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Vitamin D attenuates nucleoside reverse transcriptase inhibitor induced human skeletal muscle mitochondria DNA depletion

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Abstract

Objective—To evaluate the impact of the active metabolite of vitamin D, 1 α ,25-dihydroxycholecalciferol (1,25D3), on nucleoside reverse transcriptase inhibitor (NRTI) induced mitochondrial DNA (mtDNA) depletion in human skeletal muscle myoblasts and myotubes.

Design—mtDNA was quantified in human skeletal muscle myoblasts and myotubes following 1,25D3 and NRTI treatment using real-time PCR.

Methods—Human skeletal muscle myoblasts and myotubes were treated with didanosine (ddI), stavudine (d4T), zidovudine (ZDV), lamivudine (3TC) and abacavir (ABC) alone or in combination either in the presence or absence of 1,25D3 for 5 days. Cells were harvested, DNA extracted and mtDNA quantified.

Results—ddI and ddI-d4T significantly decreased both myoblast and myotube mtDNA in the absence of 1,25D3 compared with untreated controls ($P = 0.029$). In addition, the ZDV-3TC combination resulted in a 47% decrease in myotube mtDNA ($P = 0.005$). 1,25D3 increased myotube mtDNA levels in ddI, ZDV, 3TC, ABC, ddI-d4T, d4T-3TC, ZDV-3TC, ZDV-ABC and ZDV-3TC-ABC-containing regimens and myoblast mtDNA levels in ddI, d4T, ZDV, 3TC, ddI-d4T, ZDV-3TC and ZDV-ABC-containing regimens. Of note, 1,25D3 protected against myotube mtDNA depletion following ZDV-3TC treatment, rendering them similar to 1,25D3 untreated controls ($P = 0.62$), and increased both myotube and myoblast mtDNA two to three-fold in ddI-containing regimens ($P < 0.05$).

Conclusion—1,25D3 confers a protective effect against NRTI-induced mitochondrial toxicity in skeletal muscle myoblasts and myotubes. These findings support a protective role for vitamin D in preventing mitochondrial toxicity and suggest that supplemental vitamin D may protect against NRTI-associated mitochondrial toxicity.

Keywords

HIV; mitochondrial toxicity; mother-to-child transmission; nucleoside reverse transcriptase inhibitor; paediatrics; skeletal muscle; vitamin D

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G.R.C. and S.A.S. conceived and designed the experiments. Z.T.P. performed the experiments. G.R.C., Z.T.P. and S.A.S. performed bioinformatics analyses. S.A.S. contributed reagents/materials/analysis tools. G.R.C. and S.A.S. took the primary responsibility for writing the manuscript. All authors edited the manuscript.

Conflicts of interest

The authors declare no competing financial interests.

Introduction

Nucleoside analogue reverse transcriptase inhibitors (NRTIs) remain the backbone of many combination antiretroviral regimens (cART) used for the treatment of HIV infection. Although these drugs inhibit HIV replication, they also inhibit the catalytic subunit of the mitochondrial DNA polymerase, DNA polymerase γ (POLG). Therefore, despite cohort studies and clinical trials demonstrating the need for early cART to optimize both individual [1] and public health [2] outcomes, generalized and tissue-specific mitochondrial toxicities resulting from the depletion of mitochondrial DNA (mtDNA) are at least partially responsible for various NRTI-associated adverse effects, including peripheral neuropathy, lactic acidosis, hepatic steatosis, pancreatitis, lipodystrophy and myopathy [3].

Of the few studies that have examined the association between vitamin D [25-hydroxycholecalciferol (25D3)] status and HIV disease progression and survival, the data available suggest that HIV-infected individuals have lower levels of 25D3 and/or the vitamin D3 active metabolite, 1 α ,25-dihydroxycholecalciferol (1,25D3) than uninfected individuals [4,5], with the lowest concentrations found in persons with AIDS [4,6]. In addition, women with low levels of 25D3 have an increased risk of HIV disease progression [7] and infants born to HIV-infected mothers with low 25D3 levels have an increased risk of HIV infection and increased mortality regardless of HIV infection status [8]. Although 25D3 has no direct antiretroviral effect, its hormonally active form, 1,25D3, modulates the immune response and exerts anti-HIV effects *in vitro* [9-11]. Moreover, case studies have linked vitamin D deficiency with proximal myopathy in young children that was reversible through vitamin D supplementation [12-14].

The most sensitive marker for monitoring mitochondrial toxicity is through the measurement of mtDNA levels, as mtDNA depletion precedes all the other abnormalities in mitochondria and cell function [15]. The objective of this study, therefore, was to investigate the effects of 1,25D3 and NRTIs, used alone or in combination, on mtDNA in human skeletal muscle myoblasts and myotubes.

Materials and methods

Chemicals and reagents

2',3'-dideoxyinosine (didanosine/ddI), 2'-3'-dideoxy-2',3'-dideoxythymidine (stavudine/d4T), 3'-azido-3'-deoxythymidine (zidovudine/ZDV), lamivudine (3TC) and 1,25D3 were purchased from Sigma (St Louis, Missouri, USA). Abacavir sulphate (ABC) was purchased from Toronto Research Chemicals (Toronto, Ontario, Canada).

Human skeletal muscle myoblasts

Human skeletal muscle myoblasts from postgestational quadriceps or psoas muscle were obtained from Lonza (Walkersville, Maryland, USA) and subcultured using skeletal muscle basal medium-2 supplemented with 10 ng/ml human epidermal growth factor, 1 mg/ml insulin, 0.39 μ g/ml dexamethasone, 500 μ g/ml bovine serum albumin, 50 μ g/ml gentamicin and 50 ng/ml amphotericin B (all from Lonza). Myoblasts were differentiated into skeletal muscle myotubes using DMEM-F12 supplemented with 2% (v/v) horse serum (both BioWhittaker) for 5 days. Cultures were treated for 5 days with NRTI alone or in clinically used combinations at concentrations based on the mean peak steady-state levels in human plasma during antiretroviral therapy (C_{max}) [15]: 11.8 μ mol/l ddI; 3.6 μ mol/l d4T; 7.1 μ mol/l ZDV; 8.3 μ mol/l 3TC; and 3.0 μ mol/l ABC; either in the presence or absence of 100 pmol/l 1,25D3, the mean serum concentration found in healthy HIV-uninfected individuals

[16]. Cell proliferation/viability at the end of each treatment was estimated using WST-1 (Roche Applied Science, Indianapolis, Indiana, USA).

Real-time PCR

Mitochondrially encoded cytochrome *c* oxidase I (MT-CO1) and DNA-directed polymerase gamma 2 accessory subunit (POLG2) DNA quantification was determined using a LightCycler 480 System with the LightCycler DNA Amplification Kit HybProbe (Roche Applied Science). PCR reactions were performed in 384-well plates in a 20- μ l mixture composed of 3 mmol/l $MgCl_2$, 0.5 μ mol/l of each primer, 0.2 μ mol/l donor probes, 0.4 μ mol/l acceptor probes, 2 μ l sample and one-fold LightCycler DNA Amplification Kit HybProbe. Primers (Tib MolBiol, Adelphia, New Jersey, USA) were as previously described [17]. PCR amplification consisted of a single denaturation and enzyme activation step of 16 min at 95°C followed by 45 cycles of 10 s at 95°C, 20 s at 60°C and 20 s at 72°C, with a temperature transition rate of 4.8°C/s for all steps apart from the annealing step (2.5°C/s). A single fluorescence acquisition was performed after each annealing step. Results were calculated using the Pfaffl method [18] and are expressed as the ratio between MT-CO1 (mtDNA) and POLG2 (nuclear DNA) and normalized so that mtDNA in untreated cells equals $1.00 \pm SEM$.

Statistical analysis

Unpaired, two-tailed Student's *t*-tests were used to evaluate the significance of differences between treatments. A *P* value of 0.05 or less was considered significant.

Results

Primary myoblasts were treated with NRTI alone or in combination in the presence or absence of 100 pmol/l 1,25D3 for 5 days after which mtDNA was quantified. Importantly, at the concentrations used, cell proliferation did not differ among untreated cells and the cells treated with either NRTI or 1,25D3 (data not shown). In the absence of 1,25D3, myoblast mtDNA was significantly depleted after ddI (0.62 ± 0.06 ; $P = 0.004$) and ddI-d4T (0.53 ± 0.14 ; $P = 0.029$) treatment compared with untreated control myoblasts (Fig. 1). In contrast, there was a significant increase in myoblast mtDNA treated with ZDV-3TC-ABC (2.16 ± 0.28 ; $P = 0.014$). No other NRTI or NRTI combinations significantly changed mtDNA ratios ($P > 0.09$).

In the absence of NRTI, 100 pmol/l 1,25D3 induced a two-fold increase in myoblast mtDNA compared with untreated control myoblasts ($P = 0.04$). Similarly, 1,25D3 increased myoblast mtDNA in all NRTI regimens except for 3TC alone and ABC alone treated myoblasts. However, this increase was only significant in the ddI alone (0.62 ± 0.06 versus 1.65 ± 0.27 ; $P = 0.021$), d4T alone (1.03 ± 0.08 versus 1.95 ± 0.09 ; $P = 0.002$) and ddI-d4T combined (0.42 ± 0.08 versus 2.45 ± 0.42 ; $P = 0.047$) treated regimens (Fig. 1).

Next, the impact of NRTI on differentiated myotube mtDNA was investigated. As was observed for myoblasts, cell proliferation/viability did not differ between the untreated cells and NRTI and/or 1,25D3 treated cells (data not shown). Myotube mtDNA was significantly depleted in ddI (0.57 ± 0.04 ; $P = 0.0003$), ddI-d4T (0.53 ± 0.09 ; $P = 0.006$) and ZDV-3TC (0.53 ± 0.09 ; $P = 0.005$) regimens compared with untreated controls (1.00). No other NRTI or NRTI combinations significantly changed mtDNA ratios ($P > 0.16$; Fig. 2).

Similar to 1,25D3 treatment of myoblasts, 1,25D3 increased myotube mtDNA in all NRTI regimens except d4T alone treated myotubes compared with 1,25D3 and untreated cultures. However, unlike 1,25D3 treatment of myoblasts, this increase was not significant in myotube mtDNA in the absence of NRTI compared with untreated control myotubes (1.00

versus 1.15 ± 0.07 ; $P = 0.11$). Moreover, this increase was not significant for any tested regimen except for ddI alone (0.57 ± 0.04 versus 1.30 ± 0.16 ; $P = 0.011$), ddI-d4T (0.53 ± 0.09 versus 0.83 ± 0.05 ; $P = 0.04$) and ZDV-3TC (0.53 ± 0.09 versus 0.97 ± 0.06 ; $P = 0.015$; Fig. 2).

Discussion

The present study demonstrates that 1,25D₃ confers a protective effect against NRTI-induced mitochondrial toxicity in human skeletal muscle myoblasts and myotubes. Although a number of cross-sectional studies and randomized controlled trials have examined the specific effects of vitamin D on muscle function/strength with conflicting results, most of these studies involved elderly individuals and comparing them is difficult due to differences in the outcome measures used in each study [19]. However, of the few paediatric studies examining this relationship, there is evidence supporting the notion that vitamin D has a strong and direct effect on muscle function in children. First, proximal myopathy in vitamin D deficient young children has been shown to be reversible through vitamin D supplementation [12-14]. Second, impaired vitamin D metabolism or absorption results in profound muscle weakness that is reversible through 1,25D₃ supplementation [14,20]. Third, a positive association was observed between 25D₃ status and muscle strength/function or lean mass in older children [21-25]. Fourth, treatment of skeletal muscle with 1,25D₃ results in second messenger system activation. And, fifth, single-nucleotide polymorphisms in vitamin D receptor (*VDR*) have been associated with differences in muscle strength [19]. Despite this, there is significant controversy regarding whether the effects of vitamin D on skeletal muscle cells are mediated directly through VDR activation of second messengers or through changes in calcium absorption and parathyroid hormone secretion [26]. 1,25D₃ was used in this study, as the presence of the VDR in skeletal muscle cells provides a direct mechanism by which 1,25D₃ can act [27,28], although no study has successfully demonstrated the expression of *CYP27B1* in human skeletal muscle.

The present findings also demonstrate that infants are potentially vulnerable to mitochondrial toxicity due to ddI that could affect growth and development. In patas monkey offspring perinatally exposed to ZDV-ddI, mitochondrial damage at birth was severe and there was no improvement during the first year of life, with significant reductions of skeletal muscle mtDNA compared with levels for other NRTI regimens [29]. Of note, ddI is the only purine analogue that was commonly used in developing countries, in contrast to other NRTIs such as ZDV and d4T, which are pyrimidine analogues. This is of particular concern in developing countries, where ddI was widely used as first-line treatment, although it is now recommended as part of a second-line treatment for HIV-infected children [30]. Currently, the combination of ZDV-3TC is the preferred NRTI combination for first-line antiretroviral regimens for infants and children and is often used during pregnancy [30]. Consistent with previous research [31], we show here that this combination results in the significant depletion of mtDNA in myotubes, and that this effect was prevented in the presence of 1,25D₃.

In addition to the effects described herein, the antimicrobial effects of vitamin D are well documented and association studies have linked low levels of 25D₃ and/or 1,25D₃ with an increased risk of, or severity of, infection with HIV [4,5]. Moreover, accumulating evidence points to a role for vitamin D in both the innate and adaptive immunity [32,33].

There are some limitations to the current study, including the actual concentrations of NRTI and 1,25D₃ in human skeletal muscle may be different than those used; and although the most sensitive marker for monitoring mitochondrial toxicity is through the measurement of

mtDNA levels, as mtDNA depletion precedes all the other abnormalities in mitochondrial and cell function [15,33], actual mitochondria dysfunction was not evaluated.

In conclusion, ddI-containing regimens significantly deplete mtDNA in infant skeletal muscle myoblasts and myotubes and ZDV-3TC in myotubes and that this depletion can be halted upon administration of 1,25D3. These findings also support a protective role for vitamin D in preventing mitochondrial toxicity and suggest that supplemental vitamin D when given to pregnant women may protect infants against mitochondrial toxicity associated with NRTIs. Moreover, supplemental vitamin D may be useful in children as well as in adults in preventing adverse effects associated with NRTI-related mitochondrial toxicity. Clinical trials are required to establish the benefit of vitamin D supplementation on the prevention of NRTI-related mitochondrial toxicity.

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Fig. 1. 1 α ,25-dihydroxycholecalciferol reverses nucleoside reverse transcriptase inhibitor induced myoblast mitochondrial DNA depletion
Myoblasts were treated for 5 days with NRTI in the presence or absence of 1,25D3 after which qRT-PCR was performed for mtDNA. Results are presented as the means $\pm$ SEM, $n = 9$. 1,25D3, 1 α ,25-dihydroxycholecalciferol; mtDNA, mitochondrial DNA; NRTI, nucleoside reverse transcriptase inhibitor; BC, abacavir; ddl, didanosine; d4T, stavudine; ZDV, zidovudine; 3TC, lamivudine. * $P < 0.05$.

Fig. 2. 1 α ,25-dihydroxycholecalciferol reverses nucleoside reverse transcriptase inhibitor induced myotube mitochondrial DNA depletion

Myotubes were treated for 5 days with NRTI in the presence or absence of 1,25D3 after which qRT-PCR was performed for mtDNA. Results are presented as the means $\pm$ SEM, $n = 9$. 1,25D3, 1 α ,25-dihydroxycholecalciferol; mtDNA, mitochondrial DNA; NRTI, nucleoside reverse transcriptase inhibitor; BC, abacavir; ddI, didanosine; d4T, stavudine; ZDV, zidovudine; 3TC, lamivudine. * $P < 0.05$.