Original article

MOLECULAR METABOLISM

Opening of the mitochondrial permeability transition pore links mitochondrial dysfunction to insulin resistance in skeletal muscle

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ABSTRACT

Insulin resistance is associated with mitochondrial dysfunction, but the mechanism by which mitochondria inhibit insulin-stimulated glucose uptake into the cytoplasm is unclear. The mitochondrial permeability transition pore (mPTP) is a protein complex that facilitates the exchange of molecules between the mitochondrial matrix and cytoplasm, and opening of the mPTP occurs in response to physiological stressors that are associated with insulin resistance. In this study, we investigated whether mPTP opening provides a link between mitochondrial dysfunction and insulin resistance by inhibiting the mPTP gatekeeper protein cyclophilin D (CypD) *in vivo* and *in vitro*. Mice lacking CypD were protected from high fat diet-induced glucose intolerance due to increased glucose uptake in skeletal muscle. The mitochondria in CypD knockout muscle were resistant to diet-induced swelling and had improved calcium retention capacity compared to controls; however, no changes were observed in muscle oxidative damage, insulin signaling, lipotoxic lipid accumulation or mitochondrial bioenergetics. *In vitro*, we tested 4 models of insulin resistance that are linked to mitochondrial dysfunction in cultured skeletal muscle cells including antimycin A, C_2-ceramide, ferutinin, and palmitate. In all models, we observed that pharmacological inhibition of mPTP opening with the CypD inhibitor cyclosporin A was sufficient to prevent insulin resistance at the level of insulin-stimulated GLUT4 translocation to the plasma membrane. The protective effects of mPTP inhibition on insulin sensitivity were associated with improved mitochondrial calcium retention capacity but did not involve changes in insulin signaling both *in vitro* and *in vivo*. In sum, these data place the mPTP at a critical intersection between alterations in mitochondrial function and insulin resistance in skeletal muscle.

Keywords Glucose; Insulin resistance; Mitochondrial dysfunction; Mitochondrial permeability transition pore; Cyclophilin D; Skeletal muscle

1. INTRODUCTION

Skeletal muscle tissue has an important role in whole body glucose homeostasis by increasing glucose clearance from the blood in response to insulin. Therefore, insulin resistance in skeletal muscle is a contributing factor to glucose intolerance and type 2 diabetes. Risk factors for skeletal muscle insulin resistance include aging and obesity; however, the molecular mechanisms are unclear. Current evidence links aging and obesity to insulin resistance in skeletal muscle *via* correlations with mitochondrial dysfunction, aberrant lipid accumulation, and oxidative stress [1–6]. For example, physiological studies in both humans and rodents demonstrate that acute lipid infusion or chronic consumption of a high fat diet (HFD) is sufficient to promote skeletal muscle insulin resistance concomitant with lipid accumulation in muscle and/or mitochondrial dysfunction [7–10]. Furthermore, skeletal muscle of young insulin resistant pre-diabetic patients that are non-obese also demonstrates mitochondrial dysfunction and aberrant lipid accumulation [11]. These data led the authors to speculate that mitochondrial

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Abbreviations: MPTP, mitochondrial permeability transition pore; CYPD, cyclophilin D; HFD, high fat diet; LFD, low fat diet; WT, wild type; KO, knockout; CSA, cyclosporin A; BKA, bongkrekic acid; $O_2^{\cdot}$, superoxide; [^{3}H]-2-DOG, [^{3}H]-2-deoxyglucose; Rg′, rate of glucose transport; FFA, free fatty acid; DAG, diacylglycerol; TEM, transmission electron microscopy; PDH, pyruvate dehydrogenase; PDH_a, active PDH; PDH_t, total PDH; MCAD, medium chain acyl-CoA dehydrogenase; β-HAD, β-hydroxyacyl-CoA dehydrogenase; PM, plasma membrane; ANT, adenine nucleotide translocator; VDAC, voltage-dependent anion channel; HK2, hexokinase 2; ETC, electron transport chain; OXPHOS, oxidative phosphorylation; MnSOD, mitochondrial manganese superoxide dismutase; MIRKO, muscle insulin receptor knockout; MHC, myosin heavy chain; TBARS, thiobarbituric acid reactive substances

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inefficiency may promote lipotoxic lipid accumulation to drive skeletal muscle insulin resistance. Together, these studies and others [12] have identified an association between mitochondrial dysfunction and insulin resistance, but cause–effect relationships remain to be proven [12]. Further work is required to determine the molecular mechanisms linking skeletal muscle mitochondria to insulin sensitivity.

We have previously demonstrated that acute induction of mitochondrial superoxide ($O_2^{\bullet}$) in skeletal muscle myotubes with the mitochondrial electron transport chain (ETC) inhibitor antimycin A was sufficient to cause insulin resistance [2]. This mechanism of insulin resistance was prevented by overexpression of mitochondrial manganese superoxide dismutase (MnSOD), treatment with mitochondrial $O_2^{\bullet}$ scavengers, and inhibition of the ETC with stigmatellin at a site upstream of antimycin A. Collectively, this experiment demonstrated that mitochondrial $O_2^{\bullet}$, but not altered ATP production, is sufficient to drive insulin resistance in skeletal muscle. However, it also revealed a gap in knowledge concerning the mechanism whereby membrane impermeable mitochondrial $O_2^{\bullet}$ triggers insulin resistance, a process that occurs in the cytoplasm and at the plasma membrane (PM) [13,14]. To explore this mechanism we investigated a role for the mitochondrial permeability transition pore (mPTP). The mPTP is a multi-protein complex that spans both mitochondrial membranes and allows the passage of molecules less than 1500 Da between the cytoplasm and mitochondrial matrix. Importantly, the mPTP is triggered to open by mitochondrial $O_2^{\bullet}$ and other factors linked to insulin resistance including mitochondrial calcium overload [15–18]. Under normal physiological conditions, transient opening of the mPTP releases ions and metabolites from the mitochondrial matrix in order to maintain proper homeostasis [16,17,19–21]. To investigate whether opening of the mPTP is required for insulin resistance, we targeted the mitochondrial matrix peptidyl-prolyl *cis-trans* isomerase cyclophilin D (CypD). CypD regulates mPTP opening by directly binding to pore constituent proteins, and inhibition of CypD decreases the probability of mPTP opening [18,19,21]. Based on these data, we investigated whether the genetic or pharmacological inhibition of CypD would protect from insulin resistance.

2. MATERIALS AND METHODS

2.1. Cell culture

Maintenance and differentiation of L6 rat skeletal muscle cells expressing HA-tagged GLUT4 was performed as described [2,22]. Differentiation of myoblasts into myotubes was induced by culturing cells in MEM-α containing 2% (v/v) horse serum and 1% (v/v) penicillin–streptomycin. L6 myotubes were treated with specified drugs for the durations and concentrations noted in the figure legends. Ferutinin, bongkrekic acid (BKA), cyclosporin A (CsA), antimycin A, C_2-ceramide, and bovine serum albumin (BSA) were purchased from Sigma Aldrich (St. Louis, MO).

2.2. GLUT4 translocation assays

GLUT4 translocation assays were performed as previously described [2] in L6 myotubes treated with either 150 μM palmitic acid conjugated to BSA or ethanol (control) in DMEM. Palmitate–BSA complexes were made by combining BSA (20% BSA stock) and palmitic acid (200 mM palmitic acid stock) in DMEM while vortexing at low speed at 50 °C. The palmitate:BSA or ethanol:BSA (vehicle control) solutions were heated at 50 °C in a water bath for 20 min, then cooled to 37 °C for 15 min and diluted in DMEM to the final concentrations indicated. The diluted solutions were sterile filtered through a 0.45 μm PVDF membrane prior to treatment of myotubes.

2.3. Western blotting

Following drug treatment, cells were washed twice with ice-cold phosphate-buffered saline (PBS) and lysed with HEPES–EDTA–Sucrose lysis buffer (250 mM sucrose, 20 mM HEPES pH 7.4, and 1 mM EDTA) containing 2% sodium dodecyl sulfate (SDS). Whole cell lysates were cleared of insoluble material by centrifugation [22]. Quadriceps (~20 mg) were homogenized in 20 × volumes (~400 μL) of radioimmunoprecipitation assay (RIPA) buffer [150 mM NaCl, 10 mM nonyl phenoxypolyethoxylethanol (NP)-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris pH 7.5] containing protease inhibitors (Roche) and phosphatase inhibitors (2 mM Na-orthovanadate, 1 mM Na-pyrophosphate, 10 mM Na-fluoride, 250 nM microcystin LR). Homogenates were sonicated, rotated at 4 °C for 1 h and centrifuged at 16,000 × *g* at 4 °C for 10 min. Lysates were diluted in 4 × Laemmli buffer and denatured at 65 °C for 5 min. Cellular proteins (20 μg) were resolved on 10% SDS-polyacrylamide gels or AnykD pre-cast gels (Bio-Rad Laboratories, Hercules, CA) and electro-transferred overnight onto nitrocellulose membranes. Equal protein loading was confirmed by Ponceau staining. Protein expression was detected with the following antibodies: phospho-Akt S473, total Akt, phospho-GSK3β S9, total GSK3β, hexokinase 2, total pyruvate dehydrogenase (PDH) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Cell Signaling, Beverly, MA), phospho-insulin receptor (IR)/insulin-like growth factor 1 receptor (IGF1R) Y1158/Y1162/Y1163 (Millipore, Billerica, MA), CypD (Mitosciences, Eugene, OR), 14-3-3 (Santa Cruz Biotechnology, Dallas, Texas), phospho-PDH S293 E1α (Novus Biologicals, Littleton, CO), GLUT4 R82 (generously provided by Dr. Thurl Harris, University of Virginia Department of Pharmacology), myosin heavy chain (MHC) I (BA-F8) and MHC IIa (sc-71) (generous gifts from Dr. Zhen Yan, University of Virginia Robert M. Berne Cardiovascular Research Center). Primary antibodies were detected using goat anti-mouse IgG (DyLight 800 conjugate) or goat anti-rabbit IgG (DyLight 680 conjugate) polyclonal secondary antibodies. Membranes were visualized, and protein band intensities quantified, using the LI-COR ODYSSEY System and software (LI-COR, Lincoln, NE, USA).

2.4. Animals

Food and water were provided *ad libitum* until the date of study and all animal care was in compliance with NIH guidelines and the University of Virginia Animal Care and Use Committee. The high fat diet (45% kcal as fat) was purchased from Research Diets (D12451). Normal chow diet was purchased from Harlan Teklad (diet 7912). Animals were maintained on a 12/12 light/dark schedule at 68–72 °F and housed 4–5 per cage. The CypD KO mice were obtained from Dr. Jeffrey Molkentin [23] and maintained on an inbred C57BL/6 background as heterozygous breeding pairs. Glucose tolerance tests were performed on mice that were fasted for 5–6 h prior to intraperitoneal injection of glucose (1.5–2 g/kg). Blood glucose levels were monitored at indicated time points using an Accu-check II glucometer (Roche Diagnostics). Clearance of the glucose analog [^{3}H]-2-deoxyglucose ([^{3}H]-2-DOG) into glucose-6-phosphate and [U-^{14}C]-glucose into glycogen was measured in quadriceps muscles as described previously [2,24].

2.5. Serum and tissue analyses

Serum insulin was determined by ELISA (Crystal Chem, Downers Grove, IL). Non-esterified fatty acids were measured from serum samples by colorimetric assay (WAKO diagnostics, Osaka Japan). Transmission electron microscopy (TEM) was performed at the UVa EM facility using finely diced tibialis cranialis muscle fixed in 4% glutaraldehyde and 2.5% paraformaldehyde for 3 days prior to post-

