Available online at www.sciencedirect.com

SCIENCE DIRECT®

Biochemical and Biophysical Research Communications 324 (2004) 231–235

BBRC

www.elsevier.com/locate/ybbrc

Glutathione precursors replenish decreased glutathione pool in cystinotic cell lines

Marie Chol[a], Nathalie Nevo[a], Stéphanie Cherqui[a], Corinne Antignac[a], Pierre Rustin[b,*]

[a] *INSERM U574, Néphropathies Héréditaires et Rein en Développement, Hôpital Necker-Enfants Malades, rue de Sèvres, 75015 Paris, France*
[b] *INSERM U393, Handicaps Génétiques de l'Enfant, Hôpital Necker-Enfants Malades, 149, rue de Sèvres, 75743 Paris, Cedex 15, France*

Received 27 August 2004
Available online 25 September 2004

Abstract

Cystinosis is an inherited disorder due to mutations in the *CTNS* gene which encodes cystinosin, a lysosomal transmembrane protein involved in cystine export to the cytosol. Both accumulation of cystine in the lysosome and decreased cystine in the cytosol may participate in the pathogenic mechanism underlying the disease. We observed that cystinotic cell lines have moderate decrease of glutathione content during exponential growth phase. This resulted in increased solicitation of oxidative defences of the cell denoted by concurrent superoxide dismutase induction, although without major oxidative insult under our experimental conditions. Finally, decreased glutathione content in cystinotic cell lines could be counterbalanced by a series of exogenous precursors of cysteine, denoting that lysosomal cystine export is a natural source of cellular cysteine in the studied cell lines.

Keywords: Cystinosis; Human skin fibroblasts; Mitochondria; Glutathione; Superoxide dismutase

Cystinosis (MIM 219800) is a storage disorder characterized by lysosomal accumulation of cystine. Three forms of the disease have been described, according to severity of symptoms and age of onset [1]. First symptoms of infantile cystinosis, the most severe form of the disease, appear in the first year of life. Affected patients present photophobia, growth retardation, and the hallmarks of Fanconi syndrome, i.e., proximal tubulopathy and glomerular damage which progress to renal failure before 10 years of age, if untreated. The widespread accumulation of cystine also leads to diabetes, portal hypertension, hypothyroidism, hypogonadism, and muscular and neurological deterioration. The juvenile form (MIM 219900) which usually appears between 12 and 15 years is mostly characterized by photophobia and glomerular renal impairment but generally lacks major features of the Fanconi syndrome. Last, patients affected by the ocular form (MIM 219750) only present a mild photophobia [1].

Cystinosis results from mutations in the *CTNS* gene [2]. This latter encodes cystinosin, a lysosomal transmembrane protein, which acts as a proton–cystine symporter allowing cystine export from lysosome to cytosol [3]. Impairment of cystinosin activity leads to massive accumulation of cystine in the lysosomes. This accumulation can be successfully decreased by cysteamine which reacts with intralysosomal cystine to generate a cysteine–cysteamine mixed disulfide that exits lysosomes via the lysine transporter (Fig. 1 [1]). Providing oral cysteamine to the patients improves most of the symptoms, but is inefficient against the tubulopathy [4].

Resistance of the tubulopathy to cysteamine has been an incentive to develop studies aiming at determining the resistance mechanism. While reduced cysteamine access to renal tubules can still be advocated to account for resistance, multiple anomalies in tubules

* Corresponding author. Fax: +33147348514.
E-mail address: rustin@necker.fr (P. Rustin).

0006-291X/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2004.09.033

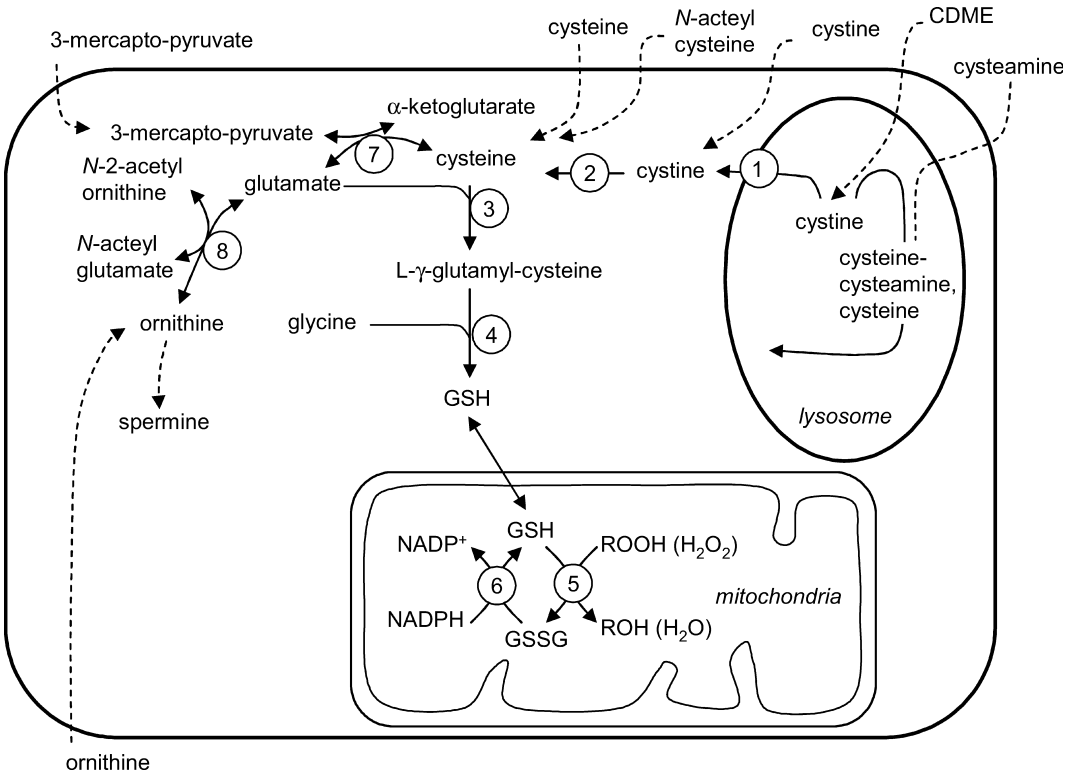

Fig. 1. A schematic view of glutathione biosynthetic pathway featuring various precursors used in this study. 1, cystinosine; 2, cystine reductase; 3, L-γ-glutamylcysteine synthetase; 4, glutathione synthetase; 5, glutathione peroxidase; 6, glutathione reductase; 7, cysteine transaminase; 8, glutamate acetyltransferase; CDME, cystine dimethylester; GSH and GSSG, reduced and oxidized glutathione, respectively; and ROOH, lipoperoxide.

have been reported mostly affecting mitochondria. In particular, swollen mitochondria have been observed in renal proximal tubules of some patients affected by cystinosis [5]. Interestingly enough, similar anomalies have been encountered when loading isolated rabbit proximal tubules with cystine using cystine dimethylester (CDME) treatment (Fig. 1 [6]). Similarly, abnormal mitochondria have been inconsistently observed in renal tubules of mice invalidated for the murine homologue of the *CTNS* gene [7]. Decreased ATP level, oxygen consumption, mitochondrial substrate consumption, and tubule absorption volume have been reported in rat isolated tubules upon CDME treatment [8–12]. Finally, altered oxidative metabolism has also been reported in phagocytic cells from cystinotic children [13].

These observations suggest mitochondrial dysfunction in cystinosis, but so far the underlying mechanism remains to be established. Decreased glutathione, normally produced from cysteine (Fig. 1), is an obvious candidate to account for mitochondrial dysfunction in this disease. However, surprisingly enough, investigation of either glutathione levels or γ-glutamyl cycle enzyme activity has failed to reveal major alteration in cystinotic fibroblasts [14–17]. We therefore decided to reinvestigate these parameters using stably transformed *CTNS*−/− cell lines.

Materials and methods

Cell line production. Fibroblasts were derived from a skin biopsy from 3 patients affected by infantile cystinosis harboring different mutations in the *CTNS* gene previously reported [2,18,19]. One of these patients was homozygous for the common 57 kb deletion of the *CTNS* gene. The second patient was heterozygous for this deletion and an in-frame insertion (1386–1387 ins 12). The third patient was heterozygous for a splicing mutation (564 + 1 G > A) and the deleterious substitution 1354 G > A. Fibroblasts used as controls were obtained from three healthy individuals. These fibroblasts were next transformed by retroviral particles containing the SV40 antigen T gene [20], in the Centre de Thérapie Génique, Nantes, France.

Cell culture. Fibroblast cell lines were grown in RPMI supplemented with 10% fetal calf serum, 2 mM glutamine, 10^5 U/L streptomycin, and 10^5 U/L penicillin under standard conditions. When indicated, cells were treated with 10 mM *N*-acetyl-cysteine (NAC), 1 mM cysteamine, 1 mM mercaptopyruvate, and 1 mM ornithine for 24 h or 0.2 mM cystine dimethylester (CDME) for 23 h followed by a last hour incubation with 1 mM CMDE. Cells were then trypsinized and washed with phosphate-buffered saline solution for biochemical investigations.

Biochemical investigations. Cystine content was assayed by radiocompetition with [^{14}C]cystine for the cystine-binding protein as described [21]. Glutathione content was measured by the enzymatic recycling assay [22]. Redox status of the glutathione pool and superoxide dismutase activity were determined according to the procedure of Anderson and Marklund, respectively [22,23]. Glutathione reductase and glutathione peroxidase activities were spectrophotometrically measured according to standard procedures [24]. Cellular respiration was polarographically studied in 250 μl cell, magnetically stirred, and thermostated at 37 °C, as described [25]. Aconitase was

spectrophotometrically measured as described [26]. Protein concentration was estimated according to Bradford [27].

Statistical analysis. Repeated measures analysis of variance and paired t tests have been used to estimate statistical significance of observed differences between CTNS−/− and CTNS+/+ cell lines. Results were considered as statistically significant for $p < 0.05$.

Results

We first determine the cystine content of the various cell lines. As predicted from the deleterious deletion/mutations identified in the *CTNS* gene in the three patients, a huge accumulation of cystine was measured in the *CTNS*−/− cell lines (1.76 ± 0.73 1/2 cystine nmol/mg protein) as compared to *CTNS*+/+ cell lines (0.11 ± 0.05 1/2 cystine nmol/mg protein).

Because glutathione is possibly synthesized in part from cytosolic cysteine originating from cystine delivered by lysosomes (Fig. 1), we first monitored glutathione content of both cystinotic and control cell lines during passages. A consistent, although limited, reduction of glutathione content was noticed in cystinotic cell lines as compared to controls during the exponential growth phase (Fig. 2A). In addition, the analysis of the glutathione pool redox status inconsistently showed increased oxidation of glutathione in CTNS−/− as compared to control (not shown).

We next examine if the observed changes in glutathione could originate from reduced activity of either the glutathione reductase or peroxidase (Table 1). Similar activities of both enzymes in cystinotic and control cell line were measured. This suggests that decreased glutathione content resulted from reduced precursor availability, rather than from a significantly increased demand for peroxide elimination in the cell. However, the decrease in glutathione content of cystinotic cell lines may in turn result in increased oxidant species in these cells. As superoxide dismutase activity level represents an endogenous sensitive marker for increased superoxide production [28], we next compared superoxide dismutase activity in both cystinotic and control cell lines. As shown in Fig. 2B, superoxide dismutase activity was moderately, but significantly, induced in cystinotic cell lines as compared to control cells. Glutathione is well known to play a major role in the control of oxidative stress in mitochondria, and mitochondrial enzymes, especially those containing iron–sulfur clusters (ISC), such as succinate dehydrogenase (associated with the mitochondrial inner membrane) and aconitase (matrix located), are among the first targets of increased superoxide production. We therefore measured both succinate oxidation and aconitase activities in *CTNS*−/− and control cell lines (Table 1). However, no differences were observed between cell lines. Accordingly, the overall respiration was not significantly affected in the *CTNS*−/− cells (Table 1).

Table 1
Biochemical investigations in cystinotic and control cell lines

	CTNS+/+ ($n = 3$) (nmol/min/mg prot)	*CTNS*−/− ($n = 3$) (nmol/min/mg prot)
Spectrophotometry		
Glutathione reductase	39.8 ± 6.8	39.7 ± 9.3
Glutathione peroxidase	44.6 ± 11.8	46.6 ± 4.8
Aconitase	2.9 ± 2.0	3.1 ± 0.1
Polarography		
Cell respiration	16.6 ± 1.4	20.6 ± 2.0
Mitochondrial succinate oxidation	22.3 ± 2.9	24 ± 2.7
Ratio		
Succinate oxidation/ respiration	1.3 ± 0.1	1.2 ± 0.1

Assays were carried out has described under Materials and methods. Mitochondrial succinate oxidation has been measured in the presence of the uncoupler *m*-Cl-CCP (carbonylcyanide-*m*-chlorophenylhydrazone) as to determined maximal rates, essentially limited by the activity of the succinate dehydrogenase.

We finally compared the ability of a series of exogenously added glutathione precursors (Fig. 1) to increase the glutathione content of cystinotic and control cell lines. In agreement with the suggestion that decreased glutathione content originated from limited precursor availability, we observed that most of the added compounds could be used as glutathione precursors in cystinotic cell lines (Fig. 3A). Cysteamine, CDME, *N*-acetyl-cysteine, ornithine, and mercaptopyruvate all tend to increase the glutathione content, this being particularly true for ornithine and CDME ($p < 0.05$). Indicative that precursor availability is also limiting in control cells, most of these compounds increased glutathione content in control cells (Fig. 3B). Noticeably, CDME increased glutathione content in both control and cystinotic cell lines, yet to a significantly lower extent in *CTNS*−/− cell lines ($p < 0.01$). Under our experimental condition, ornithine and mercaptopyruvate were

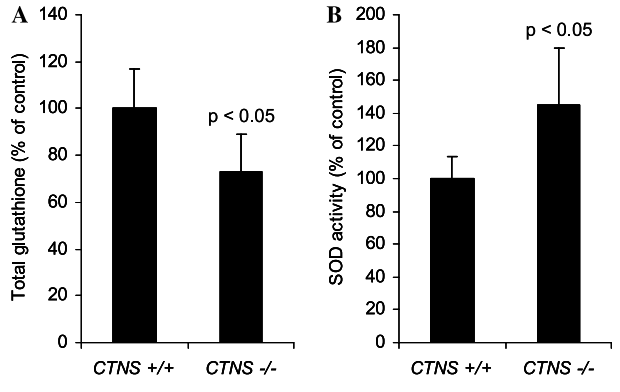

Fig. 2. Glutathione content and superoxide dismutase activity in cystinotic and control cell lines. (A) Glutathione content in *CTNS*+/+ and *CTNS*−/− cell lines. (B) Superoxide dismutase activity in *CTNS*+/+ and *CTNS*−/− cell lines. Glutathione content and enzyme activity were determined as described under Material and methods.

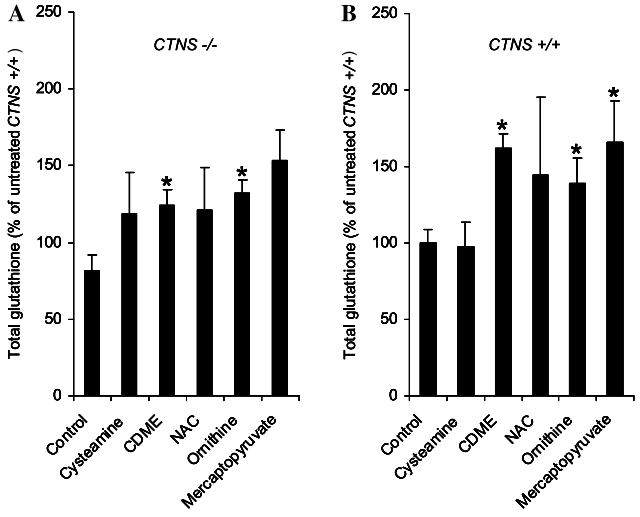

Fig. 3. Effect of glutathione precursors on glutathione content of both cystinotic (A) and control (B) cell lines. Glutathione content was determined as in Fig. 1 and values indicated as % of *CTNS*+/+ cell lines without treatment. CDME, cystine dimethylester; NAC, *N*-acetyl-cysteine; $*p < 0.05$.

found to be the most efficient compounds to trigger glutathione synthesis.

Discussion

Oxidative stress and mitochondrial dysfunction resulting in decreased ATP are elusive features of cystinosis, which might participate in the unexplained tubulopathy observed in patients and account for abnormal mitochondrial morphology occasionally observed in mouse model kidney [5,7,8,10]. The present study establishes that, under controlled conditions, exponentially growing cystinotic cell lines have lower glutathione content and increased superoxide dismutase activity as compared to controls. This indicates that cystine, sequestered in the lysosomes of affected cells, is normally utilized as a precursor for glutathione synthesis in this type of cells (Fig. 1). Cytosolic cysteine pool replenishment seems therefore to be partially dependent on cystine export from lysosomes, the degree of dependency being possibly variable according to experimental conditions and to tissues and cell types, in vivo.

Concurrent with the moderate glutathione depletion observed in cystinotic cell lines, we noticed an increase of superoxide dismutase activity denoting increased solicitation of cell oxidative defences. On the other hand, we failed to detect any significant oxidative insult to the mitochondria under our experimental condition, as shown by preserved respiratory chain and mitochondrial iron–sulfur protein activity (Table 1). Finally, we found that the glutathione pool in cystinotic cell lines could be successfully replenished by exogenous supplementation with a series of metabolic precursors of cysteine. This indicated that decreased glutathione was indeed due to decreased cysteine availability and not to impaired capacity to use cytosolic cysteine.

Although it would be hazardous to generalize our conclusion to the in vivo situation, the mechanism underlying glutathione depletion in our cell model could be at work in vivo as well. This certainly deserves to be further investigated, especially as glutathione precursors as *N*-acetyl-cysteine can be provided to patients.

Acknowledgments

We thank Philippe Moulier and Frédéric Broucque for transforming the fibroblasts. This work was supported by Vaincre les Maladies Lysosomales, Association Française contre les Myopathies, and the Cystinosis Foundation. M.C. is the recipient of a Ph.D. grant from Association pour l'Information et la Recherche sur les Maladies Rénales Génétiques.

References

[1] W.A. Gahl, J.A. Schneider, P. Aula, Lysosomal transport disorders: cystinosis and sialic acid storage disorders, in: C.R. Scriver, A.L. Beaudet, W.S. Sly, D. Valle (Eds.), The Metabolic and Molecular Basis of Inherited Disease, McGraw-Hill, New York, 2001, pp. 5085–5108.

[2] M. Town, G. Jean, S. Cherqui, M. Attard, L. Forestier, S.A. Whitmore, D.F. Callen, O. Gribouval, M. Broyer, G.P. Bates, W. van't Hoff, C. Antignac, A novel gene encoding an integral membrane protein is mutated in nephropathic cystinosis, Nat. Genet. 18 (1998) 319–324.

[3] V. Kalatzis, S. Cherqui, C. Antignac, B. Gasnier, Cystinosin, the protein defective in cystinosis, is a H(+)-driven lysosomal cystine transporter, EMBO J. 20 (2001) 5940–5949.

[4] W.A. Gahl, G.F. Reed, J.G. Thoene, J.D. Schulman, W.B. Rizzo, A.J. Jonas, D.W. Denman, J.J. Schlesselman, B.J. Corden, J.A. Schneider, Cysteamine therapy for children with nephropathic cystinosis, N. Engl. J. Med. 316 (1987) 971–977.

[5] G.S. Spear, R.J. Slusser, A.J. Tousimis, C.G. Taylor, J.D. Schulman, Cystinosis. An ultrastructural and electron-probe study of the kidney with unusual findings, Arch. Pathol. 91 (1971) 206–221.

[6] A. Sakarcan, C. Timmons, M. Baum, Intracellular distribution of cystine in cystine-loaded proximal tubules, Pediatr. Res. 35 (1994) 447–450.

[7] S. Cherqui, C. Sevin, G. Hamard, V. Kalatzis, M. Sich, M.O. Pequignot, K. Gogat, M. Abitbol, M. Broyer, M.C. Gubler, C. Antignac, Intralysosomal cystine accumulation in mice lacking cystinosin, the protein defective in cystinosis, Mol. Cell. Biol. 22 (2002) 7622–7632.

[8] C. Coor, R.F. Salmon, R. Quigley, D. Marver, M. Baum, Role of adenosine triphosphate (ATP) and NaK ATPase in the inhibition of proximal tubule transport with intracellular cystine loading, J. Clin. Invest. 87 (1991) 955–961.

[9] J.W. Foreman, L. Benson, Effect of cystine loading and cystine dimethylester on renal brushborder membrane transport, Biosci. Rep. 10 (1990) 455–459.

[10] J.W. Foreman, L.L. Benson, M. Wellons, E.D. Avner, W. Sweeney, I. Nissim, Metabolic studies of rat renal tubule cells

loaded with cystine: the cystine dimethylester model of cystinosis, J. Am. Soc. Nephrol. 6 (1995) 269–272.
[11] A. Sakarcan, R. Aricheta, M. Baum, Intracellular cystine loading causes proximal tubule respiratory dysfunction: effect of glycine, Pediatr. Res. 32 (1992) 710–713.
[12] R.F. Salmon, M. Baum, Intracellular cystine loading inhibits transport in the rabbit proximal convoluted tubule, J. Clin. Invest. 85 (1990) 340–344.
[13] G. Pintos Morell, P. Niaudet, G. Jean, B. Descamps-Latscha, Altered oxidative metabolism, motility, and adherence in phagocytic cells from cystinotic children, Pediatr. Res. 19 (1985) 1318–1321.
[14] J.D. Butler, J.D. Key, B.F. Hughes, F. Tietze, D.S. Raiford, G.F. Reed, P.M. Brannon, S.P. Spielberg, J.D. Schulman, Glutathione metabolism in normal and cystinotic fibroblasts, Exp. Cell Res. 172 (1987) 158–167.
[15] A. Larsson, A. Holmgren, I. Bratt, Thioredoxin and glutathione in cultured fibroblasts from human cases with 5-oxoprolinuria and cystinosis, FEBS Lett. 87 (1978) 61–64.
[16] J.D. Schulman, J.A. Schneider, K.H. Bradley, J.E. Seegmiller, Cystine, cysteine, and glutathione metabolism in normal and cystinotic fibroblasts in vitro, and in cultured normal amniotic fluid cells, Clin. Chim. Acta 37 (1972) 53–58.
[17] B. States, K. Scardigli, S. Segal, Glutathione in fibroblasts from normal and cystinotic children, Life Sci. 22 (1978) 31–37.
[18] V. Kalatzis, L. Cohen-Solal, B. Cordier, Y. Frishberg, M. Kemper, E.M. Nuutinen, E. Legrand, P. Cochat, C. Antignac, Identification of 14 novel CTNS mutations and characterization of seven splice site mutations associated with cystinosis, Hum. Mutat. 20 (2002) 439–446.
[19] V. Shotelersuk, D. Larson, Y. Anikster, G. McDowell, R. Lemons, I. Bernardini, J. Guo, J. Thoene, W.A. Gahl, CTNS mutations in an American-based population of cystinosis patients, Am. J. Hum. Genet. 63 (1998) 1352–1362.
[20] L.C. Racusen, P.D. Wilson, P.A. Hartz, B.A. Fivush, C.R. Burrow, Renal proximal tubular epithelium from patients with nephropathic cystinosis: immortalized cell lines as in vitro model systems, Kidney Int. 48 (1995) 536–543.
[21] R.G. Oshima, R.C. Willis, C.E. Furlong, J.A. Schneider, Binding assays for amino acids. The utilization of a cystine binding protein from *Escherichia coli* for the determination of acid-soluble cystine in small physiological samples, J. Biol. Chem. 249 (1974) 6033–6039.
[22] M.E. Anderson, Tissue glutathione, in: R.A. Greenwald (Ed.), Handbook of Methods for Oxygen Radical Research, CRC Press, Boca Raton, FL, 1985, pp. 317–323.
[23] S. Marklund, G. Marklund, Involvement of the superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase, Eur. J. Biochem. 47 (1974) 469–474.
[24] R.F. Del Maestro, W. McDonald, Oxidative enzymes in tissue homogenates, in: R.A. Greenwald (Ed.), Handbook of Methods for Oxygen Radical Research, CRC Press, Boca Raton, FL, 1985, pp. 291–296.
[25] P. Rustin, D. Chretien, T. Bourgeron, B. Gerard, A. Rotig, J.M. Saudubray, A. Munnich, Biochemical and molecular investigations in respiratory chain deficiencies, Clin. Chim. Acta 228 (1994) 35–51.
[26] J.B. Robinson, L.G. Brent, B. Sumegi, P.A. Srere, An enzymatic approach to the study of the KREBS tricarboxylic acid cycle, in: V.M. Darley-Usmar, D. Rickwood, M.T. Wilson (Eds.), Mitochondria, IRL, Oxford, 1987, pp. 153–170.
[27] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248–254.
[28] V. Geromel, N. Kadhom, I. Cebalos-Picot, O. Ouari, A. Polidori, A. Munnich, A. Rotig, P. Rustin, Superoxide-induced massive apoptosis in cultured skin fibroblasts harboring the neurogenic ataxia retinitis pigmentosa (NARP) mutation in the ATPase-6 gene of the mitochondrial DNA, Hum. Mol. Genet. 10 (2001) 1221–1228.