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# The depletion of cellular mitochondrial DNA causes insulin resistance through the alteration of insulin receptor substrate-1 in rat myocytes

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**Abstract**

Since the bioenergetic capacity of skeletal muscle mitochondria is decreased in type 2 diabetes and obesity, the reduction of mitochondrial DNA (mtDNA) content may be involved in the development of insulin resistance in skeletal muscle. To elucidate the association of cellular mtDNA content and insulin resistance, we produced L6 GLUT4myc myocytes depleted of mtDNA by long-term treatment with ethidium bromide (EtBr). L6 GLUT4myc cells cultured with 0.2 μg/ml EtBr (termed depleted cells) revealed a marked decrease in cellular mtDNA, concomitant with a lack of mRNAs encoded by mtDNA. Interestingly, the mtDNA-depleted cells showed a drastic decrease in basal and insulin-stimulated glucose uptake, indicating that L6 GLUT4myc cells develop impaired glucose utilization and insulin resistance. The repletion of mtDNA normalized basal and insulin-stimulated glucose uptake. The plasma membrane (PM) GLUT4 in the basal state was decreased, and the insulin-stimulated GLUT4 translocation to the PM was drastically reduced by mtDNA depletion. Interestingly, the expression of IRS-1 associated with insulin signaling was decreased by 90% in the depleted cells, and the insulin-stimulated phosphorylation of IRS-1 and Akt2/PKB were drastically reduced in the depleted cells. Those changes returned to control levels after mtDNA repletion. Taken together, our data suggest that PM GLUT4 content and insulin signal pathway intermediates are modulated by the alteration of cellular mtDNA content, and the reduction in the expression of IRS-1 and insulin-stimulated phosphorylation of IRS-1 and Akt2/PKB are associated with insulin resistance in the mtDNA-depleted L6 GLUT4myc myocytes.


*Keywords:* mtDNA; GLUT4; Insulin resistance; IRS-1; Akt2/PKB

## 1. Introduction

Type 2 diabetes is a polygenic disorder composed of subtypes whereby genetic susceptibility is strongly associated with environmental factors [1]. Maternally inherited mtDNA, which encodes 22 tRNAs, 13 subunits of the electron transport system and its own 16S and 26S ribosomal RNAs, has been considered one of the genetic factors for the development of diabetes [2]. Although mtDNA within the cells is heteroplasmic, it has been reported that approximately 0.5–1.5% of diabetics over the age of 40 exhibit mtDNA abnormalities such as duplications [3], point mutations [4], and large-scale deletions [5]. Furthermore, the cellular oxidative capacity representing one of mitochondrial functions is directly correlated with insulin sensitivity

*Abbreviations:* IRS, insulin receptor substrate; GLUT4, facilitative glucose transporter isoform 4; PI3K, phosphatidylinositol 3-kinase; PDK-1, 3-phosphoinositide-dependent protein kinase-1; Akt2/PKB, protein kinase B

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in skeletal muscles [6], and reduced mitochondrial oxidation and phosphorylation are associated with insulin resistance in skeletal muscle of elderly people [7], raising the possibility that impaired mitochondrial function results in insulin resistance known as the hallmark of type 2 diabetes.

Several lines of evidence suggested that the reduction of mtDNA content also correlate with the development of insulin resistance. The cellular content of mtDNA in the pancreatic islets of Goto-Kakizaki rat was markedly decreased without major deletion or restriction fragment polymorphism in mtDNA [8]. Southern blot analyses from types 1 and 2 diabetic patients also revealed that the cellular mtDNA copy number in skeletal muscles was approximately 50% of normal [9]. Lee et al. [10] also reported that the quantitative decrease of mtDNA in lymphocytes preceded the type 2 diabetic development, suggesting that the decreased content of mtDNA might be a causal factor in type 2 diabetes. In addition, several results obtained from mtDNA-depleted cells have revealed that mitochondrial stress causes substantial activation and inactivation of nuclear-encoded gene expression [11,12]. However, it is not clear whether the mtDNA-depletion is a contributing factor in the expression of molecules responsible for the insulin signaling and GLUT4 translocation.

In the present study, we report that the depletion of mtDNA in L6 GLUT4myc myocytes is directly correlated with drastic reduction in basal glucose utilization and resistance to insulin stimulation as shown by glucose uptake and GLUT4 translocation. The expression level of IRS-1 was also reduced in the depleted cells with drastic reduction in the insulin-stimulated GLUT4 translocation to the PM, and the insulin-stimulated phosphorylations of IRS-1 and Akt2/PKB were decreased in the depleted cells. These findings strongly suggest that the GLUT4 contents in the PM and insulin signal pathway intermediates are modulated by the alteration of cellular mtDNA content, and that the reductions in the expression of IRS-1 and the insulin-stimulated phosphorylation of IRS-1 and Akt2/PKB are associated with insulin resistance in the mtDNA-depleted L6 GLUT4myc myocytes.

## 2. Experimental procedures

### 2.1. Cells culture

The parent cell line used in this study was L6 GLUT4myc, an L6 cell line (provided by Dr. Amira Klip, the Hospital for Sick Children, Toronto, Ontario, Canada) expressing GLUT4-myc, constructed by inserting a human *c-myc* epitope (14 amino acids) into the first ectodomain of rat GLUT4 [13]. Cells were maintained in minimal essential medium-α (α-MEM) supplemented with 10% FBS in a humidified atmosphere of air and 5% $CO_2$ at 37 °C. The L6 GLUT4myc cell line with partially depleted mtDNA was isolated by treating L6 GLUT4myc myocytes with ethydium bromide (EtBr, 0.2 μg/ml) and uridine (50 μg/ml) for 3 weeks in α-MEM supplemented with 10% FBS. The control parental L6 GLUT4myc myocytes were maintained for the same time period in normal culture condition. Since L6 GLUT4myc myocytes without differentiation possess GLUT4 recycling compartments, insulin-sensitive glucose uptake and the insulin signaling intermediates similar to primary muscle cells [14], we used L6 GLUT4myc myocytes to study how the insulin sensitivity and glucose utilization is modulated by the alteration of cellular mtDNA content. Myocytes were deprived of serum for 5 h prior to all experimental manipulations.

### 2.2. Genomic DNA extraction and polymerase chain reaction (PCR)

Total cellular DNA was extracted according to the manufacturer's instructions by using DNeasy Tissue kit (Qiagen, Hilden, Germany). The amplification of mtDNA was performed in Perkin-Elmer 2400 PCR thermocycler using the following conditions: 94 °C for 2 min (initial denaturation); 94 °C for 30 s, 60 °C for 30 s, 72 °C for 45 s (25 cycles); 72 °C for 10 min (final extension).

### 2.3. Quantitative gene expression analysis

In order to confirm gene expression levels, quantitative real-time RT-PCR (qRT-PCR) was carried out in Rotor Gene 2000 (Corbett Research, Mortlake, Australia) using SYBR-Green PCR Master Mix according to manufacturer's instructions (Qiagen, Valencia, CA). The comparative cycle threshold ($C_T$) method was used to analyze the data by generating relative values of the amount of target cDNA. Relative quantitation for any given gene, expressed as % variation over control, was calculated after determination of the difference between $C_T$ of the given gene A and that of the calibrator gene B (β-actin) in the depleted or reverted myocytes ($\Delta C_{T1} = C_{T1A} - C_{TB}$) and control myocytes ($\Delta C_{T0} = C_{T0A} - C_{TB}$) using the $2^{-\Delta\Delta C_{T(1-0)}}$ formula. $C_T$ values are means of triplicate measurements. Experiments were repeated three to five times.

### 2.4. Determination of 3OMG uptake

3OMG uptakes were measured as described previously [15]. Briefly, 50 μM [$^{14}$C]3OMG (4 μCi/ml, NEN Life Science) was added to Hepes-buffered saline solution and uptake allowed to occur for 15 s, a period over which 3OMG uptake is known to be linear.

### 2.5. Measurement of GLUT4myc translocation

The movement of myc-tagged GLUT4 to the cell surface was measured by an antibody-coupled colorimetric assay [16].