

# CHARACTERIZATION OF 2-DEOXY-D-GLUCOSE UPTAKE IN FIBROBLAST CULTURES DERIVED FROM PATIENTS WITH A3243G MITOCHONDRIAL DNA MUTATION

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**Abstract.** We investigated cellular glucose uptake of fibroblast cultures derived from seven patients with mitochondrial DNA (mtDNA) A3243G mutation and from six healthy controls with no mtDNA mutations. Heteroplasmy of fibroblast cultures were shifted by culturing for 5 days in galactose-containing medium. The proportion of mutant mtDNA decreased by 7.7% to 10% in three patient fibroblast cultures, whereas 2-deoxy-D-glucose uptake increased 1.8-2.1-fold at basal state, 1.9-2.3-fold in the presence of 60 ng/ml of insulin, and 1.8-2.1-fold in 100 ng/ml of insulin. No significant changes in level of heteroplasmy or glucose uptake were observed in the other patients samples and control samples. This study showed that alteration in the proportion of fibroblast mtDNA A3243G mutation content directly affected basal and insulin-stimulated glucose uptake.

## INTRODUCTION

Diabetes is a group of diseases characterized by the presence of chronic hyperglycemia. Pathophysiological mechanisms leading to diabetes involve a defect in insulin secretion, insulin resistance of liver, muscle and fat, or a combination of these two defects. The risk to an individual of developing diabetes involves complex interactions between genetic and environmental factors. In addition to mutations in the nuclear genome, abnormal mitochondrial function resulting from mitochondrial DNA (mtDNA) mutations can lead to diabetes (Ballinger *et al*, 1992).

An A to G mutation in the tRNA<sup>Leu(UUR)</sup> gene at nucleotide position (np) 3243 of mtDNA (termed A3243G mutation) has been observed

in about 80% of the patients presented with mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes (MELAS) (Wallace, 1992). The A3243G mtDNA mutation and diabetes were found to cosegregate in families with maternally inherited diabetes and deafness (MIDD) (Kadowaki *et al*, 1994) and this mtDNA mutation is a hallmark in the discovery of mitochondrial diabetes.

Mitochondria play an essential role in the maintenance of glucose homeostasis (Ballinger *et al*, 1992; van den Ouweland *et al*, 1992). MtDNA mutations can cause diabetes by affecting insulin secretion from pancreatic  $\beta$ -cells (Maechler and Wollheim, 2001). In addition, mitochondrial abnormalities can also cause insulin resistance in target cells (Lowell and Shulman, 2005). Investigations of the effects of mtDNA depletion on glucose metabolism demonstrated that intracellular ATP content, glucose-stimulated ATP production, glucose uptake, cellular activities of the glucose

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metabolizing enzymes, including hexokinase, were decreased (Park *et al*, 2001; de Andrade *et al*, 2006). Indeed, defects in adipocyte metabolism, which occur in conditions such as severe lipodystrophy, can result in the elevation in plasma free fatty acid concentration (Roden *et al*, 1996). It has become increasingly evident that defects in mitochondrial fatty acid oxidation can result in an inhibition of glucose transport and/or phosphorylation with subsequent reduction in rates of glucose oxidation and muscle glycogen synthesis and may be responsible for the more common forms of insulin resistance.

The impact of A3243G mutation on insulin secretion in diabetic patients has been examined in a number of studies (Velho *et al*, 1996; Maassen *et al*, 2004). It is hypothesized that a defect in glucose recognition is an early primary abnormality in carriers of the mutation (Velho *et al*, 1996). It has also been suggested that impaired mitochondrial metabolism in cells of individuals carrying mutations in the mitochondrial genome might predispose to  $\beta$  cell dysfunction (Lowell and Shulman, 2005).

To better understand the potential role of mitochondrial dysfunction in diabetes, we have studied the effect of mitochondrial DNA mutation on cellular glucose uptake in skin fibroblast cultures from patients with A3243G mtDNA mutation. Our study is based on the fact that fibroblast cultures from patients with mtDNA mutations grow poorly when glucose is replaced by galactose as the sole carbon source (Robinson *et al*, 1992), and percent heteroplasmy among cells is shifted and 2-deoxy-D-glucose uptake is observed in these cells compared with the same samples grown in normal glucose medium (Manfredi *et al*, 1999).

## MATERIALS AND METHODS

### Fibroblast cultures and culture conditions

A total of 13 fibroblast cultures were obtained from early subcultures at Neurogenetics

laboratory, Royal North Shore Hospital, Sydney, Australia. These fibroblast cultures were from seven MELAS patients harboring the A3243G mutation, and six healthy controls. The fibroblast cultures used were of 12<sup>th</sup> and 25<sup>th</sup> passage. Fibroblast cultures were routinely grown as monolayer cultures in 100x15 mm plastic culture dishes and cultured in high-glucose (4,500 mg/l D-glucose) Dulbecco's modified Eagle's medium (DMEM, GIBCO, Invitrogen Corporation Company) supplemented with 10% fetal bovine serum (GIBCO, Invitrogen Corporation Company), 5,000 U/ml of penicillin, 5,000  $\mu$ g/ml of streptomycin (GIBCO, Invitrogen Corporation Company), 44 mM sodium bicarbonate, and 20 mM HEPES at 37°C in 5% CO<sub>2</sub> incubator. Feeding schedule was conducted twice a week by removing of 5 ml of old DMEM medium from each Petri dish, adding an equal volume of new DMEM medium and then incubating at 37°C in 5% CO<sub>2</sub> incubator. When the fibroblast cells were confluent, fibroblasts were gently washed in 1 ml of phosphate-buffered saline (PBS) before the addition of 2 ml of 0.5% trypsin-EDTA per Petri dish. The dish was gently shaken, and then incubated at 37°C in 5% CO<sub>2</sub> incubator for 5 minutes. Adhesion of fibroblast cells were monitored under an inverted microscope. Cells were added with 0.5 ml of fetal bovine serum (FBS) to inhibit trypsin digestion. One half of DMEM medium was removed, and 5 ml of DMEM medium were added and the dish was gently agitated at 37°C in 5% CO<sub>2</sub> incubator.

### Fibroblast culture in galactose medium

Fibroblast cells ( $3 \times 10^5$  per dish) were resuspended in high glucose-DMEM medium in duplicate 60 mm tissue culture dishes and incubated overnight at 37°C in 5% CO<sub>2</sub> incubator. The medium in one dish was changed to DMEM (lacking glucose, GIBCO™) containing 10% FBS and 5,000 U/ml of penicillin, 5,000  $\mu$ g/ml of streptomycin, 20 mM galactose (Sigma) and 1 mM sodium pyruvate (Sigma), and the other dish was kept in the

same medium and both dishes were then incubated at 37°C in 5% CO<sub>2</sub> incubator. Following 5 days, the galactose medium was replaced with high glucose-containing DMEM medium and incubated at 37°C in 5% CO<sub>2</sub> incubator overnight. Fibroblast cells were harvested from the dishes and subcultured for 2-deoxy-D-glucose uptake assay and quantification of mtDNA mutation.

### 2-Deoxy-D-glucose uptake

Fibroblast cells were resuspended to a density of 3x10<sup>4</sup> cells/ml in duplicate in 24-well plates and incubated at 37°C in 5% CO<sub>2</sub> incubator overnight. Cell monolayer was rinsed 3 times with PBS and incubated at 37°C in 5% CO<sub>2</sub> incubator for 2.5 hours in 450 µl of Krebs buffer (115 mM NaCl, 4.7 mM KCl, 1.25 mM CaCl<sub>2</sub>, 1.2 mM KH<sub>2</sub>PO<sub>4</sub>, 25 mM NaHCO<sub>3</sub>, 1 mM MgSO<sub>4</sub>, 10 mM HEPES pH 7.4) (Strazzabosco *et al*, 1991) supplemented with 0.5% albumin bovine serum (Sigma) and 1 mM sodium pyruvate. Then, the indicated insulin concentrations (bovine pancreas cell, Sigma) were added and the cells were incubated for 20 minutes at 37°C in 5% CO<sub>2</sub> incubator. Cells were pulsed for 5 minutes with 1 µCi of 2-[1,2-<sup>3</sup>H(N)] deoxy-D-glucose (Perkin Elmer) in a final volume of 550 µl. The reaction was terminated by removal of the reaction medium and washing 3 times with ice-cold PBS. Then, 1 ml of 0.5% sodium dodecyl sulfate was added into each well, and after rotating the plates for 15 minutes, the solubilized cell materials were transferred to scintillation vial containing 3 ml of counting scintillator (Ultima Gold, PerkinElmer). Radioactivity was determined in a Packard Tri-Carb 2900TR liquid scintillation Analyzer. Data were expressed as cpm of [<sup>3</sup>H]-2-deoxy-glucose per 5 minutes per 3x10<sup>4</sup> cells. For each sample, uptake was assayed in two to four experiments.

### DNA extraction and quantification of mtDNA mutation

DNA was extracted from cell pellets harvested from both high-glucose and glucose-

free, galactose-supplemented DMEM culture using standard phenol-chloroform procedure. Quantification of the amount of A3243G mutation in fibroblast cultures was performed using hot last cycle PCR. PCR-RFLP analysis of mtDNA A3243G point mutation was conducted using oligonucleotide primers corresponding to mtDNA positions (5'→3') 3116-3134 (CCTCCCTGTACGAAAGGAC) and 3333-3353 (GCGATTAGAATGGGTACAATG). PCR conditions were: 94°C, 30 seconds; 55°C, 30 seconds; 72°C, 30 seconds for 35 cycles. The last cycle was carried out in the presence of [α-<sup>32</sup>P] dATP. A 238 bp DNA fragment encompassing nt 3243 was amplified and 15 µl of amplicons were digested with *Hae*III. DNA fragment containing a G at nt 3243 (mutant) is cleaved by *Hae*III into four fragments of 97, 72, 37 and 32 bp, whereas that containing an A at nt 3243 (wild type) produces three fragments of 169, 37 and 32 bp. The digestion products were electrophoresed in 12% nondenaturing polyacrylamide gel, and the amounts of the *Hae*III-digested DNA fragments were quantitated by scanning the gel in a Fuji-scanner (Fujifilm, Model FLA3000). For each sample, quantification of mtDNA mutation was assayed in duplicate.

### Statistical analysis

Values of [<sup>3</sup>H]-2-deoxy-glucose uptake and quantification of mtDNA mutation were expressed as the means ± S.E.M. Where applicable, significance of difference was analyzed using *t* test in MedCalc for Windows, version 7.2.1.0 (MedCalc Software, Mariakerke, Belgium, <http://www.medcalc.be>).

## RESULTS

### Fibroblast samples

A total of 13 fibroblast cultures were studied, 7 cultures from MELAS patients harboring heteroplasmic mtDNA A3243G mutation (P1-P7), and 6 control cultures with only wild-type mtDNA (C1-C6).

### Effect of galactose medium on the proportion of mitochondrial DNA mutation in heteroplasmic cells

To shift the heteroplasmic mutation in the fibroblast cultures, fibroblasts were cultured for 5 days in glucose-free medium containing galactose as the sole carbon source (Robinson *et al*, 1992). Floating cells in the medium were visualized by microscopy in P4 and P6 cell samples whereas all control cells remained attached to the plates (Fig 1). After 5 days of culture in galactose medium, the heteroplasmic level of A3243G mutation in the fibroblasts of each sample was measured by PCR-RFLP. The proportion of mutant mtDNA decreased 10, 8.2, 7.7, 2.2, 1.5, 1.2, and 0.4% in patient fibroblast cultures P4, P5, P6, P3, P7, P1, and P2, respectively (Table 1), whereas no mutation was detected in all control cells. Fibroblast

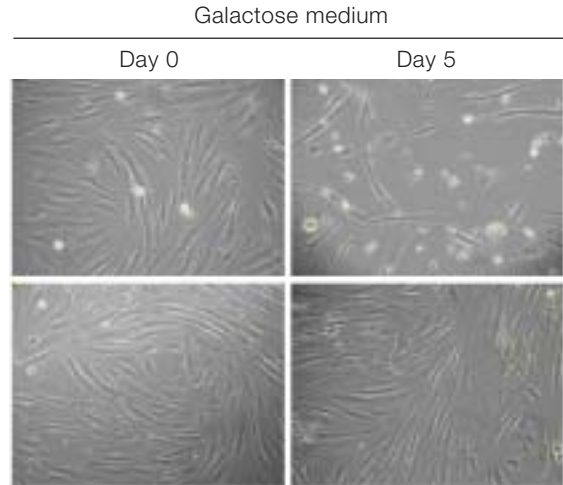


Fig 1—Morphology of fibroblast culture harboring heteroplasmic mtDNA A3243G mutation and control fibroblast culture. Day 0, before, and Day 5, after 5 days in galactose medium.

Table 1  
Percent mutant mtDNA of patient and control skin fibroblast cells cultured in normal medium and 5 days in galactose medium.

Fibroblast culture	Normal medium	Galactose medium
	%mutant mtDNA	%mutant mtDNA
P1	27.5 ± 1.3	26.3 ± 1.2
P2	59.9 ± 5.9	59.5 ± 6.4
P3	58.6 ± 1.3	56.4 ± 1.3
P4	77.5 ± 1.2	67.5 ± 1.1
P5	74.3 ± 0.4	66.1 ± 0.3
P6	84.5 ± 3.1	76.8 ± 1.6
P7	50.0 ± 5.5	48.5 ± 6.7
C1	0	0
C2	0	0
C3	0	0
C4	0	0
C5	0	0
C6	0	0

Each value is given as the mean ± SEM of duplicate experiments.

cultures P4, P5 and P6 had higher initial mutant mtDNA levels than others (77.5, 74.3 and 84.5%, respectively).

### 2-Deoxy-D-glucose uptake

To study the correlation between the amount of mutant mtDNA and 2-deoxy-D-glucose uptake, we employed 2-[1,2-<sup>3</sup>H(N)]deoxy-D-glucose for glucose uptake study in fibroblast cultures. Glucose uptake of all samples was determined in the absence and in the presence of insulin (60 ng/ml and 100 ng/ml insulin). Fig 2 shows that the 2-[1,2-<sup>3</sup>H(N)]deoxy-D-glucose uptake increased significantly in the same sample when the level of mutant mtDNA decreased (P4, P5 and P6). Glucose uptake of P4 fibroblast cultures increased 2.1-fold ( $p < 0.01$ ) at basal state, 2.3-fold ( $p < 0.05$ ) in the presence of 60 ng/ml of insulin, and 1.8-fold in 100 ng/ml of insulin; P5 fibroblast cultures increased 1.8-fold ( $p < 0.01$ ) at basal state, 2-fold ( $p < 0.001$ ) in the presence of 60 ng/ml of insulin, and 1.5-fold ( $p < 0.01$ ) in 100 ng/ml of insulin, and P6 fibroblast cultures increased 1.9-fold ( $p < 0.01$ ) at basal state, 1.9-fold ( $p < 0.01$ ) in 60 ng/ml of insulin, and 2.1-fold ( $p < 0.05$ ) in

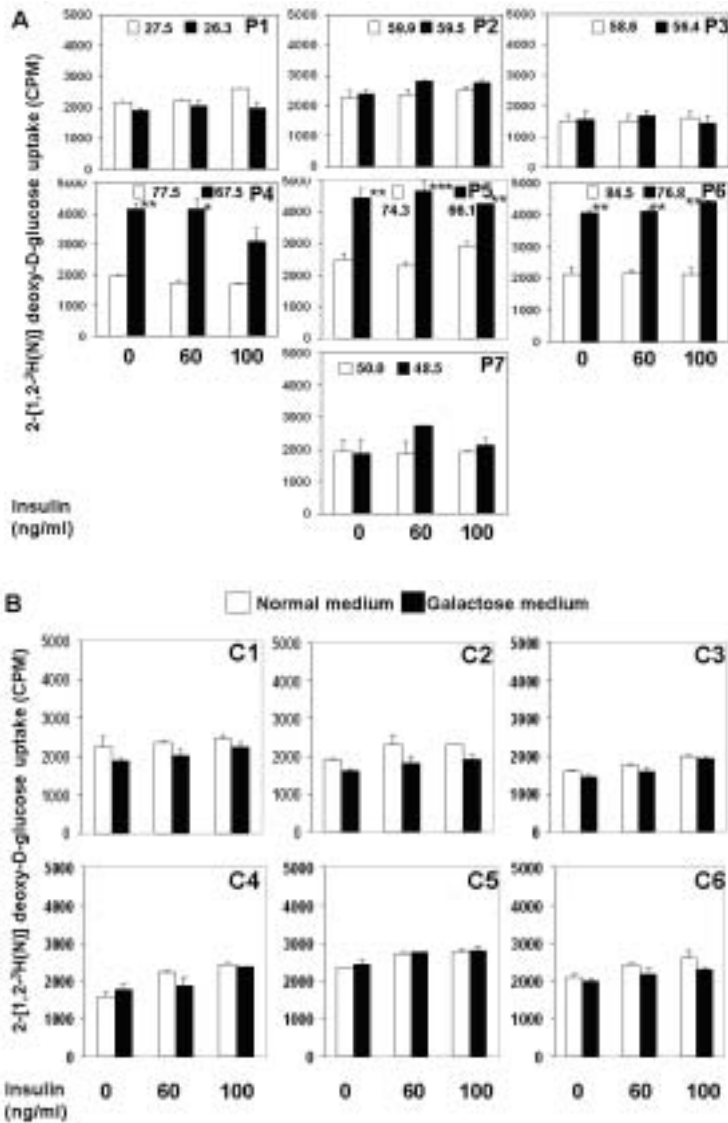


Fig 2—2-deoxy-D-glucose uptake of fibroblast cultures from MELAS patients and controls. Fibroblasts were grown in high glucose medium (open column) and for 5 days in galactose medium (solid column), in the absence (0) and in the presence of insulin (60 and 100 ng/ml) before performing uptake assay as described in Materials and Methods. The proportion of mutant mtDNA in the fibroblasts grown in each medium prior to the uptake experiment is indicated in the box, □ = normal medium, ■ = in galactose medium. Difference of uptake between fibroblast grown in galactose medium compared to normal medium was calculated using Student's *t*-test. (\*\*\*) =  $p < 0.001$ ; \*\* =  $p < 0.01$ ; \* =  $p < 0.05$ ). A, MELAS patients; B, control subjects.

100 ng/ml insulin. Glucose uptakes were not significantly different in the other patient and control samples. We observed that in the fibroblast samples containing original high levels of mutant mtDNA (P4, P5, P6), not only the levels of mutant mtDNA were significantly decreased when cultured in glucose-free medium containing galactose but also that 2-[1,2-<sup>3</sup>H(N)]deoxy-D-glucose uptakes were significantly increased.

## DISCUSSION

An A3243G mutation was demonstrated to be important for MIDD. Insulin secretion defect was reported to be the cause of the disease resulting from this mutation (Reardon *et al*, 1992; Kadowaki *et al*, 1994; Katagiri *et al*, 1994; Suzuki *et al*, 1994). However, insulin resistance was also found in patients with MIDD and the A3243G mutation (Katagiri *et al*, 1994; Kishimoto *et al*, 1995; Velho *et al*, 1996). This mutation may then be the cause of the defect in target organs involved in the transportation of glucose. The A3243G mutation detected in these diabetic patients occurs in heteroplasmic form and different patients exhibited differences in heteroplasmic levels. They also expressed different levels of disease severity. Thus, this study aimed to investigate the involvement of this mtDNA mutation in cellular glucose uptake function and to determine

whether the level of mutant mtDNA mutation played any role in this function.

To understand glucose transport and insulin action resulting from this mtDNA mutation, skin fibroblast cultures from patients with A3243G mtDNA mutation and from healthy controls with no known mtDNA mutation were examined. Although not playing any major role in the overall glucose homeostasis, skin fibroblasts represent a convenient system for this study since they can be cultured for several generations so that abnormalities in their functional properties can be attributed to genetic rather than environmental factors (Prince *et al*, 1981; Magre *et al*, 1988; Wells *et al*, 1993). This distinction cannot be as easily achieved for more regular insulin targets such as differentiated muscle tissue and adipocytes, where insulin resistance appears to be significantly contributed by secondary factors (Gulve *et al*, 1994; Zierath *et al*, 1994, 1996). Although fibroblast is not a major target organ for insulin-mediated glucose disposal *in vivo*, this system may be qualitatively reflective of insulin action to regulate glucose (Gulve *et al*, 1994).

In mammalian non-epithelial cells, facilitated transport of glucose is mediated by a family of stereo specific transport proteins known as glucose transporter (GLUT) proteins (Mueckler, 1994). GLUT expression is tissue and cell specific. GLUT1 is expressed ubiquitously and facilitates basal glucose transport in most cells (Mueckler, 1994) including fibroblast cells (Gulve *et al*, 1994). Thus, cultured fibroblasts from patients with genetically caused syndromes serve as a useful model to study the genetic contribution of defects in cellular insulin action (Kausch *et al*, 2000).

This study chose 2-deoxyglucose as the glucose analogue because it is recognized by GLUT1 and phosphorylated by hexokinase (Kimmich and Randles, 1976) but is not further metabolized owing to the absence of hydroxyl group, and thus allowing it to accumulate intracellularly and thus can be measured.

Since several factors are involved in cellular glucose uptake, it is best to use fibroblasts derived from the same patient but having different levels of mtDNA mutation in order to minimize those influencing factors. We utilized the phenomenon that fibroblast culture derived from patients with mtDNA mutation grow poorly when glucose is replaced by galactose as the sole carbon source to shift the level of heteroplasmy among cells (Robinson, 1996). Fibroblast samples containing more than 70% of mutant mtDNA tended to significantly reduce the proportion of mutant mtDNA when cultured in galactose medium. This observation can be simply explained by the fact that fibroblasts have their own threshold for mitochondrial dysfunction especially under stress conditions (using galactose as sole carbon source).

Fibroblast samples derived from the same patient but containing different percent heteroplasmy were then used to assay for 2-deoxy-D-glucose uptake both in the absence and presence of insulin.

Only fibroblasts that originally contained high levels of mutant mtDNA and the levels of which had decreased significantly after culturing in galactose media showed significant different levels of the glucose uptake between fibroblasts cultured in glucose and galactose media. These results confirmed that glucose uptake in fibroblast depends on the level of mtDNA heteroplasmy. Insulin (60 ng/ml and 100 ng/ml) had little effect in stimulating glucose uptake in control and patient fibroblast cultures. The clearance of circulating glucose depends on insulin-stimulated translocation of GLUT4 isoform to the cell surface (Pessin and Saltiel, 2000), but fibroblasts contain more GLUT1 than GLUT4. In addition, the response to insulin depends greatly on culture conditions (confluency, medium, carbon source) (Howard *et al*, 1979, 1981; Ishibashi *et al*, 1982; Cynober *et al*, 1986).

In summary, our results showed that al-

teration of the proportion of mutant mtDNA A3243G content directly affected basal and insulin-stimulated glucose uptake of fibroblasts from MELAS patients. These results support the previous study of Olsson *et al* (1998) in which the proportion of mitochondrial A3243G mutation is associated with a syndrome of diabetes and deafness.

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