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Activity profiles of deoxynucleoside kinases and 5′-nucleotidases in cultured adipocytes and myoblastic cells: insights into mitochondrial toxicity of nucleoside analogs

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Abstract

Nucleoside reverse transcriptase inhibitor (NRTI) treatment of HIV is associated with complications, including lipodystrophy (LD) and myopathy. Inhibition of mitochondrial DNA polymerase and depletion of mtDNA by NRTI triphosphates are believed to be key mechanisms in NRTI toxicity. Here, we determined the activities and mRNA levels of deoxynucleoside kinases (dNK) and 5′-nucleotidases (5′-NT) controlling the rate-limiting step in intracellular phosphorylation of NRTIs in cell models representing adipose, muscle tissue and peripheral blood cells using specific assays and Taqman RT-PCR. In vitro phosphorylation of 3′-azido-2′,3′-dideoxythymidine (AZT) and 2′,3′-didehydro-2′,3′-dideoxythymidine (d4T) in extracts was also determined. 3T3-L1 adipocytes showed similar activity of mitochondrial thymidine kinase-2 (TK2) and deoxyguanosine kinase (dGK) but 3- to 36-fold lower levels of cytosolic deoxycytidine kinase (dCK), thymidine kinase-1 (TK1) and thymidine monophosphate kinase (TMPK) and higher levels of deoxyribonucleotidase activity compared to proliferating 3T3-L1. dCK, dGK and TK2 activities correlated with their mRNA levels in proliferating, resting and differentiating 3T3-L1. Differentiated L6 myoblasts had lower activities of cytosolic dNK's and TMPK, higher dGK and similar TK2 and deoxyribonucleotidases (dNT) activities compared to proliferating myoblasts. TK2 was the limiting dNK activity while dGK was predominant in adipocytes and myocytes. Activity profiles revealed limited capacity to phosphorylate dThd and dCyd in adipocytes and myocytes compared to proliferating cells and CEM lymphocytes. Phosphorylation of AZT and d4T was low in adipocytes and myocytes, and the presence of these analogs inhibited the phosphorylation of dThd by TK2 suggesting that mitochondrial toxicity of some NRTIs in adipocytes and myocytes is due to the depletion of normal mitochondrial dNTP pools.

Keywords: NRTI; Mitochondrial toxicity; Adipocytes; Myoblasts; Deoxynucleoside kinases; 5′-Nucleotidases

1. Introduction

Currently, eight nucleoside reverse transcriptase inhibitors (NRTIs) are approved for use in the treatment of HIV. Prolonged treatment with almost all NRTIs has been associated with NRTI-specific incidences of myopathy, peripheral neuropathy, cardiomyopathy, hepatotoxicity and lactic acidosis [1,2].

Recently, a lipodystrophy syndrome (LD), consisting of changes of body shape and metabolic abnormalities (hyperlipidaemia and lactic acidosis) has been described. LD occurs relatively frequently when compared to other complications of HIV treatment, and presents a risk of atherosclerosis and other metabolic complication [3,4]. It has been suggested that the complications of NRTI treatment are the consequence of mitochondrial toxicity induced in specific tissues [5,6]. Due to the low selectivity of mitochondrial DNA (mtDNA) polymerase gamma, NRTI triphosphates (NRTI-TP) are incorporated into

Abbreviations: AZT, 3′-azido-2′,3′-dideoxythymidine; cN-1b, cytosolic 5′-nucleotidase 1b; cN-2, cytosolic 5′-nucleotidase; dCK, deoxycytidine kinase; dGK, deoxyguanosine kinase; DMEM, Dubelcco's modified Eagle's medium; DNC, deoxynucleotide carrier; dNK, deoxynucleoside kinase; d4T, 2′,3′-didehydro-2′,3′-dideoxythymidine; dNT-1, deoxynucleotidase 1; HIV, human immunodeficiency virus; LD, lipodystrophy; mtDNA, mitochondrial DNA; NRTI, nucleoside reverse transcriptase inhibitor; NRTI-DP, NRTI diphosphate; NRTI-MP, NRTI monophosphate; NRTI-TP, NRTI triphosphate; 5′-NT, 5′-nucleotidases; TK1, thymidine kinase 1; TK2, thymidine kinase 2; TMPK, thymidine monophosphate kinase

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mitochondrial DNA and cause chain termination and mitochondrial DNA depletion [5,6]. mtDNA depletion was found in biopsies from muscle, liver and adipose tissue of HIV patients with myopathy, hepatotoxicity and LD [7–10]. mtDNA depletion leads to a decrease in oxidative phosphorylation and an increase in lactate production, which result in mitochondrial dysfunction [6].

There have been many reports concerning the potency of different NRTIs to cause mtDNA depletion or the efficiency of NRTI-TP incorporation by mtDNA polymerase gamma [11–14]. However, less attention has been paid to the intracellular phosphorylation of NRTIs, which determines the size of mitochondrial NRTI-TP pools in specific tissues. The rate-limiting step in the NRTI phosphorylation cascade is catalyzed by deoxynucleoside kinases (dNK), which are represented in the cytosol by thymidine kinase 1 (TK1) and deoxycytidine kinase (dCK), and in the mitochondrial matrix by thymidine kinase 2 (TK2) and deoxyguanosine kinase (dGK) [15]. Importantly, this reaction can be reversed by 5′-nucleotidases (5′-NT), represented in the cytosol by 5′-nucleotidase 1,2 (cN-1, cN-2) and deoxynucleotidase 1 (dNT-1) and in mitochondria by deoxynucleotidase 2 (dNT-2) [16,17]. Therefore, the ratio of NK/5′-NT activities both in the cytosol and mitochondria most likely controls the amount of NRTI-MP and natural dNMP formed, which are further phosphorylated into NRTI-TP and dNTP, respectively. Cytosolic and mitochondrial NRTI-TP pools interchange through the deoxynucleotide carrier (DNC) found in the mitochondrial inner membrane [18].

Treatment with different NRTIs results in mitochondrial toxicity in specific tissues. In case of myopathy and lactic acidosis related to the treatment with 3′-azido-2′,3′-dideoxythymidine (AZT) (a thymidine analog), the mtDNA content was depleted in skeletal muscles and liver, but was not affected in blood, kidney or brain [10,19]. By analogy, an inherited mtDNA depletion syndrome, caused by mutation in mitochondrial TK2, also showed a very specific tissue distribution and resulted in infantile myopathy [20]. Mutation in the other mitochondrial kinase, dGK, affects liver and brain and leads to depletion in mtDNA content mainly in these tissues [21]. Interestingly, proliferating fibroblasts from patients with dGK mutation had normal mtDNA content, which was gradually depleted when cells stopped dividing [22]. In contrast to inherited mtDNA defects, acquired mtDNA depletion caused by NRTI can be reversed after cessation of treatment [5]. However, tissues with actively dividing cells seem to recover faster than nondividing cells from neuronal tissue [5].

It is still unclear, why NRTI-induced mitochondrial toxicity targets specific tissues, and also why differentiated nondividing cells seems to be more sensitive to mitochondrial toxicity than proliferating cells. Here, we attempt to answer some of these questions by focusing on the enzymes controlling the initial step in intracellular phosphorylation of NRTI. 3T3-L1 adipocytes and L6 myoblastic cells have extensively been used to study the biology of adipocytes and muscle cells. Therefore, we chose to use these cells as models for white adipose tissue and muscle tissue, respectively. CEM T-lymphoblastic cells were used as models for peripheral blood cells. We simultaneously compared the activity and expression of both cytosolic and mitochondrial deoxynucleoside kinases and 5′-nucleotidases as well as the expression of the DNC transporter in proliferating, resting and differentiated 3T3-L1 adipocytes and L6 myoblasts. Moreover, we compared the phosphorylation of two anti-HIV nucleoside analogs, AZT and d4T, associated with myopathy and lipodystrophy, respectively, in proliferating and differentiated 3T3-L1 and L6 cells and their effect on the phosphorylation of dThd in the studied extracts.

2. Materials and methods

2.1. Materials

[Methyl-^{3}H] thymidine and deoxy [5-^{3}H] cytidine were purchased from Amersham Pharmacia Biotech 2′-deoxyguanosine [8-^{3}H]; 3′-azido-2′,3′-dideoxythymidine [methyl-^{3}H] and thymin-1-β-D-arabinofuranoside [methyl-^{3}H(N)], [5-^{3}H] uridine were obtained from Moravec Biochemicals.

2.2. Differentiation of 3T3-L1 fibroblasts into adipocytes

3T3-L1 mouse fibroblast were obtained from LGC Promochem AB and maintained in growth media (DMEM with glutamax-1, containing pyridoxine, 4500 mg/l glucose, 10% fetal bovine serum and 1% of penicillin–streptomycin (Gibco)). To induce differentiation, confluent 3T3-L1 cells were first incubated in differentiation media #1: growth media, containing 0.5 mM isobutylmethylxanthine (IBMX) (Sigma), 100 nM dexamethasone (Sigma) and 240 nM insuline (Calbiochem). On the 3rd day media #1 was substituted with differentiation media #2: growth media, containing 240 nM insulin. Cells were maintained in media #2 for 7 days, replacing it every 2 days [23].

2.3. L6 myoblast differentiation

L6 rat myoblast (LGC Promochem AB) were cultured in growth media—DMEM containing glutamax-1, 4500 mg/ml glucose, 1 mM sodium pyruvate, 10% fetal bovine serum and 1% penicillin–streptomycin. Confluent L6 cells were differentiated into muscle cells by incubation in DMEM with 2% fetal bovine serum, 100 nM of 9-*cis* retinoic acid (Sigma) and 10 mM creatine (Sigma) for 3 days [24].

2.4. Flow cytometry

One to two million cells were washed twice with PBS and fixed for 5 min in 4% phosphate buffered formalin. After centrifugation the cell pellets were vortexed in 95% ethanol and the proportion of cells in different cell cycle phase was determined as described previously [25].

2.5. Preparation of total cell extracts

Proliferating cells at 60% confluency, resting cells at 100% confluency or differentiated cells (adipocytes on the 9th day, L6 myoblast on the 3rd day after induction of differentiation) were harvested using trypsin and washed twice with PBS. Cell pellets containing 12×10^6 of 3T3-L1 cells or 20×10^6 of L6 cells were resuspended in 0.5 ml of extraction buffer containing 50 mM Tris–HCl 7.6, 2 mM DTT, 20% glycerol, 0.5% NP-40 and complete mini protease inhibitor cocktail (Rocher Diagnostics GmbH). CEM cells were maintained in the same media as 3T3-L1 cells. Exponentially growing CEM cells were harvested, washed with PBS and the cell pellets containing 20×10^6 of cells were resuspended in 0.5 ml of extraction buffer. After incubation on ice for 30 min extracts were subjected to 3 cycles of freezing and thawing, sonication (2 times, 15 s each) to release both cytosolic and mitochondrial proteins and centrifugation for 20 min at $12{,}000 \times g$. Protein concentration was determined by the Bradford method (Biorad).

2.6. Assays for deoxynucleoside kinase activitiess

The activities of the deoxynucleoside kinases were determined in total protein extracts by a radiochemical method using a DE-81 filter paper technique with appropriate [^{3}H]-labelled substrates [26]. Briefly, 10–20 μg of total protein extract was incubated in buffer containing 50 mM Tris–HCl (pH 7.6), 5 mM $MgCl_2$, 5 mM ATP, 10 mM NaF, 2 mM DTT, 0.5 mg/ml BSA and 11 μM [^{3}H]-Thd or 22 μM [^{3}H]-[3′-azido-2′,3′-dideoxythymidine (for thymidine kinase 1-TK1), 11 μM [^{3}H]-dCyd plus 1.5 mM Thd (for deoxycytidine kinase-dCK) [27], 22 μM [^{3}H]-dGuo plus 1.5 mM dCyd (for deoxyguanosine kinase-dGK) [28], 105 μM [5′-^{3}H] uridine (for uridine kinase) [29], 50 μM [^{3}H]-TMP (for thymidine monophosphate kinase, TMPK) [30]. Phosphorylation of d4T was measured using 100 μM of [^{3}H]-d4T in the same buffer described above. Aliquots from the reactions were spotted onto the DE-81 filters at 3-time points. The filters were washed in 5 mM ammonium formate (for the TMPK assay in 4 M formic acid and 1 mM ammonium formate) and the amount of the product was quantitated by a liquid scintillation counting [26]. TK2 activity was calculated as the difference between dCK activity with and without 1.5 mM Thd [27]. Results are expressed in pmols of monophosphates formed per mg of protein per min. We also expressed the activity of mitochondrial dNKs as pmol per unit of citrate synthase activity, for the purpose of normalization to the amount of mitochondrial proteins in the total extract. Activity measurements were repeated at least twice (in duplicates). The results are presented as mean ± S.D.

2.7. Assays for 5′-nucleotidases

The activity of dNT in crude cell extracts was measured using 0.2 mM [^{3}H]-dUMP (5–15 Ci/mmol, 1 mCi/ml, Moravek Biochemicals Inc.) in 0.25 mM acetate buffer, pH 5.5, 1 mM, methyleneADP, 20 mM, $MgCl_2$, 5 mM DTT, 30 mM KCl and 0.2 mg/ml bovine serum albumin [31]. Separation of labeled deoxyuridine from dUMP was performed by HPLC as described below.

The cN-2 assay measured the dephosphorylation of 0.2 mM [^{14}C]-IMP (45–60 mCi/mmol, 100 μCi/ml, Moravek Biochemicals Inc.) in 50 mM imidazol buffer, pH 6.5, 3 mM ATP, 1 mM, methyleneADP, 10 mM $MgCl_2$, 5 mM DTT, 0.5 M NaCl and 0.2 mg/ml bovine serum albumin [31]. Separation of labeled inosine from IMP was performed by HPLC as described below.

The cN-1 assay measured the dephosphorylation of 0.2 mM [^{3}H]-AMP (15–30 Ci/mmol, 1mCi/ml, Moravek Biochemicals Inc.) in 50 mM 3-(*N*-morpholino) propanesulfonic acid (MOPS) buffer, pH 6.9, 100 mM KCl, 3 mM ADP, 6 mM $MgCl_2$, 0.1 mM DTT, 1 mM methyleneADP, and 0.2 mg/ml bovine serum albumin [31]. Separation of labeled adenosine from AMP was performed by HPLC as described below.

The reaction was started by adding 2–5 μg crude cell extracts in 25 μl final reaction and terminated after 30 min incubation by adding 5 μl 8 M ice-cold perchloric acid (PCA) and 20 μl 1.2 M KOH–0.4 M $NH_4H_2PO_4$. After centrifugation at $13{,}500 \times g$ for 15 min, 30 μl of the supernatant was injected into an HPLC system. The separation of nucleosides from nucleotides were done on a Represil, C184.6 × 80 mm, 5 μm, column (Waters). The mobile phase consisted of 0.4 M $NH_4H_2PO_4$, pH 3.6, 2 and 6 ml per min scintillation fluid during 25 min. The HPLC system consisted of a CM4000 pump (Milton Roy, LDC Division), a CMA-240 autosampler (Carneige Medicine) equipped with a variable-wavelength detector (LDC Division), and a flow scintillation analyzer 500TR (Packard). The activity of enzymes was expressed as pmol/(mg protein min).

2.8. Citrate synthase activity

Citrate synthase activity was assayed by measuring absorbance of the complex formed by released mercaptide ion with 5,5-dithiobis-(2-nitrobenzoic acid) (DNTB) at 412 nm essentially as described in [32]. One unit corresponds to 1 μmol of mercaptide ion formed per min per mg of protein.

2.9. Real-time quantitative PCR

Total RNA from proliferating (50% confluency), resting (100% confluency) cells and differentiated adipocytes and myoblasts was isolated using the RNeasy mini kit (Qiagen) and treated with DNase 1 (Qiagen) according to the manufacturer's protocol. cDNA was synthesized with 2 μg total RNA using 1 μg of random primers (Promega), 40 units of Rnasin ribonuclease inhibitor (Promega), dNTP mix (1 mM each) (Promega) and 30 units of avian myeloblastosis virus reverse transcriptase (AMV RT) (Promega) in total volume of 25 μl. 2.5 μl of 3T3-L1 cells cDNA was used for the Taqman real-time PCR assay.

Sequence specific primers (Invitrogen) and Taqman probes (MWG Biotech AG) for nine genes were designed using the Primer Express software (PE Applied Biosystem) and are presented in Table 1. Equal amounts of cDNA from each sample were amplified in the reaction mixture containing TaqMan Universal PCR master mix (Applied Biosystem), 900 nM primers and 250 nM Taqman probe in a total volume of 25 μl, and using the ABI PRISM 7700 thermocycler (PE Applied Biosystems) under the following conditions: 1 cycle at 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. The relative amounts of deoxynucleoside kinases or 5′-nucleotidase mRNAs were calculated using the standard curve method according to the User Bulletin #2 (ABI PRISM 7700 sequence detection system, Applied Biosystem). Standard curves for each gene in 3T3 cells were generated with cDNA from 3T3 cells. In 3T3-L1 cells, the relative expression of each gene was normalized to the expression of cytosolic nucleotidase 2 (cN-2), whose expression was relatively constant. Each cDNA was amplified in duplicate and the experiments were repeated twice. The results are expressed in arbitrary units.

Table 1
Sequence of primers and probes for real-time quantitative PCR

Gene	Sequence
TK1	Forward 5′-TGC CAA AGA CAC GCG CTA-3′ Reverse 5′-TGG CAA TGC GTC CAT GG-3′ Probe 5′-FAM-CAA CAG CTT CTC CAC ACA TGA TCG GAA C-TAMRA-3′
TK2	Forward 5′-GTA CGT GCA GCT CAC CAT GC-3′ Reverse 5′-GGC ATC TTC CCA CTT CTA TAC AG-3′ Probe 5′-FAM-ACC AGC ACA CGC GCC CTC AGA TG-TAMRA-3′
dCK	Forward 5′-GGA CTC TGA AAA CCA GCT TTG ATT-3′ Reverse 5′-CCA GGC TTT CGT GTT TGT CTT TA-3′ Probe 5′-FAM-AGG TGC CCG TCC TCA CAC TGG ATG-TAMRA-3′
dGK	Forward 5′-CAG TGC TGG TGC TGG ATG TC-3′ Reverse 5′-CTG TCC CAT GAG CTC TTC CTG-3′ Probe 5′-FAM-CTG AAG ACT TCT CTG AAA ATG CCG CCA-TAMRA-3′
DNC	Forward 5′-AGC TGC TCT ACC AAG CCA ACT T-3′ Reverse 5′-GCC GCC GCA CAC AAA-3′ Probe 5′-FAM-CAA ACG CAC CAG TTC TCA GCG CA-TAMRA-3′
dNT-1	Forward 5′-ATG GAC GGC GTG CTA GCT-3′ Reverse 5′-AAA GCG GCG GCG AAA-3′ Probe 5′-FAM-ATT TCG AGT CCG GCC TCC TGC AG-TAMRA-3′
cN-2	Forward 5′-CAC CTG GAC AGC AGT AGC AAT G-3′ Reverse 5′-ATG TCC ATG TCA TGA GTT ACT TTC TTA ATC-3′ Probe 5′-FAM-CGC CCG GAC ATT AGT TCC ATC CAG A-TAMRA-3′
cN-1b	Forward 5′-CGC CCA AAC CCA AGC AT-3′ Reverse 5′-ATT TTC CTG TCA TCT ACC ATA TTG AAA A-3′ Probe 5′-FAM-CAT CAC CAT TGC CGT CTC TTC TCG G-TAMRA-3′

FAM: carboxyfluorescein; TAMRA: tetramethylcarboxyrhodamine.

2.10. Statistical analysis

Statistical significance of the changes was estimated using Student's *t*-test.

3. Results

3.1. Activity and expression levels of cytosolic deoxynucleoside kinases and thymidine monophosphate kinase (TMPK) in mouse 3T3-L1 cells and CEM T-lymphoblasts

Flow cytometry revealed that 65% of proliferating 3T3-L1 cells were in S-phase of the cell cycle, whereas in resting and adipocyte-differentiated 3T3-L1 the amount of cells in S-phase decreased to 2.5 and 1.9% and the majority of cells were in G1 phase (data not shown). As seen in Table 2, activity of cytosolic TK1 was very high in extracts from proliferating 3T3-L1 cells and it was 35- and 38-fold lower in extracts from resting 3T3-L1 cells and 3T3-L1 adipocytes (Table 2). Phosphorylation of AZT, mainly carried out by TK1, was 64 times lower in 3T3-L1 adipocytes than in proliferating cells. The dCK activity in proliferating 3T3-L1 was 25-fold lower than TK1 and it decreased in resting 3T3-L1 cells and adipocytes. In contrast, the activity of uridine kinase decreased only 1.8-fold and remained high in 3T3-L1 differentiated adipocytes compared to proliferating 3T3-L1 cells. We also measured the activity of TMPK, which is rate limiting in activation of AZT. TMPK activity in dividing 3T3-L1 cells was 1049 pmol/(mg min), it decreased in resting 3T3-L1 and adipocytes 4.8- and 2.7-fold, respectively, but still remained relatively high compared to the activity of other studied kinases (Table 2).

Fig. 1A shows mRNA expression of cytosolic dNKs in proliferating resting and differentiated 3T3-L1 cells. The expression of TK1 mRNA was reduced 4.2- and 8.3-fold, respectively, in resting 3T3-L1 and adipocyte compared to proliferating cells. dCK mRNA levels were also lower in resting 3T3-L1 cells and adipocytes. There was a good

Table 2
Activity of cytosolic and mitochondrial deoxynucleoside kinases and thymidine monophosphate kinase in 3T3-L1 and CEM cells

Enzyme	Substrate	CEM lymphocytes	Proliferating 3T3-L1	Resting 3T3-L1	Differentiated adipocytes
TK1	Thd	431.6 ± 53.1	469.7 ± 28.7	13.3 ± 0.7	12.4 ± 1.3
	AZT[a]	209.0 ± 42.9	249.2 ± 39.4	5.4 ± 0.4	3.9 ± 0.7
dCK	dCyd + Thd[b]	52.1 ± 1.7	18.4 ± 2.4	6.9 ± 1.0	5.7 ± 1.0
TMPK	TMP[c]	142.6 ± 5.0	1049.1 ± 61.3	220.0 ± 26.1	385.3 ± 57.4
UK[d]	Urd	n.d.	411.9 ± 13.3	357.6 ± 16.8	232.2 ± 10.5
TK2	Ara T[e]	1.0 ± 0.1 (0.012)	1.0 ± 0.1 (0.009)	1.2 ± 0.1 (0.008)	1.6 ± 0.1 (0.009)
	dCyd-(dCyd + Thd)[f]	0.5 (0.006)	2.5 (0.023)	2.5 (0.017)	4.7 (0.026)
dGK	dGuo+dCyd[g]	3.2 ± 0.7 (0.039)	49.8 ± 4.8 (0.465)	66.5 ± 4.1 (0.44)	63.5 ± 4.4 (0.349)

Enzyme activities with the indicated substrates were assayed in total extracts prepared from proliferating 3T3-L1 cells (at 60% confluence), resting 3T3-L1 cells (100% confluent), adipocytes (on the 9th day after induction of differentiation) and from exponentially growing CEM lymphocytes. Activities are presented in pmol/(mg min) as a mean ± S.D. of two experiments performed in duplicates. Values in parenthesis represent the activities of mitochondrial enzymes expressed in pmol/unit of citrate synthase activity (1 unit of citrate synthase equals 1 nmol of mercaptide ion formed per mg of protein per min).
n.d.: has not been determined.
[a] AZT: 3′-azido-2′,3′-dideoxythymidine.
[b] dCK activity was measured with dCyd in the presence of excess of Thd to block TK2 activity present in total extracts.
[c] TMPK activity was measured with 50 μM of TMP.
[d] Activity of uridine nucleoside kinase was assayed with 105 μM of Urd.
[e] TK2 activity was measured with Thd analog—AraT, which is phosphorylated by TK2 with the same efficiency as Thd, but is a very poor substrate for TK1.
[f] TK2 activity is measured as inhibition of total activity in extract with dCyd in the presence of excess of Thd.
[g] dGK activity is assayed with dGuo in the presence of an excess of dCyd, which inhibits dCK activity in total extract.

correlation between mRNA expression and enzyme activity in case of dCK. However, the TK1 activity in adipocytes was reduced more than its mRNA levels (Fig. 1A; Table 2).

CEM lymphocytes had similar levels of TK1 and 2.8 times higher levels of dCK activity compared to proliferating 3T3-L1 cells. TMPK activity in CEM cells was 7 times lower than in proliferating 3T3-L1 cells (Table 2).

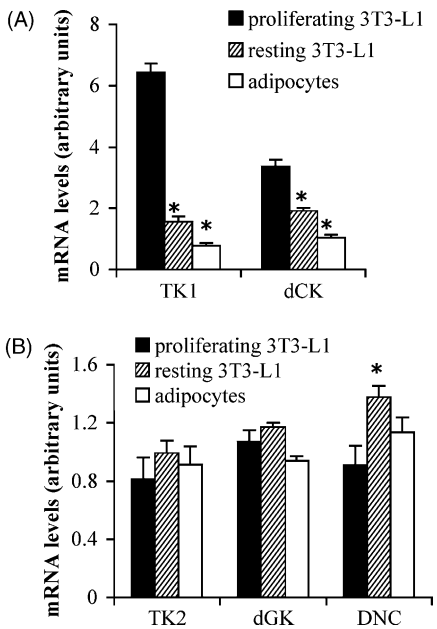

Fig. 1. Expression of deoxynucleoside kinases and 5′-nucleotidases mRNA in 3T3-L1 cells: mRNA levels of cytosolic (A), mitochondrial deoxynucleoside kinases (B), DNC (B) in proliferating 3T3-L1 cells (at 60% confluency), resting 3T3-L1 cells (100% confluent) and adipocytes (on the 9th day after induction of differentiation) were quantitated by real-time PCR using the standard curve method. Relative amounts of gene expression are normalised to cN-2 level as described in Section 2. Results are presented in arbitrary units as a mean ± S.D. of two assays run in duplicates, $^{*}p < 0.05$ compared to proliferating cells.

3.2. Activity and expression levels of mitochondrial deoxynucleoside kinases in 3T3-L1 and CEM cells

In mitochondria, there are two enzymes, TK2 and dGK, phosphorylating nucleosides and their analogs to monophosphates. Overall levels of TK2 activity in total extracts from 3T3 cells were very low compared to the levels of the cytosolic enzymes (Table 2). TK2 activity was similar in proliferating, resting and differentiated 3T3-L1 cells, as measured with the selective assays based on AraT or dCyd-(dCyd plus Thd) (Table 2). This was more clearly seen when the TK2 activity was normalized to the amount of mitochondrial protein (citrate synthase) in the total extract (Table 2, values in parenthesis). Levels of dGK activity were 20-fold higher compared to TK2 in proliferating 3T3-L1 (Table 2). dGK activity remained constant in resting 3T3-L1 cells and slightly decreased in 3T3-L1 adipocytes compared to proliferating 3T3-L1 cells (Table 2, values in parenthesis). In differentiated 3T3-L1 adipocytes the activity of dGK was higher than all other dNKs.

The levels of TK2 and dGK mRNA were similar in proliferating, resting and differentiated 3T3-L1 cells and in good correlation with the TK2 and dGK enzyme activities (Fig. 1B). The expression of the DNC transporter mRNA increased 52% in resting and 25% in differentiated 3T3-L1 adipocytes, as compared to proliferating cells (Fig. 1B).

Activities of mitochondrial TK2 and dGK in CEM lymphocytes were significantly lower than activities of cytosolic TK1 and dCK (Table 2). Moreover, CEM lymphoblastic cells had 4 times less TK2 and 9 times less dGK

Table 3
Activity and mRNA levels of 5′-nucleotidases in 3T3-L1 and CEM cells

	cN-1[a]	cN-2	dNT
CEM lymphocytes	448 ± 14	571 ± 101	265 ± 89
Proliferating 3T3-L1	761 ± 17 (0.44 ± 0.10)	743 ± 182 (4.66 ± 0.36)	224 ± 118 (1.21 ± 0.11)
Resting 3T3-L1	1341 ± 162[b] (0.31 ± 0.03)[b]	943 ± 117 (4.68 ± 0.33)	526 ± 103[b] (1.30 ± 0.07)
Differentiated adipocytes	1993 ± 39[b] (0.39 ± 0.03)	1307 ± 212[b] (5.71 ± 0.45)[b]	550 ± 130[b] (1.02 ± 0.03)[b]

Enzyme activities of 5′-nucleotidases were assayed in total protein extracts prepared from 3T3-L1 and CEM cells as described in Section 2. dNT activity represents a sum of cytosolic dNT-1 and mitochondrial dNT-2 activities, both dephosphorylating dUMP. Activities are presented in pmol/(mg min). mRNA levels shown in parenthesis were quantitated by real-time PCR as described in Materials and Methods. Relative amounts of gene expression were normalized to cN-2 mRNA levels for cN-1b and dNT-1. Results are expressed in arbitrary units as a mean ± S.D. of two experiments run in duplicates. cN-2 expression here is presented in arbitrary units per 1 μg of total RNA since it was used as a normalizer for all the other genes.

[a] cN-1b mRNA levels are multiplied by a factor of 1000.

[b] $p < 0.05$ compared to proliferating cells.

activity compared to differentiated 3T3-L1 adipocytes (Table 2, values in parenthesis).

3.3. Enzyme activity and expression of 5′-nucleotidases in total extracts from 3T3-L1 and CEM cells

Cellular 5′-nucleotidases dephosphorylate dNMP's, and are involved in the control of intracellular dNTP or NRTI-TP pools [16]. Table 3 lists the activities of two cytosolic 5′-ribonucleotidases (cN-1, AMP-specific and cN-2, IMP-specific) and the total activity of deoxyribonucleotidases dNT-1 and dNT-2 (both dUMP-specific) using selective assays as described in Section 2. cN-2 activity was constant in extracts from proliferating and resting 3T3-L1 cells and higher in differentiated 3T3-L1 cells (Table 3). The activity of cN-1 was 80 and 160% higher in resting and differentiated 3T3-L1 cells compared to proliferating 3T3-L1 cells. In addition, the total deoxyribonucleotidase activity increased in both resting 3T3-L1 cells and adipocytes as compared to proliferating cells (Table 3). In general, the activity of deoxyribonucleotidases was significantly lower than 5′-ribonucleotidases in extracts from 3T3-L1 cells (Table 3).

cN-1b, dNT-1 and cN-2 mRNA were expressed at approximately constant levels in proliferating, resting and differentiated 3T3-L1 cells (Table 3). Therefore, there was no apparent correlation between mRNA expression and enzymatic activity for all three 5′-NTs in 3T3-L1 cells. The activity of all measured 5′-nucleotidases was lower in CEM lymphocytes than in 3T3-L1 cells.

3.4. The activities of cytosolic deoxynucleoside kinases and thymidine monophosphate kinase (TMPK) in L6 rat myoblast

L6 cells were used as a model for muscle tissue [24,33]. Twenty-seven percent of the cells were in S-phase of the cell cycle in samples from proliferating L6 cells. In resting and differentiated L6 myoblasts, the proportion of cells in S-phase decreased to 11 and 3%, respectively. About 76 and 81% of cells were in the G1 phase of the cell cycle according to flow cytometry results (data not shown). Table 4 shows the activities of the deoxynucleoside kinases in L6 cells. The activity of cytosolic TK1 in proliferating L6 myoblasts was very high. It decreased in resting and differentiated L6 myoblasts 7- and 417-fold, respectively. Phosphorylation of AZT decreased 8- and 300-fold in resting and differentiated myoblasts compared to proliferating L6 cells. dCK activity was constant in resting L6 and decreased 9-fold in differentiated L6 myoblasts compared to proliferating L6 cells (Table 4). The activity of thymidine monophosphate kinase was 418.8 pmol/(mg min) in proliferating L6 myoblasts and it decreased 2- and 33-fold in resting and differentiated L6 myoblasts (Table 4). This was very different from the 3T3-L1 cells, where differentiated adipocytes showed 30-fold higher TMPK activity compared to differentiated L6 myoblasts (Tables 2 and 4). RT-PCR methods for quantitation of the rat enzyme mRNAs were not developed in this study.

Proliferating L6 myoblasts had the highest TK1 activity among proliferating 3T3-L1 and CEM lymphoblastic cells (Tables 2 and 4). Overall, TK1 and dCK activity in differentiated L6 myoblasts was 4.2- and 2.9-fold lower than in 3T3-L1 adipocytes (Tables 2 and 4).

3.5. The activities of mitochondrial deoxynucleoside kinases in L6 myoblastic cells

Table 4 shows that the activity of TK2 is very low in L6 cells compared to the activity of the cytosolic dNK's. It did not change significantly in differentiated L6 myoblasts but was 2 times lower in resting L6 cells compared to proliferating L6 (Table 4, values in parenthesis). The levels of dGK activity were higher than the TK2 activities in proliferating, resting and differentiated L6 myoblasts: 9-, 39- and 16-fold, respectively (Table 4). The activity of dGK related to the citrate synthase levels was 2-fold higher in resting and differentiated L6 cells as compared to proliferating cells (Table 4, values in parenthesis).

Overall levels of TK2 and dGK activity were 2- to 4-fold lower in proliferating, resting and differentiated L6 myoblasts as compared to proliferating, resting and differentiated 3T3-L1 adipocytes (Tables 2 and 4).

Table 4
Activity of cytosolic and mitochondrial deoxynucleoside kinases and thymidine monophosphate kinase in L6 rat myoblast

Enzyme	Substrate	Proliferating L6	Resting L6	Differentiated L6 myoblasts
TK1	Thd	1211.6 ± 100.4	168.4 ± 1.7	2.9 ± 0.3
	AZT[a]	544.3 ± 42.2	71.4 ± 7.8	1.8 ± 0.3
dCK	dCyd + Thd[a]	18.3 ± 1.6	14.6 ± 0.7	2.0 ± 0.3
TMPK	TMP[a]	418.8 ± 28.3	189.7 ± 16.1	12.5 ± 2.7
TK2	Ara T[b]	3.0 ± 0.3 (0.027)	1.1 ± 0.1 (0.008)	0.8 ± 0.1 (0.008)
	dCyd-(dCyd + Thd)[c]	1.3 (0.012)	0.9 (0.006)	1.1 (0.012)
dGK[d]	dGuo + dCyd	11.9 ± 1.6 (0.106)	33.9 ± 3.7 (0.231)	18.5 ± 1.8 (0.194)

Enzyme activities with indicated substrates were assayed in total extracts prepared from proliferating L6 cells (at 60% confluency), resting L6 cells (100% confluent), differentiated L6 myoblasts (on the 3rd day after induction of differentiation). Activities are presented in pmol/(mg min) as a mean ± S.D. of two experiments performed in duplicates. Values in parenthesis represent the activities of mitochondrial enzymes expressed in pmol/unit of citrate synthase activity.
[a] See Table 1.
[b] TK2 activity was measured with Thd analog—AraT, which is phosphorylated by TK2 with the same efficiency as Thd, but is a very pure substrate for TK1.
[c] TK2 activity is measured as inhibition of total activity in extract with dCyd in the presence of excess of Thd.
[d] dGK activity is assayed with dGuo in the presence of excess of dCyd, which inhibits dCK activity in total extract.

3.6. 5′-nucleotidases activities in total extracts from L6 myoblastic cells

The activity of cytosolic cN-1 nucleotidase increased in resting and differentiated L6 myoblasts compared to proliferating L6 cells (Table 5). The activity of cN-2 was slightly decreased in resting and differentiated L6 myoblasts compared to proliferating cells. The total activity of cytosolic and mitochondrial deoxyribonucleotidases (dNT) was significantly lower than the cN-1 and cN-2 activities in extracts from L6 myoblasts (Table 5). dNT activity increased in resting L6 cells and somewhat in differentiated myoblasts compared to proliferating L6 cells.

Overall levels of cN-1 and dNT nucleotidases activity were lower in extracts from differentiated L6 myoblasts when compared to the levels in extracts from 3T3-L1 adipocytes (Tables 3 and 5). The levels of cN-2 activity were 2-fold higher in differentiated myoblasts compared to adipocytes (Tables 3 and 5).

3.7. Ratios between the activities of deoxynucleoside kinases and 5′-deoxyribonucleotidase

Ratios between the activities of cytosolic deoxynucleoside kinases TK1, dCK and deoxyribonucleotidase dNT were significantly lower in differentiated 3T3-L1 cells compared to proliferating 3T3-L1 cells (Table 6). There was also a decrease in the ratios between the activities of mitochondrial deoxynucleoside kinases TK2, dGK and deoxyribonucleotidase dNT in differentiated 3T3-L1 versus proliferating cells (Table 6). CEM lymphocytes had as high TK1/dNT and dCK/dNT ratios as in proliferating 3T3-L1 cells, but lower TK2/dNT and dGK/dNT ratios (Table 6). The TK1/dNT and dCK/dNT ratios were much lower in differentiated L6 compared to proliferating L6 cells. However, TK2/dNT ratio was constant and dGK/dNT ratio slightly increased (Table 6).

Table 5
Activity of 5′-nucleotidases in L6 rat myoblasts

	cN-1	cN-2	dNT
Proliferating L6	684 ± 130	3319 ± 539	215 ± 2
Resting L6	1286 ± 93	2541 ± 406	550 ± 57
Differentiated L6 myoblasts	961 ± 97	2457 ± 81	299 ± 11

Enzyme activities of 5′-nucleotidases were assayed in total protein extracts prepared from L6 cells as described in Materials and Methods. dNT activity represents a sum of cytosolic dNT-1 and mitochondrial dNT-2 activities, both dephosphorylating dUMP. Activities are presented in pmol/(mg min).

3.8. Phosphorylation of antiviral nucleoside analogs in extracts from proliferating and differentiated 3T3-L1 and L6 cells and their effect on the phosphorylation dThd in these extracts

The phosphorylation of d4T was 6.1 ± 1.3 and less than 0.6 pmol/(min mg) in proliferating L6 and 3T3-L1 cells and less than 0.5 and 0.1 pmol/(min mg) in differentiated L6 myoblasts and 3T3-L1 adipocytes (data not shown). AZT phosphorylation was about 40–50% of thymidine phosphorylation in extracts from proliferating and differentiated L6 and 3T3-L1 cells as shown previously in Tables 2 and 4.

Table 7 demonstrates the effect of d4T and AZT on the phosphorylation of Thd in the extracts from L6 and 3T3-L1 cells. d4T did not affect Thd phosphorylation at low concentration. However, 500 μM of d4T inhibited Thd phosphorylation by 15 and 17% in proliferating and differentiated 3T3-L1 cells, and by 13 and 18% in proliferating and differentiated L6 cells.

AZT was a strong inhibitor of Thd phosphorylation in all extracts and 20 μM of AZT decreased phosphorylation of Thd by 59 and 42% in proliferating and differentiated 3T3-L1 cells and by 63 and 50% in proliferating and differentiated L6 cells (Table 7). Thd phosphorylation was inhibited even more by higher concentration of AZT in all extracts.

Table 6
Ratios between the activities of deoxynucleoside kinases and 5′-deoxyribonucleotidase in proliferating and differentiated 3T3-L1 and L6 cells

	TK1/dNT	dCK/dNT	TK2/dNT $\times 10^{-3}$	dGK/dNT $\times 10^{-3}$
CEM lymphocytes	1.62	0.20	0.02	0.15
Proliferating 3T3-L1	2.09	0.08	0.10	2.07
Differentiated 3T3-L1	**0.02**	**0.01**	**0.05**	**0.63**
Proliferating L6	5.63	0.09	0.05	0.49
Differentiated L6	**0.01**	**0.01**	**0.04**	**0.65**

Ratios were calculated from the values for the activities of TK1, dCK, TK2, dGK and dNT in Tables 2–5. Ratios for the differentiated 3T3-L1 and L6 cells are selected in bold.

Table 7
Inhibition of deoxythymidine phosphorylation by antiviral nucleoside analogs in total extracts from 3T3-L1 and L6 cells

	Proliferating 3T3-L1	Differentiated adipocytes	Proliferating L6	Differentiated L6
Thd (11 μM)	98.2 ± 2.9 (100)	11.1 ± 0.1 (100)	784.7 ± 6.4 (100)	2.2 ± 0.2 (100)
Thd + 100 μM d4T	91.8 ± 4.5 (93)	10.6 ± 0.1 (95)	753.0 ± 6.2 (96)	2.0 ± 0.1 (91)
Thd + 500 μM d4T	83.7 ± 1.3 (85)	9.2 ± 0.2 (83)	682.6 ± 10.7 (87)	1.8 ± 0.1 (82)
Thd + 20 μM AZT	40.73 ± 5.5 (41)	6.4 ± 0.2 (58)	288.4 ± 11.6 (37)	1.1 ± 0.1 (50)
Thd + 100 μM AZT	28.1 ± 0.1 (29)	4.3 ± 0.3 (39)	89.4 ± 5.3 (11)	0.8 ± 0.1 (36)

Phosphorylation of deoxythymidine was measured using DE-81 filter paper technique, as described in Section 2, with 11 μM dThd and the indicated amount of AZT or d4T nucleoside analogs. Results are presented in pmol/(min mg) as a mean ± S.E.M. of 2–4 measurements. Numbers in parenthesis are the percent of dThd phosphorylation compared to the control (dThd).

4. Discussion

NRTI treatment in combination with other antiviral drugs is effective in controlling HIV infection. At the same time it is complicated by a number of unwanted metabolic adverse effects, including myopathy and LD. The molecular mechanism of these side effects has been attributed to mitochondrial toxicity caused by NRTI's [5,6]. Inhibition of mtDNA polymerase by NRTI-TP is believed to be the cause of NRTI toxicity, which results in mtDNA depletion in specific tissues. However, the role of the intracellular phosphorylation of NRTI to the triphosphates has not been analyzed in great detail. The tissue specificity of enzyme expression and activity can potentially enhance our understanding of the tissue specificity of NRTI mitochondrial toxicity and suggest measures to prevent it. In the present study, we used 3T3-L1 cells as a model for white adipose tissue, L6 myoblastic cells and CEM T-lymphocytes as models for muscle tissue and peripheral blood cells to analyze the activity and expression profiles of enzymes controlling intracellular NRTI phosphorylation (activation), i.e., deoxynucleoside kinases and 5′-nucleotidases and to compare phosphorylation of commonly used nucleoside analogs in extracts from proliferating and differentiated cells.

Our results indicate that differentiated 3T3-L1 adipocytes have the same activity of mitochondrial deoxynucleoside kinases TK2 and dGK as proliferating cells, but low activity of cytosolic dCK and TK1. However, the total activity of deoxyribonucleotidases was higher in differentiated adipocytes compared to proliferating 3T3-L1 cells. In the L6 cell model for muscle we found a similar activity profile of cytosolic and mitochondrial deoxynucleoside kinases and 5′-nucleotidases. Moreover, differentiated L6 myoblasts had even lower activity of cytosolic kinases (TK1 and dCK) and 2 times lower activity of mitochondrial TK2 and dGK compared to 3T3-L1 adipocytes. The total activities of 5′-deoxyribonucleotidases were also, somewhat, lower in differentiated L6 myoblasts compared to adipocytes.

We clearly show that TK2 is the limiting mitochondrial deoxynucleoside kinase and dGK activity is approximately 10-fold higher than the TK2 levels in differentiated adipocytes and myoblasts. The TMPK levels were lower in differentiated myoblasts compared to adipocytes, but still considerably higher than the levels of TK1.

CEM lymphoblastic cells show a different activity profile. They have low activities of mitochondrial TK2 and dGK, but significantly high levels of cytosolic TK1 and dCK and low levels of 5′-deoxyribonucleotidases compared to differentiated adipocytes and myoblasts. Therefore, dNK/5′-NT ratios were significantly higher in CEM cells compared to adipocytes and myocytes.

Our results are in agreement with previous studies demonstrating that dCK activity is not cell cycle regulated, but tissue specific with highest activity found in lymphoid cells [15,34]. In contrast, TK1 activity is shown to be cell-cycle regulated with the highest levels in proliferating cells [15,27,34]. Taken together, our data demonstrate that the terminally differentiated adipocytes and myoblasts possess low but significant and probably sufficient activity of deoxynucleoside kinases to maintain dNTP pools for basal nuclear DNA repair and for mtDNA replication as compared to the proliferating cells. Considering that dGK levels are still high, adipocytes and myocytes might have limited capacity to phosphorylate dThd and dCyd compared to CEM cells.

Importantly, activity profiles of dNK and 5′-NT in these cell models can provide insights into (1) how NRTIs can be phosphorylated and dephopshorylated in adipocytes and differentiated myoblasts compared to proliferating cells and (2) how the presence of NRTIs in adipocytes and myocytes can affect normal nucleoside metabolism and dNTP pools needed for mitochondrial DNA synthesis. Our results indicate that AZT phosphorylation in adipocytes and myocytes may occur, but at lower levels compared to proliferating cells. d4T is not phosphorylated significantly in extracts from proliferating and differentiated adipocytes and L6 myoblasts. NRTI monophosphates probably also can be dephosphorylated in adipocytes and myocytes, which have high deoxyribonucleotidase activity compared to proliferating cells. However, due to low levels of dNK activity and a low ratio of dNK/5′-NT, adipocytes and myocytes should be more sensitive to NRTI interference with normal nucleoside metabolism than proliferating cells. NRTIs may be able to block the activity of some cytosolic deoxynucleoside kinases or mitochondrial dNK by serving as a substrate or inhibitor giving a limitation in the supply of dNTPs. This effect together with the low affinity of NRTIs for 5′-deoxyribonucleotidases [35] can result in time-dependent depletion of normal mtDNA precursors pools, and probably increase the NRTI-TP pools in cytosol and mitochondria of adipocytes and myoblasts.

Here, we demonstrated that AZT inhibits phosphorylation of Thd in differentiated 3T3-L1 adipocytes and L6 myocytes most likely via inhibition of both TK1 and TK2 activities (Ki values of AZT for TK1 and TK2 are 0, 6 and 2 μM, respectively) [36]. Importantly, the remaining dThd kinase activity was significantly lower in extracts from differentiated adipocytes and myocytes than in proliferating cells. Our data demonstrate that d4T inhibited dThd phosphorylation in extracts from proliferating and differentiated 3T3-L1 and L6 myoblasts to some extent albeit at very high concentrations. The Ki values of d4T for TK1 and TK2 are 2073 and 78 μM, respectively [36] suggesting that d4T inhibition of TK2 in adipocytes and myocytes would be minimal in the in vivo situation since the maximal concentration of d4T obtained in pharmacokinetic experiments is about 4 μM [1,2]. However, the inhibitory effect of AZT was much more pronounced at physiologically relevant concentrations and could be involved in myopathy side effects observed in HIV patients [7].

NRTI interference may also cause imbalance in cytosolic or mitochondrial dNTP pools and this mechanism may be analogous to the inherited mitochondrial DNA depletion syndrome caused by mutation in mitochondrial deoxynucleoside kinases TK2 and dGK [37]. Mutations in TK2 and dGK result in complete or partial loss of enzymatic activity [21,37]. It has been noticed that only the patients with TK2 or dGK activity exceeding a certain minimal threshold can survive [38,39]. A similar threshold level in the capacity of mitochondria to salvage dThd or dCyd may come into operation in case of NRTI toxicity.

We found that the expression of DNC transporter, carrying cytosolic dNDP into mitochondria, was somewhat increased in resting and differentiated 3T3-L1 cells compared to proliferating 3T3-L1 cells. DNC has a higher affinity for di-deoxynucleotides (most of NRTIs) [18]. Therefore, it may contribute to the depletion in mitochondrial dNTP pools in resting and differentiated 3T3-L1 cells.

In conclusion, activity profiles revealed that the differentiated adipocytes and myocytes have a limited capacity to phosphorylate dThd and dCyd compared to proliferating cells and CEM lymphocytes. Mitochondrial toxicity of NRTIs has been attributed to inhibition of mtDNA polymerase gamma by NRTI-TP [3]. Our present study suggests that mitochondrial toxicity of some NRTIs in adipocytes and myocytes might, at least in part, be attributed to the depletion of normal dNTP pools caused by NRTI interference with endogenous nucleoside metabolism.

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