PRELIMINARY REPORT

Respiratory Chain Activity and Mitochondrial DNA Content of Nonpurified and Purified Pancreatic Islet Cells

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Considerable interest has recently focused on the possible role of alterations in mitochondrial activity and mutations in the mitochondrial genome for the development of non–insulin-dependent diabetes. Our study aimed at investigating the normal mitochondrial respiratory chain activity of nonpurified and purified islet cells to further explore whether some diabetic states are associated with alterations of mitochondrial oxidative processes. For this purpose, pancreatic islets were isolated from Wistar rats. Unpurified islet cells were obtained in the presence of trypsin and DNAse, and purified β and non-β cells were prepared by autofluorescence-activated sorting using a flowcytometer. Intact cell respiration and substrate oxidation in digitonin-permeabilized cells were measured polarographically with a Clark oxygen electrode in a micro–water-jacketed cell. Specific activity of the individual complexes of the respiratory chain was determined spectrophotometrically in unpurified islet cells. The relative amount of mitochondrial (mtDNA) and nuclear (nDNA) DNA in all three cell populations and in rat brain and skeletal muscle was estimated by dot blotting. The intact cell respiration of unpurified islet cells corresponds to the mean of values obtained for β and non-β islet cells. Oxidation rates of different substrates by permeabilized β cells were lower than those for unpurified and non-β cells. The amount of mtDNA relative to nDNA was similar in all three groups of cells, and was also similar to that obtained from brain and skeletal muscle. In summary, we have described mitochondrial respiratory chain activity in unpurified, β, and non-β islet cells. Our results represent an initial step in investigating the potential pathogenic role that alterations in oxidative phosphorylation could play in some diabetic states.

THE EXACT MECHANISM by which glucose metabolism in β cells is coupled to insulin secretion remains to be fully established. However, it is currently believed that stimulation of insulin release by D-glucose and other nutrient secretagogues is linked to their capacity to increase adenosine triphosphate production in the mitochondria.[1] In this context, it should be expected that an impairment in mitochondrial oxidative events may result in a derangement of insulin secretion.

Defects in some mitochondrial enzymes have been related to the appearance of diabetes in animal models and humans.[2-4] In addition to this, a diminution of mitochondrial DNA (mtDNA) content has been related to long-lasting impairment of insulin secretion,[5,6] and particular attention has focused on maternally inherited diabetes and deafness syndrome, a pathological condition related to mutations of mtDNA.[7,8] However, there is no information about the characteristics of mitochondrial respiratory chain activity in pancreatic islet cells in normal and pathological conditions.

The aim of the present study was to investigate the normal mitochondrial respiratory chain activity of purified and nonpurified islet cells as a first step to further investigate whether alterations of oxidative phosphorylation play a pathogenic role in non–insulin-dependent diabetes mellitus.

MATERIALS AND METHODS

Preparation of Pancreatic Islet Cells

Pancreatic islets were isolated from fed male Wistar rats (175 to 200 g) by the collagenase technique[9] and passed through a 400-μm mesh nylon filter to discard large exocrine aggregates. The islets were then placed in iced Hanks solution and isolated in two consecutive collections conducted under the control of a dissecting microscope to minimize exocrine contamination. The islets were centrifuged for 5 minutes at 200 × *g*, and the pellet was resuspended in a Ca^{2+}-free HEPES-buffered Earle's dissociation medium (NaCl 124 mmol/L, KCl 5.4 mmol/L, $MgSO_4$ 0.8 mmol/L, NaH_2PO_4 1.0 mmol/L, $NaHCO_3$ 14.3 mmol/L, HEPES 10 mmol/L, pH 7.3 to 7.4) containing bovine serum albumin ([BSA] 2.5 mg/mL, Fraction V, RIA grade; Sigma, St Louis, MO), EGTA (1.0 mmol/L), and D-glucose (5.6 mmol/L). After gentle mechanical dispersion by suction through a polyethylene pipette, further digestion was conducted in the presence of trypsin (0.8 μg/mL Bovine pancreatic trypsin; Boehringer, Mannheim, Germany) and DNAse (0.3 μg/mL DNAse I, RIA grade; Boehringer) for 12 to 15 minutes at 30°C. At intervals, samples were monitored by phase-contrast microscopy, with digestion being stopped when about 60% of the islet cells were present as single cells. The suspension of islet cells was then passed through a 70-μm mesh nylon filter (Falcon Products, Franklin Lanes, NJ) and carefully layered on the surface of a density 1.045 Earles-HEPES-Percoll solution (6.0 mL; Pharmacia, Uppsala, Sweden). After 15 minutes' centrifugation at 300 × *g* and 6°C, the supernatant was discarded, with the cell pellet being resuspended in 5 mL dissociation medium (see above), again centrifuged (5 minutes at 200 × *g*), and eventually resuspended in an Earle's HEPES buffer (see above) containing 1.8 mmol/L $CaCl_2$, 5 mg/mL BSA, and 2.8 mmol/L

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D-glucose. Purified β and non-β islet cells were prepared by autofluorescence-activated sorting as described elsewhere, after 60 minutes' incubation of islet cells at 17°C in the presence of 2.8 mmol/L D-glucose.[10] Cell sorting was performed using a FACSTAR PLUS (Becton-Dickinson, Sunnyvale, CA).

Viability of total islet and purified cells was assessed by neutral red staining. Insulin and glucagon levels were measured by RIA (insulin, CIS Biointernational, Gif-sur-Yvette, France; and 30-K antibody for glucagon, provided by R. Unger) in cell extracts after disintegration by an ultrasonic procedure in 0.5 mL acid-alcohol solution (75% vol/vol ethanol, 23.5% bidistilled water, and 1.5% vol/vol 10N HCl). Protein concentration in islet cell preparations was determined by Bradford's method.[11]

Polarographic Studies

Oxygen utilization was measured polarographically in a standard medium (10 mmol/L KCl, 5 mmol/L $MgCl_2$, 10 mm KH_2PO_4, and 1 mg/mL BSA, pH 7.4, 37°C) with a Clark oxygen electrode in a micro–water-jacketed cell at 37°C (Hansatech Instruments Limited, Norfolk, UK) as described elsewhere.[12] We measured the intact cell respiration and the substrate oxidation in digitonin-permeabilized cells using succinate (10 mmol/L), glycerol-3-phosphate (20 mmol/L), pyruvate (10 mmol/L) plus malate (1 mmol/L), and glutamate (10 mmol/L). State 3 rate was assessed for each substrate.[12] Glucose concentration in the medium was always less than 0.05 mmol/L.

Biochemical Studies

Measurement of the specific activity of individual complexes of the respiratory chain was performed spectrophotometrically (UVIKON 922; Kontron, Zurich, Switzerland) in unpurified islet cells. Assays were performed at 37°C. The following complexes were studied: complex II (succinate-ubiquinone reductase, EC 1.3.99.1), using as acceptor 2,6-dichlorophenolindophenol (50 μmol/L) in the presence of decyl-ubiquinone (50 mmol/L) followed by assay of glycerol-3-phosphate-ubiquinone reductase activity; and complex IV (cytochrome *c* oxidase, EC 1.9.3.1) using reduced cytochrome (50 μmol/L) as donor in a medium containing 1 mg/mL BSA and 10 mmol/L KH_2PO_4 (pH 6.50).[12]

mtDNA Analysis

Total DNA was extracted from both unpurified and purified islet cells following standard procedures.[13] The relative amount of mtDNA and nuclear DNA (nDNA) was estimated by dot blotting. Each DNA sampling was spotted on the filter in different concentrations, 100, 150, and 200 ng. Three replicas of the filter were prepared on nylon membrane (Hybond+). The filters were hybridized using two different [^{32}P]dCTP-labeled probes: a total rat mtDNA probe and a nDNA probe corresponding to the cDNA gene of the placental growth hormone releasing–hormone, respectively.[14] Samples from rat brain and skeletal muscle were used for comparison.

Statistical Analysis

Values are expressed as the mean ± SEM. Comparison between more than two means was performed by ANOVA and Scheffe test. Statistically significant differences were established with P values less than .05.

RESULTS

Viability of nonpurified and purified islet cells as assessed by neutral red staining was always greater than 90%. The insulin to glucagon ratio was over 1,000 times higher in β cells than in non-β islet cells. Hormonal content in β and non-β cells was as follows: insulin 20.18 ± 1.52 and 0.40 ± 0.11 pg/cell and glucagon 0.19 ± 0.02 and 5.08 ± 0.39 pg/cell, respectively.

Polarographic Studies

Results of polarographic studies are shown in Table 1. The intact cell respiration of β cells (16.53 ± 2.20 nmol O_2/min/mg protein) was not significantly different from that of non-β cells (19.45 ± 5.97) and unpurified islet cells (17.92 ± 1.58). When the oxidation of different substrates by permeabilized cells was analyzed, β cells showed lower rates with respect to unpurified and non-β cells. However, only succinate oxidation showed significant differences between β cells (25.71 ± 5.01) and unpurified islet cells (50.08 ± 4.93, $P < .05$). Ratios between the different substrate oxidation rates were practically identical in β and non-β cells; however, the ratios where succinate oxidation was implicated were higher in purified cells compared with nonpurified cells ($P < .05$) (Table 1).

Enzymatic Activities

Values for enzymatic activities in unpurified islet cells were 156 ± 10 nmol/min/mg protein for succinate-ubiquinone reductase, 191 ± 32 for glycerol-3-phosphate-ubiquinone reductase, and 950 ± 24 for cytochrome *c* oxidase.

mtDNA Content

The relative amount of mtDNA with respect to nDNA (DO mtDNA/DO nDNA) was similar in unpurified islet cells (0.799), β cells (0.795), and non β cells (0.798), and was also similar to that of brain (0.762) and skeletal muscle (0.718) (Fig 1).

DISCUSSION

Polarographic studies in pancreatic cells showed no differences between nonpurified and purified islet cells concerning intact cell respiration, although this parameter was somehow lower in β cells. In permeabilized β cells, oxidation rates for all

Table 1. Polarographic Studies in Unpurified and Purified Islet Cells

	Unpurified Islet Cells (n = 6)	β Cells (n = 4)	Non-β Cells (n = 4)
Oxidation rates (nmol O_2/min/mg protein)			
Intact cells	17.92 ± 1.58	16.53 ± 2.20	19.45 ± 5.97
Pyruvate/malate	20.48 ± 2.72	17.51 ± 2.28	25.95 ± 7.95
Glutamate	24.00 ± 3.95	16.98 ± 2.61	22.46 ± 5.29
Succinate	50.08 ± 4.93	25.71 ± 5.01*	34.30 ± 2.98
Glycerol-3-phosphate	21.22 ± 2.70	19.45 ± 3.45	26.48 ± 1.22
Ratios			
PMox/intact cells	1.15 ± 0.11	1.06 ± 0.03	1.30 ± 0.10
PMox/Sox	0.37 ± 0.02	0.75 ± 0.15*	0.74 ± 0.11*
G3Pox/Sox	0.43 ± 0.05	0.88 ± 0.05*	0.82 ± 0.05*

NOTE. Data are expressed as the mean ± SEM. n = number of experiments; in each experiment, cells were obtained from 8 to 10 animals.

Abbreviations: PMox, pyruvate and malate oxidation; Gox, glutamate oxidation; Sox, succinate oxidation; G3Pox, glycerol-3-phosphate oxidation.

*$P < .05$, purified *v* unpurified islet cells.

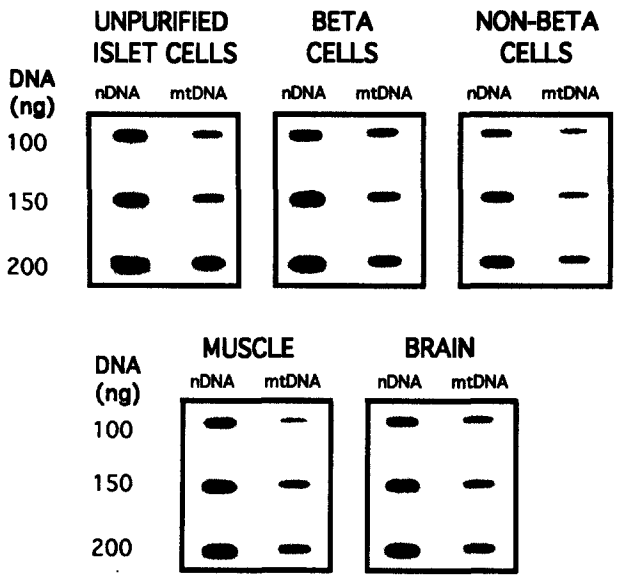

Fig 1. Quantitative slot-blot analysis of mtDNA with respect to nDNA from unpurified islet cells, β cells, non-β cells, brain, and skeletal muscle.

substrates were also lower than those observed in unpurified and non-β islet cells, although we only found significant differences for succinate oxidation. These results suggest a lower oxidative capacity for pancreatic β cells compared with total and non-β islet cells in conditions of high energy demand, which is evident in the presence of the succinate. These findings are not due to differences in mtDNA content, since it was similar in all analyzed cell groups, as well as in other tissues with high energetic capacity. We also measured the rates of succinate oxidation in the absence and presence of digitonin; this parameter resembles the respiratory control measured usually in polarographic studies in isolated mitochondria and gives a rough estimation of cell integrity. The ratios between the different substrate oxidation rates, the best parameter for measuring balanced respiratory chain activity in vivo,[12] were practically identical in β and non β cells, indicating a similar coupled state for both cell types in the absence of glucose. According to this, a lower rate of succinate oxidation could be due to succinate-dehydrogenase activity itself or to succinate transport across the cell membrane or mitochondrial membrane.

The lower mitochondrial substrate oxidative capacity of β cells under conditions of high energy demand is a remarkable finding, considering that mitochondrial oxidation represents a key event in the metabolic and hence secretory behavior of these cells. Nevertheless, these findings do not argue against this metabolic characteristic, considering that it could be masked by our experimental conditions: (1) We used a very low D-glucose concentration, which induces a preferential increase in FAD content in β cells that in turn could inhibit mitochondrial respiratory chain activity to some extent[10]; and (2) Since glucose-induced insulin release depends on functional cooperation between islet cells,[15] high energy demand could be limited in purified single β cells compared with β cells lodged within intact islets or surrounded by other islet cell types. Taking into account that succinate represents a well-recognized nutrient secretagogue,[16] it could also be argued that the latter phenomenon could explain its lower oxidation. Unfortunately, we could not measure enzymatic activity of the electron chain complexes in purified populations to test this hypothesis, because we obtained insufficient amounts of cellular material.

Interest in clinical and basic research has been raised in the investigation of an increasing number of neuromuscular and nonneuromuscular pathological conditions probably related to mitochondrial respiratory chain defects, including a subtype of diabetes mellitus, the maternally inherited diabetes and deafness syndrome.[7] Considering the large number of genes involved in the coding of respiratory chain components, molecular screening of defects is troublesome. Moreover, the pathogenic role of molecular defects is usually far from proven. In addition to this, islet cells that have been exposed to diabetogenic agents, as well as those from suitable animal models of type II diabetes, display a diminution of mtDNA content associated with an impairment of insulin secretion.[5,6] However, information about the precise alteration generated in mitochondrial respiration activity of islet cells remains scarce. In this context, we believe that an alternative approach to the study of mitochondrial disorders could be the measurement of mitochondrial respiratory chain activity in purified (β and non-β) cells, including tissue- and enzyme-specific investigations. Our results represent a step forward in the investigation of the potential pathogenic role that alterations in oxidative phosphorylation could play in some diabetic states.

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