Short Communication

Reversible Mitochondrial Respiratory Chain Impairment During Symptomatic Hyperlactatemia Associated with Antiretroviral Therapy

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ABSTRACT

Direct evidence confirming the hypothesis that a dysfunction of the mitochondrial respiratory chain (MRC) underlies the pathogenesis of hyperlactatemia associated with highly active antiretroviral therapy (HAART) is scarce. We studied mitochondrial DNA (mtDNA) content and MRC function in the skeletal muscle of an HIV-infected patient during an episode of symptomatic hyperlactatemia. Skeletal muscle biopsy was performed during the episode when the patient was symptomatic and 3 months later when the patient was clinically recovered. Assessment of mitochondria was performed using histological, polarographic, spectrophotometrical, and Southern blot and real time PCR DNA quantification methods. The histological study disclosed extensive mitochondrial impairment in the form of ragged-red fibers or equivalents on oxidative reactions. These findings were associated with an increase in mitochondrial content and a decrease in both mitochondrial respiratory capacity and MRC enzyme activities. Mitochondrial DNA content declined to 53% of control values. Mitochondrial abnormalities had almost disappeared later when the patient became asymptomatic. Our findings support the hypothesis that MRC dysfunction stands at the basis of HAART-related hyperlactatemia.

PERLACTATEMIA is observed in up to 10% of HIV-infected patients on antiretroviral therapy. In most cases, blood lactate elevation is usually mild (less than 2-fold) and patients remain asymptomatic. With higher plasma lactate concentration, patients may develop fatigue, weakness, abdominal pain, weight loss, tachycardia, and/or exertional dyspnea. Occasionally, hyperlactatemia may lead to severe lactic acidosis and death.1,2

Due to their central role in intermediary metabolism, mitochondria could participate in such a metabolic imbalance. Despite the fact that mitochondrial dysfunction has been widely investigated in other complications of antiretroviral therapy (as zidovudine-related myopathy3 and lipodystrophy syndrome4), few works have directly studied mitochondrial respiratory chain (MRC) function in patients with hyperlactatemia.5–8 Although a recent study9 has demonstrated mtDNA depletion in peripheral blood mononuclear cells (PBMCs) obtained from patients with hyperlactatemia, the clinical repercussion of such a depletion is uncertain because any abnormality in mitochondrial DNA (mtDNA) in a pathogenic role must necessarily cause overt MRC dysfunction.10

A 50-year-old HIV-1-infected man was admitted in May 2001 because of malaise, fatigue, weight loss, increasing abdominal discomfort, and dyspnea for the previous 6 weeks. He had been diagnosed with HIV infection in 1988 and treated with

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antiretroviral drugs for the past 8 years (including zidovudine for 55 months, zalcitabine for 12 months, didanosine for 55 months, stavudine for 55 months, saquinavir for 20 months, lamivudine for 12 months, ritonavir for 19 months, nelfinavir for 28 months, and nevirapine for 12 months). At the time of the study, he had been treated with stavudine 40 mg/12 hr, didanosine 400 mg/24 hr, indinavir 800 mg/12 hr, ritonavir 100 mg/12 hr, gemfibrozil 600 mg/12 hr, and allopurinol 300 mg/24 hr for at least the previous 6 months. Body fat changes consisting of abdominal accumulation, gynaecomastia, and lipatrophy, as well as hypertriglyceridaemia and hyperuricemia, had been noticed in February 1999. On admission, the patient was cachectic. Serum lactate was 49 mg/dl (normal: 5–22), but there was no academia (pH 7.39, bicarbonate 21 mmol/liter). He had mild abnormalities in liver and pancreas function tests. Serological markers for hepatitis C and B viruses were negative. CD4 cell count was 450/mm$^3$ and HIV-1 RNA was <20 copies/ml. The patient was considered to have symptomatic hyperlactataemia. A first muscle biopsy was indicated, and all antiretroviral drugs were immediately withdrawn. The condition of the patient progressively improved and serum lactate and liver and pancreas tests returned to normal concentrations 3 months after discontinuation of antiretroviral therapy. At this time, when the patient was asymptomatic and was not receiving antiretroviral drugs, a second muscle biopsy was performed.

Four samples of quadriceps were obtained in each biopsy. One was used for histological studies as explained elsewhere.$^{11}$ A second sample was used to spectrophotometrically measure enzyme activities of complexes I to V of MRC on muscle homogenate.$^{12}$ Results were corrected by citrate synthase activity in order to normalize for mitochondrial content.$^{12}$ The third sample was used to polarographically determine oxygen consumption on fresh mitochondrial suspension$^{13}$ using pyruvate-malate, succinate, and ascorbate as substrates of complexes I, II, and IV of MRC, respectively.$^{14}$ Total DNA was extracted by standard phenol-chloroform procedures from the fourth sample.

Rearrangements in mtDNA were examined by Southern blot hybridization using a mitochondrial ND4 gene as the probe.$^{15}$ For mitochondrial DNA quantification, the nuclear 18S rRNA housekeeping gene and the highly conserved mitochondrial ND2 gene were quantified separately by real time quantitative polymerase chain reaction (PCR) (LightCycler FastStart DNA Master SYBR Green I, Roche Molecular Biochemicals, Mannheim, Germany). The PCR amplification of a 500-bp fragment length of the 18S rRNA gene was performed by using the forward 5'-ACGGACCAAGCGGAACGAT-3' and the reverse 5'-GGACATCTAAAGGGCATAG-3' primers. For the mitochondrial ND2 gene, the forward 5'-GGCCCTAGAAATATATGCTA-3' and the reverse 5'-GGGCTATTCCATGTTTTTAT-3' primers were used for the amplification of a 200-bp fragment length. The PCR reactions for mitochondrial gene amplification contained 3 mM MgCl$_2$, 0.25 pmol/µl of each primer, and 10 ng of DNA in 20 µl of final volume. The PCR reactions for nuclear gene amplification contained 2 mM MgCl$_2$, 0.3 pmol/µl of each primer, and 10 ng of DNA in 20 µl of final volume. The PCR amplification program consisted of a single denaturation-enzyme-activation step of 10 min at 95°C, followed by 35 cycles (for 18S rRNA gene) and 29 cycles (for the ND2 gene). Each cycle consists of a denaturation step (2 sec at 95°C for the 18S gene and 0 sec at 94°C for the ND2 gene), an annealing step (10 sec at 66°C for the 18S rRNA gene and 10 sec at 53°C for the ND2 gene), and an extension step (20 sec at 72°C for the 18S rRNA gene and 10 sec at 72°C for the ND2 gene), with a temperature-transition rate of 20°C/sec. The fluorescent product was detected at the last step of each cycle by single acquisition. The method used a double-stranded DNA dye (SYBR Green I) to continuously monitor product formation. The sensitivity of SYBR Green I detection is limited by nonspecific product formation, which is monitored by fluorescence acquisition at temperatures at which only specific products are double stranded. After amplification, a melting curve was acquired by heating the product at 20°C/sec to 95°C, cooling at 20°C/sec to 72°C for the ND2 gene and 76°C for the 18S rRNA, and slowly heating it at 0.2°C/sec to 94°C with continuous fluorescence collection. Melting curves were used to determine the specificity of the PCR products. The results were expressed as the ratio of the mean mtDNA value of duplicate measurements to the mean nuclear DNA value of duplicate measurements (ND2/18S rRNA).$^{17}$ To also express mtDNA content by organello, we divided the ND2/18S rRNA quotient by citrate synthase activity. The second muscle biopsy from the patient rendered enough biological material to perform a Southern blot hybridization using a mitochondrial ND4 gene probe in order to detect mtDNA deletions.$^{4}$

All the results were expressed as the percentage with respect to the mean (± SD) of control values (100%) obtained from six healthy men.

The first histological study showed 5–10% of ragged-red fibers (RRF) or RRF equivalents and the presence of abundant lipid droplets in 50% of myocytes. In the second biopsy, RRF or equivalents had disappeared while only 5% of myocytes still showed an abnormal amount of lipid droplets (Fig. 1).

A marked increase (335%) in mitochondrial content during the symptomatic episode of hyperlactataemia was demonstrated. After treatment withdrawal and disappearance of clinical symptoms, mitochondrial content decreased but still remained above control values (177%, Fig. 2).

Enzyme activity decreased for all complexes of MRC during the acute phase of hyperlactataemia. The decrease was specially marked for complexes III and V, which showed only 36% and 11% of residual activity, respectively. Three months later, a general recovery of activities was observed, returning all of them into the control range (Fig. 2).

Regarding respiratory activity (Fig. 2), oxidation of all substrates tested in the symptomatic phase was found to be decreased (21% for pyruvate, 41% for succinate, 54% for ascorbate). However, the second study of the patient was not associated with a full recovery of respiratory activity.

Analysis of mtDNA relative abundance disclosed 47% of deletion with respect to control values in the first study when expressed by cell, which reached 83% when expressed by organello. Complete restoration of mtDNA content was ascertained in the second biopsy, 3 months after highly active antiretroviral therapy (HAART) discontinuation (Fig. 3). The Southern blot study of the second skeletal muscle specimen disclosed the presence of several mtDNA deletions of different size (Fig. 3).

We have demonstrated mitochondrial proliferation, deficient MRC complexes III and V activities, a general decay of mito-
FIG. 1. Histological study of skeletal muscle during symptomatic hyperlactatemia (left) and after clinical and analytical recovery (right). First line: Gomori’s trichrome staining demonstrating ragged-red fibers (RRF, arrows). Second line: Cytochrome c oxidase reaction demonstrating negative fibers (RRF equivalents, asterisks). Third line: Succinate dehydrogenase reaction demonstrating hyperactive fibers (RRF equivalents, asterisks). Forth line: Oil red O staining demonstrating deposition of neutral lipid droplets, which are more prominent in the first skeletal muscle biopsy (left).
Mitochondrial respiratory capacity, and mtDNA depletion during a symptomatic episode of hyperlactatemia in an HIV-infected patient. Mitochondrial DNA depletion was even more evident when expressed by organello than by cell. A similar study performed 3 months later, when the patient became asymptomatic, showed a marked improvement in most of these mitochondrial parameters. At that time, the patient was receiving no antiretroviral drugs, while the rest of the treatments were maintained. This fact argues against the hypothetical role of gemfibrozil in our pathological findings because although gemfibrozil use has been described as causing decreased state 3 respiration stimulated by malate-pyruvate, the drug was maintained during the time that most of the abnormal mitochondrial parameters returned to normal.

FIG. 2. Results of biochemical studies of mitochondrial respiratory chain function. y-axis are arbitrary values, where 100 is assigned to the mean of control group for each variable. Bars denote SD, which was obtained from six healthy men. CS, citrate synthase; MRC, mitochondrial respiratory chain; C-I, complex I; C-II, complex II; C-III, complex III; C-IV, complex VI; C-V, complex V.

MTDNA content

FIG. 3. Results of genetic analysis. Left: Results of mtDNA quantification (by cell and by organello) using real time PCR methodology (bars denote SD, which was obtained from six healthy men). CS, citrate synthase activity. Right: Radioactive Southern blot analysis of muscle mtDNA from the patient (second biopsy) and a healthy control. DNA samples were PvuII digested, electrophoretically separated, and probed with the mitochondrial ND4 gene. Arrow indicates the wild-type mtDNA molecule (right).
As a whole, our data confirm that multiple mitochondrial dysfunction is involved in the pathogenesis of this syndrome and are in accordance with two previous reports investigating mitochondrial function of skeletal muscle in hyperlactemic HIV-infected patients. Gérard et al.\textsuperscript{6} biopsied skeletal muscle in 6 of 14 patients with symptomatic hyperlactemia and in four of them found complex IV deficiency, with the residual activity ranging from 16% to 36% of control values. Unfortunately, there is a lack of data concerning the evolution after resolution of symptomatic hyperlactatemia in that study. More recently, Church et al.\textsuperscript{8} reported abnormal mitochondrial morphology and reduced activities (from 11% to 38%) for all MRC complexes (from I to IV) in skeletal muscle of an HIV-infected child with lactic acidosis and liver failure. Two months after discontinuing treatment with nucleoside analogs, a marked recovery was seen in both clinical status and MRC enzyme activities. Two additional mitochondrial studies\textsuperscript{7,13} also found different MRC complex deficiencies on hepatic biopsies. Consistent with all these facts, our demonstration that not only MRC enzyme activities, but also respiration are concurrently disturbed heightens the functional relevance of such MRC enzyme deficiencies in HAART-related hyperlactatemia. However, our documentation of skeletal muscle mitochondrial dysfunction in the presence of hyperlactatemia does not necessarily mean that this is the only tissue affected, or the main source of increased lactate production in hyperlactatemic patients. In fact, Roge et al.\textsuperscript{19} have shown that skeletal muscle function and response to exercise remained relatively normal in patients with hyperlactatemia. Côté et al.\textsuperscript{9} demonstrated that mtDNA was significantly depleted in PBMCs from HIV-infected patients with symptomatic hyperlactatemia who were receiving HAART. The decrease in mtDNA preceded the rise in venous lactate levels, an observation suggesting that hyperlactatemia is a consequence of mtDNA depletion. However, that study lacks MRC functional studies. Lactate is the product of anaerobic glycolysis. Therefore, hyperlactatemia in normal aerobic conditions may indicate mitochondrial dysfunction. In addition, if mtDNA depletion plays a pathogenic role it must necessarily be via MRC dysfunction, since mtDNA encodes only for certain subunits of certain complexes constituting MRC. Therefore, polarographic and spectrophotometrical studies of MRC must be the clue to link genetic and clinical findings.\textsuperscript{10} The case described in the present study, extensively evaluated from an MRC point of view, is consistent with the hypothesis of Côté et al.\textsuperscript{9} that mitochondrial toxicity stands at the basis of HAART-related hyperlactatemia.

An interesting aspect of our patient is that in addition to mtDNA depletion, multiple mtDNA deletions were present. One hypothetical mechanism to explain these gene defects, in addition to $\gamma$-polymerase inhibition, which causes mtDNA depletion, could be the coexistence of inhibition of mitochondrial processing peptidases by protease inhibitors included in HAART. Although not demonstrated to date, such a hypothetical effect of HAART regimens has also been postulated for mtDNA multiple deletions found in patients with lipodystrophy.\textsuperscript{4,20} Irrespective of all these speculations, all the above-mentioned data seem to confirm that mitochondrial dysfunction is the basis of hyperlactataemia occurring during HAART.

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