The Stimulus-Secretion Coupling of Amino Acid-induced Insulin Release

Secretory and Oxidative Response of Pancreatic Islets to L-Asparagine

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SUMMARY

L-Asparagine (2–10 mM) failed to affect insulin secretion from rat pancreatic islets incubated in the absence of exogenous nutrient or presence of D-glucose, but caused a dose-related and progressive enhancement of insulin release evoked by L-leucine, 2-aminobicyclo[2,2,1]heptane-2-carboxylate, or 2-ketoisocaproate. The secretory response to the combination of L-asparagine and L-leucine was augmented by theophylline and inhibited in the absence of extracellular Ca^{2+} or presence of either menadione or methylamine. L-Asparagine augmented leucine-stimulated ^{45}Ca net uptake. The ATP content, rate of O_2 uptake, and malate/pyruvate ratio were not significantly different in islets exposed to L-leucine alone or to both L-asparagine and L-leucine, respectively. In the sole presence of L-asparagine, however, the malate/oxalacetate ratio was decreased and the malate/pyruvate ratio increased, relative to basal values. It is proposed that the enhancing action of L-asparagine upon insulin release evoked by L-leucine might be due to an accelerated generation rate of cytosolic NADPH, rather than to any sizable increase in either islet respiration or steady-state cytosolic NADPH/$NADP^+$ ratio. DIABETES 33:464–469, May 1984.

Our understanding of the mechanism by which certain amino acids stimulate insulin release from the pancreatic B-cell has gained from studies in which L-glutamine was used in combination with keto acids[1–3] or other amino acids.[4–8] Indeed, by investigating both the metabolism of L-glutamine in pancreatic islets[9,10] and the reciprocal metabolic effects of L-glutamine, on one hand, and either L-leucine,[11] its nonmetabolized analogue 2-aminobicyclo[2,2,1]heptane-2-carboxylic acid,[12,13] or its metabolite 2-ketoisocaproate,[14] on the other hand, it became evident that the secretory response to these nutrients always coincides with an increase in oxidative fluxes and O_2 uptake. In the course of these investigations, it was observed that L-asparagine, like L-glutamine, augments insulin secretion evoked by either L-leucine[5] or 2-ketoisocaproate,[1] while failing to affect insulin release in the absence of another exogenous nutrient or in the presence of D-glucose.[5] In the present study, we have scrutinized in greater detail the secretory and ionic response to L-asparagine and its possible dependency on changes in the redox state and respiration of islet cells.

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MATERIALS AND METHODS

All experiments were performed with pancreatic islets removed from fed albino rats. The methods used to measure insulin release from incubated[15] or perifused[16] islets, ^{45}Ca net uptake,[17] O_2 consumption,[11,18] the islet content[19] and/or output[9,20] of malate, pyruvate, and lactate, and islet content of ATP and ADP[21] have been described in detail previously. Briefly, insulin release in static experiments was measured by incubating groups of 8 islets for 90 min in 1.0 ml of medium.[15] In dynamic experiments,[16] groups of 100 islets were perifused (flow rate, 1.0 ml/min) and the insulin measured in the effluent over successive periods of 1 min (or more). The data were corrected for the dead space of the system (ca. 1.0 ml). The net uptake of ^{45}Ca was measured after 30–90 min incubation, the islets being then washed repeatedly to remove extracellular radioactivity.[17] The steady-state islet content in malate and pyruvate was measured at the 60th min of incubation, the islets being separated from the medium by centrifugation through a layer of silicone oil.[19] The total production of malate, pyruvate, and lactate, as measured in both islets and incubation media, was assessed by incubating groups of 15–20 islets for 120 min in 30–60 μl of medium.[9,20] The steady-state value for the ATP and ADP content was measured at the 30th min of incubation in groups of 10 islets each placed in 0.1 ml of medium.[21] In all

TABLE 1
Effect of L-asparagine on insulin release (μU/90 min/islet) evoked by distinct nutrients

	L-Asparagine	
	Nil	10 mM
Nil	10.1 ± 1.8 (50)	15.1 ± 1.5 (60)
D-Glucose (5.6 mM)	18.4 ± 2.4 (9)	15.1 ± 2.7 (9)
D-Glucose (8.3 mM)	60.8 ± 3.7 (18)	62.9 ± 5.2 (18)
D-Glucose (16.7 mM)	214.0 ± 6.1 (9)	209.9 ± 9.9 (9)
L-Leucine (1.0 mM)	11.5 ± 1.1 (8)	9.2 ± 1.2 (8)
L-Leucine (3.0 mM)	15.2 ± 3.7 (9)	39.6 ± 2.9 (9)*
L-Leucine (5.0 mM)	22.0 ± 2.1 (21)	44.1 ± 1.9 (44)*
(±)BCH (10.0 mM)	19.0 ± 1.8 (18)	46.2 ± 3.3 (46)*
L-Leucine (10.0 mM)	47.7 ± 2.7 (53)	96.2 ± 5.8 (120)*
2-Ketoisocaproate (3.0 mM)	17.4 ± 2.5 (9)	16.9 ± 3.2 (9)
2-Ketoisocaproate (5.0 mM)	25.5 ± 1.9 (8)	83.9 ± 9.0 (7)*
2-Ketoisocaproate (10.0 mM)	86.6 ± 3.4 (17)	147.9 ± 2.7 (18)*

*$P < 0.001$.

these experiments, the islets were placed in bicarbonate-buffered media (Na^+ 139 mM, K^+ 5 mM, Ca^{2+} 1 mM, Mg^{2+} 1 mM, Cl^- 124 mM, and HCO_3^- 24 mM) equilibrated against a mixture of CO_2 (5%) and O_2 (95%) and containing bovine albumin (5 mg/ml). Except if otherwise mentioned, these media contained no exogenous nutrient. However, for the measurement of O_2 uptake, groups of 8–16 islets were allowed to respire during an initial period of 60 min in a Krebs-Ringer phosphate buffer equilibrated against ambient air and deprived of exogenous nutrient.[11] The exogenous nutrients were then added from a side drop[18] and the respiration followed for another hour, so that the paired increment in O_2 uptake above basal value could be established in each case.

For measuring the islet content in oxalacetate, groups of 25 islets were incubated for 60 min in 0.1 ml of our usual bicarbonate-buffered incubation medium.[15] After removal of the incubation medium and addition of 40 μl of perchloric acid (2.5%, v/v), the tubes containing the islets were placed in liquid N_2, the islets being disrupted by mechanical vibration.[21] Aliquots (30 μl) of the islet extract were neutralized with KOH (15 μl, 0.94 M), and mixed with 200 μl of a potassium phosphate buffer (100 mM, pH 7.4) containing a tracer amount of [acetyl-^{3}H]acetyl coenzyme A (25–30 nM; 1.1 Ci/mmol) and citrate synthase (0.12 mg/ml). After 60-min incubation at room temperature, the reaction was halted by addition of 0.6 ml of a charcoal mixture.[22] The tubes were vortexed four times for 15 s each time at 2-min intervals, briefly centrifuged, and 0.4 ml of the supernatant examined for its radioactive content by liquid scintillation. Standards (containing 0.0–2.0 pmol of oxalacetate/tube) were prepared in perchloric acid and treated in an identical manner.

The leucine analogue 2-aminobicyclo[2,2,1]heptane-2-carboxylic acid (BCH) was used as the (±) stereoisomeric mixture (Calbiochem-Behring Corp., La Jolla, California).

All results are expressed as the mean (± SEM) together with the number of individual observations (N). The SEM on a ratio between mean values was calculated from their relative errors (SEM/mean value), which were combined to establish the relative error on the ratio by the same procedure as that classically used to calculate the SEM for the difference between two sets of values.[23] The statistical significance of differences between mean values was tested by use of Student's *t* test.

RESULTS

Secretory and ionic data. As shown in Table 1, L-asparagine (10 mM) failed to significantly affect insulin release in the absence of another exogenous nutrient, in the presence of D-glucose (5.6–16.7 mM), or at non-insulinotropic concentrations of L-leucine (1.0 mM) or 2-ketoisocaproate (3.0 mM). L-Asparagine markedly enhanced insulin release evoked by stimulating concentrations of L-leucine, b(±)BCH, or 2-ketoisocaproate. L-Asparagine apparently lowered the threshold concentration of L-leucine required for stimulation of insulin release. In the absence of any other exogenous nutrient, such a threshold concentration exceeds 3 mM.[4] In the presence of L-asparagine (10 mM), however, L-leucine

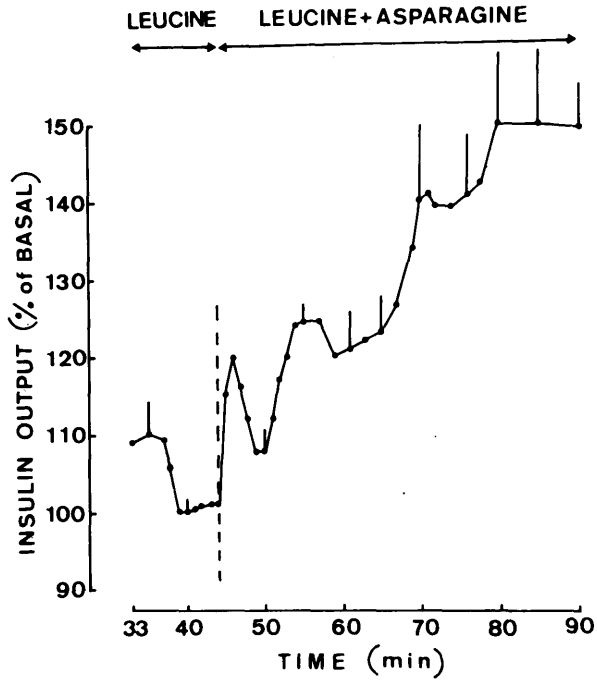

FIGURE 1. Effect of asparagine on leucine-induced insulin release in perifused islets. L-Leucine (10 mM) was administered throughout the perifusion period, and L-asparagine (10 mM) from min 45 onward. In each of 4 experiments, the rate of insulin release was expressed in percent of the value recorded 5 min before L-asparagine administration. Such a control value averaged 0.22 ± 0.05 μU/min/islet.

TABLE 2
Effect of various agents on insulin release (μU/90 min/islet) evoked by L-leucine and L-asparagine (10 mM each)

Control	96.2 ± 5.8 (120)
No $CaCl_2$	31.9 ± 7.0 (9)
Menadione (15 μM)	12.1 ± 7.6 (8)
Theophylline (1.4 mM)	527.2 ± 51.1 (9)
Methylamine (2.0 mM)	51.1 ± 7.7 (18)

(3 mM) markedly enhanced insulin output. As little as 2 mM L-asparagine was sufficient to increase insulin release evoked by L-leucine (10 mM) from 47.7 ± 2.7 (N = 53) to 61.2 ± 4.1 (N = 36) μU/90 min per islet ($P < 0.01$). Likewise, L-asparagine at a 2-mM concentration enhanced insulin release evoked by 2-ketoisocaproate (10 mM) from 86.6 ± 3.4 (N = 17) to 100.1 ± 3.4 (N = 18) μU/90 min per islet ($P < 0.01$).

L-Aspartate (10 mM) failed to significantly affect insulin release evoked by 10 mM L-leucine, the secretory rate averaging 46.9 ± 3.3 and 37.7 ± 3.7 μU/90 min per islet (N = 21–22) in the sole presence of L-leucine and presence of both L-leucine and L-aspartate, respectively.

In a perifusion system, the release of insulin after 40-min exposure to L-leucine (10 mM) averaged 0.22 ± 0.05 μU/min per islet (Figure 1). L-Asparagine (10 mM), when administered together with L-leucine, first provoked a modest and transient increase in insulin output (paired peak increment: +20.1 ± 1.6%, N = 4), followed by a progressive build-up in secretory rate. In 4 individual experiments, the mean rate of insulin release in the last 7 samples (collected after 28–46 min of exposure to both amino acids) was 44.3 ± 4.8% higher ($P < 0.005$) than that recorded in the last 7 samples before L-asparagine administration. In static experiments performed over 30, 60, or 90 min incubation in the presence of both L-leucine and L-asparagine (10 mM each), the rate of insulin release was lower during the initial 30 min (22.6 ± 4.3 μU/30 min/islet; N = 9) than over the ensuing 60 min (40.6 ± 3.3 μU/30 min/islet; N = 18) of incubation.

The release of insulin evoked by the association of L-leucine and L-asparagine was markedly reduced by methylamine or when no $CaCl_2$ was added to the incubation medium, abolished by menadione, and dramatically potentiated by theophylline (Table 2).

L-Asparagine failed to affect ^{45}Ca net uptake (Table 3). L-Leucine stimulated ^{45}Ca net uptake. L-Asparagine enhanced significantly ($P < 0.02$ or less) L-leucine-stimulated ^{45}Ca net uptake, whether over 30, 60, or 90 min of incubation. There was a tight correlation ($r = 0.987$, $P < 0.02$) between the mean values for ^{45}Ca net uptake and insulin release after 90-min incubation in the presence or absence of L-leucine and/or L-asparagine (N = 4).

Oxidative data. The basal rate of O_2 uptake averaged 2.24 ± 0.12 nl/60 min per islet (N = 65). All nutrients and combination of nutrients examined in the present series of experiments augmented O_2 uptake above basal value (Table 4). In all cases the increase in O_2 consumption was a sustained phenomenon, persisting throughout the period of observation (≥60 min). As shown in Figure 2, there were significant correlations ($P < 0.01$) between the rate of insulin release and either the absolute values for O_2 uptake ($r = 0.916$) or the nutrient-induced increment in O_2 consumption relative to basal value ($r = 0.896$). L-Asparagine, which slightly augmented O_2 uptake in the absence of another exogenous nutrient, failed to significantly affect the respiratory response to L-leucine or 2-ketoisocaproate, whether such a response was judged from the absolute values for O_2 uptake or from the percent increase above the paired basal value (Table 4). These negative data contrast with the capacity of L-glutamine to augment the respiratory response to L-leucine.[11]

L-Asparagine failed to prevent the fall in ATP content and ATP/ADP ratio normally seen in islets deprived of exogenous nutrient (Table 5). L-Asparagine also failed to significantly affect the higher ATP content or ATP/ADP ratio ($P < 0.01$) found in islets exposed to L-leucine. Thus, in both the absence or presence of L-leucine, L-asparagine failed to significantly affect the ATP, ADP, ATP + ADP, or ATP/ADP values.

L-Asparagine decreased ($P < 0.001$) and L-leucine increased ($P < 0.02$) the islet malate/oxalacetate ratio (Table 5). This was paralleled by comparable changes in the lactate/pyruvate ratio for the content and/or output of these metabolites (total production) after 120-min incubation. In the presence of both amino acids, the islet malate/oxalacetate ratio was identical to that found in the absence of exogenous nutrient, being higher ($P < 0.001$) than in the sole presence of L-asparagine and lower ($P < 0.02$) than in the sole presence of L-leucine. The malate/oxalacetate ratios, when multiplied by the equilibrium constant for malate dehydrogenase (2.78×10^{-5}, see ref. 24) yielded mean values ranging from 0.0007 to 0.0030, which is close to the cytosolic $NADH/NAD^+$ ratio in rat liver (0.0005–0.0023, see ref. 24) as calculated from the lactate/pyruvate ratio.

L-Asparagine tended to increase the malate/pyruvate ratio, whether these metabolites were measured in the islet cells ($P < 0.07$) or in both islets and incubation medium ($P < 0.02$). L-Leucine clearly increased the malate/pyruvate ratio ($P < 0.02$). In the presence of both amino acids, the

TABLE 3
Effects of L-leucine and L-asparagine on ^{45}Ca net uptake (pmol/islet)

	Time (min)		
	30	60	90
Nil	0.85 ± 0.10 (10)	—	1.25 ± 0.28 (10)
L-Asparagine (10 mM)	—	—	1.22 ± 0.10 (10)
L-Leucine (10 mM)	1.62 ± 0.09 (10)	2.60 ± 0.22 (10)	2.71 ± 0.26 (10)
L-Asparagine (10 mM) + L-leucine (10 mM)	1.99 ± 0.09 (20)	3.29 ± 0.16 (20)	3.84 ± 0.20 (10)

TABLE 4
Effect of nutrients on O_2 uptake by pancreatic islets

Nutrient(s) (mM)	O_2 uptake (nl/60 min/islet)		Paired increment (%)	N
	Basal	Stimulated		
D-Glucose (16.7)	2.16 ± 0.20	3.73 ± 0.34	73.7 ± 7.8‡	(5)
L-Asparagine (10)	2.34 ± 0.50	2.48 ± 0.53	4.6 ± 1.6*	(6)
L-Leucine (10)	2.18 ± 0.39	3.11 ± 0.58	40.5 ± 7.3†	(6)
L-Leucine (10) + L-asparagine (10)	2.54 ± 0.34	3.48 ± 0.48	36.9 ± 4.2‡	(10)
L-Leucine (10) + L-glutamine (10)	1.96 ± 0.43	3.77 ± 1.02	88.8 ± 27.4*	(6)
2-Ketoisocaproate (10)	2.02 ± 0.29	3.09 ± 0.49	52.5 ± 8.3†	(6)
2-Ketoisocaproate (10) + L-asparagine (10)	2.45 ± 0.18	3.54 ± 0.29	45.0 ± 4.5‡	(11)

*$P < 0.04$, †$P < 0.005$, and ‡$P < 0.001$.

malate/pyruvate ratio was again much higher than in the basal state ($P < 0.01$), but not significantly different from that found in the sole presence of L-leucine. The malate/pyruvate ratios, which were of the same order of magnitude as that found in hepatocytes,[25] when multiplied by the equilibrium constant for the malic enzyme (3.44×10^{-2} M) and divided by the value used by Veech et al.[24] for the concentration of CO_2 in rat liver (1.16×10^{-3} M) yielded mean values ranging from 8.5 to 15.4, close to those found by Ashcroft et al. (15.7–26.9) in rat islets exposed to a low or high concentration of D-glucose.[26]

DISCUSSION

The present results indicate that the secretion of insulin evoked by the combination of L-asparagine and L-leucine displays features usually encountered in the process of nutrient-induced insulin release. Thus, the secretory response to this combination of amino acids was impaired in the absence of extracellular Ca^{2+}, was enhanced by theophylline (which apparently affects the intracellular distribution of Ca^{2+}),[27] coincided with an increased ^{45}Ca uptake, and required the integrity of the Ca^{2+}-responsive transglutaminase system, being inhibited by methylamine.[28] The secretory response to the combination of L-asparagine and L-leucine may depend on the catabolism of these amino acids, resulting in an increased generation rate of both reducing equivalents and ATP. Indeed, relative to basal values, the association of L-asparagine and L-leucine augmented both O_2 uptake and the ATP content or ATP/ADP ratio. Moreover, the release of insulin evoked by L-asparagine and L-leucine was suppressed by menadione, which lowers the islet content in reduced pyridine nucleotides.[21]

In the absence of L-leucine, L-asparagine slightly augmented O_2 uptake. However, the capacity of L-asparagine to act as a fuel in islet cells was apparently not sufficiently marked to either prevent the fall in ATP/ADP ratio normally seen in islets deprived of exogenous nutrient or to provoke insulin release in the absence of such exogenous nutrient.

Although our measurements of O_2 uptake confirm that there is usually a fair correlation between the effects of nutrients on O_2 consumption and insulin release, respectively,[18,29] the present data do not suggest that the enhancing action of L-asparagine upon leucine-induced insulin release is attributable to any obvious increase in O_2 uptake by leucine-stimulated islets. This is in sharp contrast to the situation found in the presence of L-glutamine, which markedly increased the respiratory response to L-leucine, as already noted in a prior study.[11]

An alternative explanation for the enhancing action of L-asparagine upon leucine-induced insulin release could be that L-asparagine alters the intracellular distribution or site of generation of reducing equivalents. The response to L-asparagine would then be in mirror of that evoked by aminooxyacetate.[19]

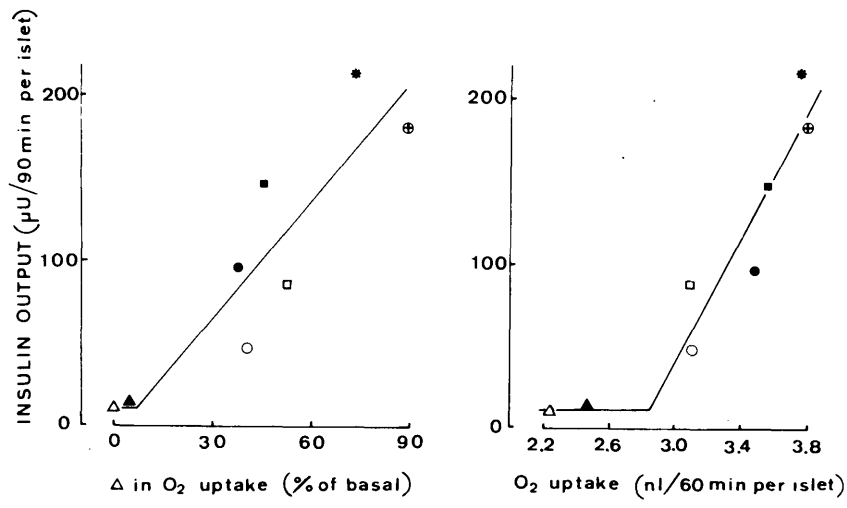

FIGURE 2. Correlation between mean values for insulin output and O_2 uptake (expressed as either the paired increment relative to basal O_2 consumption, or an absolute value) in the absence of exogenous nutrient (open triangle) and presence of either L-asparagine (closed triangle), L-leucine (open circle), 2-ketoisocaproate (open square), or D-glucose (asterisk), or the association of L-asparagine and L-leucine (closed circle), L-asparagine and 2-ketoisocaproate (closed square), or L-glutamine and L-leucine (circled cross). The concentration of each nutrient amounted to 10 mM, except for D-glucose (16.7 mM). The regression lines were calculated for all conditions in which insulin release exceeded basal value.

TABLE 5
Effects of L-asparagine and L-leucine on metabolic variables in pancreatic islets

L-Asparagine (mM) L-Leucine (mM)	None None	10.0 None	None 10.0	10.0 10.0	N
ATP (pmol/islet)	6.68 ± 0.68	7.81 ± 0.26	9.50 ± 0.66	9.32 ± 0.61	(19)
ADP (pmol/islet)	4.46 ± 0.42	4.96 ± 0.62	3.20 ± 0.31	3.05 ± 0.32	(19)
ATP/ADP (ratio)	1.71 ± 0.20	1.78 ± 0.20	3.07 ± 0.41	3.27 ± 0.46	(19)
Islet oxalacetate (fmol/islet)	14.0 ± 1.7	42.1 ± 5.0	10.0 ± 1.4	18.1 ± 2.1	(16–18)
Islet malate (fmol/islet)	892 ± 47	1050 ± 51	1078 ± 65	1145 ± 84	(30)
Islet pyruvate (fmol/islet)	3018 ± 331	2633 ± 235	2080 ± 87	2421 ± 222	(21)
Islet malate/oxalacetate (ratio)	63.7 ± 8.4	24.9 ± 3.2	107.8 ± 16.4	63.3 ± 8.7	(30/16–18)
Islet malate/pyruvate (ratio × 10^3)	296 ± 36	399 ± 41	518 ± 78	473 ± 56	(30/21)
Total lactate/pyruvate (ratio)	3.38 ± 0.27	2.77 ± 0.28	4.38 ± 0.65	4.92 ± 0.51	(15–16/15)
Total malate/pyruvate (ratio × 10^3)	184 ± 25	289 ± 35	390 ± 63	482 ± 67	(17–18/15)

L-Asparagine and L-leucine exerted opposite effects upon the redox state of the cytosolic NADH/NAD^+ couple, as judged from the malate/oxalacetate ratio. Although the NAD-dependent malate dehydrogenase is found both in cytosol and mitochondria, there are several indications that the malate/oxalacetate ratio refers to the cytosolic enzyme. First, the NADH/NAD^+ ratio being at least two orders of magnitude higher in the mitochondria than in the cytoplasm,[24] it is obvious that the value here derived from the malate/oxalacetate ratio is representative of the cytosolic system. Second, the latter value was not vastly different from that (0.0004–0.0005) which could be derived from the lactate/pyruvate ratio for the total production of these metabolites. Last, L-leucine increased the malate/oxalacetate ratio, although this amino acid lowers the mitochondrial NADH/NAD^+ ratio (Sener, A., and Malaisse, W. J., unpublished observation).

At variance with its effect to decrease the cytosolic NADH/NAD^+ ratio, L-asparagine enhanced the cytosolic NADPH/$NADP^+$ ratio, as judged from the islet content or total production of malate and pyruvate. This dissociated behavior can be attributed to the fact that oxalacetate, derived from exogenous L-asparagine, is converted, to a large extent, to malate and then to pyruvate in the cytosol of islet cells.[30] This sequence of reactions results in the consumption of cytosolic NADH and production of cytosolic NADPH. Since L-leucine also augmented the cytosolic NADPH/$NADP^+$ ratio, it is tempting to suggest that, in the presence of both L-asparagine and L-leucine, the two amino acids acted synergistically in increasing the cytosolic generation rate of NADPH. This could account for the enhancing action of L-asparagine upon leucine-induced insulin release, since the NADPH-dependent reduction of glutathione apparently participates in the stimulus-secretion coupling of nutrient-induced insulin release by modulating the thiol-disulfide balance in membrane-associated target systems.[31]

It could be argued that the malate/pyruvate ratio, whether measured solely in the islets or in both the islets and incubation medium, was not significantly different in the simultaneous presence of L-asparagine and L-leucine or in the sole presence of the branched-chain amino acid, respectively. At high concentrations of nutrient secretagogues, it is not exceptional, however, that no sizable increase in the islet content of reduced pyridine nucleotides can be detected, despite further increases in both their generation rate and insulin output. Such a situation was previously encountered at high concentrations of D-glucose,[32] high concentrations of 2-ketoisocaproate[33] and in the combined presence of 2-ketoisocaproate and L-glutamine.[14] The present data are not incompatible, therefore, with the knowledge that L-asparagine, when administered to leucine-stimulated islets, further increases the generation rate of cytosolic NADPH.[34]

In conclusion, the enhancing action of L-asparagine upon insulin release evoked by L-leucine could be due to an accelerated generation of cytosolic NADPH rather than to an increase in oxidative fluxes and O_2 consumption. This conclusion does not rule out the view that the initiation of insulin release by nutrient secretagogues requires an increased production of ATP.[35] Quite the contrary, the data here obtained in the sole presence of L-asparagine clearly indicate that the induction of a more reduced state in the cytosolic NADPH/$NADP^+$ couple fails to provoke insulin release when it does not coincide with a sufficient increase in O_2 uptake.

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