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Glucose-dependent Functional Heterogeneity In β-TC-6 Murine Insulinoma

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***Abstract*—**To determine if the murine insulinoma, β-TC-6, is a suitable substitute for primary pancreatic β-cells in the study of β-cell functional heterogeneity, we used three distinct functional assays to ascertain the cell line's response to glucose or a glucose analog. These assays include: (i) a 2-NBDG uptake assay; (ii) a calcium influx assay, and; (iii) a quinacrine secretion assay. We show that a population of β-TC-6 cells endocytoses the glucose analog, 2-NBDG, at different rates, has non-uniform intracellular calcium ion concentrations and releases quinacrine at different rates when challenged with glucose. We also measured the K_m for β-TC-6 glucose uptake to be 46.9 mM and the V_m to be 8.36 x 10^{-5} mmole/million cells/min. These data suggest that β-TC-6 might be used as an alternative to primary pancreatic β-cells for the study of glucose-dependent β-cell functional heterogeneity.

***Keywords*—**2-NBDG, Fura-2/AM, functional heterogeneity, quinacrine.

I. Introduction

PANCREATIC β-cells in the Islets of Langerhans play a pivotal role in maintaining homeostatic concentrations of glucose in the systemic circulation. When exposed to elevated levels of glucose in the blood, they endocytose the molecule, whose metabolism ultimately results in the release of insulin from cytoplasmic granules [1], [2]. This glucose-dependent response is based on the cells' ability to sense glucose and respond accordingly, the failure of which typically results in diabetic pathologies [1], [3].

Studies have demonstrated that although the β-cells in an islet respond in a synchronized manner to a glucose challenge, not all of the population secretes insulin [4]–[9]. Of those that do, variation is observed in the enzymatic activity as well as quantity of insulin released. This functional heterogeneity is a characteristic of glucose-dependent pancreatic β-cell function and allows the tissue fine control over the response. Interestingly, this heterogeneity is plastic and appears to be modulated by glucose itself [3], [7], [10]–[15].

Our laboratory is interested in the study of pancreatic β-cell functional heterogeneity. However, owing to the difficulty in isolating, purifying and maintaining primary β-cell cultures, a hardier and more easily managed alternative was sought to allow the optimization of experimental setups and protocols.

The murine SV40 T-antigen-transformed pancreatic β-cell line, β-TC-6, was selected for our purpose because it retains major functional characteristics of pancreatic β-cells, in addition to being easier to maintain in culture [16]. However, it was necessary to determine if β-TC-6 retains the functional heterogeneity observed in primary pancreatic β-cells.

To ascertain this, we performed three distinct functional assays to observe: (i) its ability to endocytose and metabolize the fluorescent glucose analog, 2-[*N*-(7-nitrobenz-2-oxa-1,3-diaxol-4-yl)amino]-2-deoxyglucose (2-NBDG) [17]–[20]; (ii) its ability to respond to a glucose challenge by increasing membrane permeability to calcium ions [7]; (iii) as well as its ability to secrete quinacrine, and hence insulin, dose-dependently [7]. Finally, the Michaelis-Menten constant, as well as the maximum glucose uptake rate of β-TC-6 was measured for future reference.

II. Materials and Methods

A. β-TC-6 culture

β-TC-6 cells were purchased from American Type Culture Collection (ATCC) and cultured in Dulbecco's Modified Eagle Medium. Cultures were maintained at 37 °C and under a 5 % CO_2 atmosphere inside a humidified incubator. The growth medium was changed every three days.

B. 2-NBDG uptake and Metabolism Assay

β-TC-6 at 1.0 x 10^5 cells/mL was seeded onto a 24-well plate and allowed to attach, spread and proliferate to near confluence at 37 °C, 5 % CO_2. The complete medium was then removed and the cells were washed with PBS. The PBS was then replaced with 2.5 mM glucose in basal medium comprising Dulbecco's Modified Eagle medium (DMEM) with neither glucose nor pyruvate (Product No. 11966, GIBCO, Invitrogen, USA), supplemented with L-glutamine and 10 % (v/v) fetal bovine serum (final serum glucose concentration of about 0.25 mM). Conditioning of the cells proceeded at 37 °C, 5 % CO_2 for 60 mins. The conditioning medium was then removed and replaced with 200 μM 2-[*N*-(7-nitrobenz-2-oxa-1,3-diaxol-4-yl)amino]-2-deoxyglucose (2-NBDG, Invitrogen, USA) in basal medium. The cells were then incubated further at 37 °C, 5 % CO_2 for 30 mins to allow them to endocytose the glucose analog. The 2-NBDG in basal medium was then removed and the cells were washed with

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PBS. They were then observed for intra-cellular fluorescence at excitation and emission wavelengths of 467 nm and 542 nm respectively.

C. Glucose-dependent Calcium Ion Influx

The complete medium was removed from a T-25 culture of β-TC-6 at about 50 % confluence and the cells were washed with PBS. The PBS was then replaced with 2.5 mM glucose in basal medium comprising Dulbecco's Modified Eagle medium (DMEM) with neither glucose nor pyruvate (Product No. 11966, GIBCO, Invitrogen, USA), supplemented with L-glutamine and 10 % (v/v) fetal bovine serum (final serum glucose concentration of about 0.25 mM). Conditioning of the cells proceeded at 37 °C, 5 % CO_2 for 60 mins. The conditioning medium was then removed and replaced with 2 μM fura-2/acetoxymethyl ester (Fura-2/AM, Sigma-Aldrich, USA) in basal medium supplemented with 10 % (v/v) fetal bovine serum. The cells were then incubated at 37 °C, 5 % CO_2 for 30 mins after which they were washed twice with PBS. 25 mM glucose in PBS supplemented with 1 mM $CaCl_2$ was added and the cells were observed for intra-cellular fluorescence at excitation and emission wavelengths of 360 nm and 500 nm respectively.

D. Quinacrine Secretion Assay

β-TC-6 at 1.0 x 10^5 cells/mL was seeded onto a 24-well plate and allowed to attach, spread and proliferate to near confluence at 37 °C, 5 % CO_2. The complete medium was then removed and the cells were washed with PBS. The PBS was then replaced with 500 μL of glucose-free DMEM supplemented with 0.1 % bovine serum albumin (BSA). Conditioning of the cells proceeded at 37 °C, 5 % CO_2 for 30 mins. The basal medium was then replaced with a fresh aliquot. Incubation at 37 °C, 5 % CO_2 was resumed for a further 30 mins. The basal medium was then replaced with 100 nM quinacrine dihydrochloride (Aldrich, USA) and the cells were incubated at 37 °C, 5 % CO_2 for 15 mins. The cells were then washed twice with PBS and the center of each well was imaged under fluorescence excitation at 360 nm and emission at 500 nm for 0.5 s. The PBS was then replaced with DMEM supplemented with 0.1 % BSA and 0.5 mM, 1.0 mM, 2.8 mM, 5.6 mM or 16.5 mM glucose. The cells were then incubated at 37 °C, 5 % CO_2 for 60 mins. Subsequently, the cells were washed with PBS and imaged under fluorescence excitation at 360 nm and emission at 500 nm for 0.5 s. The fluorescence intensity of the cells in these images was then analysed using *ImagePro Plus* software.

E. β-TC-6 Glucose Uptake Rate

β-TC-6 at 1.0 x 10^5 cells/mL was seeded onto a 24-well plate and allowed to attach, spread and proliferate at 37 °C, 5 % CO_2. The complete medium was then removed and the cells were washed with PBS. The wells were then filled with 500 μL of basal media containing 5.8 mM or 2.9 mM glucose. The plate was then incubated at 37 °C, 5 % CO_2 for 120 mins with gentle agitation at 100 rpm. Aliqouts of 10 μL from each well were taken at t = 0, 10, 20, 30, 40, 50, 60, 80, 100 and 120 mins for glucose quantitation using an ACCU-CHEK glucose quantitation kit (Roche Diagnostics). Finally, the cells in each well were harvested by trypsinisation and counted using a haemocytometer.

III. Results

A. 2-NBDG uptake and Metabolism Assay

The data show that β-TC-6 can endocytose the fluorescent glucose analog, 2-NBDG, and also shows increased intracellular 2-NBDG fluorescence in distinct groups of cells (Fig. 1). Fluorescence appears to be restricted to the cytoplasm. As the time allowed for 2-NBDG uptake should be sufficient for 2-NBDG uptake and metabolism to stabilise [20], this heterogeneous intracellular 2-NBDG fluorescence suggests that β-TC-6 retains the heterogeneous glucose uptake activity of native β-cells.

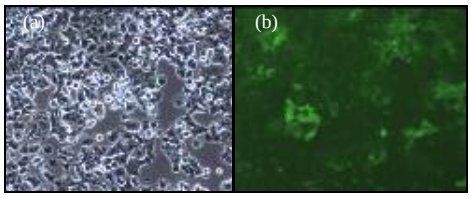

Fig. 1 Brightfield (a) and fluorescence (b) images of β-TC-6 cells in 24-well plate loaded with 2-NBDG showing heterogeneous 2-NBDG uptake and metabolic activity

B. Glucose-dependent Calcium Ion Influx

Loading β-TC-6 with the calcium chelator, Fura-2/AM and then challenging them with glucose resulted in an increase in intracellular fluorescence at 500 nm (Fig. 2) compared to controls (not shown). This demonstrated that Fura-2/AM was able to penetrate the β-TC-6 cell membrane and detect an increase in intracellular calcium ion concentration as previously reported by Holz and co-workers [21]. As the intracellular Fura-2/AM is not uniform, β-TC-6 appears to display different calcium ion influx rates in different cells.

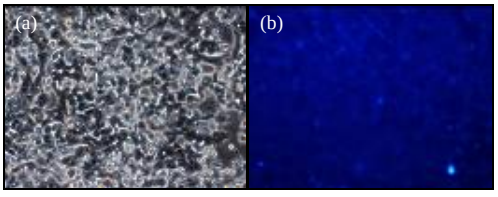

Fig. 2 Brightfield (a) and fluorescence (b) images of β-TC-6 cells in T-25 flask loaded with Fura-2/AM showing intracellular fluorescence following a glucose challenge

C. Quinacrine Secretion Assay

Quinacrine dihydrochloride was able to penetrate and accumulate in the β-TC-6 cells. This resulted in a marked increase in intracellular fluorescence at 500 nm (Fig. 3).

Furthermore, the intracelluar fluorescence appears to be punctate and the specks of increased fluorescence are likely to be quinacrine-loaded insulin granules. Non-uniform intracellular fluorescence suggests that β-TC-6 has retained heterogeneous insulin content.

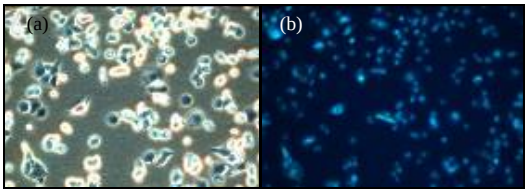

Fig. 3 Brightfield (a) and fluorescence (b) images of β-TC-6 cells in T-75 flask loaded with quinacrine

Furthermore, subsequent incubation with medium containing glucose resulted in a decrease in intracellular fluorescence intensity, suggesting that the quinacrine had localised within the insulin granules and been co-secreted with the insulin upon glucose stimulation of the β-TC-6 cells.

Quantitative assays performed in 24-well plates also showed that there was a dose-dependent decrease in intracellular quinacrine fluorescence (Fig. 4). The release profile so-obtained is similar to the insulin secretion profile described by Poitout and co-workers for β-TC-6 cells, with the half-maximal seceretion rate at about 0.5 mM glucose [16].

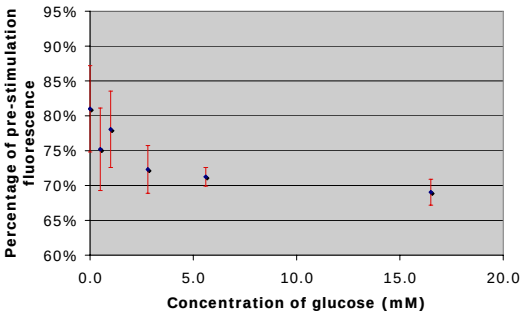

Fig. 4 Graph showing glucose-dependent secretion of quinacrine from β-TC-6 (*P<0.005, 2-Factor ANOVA, α =0.05*).

Unfortunately, direct exposure of quinacrine-loaded cells to UV irradiation sometimes led to loss of viability. During the assays, exposure to UV resulted in a circular area of cell death which seemed to correspond to the field of view of the objective lens. However, since this effect was not consistent, it was dififcult to determine the root cause of the cell death as well as to resolve the problem.

D. β-TC-6 Glucose Uptake Rate

The data obtained from the glucose quantitation kit were analyzed using non-linear regression and made to fit a Michaelis-Menten type equation. This allowed us to determine the Michaelis-Menten constant to be, K_m = 46.9 mM, and the maximum glucose uptake rate to be, V_m = 8.36 x 10^{-5} mmole/million cells/min.

IV. Discussion

The glucose analog, 2-NBDG, has been used to determine cell viability as well as to estimate glucose uptake rates in a variety of cell types [17]-[20]. As β-TC-6 is a β-cell derivative, it is expected to be able to endocytose the fluorescent analog. This ability is not uniform and certain groups of cells were observed to have higher uptake rates or lower metabolic rates, which resulted in regions of increased intracellular 2-NBDG fluroescence. This demonstates the heterogeneous nature of the 2-NBDG, and hence glucose, processing machinery in the cell line. This heterogeneity was also observed, albeit less distinctly, when the cells were stained with quinacrine. Quinacrine is a fluorescent probe that readily diffuses across cell membranes and accumulates in regions of low pH, such as secretory vesicles. In β-TC-6 cells, it is expected to co-localise with the insulin granules and has been reported to do so in primary pancreatic β-cells. Our data showing non-uniform quinacrine staining demonstrates the heterogeneous insulin content among β-TC-6 cells. The secretion assay data also confirms Poitout's characterisation of glucose-dependent insulin secretrion in β-TC-6 [16]. In pancreatic β-cells, glucose-dependent insulin secretion has been shown to be concomitant with a calcium ion influx [14], [18]. The calcium ion chelator Fura-2/AM, used in these studies, readily penetrates cell membranes and fluoresces when in the presence of Ca^{2+}. Our data showing non-uniform intracellular Fura-2/AM fluorescence, following a glucose challenge, suggests that β-TC-6 retains heterogeneous insulin secretion rates among its cells. The ability of β-TC-6 to respond appropriately to a glucose challenge supports its use as a viable alternative to primary pancreactic β-cells in the study of glucose-dependent functional heterogeneity.

Our estimate of the Michaelis-Menten constant for β-TC-6 appears to be higher than reported values for the predominant GLUT2 receptor as well as the metabolic enzyme, glucokinase in pancreatic β-cells [3], [15]. Our assay does not distinguish between the affinity of either GLUT2 or glucokinase for glucose, but does provide a value that might be a resultant value for both activities. A more rigorous measurement of the K_m for β-TC-6 might yield results closer in agreement to those reported. However, it should be noted that β-TC-6, being a transformed derivative of primary pancreatic β-cells, might not have retained its native affinity for glucose. Furthermore, our cultures were maintained in growth media containing 21 mM glucose, a supra-physiological concentration which might have altered β-TC-6 glucose dependence.

We aim to carry out further studies of glucose-dependent functional heterogeneity in β-TC-6 in a microfluidic channel system. This would make it possible for us to employ microfluidic phenomena to manipulate the extracellular

microenvironment. This would also allow us to generate specific microenvironemnts over length-scales mimicking those *in vivo*, so rendering our observations physiologically relevant. A possible experiment would be to generate a micrometer-scale glucose concentration gradient over a monolayer of the β-TC-6 cells, and then to determine how conditioning with such a gradient affects the cells' ability to respond to a glucose challenge. Preliminary work in this direction is currently in progress.

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