The coupling of metabolic to secretory events in pancreatic islets

The cytosolic redox state

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(Received 5 December 1983/Accepted 20 February 1984)

1. NADP-linked isocitrate dehydrogenase and malic enzyme [malate dehydrogenase (decarboxylating) (NADP)] activities were characterized in the cytosol of pancreatic islet cells. 2. D-Glucose and L-leucine augmented the cytosolic NADPH/NADP⁺ ratio, as judged from the isocitrate/2-oxoglutarate and malate/pyruvate islet contents. 3. The flow rate through the malic enzyme was judged from the output of labelled pyruvate by islets exposed to either L-[U-¹⁴C]glutamine or L-[U-¹⁴C]leucine. 4. The cytosolic generation of NADPH, e.g. at the level of the malic enzyme, may play a role in the coupling of metabolic to secretory events in the process of nutrient-stimulated insulin release.

It is now established that the capacity of nutrients to stimulate insulin release from the pancreatic B-cell reflects their capacity to augment oxidative fluxes in the islet cells (Malaisse *et al.*, 1979c; Ashcroft, 1980; Hedekov, 1980). It has been proposed that the coupling of metabolism to distal events in the secretory sequence represents a multifactorial process (Malaisse *et al.*, 1979a), including changes in the generation rate and/or availability of reducing equivalents (Hellman *et al.*, 1974; Ammon & Verspohl, 1976), protons and high-energy phosphate intermediates. However, the precise role of each of these coupling factors remains to be evaluated. In the present work, emphasis is given to the nutrient-induced changes in the cytosolic NADPH/NADP⁺ ratio, and their possible link to the reactions catalysed by isocitrate dehydrogenase and malic enzyme.

Materials and methods

All experiments were performed with pancreatic islets removed from fed albino rats.

Enzyme activity

The activities of lactate dehydrogenase (EC 1.1.1.27) and glutamate dehydrogenase (EC 1.4.1.3) were measured by methods described elsewhere (Malaisse *et al.*, 1979b; Sener & Malaisse, 1980a). The activity of citrate synthase (EC 4.1.3.7) was measured in 0.2 ml of Tris/HCl buffer (100 mM, pH 8.0) containing oxaloacetate (0.2 mM) and [acetyl-³H]acetyl-CoA (0.1 mM). After 10 min

incubation at room temperature, the reaction was halted by addition of 0.6 ml of a charcoal mixture (Parvin *et al.*, 1980), and [³H]citrate was measured as described elsewhere (Malaisse-Lagae *et al.*, 1984).

For measuring the activities of malic enzyme (EC 1.1.1.40) and isocitrate dehydrogenase (EC 1.1.1.42) in islet homogenates, groups of 400–500 islets were sonicated (2 × 10 s) in 0.8 ml of triethanolamine hydrochloride/NaOH buffer (50 mM, pH 7.4) containing MnCl₂ (3.0 mM) and bovine serum albumin (0.02 mg/ml). For malic enzyme determination, a portion of this homogenate (50 µl) was mixed with 450 µl of the same buffer containing NADP⁺ (final concn. 0.4 mM) and malate (0.1–1.0 mM). The NADPH formed was monitored by spectrophotometry over 20 min incubation at 37°C. For isocitrate dehydrogenase determination, a portion of the homogenate (50 µl) was mixed with 550 µl of imidazole/HCl buffer (50 mM, pH 8.0) containing MnCl₂ (5.0 mM), EDTA (1.0 mM), bovine albumin (0.1 mg/ml), NADP⁺ (final concn. 0.4 mM) and DL-isocitrate (0.05–4.0 mM). The formation of NADPH was monitored as described above.

In order to study the subcellular distribution of enzyme activity, groups of 1100 islets were homogenized in Potter–Elvehjem tubes (eight strokes) in 2.0 ml of Tris/HCl buffer (10 mM, pH 7.4) containing sucrose (250 mM), EDTA (2.5 mM), cysteine (2.0 mM) and bovine albumin (0.02 mg/ml). After centrifugation at 600g for 5 min at 4°C to deposit nuclei and cell debris, a portion (0.6 ml) of the

supernatant was sonicated (2×10 s) and used for measurement of total enzyme activity, and another portion (1.2 ml) of the supernatant was centrifuged at $27600g$ for 10 min at 4°C . The supernatant (1.2 ml) and the pellet (which was resuspended in 1.2 ml of the same buffer) from this second centrifugation were both sonicated (2×10 s) and also examined for their enzyme activity. The activity of isocitrate dehydrogenase in the cytosolic supernatant and mitochondrial pellet was corrected for both the contamination of the cytosolic supernatant by mitochondrial material and the incomplete recovery of enzyme activity from the mitochondrial pellet. For this purpose, the contamination of the cytosolic supernatant by mitochondrial material and the recovery of mitochondrial activity were judged from the mean values obtained within the same experiments for the subcellular distribution of glutamate dehydrogenase and citrate synthase, used as mitochondrial markers. For the latter two enzymes, the contamination of the cytosol did not exceed $18.2 \pm 4.2\%$, and the total recovery (mitochondria plus cytosol) was not less than $91.2 \pm 8.7\%$ of the paired value found in the total homogenate. After correction, the sum of the cytosolic and mitochondrial activities for isocitrate dehydrogenase averaged $101.1 \pm 6.1\%$ ($n = 4$) of the paired value found in the total homogenate.

Islet contents of metabolites

The method used to measure the islet content of pyruvate was described by Malaisse *et al.* (1982a).

For measuring the malate content, groups of eight islets were incubated for 60 min at 37°C in 0.2 ml of a bicarbonate-buffered medium (Malaisse *et al.*, 1970) and, after removal of the incubation medium, mixed with HClO_4 (2.5%, v/v; 0.1 ml), placed in liquid N_2 and disrupted by mechanical vibration (Malaisse *et al.*, 1978). After 1 min centrifugation (Beckman Microfuge), a sample (30 μl) of the supernatant was mixed with 15 μl of KOH (1.0 M) containing Tris (50 mM) and 200 μl of Tris/HCl buffer (100 mM, pH 8.0) containing NAD^+ (0.4 mM), [*acetyl*- ^3H] acetyl-CoA (0.12 μM), malate dehydrogenase (20 $\mu\text{g}/\text{ml}$; EC 1.1.1.37; from pig heart; Boehringer Mannheim) and citrate synthase (120 $\mu\text{g}/\text{ml}$; from pig heart; Boehringer Mannheim). After 60 min incubation at room temperature, the reaction was halted by addition of a charcoal mixture and [^3H]citrate was measured as described above. Malate standards (3–24 pmol/sample) prepared in HClO_4 were treated in exactly the same manner.

For measuring the islet content of 2-oxoglutarate, groups of 25 islets were incubated for 60 min at 37°C in 30 μl of the usual bicarbonate-buffered medium and then mixed with 10 μl of

NaOH (80 mM), placed in liquid N_2 , disrupted, and centrifuged (see above). The supernatant was heated for 10 min at 60°C , and a sample (30 μl) mixed with 30 μl of imidazole/HCl buffer (100 mM, pH 6.3; yielding a final pH of 6.8) containing ammonium acetate (100 mM), ADP (1.0 mM), NADH (0.01 mM) and glutamate dehydrogenase (20 $\mu\text{g}/\text{ml}$; from ox liver; Boehringer Mannheim). After 15 min incubation at room temperature, the reaction was halted by addition of 0.3 ml of HCl (0.5 M). After 20 min incubation at room temperature to destroy NADH, the NAD^+ formed was converted into a fluorescent product by addition of 1.0 ml of NaOH (6 M) and heating for 10 min at 60°C . Standards of 2-oxoglutarate (20–80 pmol/sample) prepared in the incubation medium were treated in the same way.

For measuring the islet content of isocitrate, groups of 12 islets were incubated for 60 min at 37°C in 100 μl of medium and, after removal of the incubation medium, mixed with HClO_4 , placed in liquid N_2 , disrupted and centrifuged as described above for malate measurement. A sample (30 μl) of the supernatant was mixed with 15 μl of KOH (1.0 M) containing Tris (50 mM). From this mixture, a sample (20 μl) was mixed with 20 μl of Tris/HCl buffer (100 mM, pH 8.1) containing MgCl_2 (0.4 mM), NADP^+ (0.4 mM), bovine albumin (0.02 mg/ml) and isocitrate dehydrogenase (0.1 mg/ml; from pig heart; Boehringer Mannheim). After 20 min incubation at room temperature, the reaction was halted by addition of 20 μl of NaOH (200 mM), and the NADPH formed was measured (in duplicate) by a bioluminescence technique (Malaisse *et al.*, 1978). Standards of D-isocitrate (0.1–2.0 pmol/sample) prepared in HClO_4 were treated in the same manner.

Measurement of [^{14}C]pyruvate output

For this, groups of 100 islets each were incubated for 120 min at 37°C in 0.15 ml of our usual bicarbonate-buffered medium containing bovine albumin (1 mg/ml) and either 25 μM - or 0.25 mM-L-[U- ^{14}C]glutamine (10 $\mu\text{Ci}/\text{ml}$). A sample (40 μl) of the incubation medium was used for the determination of total pyruvate output, pyruvate being measured by an enzymic procedure (Malaisse *et al.*, 1978). A second sample (100 μl) of the incubation medium was immediately placed on a Dowex 50 (H^+ form) column (Hutton *et al.*, 1979). After elution with water (0.4 ml) and neutralization to pH 7.0–7.5 with NaOH (0.1 M), the eluate was mixed with 0.4 ml of Tris/HCl buffer (100 mM, pH 8.1) containing L-glutamate (40 mM) and glutamate:pyruvate transaminase (50 $\mu\text{g}/\text{assay}$; EC 2.6.1.2; from pig heart; Boehringer Mannheim). After 20 min incubation at room temperature, the reaction mixture was placed on a Dowex 50 (H^+

form) column. The column was washed with 10 ml of water, and the [^{14}C]alanine generated from [^{14}C]pyruvate was eluted with 2 ml of NH_3 (1.0M). Blank values were obtained by treating media incubated in the absence of islets in an identical manner. When standard amounts of pyruvate (0.1–10.0 nmol/sample) were treated in a comparable manner, but with L-[1- ^{14}C]glutamate as transamination partner, the recovery of 2-oxo[^{14}C]glutamate was never below 90%. None of the agents added to the incubation medium and listed in the Results section interfered with the measurement of [2- ^{14}C]pyruvate by the above procedure. However, when amino-oxyacetate (5.0 mM) was present in the incubation medium, the recovery of [2- ^{14}C]pyruvate was decreased to $67.1 \pm 3.2\%$ ($n = 3$) of its paired control value.

A comparable procedure was used to measure the formation of [^{14}C]pyruvate by islets incubated in the presence of L-[U- ^{14}C]leucine (0.25 mM; 40 $\mu\text{Ci/ml}$). Since the islets exposed to L-leucine may produce 4-methyl-2-oxopentanoate, it was duly verified that, in the presence of 4-methyl-2-oxopentanoate (2.5–10 nmol/sample), no conversion of L-[1- ^{14}C]glutamate into 2-oxo[^{14}C]glutamate could be detected in the present system.

In these experiments, [^{14}C]pyruvate was purposely measured solely in the incubation medium to avoid the interference by intracellular 2-oxoglutarate. Indeed, when 2-oxo[1- ^{14}C]glutamate (10–250 pmol/sample) was added to the transamination reaction mixture, $79.6 \pm 1.4\%$ ($n = 3$) was recovered as L-[1- ^{14}C]glutamate, in either the absence or the presence of unlabelled pyruvate (1–10 nmol/sample). Incidentally, after 120 min incubation in the presence of 0.25 mM-L-glutamine, the content of 2-oxoglutarate in the incubation medium was close to the lower limit of sensitivity of our assay and, in any case, did not exceed $12 \pm 3\%$ ($n = 4$) of the total islet content and/or output of 2-oxoglutarate (1.86 ± 0.16 pmol/islet; $n = 4$). Therefore, and because of the isotopic dilution of 2-oxo[^{14}C]glutamate (formed in the islet cells from exogenous L-[U- ^{14}C]glutamine) by endogenous 2-oxoglutarate (such as that continuously enriched in ^{12}C atoms by circulation in the Krebs cycle), it appears that the contribution of extracellular 2-oxo[^{14}C]glutamate, if any, to our estimation of [^{14}C]pyruvate output is negligible.

Presentation of results

All results, including those mentioned above, are expressed as means $\pm$ S.E.M., together with either the number of individual observations (n) or the degree of freedom (d.f.). Control and test values were collected within the same experiments and, as a rule, with equal numbers of individual observations in each group. When several distinct

agents were tested (see Tables 1 and 2), the results are presented as normalized values in order to avoid the cumbersome tabulation of control values for each experiment. The S.E.M. of the ratio between mean values was calculated from their relative errors (S.E.M./mean value), which were combined, to establish the relative error of the ratio, by the same procedure as that classically used to calculate the S.E.M. for the difference between two sets of values (Snedecor, 1962). The statistical significance of differences between mean values was tested by Student's t test.

Results

Enzyme activity

In crude homogenates, the activity of malic enzyme averaged 8.01 ± 0.92 pmol/min per islet ($n = 7$), as measured in the presence of 1.0 mM-malate and 0.4 mM-NADP $^+$. The K_m of malic enzyme was close to 0.15–0.16 mM for malate and 0.01–0.02 mM for NADP $^+$, and its maximal velocity was close to 9.0 pmol/min per islet (Fig. 1). On average, $92.0 \pm 1.7\%$ ($n = 4$) of the enzyme activity was recovered in the cytosolic supernatant, compared with $93.2 \pm 2.5\%$ ($n = 4$) for lactate dehydrogenase.

In crude homogenates, the activity of isocitrate dehydrogenase averaged 25.10 ± 1.48 pmol/min per islet ($n = 6$), as measured in the presence of 2.0 mM-DL-isocitrate and 0.4 mM-NADP $^+$. The velocity of isocitrate conversion into 2-oxoglutarate in the presence of 0.4 mM-NADP $^+$ and increasing concentrations of D-isocitrate (0.025–2.0 mM) suggested that the islet isocitrate dehydrogenase displays two K_m values for isocitrate, the first close to 0.04 mM and the second close to 2.0 mM (Fig. 2).

Fig. 1. Malic enzyme activity (v) in islet homogenates as a function of malate (a) and NADP $^+$ (b) concentrations. The concentration of malate and NADP $^+$ was fixed at 1.0 mM (b) and 0.4 mM (a) respectively. Values are means $\pm$ S.E.M. for four individual measurements.

The low- K_m activity accounted for about 80% of the maximal velocity. After correction for mitochondrial contamination, the enzymic activity in the cytosolic supernatant represented $21.9 \pm 2.4\%$ ($n=4$) of the total activity (cytosol + mitochondria).

Fig. 2. Isocitrate dehydrogenase activity (v) in islet homogenates at a fixed concentration of $NADP^+$ (0.4 mM) and increasing concentrations of D-isocitrate

The reaction velocity is plotted as a function of the ratio between reaction velocity and substrate concentration (v/s). Values are means $\pm$ S.E.M. for three to seven individual measurements.

Cytosolic $NADPH/NADP^+$ ratio

The cytosolic $NADPH/NADP^+$ ratio in islets deprived of exogenous nutrient or exposed to either D-glucose (11.1 mM) or L-leucine (10.0 mM) was estimated from either the malate/pyruvate or the isocitrate/2-oxoglutarate contents of the islets. As illustrated in Table 1, both D-glucose and L-leucine significantly augmented the cytosolic $NADPH/NADP^+$ ratio. Virtually identical results were obtained whether the $NADPH/NADP^+$ ratio was judged from the malate/pyruvate or isocitrate/2-oxoglutarate ratio.

Production of [^{14}C]pyruvate from L-[$U-^{14}C$]glutamine

When only a tracer amount of L-[$U-^{14}C$]glutamine (25 μ M) was added to the incubation medium, the output of pyruvate was not significantly different from the basal value (5.63 ± 0.41 pmol/120 min per islet; $n=10$). The output of [^{14}C]pyruvate represented $2.89 \pm 0.48\%$ ($n=5$) of the total pyruvate output. When the concentration of L-[$U-^{14}C$]glutamine was raised to 0.25 mM, the outputs of both labelled and total pyruvate were significantly increased. The output of [^{14}C]pyruvate now represented $13.43 \pm 1.00\%$ ($n=33$) of the total pyruvate output (7.76 ± 0.75 pmol/120 min per islet; $n=38$). At the high concentration of L-glutamine (0.25 mM), the total pyruvate output represented $137.8 \pm 14.3\%$ ($P < 0.05$) of the paired basal value recorded within the same experiments. The ratio of pyruvate output to total production (islets plus medium) was not significantly different in islets deprived of exogenous nutrient (0.699 ± 0.046 , $n=5$) and in those exposed to 0.25 mM-L-glutamine (0.735 ± 0.103 , $n=5$). Incidentally, in the presence of 0.25 mM-L-[$U-^{14}C$]glutamine, the oxidation of the amino acid averaged 29.7 ± 1.7 pmol/120 min per islet ($n=15$).

Further experiments, designed to investigate the effect of environmental factors on the production of [^{14}C]pyruvate from L-[$U-^{14}C$]glutamine, were

Table 1. Effects of D-glucose and L-leucine on the cytosolic $NADPH/NADP^+$ ratio

The $NADPH/NADP^+$ ratio was calculated from ^athe equilibrium constant of malic enzyme (3.44×10^{-2} M), assuming a $[CO_2]$ of 1.16 mM (Veech *et al.*, 1969) and ^bthe equilibrium constant of isocitrate dehydrogenase (1.17 M), assuming the same $[CO_2]$ as above. The other indices (^c $P < 0.1$, ^d $P < 0.05$, ^e $P < 0.025$, ^f $P < 0.01$, ^g $P < 0.005$ and ^h $P < 0.001$) refer to the statistical significance of differences from basal values.

	Nutrient ...	None	D-glucose (11.1 mM)	L-leucine (10.0 mM)
Metabolites				
Malate (pmol/islet)		1.49 ± 0.07 (56)	2.82 ± 0.13 (41) ^h	1.75 ± 0.10 (40) ^d
Pyruvate (pmol/islet)		1.84 ± 0.16 (84)	2.40 ± 0.15 (16)	1.27 ± 0.05 (21) ^c
Isocitrate (fmol/islet)		46.0 ± 3.1 (23)	83.7 ± 7.0 (23) ^h	78.0 ± 5.1 (22) ^h
2-Oxoglutarate (pmol/islet)		1.74 ± 0.14 (71)	2.11 ± 0.32 (24)	1.71 ± 0.14 (22)
Cytosolic $NADPH/NADP^+$				
Malate/pyruvate ^a		24.02 ± 2.72	34.84 ± 2.91 ^e	40.85 ± 3.42 ^h
Isocitrate/2-oxoglutarate ^b		26.61 ± 3.91	39.97 ± 7.01 ^c	45.99 ± 4.80 ^g

conducted at the high concentration (0.25 mM) of the amino acid, to avoid the shortcomings of experiments performed in the presence of a tracer amount of L-[U- 14 C]glutamine (Sener *et al.*, 1982a). The total output of pyruvate was increased by glucose ($+298.4 \pm 34.9\%$, d.f. = 14, $P < 0.001$) and, to a lesser extent, by menadione ($+68.1 \pm 11.2\%$, d.f. = 15, $P < 0.001$), slightly decreased by L-leucine ($-31.8 \pm 11.9\%$, d.f. = 6, $P < 0.05$), and not significantly affected by either 4-methyl-2-oxopentanoate or L-asparagine. The output of [U- 14 C]pyruvate was markedly increased by menadione ($P < 0.005$), severely inhibited by L-leucine ($P < 0.001$) and not significantly affected by D-glucose, 4-methyl-2-oxopentanoate or L-asparagine (Table 2). As a result of these changes, the ratio of [U- 14 C]pyruvate to total pyruvate output was increased by menadione ($P < 0.05$) and decreased by D-glucose and L-leucine ($P < 0.005$ in both cases), but not significantly affected by other agents (Table 2).

Production of [U- 14 C]pyruvate from L-[U- 14 C]leucine

By comparison with basal values, L-leucine (0.25 mM) failed to affect pyruvate output significantly, whether the results were expressed in absolute terms or relative to the total content of the islets and incubation medium. Thus, in the presence of L-leucine (0.25 mM), the pyruvate output averaged 5.67 ± 0.52 pmol/120 min per islet ($n = 14$), which represented $71.5 \pm 6.0\%$ ($n = 5$) of the total amount of pyruvate recovered in the islets and incubation medium. In the presence of L-[U- 14 C]leucine (0.25 mM), the production of [U- 14 C]pyruvate corresponded to 243.2 ± 40.3 fg-atoms of 14 C/120 min per islet ($n = 14$). If the assumption is made that the incorporation of 14 C derived from L-[U- 14 C]leucine into pyruvate results from the production of [U- 14 C]acetyl-CoA, its incorporation into citrate, the stepwise conversion of citrate into malate in the Krebs cycle and the eventual conversion of malate into pyruvate, half of the labelled molecules of pyruvate should contain one 14 C atom and the other half should contain two 14 C atoms. Hence, the production of [U- 14 C]pyruvate would amount to 162.1 ± 26.8 fmol/

120 min per islet. This represents $2.83 \pm 0.41\%$ ($n = 14$) of the total pyruvate output. As observed above with L-[U- 14 C]glutamine, addition of D-glucose (16.7 mM) to the incubation medium augmented the output of both unlabelled and labelled pyruvate from islets exposed to L-[U- 14 C]leucine (results not shown).

Conversion of malate into pyruvate

The relative contribution of 14 C-labelled metabolites, derived from exogenous L-[U- 14 C]glutamine or L-[U- 14 C]leucine, to the total generation rate of malate and pyruvate in the islets was estimated as follows. For L-[U- 14 C]glutamine (25 μ M or 0.25 mM), the output of 14 CO₂, measured over 120 min incubation, was used to calculate the generation rate of [14 C]-malate and -pyruvate, by reference to a previously defined model for the metabolism of exogenous L-glutamine in pancreatic islets (see Fig. 3 in Sener *et al.*, 1982b). For reasons given elsewhere (Malaisse *et al.*, 1980), the generation rate of [14 C]acetyl-CoA from L-[U- 14 C]leucine was taken as twice its rate of oxidation. The generation rate of malate and pyruvate from endogenous nutrients was taken from a model based on the study of metabolic events in islets deprived of exogenous nutrient (Malaisse, 1983; Malaisse *et al.*, 1983). As illustrated in Table 3, there was a close agreement between the 14 C-labelled/total ratio for pyruvate generation rate, as derived from these calculations, and the experimental value for the 14 C-labelled/total ratio for pyruvate output.

Discussion

Previous studies performed in extra-pancreatic tissues have established the validity of the malate-pyruvate and isocitrate/2-oxoglutarate ratios as indicators of the cytosolic NADPH/NADP⁺ redox state (Veech *et al.*, 1969; Greenbaum *et al.*, 1971; Sies *et al.*, 1977). The present results confirm and extend previous observations indicating that D-glucose (Ashcroft & Christie, 1979; Malaisse *et al.*, 1982a; Hedeskov *et al.*, 1983) and L-leucine (Malaisse-Lagae *et al.*, 1984) augment the cytosolic

Table 2. Effect of various agents on the output of pyruvate by islets exposed to L-[U- 14 C]glutamine (0.25 mM)
For details see the text.

Agent (mM)	Total pyruvate (pmol/120 min per islet)	[U- 14 C]Pyruvate (pmol/120 min per islet)	Labelled/total (%)
None	7.76 ± 0.75 (38)	1.19 ± 0.15 (33)	13.43 ± 1.00 (33)
D-Glucose (16.7)	30.28 ± 3.47 (4)	1.85 ± 0.36 (4)	5.25 ± 1.04 (4)
L-Leucine (10.0)	5.25 ± 0.55 (4)	0.47 ± 0.03 (4)	8.62 ± 0.61 (4)
4-Methyl-2-oxopentanoate (10.0)	7.44 ± 0.53 (4)	0.95 ± 0.07 (4)	11.23 ± 0.90 (4)
L-Asparagine (10.0)	8.12 ± 0.44 (4)	1.09 ± 0.08 (4)	11.69 ± 0.09 (4)
Menadione (0.01)	13.81 ± 1.04 (5)	3.16 ± 0.28 (5)	19.99 ± 1.75 (5)

Table 3. Contributions of L-glutamine and L-leucine to pyruvate production

The relative contribution of each amino acid to total malate production (first line) and the relative contribution of pyruvate derived from malate to total pyruvate production (second line) were used to establish the relative contributions of each amino acid to total pyruvate production (third line). The contribution of exogenous L-glutamine to malate production and pyruvate production was calculated, as indicated in the text, from the rate of L-[U- 14 C]glutamine oxidation (3.26 and 14.85 pmol/60 min per islet in the presence of 25 μ M- and 0.25 mM-L-[U- 14 C]glutamine respectively). The production of acetyl-CoA from L-leucine was judged from the rate of L-[U- 14 C]leucine oxidation (3.36 pmol/60 min per islet). The basal value for flow rates (all expressed as pmol/60 min per islet) is taken from a previous report (Malaisse, 1983).

Amino acid ...	Glutamine (25 μ M)	Glutamine (0.25 mM)	Leucine (0.25 mM)
Malate production ratio			
Amino acid-derived/total	7.05/(127.97 + 20.97 + 7.05)	32.10/(127.97 + 20.97 + 32.10)	6.73/(127.97 + 20.97 + 6.73)
Pyruvate production ratio			
Malate-derived/total	(20.97 + 4.38)/(35.64 + 4.38)	(20.97 + 19.95)/(35.64 + 19.95)	20.97/35.64
Amino acid-derived/total	2.86%	13.05%	2.54%
14 C-labelled/total	2.89 $\pm$ 0.48%	13.43 $\pm$ 1.00%	2.83 $\pm$ 0.41%

NADPH/NADP $^{+}$ ratio in pancreatic islets. It is conceivable, but remains to be proved, that the generation rate or availability of cytosolic NADPH, in addition to its role in the nutrient-induced stimulation of fatty acid synthesis in the islets (Sener *et al.*, 1978; Hutton *et al.*, 1979; Malaisse *et al.*, 1984), conditions the rate of conversion of oxidized into reduced glutathione and, through this or another pathway, affects the thiol-disulphide balance of proteins participating in distal events in the process of nutrient-stimulated insulin release (Anjaneyulu *et al.*, 1982). The fact that, at the concentrations here used, L-leucine (10.0 mM) augmented more than D-glucose (11.1 mM) the cytosolic NADPH/NADP $^{+}$ ratio, although D-glucose is a more potent insulin secretagogue than L-leucine, suggests that factors other than the cytosolic NADPH/NADP $^{+}$ ratio modulate the magnitude of insulin release. In this respect, it should be noted that the ratio of insulin output to 45 Ca net uptake is also lower in islets stimulated by L-leucine than in those exposed to D-glucose, D-glyceraldehyde or 4-methyl-2-oxopentanoate, all nutrients being used at concentrations of equal insulinotropic potency (Sener & Malaisse, 1980b).

Four sites of cytosolic NADPH production have now been identified in the islets, namely at the level of glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, NADP-linked isocitrate dehydrogenase and malic enzyme (Ashcroft & Randle, 1970). NADP-linked isocitrate dehydrogenase activity was previously observed in mouse pancreatic islets (Ashcroft & Randle, 1970), but its possible cytosolic location was not assessed. Most studies suggest that the generation of NADPH in the pentose phosphate pathway is relatively modest, even in glucose-stimulated islets

(for a review, see Sener & Malaisse, 1978). For instance, in the present system, the production of NADPH in the pentose phosphate pathway does not exceed 8 pmol/60 min per islet at 16.7 mM-D-glucose (Malaisse *et al.*, 1976). The flux through the cytosolic NADP-linked isocitrate dehydrogenase remains to be evaluated, if this is feasible. In the present work, we have attempted to quantify the flux through malic enzyme.

The method used for such a purpose was based on the measurement of [14 C]pyruvate output from islets exposed to L-[U- 14 C]glutamine or L-[U- 14 C]leucine. The measurement of [14 C]pyruvate was restricted to the incubation medium, to eliminate the participation of intracellular radioactive 2-oxoglutarate. Several findings support the validity of this experimental approach. First, D-glucose augmented the output of both total and 14 C-labelled pyruvate, but decreased the 14 C-labelled/total pyruvate ratio. It seems likely that D-glucose, acting as a precursor of pyruvate, caused isotopic dilution of [14 C]pyruvate derived from the exogenous amino acids, and hence prevented the conversion of [14 C]pyruvate into such metabolites as lactate, alanine and CO $_2$. Second, L-leucine decreased the output of both 14 C-labelled and total pyruvate, as well as the 14 C-labelled/total pyruvate ratio. This is consistent with the knowledge that L-leucine decreases the oxidative catabolism of 2-oxoglutarate derived from exogenous L-glutamine, by stimulating the recirculation of 2-oxoglutarate to glutamate (Malaisse *et al.*, 1982b). The 2-oxo acid 4-methyl-2-oxopentanoate, which fails to simulate the inhibitory action of L-leucine on the oxidation of exogenous L-[U- 14 C]glutamine (Malaisse *et al.*, 1981), also failed to reproduce the inhibitory effect of L-leucine on [14 C]pyruvate output. Third, exogenous L-asparagine, which is

metabolized, in part at least, through malic enzyme (Sener *et al.*, 1984), also failed to affect output of ^{14}C -labelled or total pyruvate. The results obtained with 4-methyl-2-oxopentanoate and L-asparagine indicate that agents known to increase the cytosolic NADPH/NADP⁺ ratio (Malaisse *et al.*, 1982a; Malaisse-Lagae *et al.*, 1984) did not impair the exit of malate from the mitochondria and its conversion into pyruvate. Last, menadione markedly increased the output of both total and ^{14}C -labelled pyruvate, as well as the ^{14}C -labelled/total pyruvate ratio. Menadione could impair the conversion of pyruvate into lactate, but such a change was only observed at a higher concentration of the drug (0.05 mM) in glucose-stimulated islets (Malaisse *et al.*, 1978). Alternatively, menadione is known to facilitate the oxidative catabolism of exogenous L-[U- ^{14}C]glutamine (Sener *et al.*, 1982b), and this would account for the increased ^{14}C -labelled/total pyruvate ratio. Moreover, it is conceivable that, by increasing the availability of NADP⁺ (Malaisse *et al.*, 1978), menadione facilitates the conversion of malate into pyruvate. The view that menadione may simultaneously increase the rate of generation of malate and its rate of conversion into pyruvate is compatible with the fact that, in the presence of L-glutamine (0.25 mM), menadione failed to affect the islet malate content (results not shown).

The significance of the present results was further assessed by comparison with values derived from a model for the metabolism of endogenous nutrients in the islets. Under three different conditions, there was a close analogy between the present experimental results and the values derived from the model. In this model, the flow rate through the malic enzyme amounts to 21.0 pmol/60 min per islet in the absence of exogenous nutrient.

In conclusion, these converging observations indicate that, in addition to the cytosolic conversion of isocitrate into 2-oxoglutarate, which remains to be quantified, the conversion of malate into pyruvate represents a major source of cytosolic NADPH, providing at least as much NADPH as that attributable to the conversion of glucose 6-phosphate into 6-phosphogluconate and ribulose 5-phosphate in glucose-stimulated islets. This quantitative information may prove helpful in the perspective of further investigations on the possible role of cytosolic NADPH in the coupling of metabolic to secretory events in the process of nutrient-stimulated insulin release.

This work was supported by grants from the Belgian Ministry of Scientific Policy and Belgian Foundation for Scientific Medical Research. J. Schoonheydt and M. Urbain are thanked for technical assistance and C. Demesmaeker for secretarial help.

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