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Metabolomic analysis of pancreatic beta cells following exposure to high glucose

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ABSTRACT

Background: Chronic exposure to hyperglycaemic conditions has been shown to have detrimental effects on beta cell function. The resulting glucotoxicity is a contributing factor to the development of type 2 diabetes. The objective of this study was to combine a metabolomics approach with functional assays to gain insight into the mechanism by which glucotoxicity exerts its effects.
Methods: The BRIN-BD11 and INS-1E beta cell lines were cultured in 25 mM glucose for 20 h to mimic glucotoxic effects. PDK-2 protein expression, intracellular glutathione levels and the change in mitochondrial membrane potential and intracellular calcium following glucose stimulation were determined. Metabolomic analysis of beta cell metabolite extracts was performed using GC–MS, ^{1}H NMR and ^{13}C NMR.
Results: Conditions to mimic glucotoxicity were established and resulted in no loss of cellular viability in either cell line while causing a decrease in insulin secretion. Metabolomic analysis of beta cells following exposure to high glucose revealed a change in amino acids, an increase in glucose and a decrease in phospho-choline, n−3 and n−6 PUFAs during glucose stimulated insulin secretion relative to cells cultured under control conditions. However, no changes in calcium handling or mitochondrial membrane potential were evident.
Conclusions: Results indicate that a decrease in TCA cycle metabolism in combination with an alteration in fatty acid composition and phosphocholine levels may play a role in glucotoxicity induced impairment of glucose stimulated insulin secretion.
General significance: Alterations in certain metabolic pathways play a role in glucotoxicity in the pancreatic beta cell.

1. Introduction

Type 2 diabetes is characterised by failure to maintain glucose homeostasis due to impaired insulin secretion and insulin resistance. Impaired insulin secretion is the result of multiple factors on beta cells, including reduced beta cell mass, glucotoxicity and lipotoxicity [1]. In vitro studies on beta cell lines and isolated rat islets have shown that chronic exposure to hyperglycaemic conditions has detrimental effects on expression of genes related to insulin production and secretion, insulin content, glucose stimulated insulin secretion, mitochondrial function and cell viability [2–6]. Several molecular mechanisms have been proposed to explain the effects of chronic hyperglycaemia on pancreatic beta cell function including oxidative stress, endoplasmic reticulum stress [7], alterations in glucose metabolism [8] and impairment of insulin exocytosis [9–13]. Oxidative stress due to reactive oxygen species (ROS) generated by mitochondrial metabolism and plasma membrane bound NADPH oxidases has been extensively researched and is proposed to play a key role in glucotoxicity [12,14–16]. In support of the role of oxidative stress, studies have shown that oxidative stress markers are elevated in the pancreatic islets of type 2 diabetic patients [14], hyperglycaemic conditions result in increased ROS production [12,15] and antioxidants or overexpression of antioxidant enzymes protect beta cells from the detrimental effects of chronic hyperglycaemia [11,14,16]. Furthermore, beta cells are inherently susceptible to oxidative stress as they have reduced levels of antioxidant enzymes compared to other cell types [17–19]. Despite these detailed studies and others relating to molecular mechanisms of beta cell dysfunction further research is needed in order to identify the initiating factors and key modulators of glucotoxicity in beta cells.

Metabolomics is the study of the metabolite composition of a sample such as a biofluid or cellular extract and has been used to gain insight into the pathogenesis of many diseases including diabetes [20–24]. As metabolism of glucose is crucial to glucose stimulated insulin secretion, metabolomic analysis of beta cells may give further insight into the mechanisms by which glucotoxicity exerts its effects on beta cell function and specifically glucose stimulated insulin secretion (GSIS). Previous GC–MS based metabolomic studies investigated the effect of exposure to 16.7 mM glucose on the beta cell line INS-1

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832/13 relative to exposure to 2.8 mM glucose and compared the INS-1 832/13 cell line which is a glucose responsive cell line to the glucose unresponsive cell line INS-1 832/2 and found differences in glycolysis and TCA cycle metabolism in both instances [25,26]. More recently, GC–MS based analysis of the beta cell line INS-1E resulted in the finding that pentose phosphate pathway metabolites accumulate during chronic (48 h) treatment with 16 mM glucose [27]. While these studies demonstrate that metabolomic analysis is valuable in the study of the beta cell, employment of a broader metabolomic approach using multiple platforms and ^{13}C labelling experiments in conjunction with functional assays may give further insight into the mechanisms of glucotoxicity.

The aim of the present study was to employ both GC–MS and NMR based metabolomics to study the effect of exposure to high glucose on pancreatic beta cell fatty acid and metabolite composition. This analysis was combined with functional assays to aid in the elucidation of the mechanisms by which glucotoxicity exerts its effects.

2. Materials and methods

2.1. Materials

All chemicals were obtained from Sigma-Aldrich Ireland. Culture media and its components were obtained from Gibco (Glasgow, UK).

2.2. Cell culture and treatment

The BRIN-BD11 cell line [28] and INS-1E cell line [29,30] were employed in this study. BRIN-BD11 cells were maintained in RPMI-1640 containing 11.1 mM glucose, supplemented with 10% (v/v) foetal calf serum, 2 mM glutamine, 50 IU/ml penicillin, 0.05 mg/ml streptomycin and incubated at 37 °C in a humidified atmosphere containing 5% CO_2 and 95% air. INS-1E cells were maintained in RPMI-1640 containing 11.1 mM glucose, supplemented with 5% (v/v) foetal calf serum, 2 mM glutamine, 100 IU/ml penicillin, 0.1 mg/ml streptomycin, 1 mM sodium pyruvate, 10 mM HEPES, 50 μM 2-mercaptoethanol and incubated at 37 °C in a humidified atmosphere containing 5% CO_2 and 95% air. Two cell lines were employed to demonstrate that the results were not cell line specific.

Cells were seeded as indicated for individual assays below. Once confluent, cells were incubated with 25 mM glucose for 20 h. Control experiments in media containing 11.1 mM glucose were run in parallel.

2.3. Metabolite extraction, insulin secretion, glucose uptake and glutathione content

BRIN-BD11 cells and INS-1E cells were seeded in T175 flasks at a density of 7×10^6 cells. Following the treatment period, the culture medium was removed and the cells were washed with PBS. The cells were then incubated with Krebs-Ringer bicarbonate (KRB) buffer (115 mM NaCl, 4.7 mM KCl, 1.28 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4\cdot7H_2O$, 10 mM $NaHCO_3$, 5 g/l BSA, pH 7.4) supplemented with 1.1 mM glucose for 20 min. The media were then replaced with KRB buffer supplemented with 14 mM glucose and incubated for 1 h prior to metabolite extraction. Following the treatment period, the KRB buffer was removed and stored at −20 °C until measurement of insulin and glucose concentration.

For NMR analysis, cells underwent a perchloric acid (PCA) metabolite extraction. Cells were washed with ice cold PBS and then 6 ml of ice cold perchloric acid (6%) was added, cells were scraped and the extracts of each flask were centrifuged at 260 *g* for 5 min. The resulting supernatant was neutralised with KOH (5 M, 1 M and 0.1 M solutions). Following centrifugation at 260 *g* for 5 min, the supernatant was retained, lyophilised and stored at −20 °C until further analysis. For the ^{13}C NMR experiments the same procedure was followed with the exception that the 14 mM glucose was labelled (U-^{13}C glucose).

For GC–MS analysis, cells underwent a methanol:chloroform metabolite extraction. Cells were collected in PBS, centrifuged (771 *g* for 3 min) and resuspended in 5 ml of ice-cold PBS. Following centrifugation (433 *g* for 5 min), the resulting cell pellet was resuspended in 1 ml of a 2:1 methanol:chloroform mixture. The sample was vortexed and allowed to rest on ice for 2 min prior to the addition of 300 μl of chloroform and MilliQ water. The sample was centrifuged (433 *g* for 5 min), following which clear separation of the phases into an aqueous upper layer, an interphase containing protein precipitate and an organic lower layer was visible. The upper aqueous layer was removed and the organic layer was retained for derivatisation as detailed in Section 2.5. The protein pellet was frozen at −20 °C until determination of protein concentration using a bicinchoninic (BCA) assay.

Insulin secretion was determined via measurement of the insulin content of the KRB buffer using the Mercodia Ultrasensitive Rat Insulin ELISA kit (Mercodia AB, Uppsala, Sweden). Glucose uptake was determined via analysis of the glucose concentration of the KRB buffer following treatment using the YSI amino acid analyser (YSI life sciences, Ohio, USA) which employs an enzyme based sensor to detect glucose in the range of 0.3–99.9 mM with a precision of 0.1 mM. The insulin content of both cell lines was determined in a 24 well plate seeded at a density of 1×10^5 cells/well. Following treatment as outlined above, 1 ml ice-cold acid ethanol (75% v/v ethanol, 1·5% v/v concentrated HCl) solution was added to each well and incubated overnight at 4 °C. The insulin content was measured using the Mercodia Ultrasensitive Rat Insulin ELISA kit (Mercodia AB, Uppsala, Sweden). Cellular viability of both cell lines following treatment was assessed by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide) assay.

The glutathione (GSH) content of BRIN-BD11 cells was determined in a 96 well plate seeded at a density of 3×10^4 cells/well using the fluorescent probe monochlorobimane (mBCl). Following the above glucose treatment, cells were incubated with 0.1 mM mBCl and 1 U/ml glutathione-S-transferase in KRB buffer for 30 min at 37 °C. Fluorescence readings were measured using an excitation wavelength of 380 nm and an emission wavelength of 470 nm.

2.4. NMR spectroscopy

The lyophilised cell extracts were prepared by re-suspension in 750 μl of phosphate buffer (0.2 M K_2HPO_4, 0.2 M KH_2PO_4 in D_2O, pH 7.4), followed by centrifugation at 380 *g* for 10 min and addition of 10 μl of sodium trimethylsilyl [2,2,3,3-$^2H_4$] proprionate (TSP) to the resultant supernatant. ^1H NMR spectra of INS-1E and BRIN-BD11 cells were acquired on a 600 MHz NMR spectrometer (Varian) and a 500 MHz DRX NMR spectrometer (BrukerBiospin, Karlsruhe, Germany) respectively using a Noesypresat pulse sequence at 25 °C. Spectra were acquired with 32 K data points and 256 scans or 512 scans over a spectral width of 8 kHz. Water suppression was achieved during the relaxation time and mixing time. Spectra were referenced to TSP at 0.0 ppm and processed using the processor on chenomx NMR suite 5.1 or XWIN NMR using a line broadening of 0.2 Hz. The spectra were integrated into bins consisting of spectral regions of 0.02 ppm (INS-1E) or 0.001 ppm (BRIN-BD11). The water region (4.5–5.5 ppm) was excluded and the data was normalised to the sum of the spectral integral.

^{13}C NMR spectra of BRIN-BD11 cells were acquired and analysed as previously described [31,32]. Typically spectra were acquired at 25 °C with 32 K data points using 90° pulses, 260 ppm spectral width, 2.5 s relaxation delay and 12,000–24,000 scans on a Bruker 500 MHz machine. Chemical shifts were referenced to tetramethylsilane at 0 ppm. The fraction of acetyl-CoA labelled from [U-^{13}C]glucose was calculated using the following equation for the C4 peak: (3,4,5-^{13}C)∗C4/C3 [33,34].