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The effect of glucose concentration and sodium phenylbutyrate treatment on mitochondrial bioenergetics and ER stress in 3T3-L1 adipocytes

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ABSTRACT

While the 3T3-L1 adipocyte model is routinely used for the study of obesity and diabetes, the mitochondrial respiratory profile in normal versus high glucose has not been examined in detail. We matured adipocytes in normal (5 mM) or high (30 mM) glucose and insulin and examined the mitochondrial bioenergetics. We also assessed the requirement for the Unfolded Protein Response (UPR) and ER stress under these conditions. Basal respiration was ~1.7-fold greater in adipocytes that had matured in 30 mM glucose; however, their ability to increase oxygen consumption in response to stress was impaired. Adipogenesis proceeded in both normal and high glucose with concomitant activation of the UPR, but only high glucose was associated with increased levels of ER stress and mitochondrial stress as observed by parallel increases in CHOP and protein succination. Treatment of adipocytes with sodium phenylbutyrate relieved mitochondrial stress through a reduction in mitochondrial respiration. Our data suggests that mitochondrial stress, protein succination and ER stress are uniquely linked in adipocytes matured in high glucose.

1. Introduction

Adipose tissue is composed of a network of cell types including adipocytes and macrophages that play a central role in the maintenance of nutrient homeostasis and hormonal secretion [23,27]. Increased adipose tissue mass during the development of type 2 diabetes is associated with increased macrophage infiltration and hypertrophied adipocytes that have compromised metabolic and endocrine functions. We have identified a chemical modification of proteins that appears to increase selectively in adipose tissue during type 2 diabetes [29]. This protein modification, known as S-2-succinocysteine (2SC), is derived from the irreversible reaction between the Krebs cycle metabolite fumarate and cysteine residues on proteins, and is also termed protein *succination* [1,3,21]. Mechanistically, the increase in succination is related to the nature of adipocyte metabolism; in the presence of nutrient excess (glucotoxicity) the adipocyte produces more ATP than is energetically required and this leads to an increase in the mitochondrial membrane potential ($\Delta\Psi_m$) and the NADH/NAD^+ ratio [9]. Consequently, the NAD^+-dependent enzymes of the Krebs cycle are inhibited resulting in an increase in fumarate and protein succination [9]. We have further confirmed this by demonstrating that chemical uncouplers, which can relieve the increased $\Delta\Psi_m$, are able to reduce mitochondrial stress and the accumulation of succinated proteins [9]. We have identified ~40 succinated proteins in adipocytes ([20,21,26],) and we have demonstrated that succination appears to impair protein function or regulation, e.g. increased succination of adiponectin in the presence of high glucose can reduce the polymerization and secretion of this adipokine [8]. Interestingly we have observed that several endoplasmic reticulum (ER) proteins, including the oxidoreductase protein disulfide isomerase (PDI), are succinated in the adipocyte during diabetes [21]. This may be significant considering that the unfolded protein response (UPR, a response to the accumulation of misfolded proteins) and ER stress develop in 3T3-L1 adipocytes grown in 30 mM glucose [2,14]. ER stress has also been documented in the adipose tissue of diet induced obese (DIO) [17], *ob*/*ob* [25], *db*/*db* mice [16] and in the adipose tissue of obese insulin-resistant humans [4,11]. However, although these studies document the presence of ER stress in diabetes, the direct initiator of adipocyte ER stress remains to be elucidated. We propose that increased succination of proteins may contribute to the accumulation of misfolded proteins and that this links mitochondrial stress to ER stress. We investigated the effect of sodium phenylbutyrate (PBA), an agent that reduces ER stress in adipocytes [2,25], to determine if this compound may also be acting on the mitochondria and potentially limiting protein succination and ER stress in a different manner.

The 3T3-L1 adipocyte model is a widely accepted model for the study of adipocyte function in obesity and diabetes. These studies are often

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conducted in the presence of high (25–30 mM) glucose concentrations as many cell lines are commonly maintained under these conditions. However, recent reports have documented that extracellular glucose concentrations have a significant impact on the measurement of mitochondrial bioenergetics [30] and this is of importance for experimental conditions examining mitochondrial function in diabetes. While several studies have examined adipocytes cultured in both 5 mM (normal) and 25–30 mM (high) glucose [10,12,18] the majority of published studies with 3T3-L1 adipocytes appear to be conducted in high glucose medium. We have previously demonstrated that the levels of protein succination are dependent on the glucose concentration; 2SC is increased in a concentration dependent manner in adipocytes cultured in 10–30 mM glucose [12]. In this study, we examined the impact of glucose and insulin concentrations on mitochondrial bioenergetics, markers of ER stress and protein succination in adipocytes. Our data highlights the role of mitochondrial stress and ER stress in the adipocyte as a function of nutrient conditions. In addition, we demonstrate that PBA alters mitochondrial bioenergetics in the adipocyte during maturation in high glucose medium.

2. Materials and methods

2.1. Chemicals

Unless otherwise noted, all chemicals were purchased from Sigma/Aldrich Chemical Co (St. Louis, MO & Milwaukee, WI). DNA extraction solvents were from Life Technologies (Grand Island, NY). Criterion polyacrylamide gels and Precision Plus protein ladder were purchased from BioRad Laboratories (Richmond, CA). PVDF membrane and ECL Plus chemiluminescent substrate were from GE Healthcare (Piscataway, NJ). Pierce® ECL 2 Western Blotting Substrate was from Thermo Scientific (Rockford, IL). Sodium phenylbutyrate (PBA) was from Enzo Life Sciences. Saturated phenol and phenol/chloroform/isoamyl alcohol were from Life Technologies (Grand Island, NY). The synthesis of 2-succinocysteamine (2SCEA) and preparation of polyclonal anti-2SC antibody have been described previously [21].

2.2. 3T3-L1 adipocyte culture

Murine 3T3-L1 fibroblasts were obtained from American Type Cell Culture (Manassas, VA). Confluent monolayers were differentiated into adipocytes in DMEM containing 255 nM dexamethasone, 500 μM IBMX and 160 μM insulin as described previously [21]. After differentiation, the adipocytes were matured in DMEM containing either 5 mM glucose and 0.3 nM insulin or 30 mM glucose and 3 nM insulin. Cells cultured in 5 mM glucose were supplemented with 5 mM glucose daily and several hours prior to protein harvest to maintain glucose levels. Cells were harvested in 500 μl radioimmunoprecipitation assay (RIPA) lysis buffer (50 mM Tris–HCl, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate, pH 7.4, 2 mM ethylenediaminetetraacetic acid (EDTA), 2 mM sodium fluoride, 2 mM sodium orthovanadate, and protease inhibitors). The cell lysate was pulse sonicated at 2 W RMS using a Model 100 sonic dismembrator (Fisher Scientific, Fair Lawn, NJ) for 3 s 3 times each. The protein was precipitated with 9 volumes of cold acetone for 10 min on ice. After centrifugation at 2000 ×*g* for 10 min and removal of the acetone the protein pellet was resuspended in RIPA buffer. The protein content was determined by the Lowry assay [19].

2.3. Western immunoblotting

Western blotting was performed using the Bio-Rad Criterion System. Proteins were resolved on Criterion TGX gels at 200 V for 1 h and transferred to PVDF membranes at 40 mA overnight or 250 mA for 100 min. Detection of protein succination was performed as previously described using a polyclonal anti-2SC antibody [21]. Antibodies for PPARγ, cleaved caspase 3, GAPDH, eIF2α, p-eIF2α, succinate dehydrogenase (SDHA), fumarase, PDI, calreticulin, and Ero1-Lα were from Cell Signaling Technologies (Danvers, MA). NDUFS4 was from Abcam (Cambridge, MA). β-tubulin and Grp78 were from Santa Cruz Biotech (Dallas, TX). Monoclonal anti-CHOP was from Thermo Fisher Scientific (Waltham, MA). Anti-adiponectin was from R&D systems (Minneapolis, MN). Chemiluminescent signals were captured on photographic film (Denville Scientific, Metuchen, NJ). Image J software (NIH) was used to quantify band intensity by densitometry.

2.4. Measurement of oxygen consumption rate (OCR)

Murine 3T3-L1 fibroblasts were seeded on V7 cell culture microplates coated with 0.2% gelatin at a density of 10,000 cells/well. After 72 h, the cells were differentiated and matured as described in Section 2.2. The Seahorse extracellular flux analyzer 24 XF-24 was used to measure the oxygen consumption rate (OCR). XF Assay Medium was supplemented with 1 mM sodium pyruvate and the glucose and insulin concentrations described above. After measurement of basal respiration, oligomycin (5 μg/mL), carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) (1.25 μM), and rotenone/antimycin A (3 μM/4 μM) were added sequentially to determine ATP production/proton leak, spare respiratory capacity and non-mitochondrial respiration. After completion of the assay, the medium was removed and the cells were washed 3 times with cold PBS. The plate was stored at −70 °C prior to the measurement of the total DNA content using the CyQuant® Assay (Invitrogen, Grand Island, NY).

2.5. Measurement of mitochondrial membrane potential

Mitochondrial membrane potential ($\Delta\Psi_m$) was measured using the fluorescent potentiometric dye 5,5′,6,6′-tetra-chloro-1,1′,3,3′-tetraethylbenzimidazolyl-carbocyanine iodide (JC-1) (Molecular Probes, Eugene, OR). Briefly, 3T3-L1 adipocytes were cultured in 24-well plates and treatments in maturation medium were continued for 2 days, which was the optimal time point for measurement of $\Delta\Psi_m$. After 2 days in various treatments, the cells were incubated with 10 μg/ml JC-1 dye for 20 min at 37 °C. The cells were then washed and resuspended in 200 μl PBS and JC-1 fluorescence was measured on a Tecan Safire 2 microplate reader (Tecan, Zurich, Switzerland). The fluorescence of the JC-1 monomer was measured at 485 nm excitation/535 nm emission and the fluorescence of the JC-1 aggregate was measured at 550 nm excitation/600 nm emission. The aggregate/monomer ratios (600/535) were used to assess $\Delta\Psi_m$. Cells treated with 10 μM CCCP were used as a positive control to demonstrate partial depolarization of the mitochondrial membrane.

2.6. Measurement of triglycerides, glucose, and inflammatory markers

Triglyceride content was measured in 10 μl aliquots of cell lysates using the Infinity™ Triglycerides assay kit (Thermo Fisher Scientific, Waltham, MA), according to the manufacturer's instructions. Glucose concentration was measured in phenol red free medium using the Amplex® Red Glucose/Glucose Oxidase Assay kit (Invitrogen, Grand Island, NY). The media on 3T3-L1 adipocytes was replaced with serum-free DMEM for 18 h prior to collection of the media. Pro-inflammatory cytokines in the conditioned media were analyzed by ELISA according to the manufacturer's instructions (Mouse Obesity ELISA, Signosis, Santa Clara, CA).

2.7. Mitochondrial DNA isolation and content analysis

Cell lysates were collected in 500 μL PBS and centrifuged at 2000 rpm for 5 min to yield a pellet which was resuspended in 350 μL DNA lysis buffer (10 mM Tris–HCl, pH 7.5, 400 mM NaCl, 2.5 mM EDTA, and 0.1% SDS) and 50 μL of 50 mg/mL proteinase K and heated at 55 °C