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Glucose-induced heat production, insulin secretion and lactate production in isolated *Wistar* rat pancreatic islets

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ABSTRACT

Transplantation of pancreatic islets is efficient in improving the metabolic control and quality of life and in preventing severe hypoglycemia in patients with brittle type 1 diabetes mellitus. More accurate methods to assess islet viability would be extremely useful in designing target interventions for islet cytoprotection and in reducing the number of islets required to achieve insulin independence. Here we report on an application of calorimetry to evaluate the metabolic response of pancreatic islets to glucose stimulation. A significant increase in metabolic heat was produced by islet samples when consecutively subjected to 2.8 and 16.3 mmol L^{-1} glucose. Under these glucose concentrations, 1000 islets released average heat values of 9.16 ± 0.71 mJ and 14.90 ± 1.21 mJ over 50 min, respectively. Additionally, the glucose stimulation indexes were 1.67 ± 0.30 for insulin, 1.72 ± 0.13 for heat and 2.91 ± 0.50 for lactate, raising the important possibility of substituting the secreted insulin index/ratio by the index/ratio of the heat released in the evaluation of Langerhans islets viability for transplantation. Altogether, our results demonstrate the applicability of calorimetry to assess the quality of isolated pancreatic islets and to study vital islet functions.

1. Introduction

Pancreatic islet transplantation has proven to be efficient in improving the metabolic control and quality of life and in preventing severe hypoglycemia in patients with brittle type 1 diabetes mellitus [1]. This procedure provides a safe and minimally invasive [2–4] alternative for β-cell replacement, which can be indicated earlier in the course of type 1 diabetes mellitus to allow a more physiological control of glucose metabolism than exogenous insulin therapy, helping to prevent the development of secondary complications of diabetes [5,6]. New clinical islet transplant programs are being established worldwide and the annual number of transplants continues to increase. However, it is still difficult to evaluate the viability of isolated islets and to ensure that viable islets will correspond to a successful transplantation [7].

Improved methods for islet assessment are being developed to predict islet potency. Better understanding of the causes of islet dysfunction is crucial to design new target interventions for islet cytoprotection and to reduce the number of islets required to achieve insulin independence [2–5,8]. Several methods of assessing viability and function of human islet preparations have recently been reported. Current practice includes islet quantification (including a measurement of purity and number of islets) and islet viability assessment (by membrane integrity staining assays and other methods). Additionally, glucose-stimulated insulin secretion by purified islets can be assessed by static incubation assays or perfusion studies. The glucose stimulation index is calculated as the ratio between the amount of insulin secreted during rat islet incubation in medium containing 16.3 mmol L^{-1} glucose and the basal level of insulin secretion in medium containing 2.8 mmol L^{-1} glucose. Glucose-stimulated insulin secretion is the standard assay used worldwide to evaluate islet viability. Stimulation indexes of islets that are considered ideal for transplantation typically range from approximately 2 to 4 [9]. These values indicate that the iso-

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lated islets preserved their ability to perform glucose metabolism, protein synthesis and secretion [10], crucial processes that must be intact in islets that will be used for transplantation. Indirect methods routinely used to quantify the insulin secreted by the islets β-cells, such as ELISA (immunoenzymatic absorption assay) and RIA (radioimmunoassay), usually require long periods of time for complete processing [11]. However, the main problem of using these methods is the finding that islet preparations that failed to reverse diabetes were indistinguishable from those that resulted in excellent function, considering their glucose stimulation indexes. There have been reports of failures occurring in the very early post-transplant period. This disappointing observation could be related to the use of islet preparations displaying a sub-optimal quality, since insulin release assays reflect the function of only a part of the metabolic pathways within the islets, rendering this parameter not very useful when global changes in islet function or viability need to be detected.

Other methods include DNA-binding dye exclusion, which can only reveal cells that have lost membrane selectivity. Dithizone staining only provides an estimate of β-cell content in islet preparations. A more predictive pre-transplant test is the reversal of diabetes in immunodeficient mice; however, several days are required to assess the outcome, rendering it a non-practical pre-transplant quality control criterion [12–14].

The aim of the present work is to use calorimetry to evaluate the amount of heat produced by isolated *Wistar* rat pancreatic islets upon glucose stimulation and to identify factors that contribute to heat production. This approach may lead to a new technique to assess islet metabolism that has the potential to be more accurate, rapid and efficient than the currently available methods.

2. Materials and methods

2.1. Reagents

Bovine serum albumin (BSA), collagenase (type V), dithizone (diphenylthiocarbazone), RPMI 1640 (with L-glutamine, 2.0 g L^{-1} D-glucose, without sodium bicarbonate) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Rat insulin determination kits were purchased from Linco (Linco Research, MO, USA). Molecular Biology grade Ficoll 400 was purchased from Calbiochem (San Diego, CA, USA). Acridine orange and propidium iodide were purchased from Sigma–Aldrich.

2.2. Islet isolation and purification

The islets were isolated from male *Wistar* rats with 220–280 g body weight, anesthetized with ketamine chloridrate associated with xylazine hydrochloride (10:7.5, v/v) in the concentration of 0.2 mL/100 g body weight. After performing an abdominal midline incision, mosquito clamps were applied to the distal bile duct at its entry points into the duodenum, and before the bifurcation of right and left hepatic ducts. The pancreatic duct was incised with microsurgery scissors and cannulated before the bifurcation with PE50 tubing attached to a 26-gauge needle and syringe [15,16]. The tubing was kept in place, with extra care being taken to position the tip 2–3 mm before the point where the pancreatic duct emerges from the pancreas to allow adequate distention of the pancreatic head.

After ductal cannulation, pancreata were completely distended with 10 mL of cold Hanks supplemented with 1 mg mL^{-1} albumin, 2.8 mmol L^{-1} D-glucose, 0.7 mg mL^{-1} type V collagenase, using a pulsed infusion technique. After complete distention, pancreata were excised and placed in glass Petri dishes containing cold Hanks (without glucose), rapidly dissected to extract visible vessels and lymph nodes, minced into 1–2 mm fragments with curved scissors, and stored in Falcon 50 mL tissue culture tubes. These tubes were incubated in a 37 °C water bath for stationary digestion during 25 min. The tubes were then manually and vigorously shaken to dissociate the islets from adherent acinar elements. Digestion was interrupted by the addition of 15 mL of cold Hanks followed by centrifugation at 162.4 × *g* (Sorvall® RT 6000B refrigerated centrifuge, 14.5 cm radius rotor), for 5 min. Three serial rinse cycles were performed prior to resuspension in 10 mL of Euro-Ficoll (1.037 density). Purification was carried out in a discontinuous density gradient (1.037, 1.069, 1.096 and 1.110) at 649.6 × *g* and 4 °C for 15 min. The islets were located between the 1.069 and 1.096 layers, and between the 1.096 and 1.110 layers. The 1.037 layer and the top portion of the 1.069 layer were slowly aspirated and discarded with an automatic pipette. The third layer (1.096), with overlying and underlying islets, was aspirated and placed into 50 mL tubes containing cold Hanks supplemented with 10% fetal calf serum (FCS) and washed at 469.3 × *g* and 4 °C for 5 min. During washing, the pellets were resuspended and a sample was taken and checked for islets with dithizone staining. The supernatants were discarded and the pellets were resuspended in 20 mL of RPMI containing 10% FCS and washed by centrifugation at 274.5 × *g* and 4 °C for 5 min. Finally, the islet-containing pellet was suspended in RPMI with 10% FCS and transferred to tissue culture-treated 60 mm Petri dishes. The islets were incubated overnight at 37 °C in 5% CO_2 before the calorimetric assays.

2.3. Islet viability

Islet samples were assessed for morphological viability immediately before and after the calorimetric assays with a simultaneous Live-Dead double staining method consisting of: 290 μmol L^{-1} of acridine orange (Sigma), a weak base capable of penetrating living cells and binding to nucleic acids, causing a green fluorescence, and 150 μmol L^{-1} of propidium iodide, an exclusion dye that cannot penetrate viable cells, but binds to the nucleic acids of dead cells, producing a bright red fluorescence [17], which was observed and registered under an Olympus IX70-S870/BH2 RFL-T3 fluorescence microscope (Olympus Optical Co., Japan).

2.4. Islet sample preparation

After the overnight incubation, cultured islets were checked for morphological viability and counted before a volume containing 1000 islets was transferred to a 15 mL conical tube and centrifuged at 162.4 × *g* during 1 min at room temperature. The supernatant was discarded and the pellet was washed once in a glucose-free Krebs–Ringer solution containing 1 mg mL^{-1} of BSA by centrifugation at 162.4 × *g* during 1 min at room temperature. The supernatant was discarded and the resulting pellet resuspended in 1.66 mL of glucose-free Krebs–Ringer solution, before being introduced into the calorimeter sample cell.

2.5. Isothermal calorimetry

The calorimetric experiments with islets treated with glucose were performed at 37 °C in a VP-ITC system (Microcal Inc., Northampton, MA, USA), with a 1.7 mL reaction vessel.

The calorimetric sample cell was gently filled with 1000 rat islets suspended in 1.66 mL of glucose-free Krebs–Ringer solution. The reference cell was filled with ultrapure Milli-Q water (Millipore, Billerica, USA). The titration syringe was filled with Krebs–Ringer solution containing 119 mmol L^{-1} of glucose. The sample cell was then closed and the islets were kept in suspension by stirring at 270 rpm until a stable baseline was observed. When these condi-

tions were reached, a single 40 μL injection of the 119 mmol L^{-1} glucose solution was made, resulting in a final glucose concentration of 2.8 mmol L^{-1} in the sample cell. The heat evolved in the reaction was monitored during 50 min with constant stirring at 270 rpm. After the assay, the contents of the sample cell were gently removed with a syringe and centrifuged during 1 min at 162.4 × *g* at room temperature. The supernatant was frozen and kept at −30 °C for measurement of insulin content. The pellet was resuspended in 1.66 mL of glucose-free Krebs–Ringer solution to be used in the next assay, with a higher glucose concentration. The injector syringe was washed with ultrapure water and filled with Krebs–Ringer solution containing 692.75 mmol L^{-1} glucose. The sample cell was filled with the resuspended pellet containing the same islets from the previous run. After reaching thermal equilibrium, 40 μL of the 692.75 mmol L^{-1} glucose solution was injected resulting in a final glucose concentration of 16.3 mmol L^{-1} in the sample cell. The heat evolved in the reaction was monitored during 50 min with constant stirring at 270 rpm. After the run, the contents of the sample cell were removed, centrifuged during 1 min at 162.4 × *g* at room temperature and the supernatant was frozen at −30 °C for measurements of insulin and lactate contents.

2.6. Determination of insulin and lactate content

The insulin content of the frozen supernatants obtained after each calorimetric run was determined by radioimmunoassay. These supernatants were also used for determination of the lactate content with a 2300 STAT PLUS Glucose & L-Lactate Analyzer (YSI Incorporated, Yellow Springs, Ohio). All determinations were performed in duplicate.

2.7. Statistical analysis

All statistical analyses were performed in the R Statistical System software [18], using linear mixed effects models [19] with the *lme* function from the *nlme* package [20]. The model was simplified by the backwards process, and the minimal adequate model was obtained by withdrawing all the non-significant terms ($p < 0.05$) from the full model presented below [21]. The graphs were generated with the software GraphPad Prism version 3.0 (GraphPad Software, Inc., San Diego, USA).

Statistical models:

$$\begin{aligned}\text{heat} = {} & \text{insulin} + \text{condition} + \text{lactate} + \text{block} + \text{insulin} : \text{condition} \\ & + \text{insulin} : \text{lactate} + \text{condition} : \text{lactate} \\ & + \text{insulin} : \text{condition} + \text{insulin} : \text{condition} : \text{lactate} \end{aligned} \quad (1)$$

$$\text{lactate} = \text{condition} + \text{insulin} + \text{block} + \text{condition} : \text{insulin} \quad (2)$$

$$\text{insulin} = \text{condition} + \text{lactate} + \text{block} + \text{condition} : \text{lactate} \quad (3)$$

where insulin is the concentration of insulin produced per islet; lactate is the concentration of lactate produced per islet; condition is the glucose concentration (2.8 or 16.3 mmol L^{-1}) used in the assays; block is the specific lot of animals used in the experiment.

In the models presented above, the (+) signal indicates the addition of a term to the model, while the (:) signal indicates a statistical interaction between the terms.

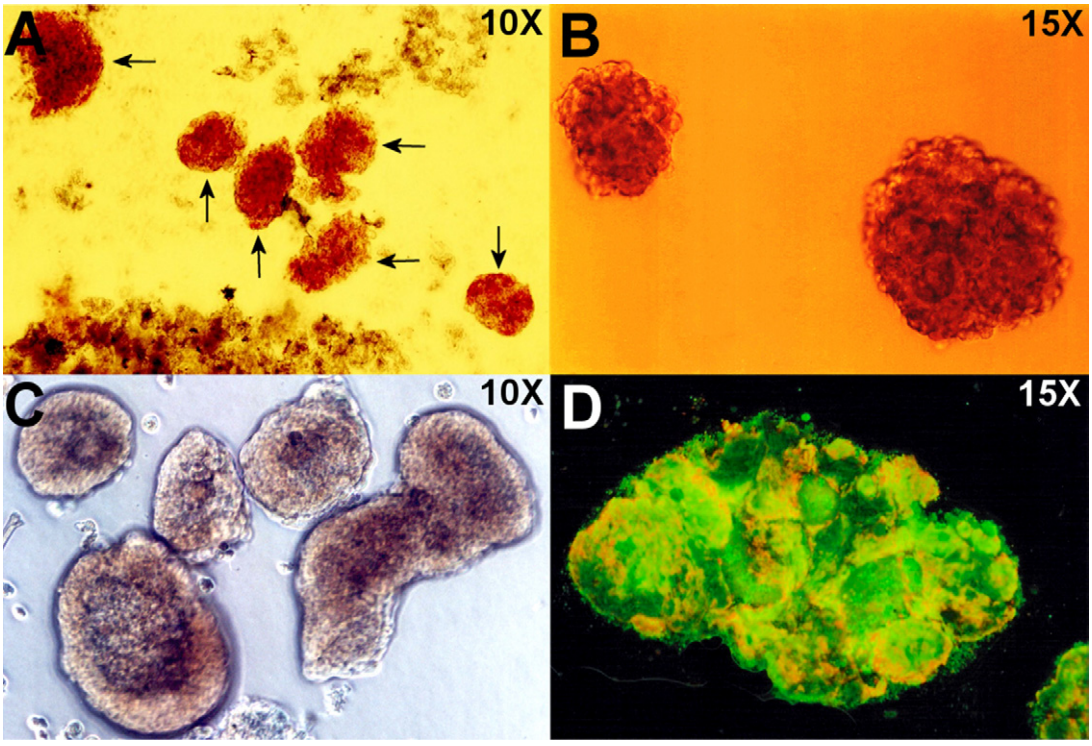

Fig. 1. Rat pancreatic islets before (A) and after (B) the purification step, stained with dithizone. Islets are indicated by arrows in (A), to differentiate them from the brown acinar tissue. Unstained cultured islets (C) after overnight incubation at 37 °C in 5% CO_2. (D) Single islet doubly stained for morphological viability with acridine orange (live cells in green) and propidium iodide (dead cells in red) immediately after the calorimetric experiment. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

3. Results and discussion

After digesting the rat pancreata, free islets could be visualized amongst the digested acinar tissue (Fig. 1A). As can be seen in Fig. 1B, the purification step successfully eliminated this acinar tissue, resulting in highly pure islet preparations. These islets were stained with dithizone, displaying a strong red color, indirectly indicating the presence of insulin stored inside the secretory granules of the beta cells (Fig. 1A and B).

The average islet yield obtained after murine pancreatic islets isolation varies depending on the technique employed. A comparative study of two different procedures [16] reported yields of 488 [15] and 719 [16] islets per pancreas. Our results are in good agreement with these and other reports [16,22], since the average yield obtained for our preparations was around 600 islets per pancreas. These purified islet preparations were cultured for 18–20 h prior to the calorimetric experiments exhibiting intact structures with well-defined borders (Fig. 1C).

A typical thermogram of 1000 murine pancreatic islets treated with 2.8 mmol L^{-1} glucose is shown in Fig. 2A. Two exothermic peaks can be distinguished in the graph, the first of which corresponds to the heat released by the glucose dilution. The second peak, more intense and broader than the first one, corresponds to the initial heat released by the islets upon treatment with 2.8 mmol L^{-1} glucose. The total heat released was calculated by integration of the area over 50 min. A blank ITC run in the absence of islets in the sample cell is shown in Fig. 2B, where only the peak corresponding to the dilution of 2.8 mmol L^{-1} glucose can be observed. To obtain only the heat released by the islets, the data from Fig. 2B were point-by-point subtracted from Fig. 2A, generating the graph shown in Fig. 2C. Similarly to the experiment with 2.8 mmol L^{-1} glucose, the 16.3 mmol L^{-1} glucose dilution heat was also subtracted from the experimental data (data not shown).

Immediately after the calorimetric experiment, the islets were removed from the calorimeter sample cell and checked for morphological viability (Fig. 1D). The islets presented a predominant green color, indicating that they were still viable at the end of the experimental procedures.

Twenty-three complete experiments, each with 1000 islets, were performed. The averages ± standard errors of heat released per 1000 islets during 50 min were 9.16 ± 0.71 mJ after the injection of 2.8 mmol L^{-1} glucose and 14.90 ± 1.21 mJ following the injection of 16.3 mmol L^{-1} glucose (Table 1).

Heat production is strongly influenced by the glucose concentration ($p < 0.001$). According to the linear mixed effects models statistical analysis, none of the other variables (insulin secretion and lactate production) influenced heat production. Previous studies on glucose metabolism of rat pancreatic islets demonstrated that most of the glucose utilized is accounted for by lactate production, which occurs at a constant rate throughout the 90 min period in which the islets were incubated with glucose [23].

The concentration of lactate was determined after the end of each calorimetric run. Eq. (2) was used to verify which variables would present a positive or negative correlation with the lactate output. The production of lactate is affected only by the glucose concentration used to stimulate the islets. At 2.8 mmol L^{-1} glucose, 20.53 ± 3.43 μmol L^{-1} lactate was produced and at 16.3 mmol L^{-1} glucose 41.6 ± 4.35 μmol L^{-1} lactate was produced (Table 1).

The model represented by Eq. (3) was also tested to verify which variables would contribute to insulin secretion. Surprisingly, none of the variables, including the glucose concentration, were found to influence insulin secretion.

The mean values for secreted insulin are statistically equal for the two glucose concentrations tested (Table 1). These are global mean values, from data pooled from all islet samples tested in low

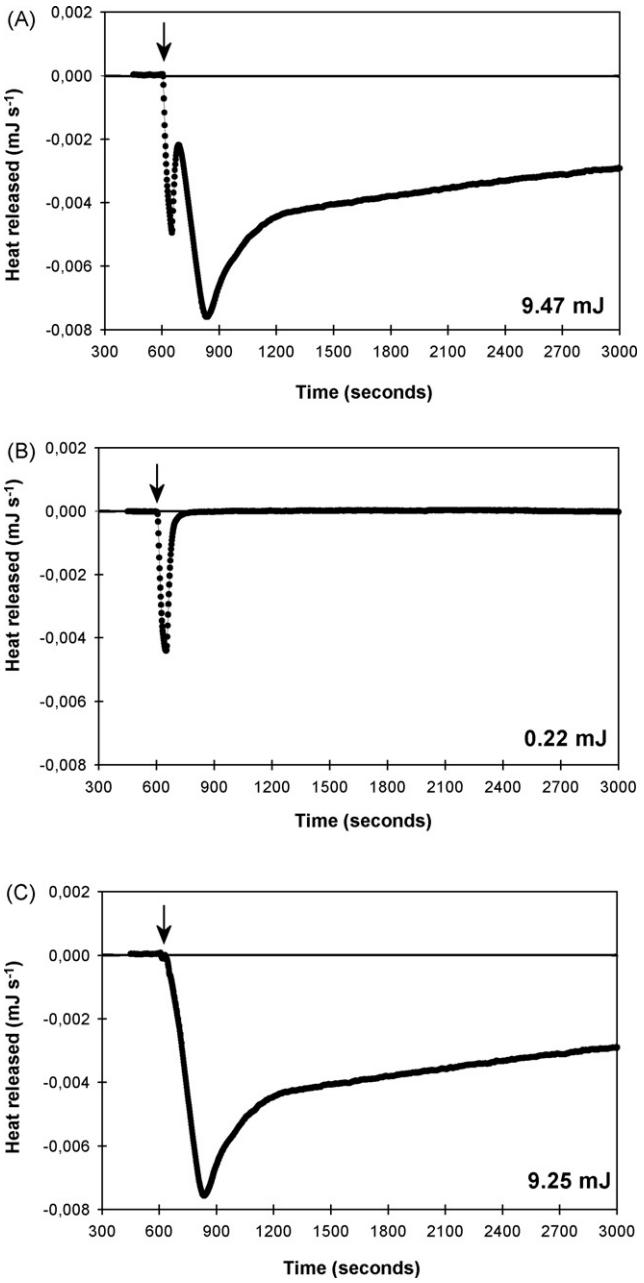

Fig. 2. Thermograms produced by (A) treatment of 1000 islets with 2.8 mmol L^{-1} glucose, (B) the dilution heat due to 2.8 mmol L^{-1} glucose and (C) thermogram obtained upon subtraction of B from A, resulting in the heat released by the islets without the dilution heat of glucose. The arrows in the thermograms indicate glucose injection. The total heat released is shown in the lower right corner of each graph.

Table 1
Heat, insulin and lactate released by 1000 rat pancreatic islets during 50 min in the calorimeter, under treatment with 2.8 or 16.3 mmol L^{-1} glucose

Experimental condition	Heat (mJ)	Insulin (nmol L^{-1})	Lactate (μmol L^{-1})
2.8 mmol L^{-1} glucose	9.16 ± 0.71[a]	0.36 ± 0.07[a]	20.53 ± 3.43[a]
16.3 mmol L^{-1} glucose	14.90 ± 1.21[b]	0.36 ± 0.05[a]	41.60 ± 4.35[b]
Glucose stimulation index*	1.72 ± 0.13	1.67 ± 0.30	2.91 ± 0.50

Different letters on the same column differ statistically ($p < 0.001$). Values are represented as mean ± standard error.

* The glucose stimulation index is the ratio of the parameter values for 16.3 mM/2.8 mM glucose, for the same islet preparation.

and high concentrations of glucose, and show the natural variability of the data. But, in a real test of islet functional viability, the important information is the ratio or index of parameters observed with high glucose concentration divided by the value observed with low glucose concentration for the same islet preparation (glucose stimulation index). The data for the heat evolved, secreted insulin and produced lactate ratios are shown in Table 1. An important feature becomes apparent: all three ratios are statistically larger than unity. The produced lactate ratio value (2.91 ± 0.50) suggests that an increase in glucose concentration leads to increased amounts of lactate produced by the islet, a result supported by the literature [23,24]. Another important observation relates the other two ratios: the value for the released heat ratio (1.72 ± 0.13) is numerically similar to the value of secreted insulin index (1.67 ± 0.30), a fact that raises the important possibility of replacing the secreted insulin ratio by the released heat ratio in the evaluation of the viability of the pancreatic islets for transplants, with several possible practical advantages relative to the currently used methods.

Our results demonstrate that the calorimetric assay is an excellent method for assessing the viability of isolated rat islets. The complete evaluation required approximately 5 h, constituting a substantial reduction in the time required to obtain the results, when compared to RIA or ELISA. We also demonstrate that calorimetry is useful for studies of pancreatic islets metabolism, since a strong correlation was obtained between heat production and the concentration of glucose used to stimulate the islets. Glucose stimulation is also correlated to the lactate output from the islets, as already described in the literature [23,24]. In contrast to other methods, calorimetry avoided false-negative results since the evaluated islets were metabolically viable. The secreted insulin ratio or glucose stimulation index value was numerically similar to the released heat ratio or index value, observed calorimetrically. We are currently testing this calorimetric procedure to assess the viability of human Langerhans islets for human transplant.

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