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Lipid content and response to insulin are not invariably linked in human muscle cells

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ABSTRACT

In type 2 diabetes, a strong correlation between intramyocellular lipid accumulation and insulin resistance exists but whether intramyocellular accumulation is a cause or a consequence of insulin resistance is not clear. Lipid accumulation and response to insulin were evaluated in primary human myotubes derived from non-diabetic subjects and type 2 diabetic patients. Myotubes derived from type 2 diabetic patients had a defective response to insulin without showing a significant increase in lipid accumulation compared to myotubes derived from non-diabetic subjects. In myotubes derived from non-diabetic subjects, response to insulin stimulation (Akt phosphorylation) was abrogated and lipid content was increased after palmitate treatment. However, chronic exposure to insulin or inhibition of mitochondrial activity by antimycin led to independent changes of lipid content and response to insulin in myotubes derived from non-diabetic subjects. Altogether these results suggest that lipid accumulation and response to insulin are not invariably linked.

1. Introduction

Obesity-associated insulin resistance is a major risk factor for type 2 diabetes and cardiovascular disease. Skeletal muscle accounts for the bulk of insulin-mediated glucose disposal and thus is important for systemic energy metabolism. Intramyocellular lipid (IMCL) accumulation in skeletal muscle is abnormally high in obesity, type 2 diabetes and other metabolic conditions. Elevated IMCL stores in the obese and/or type 2 diabetic patient seem to be secondary to a structural imbalance between plasma free fatty acid (FFA) availability, fatty acid (FA) storage and oxidation (van Loon and Goodpaster, 2006; Kriketos et al., 2003). Numerous studies have reported a strong relationship between IMCL content and insulin resistance (Kriketos et al., 2003; Perseghin et al., 1999; Pan et al., 1997). Lipid infusion or high fat diet applied on healthy persons induced an increase in both plasma FFA level and IMCL content in relation with a significant decrease in insulin sensitivity (Bachmann et al., 2001; Boden et al., 2001). Moreover, addition of FFA on myotubes established from rodent cell lines induced insulin resistance in relation with IMCL accumulation (Pedrini et al., 2005; Perdomo et al., 2004; Schmitz-Peiffer et al., 1999). However, most of the investigations have been correlative in nature and IMCL accumulation within skeletal muscle may not be invariably linked to insulin resistance since endurance-trained athletes have an enhanced storage of IMCL despite their high insulin sensitivity (Goodpaster et al., 2001; van Loon, 2004)

Gene–environment interaction can be viewed as a departure from an otherwise expected additivity of genetic and environmental factors on a given outcome measure. Human satellite cells have been shown to display the majority of the defects previously described for type 2 diabetic muscle in vivo including defective insulin signaling pathway (Bouzakri et al., 2003; Jackson et al., 2000; Nikoulina et al., 2001), reduced insulin-stimulated glycogen synthase (GS), glucose transport activities (Nikoulina et al., 2001; Gaster et al., 2002) and reduced lipid oxidation (Gaster et al., 2004, 2005). In this context, cell culture of primary human myotubes offers an excellent model to evaluate the causality of the relation between IMCL accumulation and response to insulin by measuring inherited intrinsic defects under standardized conditions.

In the present study, we used primary human muscle cell cultures grown from satellite cells taken from non-diabetic subjects and type 2 diabetic patients, to determine the gene–environment interaction between IMCL accumulation and response to insulin.

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2. Materials and methods

2.1. In vivo human experimental procedures

2.1.1. Subjects

A total of 13 volunteers (5 male subjects with no family history of type 2 diabetes and 8 male type 2 diabetic patients) participated to this study. Subjects were matched on age and physical activity measured by VOORRIPS index (Voorrips et al., 1991). The experimental protocol was approved by the local ethic committee (03/10/GESE, Montpellier, France). Informed and written consent was obtained from all subjects after explanation of the protocol.

2.2. In vitro experimental procedures

2.2.1. Primary human muscle cell culture: process of purification

A skeletal muscle biopsy of the *vastus lateralis* was performed according to the percutaneous Bergström technique after local anesthesia (xylocaine) (Thomas et al., 2004; Thomas et al., 2005). 50 mg of muscle biopsy were scissor-minced and tissue explants were plated into collagen-coated dishes as previously described (Kitzmann et al., 2006). After 10–15 days, cells migrated out of explants. Cells were purified using an immunomagnetic bead based sorting system (Sinanan et al., 2004) with MACS microbeads directly linked to an antibody to NCAM (CD56) (Kitzmann et al., 2006). Cultures were maintained in a growth medium composed of (DMEM, 10% FBS and 1% Ultroser G) and when $CD56^+$ cells reached confluence, medium was changed (growth medium minus Ultroser G) and the differentiation process occurred until fusion and terminal differentiation into contractile myotubes (8 days).

2.2.2. Myotubes treatments

Treatments performed on myotubes are detailed in the figure legends and were realized in triplicate for each of the 13 independent cultures. The following reagents: L-glutamine, DMEM, palmitate and antimycin were purchased from Sigma–Aldrich (Saint Quentin Fallavier, France), FBS from Hyclone (Brebières, France), insulin from Lilly (Suresnes, France). Palmitate was dissolved in chloroform (0.6 mol/l), diluted to 0.6 mmol/l in DMEM containing 10% of FBS and added to the myotubes. Untreated myotubes were incubated in DMEM containing 10% FBS and 0.1% chloroform without palmitate.

2.2.3. IMCL content

Lipid content in myotubes was visualized by oil red O (Sigma–Aldrich, Saint Quentin Fallavier, France) staining. Myotubes were firstly washed with PBS. Oil red O staining was carried for 1 h followed by 2 or 3 washes with distilled water. In order to quantify IMCL content in myotubes, oil red O was extracted using isopropanol for 10 min. 1 ml of sample was transferred into spectrophotometer cuvettes. The absorbance value was measured using a spectrophotometer set at 490 nm (Gaster and Beck-Nielsen, 2006) and blanked to untreated cells.

2.2.4. Western blots

Cell lysates were quantified (Biorad protein assay, Biorad, Paris, France), 30 μg of total proteins were separated by SDS-PAGE and then transferred to nitrocellulose membranes (Schleicher and Schuell Bioscience, Dassel, Germany). Western blots were realized as previously described (Kitzmann et al., 1998). Following primary antibodies were used: polyclonal anti-phospho-Glycogen Synthase (Ser641) (Cell Signaling Technology, Ozyme, Saint-Quentin-en-Yvelines, France) diluted 1/200, monoclonal anti-phospho-Akt (Ser473) (587F11)(Cell Signaling Technology, Ozyme, Saint-Quentin-en-Yvelines, France) diluted 1/200, polyclonal anti-Akt (Cell Signaling Technology, Ozyme, Saint-Quentin-en-Yvelines, France) diluted 1/200, monoclonal anti-troponin T and monoclonal anti-α-tubulin (Sigma–Aldrich, Saint Quentin Fallavier, France) diluted 1/500. The secondary antibodies were anti-rabbit and anti-mouse antibodies coupled to horseradish peroxidase (Amersham Bioscience, Orsay, France). Proteins were visualized using an enhanced luminescent reagent (Santa Cruz Biotechnology, Tebu-Bio, Le Perray en Yvelines, France), and exposed to autoradiograph film (Amersham Bioscience, Orsay, France). Expressions of α-tubulin and Troponin T were used as a loading charge control and a marker of myotube differentiation, respectively. Expression of proteins was quantified by density analysis using ImageJ Launcher Software.

2.2.5. Statistical analyses

Data are presented as mean ± SEM. Statistical analyses were performed using Statview 5.0. Student's *t* test for unpaired and paired comparison or a two-way ANOVA were used to assess statistical differences. $p < 0.05$ was considered to be significant.

3. Results

Following insulin stimulation, activation of Akt is known to phosphorylate and inactivate Glycogen Synthase Kinase-3 (GSK-3), resulting in the dephosphorylation of Glycogen Synthase (GS) that controls the rate of glycogen metabolism. Akt activation is known to trigger the translocation of Glucose Transporter 4 (GLUT4) to plasma membrane in response to insulin. Thus, increased Akt phosphorylation and decreased GS phosphorylation in response to insulin stimulation are positive markers of GLUT4 translocation and glycogen synthesis.

Fig. 1A shows the representative western blots of Ser473 phosphorylation of Akt and of Ser641 phosphorylation of GS, with or without insulin stimulation, performed on myotubes derived from non-diabetic subjects (ND) and type 2 diabetic patients (T2D). ND myotubes show a response to insulin as attested by the significant increase in Ser473-Akt (Fig. 1B) and the significant decrease in Ser641-GS (Fig. 1B) phosphorylation levels after insulin stimulation. In contrast, phosphorylation of these proteins was not significantly modified after insulin stimulation in T2D myotubes (Fig. 1B). This difference in the response to insulin between ND and T2D myotubes was not the result of a difference in the loading charge as quantification of Ser473-Akt and of Ser641-GS was normalized to α-tubulin expression. Troponin T is only expressed in differentiated muscle cells and was used to show that satellite cells (ND or T2D) derived from muscle biopsies were able to form myotubes. As shown on Fig. 1A, Ser473-Akt level was increased in some myotubes derived from T2D patients. We have thus monitored P-Akt to Akt ratio in ND and T2D myotubes in basal state and after insulin stimulation (Fig. 1C). ND myotubes show a response to insulin as attested by the significant increase in P-Akt to Akt ratio (Fig. 1D). In contrast, P-Akt to Akt ratio was neither significantly modified after insulin stimulation in T2D myotubes (Fig. 1D) nor significantly different in the basal state between ND and T2D myotubes (Fig. 1D). IMCL content was not significantly different between ND and T2D myotubes neither after palmitate treatment ($p > 0.05$) nor after insulin and palmitate treatment ($p > 0.05$) (Fig. 1E).

The importance of FFA in the development of type 2 diabetes is well reported (for review see Wilding, 2007). Studies on muscle cell lines showed that palmitate (saturated FFA) treatment induced lipid accumulation and insulin resistance (Pedrini et al., 2005; Perdomo et al., 2004; Schmitz-Peiffer et al., 1999). Fig. 2A shows the representative western blots of Ser473 phosphorylation of Akt and of Ser641 phosphorylation of GS, with or without insulin stimulation, performed on myotubes derived from two different non-diabetic subjects (ND1 and ND2) treated or not by palmitate. Response to insulin stimulation at the level of Ser473-Akt phosphorylation was abrogated after palmitate treatment (Fig. 2B) whereas response to insulin at the level of Ser641-GS phosphorylation was unchanged. Quantification of IMCL after palmitate treatment for 16 h in C myotubes is presented in Fig. 2C. Palmitate treatment induced a significant increase in IMCL content ($p < 0.05$).

Chronic insulin treatment is known to decrease insulin-stimulated PI3K activity and glucose uptake in mouse cell lines (C2C12) (Kumar et al., 2004). We have confirmed that chronic insulin exposure (64 h) abrogated the response to insulin in ND myotubes, at the level of Ser473-Akt and Ser641-GS phosphorylation (Fig. 3A and B). To know if the induction of insulin resistance in these cells was associated with an increase in IMCL content, we have measured the change in IMCL content after chronic insulin exposure before palmitate treatment (Fig. 3C). No significant modification in the content of IMCL was observed in myotubes derived from non-diabetic subjects after chronic insulin exposure.

A current hypothesis to explain the increase in IMCL accumulation in insulin resistant state is a reduced lipid oxidative capacity commonly associated with mitochondrial dysfunction (Kelley et al., 2002; Mogensen et al., 2007). In order to evaluate whether mitochondrial activity inhibition could decrease insulin sensitivity by increasing IMCL content in ND myotubes, cells were exposed to an inhibitor of oxidative phosphorylation (antimycin) before palmitate treatment. Mitochondrial activity inhibition by antimycin