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The coupling of metabolic to secretory events in pancreatic islets. Glucose-induced changes in mitochondrial redox state

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Abstract

Mitochondrial NAD^+, NADH, $NADP^+$ and NADPH were measured in dispersed pancreatic islet cells incubated in the absence or presence of D-glucose and then exposed for 20 s to 0.5 mg/ml digitonin. The latter treatment resulted in the full release of lactate dehydrogenase without any detectable loss of glutamate dehydrogenase. The permeabilized cells were separated from the incubation medium by centrifugation through an oil layer and their content in pyridine nucleotides measured by a radioisotopic procedure coupled to the classical cycling technique. Relative to basal value, D-glucose, in concentrations of 2.8 and 16.7 mM, caused a concentration-related increase in both the NADH/NAD^+ and NADPH/$NADP^+$ ratio. These findings provide the first direct evidence for the induction of a more reduced mitochondrial redox state in glucose-stimulated pancreatic islets.

Keywords: Pancreatic islet; Mitochondrial redox state; Dehydrogenase; Glucose; Metabolism

1. Introduction

A change of mitochondrial redox state in glucose-stimulated pancreatic islets may participate to the coupling of metabolic to secretory events. It could indeed affect such variables as the distribution of Ca^{2+} ions between the cytosolic and mitochondrial domains or the activity of key mitochondrial dehydrogenases [1]. However, only indirect, and apparently conflicting, information is so far available on the effect of D-glucose and other nutrients upon the mitochondrial redox state in islet cells. On the one hand, all nutrient secretagogues examined for such a purpose were shown to increase the total NAD(P)H autofluorescence [2] and to decrease the fluorescence of oxidized flavoproteins, half of which are, however, located in the cytosolic compartment [3,4]. On the other hand, measurements based on the steady-state islet content of L-glutamate, 2-ketoglutarate and NH_4^+ suggested, assuming near equilibrium between the glutamate dehydrogenase reaction and NAD system, that nutrient secretagogues may lower the mitochondrial NADH/NAD^+ ratio [1,5,6].

In the present study, the effect of D-glucose upon both the NADH/NAD^+ and NADPH/$NADP^+$ mitochondrial ratios was assessed by separation of the cytosolic and mitochondrial domains through rapid permeabilization of dispersed islet cells by digitonin, and subsequent direct measurement of pyridine nucleotides in the mitochondria-rich cell pellet.

2. Materials and methods

2.1. Pancreatic islets and dispersed islet cells

All experiments were conducted in pancreatic islets or dispersed islet cells prepared from fed female Wistar rats (Proefdierencentrum, Heverlee, Belgium). The islets were isolated by the collagenase procedure [7].

For dispersion of islet cells, groups of 800 islets each were washed thrice and then incubated for 30 min at 30°C in 0.5 ml of a Hepes-NaOH buffer (10 mM, pH 7.4) containing 124 mM NaCl, 5.4 mM KCl, 0.8 mM $MgSO_4$, 1.0 mM NaH_2PO_4, 14 mM $NaHCO_3$, 1.0 mM EGTA and 2.8 mM D-glucose. This islet suspension was then sucked up and driven back 30 times with a 400 μl Eppendorf pipette, and the separation of islet cells completed by 1–3 min incubation at 20°C in 1.0 ml of the same buffer containing 5 mg neutral proteinase (EC 3.4.24.4) from *Bacillus polymyxa* (Dispase II; Boehringer-Mannheim, FRG). After addition of 4 ml of a bicarbonate-buffered

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salt-balanced medium [8] and centrifugation for 90 s at $800 \times g$ and 20°C, the cell pellet was washed with the same bicarbonate buffer and eventually resuspended in it in a ratio equivalent to one islet/μl. The cell number was determined in a haemocytometer. In this procedure, the yield averaged $(1.49 \pm 0.19) \cdot 10^3$ cells per islet ($n = 10$). Trypan blue exclusion test indicated the presence of, at least, 95% viable cells.

2.2. Digitonin treatment

Pancreatic islets or dispersed islet cells were incubated in a bicarbonate-buffered salt-balanced medium containing 5 mg/ml bovine serum albumin [8]. Groups of 300–400 islets each or $(0.2–0.4) \cdot 10^6$ cells were incubated in 80 μl of such a medium, which was placed in polythene tubes on top of 150 μl of silicone oil (Versilube F-50; General Electrics, Waterford, NY), itself layered over 70 μl of the same bicarbonate buffer enriched with 640 mM CsCl. After addition of 80 μl of a solution of digitonin (0.5 to 10.0 mg/ml) prepared in the same buffer and after a further incubation of 20 to 40 s at 20–24°C, the islets or cells were centrifuged for 3 min at $5000 \times g$ (Beckman Microfuge, model 152; Beckman Instruments, Palo Alto, CA) through the oil layer into the solution of CsCl. Aliquots of either the supernatant incubation medium (140 μl) or the suspension of permeabilized islets or cells (70 μl) were brought to a final volume of 280 μl by mixing with the same buffer containing when required CsCl and digitonin, in order to reach the same final concentration of CsCl (160 mM) and digitonin (0.125 to 2.5 mg/ml) in all samples derived from the same experiment. These diluted samples were then sonicated on ice for 3×10 s and eventually examined for their activity in both lactate dehydrogenase (LDH) and glutamate dehydrogenase (GlDH) by methods described elsewhere [9,10].

2.3. D-Glucose metabolism

The generation of ^{3}HOH from D-[5-^{3}H]glucose and that of $^{14}CO_2$ from D-[U-^{14}C]glucose by dispersed islet cells incubated in the same bicarbonate-buffered medium as mentioned above were measured as previously described [11].

2.4. Assay of mitochondrial pyridine nucleotides

For measurement of mitochondrial pyridine nucleotides, dispersed islet cells were preincubated for 60 min at 37°C in a bicarbonate-buffered solution containing 5 mg/ml bovine serum albumin and 2.8 mM D-glucose, and equilibrated with a CO_2/O_2 mixture (1:19, v/v). After centrifugation for 90 s at $800 \times g$ and after removal of the supernatant, the cells were resuspended in the same buffer, except for the absence of D-glucose, in a ratio of about 10^7 cells per ml. Aliquots (40 μl each) of this cell suspension were placed in polythene tubes already containing 80 μl of a solution of NaOH (40 mM) and CsCl (640 mM) topped by 150 μl of silicone oil, mixed with 40 μl of the bicarbonate buffer containing D-glucose in increasing concentrations and eventually incubated for 60 min at 37°C. After addition of the solution of digitonin (80 μl; 1.0 mg/ml) and a further incubation for 20 s at 37°C, the permeabilized cells were separated from the incubation medium, as described above. The incubation medium and most of the oil was then removed by aspiration, and the tubes placed in liquid N_2. The permeabilized cells were then disrupted by mechanical vibration [12]. After centrifugation, aliquots (32 μl each) of the cell extract were either mixed with 16 μl of H_2O and incubated for 10 min at 60°C (assay of reduced pyridine nucleotides) or mixed with 16 μl of 120 mM HCl and incubated for 30 min at 30°C (assay of oxidized pyridine nucleotides). The alkalinized or acidified extracts were then stored at −20°C, until examined for their $NAD(P)^+$ and NAD(P)H content.

The assay of pyridine nucleotides was achieved by coupling the classical cycling technique [13] to a highly sensitive radioactive procedure for the assay of NADH and NADPH [10]. Standards containing increasing amounts (0.5 to 8.0 pmol/20 μl) of all four nucleotides (NAD^+, NADH, $NADP^+$ and NADPH) were prepared in the solution of NaOH (40 mM) and CsCl (640 mM) and treated in the same manner as the cell extracts. Samples (20 μl each) of the acidified or alkalinized cell extracts (corresponding to $(72.9 \pm 1.4) \cdot 10^3$ cells; $n = 8$) and standards were mixed with 80 μl of a Tris-HCl buffer (125 mM, pH 8.4 for NAD cycling and pH 8.2 for NADP cycling) containing 0.38 mM ADP, 2.5 mM 2-[U-^{14}C]ketoglutarate, 12.5 mM ammonium acetate, 0.3 mg/ml bovine serum albumin and either 10 U/ml (NADP cycling) or 22 U/ml (NAD cycling) beef liver glutamate dehydrogenase (EC 1.4.1.3). In the NAD cycling procedure, this reaction mixture also contained 125 mM sodium L-lactate and 11 U/ml beef heart lactate dehydrogenase (EC 1.1.1.27). In the NADP cycling procedure, it contained 1.25 mM D-glucose 6-phosphate and 7 U/ml yeast glucose-6-phosphate dehydrogenase (EC 1.1.1.49). After 120 min incubation at 38°C, the L-[U-^{14}C]glutamate formed during the cycling procedure was separated by ion-exchange chromatography [10]. In this procedure, the solutions of L-lactate, ADP, glutamate dehydrogenase and lactate dehydrogenase were treated as recommended elsewhere [13] to remove contaminants such as pyruvate and pyridine nucleotides.

2.5. Presentation of results

All results are expressed as mean values (±S.E.), together with the number of individual determinations (n), each performed in a separate group of islets or cells. The data relative to the islet cell content of mitochondrial pyridine nucleotides were derived from 6–8 separate experiments in all cases. The statistical significance of differ-

ences between mean values was assessed by use of Student's *t*-test.

3. Results

3.1. Efficiency of digitonin treatment

After exposure to digitonin and separation of the incubation medium from the permeabilized islets or cells by centrifugation through an oil layer, the activity of GlDH and LDH was measured in both these two samples.

In pancreatic islets, the bulk of GlDH activity (96.7 ± 0.6%; $n = 32$) was recovered in the islet pellet, whatever the length (20–40 s) of exposure to digitonin and whatever its final concentration (0.25 to 5.0 mg/ml). However, the recovery of LDH in the incubation medium ranged from only 31.7 ± 2.1% (after 20 s exposure to 0.25 mg/ml digitonin) to no more than 58.9 ± 0.4% (after 40 s exposure to 5.0 mg/ml digitonin). After 20 s exposure to 0.5 mg/ml digitonin, i.e., the standard conditions eventually used in the experiments conducted with dispersed islet cells, the recovery of LDH in the incubation medium averaged 40.3 ± 2.9% ($n = 5$). These results indicate that, under the present experimental conditions, the exposure of intact islets to digitonin was far from optimal to cause the full release of cytosolic material, possibly because of insufficient permeabilization of the cells located in the core of the islets and representing mostly insulin-producing cells. Incidentally, in these experiments, the total activity (islets and incubation medium) of GlDH and LDH averaged, respectively, 20.74 ± 0.99 and 9.21 ± 0.58 pmol/min per islet ($n = 20$ in both cases).

A vastly different situation prevailed in dispersed islet cells. After 20 s exposure to 0.5 mg/ml digitonin, the recovery of GlDH in the cell pellet averaged 95.5 ± 0.3% ($n = 6$), as compared to 97.9 ± 0.5% ($n = 10$) in control cells not exposed to digitonin and 91.1 ± 1.4% ($n = 10$) after exposure to 1.0 mg/ml digitonin for 20–40 s. All LDH was now recovered in the incubation medium, the residual activity in the cell pellet being below the limit of detection whatever the length (20–40 s) of exposure to digitonin and whatever its concentration (0.5 to 1.0 mg/ml). Inversely, all LDH activity was recovered in the cell pellet when the islet cells had not been exposed to digitonin. In these experiments, the total activity of GlDH and LDH averaged, respectively, 7.70 ± 1.07 and 2.11 ± 0.36 pmol/min per 10^3 cells ($n = 14$ in both cases).

In the light of these findings, further experiments were conducted in dispersed islet cells exposed for 20 s to 0.5 mg/ml digitonin.

3.2. Metabolic behaviour of dispersed islet cells

The metabolic integrity of dispersed islet cells was assessed by measuring both the generation of ^{3}HOH from D-[5-^{3}H]glucose and oxidation of D-[U-^{14}C]glucose. A rise in D-glucose concentration from 2.8 to 16.7 mM increased ($P < 0.001$) the generation of ^{3}HOH from 5.66 ± 0.25 to 20.41 ± 2.36 pmol/10^3 cells per 120 min ($n = 27$ in both cases) and the oxidation of D-[U-^{14}C]glucose from 1.67 ± 0.18 to 7.01 ± 1.30 pmol/10^3 cells per 120 min ($n = 25$–26).

3.3. Measurement of pyridine nucleotides

The combination of the classical cycling procedure for the assay of pyridine nucleotides [13] with a highly sensitive radioisotopic technique based on the NAD(P)H-depen-

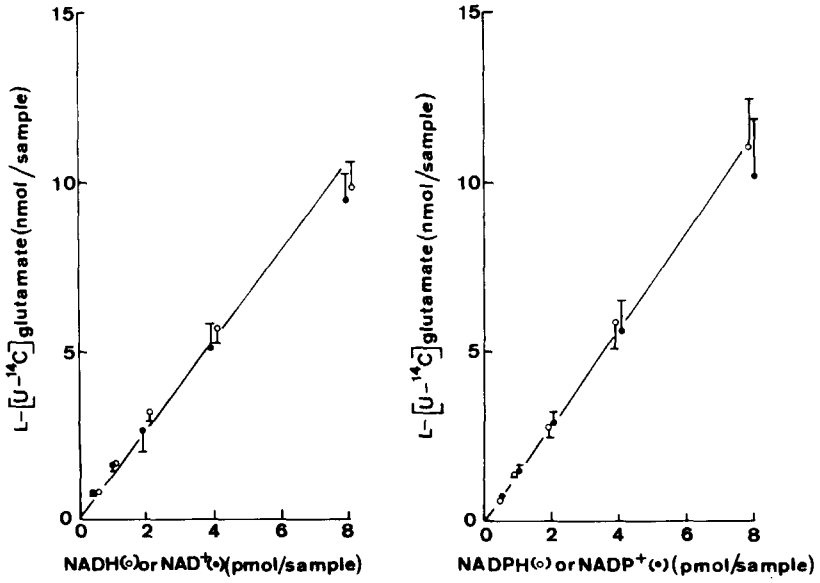

Fig. 1. Generation of L-[U-^{14}C]glutamate in the assay of NAD (left panel) and NADP (right panel) standards. Mean values (±S.E.) are derived from 6–7 individual experiments in the left panel and 4 experiments in the right panel.

Table 1
Effect of D-glucose upon mitochondrial redox state in dispersed islet cells

	D-Glucose		
	Nil	2.8 mM	16.7 mM
NADH (pmol/10^6 cells)	4.51 ± 0.48 (14)	4.27 ± 0.45 (13)	5.26 ± 0.46 (19)
NAD^+ (pmol/10^6 cells)	11.20 ± 1.06 (14)	7.95 ± 0.64 (13)	8.54 ± 0.92 (19)
NADH + NAD^+ (pmol/10^6 cells)	15.71 ± 1.42 (14)	12.21 ± 0.86 (13)	13.80 ± 1.16 (19)
NADH/NAD^+	0.414 ± 0.034 (14)	0.560 ± 0.067 (13)	0.698 ± 0.086 (19)
NADPH (pmol/10^6 cells)	5.09 ± 0.47 (14)	6.65 ± 0.45 (15)	10.73 ± 0.61 (15)
$NADP^+$ (pmol/10^6 cells)	4.09 ± 0.41 (14)	3.31 ± 0.27 (15)	3.65 ± 0.21 (15)
NADPH + $NADP^+$ (pmol/10^6 cells)	9.18 ± 0.67 (14)	9.96 ± 0.47 (15)	14.39 ± 0.66 (15)
NADPH/$NADP^+$	1.39 ± 0.19 (14)	2.26 ± 0.26 (15)	3.09 ± 0.27 (15)

dent generation of L-[U-^{14}C]glutamate from 2-[U-^{14}C]ketoglutarate [10] permitted the reliable measurement of picomolar amounts of NAD^+, NADH, $NADP^+$ and NADPH. In this procedure, neither NAD^+ nor NADH reacted in the NADP cycle, and neither $NADP^+$ nor NADPH did so in the NAD cycle. It was also verified that the acid and alkaline treatment selectively removed NAD(P)H and $NAD(P)^+$, respectively. In each individual experiment, all standards were measured in triplicate. The coefficient of variation (S.D./mean) for such triplicates averaged 2.2 ± 0.4 and 2.2 ± 0.3% in the assay of NAD^+ and NADH and 2.6 ± 0.4 and 2.8 ± 0.4% in the assay of $NADP^+$ and NADPH ($n = 20$ in all cases).

In the NAD cycling procedure, the specific radioactivity of 2-[U-^{14}C]ketoglutarate averaged 954.2 ± 19.3 cpm/pmol ($n = 7$). The blank value represented 4.1 ± 0.5% ($n = 7$) of the total radioactive content of each sample. Such a relatively high blank value is probably attributable to the contamination of enzymes, e.g., lactate dehydrogenase, by NAD [13]. The results obtained with NAD^+ and NADH standards were similar to one another. The amount of L-[U-^{14}C]glutamate generated from 2-[U-^{14}C]ketoglutarate was grossly proportional to the sample content in NAD^+ or NADH (Fig. 1, left panel). The rate of cycling over 120 min incubation averaged $(1.52 \pm 0.11) \cdot 10^3$ ($n = 15$).

In the NADP cycling procedure, the specific radioactivity of 2-[U-^{14}C]ketoglutarate averaged 943.5 ± 18.7 cpm/pmol ($n = 4$). The blank value represented no more than 0.23 ± 0.03% ($n = 4$) of the total radioactive content of each sample. The readings obtained with the $NADP^+$ and NADPH standards were virtually identical to one another. The amount of L-[U-^{14}C]glutamate generated in the assay was proportional to the amount of $NADP^+$ or NADPH present in each sample (Fig. 1, right panel). The rate of cycling over 120 min incubation ranged in individual experiments from $1.08 \cdot 10^3$ to $1.88 \cdot 10^3$, with a mean value of $(1.43 \pm 0.13) \cdot 10^3$ ($n = 8$).

When duplicate measurements of the NADH/NAD^+ ratio were conducted in the same cell extract, the coefficient of variation for such duplicates averaged 2.1 ± 0.4% ($n = 29$). In the case of NADPH/$NADP^+$ ratio, the coefficient of variation averaged 4.6 ± 0.6% ($n = 12$).

3.4. Effect of D-glucose on mitochondrial redox state

The total amount of NADH and NAD^+ was comparable in cells incubated in the absence of D-glucose or at a low concentration (2.8 mM) of the hexose (14.02 ± 0.90 pmol/10^6 cells; $n = 27$) and in cells exposed to a much higher concentration (16.7 mM) of D-glucose (13.80 ± 1.6 pmol/10^6 cells; $n = 19$). The rise in D-glucose concentration caused an increase in the NADH/NAD^+ ratio (Table 1). Relative to basal value, the glucose-induced increment in such a ratio was of borderline statistical significance ($P = 0.06$) in cells exposed to 2.8 mM D-glucose, but highly significant ($P < 0.015$) in cells incubated at 16.7 mM D-glucose. Nevertheless, no significant difference ($P > 0.2$) in NADH/NAD^+ ratio was found between cells incubated at 2.8 and 16.7 mM D-glucose.

The total mitochondrial pool of NADP was similar in cells incubated either in the absence of D-glucose or at a low concentration of the hexose (2.8 mM). It was significantly increased, however, after 60 min exposure to 16.7 mM D-glucose ($P < 0.001$). As little as 2.8 mM D-glucose increased significantly ($P < 0.02$) the mitochondrial NADPH/$NADP^+$ ratio above basal value. Such a ratio was further increased ($P < 0.05$) in the presence of 16.7 mM D-glucose.

4. Discussion

The present results suggest that a rise in D-glucose concentration increases both the NADH/NAD^+ and NADPH/$NADP^+$ mitochondrial ratio in isolated pancreatic islet cells.

The experimental procedure used in the present study offers three major advantages. First, the incubation of dispersed islet cells, as distinct from intact islets, made it possible to obtain, over a relatively short period (20 s) of exposure to digitonin (0.5 mg/ml) a full release of lactate dehydrogenase, whilst avoiding any significant loss of glutamate dehydrogenase. Second, the separation of the permeabilized islet cells from the surrounding incubation medium containing cytosolic pyridine nucleotides, by centrifugation through an oil layer, permitted the rapid collec-

tion of the permeabilized cells in an alkaline medium designed to avoid further changes in the redox state of the pyridine nucleotides. Last, the combination of classical cycling procedures with a highly sensitive radioisotopic method for the assay of pyridine nucleotides allowed their reliable measurement in picomolar amounts.

A shortcoming of the present procedure, however, consists in the fact that our measurements presumably refer to the total amount of either reduced or oxidized NAD and NADP, rather than the true concentration of free nucleotides.

A prior attempt to measure the mitochondrial $NADH/NAD^+$ ratio was based on the measurement of the islet content in L-glutamate, NH_4^+ and 2-ketoglutarate and, hence, relied, inter alia, on the assumption that the reaction catalyzed by glutamate dehydrogenase was close to equilibrium in islet mitochondria [1]. Since the results obtained with this prior and indirect approach suggested that D-glucose and other nutrients may paradoxically induce a more oxidized state of the NAD system [1,5,6], it was felt necessary to extend these investigations to a direct measurement of oxidized and reduced pyridine nucleotides.

Whilst keeping in mind the above-mentioned reservation on the free versus total amount of pyridine nucleotides, the comparison between prior findings and the present results argue against the view that the glutamate dehydrogenase is indeed close to equilibrium in islet cell mitochondria. This is compatible with the knowledge that a sizeable amount of NH_4^+ is generated during incubation of islet cells in media deprived of exogenous nutrient and that such a production of NH_4^+ is only partially decreased in the presence of D-glucose [14].

Two striking findings in the present study consisted in the fact that as little as 2.8 mM D-glucose was sufficient to induce a more reduced state of the NAD and NADP system relative to basal value and that, at this low concentration or at a higher concentration of the hexose, the mitochondrial $NADPH/NADP^+$ ratio was as markedly affected as the $NADH/NAD^+$ ratio. The first of these two findings imply that a change in mitochondrial redox state is not sufficient per se to cause stimulation of either proinsulin biosynthesis or insulin release, which are both enhanced by D-glucose at concentrations in excess of 2.8 mM. The second finding suggests that the redox state of the NAD and NADP systems are somehow linked together in islet mitochondria, possibly at the intervention of an energy-linked nicotinamide nucleotide transhydrogenase, of $NADP^+$- and NAD^+-linked isocitrate dehydrogenases and of glutamate dehydrogenase [15].

In conclusion, the present data strongly suggest that glucose increases both the $NADH/NAD^+$ and $NADPH/NADP^+$ mitochondrial ratio in islet cells. The relevance of these changes to such variables as the activity of key mitochondrial dehydrogenases and the efflux of Ca^{2+} from the mitochondria remain, however, to be investigated.

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