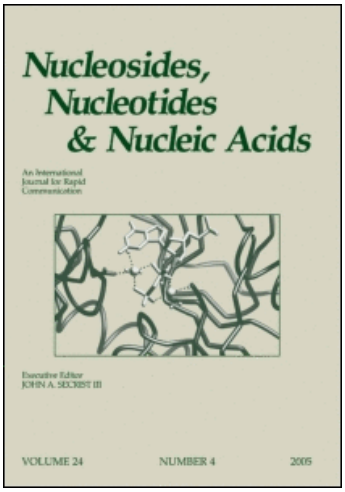

Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

The Role of Mitochondrial dNTP Levels in Cells with Reduced TK2 Activity

C. Desler[a]; B. Munch-Petersen[a]; L. J. Rasmussen[a]
[a] Department of Life Sciences and Chemistry, Roskilde University, Roskilde, Denmark

To cite this Article Desler, C. , Munch-Petersen, B. and Rasmussen, L. J.(2006) 'The Role of Mitochondrial dNTP Levels in Cells with Reduced TK2 Activity', Nucleosides, Nucleotides and Nucleic Acids, 25: 9, 1171 — 1175
To link to this Article: DOI: 10.1080/15257770600894501
URL: http://dx.doi.org/10.1080/15257770600894501

Nucleosides, Nucleotides, and Nucleic Acids, 25:1171–1175, 2006

ISSN: 1525-7770 print / 1532-2335 online
DOI: 10.1080/15257770600894501

Taylor & Francis
Taylor & Francis Group

THE ROLE OF MITOCHONDRIAL dNTP LEVELS IN CELLS WITH REDUCED TK2 ACTIVITY

C. Desler, B. Munch-Petersen, and L. J. Rasmussen □ *Department of Life Sciences and Chemistry, Roskilde University, Roskilde, Denmark*

□ *Both the nuclear and mitochondrial DNA (mtDNA) depend on separate balanced pools of dNTPs for correct function of DNA replication and repair of DNA damage. Import of dNTPs from the cytosolic compartment to the mitochondria has been suggested to have the potential of rectifying a mitochondrial dNTP imbalance. Reduced TK2 activity has been demonstrated to result in mitochondrial dNTP imbalance and consequently mutations of mtDNA in non-dividing cells. In this study, the consequences of a reduced thymidine kinase 2 (TK2) activity were measured in proliferating HeLa cells, on both whole-cell as well as mitochondrial dNTP levels. With the exception of increased mitochondrial dCTP level no significant difference was found in cells with reduced TK2 activity. Our results suggest that import of cytosolic dNTPs in mitochondria of proliferating cells can compensate a TK2 induced imbalance of the mitochondrial dNTP pool.*

Keywords Thymidine kinase 2; Mitochondrial dNTP imbalance; dNTP transport

INTRODUCTION

Imbalanced levels of dNTP result in misincorporation of nucleotides into the DNA, frameshift mutations and stalling of replication fork.[1–3] Mitochondrial dNTP pool imbalance has been demonstrated to induce deletions and depletions in mtDNA,[4] which may cause a number of mitochondrial related diseases such as mitochondrial neurogastrointestinal encephalomyopathy (MNGIE) and mtDNA depletion syndrome (MDS).[4,5] Among the possible mediators of a mtDNA imbalance are dysfunctions of either of the mitochondrial deoxyribonucleoside kinases: thymidine kinase 2 (TK2) or deoxyguanosine kinase (dGK).[1,6]

Recently, the importance of dNTPs from the cytosol to the mitochondria has been demonstrated.[7] Once putative function of this transport is to maintain the balance of the mitochondrial dNTP pool. In nondividing tissue

Address correspondence to B. Munch-Petersen, Department of Life Sciences, Roskilde University, DK-4000, Roskilde, Denmark. E-mail: bmp@virgil.ruc.dk

though, loss of function mutations in either TK2 or dGK have been demonstrated to result in a mitochondrial dNTP imbalance and the consequential deletions and depletions of mtDNA,[1,6] indicating that import of dNTP to the mitochondria is insufficient in these cells. The cytosolic dNTP levels in proliferating cells are however approximately 10-fold higher compared to the levels of nondividing cells.[8] The increased concentration of cytosolic dNTP could allow a putative import of dNTP to the mitochondria from the cytosol, which would contribute to the maintenance of the mitochondrial dNTP balance in dividing cells.

In the present study, we have modelled a loss of function mutation in TK2 by generating a cell line that can be induced to express lower levels of TK2, and we have measured both the cytosolic and mitochondrial dNTP levels in these proliferating cells.

MATERIALS AND METHODS

Transfection of conditional TK2 antisense plasmids: The doxycyline-responsive (Tet-On) vector pTRE2 (Clontech, Medinova Scientific A/S, Denmark) was used to construct a plasmid expressing the TK2 cDNA in antisense orientation. HeLa cells were transfected with the plasmid using Lipofectamine 2000 (Invitrogen, A/S, Denmark), according to manufacturer's instructions. As selectable marker the plasmid has the puromycin resistance gene and stable clones were selected by incubation in culture medium containing 1 μg/ml puromycin for 10 days. Subsequently, the cells where trypsinated and transferred to 24-well microtiter plates at a density of 50 cells per well. The cells were further incubated 10 days in culture medium without puromycin and finally 7 days in culture medium containing 1 μg/ml puromycin. At each treatment with puromycin, a control culture containing HeLa Tet-On cells not transfected with plasmid, was grown at similar conditions. Before the puromycin treatment was ended, it was made sure that all cells in the control culture had succumbed to the treatment.

Thymidine kinase assay: The activity of TK2 was assayed by its unique ability to phosphorylate thymine-β-D-arabinoside (Ara-T). TK2 phosphorylates AraT with 30–50% of its substrate specificity toward thymidine.[9] Stably transfected HeLa cells was screened by growing cells for 1 week both with and without doxycyline present the in growth media and comparing the TK2 activity of the 2 as described in the following. Cells where lysed by sonication and cell extracts were assayed by adding 15 μl cell extract to a final volume of 50 μl reaction mixture containing: 50 mM Tris-HCl pH 7.5, 10 mM DTT, 2.5 mM ATP, 2.5 mM MgCl, 3 mg/ml BSA, 6 mM NaF, 0.5 mM Chaps and 20 mM [^{3}H] AraT (3 ci/mmol, Morevek Biochemicals, Inc., Brea, CA, USA). The mix was incubated at 37°C, and 10 μl aliquots were removed from the solution at 4, 8 12, and 16 minutes after reaction start, and

spotted onto discs of Whatman DE81 filters. The filters were dried, washed (3 × 10 minutes) with ammonium formate and 5 minutes with autoclaved MilliQ water. Finally the filters were eluted in 500 μl of 0.1 M HCl and 0.2 M KCl and the radioactivity quantified by scintillation counting.

dNTP determination: The whole-cell and mitochondrial dNTP levels were measured using the method of Pontarin et al.[7] and Sherman and Fyfe.[10]

Statistics: Single classification analysis of variance (ANOVA) was used to test for differences in dNTP levels among the different cell lines. Assumptions of normality were checked by visual inspection prior to ANOVA. When the ANOVA indicated significant differences, Tukeys honestly significant method was used to test for differences between the dNTP pools of individual cell lines.

RESULTS

Screening of HeLa cells expressing TK2 antisense RNA in the presence of doxycycline revealed several clones with reduced TK2 activity. One clone that showed approximately 47% reduction of whole-cell TK2 activity upon treatment with doxycycline for a week was chosen for further analysis (data not shown). This cell line was named HeLa TK2$^-$.

The whole-cell dNTP levels were determined in HeLa TK2$^-$ cells grown in the presence of doxycycline for a week, and compared with isogenic cells grown in absence of doxycycline. As illustrated in Figure 1, the doxycycline induced reduction of TK2 activity did not result in a significant difference in dNTP levels compared to cells with a wild type TK2 activity. This indicates

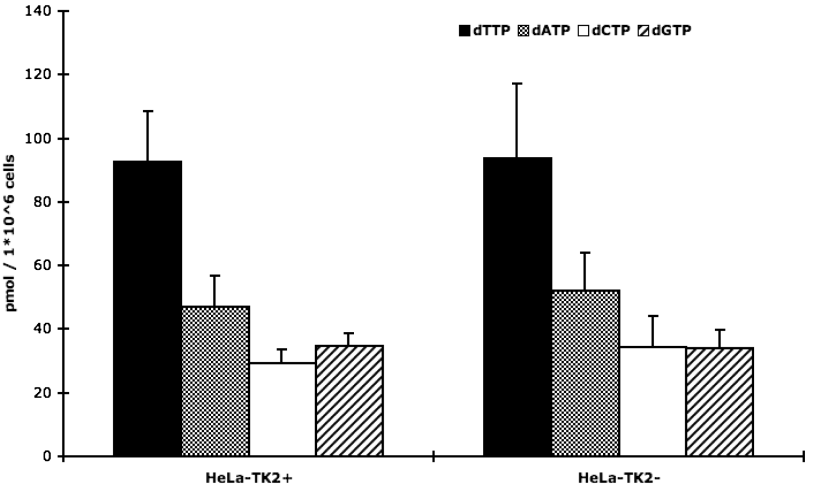

FIGURE 1 The whole-cell levels of dNTP are measured for the dividing cell lines HeLa-TK2$^+$ and HeLa-TK2$^-$ ($n = 12$; error bars indicate SD).

that reduced TK2 activity does not have an effect on the whole-cell dNTP levels.

Similarly, when we determined the mitochondrial dNTP levels of cells grown in the presence as well as the absence of doxycycline, the difference in TK2 activity did not result in a significant difference in the mitochondrial levels of dTTP, dATP and dGTP. The levels of dCTP were significantly higher with an 1.4-fold increase in cells with a reduced TK2 activity (Tukey; $n = 6$; $p < 0.001$) (Figure 2).

DISCUSSION

Even subtle impairments of the TK2 activity have been reported to have an effect on the balance of the mitochondrial dNTP pool. A His-121 to Asn substitution in human TK2 resulted in vitro in a normal activity with dThd as substrate but a 70% reduced activity with dCyd.[11] Furthermore nonreplicating fibroblast cell lines homozygous for the His-121 to Asn TK2 mutation showed decreased levels of mitochondrial dNTP content compared to control cell lines.[1] Our results indicate that a mitochondrial dNTP imbalance is rectified in dividing cells by an import of dNTP from the cytosolic pool. This confirms the initial theory of the potential of such a transport brought forth by both Pontairn et al.[7] and Saada et al.[1]

The mitochondrial levels of dTTP, dATP, and dGTP are not altered by a reduced activity of TK2. If the two salvage kinases TK2 and dGK were the sole providers of mitochondrial dNTP, reduced levels of dCTP and especially dTTP would be expected as a result of reduced TK2 activity. Since no other mitochondrial mediators of dNTP synthesis have been identified,

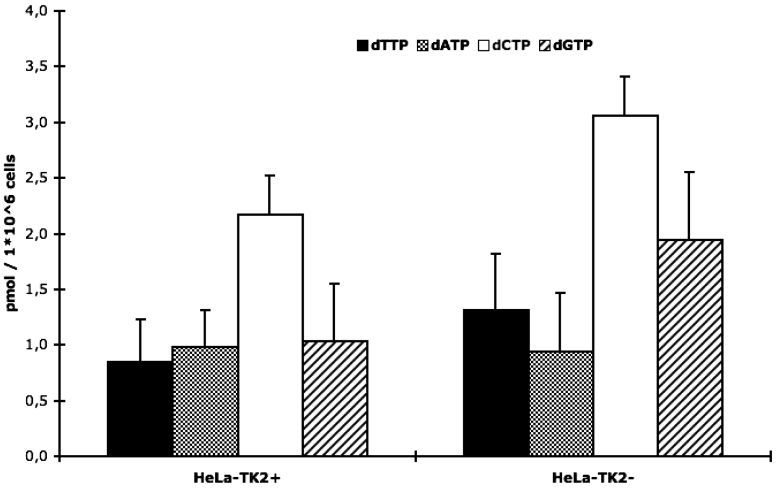

FIGURE 2 The mitochondrial levels of dNTP are measured for the dividing cell lines HeLa-TK2$^+$ and HeLa-TK2$^-$ ($n = 6$; errorbars indicate SD).

an import of dNTP is the most likely explanation for the normal levels of dTTP, dATP, and dGTP observed in the TK2$^-$ cell line.

The significant increase of dCTP levels in response to a reduced TK2 activity is intriguing however the biological explanation and significance of this remains unclear as TK2 also phosphorylates deoxycytidine.

A reduction of TK2 activity did not result in a significant difference between the whole cell levels of dNTP from the levels in cells with wild type TK2 activity. This indicates that the extra contribution needed to sustain the mitochondrial dNTP levels are well within the capacity of the cytosolic de novo and salvage synthesis of dNTP.

The rectifying effect of a dNTP import from the cytosol to the mitochondria in dividing cells serves as an excellent explanation as to why infants harbouring loss of function mutations in proteins governing the mitochondrial dNTP balance, often escape related symptoms until reaching a certain age. At this time many of the cells go into a nondividing state, which results in a 10-fold decrease of the cytosolic dNTP levels. With the decreased import of dNTP from the cytosol, the mitochondrial dNTP levels become imbalanced resulting in accumulating deletions and depletion of mtDNA giving rise to the symptoms of the patients.

REFERENCES

1. Meuth, M. The molecular basis of mutations induced by deoxyribonucleoside triphosphate pool imbalances in mammalian cells. *Exp. Cell. Res.* **1989**, 181, 305–316.
2. Bebenek, K.; Kunkel, T.A. Frameshift errors initiated by nucleotide misincorporation. *Proc. Natl. Acad. Sci. USA* **1990**, 87, 4946–4950.
3. Wickremasinghe, R.G.; Hoffbrand, A.V. Reduced rate of DNA replication fork movement in megaloblastic anemia. *J. Clin. Invest.* **1980**, 65, 26–36.
4. Song, S.; Wheeler, L.J.; Mathews, C.K. Deoxyribonucleotide pool imbalance stimulates deletions in HeLa cell mitochondrial DNA. *J. Biol. Chem.* **2003**, 278, 43893–43896.
5. Saada, A.; Shaag, A.; Elpeleg, O. mtDNA depletion myopathy: elucidation of the tissue specificity in the mitochondrial thymidine kinase (TK2) deficiency. *Mol. Genet. Metab.* **2003**, 79, 1–5.
6. Mandel, H.; Szargel, R.; Labay, V.; Elpeleg, O.; Saada, A.; Shalata, A.; Anbinder, Y.; Berkowitz, D.; Hartman, C.; Barak, M.; Eriksson, S.; Cohen, N. The deoxyguanosine kinase gene is mutated in individuals with depleted hepatocerebral mitochondrial DNA. *Nat. Genet.* **2001**, 29, 337–341.
7. Pontarin, G.; Gallinaro, L.; Ferraro, P.; Reichard, P.; Bianchi, V. Origins of mitochondrial thymidine triphosphate: dynamic relations to cytosolic pools. *Proc. Natl. Acad. Sci. USA* **2003**, 100, 12159–12164.
8. Gao, W.; Agbaria, R.; Driscoll, J.S.; Mitsuya, H. Divergent Anti-human immunodeficiency virus activity and anabolic phosphorylation of 2′,3′-dideoxynucleoside analogs in resting and activated human cells. *J. Biol. Chem.* **1994**, 269, 12633–12638.
9. Arner, E.; Spasokouktskaja, T.; Eriksson, S. Selective assays for thymidine kinase 1 and 2 and deoxycytidine kinase and their activities in extracts from human cells and tissues. *Biochem. Biophys. Res. Commun.* **1992**, 188, 712–718.
10. Sherman, P.; Fyfe, J. Enzymatic assay for deoxyribonucleoside triphosphates using synthetic oligonucleotides as template primers. *Anal. Biochem.* **1989**, 180, 222–226.
11. Wang, L.; Saada, A.; Eriksson, S. Kinetic properties of mutant human thymidine kinase 2 suggest a mechanism for mitochondrial DNA depletion myopathy. *J. Biol. Chem.* **2003**, 278, 6963–6968.