Original article

MOLECULAR METABOLISM

Mitochondrial dysfunction has divergent, cell type-dependent effects on insulin action

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ABSTRACT

The contribution of mitochondrial dysfunction to insulin resistance is a contentious issue in metabolic research. Recent evidence implicates mitochondrial dysfunction as contributing to multiple forms of insulin resistance. However, some models of mitochondrial dysfunction fail to induce insulin resistance, suggesting greater complexity describes mitochondrial regulation of insulin action. We report that mitochondrial dysfunction is not necessary for cellular models of insulin resistance. However, impairment of mitochondrial function is sufficient for insulin resistance in a cell type-dependent manner, with impaired mitochondrial function inducing insulin resistance in adipocytes, but having no effect, or insulin sensitising effects in hepatocytes. The mechanism of mitochondrial impairment was important in determining the impact on insulin action, but was independent of mitochondrial ROS production. These data can account for opposing findings on this issue and highlight the complexity of mitochondrial regulation of cell type-specific insulin action, which is not described by current reductionist paradigms.

Keywords Mitochondria; Insulin action; Reactive oxygen species; Adipocyte; Hepatocyte

1. INTRODUCTION

Type 2 diabetes (T2D) involves insulin resistance in skeletal muscle, the liver and adipose tissue [1]. One of the most contentious issues in metabolic research is the role of mitochondrial dysfunction in the development of insulin resistance. The term mitochondrial dysfunction can describe impairments in numerous mitochondrial function indices, including respiration, ATP production, membrane potential, proton leak and reactive oxygen species (ROS) production [2]. Impaired mitochondrial function has been observed in skeletal muscle [3–6], the liver [7–9] and adipose tissue [10–12] of T2D patients and animal models of T2D. Similar impairments have been observed in the skeletal muscle of insulin resistant offspring of T2D patients [13]. This could suggest a role for impaired mitochondrial function in the development of insulin resistance. This is supported by observations of insulin resistance in humans with mitochondrial DNA mutations that result in impaired mitochondrial function [14–16]. Mitochondrial dysfunction has been proposed to induce insulin resistance through ectopic lipid accumulation secondary to reduced β-oxidation, which impairs insulin signalling [17,18]. More recently, production of ROS has emerged as a direct link between mitochondrial dysfunction and insulin resistance driven by numerous insults such as saturated fatty acids, inflammatory cytokines and glucocorticoids [19,20]. However, numerous animal models with impaired mitochondrial function have either unchanged or increased insulin sensitivity [21–23], questioning both the causality of this relationship and whether mitochondrial dysfunction is necessary and/or sufficient for insulin resistance. Further adding to controversy on this issue, anti-diabetic agents such as the biguanide and thiazolidinedione family of compounds, which enhance insulin action primarily in the liver and adipose tissue respectively, have been reported to inhibit complex I of the electron transport chain and/or the mitochondrial pyruvate carrier (MPC), which impairs mitochondrial function [24–26]. These counterintuitive findings have been balanced by evidence that biguanides can prevent ROS production by complex I under conditions of electron backflow from complex II, such as during high fat feeding [27]. However, given that most of our knowledge regarding the role of mitochondria in the regulation of insulin action has been generated from studies of skeletal muscle, coupled with the fact that the primary tissues of action of these anti-diabetic drugs are not skeletal muscle, could raise the possibility that there are cell/tissue type-specific responses in this relationship that are not yet fully understood. Indeed, studies of either insulin resistant humans or animal models of mitochondrial dysfunction have not been able to mechanistically dissect this relationship with any certainty. This is due to reasons such as the non-physiological nature of gene ablation, the markedly different mitochondrial respiratory rates and metabolic function of tissues involved in whole body insulin action, the complexity in controlling substrate flux to individual insulin-sensitive tissues and inter-tissue cross-talk *in vivo*. While these factors are important for the development of the whole body metabolic phenotype in insulin resis-

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Abbreviations: AMPK, AMP-activated protein kinase; AS160, Akt substrate of 160 kDa; BSA, bovine serum albumin; ECAR, extracellular acidification rate; FoxO1, forkhead box protein O1; GP, glucose production; HI-FBS, heat-inactivated foetal bovine serum; IRS1, insulin receptor substrate 1; GLUT4, facilitative glucose transporter isoform 4; G.O., glucose oxidase; LDH, lactate dehydrogenase; MMP, mitochondrial membrane potential; MnTBAP, manganese (III) tetrakis (4-benzoic acid) porphyrin chloride; PI3K, phosphatidylinositol 3-kinase; ROS, reactive oxygen species; SOD, superoxide dismutase; T2D, type 2 diabetes; TNFα, tumour necrosis factor alpha

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tance, they are nonetheless confounding variables when examining the fundamental link between mitochondrial dysfunction and insulin action.

The fundamental biology underpinning these paradoxical findings describing the relationship between mitochondria and insulin action is poorly understood. Indeed, the potential importance of specific mitochondrial enzyme impairments and the tissue/cell type in which impairments occur are unknown. Furthermore, it is unknown how alterations in many of the interdependent indices of mitochondrial function impact on cellular insulin action. As *in vivo* studies have been unable to dissect these mechanisms, fundamental studies in cellular systems that define the biological complexity in the relationship between mitochondrial function and insulin action in multiple cell types are required before our understanding of the physiological role of mitochondria in the development of insulin resistance can advance. Therefore, the aims of this study were to: 1. determine whether mitochondrial dysfunction is necessary and/or sufficient for cellular insulin resistance; 2. establish whether specific mitochondrial enzyme impairment is important for this response in a cell type-dependent manner; and 3. assess whether ROS production is a universal link between impaired mitochondrial function and insulin resistance. We used 3T3L1 adipocytes and FAO hepatoma cells, as models of adipocytes and hepatocytes, respectively, to address these aims, assessing glucose uptake and suppression of glucose production as measures of insulin action. These cell lines have been used extensively to study insulin action and these cell types are characterised by impaired mitochondrial function in insulin resistant states [7,12]. However, few studies have utilised these cell types to mechanistically examine the role of mitochondria in insulin action.

2. MATERIALS AND METHODS

2.1. Cell culture

Mouse immortalised 3T3L1 fibroblasts were cultured in 10% CO_2 at 37 °C in growth media consisting of DMEM (4.5 g/L glucose; Invitrogen), 10% heat-inactivated foetal bovine serum (HI-FBS; Thermo Scientific) and antibiotics (100 units/mL penicillin and 100 μg/mL streptomycin; Life Technologies). Cells were induced to differentiate 2 days after reaching confluence (day 0), by supplementing growth media with 3 nM insulin (Humulin R; Eli Lilly), 0.25 μM dexamethasone (Sigma—Aldrich) and 0.5 mM 1-methyl-3-isobutyl-xanthine (Sigma—Aldrich). From day 3 until day 7, cells were maintained in growth media supplemented with 3 nM insulin after which the mature adipocytes were maintained in growth media. All treatments were for 24 h in growth media unless stated otherwise. Rat FAO immortalised hepatoma hepatocytes [28] were cultured in 5% CO_2 at 37 °C in growth media consisting of RPMI 1640 medium (2 g/L glucose; Invitrogen) and 10% foetal bovine serum (FBS; Thermo Scientific). Assays were carried out when cells were ~90% confluent. All treatments of hepatocytes were for 24 h in glucose- and serum-free RPMI 1640 media supplemented with 2 mM sodium pyruvate, 20 mM sodium L-lactate and 0.1% BSA (glucose production media; GP media), except where indicated. For 3T3L1 models of insulin resistance, cells were treated with 25 mU/mL glucose oxidase (G.O.; Sigma—Aldrich), 10 ng/mL tumour necrosis factor-α (TNFα; Peprotech) or 10 nM chronic insulin. For chronic insulin treatments, cells were returned to growth media containing no insulin 2 h before beginning glucose uptake assays or protein collection. Treatment doses for oligomycin (Sigma—Aldrich) and rotenone (Sigma—Aldrich) models of mitochondrial dysfunction as well as Antimycin A (Sigma—Aldrich) and FCCP (Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone; Sigma—Aldrich) in both 3T3L1 and FAO cells are as stated in Figures 2 and 3, Figures S2 and S3 . The doses of rosiglitazone and phenformin are stated in Figure 4 and Figure S4. MnTBAP (Manganese (III) tetrakis (4-benzoic acid) porphyrin chloride; Enzo Life Sciences) co-treatments were at a dose of 300 μM and wortmannin (wort; Sigma—Aldrich) co-treatments were at a dose of 100 nM.

2.2. Bioenergetics and respiration analyses

The cellular bioenergetics profile of 3T3L1 adipocytes and FAO hepatocytes was assessed using the Seahorse XF24 Flux Analyzer (Seahorse Bioscience). 3T3L1 fibroblasts were seeded into a 24-well XF24 cell culture microplate (Seahorse Bioscience) and were differentiated to maturity, as described above, at which time the cells were treated for 24 h. FAO hepatocytes were also seeded into a XF24 microplate at a density of 50,000 cells per well and 4 h later, 24 h treatments were begun in growth media. Cells were washed and incubated in 600 μl unbuffered DMEM (containing 25 mM glucose, 1 mM pyruvate and 1 mM glutamate) pH 7.4, at 37 °C in a non-CO_2 incubator (1 h prior to bioenergetics assessment). Three basal oxygen consumption rate (OCR) measurements were performed using the Seahorse analyzer, and measurements were repeated following injection of oligomycin (1 μM), FCCP (1 μM) and Antimycin A (1 μM). Basal extracellular acidification rate (ECAR) was determined from data collected at basal measurement points. Calculations of respiratory parameters of mitochondrial function were performed as previously described [29] and included subtraction non-mitochondrial respiration from all mitochondrial respiration parameters. Following completion of the assay cell number was determined using the CyQuant® Cell Proliferation Assay kit (Molecular Probes) according to manufacturer's instructions.

2.3. Glucose uptake assay

Mature adipocytes in 24-well plates were treated for 24 h with insults, mitochondrial inhibitors or anti-diabetic agents, as described above, in serum-starve media consisting of DMEM (4.5 g/L glucose) supplemented with 0.2% fatty acid-free bovine serum albumin (BSA; USB Corporation). To begin the assay, cells were washed twice in 1 × Dulbecco's Phosphate-Buffered Saline, pH 7.4 (Life Technologies), containing 0.5 mM $MgCl_2$, 0.5 mM $CaCl_2$ and 0.2% fatty acid-free BSA, and then incubated in the presence or absence of 10 nM insulin at 37 °C for 30 min. Uptake of 50 μM 2-deoxyglucose and 0.2 μCi 2-deoxy-D-[^{3}H]-glucose (PerkinElmer) per well was measured over the final 10 min of this incubation. Cells were washed twice in ice-cold phloretin (80 μg/mL) in PBS and lysed in 0.03% SDS before being analysed by scintillation counting. Protein content per well was measured and results expressed as pmol glucose transported per minute per μg of protein.

2.4. Glucose production assay

FAO hepatocytes in 48-well plates were treated for 24 h with mitochondrial inhibitors or insulin sensitising agents in GP media in the presence or absence of 0.1 nM insulin. To measure the glucose produced by the cells, 40 μl of media was collected from each well and combined with 250 μl of Assay Buffer consisting of 0.12 M $NaH_2PO_4 \cdot 2H_2O$ pH 7.0, 1 mg/mL phenol, 0.5 mg/mL 4-aminoantipyrine, 1.6 units/mL peroxidise and 10 units/mL glucose oxidase. This was incubated for 25 min at 37 °C after which absorbance was measured at 490 nm. Cells were lysed in 100 μl 0.03% SDS and protein was quantified using a BCA Protein Assay kit (Pierce). Results are expressed as μg glucose per mg protein.

