Reversible Inhibition of Mitochondrial Protein Synthesis during Linezolid-Related Hyperlactatemia

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The objective of the present study was to determine the mitochondrial toxicity mechanisms of linezolid-related hyperlactatemia. Five patients on a long-term schedule of linezolid treatment were studied during the acute phase of hyperlactatemia and after clinical recovery and lactate normalization following linezolid withdrawal. Mitochondrial studies were performed with peripheral blood mononuclear cells and consisted of measurement of mitochondrial mass, mitochondrial protein synthesis homeostasis (cytochrome c oxidase [COX] activity, COX-II subunit expression, COX-II mRNA abundance, and mitochondrial DNA [mtDNA] content), and overall mitochondrial function (mitochondrial membrane potential and intact-cell oxidative capacity). During linezolid-induced hyperlactatemia, we found extremely reduced protein expression (16% of the remaining content compared to control values [100%], P < 0.001) for the mitochondrially coded, transcribed, and translated COX-II subunit. Accordingly, COX activity was also found to be decreased (51% of the remaining activity, P < 0.05). These reductions were observed despite the numbers of COX-II mitochondrial RNA transcripts being abnormally increased (297%, P = 0.10 [not significant]) and the mitochondrial DNA content remaining stable. These abnormalities persisted even after the correction for mitochondrial mass, which was mildly decreased during the hyperlactatemic phase. Most of the mitochondrial abnormalities returned to control ranges after linezolid withdrawal, lactate normalization, and clinical recovery. Linezolid inhibits mitochondrial protein synthesis, leading to decreased mitochondrial enzymatic activity, which causes linezolid-related hyperlactatemia, which resolves upon discontinuation of linezolid treatment.

Linezolid belongs to a family of antibiotics (oxazolidinones) that inhibit bacterial protein synthesis by binding to 23S rRNA in the large ribosomal subunit and preventing the fusion of 30S and 50S ribosomal subunits and the formation of the translation initiation complex (1). It has shown excellent efficacy against gram-positive cocci, including Staphylococcus aureus, coagulase-negative staphylococci, enterococci, and streptococci, with MICs ranging from 0.5 to 4 mg/liter (16). Furthermore, linezolid has 100% oral bioavailability and reaches high concentrations at different sites (skin, synovial fluid, bone, cerebrospinal fluid, or lung), thus being a good alternative for the long-term treatment of orthopaedic implant infections, ventriculo-peritoneal shunts, and other infections related to foreign bodies in which gram-positive cocci are the main pathogens.

However, a major concern with this antibiotic is its safety profile, especially when it is administered for more than the 28-day period approved by the U.S. Food and Drug Administration (6, 15). Adverse events of linezolid include hematological disturbances (especially thrombocytopenia and anemia), peripheral neuropathy, hyperlactatemia, and metabolic acidosis (1, 2, 4, 6, 11, 12, 15, 23, 30). Some of these events could be related to the capacity of linezolid to interfere with mitochondrial function (25, 29) due to similarities between bacterial and mitochondrial ribosomes. Experimental reports support this hypothesis. Nagiec et al. reported the inhibition of mitochondrial protein synthesis in human erythroleukemia cells exposed to eperezolid, another oxazolidinone (24). More recently, other authors published reports of decreased numbers of mitochondrial respiratory chain subunits (10) or mitochondrial protein synthesis (19) in different tissue homogenates or isolated mitochondria, respectively, for rats treated with linezolid. All these studies also suggest that this effect is time and concentration dependent, although their cross-sectional experimental design did not allow direct information regarding the capacity of mitochondrial recovery after discontinuation of linezolid treatment to be obtained. Moreover, there are very few data for humans receiving treatment with linezolid that confirm experimental hypotheses. We recently reported a decrease in mitochondrial enzyme activities in peripheral blood mononuclear cells (PBMCs) from three patients on linezolid therapy during the acute phase of symptomatic hyperlactatemia (29). In addition, De Vriese et al. reported decreased enzyme activities for some mitochondrial respiratory chain proteins partially synthesized by mitochondrial ribosomes in the skeletal muscle, liver, and kidney of a patient with severe hyperlactatemia (10). In none of these four cases were data on a mitochondrial outcome after hyperlactatemia resolution presented. We have conducted a prospective longitudinal study to assess the homeostasis of mitochondrial protein synthesis and function in selected patients developing linezolid-related hy-
perlactatemia both during treatment and after discontinuation of linezolid treatment.

**MATERIALS AND METHODS**

Patients. In our hospital, all patients who receive linezolid for more than 1 month at the standard oral dosage (600 mg/12 h) are closely followed. Clinical symptoms of infection and linezolid-related or possibly related adverse events (anemia, thrombocytopenia, gastrointestinal discomfort, etc.) were monitored, and a hemogram was performed weekly. For the present study, blood lactate (as a surrogate marker of mitochondrial toxicity) was determined at least once a month or when hyperlactatemia was suspected based on clinical findings. When the patients developed hyperlactatemia, with or without clinical symptoms of metabolic acidosis, a blood sample was obtained for mitochondrial studies. In order to avoid confounders of mitochondrial toxicity, critically ill patients and those taking other potentially toxic drugs for mitochondria (i.e., aminoglycosides or antiretrovirals) were excluded from the study. Linezolid was withdrawn when a severe adverse event was observed (a platelet count of <100,000 cells/mm³, a hemoglobin concentration of <9 g/liter, or severe gastrointestinal manifestations). An asymptomatic increase in serum lactate was not a criterion for stopping linezolid treatment. For those patients who developed hyperlactatemia, a second sample was obtained for mitochondrial studies once linezolid treatment was discontinued, the patient became asymptomatic, and lactate levels returned to normal. For mitochondrial studies, six healthy volunteers matched by age and gender were included as controls. All individuals signed written consents to be included in the protocol.

Sample collection. For mitochondrial analysis, we obtained 20-ml samples of venous blood during the hyperlactatemic episode during linezolid use and after linezolid discontinuation and hyperlactatemia resolution. PBMCs were isolated by Ficoll’s gradient (27), and protein content was measured according to the Bradford protein-dye binding-based method (5).

Determination of mitochondrial mass. The quantity of mitochondria was estimated by two different methodologies. One was spectrophotometric measurement of the activity of citrate synthase (EC 4.1.3.7, nmol/min/mg protein), a mitochondrial matrix enzyme of the Krebs cycle, which remains highly constant in mitochondria and is considered to be a reliable marker of mitochondrial content (3, 26, 27). The other method for measurement of mitochondrial mass was Western blot analysis of V-DAC (a nuclear-encoded mitochondrial structural protein) amount, corrected by β-actin (a nuclear-encoded cytoplasmic protein) content (22).

Studies on mitochondrial protein synthesis homeostasis. (i) Mitochondrial respiratory chain complex IV (COX; EC 1.9.3.1) enzyme activity. Enzyme activity was measured spectrophotometrically according to the methodology of Rustin et al. (28), slightly modified for complex IV for minute amounts of biological samples (20). Cytochrome c oxidase (COX) activity was expressed in absolute values (nmol/min/mg protein) as well as in values relative to mitochondrial mass (by dividing absolute values by citrate synthase activity values).

(ii) Mitochondrial protein synthesis. We assessed the mitochondrial protein synthesis of the COX-II subunit (mitochondrially encoded, transcribed, and translated) by Western blot analysis (22). This expression was normalized by the content of the β-actin signal to establish the relative COX-II abundance per overall cell protein as well as per V-DAC content to establish the relative COX-II abundance per mitochondrion.

(iii) mtRNA quantification. Total RNA was obtained by an affinity column-based procedure (RNeasy; QIAGEN). RNA (1 μg/sample) was reverse transcribed (TaqMan reverse transcriptase; Applied Biosystems) using random primers. The real-time PCR was performed using TaqMan universal master mix (Applied Biosystems) in an ABI PRISM 7700 sequence detection system, in a total reaction volume of 25 μL. Quantification of COX-II mRNA was performed using the amplification conditions indicated by the supplier (Assay-by-Design; Applied Biosystems); the primers were CAAACCACTTTCACCGCTACAC (forward) and GGACGATGGGCACTAAACTCT(GT(reverse), and the β-carboxyfluorescein-labeled probe was AAATCTGTGGAGCAAACC. As a reference control, housekeeping nuclear-encoded 18S rRNA was determined using a premade kit (Assay-on-Demand, Hs9999901_s1; Applied Biosystems). Absolute COX-II mitochondrial RNA (mtRNA) content was expressed as the ratio of the mean COX-II mRNA value to the mean 18S rRNA value (13), while COX II mtRNA content relative to mitochondrial mass was calculated by dividing absolute values by citrate synthase activity values.

(iv) mtDNA quantification. Total DNA was obtained by a standard phenol-chloroform extraction procedure. Fragments of the mitochondrial ND2 gene and the nuclear rRNA 18S gene were amplified in duplicate and separately by quantitative real-time PCR as previously reported (21). The absolute content of mtDNA was expressed as the mtDNA-to-nuclear-DNA ratio (ND2 mtDNA/18S
nuclear DNA), and mtDNA content relative to mitochondrial mass was obtained after absolute values were divided by citrate synthase activity values.

**Studies on overall cellular function. (i) Mitochondrial membrane potential.** Fresh PBMCs (750,000) were stained with 2.5 μg/ml of JC-1 probe (T-3168; Molecular Probes, Eugene, OR) in 10% fetal bovine serum-complete RPMI medium. The resulting cells were washed and analyzed by flow cytometry (FACScan cytometer; Becton Dickinson, San Jose, CA) on FL1 and FL2 channels (9). The results were expressed as percentages of PBMCs with depolarized mitochondria with respect to the total number of PBMCs analyzed.

(ii) Spontaneous mitochondrial oxidative activity. Oxygen usage was measured polarographically using a Clark electrode in a water-jacketed cell magnetically stirred at 37°C (Hansatech Instruments Limited, Norfolk, England) in 250 μl of respiration buffer containing 100 to 200 μg of fresh cells. Intact-cell endogenous substrate oxidation was assessed as spontaneous PBMC oxygen uptake. The complete methodology is reported elsewhere (21, 28).

**Statistical analysis.** Results were expressed as means ± standard deviations (SD) and/or the percentages compared to control values (which were arbitrarily assigned values of 100%). The t test for independent or paired measures (as needed) was used to search for differences.

**RESULTS**

From January to December 2005, 5 out of 13 patients receiving linezolid therapy for more than 1 month developed hyperlactatemia and were included in the present study. Mitochondrial enzymatic activity and spontaneous cell oxidation during the acute phase of hyperlactatemia in two patients have previously been reported (29). In those two cases, results for mitochondrial DNA, RNA, and protein synthesis as well as complete mitochondrial functionality studies were added during and after hyperlactatemia resolution. The clinical characteristics and serum lactate values determined during and after discontinuation of linezolid treatment are shown in Table 1 and Fig. 1. As can be seen, three patients were symptomatic, and in two, linezolid treatment was prematurely interrupted due to adverse events.

The mitochondrial masses were similar irrespective of the methodology employed and were decreased during the hyperlactatemic phase (59% of control values for the citrate synthase method [P < 0.01] and 60% of control values for the V-DAC method [P < 0.05]). After linezolid discontinuation and hyperlactatemia resolution, a trend toward normalization of mitochondrial mass was observed (from 59% and 60% to 80%, respectively; P = 0.17 for both methods).

We found a marked reduction in COX activity (a mitochondrial respiratory chain complex which is partially encoded by mtDNA) during the acute phase of hyperlactatemia in patients receiving linezolid compared to what was found for controls (51% with respect to control values, P < 0.05) (Fig. 2) and an even greater reduction was observed for the expression of COX-II protein (one of the COX subunits encoded by mtDNA and translated by mitochondrial ribosomes), which was reduced to 16% of control values when assessed per cell (P < 0.001) (Fig. 2). These changes were observed despite an increase in COX-II mtRNA expression (297% of control values, P = 0.1 [not significant]) and no change in mtDNA levels (82% of control values, P = 0.57) during the hyperlactatemic phase (Fig. 2). After linezolid discontinuation and normalization of lactate levels, mitochondrial abnormalities showed a significant trend toward normalization (from 51% to 130% for COX activity [P < 0.01], from 16% to 72% for COX-II expression per cell [P < 0.05], and from 297% to 210% for COX-II mtRNA abundance [P < 0.05]) (Fig. 2 and 3). It is important to note that mitochondrial parameters conserved tendencies even after the correction for the decrease in mitochondrial mass present in patients developing linezolid-related hyperlactatemia (Fig. 3).

No significant changes were found in the overall PBMC function, irrespective of whether it was considered by spontaneous cell oxidation capacity or by mitochondrial membrane potential (Fig. 4).

**DISCUSSION**

In the present study, we evaluated the impacts of standard dosages of 600 mg/12 h of linezolid on mitochondrial activities by using lactic acid serum levels as a surrogate marker. We studied five patients developing hyperlactatemia, in whom we demonstrated reductions in COX activity and COX-II protein synthesis in PBMCs, despite an increase in COX-II mtRNA production. All these abnormalities tended to disappear after
linezolid treatment was discontinued and serum lactate levels returned to normal. Our findings for humans offer a complete picture of what occurs in mitochondria during linezolid-related hyperlactatemia and corroborate previous experimental data suggesting the inhibitory action of linezolid on mitochondrial protein synthesis. Few previous partial data regarding the in vivo effects of linezolid therapy had been reported to date. De Vriese et al. recently reported abnormally reduced enzymatic mitochondrial respiratory chain activity in the liver, kidney, and muscle of one patient under severe-symptomatology treatment after a 4-month course of linezolid treatment (10). All of our hyperlactatemic patients were studied before a 3-month linezolid treatment and presented null or mild clinical manifestations. De Vriese et al. performed no further studies to determine the causes of such a reduction of COX activity (10). In this case, no mitotoxic effects were found in PBMCs. Although some authors have argued against the validity of mitochondrial studies on PBMCs, mainly because these cells are not the typical target of mitochondrial disturbances, in our experience, most mitochondrial defects can reliably be demonstrated in PBMCs (17, 21, 22). In addition, very recently, McKee et al. (19) demonstrated equal mitochondrial ribosomal structures and antibiotic effects in all studied tissues. We believe that to find mitochondrial disturbances in PBMCs, it may be necessary to control crude results for mitochondrial

FIG. 2. Comparison of mitochondrial parameters between controls (100%) (dashed line) and patients developing linezolid-related hyperlactatemia (columns) (means ± standard errors of the means). $P$ values correspond to comparisons between values measured during and after linezolid treatment. Asterisks correspond to significant differences (*, $P < 0.05$; **, $P < 0.01$) between values for each column and the control values. NS, not significant; CS, citrate synthase.

FIG. 3. (Top) Individual values of mitochondrial protein synthesis homeostasis parameters measured in the study. Dashed lines correspond to the means for the controls and shaded squares the normality interval (±2 SD). $P$ values refer to the statistical significance of the changes observed in patients developing linezolid-related hyperlactatemia after discontinuation of linezolid treatment (n, nuclear). (Bottom) Western blot analysis of the mitochondrially coded cytochrome c oxidase subunit II (COX-II) expression with respect to both nuclear-coded porin V-DAC (mitochondrial marker) and $\beta$-actin (cellular marker) (L denotes patients receiving linezolid, C denotes control individuals, and B corresponds to the second sample after hyperlactatemia resolution).
content, which may increase or decrease in response to functional defects. Actually, we also found important reductions in PBMC mitochondrial masses in all the hyperlactatemic patients analyzed, irrespective of the methodology employed. These reductions in mitochondrial mass may not be the cause of the mitochondrial dysfunctions observed in these patients. Conversely, we believe that the reductions could be the consequence of linezolid mitochondrial protein synthesis inhibition, since mitochondrial parameters remained abnormal (or, alternatively, showed a clear tendency to be lower than control values) when they were normalized by the mitochondrial mass. In this sense, a reduction of mitochondrial mass could suggest that linezolid protein synthesis inhibition is limiting mitochondrial biogenesis. Finally, it is important to note that mitochondrial disturbances identified in the PBMCs of patients with linezolid-related hyperlactatemia could be even more pronounced in other tissues located in more-central places of lactate homeostasis, such as the liver and kidney, which are, in the end, the main organs responsible for lactate level increases in plasma.

Additionally, in these linezolid-induced-hyperlactatemia patients, mtRNA abundance is increased, probably to compensate for the linezolid inhibition of mitochondrial protein synthesis. Up-regulation of mtRNA levels in response to experimental inhibition of mitochondrial protein synthesis by thiamphenicol has previously been reported (8, 14). Nevertheless, a reduced enzymatic activity of the partially mitochondrion-coded COX was detected in our patients, suggesting that the up-regulatory mechanisms leading to increased mitochondrial DNA-encoded transcripts are not able to compensate for linezolid-mediated mitochondrial translation inhibition.

Linezolid exerts a time-dependent antimicrobial action, but an influence of linezolid concentration on adverse effects has previously been suggested by hematological alterations in patients with renal failure (18), which is provably associated with linezolid or metabolite accumulation (10). Although serum linezolid concentration was not measured, no hepatic or renal impairment which could influence linezolid pharmacokinetics was present among the studied patients. Therefore, individual susceptibilities to mitochondrial toxicity could be related to mtDNA mutations on mitochondrial rRNA genes coding for mitochondrial ribosomal subunits (25) or particular linezolid metabolism. McKee et al. (19) reported that the linezolid concentration that inhibits 50% of the mitochondrial protein synthesis (IC50) in rat and rabbit heart and liver mitochondria is between 3.37 and 5.26 mg/liter. Considering that linezolid trough serum concentrations range from 1 to 6 mg/liter, we hypothesize that patients with trough levels above the IC50 could maintain permanently inhibited mitochondrial protein syntheses and could be especially vulnerable to mitochondrial toxicity with respect to those patients with trough levels below the IC50. Since linezolid has a 100% oral bioavailability and achieves trough serum levels above the MIC for most susceptible gram-positive microorganisms, it is necessary to further evaluate whether guiding linezolid dosage by using the linezolid “trough level/MIC ratio” (7, 10) could prevent the development of the adverse events related to mitochondrial protein synthesis inhibition while maintaining the clinical efficacy, especially in patients receiving prolonged courses of linezolid treatment.

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