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Influence of mitochondrial DNA level on cellular energy metabolism: implications for mitochondrial diseases

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Abstract The total amount of cellular mitochondrial DNA (mtDNA) varies widely and seems to be related to the nature and metabolic state of tissues and cells in culture. It is not known, however, whether this variation has any significance *in vivo*, and to which extent it regulates energy production. To better understand the importance of the cellular mtDNA level, we studied the influence of a gradual reduction of mtDNA copy number on oxidative phosphorylation in two models: (a) a control human cell line treated with different concentrations of 2′, 3′-dideoxycytidine, a nucleoside analogue that inhibits mtDNA replication by interfering with mitochondrial DNA polymerase γ, and (b) a cell line derived from a patient presenting mtDNA depletion. The two models were used to construct biochemical and phenotypic threshold curves. Our results show that oxidative phosphorylation activities are under a tight control by the amount of mtDNA in the cell, and that the full complement of mtDNA molecules are necessary to maintain a normal energy production level.

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Keywords Mitochondrial DNA depletion syndrome · Oxidative phosphorylation · Respiratory chain · Threshold effect

Abbreviations

mtDNA	mitochondrial DNA
OXPHOS	Oxidative phosphorylation
ddC	2′, 3′-dideoxycytidine
DAPI	4,6-diamidino-2-phenylindole
TMPD	*N*, *N*, *N*′, *N*′,-tetramethyl-*p*-phenylenediamine

Introduction

The role of mitochondria in mammalian cells is generally presented as a "central pathway" for energy metabolism, but mitochondria house many additional metabolic pathways and play a key role in apoptosis, free radical production, thermogenesis and calcium signaling (Wallace 1999). As a consequence, impairment of mitochondrial function is associated with a clinically heterogeneous group of human disorders, often referred to as mitochondrial cytopathies (Wallace 1999).

Mitochondria are eukaryotic intracellular organelles that have their own genome, a circular double-stranded molecule of about 16 kb (Taanman 1999). Mitochondrial DNA (mtDNA) exists as multiple copies per cell (Brown and Clayton 2002), but the total amount of mtDNA per cell differs widely between cell types, tissues and metabolic state (Bogenhagen and Clayton 1974; Robin and Wong 1988). It is not clear if this variation has any significance in vivo, especially on the energy production level through the oxidative phosphorylation (OXPHOS) enzymes, which are in part encoded by mtDNA. Williams et al. (1986) have reported that for mammalian striated muscle cells the

concentration of mtDNA is proportional to the oxidative capacity of the cell, while Shay et al. (1990) have shown that the fluctuation of mtDNA per cell may be related to cell growth conditions. Tissue-specific depletion of mtDNA has been documented in neonates and infants as a cause of a partial oxidative phosphorylation deficit and severe pathologies (Bakker et al. 1996; Blake et al. 1999; Bodnar et al. 1993; Ducluzeau et al. 1999; Mariotti et al. 1995; Moraes et al. 1991; Morris et al. 1998; Poulton et al. 1994), and decreases in mtDNA content have also been reported in other pathological conditions, such as hypoxia and cancer (Arnaudo et al. 1991; Duclos et al. 2004; Nouette-Gaulain et al. 2005; Wallace 2005). These observations emphasize the importance of the amount of mtDNA on OXPHOS activities and, consequently, on cellular metabolism.

Although the mtDNA variation effect on mitochondria activity has already been studied, only few mitochondrial parameters were analyzed and/or a few different mtDNA levels were used (Benbrik et al. 1997; Chen and Cheng 1989; Nelson et al. 1997). In order to better define the correlation between the mtDNA variation in cells and the mitochondrial bioenergetics, we used two different cell models that allowed us to have several cell lines with a wide range of mtDNA quantity: (a) control cell cultures in which mtDNA was depleted by 2′, 3′-dideoxycytidine (ddC) treatment and (b) cell cultures obtained from a patient affected by mtDNA depletion syndrome. We studied the effect of varying mtDNA quantities on the OXPHOS system by determining the enzyme activity of each respiratory chain complex, and by measuring respiration rates on permeabilized cells. We used a phenotypical and biochemical threshold curve (Rossignol et al. 2003) to determine precisely effects of mtDNA quantity on OXPHOS activity. Our data show that the total amount of mtDNA is one of the major parameters that regulates energy metabolism. Furthermore, in our two models, we observed that cells do not have a sizeable reserve of mtDNA molecules. Indeed, a slight decrease of the mtDNA amount in the cell may have dramatic consequences for mitochondrial respiration and, hence, energy production.

Experimental procedures

Patient The patient was the second child of healthy non-consanguineous parents. The family history was not contributory. The patient was born at full-term after a normal pregnancy (birth weight of 3,900 g). Soon after birth, he developed hypotonia and feeding difficulties. At the age of 14 weeks, failure to thrive was noted (weight loss of 400 g) and laboratory investigations revealed elevated activities of aspartate aminotransferase (5× upper normal limit), alanine aminotransferase (2× upper normal limit), creatine phosphokinase (3× upper normal limit) and aldolase (4× upper normal limit). Two weeks later, he was admitted to hospital where poor spontaneous movements were noted. Hypotonia was still present but tendon reflexes and consciousness were normal. Liver was palpable 3 cm below the costal margin. After 12 h of force-feeding, respiratory arrest with bradycardia occurred. He was incubated and required ventilation. Laboratory tests revealed hyperglycemia (17 mmol/L) with glycosuria and ketonuria. Blood lactate was 3.2 mmol/L and CSF lactate was 2.2 mmol/L. Urinary organic acids screening showed massive excretion of fumaric acid, α-ketoglutaric acid and 3-hydroxybutyrate. Plasma and urinary concentrations of amino acids were normal. EEG and magnetic resonance imaging of the brain were normal. A skeletal muscle biopsy showed increased lipid droplets. The histochemical reaction for cytochrome-*c* oxidase was absent in all fibers, but ragged-red fibers were not observed and succinate dehydrogenase staining was normal. Electron microscopic examination of the muscle tissue revealed an accumulation of mitochondria with abnormal size and shape. There was a progressive deterioration with the onset of seizures at the age of seven months. Tracheostomy and gastrostomy were deemed necessary. A sudden and fatal cardiac arrest occurred at the age of one year.

The polarographic study of permeabilized muscle fibers indicated that mitochondrial oxygen uptake was severely affected for all respiratory substrates in the patient compared to controls. This decrease in mitochondrial respiratory rate was coupled with a notable decline in mitochondrial ATP synthesis, associated with a low [produced ATP]/[consumed oxygen] ratios when pyruvate+ malate or succinate were used as substrate. The spectrophotometric study of muscle homogenates revealed that the succinate dehydrogenase activity of the patient's muscle did not differ from control tissues. Activities of all respiratory chain enzyme complexes containing mtDNA-encoded subunits were, however, significantly decreased in the patient's muscle compared to controls. mtDNA in the patient's muscle tissue, determined by Southern Blot, was barely discernable on the blot (<1% of control levels).

Cell cultures Patient and control lymphoblastoid cell lines were generated by Epstein Barr Virus transformation of blood lymphocytes at the Généthon Laboratory in Paris. Lymphoblastoid ρ^0 cells WAL-2A (Trounce et al. 1996) were a gift from Prof D.C. Wallace. All cell lines were cultured in RPMI 1640 medium, complemented with 100 U/ml of penicillin, 100 μg/ml of streptomycin, 10% of hi-FCS, 50 μg/ml of uridine and 1 mM pyruvate. Cultures were grown at 37 °C in a humidified atmosphere of 5% CO_2 in air. ddC (Sigma) was added to the cell culture medium at the desired final concentration. Growth curve were determined by counting living cells stained with trypan blue.

DNA analysis Total genomic DNA was isolated from homogenates of muscle tissue and ~10^5 lymphoblastoid cells according to standard procedures. Approximately 5 μg of DNA was digested with either *Bam*H1 or *Pvu*II. Samples were resolved on 0.7% agarose gels by electrophoresis, transferred to nylon membranes and hybridized simultaneously with ^{32}P-labeled probes for mtDNA and the nuclear 28S rRNA gene (Moraes et al. 1991). Signals were quantified with a PhosphorImager™ using ImageQuant™ software (Molecular Dynamics, Inc.). To correct for quantitative variations among the samples, mtDNA signals were normalized relative to nuclear DNA signals.

Biochemical studies Permeabilized cells (10^8 lymphoblastoid cells) were used to simultaneously monitor oxygen consumption polarographically and ATP production by bioluminometry. Muscle homogenates were prepared to measure respiratory chain enzyme activities spectrophotometrically (Letellier et al. 1992; Ouhabi et al. 1998). We also used whole cell homogenates because of the low number of cells obtained after ddc treatment. No complex I activity was determined due to the difficulty in obtaining reliable values with cell homogenates. Normalization was done by SDH activity. We also normalized by citrate synthase (C.S.) activity, but results obtained were the same as SDH normalization. To have the clearest results we decided to take in account only the SDH normalization.

Construction of the threshold curves Phenotypic threshold curves for mtDNA (Wallace 1986) were obtained by plotting the state 3 respiratory rate value as a function of the measured mtDNA amount. Biochemical threshold curves (Rossignol et al. 1999) for respiratory chain complex IV were obtained by plotting the state 3 respiratory rate value as a function of the measured complex IV activity.

Results

Control lymphoblastoid cell cultures with different depleted levels of mtDNA obtained by ddC treatment

Characterization of treated cell cultures

In order to obtain cell cultures with various levels of mtDNA depletion, we treated a control lymphoblastoid cell line with different concentrations of ddC and followed the effect of the treatment during 2 weeks. For each ddC concentration, the growth rate and mtDNA amount were measured. At concentrations between 0 and 10 μM, ddC treatment did not affect cell proliferation (Fig. 1), but did have a dramatic effect on mtDNA levels (Fig. 2). The lack of an effect on growth rate

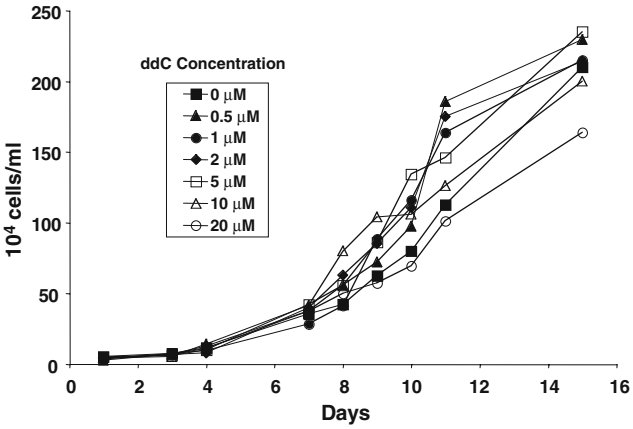

Fig. 1 Effect of ddC treatment on the proliferation of a control lymphoblastoid cell line. ddC was added to the culture media for 15 days and media was changed every 2 days to maintain a constant ddC concentration

can be explained by the fact that the cells were cultured in the presence of pyruvate and uridine, which allows OXPHOS deficient cells to grow normally (King and Attardi 1996). In contrast to cell cultures treated with ≤10 μM ddC, we did observed a slight decrease in growth rate for cell cultures treated with 20 μM of ddC. This may be due to cytotoxicity of ddC at high concentrations.

We visualized the ddC effect by plotting the relative mtDNA quantity contained in cells treated with different concentrations of ddC as a function of the time (Fig. 3). The mtDNA amount decreased gradually over time in a dose-dependent fashion during the first 8 days of treatment and then reached a level which remained constant during the remaining 7 days of treatment. Thus, by treating cell cultures with different concentrations of ddC, we were able to obtain cultures with different, stably depleted levels of mtDNA. At high concentrations of ddC, the decrease of mtDNA reached a minimum value of 20% of normal levels (Fig. 4). This suggests that, by treatment with ddC, it is not

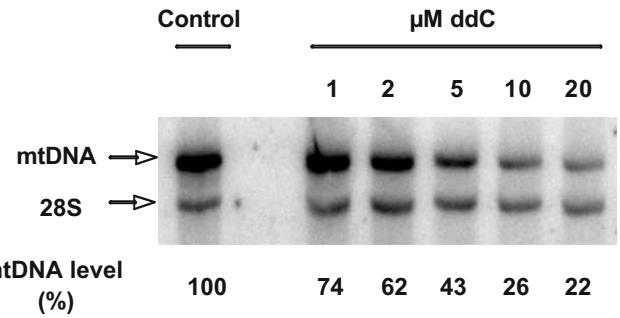

Fig. 2 Autoradiogram of a Southern blot of *Pvu*II-digested DNA from a control lymphoblastoid cell line hybridized with mitochondrial and nuclear probes. DNA, isolated from cell cultures treated with 1 to 20 μM ddC for 15 days (*lanes 1* to *20*), is compared with an untreated culture (*control*). Signals for mtDNA and nuclear DNA (*28S*) are indicated. For each cell culture, the mtDNA signal relative to the nuclear DNA signal is indicated in percentages relative to the untreated cell culture (100%)

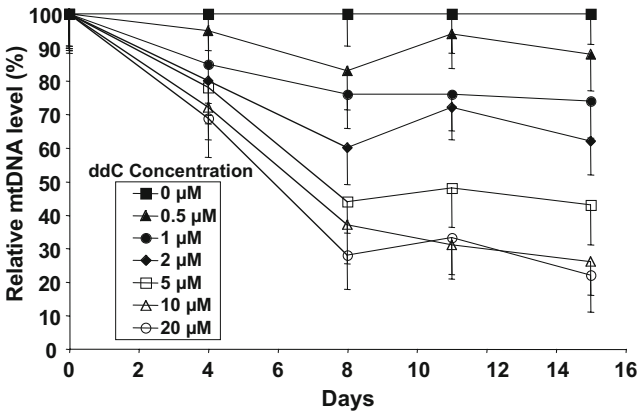

Fig. 3 Relative mtDNA content of a control lymphoblastoid cell line during exposure to different concentrations of ddC. For each time point, three measurements of the level of mtDNA were carried out. Levels of mtDNA are expressed relative to the level in untreated cells (100%)

possible to obtain cell cultures that are depleted more than 80%. Brown and Clayton have reported a similar observation (Brown and Clayton 2002). In our study, we reached this minimum value at a ddC concentration of 10 µM. The ddC concentration needed to obtain a 50% decrease of the amount of mtDNA was close to 2 µM.

Effect of mtDNA depletion on energy production

For all cell cultures treated for 15 days with different ddC concentrations, we measured mitochondrial O_2 consumption on permeabilized cells, using succinate and ascorbate + TMPD as energy substrates. We also determined the enzyme activities of complexes III and IV of the mitochondrial respiratory chain, and of succinate dehydrogenase. The results of these two studies are summarized in Tables 1 and 2, and are expressed as a percentage of the control value. While the succinate dehydrogenase activities were unchanged, the respiratory flux, as well as the

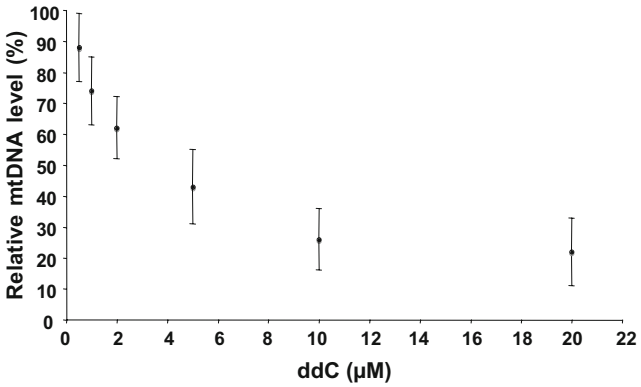

Fig. 4 Quantity of mtDNA in control lymphoblastoid cells versus the ddC concentration used during their treatment. All measurements were performed in triplicate after 15 days of treatment. The quantity of mtDNA is expressed as a percentage relative to untreated cells

Table 1 Biochemical characterization and mtDNA levels of treated control lymphoblastoid cells for different ddC concentrations

ddC[a]	Complex activities[b]			
	SDH	Complex III	Complex IV	mtDNA[c]
0.5	99.6±10.9	100.5±3.1	94.5±7.9	88±11
1	91.7±11.7	76.7±5.1	78.9±5.3	74±11
2	101.3±9.4	79.9±8.9	88.9±4.1	62±10
5	96.5±16.9	70.3±8.3	65.4±11.3	43±12
10	103.6±7.4	53.1±8.9	57.1±7.4	26±10
20	98.6±9.1	47.8±5.2	51.7±7.1	22±11

Results are presented as mean ± SD (n=3)
[a] ddC concentration in micromolar
[b] % of untreated cells
[c] Signal of mtDNA relative to signal of nuclear DNA on Southern blot

complex III and IV activities, showed a ddC dose-dependent decrease similar to the dose-dependent decrease in mtDNA levels. However, the correlation between mtDNA and OXPHOS levels was not linear.

To study this relationship in more detail, we constructed phenotypic and biochemical threshold curves. The phenotypic curve is used to depict how a mtDNA defect (mutation, deletion or depletion) affects the phenotype of a cell culture or tissue (Wallace 1986). We used the mitochondrial respiratory rate as phenotypic parameter, because the respiratory rate is one of the main factors that describes the yield of OXPHOS. The phenotypic threshold curve showed a small but clear deviation from a direct linear relationship between the mtDNA level and the respiratory rate (Fig. 5). For instance, a 50% decrease of mtDNA led only to a 30% decrease of the respiratory rate. This deviation indicates the existence of a threshold effect, and suggests that there is a certain compensation for the reduced mtDNA content at the level of respiration.

The biochemical threshold curve describes how a respiratory chain complex activity defect may affect the mitochondrial respiratory rate (Letellier et al. 1994). By plotting the respiratory rate as a function of the complex IV activity, one is able to determine the extent of a biochemical threshold effect (Rossignol et al. 2003). We obtained a straight line (Fig. 6), which indicates that for mtDNA depletion, there is no biochemical threshold. We obtained the same straight line by using complex III activity (Fig. 7). This means that complex III and IV activities are directly related to the respiratory rate.

Patient lymphoblastoid cell line

mtDNA analysis and biochemical characterization

In addition to the control lymphoblastoid cell line, we studied a patient lymphoblast cell culture with depleted

Table 2 Polarographic characterization of treated control lymphoblastoid cells for different ddC concentration

ddC[a]	Succinate			Ascorbate + TMPD		
	Oxygen[b]	ATP[c]	ATP/O[d]	Oxygen	ATP	ATP/O
0.5	95.4±5.0	94.5±14.9	101±24	87.3±3.4	93.6±13.8	111±14
1	85.9±8.3	86.8±13.5	108±23	74.8±10.4	76.2±15.3	103±13
2	85.6±5.5	84.1±14.6	104±25	84.9±3.4	86.1±12.4	104±24
5	72.9±3.8	71.7±15.7	105±21	59.6±4.3	62.4±11.0	109±20
10	47.9±4.9	45.7±14.8	106±26	55.6±3.9	55.3±13.2	101±22
20	46.8±3.2	44.2±14.1	98±23	43.7±1.8	40±12.1	97±21

Results are presented as mean ± SD (n=3)
[a] ddC concentration in micromolar
[b] Oxygen uptake in state 3 respiration, expressed as % of untreated cells
[c] ATP synthesis as % of untreated cells
[d] ATP synthesized divided by oxygen consumed in state 3 respiration

levels of mtDNA. The patient cells contained mtDNA of the same size as control cultures and no mtDNA mutations known to be involved in mitochondrial diseases were found, but the mtDNA signal relative to the nuclear DNA signal was 43% of the mean control value in the earliest investigated cell passage. In later cell passages, the levels of mtDNA progressively increased and became similar to control values after cell passage 14 (Table 3).

Permeabilized cells of early cell passages of the patient's lymphoblastoid culture showed a decrease in oxygen consumption and ATP synthesis compared to control cultures (Table 4). In contrast, later cell passages exhibited normal oxygen consumption and ATP synthesis leading to a normal [produced ATP]/[consumed oxygen] ratio (Table 4). In spectophotometric assays, cell homogenates of early cell passages of the patient showed a significant decrease in succinate dehydrogenase activity, and the activities of the respiratory chain enzyme complexes, whereas in later cell passages, activities of all respiratory chain complexes were similar to control values (Table 3). Indeed, we can assume that SDH activity, like citrate synthase (C.S.) activity is an indicator of the mitochondrial content in the cell.

Effect of mtDNA depletion on energy production

Our results show that the decrease of mtDNA causes a decrease in mitochondrial respiration and enzyme activities of the respiratory chain, and that succinate dehydrogenase activity is decreased in parallel with the activities of the respiratory chain. The phenotypic threshold curve (Fig. 8) shows that there is a very strong proportional dependence of mitochondrial respiration on the number of mtDNA copies. Indeed, there is a nearly linear relation between these two parameters between 60% to 100% of mtDNA. Below 60% of normal levels, mtDNA depletion causes a dramatic decrease of the respiratory rate. The relation

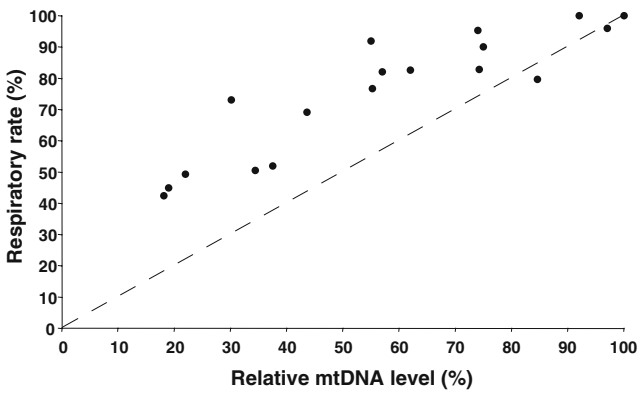

Fig. 5 Phenotypical threshold curve of ddC-treated control lymphoblastoid cells. The respiratory rate and quantity of mtDNA are both expressed as a percentage relative to untreated cells. Each point represents one measurement. A *straight dotted line* was drawn to show a putative direct linear relationship between the two parameters

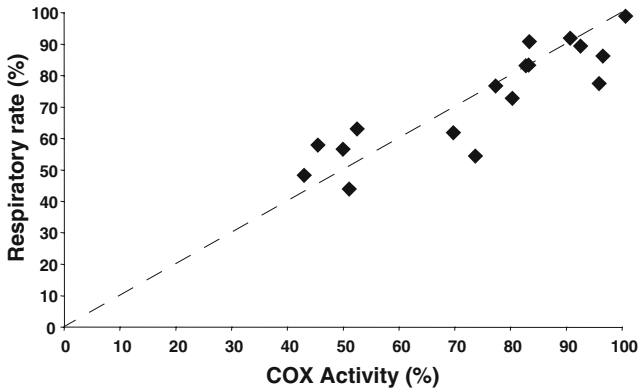

Fig. 6 Complex IV biochemical threshold curve of ddC-treated control lymphoblastoid cells. The respiratory rate and complex IV activity are both expressed as a percentage relative to untreated cells. Each point represents one measurement. A *straight dotted line* was drawn to show a putative direct linear relationship between the two parameters

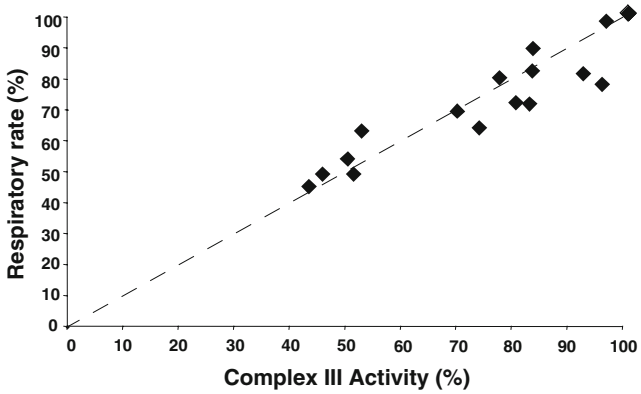

Fig. 7 Complex III biochemical threshold curve of ddC-treated control lymphoblastoid cells. The respiratory rate and complex III activity are both expressed as a percentage relative to untreated cells. Each point represents one measurement. A *straight dotted line* was drawn to show a putative direct linear relationship between the two parameters

between complex IV activity and mitochondrial respiration follows a linear relationship, indicating the absence of a biochemical threshold effect (Fig. 9). Similar results were obtained for complex III (Fig. 10).

Discussion

As mentioned in the introduction, the amount of mtDNA varies according to the nature and metabolic state of a particular tissue. This property not only raises the question of the physiological importance of these variations, but also of the role of the amount of mtDNA in the regulation of cellular metabolism, and more particularly in the regulation of mitochondrial energy metabolism.

The purpose of this study was to vary mtDNA amounts in various cell cultures and to study respiration and enzyme activities of the respiratory chain of these cultures in order to determine to what extent mtDNA copy number affects mitochondrial energy metabolism. We visualized the effect in phenotypic and biochemical threshold curves. These curves allow us to characterize and to quantify a relationship between the amount of mtDNA and mitochondrial respiratory or activities of the complexes of the respiratory chain (Rossignol et al. 2003).

Our study was conducted on two different cell culture models in which the decrease of mtDNA was obtained either by inhibiting mtDNA replication with ddC, or by selecting various lymphoblastoid cell culture passages from a patient presenting mtDNA depletion. In the ddC-treated cultures, the decrease in the quantity of mtDNA was dose-dependent. Moreover, for ddC concentration between 0 and 10 μM, we found a degree of depletion, which was stable after 8 days of treatment, with no effect on cell growth (Fig. 1). This ddC effect is in agreement with data obtained previously in several laboratories (Brown and Clayton 2002; Chen and Cheng 1989; Nelson et al. 1997).

Regarding OXPHOS activities, we noticed that ddC treatment leads to a similar inhibition of complexes III and IV (Table 2). In addition, we observed a decrease of respiratory flux and a reduction of ATP synthesis with succinate and ascorbate + TMPD as substrates, but succinate dehydrogenase activity remained unchanged. As ATP synthesis and O_2 uptake decreased in parallel, the ATP/O ratio remained constant for each ddC concentration used, and was similar to that obtained for the control cell line. Thus, it appears that the ddC-induced decrease of mtDNA leads to decreased activities of the respiratory

Table 3 Biochemical characterization and mtDNA levels of lymphoblastoid cell from a patient presenting with mtDNA depletion at different cell culture passages

	Complex activities[a]			mtDNA	
	SDH	Complex III	Complex IV	Ratio mtDNA/nDNA[b]	Percentage
Lymphoblastoid cells					
Control	19.5±5.9	28.08±7.41	30.03±1.95	1.36±0.25	
Patient cells					
Passage 6	12.4±4.72	9.59±6.19	8.23±2.26	0.58±0.17	43±13
Passage 9	13.36±2.64	19.91±4.32	13.28±1.87	0.72±0.16	53±12
Passage 12	13.76±3.45	21.06±3.56	19.85±2.38	0.91±0.21	67±15
Passage 14	14.37±3.47	19.68±2.68	25.53±1.69	1.02±0.24	75±18
Passage 17	17.4±5.78	24.71±5.67	24.97±1.78	1.34±0.14	98±10
Passage 20	18.84±2.84	27.07±2.84	25.01±2.48	1.22±0.18	90±13
Passage 29	16.7±3.98	22.71±7.64	26.72±2.82	1.17±0.25	86±18
Passage 33	19.24±2.69	27.52±2.94	27.3±1.83	1.29±0.22	95±16

Results are presented as mean ± SD (n=3)
[a] Enzyme activities were calculated in nanomol substrate per minute per milligram protein.
[b] Signal of mtDNA relative to signal of nuclear DNA on Southern blot

Table 4 Polarographic characterization of lymphoblastoid cell from a patient presenting with mtDNA depletion at different cell culture passages

	Succinate			Ascorbate + TMPD		
	Oxygen[a]	ATP[b]	ATP/O[c]	Oxygen	ATP	ATP/O
Lymphoblastoid cells						
Control	4.92±0.39	7.39±1.60	1.39±0.17	7.89±1.96	4.15±2.91	0.72±0.25
Patient cells						
Passage 6	1.03±0.43	0.55±0.11	0.53±0.04	1.52±0.51	1.11±0.49	0.72±0.08
Passage 9	2.11±0.28	3.18±1.75	0.60±0.15	4.57±1.57	2.41±2.73	0.42±0.27
Passage 12	3.47±0.34	5.21±1.82	0.98±0.22	5.68±1.41	2.99±3.55	0.52±0.21
Passage 14	3.63±0.37	5.47±1.92	1.03±0.19	6.28±1.72	3.32±3.12	0.56±0.30
Passage 17	3.93±0.31	6.97±1.73	1.65±0.18	7.92±0.28	3.94±2.22	0.84±0.29
Passage 20	4.75±0.32	6.72±1.87	1.45±0.25	8.24±1.69	4.78±3.21	0.65±0.33
Passage 29	4.50±0.27	6.69±1.52	1.51±0.34	9.02±1.38	5.23±4.39	0.57±0.31
Passage 33	4.85±0.29	7.21±1.69	1.35±0.28	7.62±1.27	4.20±2.88	0.75±0.23

Results are presented as mean ± SD (n=3)
[a] Oxygen uptake in state 3 respiration, in nanoatoms per minute per 10^6 cells
[b] ATP synthesis in nanomol per minute per 10^6 cells
[c] ATP synthesized divided by oxygen consumed in state 3 respiration

chain enzyme complexes III and IV, that are in part encoded by the mtDNA, but does not lead to a decrease in the mass of mitochondria, because succinate dehydrogenase activity was not affected. Although there are very few reports that investigated the ddC effect in the mitochondrial metabolism, our results are directly comparable to those of Benbrik and colleagues (Benbrik et al. 1997). In their article, the authors show a 30% decrease of complex IV activity in myoblasts treated for 10 days with 1 μM ddC and a 50% decrease in activity in myoblasts treated with 10 μM ddC.

In our second model, we took advantage of the drift to normal mtDNA levels of a patient lymphoblastoid cell line to obtain lymphoblastoid cell passages with different levels of mtDNA (43% to 100%). To investigate the importance of mtDNA levels, we simultaneously measured the amount of mtDNA, mitochondrial respiration and respiratory complex enzyme activities in subsequent cell passages. The results we obtained showed a clear increase of the total of mitochondrial OXPHOS activities that matched the increasing levels of mtDNA in subsequent cell passages (Tables 3 and 4). The increase of succinate dehydrogenase activity in patient cells (Table 3) suggests that the mutation responsible for mtDNA depletion may, in addition, lead to reduce mass of mitochondria per cell.

In order to quantify the effects of mtDNA decrease on mitochondrial respiration and energy metabolism, we

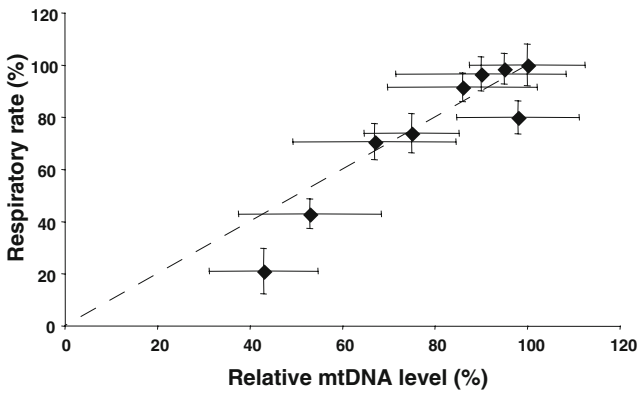

Fig. 8 Phenotypical threshold curve of lymphoblastoid cell cultures from a patient presenting mtDNA depletion. The respiratory rate and quantity of mtDNA in the patient cells are both expressed as a percentage relative to control lymphoblastoid cells. Each point is the mean of three different measurements. A *straight dotted line* was drawn to show a putative direct linear relationship between the two parameters

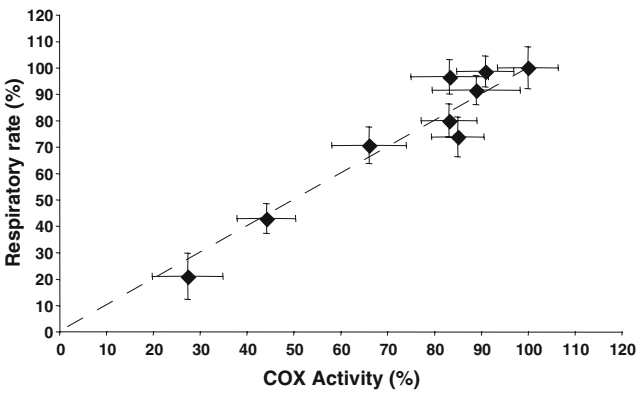

Fig. 9 Complex IV biochemical threshold curve of lymphoblastoid cell cultures from a patient presenting with mtDNA depletion. The respiratory rate complex IV activity in the patient cells are both expressed as a percentage relative to control lymphoblastoid cells. Each point is the mean of three different measurements. A *straight dotted line* is drawn to show a putative direct linear relationship between the two parameters

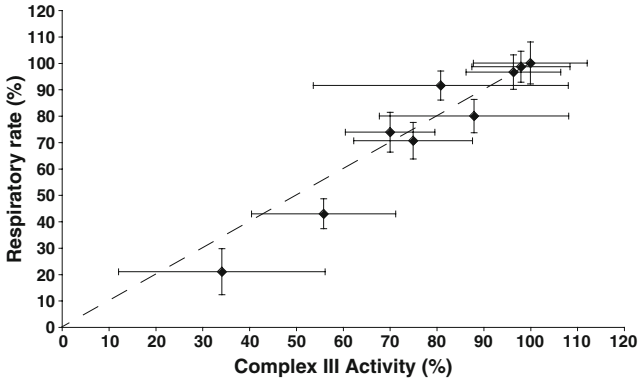

Fig. 10 Complex III biochemical threshold curve of lymphoblastoid cell cultures from a patient presenting mtDNA depletion. The respiratory rate complex III activity in the patient cells are both expressed as a percentage relative to control lymphoblastoid cells. Each point is the mean of three different measurements. A *straight dotted line* is drawn to show a putative direct linear relationship between the two parameters

constructed phenotypic and biochemical threshold curves. The phenotypic threshold curves revealed that the effect of the relative mtDNA level on respiratory rate was greater for the patient cell culture than for the culture treated with ddC (compare Fig. 5 with Fig. 8). This difference may be due to the mechanism leading to the mtDNA depletion. In the patient cell line, the decrease of the mtDNA copy number is combined with a decrease in the mass of mitochondria, while in the ddC treated cell line, only a decrease of the mtDNA copy number was observed. Thus, in the patient cell line, the decrease of mitochondrial mass appears to exacerbate the effect of the amount of mtDNA on mitochondrial respiration. In the phenotypic threshold curve obtained with the ddC-treated cells, we did not obtain a straight line but a slight curvature. Thus, there is no direct correlation between mtDNA quantity and its effect on the respiratory flux, aside from a limited partial compensation of the mtDNA quantity decrease versus the respiratory flux. Figure 5 shows that a ddC-induced mtDNA depletion of 60% results in a 20% reduction of mitochondrial respiration. A mtDNA point mutation in a gene coding for a respiratory chain complex subunit results in a similar compensation, but at a higher level (Rossignol et al. 2000). The effect of the point mutation can be compensated by a biochemical threshold effect. This means that the activity of a particular OXPHOS complex can be considerably decreased with no effect on either O_2 consumption or mitochondrial ATP synthesis. This phenomenon, which can be explained by the presence of an activity backup for most of the oxidative phosphorylation complexes, is shown to be of major importance for the shape of the phenotypic threshold curves for mtDNA point mutations (Faustin et al. 2004; Rossignol et al. 1999). Because we found some compensation in the phenotypic threshold curve for the ddC-treated cells, we wanted to know if this was due to the presence of a biochemical threshold effect as described for mtDNA point mutations (Rossignol et al. 1999). The results, illustrated in Figs. 6 and 7, show a linear relation between the activity of complex IV and III, respectively, and the rate of respiration with succinate as substrate. This implies that there is no threshold effect at the biochemical level. However, the threshold effect observed at the phenotypic level should be a combination of several factors occurring at each level of mtDNA expression (Rossignol et al. 2003). The absence of a biochemical threshold effect suggests that the low compensation observed in the ddC-treated cells is located upstream of the biochemical expression, e.g. at the transcription or translation level, but it could also be due to the presence of low level of excess mtDNA molecules in the cell. This compensation may vary depending on the tissue and the metabolic state studied (Taanman et al. 2003; Van den Bogert et al. 1993).

For point mutations affecting mitochondrial tRNA genes, and also for deletions of mtDNA, the phenotypic threshold effect is essentially localized at the level of mitochondrial translation (Hayashi et al. 1991; Lecher et al. 1994). However, in the case of mutations that result in a decrease in the quantity of mitochondrial protein encoding genes but not tRNA genes, it seems that mitochondrial metabolic flux is decreased only when the quantity of the encoded proteins becomes lower than a certain threshold value. For instance, Spelbrink et al. (1994) showed that for subunit II of complex IV this value is at least equal to half of the normal quantity. In the case of mtDNA depletion there would be a minimal quantity of mtDNA copies required to allow sufficient translation of mitochondrial proteins (Spelbrink et al. 1994). According to our results, this mtDNA quantity is very close to the amount actually present in cells, hence the presence of a low threshold effect. Therefore, we believe that the variations in the amount of mtDNA, which are observed in different tissues and under different metabolic conditions, play a fundamental role in vivo. They probably represent an adaptation of the mitochondrial metabolism to cellular metabolism. We think that this mechanism is in addition to the metabolic adaptations, which were revealed during the description of the biochemical threshold effects (Rossignol et al. 1999).

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