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Insulin acutely improves mitochondrial function of rat and human skeletal muscle by increasing coupling efficiency of oxidative phosphorylation☆

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ABSTRACT

Insulin is essential for the regulation of fuel metabolism and triggers the uptake of glucose by skeletal muscle. The imported glucose is either stored or broken down, as insulin stimulates glycogenesis and ATP synthesis. The mechanism by which ATP production is increased is incompletely understood at present and, generally, relatively little functional information is available on the effect of insulin on mitochondrial function. In this paper we have exploited extracellular flux technology to investigate insulin effects on the bioenergetics of rat (L6) and human skeletal muscle myoblasts and myotubes. We demonstrate that a 20-min insulin exposure significantly increases (i) the cell respiratory control ratio, (ii) the coupling efficiency of oxidative phosphorylation, and (iii) the glucose sensitivity of anaerobic glycolysis. The improvement of mitochondrial function is explained by an insulin-induced immediate decrease of mitochondrial proton leak. Palmitate exposure annuls the beneficial mitochondrial effects of insulin. Our data improve the mechanistic understanding of insulin-stimulated ATP synthesis, and reveal a hitherto undisclosed insulin sensitivity of cellular bioenergetics that suggests a novel way of detecting insulin responsiveness of cells.

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1. Introduction

Skeletal muscle plays an important role in the maintenance of blood glucose homeostasis by taking up excess glucose in response to insulin [1]. Defects in insulin responsiveness, as for example manifested in obese subjects, contribute to the development of type 2 diabetes [2]. Obesity-related insulin resistance is at least partly caused by high levels of circulating non-esterified fatty acids [3], and mitochondrial dysfunction has been implicated in this resistance [4]. Insufficient mitochondrial capacity to burn excess fatty acids has been held responsible for obesity-blunted insulin sensitivity [5], but this notion is highly controversial [6]. In healthy muscle, insulin stimulates the storage of imported glucose as glycogen, an anabolic effect that has been recognised for some time [7]. It is becoming increasingly clear, however, that insulin signalling also affects mitochondrial metabolism [8], suggesting a possible additional regulatory role for insulin in oxidative nutrient metabolism. Several independent studies have demonstrated that insulin indeed stimulates ATP synthesis in human muscle, and that this catabolic effect is annulled under pathological conditions relating to type 2 diabetes [9–11]. Insulin-mediated ATP production has been associated with enhanced mitochondrial protein synthesis [9,12] and with increased mRNA levels and activities of mitochondrial enzymes involved in fuel oxidation [9]. Consistently, insulin has been shown to promote glucose oxidation [13]. Insulin is thus thought to stimulate ATP synthesis by increasing the capacity of oxidative phosphorylation [9], but the experimental evidence for this notion is largely circumstantial. Generally, direct functional measurements of the effect of insulin on mitochondrial function in skeletal muscle are relatively scarce.

In this paper, we have exploited recently developed extracellular flux technology [14] to establish insulin effects on the energy metabolism of intact rat and human skeletal muscle cells. Real-time measurements of mitochondrial function reveal that insulin instantly improves the coupling efficiency of oxidative phosphorylation and increases cell respiratory control. We show that these related beneficial insulin effects on mitochondrial function are due to acutely decreased proton leak, and that they are annulled completely in fatty-acid-exposed cells.

Abbreviations: 2DG, 2-deoxyglucose; BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle medium; ECAR, extracellular acidification rate; FCCP, trifluorocarbonylcyanide phenylhydrazone; FCS, fetal calf serum; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; UCP, uncoupling protein

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2. Materials and methods

2.1. Cell culture

2.1.1. Rat cells

L6 myoblasts were obtained from the European Collection of Cell Culture, and were maintained at 37 °C under a humidified carbogen atmosphere in Dulbecco's Modified Eagle Medium (DMEM) containing 25 mM glucose and 20 mM Hepes, and supplemented with 10% (v/v) fetal calf serum (FCS), 100 U/mL penicillin, and 100 μg/mL streptomycin. Cells between passages 9 and 18 were used for experimentation.

2.1.2. Human cells

Skeletal muscle tissue (~350 mg) was removed from *vastus lateralis* through locally anaesthetised needle biopsy with informed donor consent and approval from the Ethics Committee of the Department of Sport and Health Sciences, College of Life and Environmental Sciences, University of Exeter, UK. Myoblasts were isolated and cultured at the University of Exeter Medical School, St Luke's Campus (Human Tissue Authority licence 12104) according to [15], and cells were passaged at least twice before off-site analysis. Briefly, tissue was collected in cold DMEM containing 5 mM glucose and 20 mM Hepes, minced finely with a sterile scalpel, and cells were dissociated via agitated incubation at 37 °C in digestion medium containing 0.25% (w/v) trypsin, 0.1% (w/v) type IV collagenase, and 0.1% (w/v) bovine serum albumin (BSA). Every 10 min for 30 min in total, dislodged cells were harvested: undigested tissue was spun down gently at 80 *g* and was then re-incubated with fresh digestion medium; the supernatant was subsequently spun at 150 *g* to collect dissociated cells. Pooled cells were incubated for 1 h in DMEM (containing 5 mM glucose, 20 mM Hepes, 20% (v/v) FCS, 0.5% (v/v) chick embryo extract, 2 nM insulin, 100 U/mL penicillin, and 100 μg/mL streptomycin) in an uncoated 25 cm^2 culture flask to facilitate selective attachment of fibroblasts. Subsequently, the myoblast-enriched supernatant was transferred to a 60 mm collagen-coated culture dish. Attached cells were allowed to reach 80% confluence (5 days' growth approximately), and were then trypsinised for further enrichment: 20-min incubation in a collagen-coated dish to remove relatively rapidly attaching fibroblasts [16], followed by supernatant transfer to another collagen-coated dish. As confirmed by myosin-staining, this method yielded monolayers with more than 95% myoblasts. The initial cell population was allowed to double 4× before experimentation, and all assays were performed before the population had doubled 8×.

2.1.3. Differentiation

Human and L6 myoblasts were seeded on XF24 (Seahorse Bioscience) tissue culture plates at 2×10^4 and 4×10^4 cells/well, respectively, and grown for 48 h in fully supplemented, cell-specific DMEM (specified above). At this point, the FCS level in the respective growth media was lowered to just 2% (v/v) and this 'light-serum' medium was refreshed every 2–3 days until myoblasts had turned to myotubes. Visual inspection using a light microscope indicated that complete differentiation took 8–10 days.

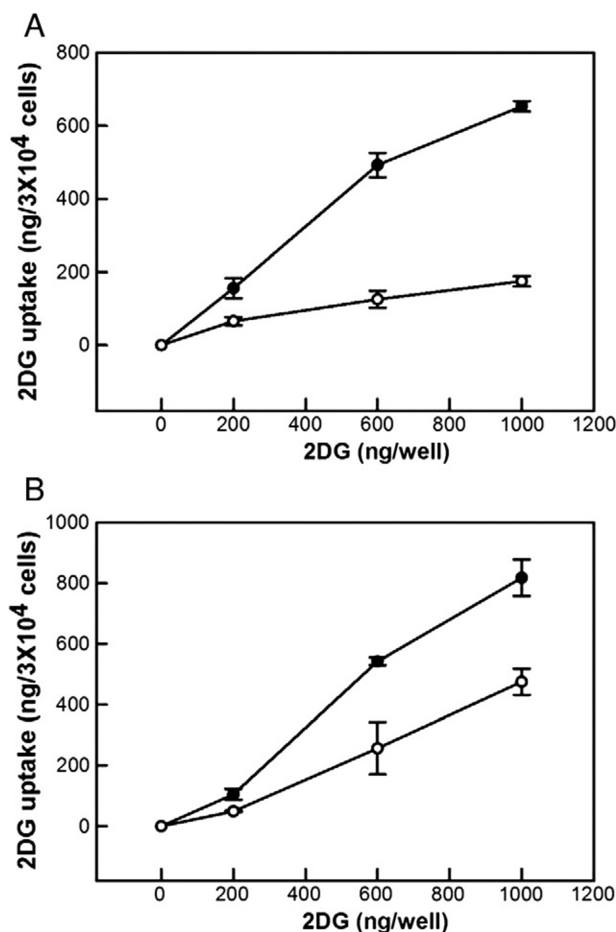

Fig. 1. Glucose uptake. 2-Deoxyglucose (2DG) accumulation was measured in L6 and human myoblasts (panels A and B, respectively) without (white symbols) or with 100 nM insulin (black symbols) as fully described in Section 2.5. The data shown are means ± SEM of 3 independent experiments. The differences between insulin-exposed and control cells are statistically significant ($P < 0.05$) at applied 2DG levels of 600 and 1000 ng/well.

2.2. Palmitate

2.2.1. Conjugation

Fatty acid-free BSA (Sigma A7030) was dissolved at 1.6 mM in medium containing 135 mM NaCl, 3.6 mM KCl, 10 mM Hepes (pH 7.4), and 0.5 mM $MgCl_2$. Palmitate (8 mM) was added as powder and stirred continuously for 24–48 h at 35–38 °C until it had dissolved completely. Cooled BSA:palmitate conjugations were filter-sterilised and stored at 4 °C. When added to cultures, the conjugations were diluted 40× and the total unbound free palmitate level was around 20 nM as estimated from previously published binding parameters [17].

2.2.2. Exposure

Cells were cultured in DMEM containing just 5 mM glucose and 2% (v/v) FCS. This nutrient restriction sensitised the cells to insulin (*cf*. [18]), and was applied for 10 h in case of glucose uptake assays (Section 2.5) and 24 h in case of the cellular bioenergetics experiments (Section 2.3). At this point, insulin sensitivity was measured either immediately or after a 16-h exposure to palmitate, in which case medium was replaced with FCS-free DMEM containing BSA-conjugated palmitate, or BSA alone.

2.3. Mitochondrial respiration

Mitochondrial bioenergetics were measured in attached cells as described before [19]. Briefly, L6 myoblasts seeded at 4×10^4 cells/well, differentiated, and exposed to palmitate on XF24 plates were washed 4× with a Krebs Ringer buffer (KRPH) comprising 136 mM NaCl, 3.7 mM KCl, 10 mM Hepes, (pH 7.4), 2 mM NaH_2PO_4, 1 mM $MgCl_2$, 1.5 mM $CaCl_2$, and 0.1% (w/v) BSA, and were then incubated in this buffer for 1 h at 37 °C under air. Human cells were treated similarly, but were seeded at 2×10^4 cells/well, were not exposed to palmitate, and were not washed into KRPH, but into serum-free DMEM containing 2 mM glucose and 10 mM Hepes. At this point, up to 100 nM insulin was added and cells were incubated for another 20 min. Subsequently, the plates were put in a Seahorse XF24 extracellular flux analyser (controlled at 37 °C) for a 10-min calibration and 3 measurement cycles to record basal cellular respiration. Glucose (2 mM), oligomycin (5 μg/mL), FCCP (2 μM and 20 μM for human and L6 cells, respectively), and a mixture of rotenone

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