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TRANSPORT OF L-LEUCINE AND D-LEUCINE INTO PANCREATIC β-CELLS WITH REFERENCE TO THE MECHANISMS OF AMINO ACID-INDUCED INSULIN RELEASE

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SUMMARY

The transport of L-leucine and D-leucine was studied in microdissected pancreatic islets of obese-hyperglycemic mice. These islets consist to more than 90% of β-cells and are known to release insulin in response to L-leucine but not in response to D-leucine.

Both L-leucine and D-leucine were distributed in a space that was larger than that occupied by sucrose, suggesting that both leucine isomers penetrated into the β-cells. The uptake of either isomer was inhibited by L-isoleucine, L-tryptophan, L-phenylalanine and glycine. L-Leucine inhibited the uptake of D-leucine, and D-leucine inhibited the uptake of L-leucine. There was no detectable difference between L-leucine and D-leucine with respect to the concentration dependence of uptake as measured in relation to sucrose. A significantly greater uptake of L-leucine than of D-leucine may be due to the fact that L-leucine was incorporated into islet protein.

It appears that D-leucine and L-leucine are largely transported by the same system in pancreatic β-cells. Consequently, it may be necessary to somewhat qualify the current hypothesis suggesting that the site signalling insulin release in response to L-leucine is identical with the receptor site of transport system L.

INTRODUCTION

Several amino acids can stimulate insulin release through a direct action on the pancreatic islets. The stimulatory effect of non-metabolizable model amino acids[1–3] supports the suggestion[4] that the recognition of amino acids as insulin secretagogues does not require their metabolic degradation. It has been proposed that amino acids stimulate insulin release by binding to specific receptors in the β-cell plasma membrane[1]. As transport sites represent recognition sites which do not necessarily degrade their substrate, it was suggested that the signal for insulin release is mediated by a transport receptor site[1]. This idea was encouraged by the observation that microdissected pancreatic islets exhibited a vigorous transport of non-metabolizable leucine and arginine analogues, which were also found to stimulate insulin release[3].

Abbreviations: BCH, 2-amino-bicyclo(2,2,1)heptane-2-carboxylic acid; GPA, 4-amino-1-guanylpiperidine-4-carboxylic acid.

In the present study we test the hypothesis further by comparing the transport of D-leucine with that of L-leucine in micro-dissected islets of obese-hyperglycemic mice. These islets consist to more than 90 % of β-cells and are known to release insulin in response to L-leucine but not in response to D-leucine[5].

MATERIALS AND METHODS

Chemicals

L-[U-^{14}C]Leucine, D-[4,5-$^{3}H_2$]leucine, [U-^{14}C]sucrose, and [6,6'-$^{3}H_2$]sucrose were obtained from the Radiochemical Centre, Amersham, England. Non-radioactive amino acids were from Sigma Chemical Co., St. Louis, Mo., U.S.A., and non-radioactive sugars were from British Drug Houses Ltd, Pool, England. All other chemicals were commercially available reagents of analytical grade. Distilled and deionized water was used throughout.

Isolation of islets

Adult obese-hyperglycemic mice (gene symbol: *obob*) from the Umeå colony were starved overnight. Fresh pancreatic islets were microdissected free-hand[6] in Krebs-Ringer bicarbonate buffer containing 3 mM glucose as well as 0.3 % (w/v) human serum albumin and equilibrated with O_2–CO_2 (95:5, v/v). No collagenase was used in the isolation procedure.

Incubations

All incubations were performed at 37 °C, using Krebs-Ringer bicarbonate buffer supplemented with 0.3 % human serum albumin as the basal medium. After preincubation for 30 min, batches of 3 islets were incubated in a medium containing radioactive leucine. In some experiments the medium contained either L-[U-^{14}C]-leucine or D-[4,5-$^{3}H_2$]leucine, whereas in others the two isomers were simultaneously present in labelled form. When only one labelled leucine isomer was used, the medium also contained [6,6'-$^{3}H_2$]sucrose or [U-^{14}C]sucrose to permit correction for extracellular label in each batch of islets. The detailed compositions of the incubation media as well as the incubation times are given in the legends to the figures and tables.

Weighing of islets

Incubated islets were placed on bits of aluminium foil and were freed of as much contaminating fluid as possible with the aid of a micro-pipette. This procedure was standardized to take only a few seconds. The islets were then plunged into melting isopentane, freeze-dried (—40 °C, 0.001 mm Hg) overnight, and weighed on a quartz-fibre balance.

Determination of amino acid uptake

After weighing, the islets were dissolved by incubation in 100 μl of hyamine for 45 min at room temperature. After the addition of 10 ml of scintillation liquid (5 g of PPO and 50 mg of POPOP in 1 l of toluene), counting was carried out in a liquid scintillation spectrometer. The observed cpm values were translated into mmoles of labelled substrate by comparison with external standards counted in parallel with the islets. The standards consisted of 5 μl incubation medium dissolved in 100 μl

of hyamine. The setting of the discriminators was such that less than 0.5 % of the ^{3}H counts were also counted in the ^{14}C channel. The spill-over to the ^{3}H channel was 25 % of the counts in the ^{14}C channel. The specific radioactivities were chosen to give approximately the same cpm in either channel.

Incorporation of leucine into islet protein

Batches of 10 islets were incubated for 3 h with 1 mM L-[U-^{14}C]leucine (15.0 mCi/mmole) or 1 mM D-[4,5-$^{3}H_2$]leucine (50.0 mCi/mmole). The incubation medium also contained 0.1 mM [6,6'-$^{3}H_2$]sucrose (250.0 mCi/mmole) or [U-^{14}C]sucrose (100.0 mCi/mmole). After homogenization in 55 μl of 0.32 M sucrose, 15 μl was used for the determination of protein[7]. The proteins in the remaining 40 μl were precipitated by the addition of 150 μl of 10 % (w/v) trichloroacetic acid. After washing twice with trichloroacetic acid, the precipitate was suspended in 20 μl of 0.32 M sucrose. Samples of 5 μl were taken for counting of radioactivity. When calculating the amount of leucine incorporated, correction was made for label residing in the sucrose space of the precipitated protein pellet.

RESULTS

Time course of leucine uptake

Fig. 1 shows the islet uptake of L-leucine and D-leucine with time. Labelled sucrose was used as extracellular marker to permit correction for extracellular and contaminating radioactivity within each batch of islets. Since equilibration of sucrose takes about 15 min[8,9], the values obtained at shorter incubation times are probably

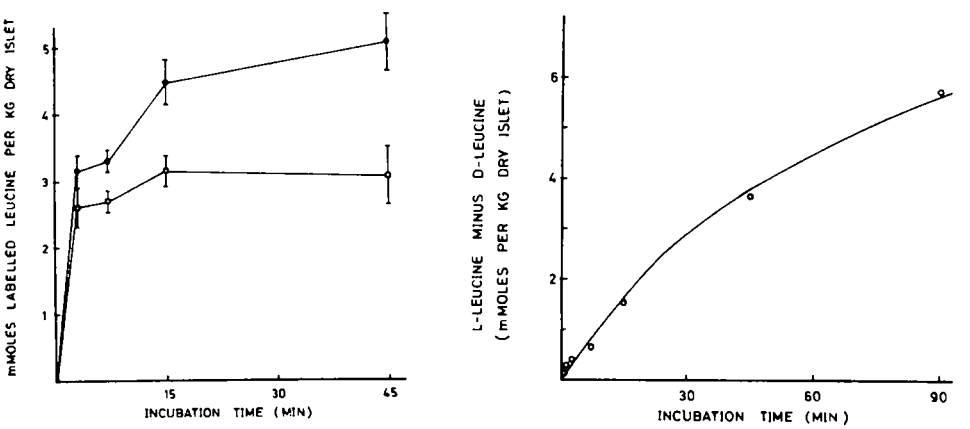

Fig. 1. Islet uptake of L-leucine (●) and D-leucine (□) with time. After preincubation for 30 min in the presence of 3 mM glucose, islets were incubated for different periods of time in glucose-free media containing either 1 mM L-[U-^{14}C]-leucine (7.5 mCi/mmole) or 1 mM D-[4,5-$^{3}H_2$] leucine (25.0 mCi/mmole), as well as 0.1 mM [6,6'-$^{3}H_2$]sucrose (250.0 mCi/mmole) or 0.1 mM [U-^{14}C]-sucrose (100.0 mCi/mmole). Amounts of leucine taken up are given after correction for label in the sucrose space. Each point represents the mean of 6 different experiments. Bars denote S.E.

Fig. 2. Time course of the uptake of L-leucine in relation to the uptake of D-leucine. After preincubation as in Fig. 1, islets were incubated for different periods of time in glucose-free medium containing 1 mM L-[U-^{14}C]leucine (7.5 mCi/mmole) as well as 1 mM D-[4,5-$^{3}H_2$]leucine (25.0 mCi/mmole). Each point represents the uptake of L-leucine *minus* the uptake of D-leucine. Mean values of 2–7 different experiments.

higher than the true β-cell uptake of leucine. However, it is evident from Fig. 1 that both L-leucine and D-leucine were ultimately distributed in a much larger space than was sucrose, suggesting that both isomers penetrated into the β-cells. The uptake of L-leucine appeared to be greater than the uptake of D-leucine at all times, and at 15 and 45 min this difference was statistically significant ($P < 0.01$). If the intracellular water content of incubated islets is taken to be 1.2 times their dry weight[8], the distribution ratio at 45 min can be estimated as 2.6 for D-leucine.

The time course of leucine uptake was also studied by incubating islets in media containing both L-[U-^{14}C]leucine and D-[4,5-$^{3}H_2$]leucine (Fig.2). These experiments confirmed that the net uptake of L-leucine by islet cells is significantly greater than the net uptake of D-leucine.

Concentration dependence of leucine uptake

The effect of the concentration of leucine on its uptake was studied by incubating islets for 90 s in media containing labelled sucrose and different concentrations of either L-[U-^{14}C]leucine or D-[4,5-$^{3}H_2$]leucine (Fig. 3). Because of the comparatively slow equilibration of sucrose the results do not allow estimation of true kinetic parameter values. It is nevertheless notable that there was no detectable difference between L-leucine and D-leucine with respect to the concentration dependence of uptake.

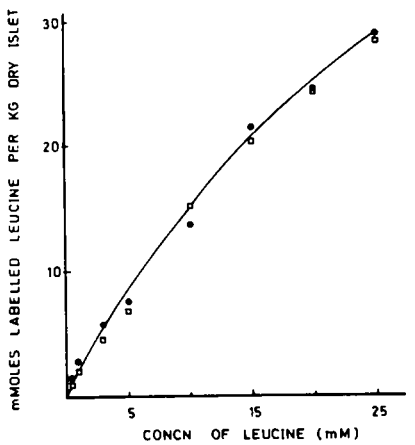

Fig. 3. Concentration dependence of leucine uptake. After preincubation as in Fig. 1, islets were incubated for 90 s in media containing either L-[U-^{14}C]leucine (0.3–15.0 mCi/mmole) or D-[4,5-$^{3}H_2$]leucine (1.0–50.0 mCi/mmole) at different concentrations. In addition, the media contained labelled sucrose as in Fig. 1. Amounts of L-leucine (●) and D-leucine (□) taken up are given after correction for label in the sucrose space. Each point represents the mean of 2–4 different experiments.

Effects of amino acids on leucine uptake

Table I shows the effect of various amino acids on the uptake of L-leucine and D-leucine. In these experiments incubation was performed for 90 s using labelled sucrose as extracellular marker. Strikingly similar results were obtained for the two leucine isomers. The uptake of both L-leucine and D-leucine was significantly inhibited by L-isoleucine, L-tryptophan, L-phenylalanine, and glycine. Moreover,

the degree of inhibition exerted by each of these amino acids was quite similar for the two leucine isomers. Finally, L-leucine inhibited the uptake of D-leucine, and D-leucine inhibited the uptake of L-leucine.

TABLE I

EFFECTS OF AMINO ACIDS ON THE UPTAKE OF L-LEUCINE OF D-LEUCINE

After preincubation as in Fig. 1, islets were incubated for 90 s in media containing 1 mM L-[U-^{14}C]-leucine (7.5 mCi/mmole) or 1 mM D-[4,5-$^{3}H_2$]leucine (25.0 mCi/mmole), as well as labelled sucrose as in Fig. 1. Parallel incubations were performed in medium containing no further additions (control) and in media also supplemented with 20 mM non-radioactive amino acid as listed. Amounts of labelled leucine taken up are given after correction for label in the sucrose space. Results are presented as mean values ± S.E. for each medium as well as for the differences between test and control media. The numbers of experiments are given within parentheses.

Amino acid tested	*mmoles of labelled* L-*leucine per kg dry islet*		*mmoles of labelled* D-*leucine per kg dry islet*	
		Test minus control		*Test minus control*
Experimental series I				
None (control)	2.42 ± 0.14 (6)	—	1.97 ± 0.18 (7)	—
D-Leucine	1.58 ± 0.08 (6)	0.83 ± 0.20** (6)	—	—
L-Leucine	—	—	1.27 ± 0.10 (7)	0.70 ± 0.21*
L-Alanine	1.89 ± 0.16 (6)	−0.53 ± 0.24 (6)	1.73 ± 0.14 (7)	−0.24 ± 0.24
L-Isoleucine	1.28 ± 0.06 (6)	−1.14 ± 0.14*** (6)	1.21 ± 0.04 (7)	−0.77 ± 0.20**
Experimental series II				
None (control)	2.00 ± 0.09 (5)	—	2.21 ± 0.14 (7)	—
L-Tryptophan	1.17 ± 0.16 (5)	−0.83 ± 0.15** (5)	1.38 ± 0.16 (7)	−0.83 ± 0.17**
Glycine	1.67 ± 0.04 (5)	−0.33 ± 0.06** (5)	1.87 ± 0.15 (7)	−0.39 ± 0.10**
L-Phenylalanine	1.21 ± 0.15 (5)	−0.79 ± 0.18* (5)	1.22 ± 0.32 (5)	−0.98 ± 0.17**

* $P < 0.025$.
** $P < 0.01$.
*** $P < 0.001$.

To further study whether any difference could be detected between L-leucine and D-leucine with respect to inhibition of uptake by amino acids, islets were incubated in media containing both L-[U-^{14}C]leucine and D-[4,5-$^{3}H_2$]leucine. As shown in Table II, the difference in uptake between L-leucine and D-leucine was not significantly altered by any of the other amino acids tested. A significant reduction of the difference was, however, observed upon the addition of large amounts of non-radioactive L-leucine to the medium.

Effects of sugars and drugs on leucine uptake

D-Glucose, 3-*O*-methyl-D-glucose, D-mannoheptulose, diazoxide, and glibenclamide had no significant effects on the islet uptake of L-leucine or D-leucine (Table III).

Incorporation of leucine into islet protein

The islets incorporated L-leucine at a rate of 94 ± 38 μmoles/kg protein per hour (mean value ± S.E. for 8 experiments), whereas under the same conditions D-leucine was not incorporated into islet protein at all. With the latter amino acid the incorporation rate was calculated as −6 ± 6 μmoles/kg protein per hour (mean

TABLE II

EFFECTS OF AMINO ACIDS ON THE SIMULTANEOUS UPTAKE OF L-LEUCINE AND D-LEUCINE

After preincubation as in Fig. 1, islets were incubated for 90 s in media containing 1 mM L-[U-^{14}C]-leucine (7.5 mCi/mmole) as well as 1 mM D-[4,5-$^{3}H_2$]leucine (25.0 mCi/mmole). Parallel incubations were performed in medium containing no further additions (control) and in media also supplemented with 20 mM non-radioactive amino acid as listed. The values denote uptake of labelled L-leucine minus the uptake of D-leucine. When calculating the effect of 20 mM nonradioactive L-leucine, the reduction of specific radioactivity in the medium was ignored in order to make possible a direct comparison with the other non-radioactive amino acids tested. Results are presented as mean values ± S.E. for each medium as well as for the differences between test and control media. The numbers of experiments are given within parentheses.

Amino acid tested	*L-Leucine minus D-leucine (mmole/kg dry islet)*	
		Test minus control
Experimental series I		
None (control)	0.59 ± 0.26 (6)	—
L-Alanine	0.56 ± 0.20 (6)	−0.03 ± 0.14 (6)
L-Isoleucine	0.49 ± 0.33 (6)	−0.10 ± 0.28 (6)
L-Tryptophan	0.61 ± 0.31 (6)	0.02 ± 0.33 (6)
Experimental series II		
None (control)	0.59 ± 0.32 (6)	—
Glycine	0.38 ± 0.32 (6)	−0.21 ± 0.16 (6)
L-Phenylalanine	0.24 ± 0.44 (6)	−0.35 ± 0.31 (6)
L-Leucine	0.23 ± 0.29 (6)	−0.37 ± 0.11 * (6)

* $P < 0.025$.

TABLE III

EFFECTS OF SUGARS AND DRUGS ON THE UPTAKE OF L-LEUCINE OR D-LEUCINE

Experimental design and presentation of results are the same as in Table I except for the fact that effects of sugars (20 mM of either D-glucose, 3-*O*-methyl-D-glucose or D-mannoheptulose) and drugs (50 μg/ml of glibenclamide or 125 μg/ml of diazoxide) instead of amino acids were tested.

Substance tested	*mmoles of labelled L-leucine per kg dry islet*		*mmoles of labelled D-leucine per kg dry islet*	
		Test minus control		*Test minus control*
Experimental series I				
None (control)	2.16 ± 0.07 (6)	—	2.12 ± 0.19 (7)	—
D-Glucose	2.06 ± 0.12 (6)	−0.10 ± 0.12 (6)	2.02 ± 0.14 (7)	−0.10 ± 0.18 (7)
3-*O*-Methyl-D-glucose	2.14 ± 0.09 (6)	−0.02 ± 0.06 (6)	2.03 ± 0.14 (7)	−0.09 ± 0.26 (7)
Mannoheptulose	2.19 ± 0.17 (6)	0.03 ± 0.12 (6)	1.90 ± 0.08 (7)	−0.22 ± 0.20 (7)
Experimental series II				
None (control	2.03 ± 0.12 (6)	—	2.33 ± 0.16 (5)	—
Glibenclamide	2.21 ± 0.08 (6)	0.18 ± 0.12 (6)	2.62 ± 0.14 (5)	0.29 ± 0.16 (5)
Diazoxide	2.26 ± 0.11 (6)	0.23 ± 0.11 (6)	2.21 ± 0.16 (5)	−0.12 ± 0.25 (5)

value ± S.E. for 9 experiments) after correction for label in the sucrose space of the precipitated protein pellet.

DISCUSSION

The present study was undertaken to test the hypothesis that amino acids stimulate insulin release by binding to transport receptors in the β-cell plasma membrane. This hypothesis was proposed by Christensen and Cullen[1], who observed that a transport-specific and non-metabolizable leucine analogue, 2-amino-bicyclo(2,2,1)-heptane-2-carboxylic acid (BCH), exerted a hypoglycemic action in the rat. It was subsequently encouraged by the demonstration that not only BCH but also 4-amino-1-guanylpiperidine-4-carboxylic acid (GPA), a non-metabolizable arginine analogue, was vigorously transported into the β-cells and stimulated insulin release *in vitro*[3]. These *in vitro* results were somewhat ambiguous, however, in that only one out of four different BCH isomers was an effective insulin secretagogue, although two isomers were found to be readily transported. It was not known whether the two BCH isomers were transported by the same system.

In contrast to L-leucine, D-leucine does not stimulate insulin release from the pancreatic islets of obese-hyperglycemic mice[5]. Neither does D-leucine inhibit insulin release in response to L-leucine (Å. Lernmark, personal communication). If amino acid transport sites and the sites signalling insulin release are identical, one would expect that D-leucine is not transported exactly like L-leucine. In a previous study we failed to demonstrate a clear-cut interference by D-leucine with the uptake of L-leucine[10]. It seems likely that the statistically significant effects observed in the present investigation were obtained because of the use of shorter incubation times and the use of double labelling instead of washing in order to correct for extracellular label. With the present technique, either leucine isomer significantly inhibited the uptake of the other. Moreover, all other amino acids tested inhibited the uptake of D-leucine to the same extent as they inhibited the uptake of L-leucine. The relationship between leucine concentration and uptake was very similar for both isomers. The somewhat greater uptake of L-leucine than of D-leucine may be due to a difference in metabolism, since L-leucine but not D-leucine was incorporated into islet protein. Such an explanation is compatible with the observation that the difference between L-leucine and D-leucine uptake could be significantly reduced by adding large amounts of L-leucine to the medium. The simplest interpretation of all these data is that D-leucine and L-leucine are largely transported by the same system in the pancreatic β-cells. Studies on the gut have previously suggested that some D-amino acids, including D-leucine, are able to utilize the same transport systems as the corresponding L-isomers[11,12].

The present results call for some qualification of the hypothesis subject to test. In particular, the observed competition between D-leucine and L-leucine makes it difficult to maintain that transport by the L system is a sufficient condition for the triggering of insulin release. It is not known whether the entry of L-leucine into its transport site produces two different effects, one associated with transport as such and another responsible for the signalling of insulin release. Although such a modification of the hypothesis could explain why D-leucine is transported without giving rise to insulin release, it does not account for the failure of D-leucine to inhibit

insulin release in response to L-leucine. In this context it is worth noting that L-isoleucine, which inhibited the uptake of both D-leucine and L-leucine, does not inhibit leucine-stimulated insulin release[5]. Nor is L-isoleucine effective as an insulin secretagogue with the present kind of islets[5]. It may thus seem tempting to conclude that the receptor site of transport system L is not identical with the site signalling insulin release. It is possible, however, that such a conclusion follows only from an overly simplistic interpretation of the molecular events involved in transport phenomena. The question whether the hypothesis can be fruitfully developed rather than rejected might be better answered when detailed data on the kinetics of L- and D-leucine transport become available.

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