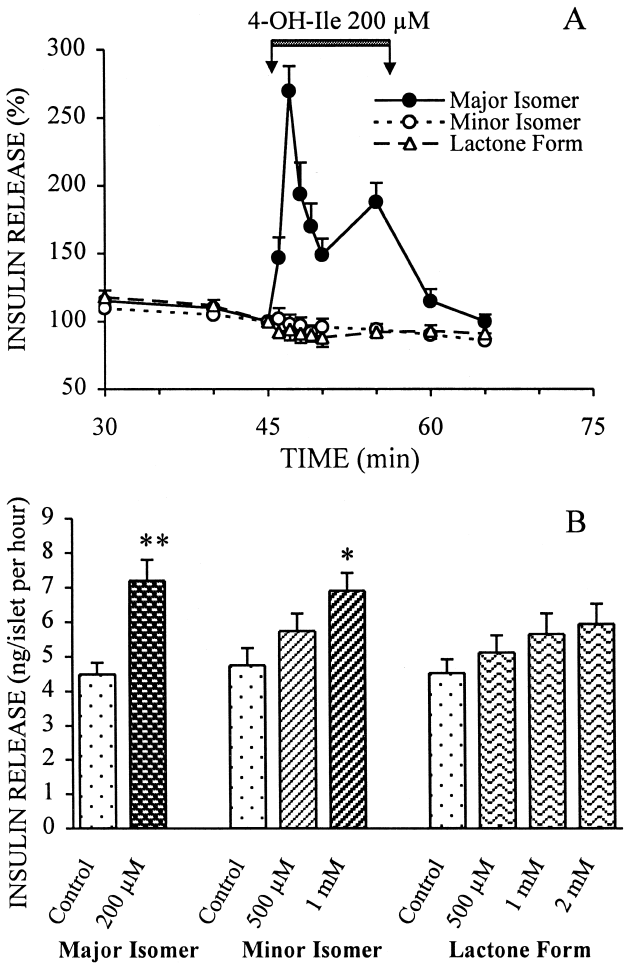

Fig. 3. (A) Effects of 4-hydroxyisoleucine major isomer (●), minor isomer (○) and lactone form (Δ) at the same concentration of 200 μM on insulin released by the isolated rat pancreas perfused in the presence of a slightly stimulating glucose concentration (8.3 mM). Values at min 45 were 10.1 ± 2.1 ng/min for the major isomer ($n = 6$), 15.2 ± 3.1 ng/min for the minor isomer ($n = 5$) and 18.2 ± 3.8 ng/min for the lactone form ($n = 3$). (B) Effect of increasing concentrations of 4-hydroxyisoleucine minor isomer or lactone form on 8.3 mM-glucose-induced insulin release from isolated incubated rat islets compared with 200 μM 4-hydroxyisoleucine major isomer. $^{*}P < 0.05$, $^{**}P < 0.01$, ($n = 10$ to 15). *n* represents the number of observations.

In the isolated rat pancreas, the lactone form of 4-OH-Ile perfused at the concentration of 200 μM for 10 min was completely ineffective (Fig. 3A). When tested in incubated isolated islets between 200 μM and 2 mM, no significant increase in insulin release could be observed (Fig. 3B).

3.2. Synthetic monomethylated analogs

Five monomethylated derivatives, lacking a methyl group on either the γ carbon (2*S*,4*R*) and (2*S*,4*S*) γ-hydroxynorvalines or the β carbon (2*R*,3*R*), (2*S*,3*R*) and (2*S*,3*S*) γ-hydroxyvalines of the 4-OH-Ile major isomer carbon chain, were tested on insulin secretion. In the

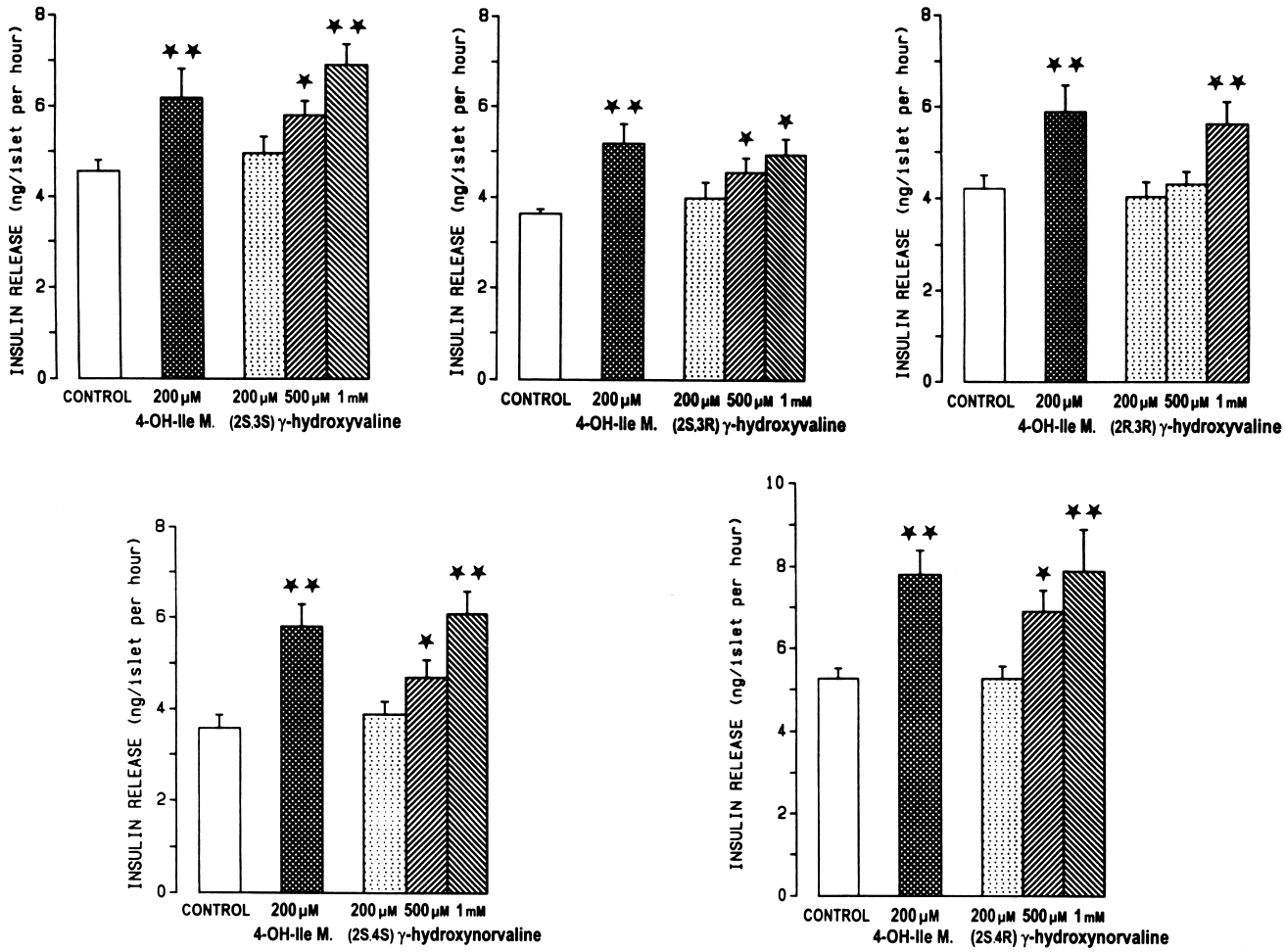

Fig. 4. Effect of increasing concentrations of synthetic monomethylated derivatives of 4-hydroxyisoleucine on 8.3 mM-glucose-induced insulin release from isolated rat islets compared with 200 μM 4-OH-Ile major isomer. $^{*}P < 0.05$, $^{**}P < 0.01$. Data are means ± S.E.M. of 13 to 25 determinations.

isolated rat pancreas perfused with 8.3 mM glucose, the effects of these monomethylated derivatives were compared to those observed with the major isomer of 4-OH-Ile at the same 200 μM concentration. Whereas the major isomer (2*S*,3*R*,4*S*) of 4-OH-Ile always provoked an immediate significant response ($+220 \pm 25\%$ at min 2, $P < 0.05$), none of the monomethylated derivatives tested was able to stimulate insulin secretion (data not shown).

In incubated isolated islets (Fig. 4), the effects of monomethylated derivatives on 8.3 mM glucose-induced insulin release were studied in the 2×10^{-4} to 10^{-3} M concentration range. In comparison with that of the major isomer of 4-OH-Ile (significant stimulation at 200 μM: $+50 \pm 5\%$, $P < 0.01$), their threshold concentration for a clear insulin potentiation ($P < 0.05$) was 500 μM for (2*S*,4*R*) and (2*S*,4*S*) γ-hydroxynorvalines as well as for

Table 1
Effect of 4-hydroxyisoleucine and classical analogues at increasing concentrations on 8.3 mM-glucose-induced insulin release of isolated rat islets
Data are means ± S.E.M. of 15 to 25 determinations.

Insulin release (ng/islet h)							
Concentration (mmol/l)	Nil	0.2	0.5	1	3	5	10
4-OH-lle (2*S*,3*R*,4*S*)	3.45 ± 0.24	5.10 ± 0.45[a]	5.43 ± 0.48[a]	5.82 ± 0.58[a]			
L-lle (2*S*,3*S*)	3.74 ± 0.30		3.98 ± 0.24	4.65 ± 0.40	5.61 ± 0.68[a]	5.97 ± 0.37[a]	6.47 ± 0.23[a]
L-Allo-lle (2*S*,3*R*)	3.66 ± 0.28		3.92 ± 0.38	3.84 ± 0.34	6.20 ± 0.56[a]	6.40 ± 0.80[a]	6.39 ± 0.34[a]
L-Leu (2*S*)	3.50 ± 0.23		3.80 ± 0.30	3.87 ± 0.36	4.67 ± 0.43[b]	5.39 ± 0.66[a]	5.30 ± 0.30[a]

[a] $P < 0.01$.
[b] $P < 0.05$.

(2*S*,3*S*) and (2*S*,3*R*) γ-hydroxyvalines. For (2*R*,3*R*) γ-hydroxyvaline, the threshold concentration was 1 mM.

3.3. Major isomer and its classical structurally related amino acids

Various structural amino acid analogues of 4-OH-Ile were tested between 10^{-4} and 2×10^{-2} M with incubated isolated islets (Table 1). The major isomer of 4-OH-Ile always stimulated 8.3 mM glucose-induced insulin release at 200 μM ($+60 \pm 5\%$, $P < 0.01$). The threshold concentrations for a significant insulin releasing effect were 5 mM for L-leucine (2*S*), 3 mM for L-isoleucine (2*S*,3*S*) and L-allo-isoleucine (2*S*,3*R*). D-isoleucine (2*R*) and L-homoserine (2*S*) were totally ineffective (data not shown).

4. Discussion

The present study showed clearly that the 4-OH-Ile major isomer (2*S*,3*R*,4*S*) extracted from fenugreek seeds is the most potent insulinotropic agent of all the 12 different structurally related amino acids we tested.

First, (2*S*,3*R*,4*S*) 4-OH-Ile is much more potent than (2*R*,3*R*,4*S*) 4-OH-Ile, the minor isomer also extracted from fenugreek seeds, since their insulin releasing effects appeared respectively at 200 μM and 1 mM. Thus the sole presence of the asymmetric carbon α in *S*-configuration appears to be a determinant feature for the insulinotropic effect induced by this molecule. Moreover these data suggest that, due to their insulinotropic activity, both (2*S*,3*R*,4*S*) and to a lesser extent (2*R*,3*R*,4*S*) 4-OH-Ile are the active principles implicated, at least in part, in the antidiabetic properties of fenugreek seeds (Ribes et al., 1984, 1986; Madar et al., 1988).

The lactonization of 4-OH-Ile (2*S*,3*R*,4*S*) appears relatively easy to perform in vitro and is likely to occur in vivo under specific pH conditions, e.g., during gastrointestinal transit. This prompted us to investigate a possible insulinotropic effect of the cyclic form of 4-OH-Ile (2*S*,3*R*,4*S*). Our results showed clearly that, in vitro, the lactone form of 4-OH-Ile is ineffective at 200 μM as well as at higher concentrations. These data could be confirmed in vivo (data not shown). Thus the linear form is required for this amino acid to stimulate efficiently insulin secretion.

The comparison with classical structurally related amino acids also yields interesting information. Among circulating amino acids in mammals, L-leucine and L-isoleucine are, with L-arginine, the most potent insulinotropic ones in the presence of a stimulating glucose concentration (Sener et al., 1981). They are also partly responsible for the increased in vivo versus in vitro glucose sensitivity of pancreatic B cells (Bolea et al., 1997). Our results first confirm that the insulin secretory effects of L-leucine and L-isoleucine appear at millimolar concentrations as previously reported by Sener et al. (1981) and others. The results further show that the threshold for (2*S*,3*R*,4*S*) 4-OH-Ile insulinotropic activity is in fact at least 10-fold lower than the thresholds of these two classical branched-chain amino acids. An additional important finding is that L-homoserine, with preserved hydroxylation on carbon γ but lacking methylation, is totally ineffective as was previously demonstrated for L-serine (Sener et al., 1981). Taken together, these observations suggest that both hydroxylation and methylation on carbon γ are required for the insulin releasing effect of 4-OH-Ile. Moreover, the branched-chain amino acids L-leucine and L-isoleucine as well as some other insulinotropic nutrients potentiate glucose-induced insulin release in the millimolar range, mainly through acceleration of oxidative fluxes depending on their catabolism (Malaisse et al., 1980). It thus seems unlikely that 4-hydroxyisoleucine at the concentration of 200 μM and even 100 μM in human islets (Sauvaire et al., 1998) could act in a similar way.

Among the monomethylated derivatives tested, none was able, at 200 μM, to potentiate glucose-induced insulin release from the isolated perfused rat pancreas. However they were all able to stimulate insulin release with a threshold concentration lower than that of classical branched chain amino acids. Moreover, our results showed that (2*S*,4*R*) and (2*S*,4*S*) γ-hydroxynorvalines as well as (2*S*,3*S*) and (2*S*,3*R*) γ-hydroxyvalines are more potent than (2*R*,3*R*) γ-hydroxyvaline to stimulate insulin release. This difference appears to be due to the *S*- or *R*-configuration of the asymmetric carbon α and could account for the similar lower potency of the minor isomer (2*R*,3*R*,4*S*) of 4-OH-Ile and (2*R*,3*R*) γ-hydroxyvaline. This agrees with and could be related to the well-known greater utilisation of L-amino acids within the islets of Langerhans. In addition, removal of the methyl group from asymmetric carbons γ or β provides evidence that they are equally important since (2*S*,4*R*) and (2*S*,4*S*) γ-hydroxynorvalines as well as (2*S*,3*S*) and (2*S*,3*R*) γ-hydroxyvalines exhibit a similar intermediate concentration threshold for the stimulation of insulin release. These results, therefore, suggest that, more than side-chain length, full branching along the carbon skeleton is an important feature which constitutes, with carbon γ hydroxylation and carbon α *S*-configuration, the three major characteristics that determine the (2*S*,3*R*,4*S*) 4-OH-Ile insulinotropic effect and should be shared by other potential antidiabetic candidates structurally related to the major isomer.

In conclusion, the present data demonstrated that the linear form of the major isomer (2*S*,3*R*,4*S*) of 4-OH-Ile is the most potent of all the analogues tested. This study also provided evidence that the carbon α *S*-configuration, carbon γ hydroxylation, and full methylation are essential structural features required for the interesting insulinotropic activity of 4-hydroxyisoleucine involved in fenugreek seed antidiabetic properties.

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