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Short and prolonged exposure to hyperglycaemia in human fibroblasts and endothelial cells: Metabolic and osmotic effects

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ABSTRACT

High blood glucose levels are the main feature of diabetes. However, the underlying mechanism linking high glucose concentration to diabetic complications is still not fully elucidated, particularly with regard to human physiology. Excess of glucose is likely to trigger a metabolic response depending on the cell features, activating deleterious pathways involved in the complications of diabetes. In this study, we aim to elucidate how acute and prolonged hyperglycaemia alters the biology and metabolism in human fibroblasts and endothelial cells.

We found that hyperglycaemia triggers a metabolic switch from oxidative phosphorylation to glycolysis that is maintained over prolonged time. Moreover, osmotic pressure is a major factor in the early metabolic response, decreasing both mitochondrial transmembrane potential and cellular proliferation. After prolonged exposure to hyperglycaemia we observed decreased mitochondrial steady-state and uncoupled respiration, together with a reduced ATP/ADP ratio. At the same time, we could not detect major changes in mitochondrial transmembrane potential and reactive oxygen species.

We suggest that the physiological and metabolic alterations observed in healthy human primary fibroblasts and endothelial cells are an adaptive response to hyperglycaemia. The severity of metabolic and bioenergetics impairment associated with diabetic complications may occur after longer glucose exposure or due to interactions with cell types more sensitive to hyperglycaemia.

1. Introduction

Type II diabetes has reached pandemic proportions worldwide and its prevalence is increasing in developing and Western countries. In 2004, an estimated 3.4 million people died from diabetes complications and the projections indicate an increase by two thirds between 2008 and 2030 (WHO). Complications arise predominantly in tissues that are not, or only in part, insulin dependent with regard to glucose uptake. Neuropathy, non-healing foot ulcers, nephropathy, accelerated atherosclerosis and retinopathy are the most common features in the progression of the disease. Tight glycaemic control is critical for quality of life as well as life expectancy. Thus, frequent exposure to blood glucose concentrations exceeding normoglycaemic levels appears to be one of the main factors altering normal metabolism and function of cells and tissues.

Small alterations in cellular bioenergetics and metabolite levels can affect cell function as well as intercellular interaction thus exacerbating diabetic complications. It has been suggested that an imbalance of pyridine nucleotides, as consequence of elevated intracellular glucose concentration, can alter the activity levels of several key cellular enzymes (Ido, 2007; Williamson et al., 1993). In addition to glucose, aminoacids (AA), free fatty acids (FFA) and lactate could have similar effects and, in diabetes, elevated blood levels of FFA and lactate have been documented (Ido, 2007). More recently, mitochondria have been proposed to be involved in

Abbreviations: GLUT, glucose transporter; TCA, tricarboxylic acid cycle; ETC, electron transport chain; $\Delta\psi_m$, mitochondrial transmembrane potential; ROS, reactive oxygen species; HDF, human dermal fibroblasts; HDMEc, human dermal microvascular endothelial cells; OCR, oxygen consumption rate; ECAR, extracellular acidification rate; ATP, adenosine triphosphate; ADP, adenosine diphosphate; NAD+/NADH, nicotinamide adenine dinucleotide; PDH, pyruvate dehydrogenase; LDH, lactic dehydrogenase.

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diabetic complications through a signalling cascade activating several deleterious pathways (Brownlee, 2005). An increase of glucose availability accelerates the flux through glycolysis and the Krebs cycle generating more substrates for the mitochondrial electron transport chain (ETC) (Nishikawa et al., 2000). In turn, this raises the mitochondrial transmembrane potential ($\Delta\psi_m$) and upregulates the production of reactive oxygen species (ROS) (Brownlee, 2005; Du et al., 2001). The exact role of oxidative stress in diabetes is not fully understood even though markers of oxidative stress, such as lipid peroxidation, antioxidant enzyme levels and activities in blood and tissues, measured in diabetes patients and animal models of diabetes, indicate a strong relationship between these markers and the disease (Avila et al., 2011; Hernandez-Mijares et al., 2011; Ihnat et al., 2007b; Kamboj and Sandhir, 2011; Kowluru, 2003; Widlansky et al., 2010). Some cell types produce ROS, *in vitro*, in the presence of hyperglycaemia (Bellin et al., 2006; Ihnat et al., 2007b; Inoguchi et al., 2000; Paltauf-Doburzynska et al., 2004; Xiao et al., 2011; Yu et al., 2006) or high free fatty acids (Bellin et al., 2006; Inoguchi et al., 2000), while in others ROS were not detected in the presence of high concentrations of these substrates (Busik et al., 2008; Fink et al., 2012; Zhang et al., 2010).

To the best of our knowledge, the metabolic and cellular events that form the basis for diabetic complications are still unclear and are most likely cell- and tissue specific. Moreover, the effects of high glucose exposure on human cells metabolism and bioenergetics have been poorly studied. In this paper, we investigated metabolism and bioenergetics of human primary fibroblasts and endothelial cells exposed to short and prolonged high glucose concentrations.

2. Materials and methods

2.1. Cell culture and treatments

Primary human dermal fibroblasts (HDF) (three individual donors) and dermal endothelial cells (HDMECs) (two individual donors) from healthy subjects were purchased from Promocell GmbH (Heidelberg, Germany). All chemicals were purchased form Sigma (St. Louis, MO, USA) unless stated otherwise. Mouse embryonic H9c2 cell line was donated by Prof. Natalia Calonghi (FaBiT). HDF and H9c2 were cultured in DMEM 1 g/L glucose, supplemented with 10% FBS (Gibco Carlsbad, CA, USA), penicillin 100 IU/ml and streptomycin 100 μg/ml. HDMEc were cultured in endothelial cell growth medium supplemented with growth factor (Promocell), penicillin 100 IU/ml and streptomycin 100 μg/ml. Cells with population doubling lower than 20 were used, incubated at 37 °C and 5% CO_2, and media were changed every second day. For prolonged hyperglycaemia experiments, cells were incubated with concentrations of glucose or mannitol (Merck KGaA, Darmstadt, Germany) as follow: control C (5.5 mM glucose), hyperglycaemia HG (25.5 mM glucose) or hyperosmotic control M (5.5 mM glucose + 20 mM mannitol). All experiments were performed in triplicate with a minimum of $n = 3$.

2.2. Polarographic assay

HDF and H9c2 cell lines were harvested, resuspended in DMEM, and assayed for oxygen consumption at 30 °C in DMEM using a thermostatically controlled oxygraph chamber (Instech Mod.203, Plymouth Meeting, PA, USA) (Bergamini et al., 2012).

2.3. XF extracellular flux analysis

Metabolic analysis was performed using Seahorse XF24 Flux Analyser (Seahorse Biosciences, Billerica, MA, USA). Cells were allowed to reach confluence. The day of the experiment the growing medium was replaced with XF medium, containing 2 mM GlutaMax (Gibco), 1 mM sodium pyruvate, glucose and/or mannitol, 2% FBS (HDF) or 1.8 ml of MV growth factors (HDMEc). Cells were incubated at 37 °C without CO_2 1 h prior to the experiment. In the acute hyperglycaemia experiments, we measured the rates before and after addition of glucose or mannitol. Cytochalasin B was added to final concentration of 20 μM. Data are presented as ratio against basal OCR and ECAR.

In experiments after prolonged treatment we measured the basal metabolism in their respective media (described above), and following injection of oligomycin (1 μM), FCCP (4–6 μM), and antimycin A (8 μM). Data were normalized to the number of cells per well after Hoechst 33342 probe staining (Life Technologies) using BD pathway 855 microscope (BD Biosciences, Franklin Lakes, USA).

2.4. Enzymatic assays

The rotenone-sensitive NADH:O_2 activity was assayed as described by Birch-Machin and Turnbull (2001) with 0.1 μM rotenone by following the decrease in absorbance due to the oxidation of NADH at 340 nm using a Jasco v-550 spectrophotometer.

Citrate synthase activity was assessed as described by Trounce et al. (1996) using thawed samples in a SAFAS UVmc2 spectrophotometer (SAFAS S.A., Monaco).

Total lactic dehydrogenase activity in cell lysate was measured using In Vitro Toxicology Assay Kit (Sigma) following manufacturer instructions. The values (absorbance 492 nm) were normalized on total DNA content (Accublue dsDNA quantitation kit Biotium, Hayward, CA, USA) (ex/em 485/530) using Pherastar Microplate Reader (Ortemberg, Germany).

2.5. mtDNA copy number

Total DNA was extracted using QIamp DNA Blood Mini kit (QiAgen, Venlo, Netherlands) according to the manufacturer' instructions. The ratio of mtDNA to gDNA was determined with AB 7300 unit (BD Biosciences, Foster City, CA, USA). Primers (Supplemental table) were purchased from Eurofins MWG Operon.

2.6. ROS measurement

Cells were washed with PBS and loaded with 10 μM carboxymethyl-2′-7′-dichlorofluorescin diacetate (CMDCF-DA, Life Technologies, Paisley, UK) or 10 μM Mitosox (Life Technologies) in DMEM serum free. Cells were then harvested, washed twice with PBS and fluorescence was detected by flow cytometry (Cyan, Beckman Coulter, Brea, CA, USA). Cells treated with 100 μM tert-butyl hydroperoxide (TBH) for 30 min or 1 μM rotenone for 48 h served as a positive control.

2.7. Mitochondrial transmembrane potential ($\Delta\psi_m$)

Transmembrane potential was measured using a SP5 confocal microscope (Leica, Mannheim, Germany) with a heated stage. On the day of the experiment, the growth medium was replaced with XF media as described above, containing 25 nM tetramethylrhodamine-ethyl-ester (TMRE, Molecular probes). Microscope settings: 4% laser power, 512 × 512 pixel, λex514 and λem565/600, pinhole 1 Airy, Optimized Z stack. Images were analysed with Image J software (National Institutes of Health, Bethesda, MD, USA). Total intensity of the identified objects per field (above 300 voxels) was divided for total objects volume. For each sample 3 different fields were averaged. Representative images are shown in Supplemental Fig. 3C and D. For images presented in the paper, Mitotracker green (Life Technologies) at a final concentration of 300 nM was loaded

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