FEBS Letters 580 (2006) 4539–4543

cAMP controls oxygen metabolism in mammalian cells

Claudia Piccoli[a], Salvatore Scacco[b], Francesco Bellomo[b], Anna Signorile[b], Arcangela Iuso[b], Domenico Boffoli[a], Rosella Scrima[a], Nazzareno Capitanio[a], Sergio Papa[b,c,*]

[a] *Department of Biomedical Science, University of Foggia, Foggia, Italy*
[b] *Department of Medical Biochemistry, Biology and Physics, University of Bari, Bari, Italy*
[c] *Institute of Bioenergetics and Biomembranes, CNR, Bari, Italy*

Received 3 May 2006; revised 27 June 2006; accepted 29 June 2006

Available online 7 July 2006

Edited by Vladimir Skulachev

Abstract The impact of cAMP on ROS-balance in human and mammalian cell cultures was studied. cAMP reduced accumulation of ROS induced by serum-limitation, under conditions in which there was no significant change in the activity of scavenger systems. This effect was associated with cAMP-dependent activation of the NADH-ubiquinone oxidoreductase activity of complex I. In fibroblasts from a patient a genetic defect in the 75 kDa FeS-protein subunit of complex I resulted in inhibition of the activity of the complex and enhanced ROS production, which were reversed by cAMP. A missense genetic defect in the NDUFS4 subunit, putative substrate of PKA, suppressed, on the other hand, the activity of the complex and prevented ROS production.

Keywords: Mitochondria; cAMP; Cellular respiration; ROS balance; Complex I

1. Introduction

The life of mammals depends on the free energy made available through cellular oxidation of food-stuffs by oxygen. In mitochondria the redox enzymes of the respiratory chain are organised in the inner membrane so as to oxidise reduced nicotinamide nucleotides and flavin coenzymes, in a stepwise process, with final reduction of O_2 to H_2O and conservation of free energy as a transmembrane electrochemical proton gradient, utilised to drive ATP synthesis from ADP and Pi [1]. In addition to reduction to H_2O, some oxygen can be partially reduced to reactive oxygen (ROS) [2]. Cellular ROS production, which generally accounts for about 1–2% of the overall cellular oxygen consumption, under certain pathophysiological conditions can increase significantly. ROS are involved in cell signalling [2]. Oxidative stress, resulting from disturbed free-radical and cellular redox balance, is associated with ageing and several human diseases [2].

Mitochondria are quantitatively the primary source of ROS [3]. Oxygen superoxide ($O_2^{\cdot-}$) is produced in the mitochondrial matrix by complex I (NADH ubiquinone oxidoreductase) [3] and complex III (ubiquinone cytochrome *c* oxidoreductase) [4]. The latter also produces $O_2^{\cdot-}$ in the intermembrane mitochondrial space [4]. Complex IV (cytochrome c oxidase), due to its high catalytic capacity of O_2 reduction to H_2O, does not produce ROS, rather prevents their formation by keeping the cellular oxygen concentration low [5]. ROS can also be generated in other cell compartments by different systems; among these the family of plasma membrane NADPH oxidases, which are involved in cell signalling [2]. $O_2^{\cdot-}$, which with a pK around 5.0 exists at physiological pH essentially in the membrane impermeable anionic form, is converted by superoxide dismutases to H_2O_2. H_2O_2 diffuses across cellular membranes and can be neutralised by a set of endogenous scavenger systems [2,6] as well as by nutritional antioxidants.

cAMP, produced by the plasma membrane adenylyl cyclase in response to hormones and neurotransmitters [7] and by the bicarbonate-activated soluble adenylyl cyclase, localised to the nucleus, mitochondria and other intracellular structures [8], plays a central regulatory role in energy-supplying and energy-requiring processes. cAMP dependent protein kinase (PKA), which is present in different subcellular compartments, including mitochondria [9], activates the utilisation of glycogen and lipid energy stores as well as energy-requiring processes, like cell growth and development, neuronal activity, etc. [7]. It has recently been found that activation of the cAMP cascade reverts accumulation of H_2O_2 and depression of the activity of complex I observed in serum-limited cell cultures [10]. Here, we show that activation of complex I and prevention of reactive oxygen species accumulation by cAMP is a general phenomenon observed in a variety of mammalian and human cell cultures under different pathophysiological conditions.

2. Materials and methods

2.1. Cell culture and mitoplast preparation

Neonatal normal human dermal fibroblasts (NHDF-neo, Cambrex# CC-2509), Hela (ATCC# CCL-2), Rabdomiosarcoma cells (RD, ATCC# CCL-136), Hepatoma cells (HepG2, ATCC# HB-8065), Balb/c 3T3 (ATCC# CCL-163), NIH 3T3 (ATCC# CRL-1658) and fibroblasts from the patients with NDUFS1 and NDUFS4 mutations were grown in the exponential phase in high glucose Dulbecco's modified Eagle's medium (DMEM, EuroClone) supplemented with 10% fetal bovine or calf serum, plus 2 mM glutamine (Euroclone), 100 IU/ml penicillin (Euroclone) and 100 IU/ml streptomycin (Euroclone). Further conditions are specified in the legends to figures. Fibroblasts harvested and mitoplast preparation as described in [20].

*Corresponding author. Present address: Department of Medical Biochemistry, Biology and Physics, University of Bari, Policlinico Piazza G. Cesare 70124, Bari, Italy. Fax: +39 (0) 80/5478109.
E-mail address: papabchm@cimedoc.uniba.it (S. Papa).

0014-5793/$32.00
doi:10.1016/j.febslet.2006.06.085

2.2. Apoptosis assays

DNA ladder detection was performed by the Roche kit. For the CFDA (6-carboxyfluorescein diacetate)/annexin V–Cy3 apoptotic assay the LSCM analysis was performed according to the manufacturer (Sigma). Live cells label only with the intracellular-esterase-deacetylated CFDA (carboxyfluorescein (6-CF), green fluorescence), whereas cells in the early stage of apoptosis spot with both annexin V–Cy3 (red) and 6-CF. The quiescence-state of serum-limited cells was verified by propidium iodide-based cytofluorimetry.

2.3. Laser scanning confocal microscopy analysis (LSCM)

Cells were seeded onto fibronectin coated glass bottom dishes. After adhesion, living cells were incubated for 20 min at 37 °C with: MitoCapture (Biovision, 1/1000 dilution) to monitor mitochondrial membrane potential; 10 μM dichlorofluorescein-diacetate DCF-DA (Oregon Probes) or 3 μM MitoSOX (Oregon Probes) for detection of H_2O_2 and $O_2^{\cdot-}$ respectively. Stained cells were washed with PBS and examined by a Nikon TE 2000 microscope (images collected using a 60× objective (1.4 NA)) coupled to a Radiance 2100 dual laser (four-lines Argon–Krypton, single-line Helium–Neon) scanning confocal microscopy system (Biorad). The fluorescent signal of the MitoCapture double-emitter probe was examined sequentially, exciting first with the Ar–Kr laser beam (λ_{ex} = 488 nm) and then with the He–Ne laser beam (λ_{ex} = 543 nm). The green fluorescence of DCF was analysed by exciting the sample with the Ar–Kr laser beam (λ_{ex} = 488 nm). Confocal planes of 0.2 μm in thickness were examined along the *z*-axis, from the top to the bottom of the cells. Acquisition, storage and analysis of data were made by using LaserSharp and LaserPix Biorad software.

2.4. Measurement of glutathione and glucose-6-phosphate

Cells were suspended in PBS and homogenized. After protein precipitation with 2% sulfosalycilic acid, total glutathione was determined in the supernatant as in [10]. For measurement of reduced glutathione (GSH), proteins were precipitated with 10% perchloric acid (PCA) and the supernatant analysed by HPLC. Glucose-6-phosphate was determined as in [25].

2.5. Determination of the activity of ROS scavenger enzymes

Aliquots of sonicated cell suspension, were used for spectrophotometric measurement of glucose-6-phosphate dehydrogenase (G6PDH) [26], GSSG reductase, GSH peroxidase and catalase [27]. The Superoxide dismutase activity was determined with the Calbiochem® assay kit. The Mn-SOD activity represented the residual activity after inhibition of Cu/Zn-SOD with 0.2 mM phenylglyoxal.

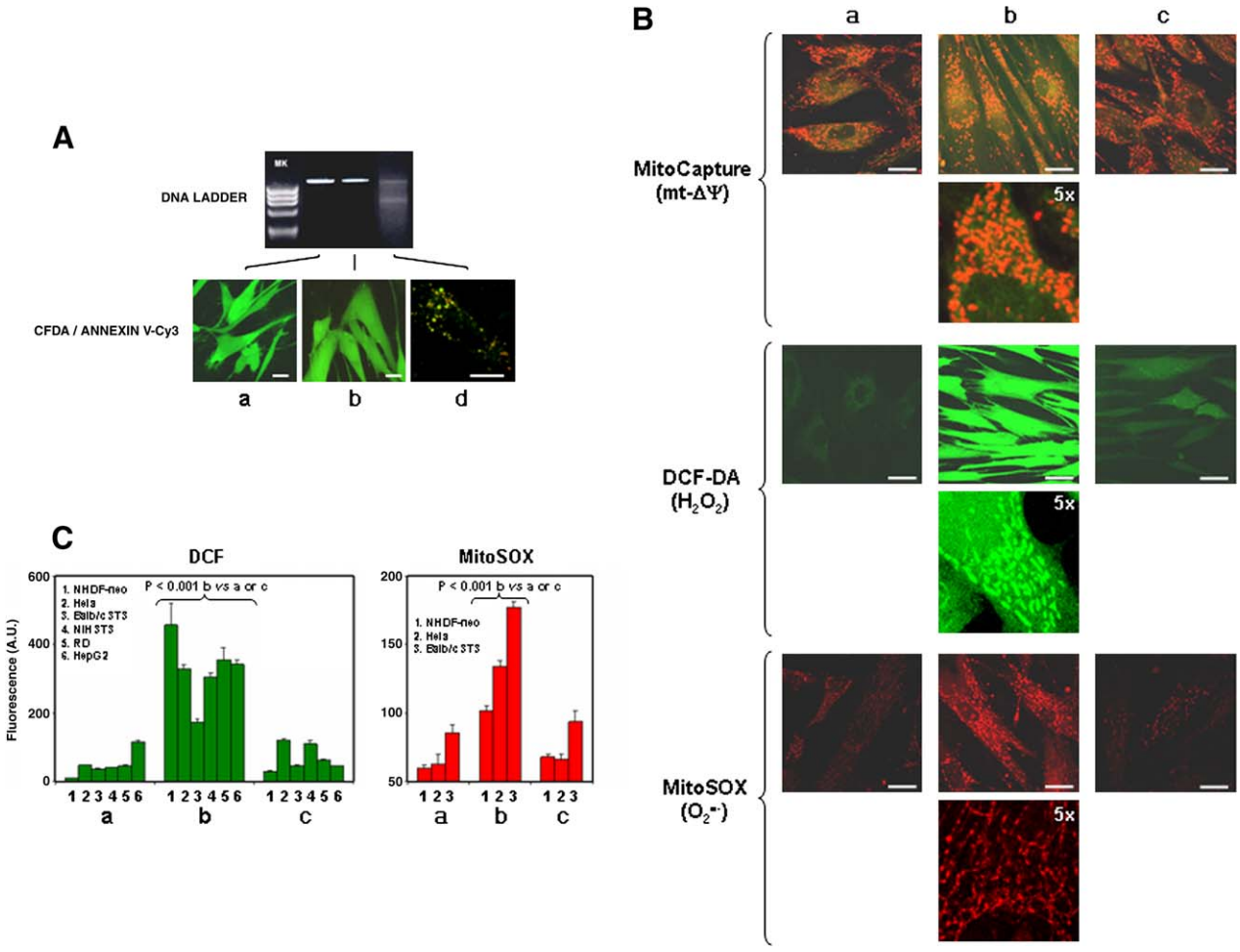

Fig. 1. Cellular ROS pattern in human and murine cell cultures. (A) Apoptosis assays. Neonatal normal human dermal fibroblasts (NHDF-neo) were grown in the exponential phase (a) (10% fetal bovine serum in DMEM), followed, where indicated, by 48 h cultivation under serum-limitation (0.5% FBS in DMEM) (b) or 2 h treatment with 500 μM H_2O_2 (apoptosis-induction) (d). Live cells were detected by the green fluorescence of intracellular esterase-deacetylated 6-carboxy fluorescein diacetate (CFDA), apoptotic cells were detected by DNA fragmentation and the red-fluorescence of annexinV–Cy3. Bars: 20 μm. (B) NHDF-neo assayed by laser scanning confocal microscopy (LSCM) for: mitochondrial potential detected by the red-fluorescence of the positively charged Mitocapture dye, H_2O_2 by the green fluorescence of 2′,7′-dichlorofluorescin diacetate (DCF-DA), $O_2^{\cdot-}$ by the red-fluorescence of the MitoSOX dye. Where indicated, serum-limited cell cultures (b) were incubated with 100 μM dibutyryl cAMP (c). LSCM images of the serum-limited cells (b) are given at low and high magnification. Bars: 20 μm. (C) Semi-quantitative analysis of H_2O_2 and $O_2^{\cdot-}$ levels in NHDF-neo, Hela cells, murine fibroblasts (Balb/C 3T3 and NIH 3T3), rabdomiosarcoma cells (RD) and hepatoma cells (HepG2). For details see Section 2.

2.6. Complex I and complex IV assay

Mitoplasts were exposed to ultrasound energy for 15 s at 0 °C and the V_{max} of NADH–UQ oxidoreductase activity was measured as described in [20]. Cytochrome *c* oxidase activity was measured as in [20].

3. Results and discussion

3.1. Serum-limitation in cultured cell lines induces mitochondrial ROS production which is abrogated by cAMP

Different human and murine cell lines were analysed, either in their exponential growth phase (serum-saturated), or when brought in a quiescent state by 48 h cultivation under serum-limitation. This period of serum limitation did not impair cell viability nor produced signs of apoptosis (Fig. 1A) or impairment of the mitochondrial potential (Fig. 1B). In the serum-limited cells there was, however, a large accumulation of H_2O_2 as compared to the serum-saturated cells which showed little, if any H_2O_2 (Fig. 1B, see also [10]). H_2O_2 was localised in spots with a cellular distribution similar to that of mitochondria. Serum-limited cells showed, also, a large accumulation of $O_2^{\cdot-}$ in the mitochondrial matrix. Exposure (60 min) of serum-limited cell cultures to dibutyryl cAMP, a permeant derivative of cAMP, resulted in the disappearance of H_2O_2 from cells and $O_2^{\cdot-}$ from mitochondria, reproducing the situation observed in the serum-saturated cells (Fig. 1B). Semi-quantitative analysis showed that the increase in the level of H_2O_2 and $O_2^{\cdot-}$, observed in serum-limited cells, and their disappearance upon cAMP treatment is a general phenomenon, observed in six different lines of tested human and murine cells (Fig. 1C). It was previously found that addition to cell cultures of DPI, inhibitor of plasma membrane NADPH oxidase, or of allopurinol, inhibitor of xanthine oxidase, did not prevent accumulation of ROS in serum-limited cells. H89, a specific inhibitor of cAMP-dependent protein kinase (PKA) depressed, on the other hand, the ROS-removing effect of cAMP [10]. Thus the cyclic effect of the nucleotide involves PKA.

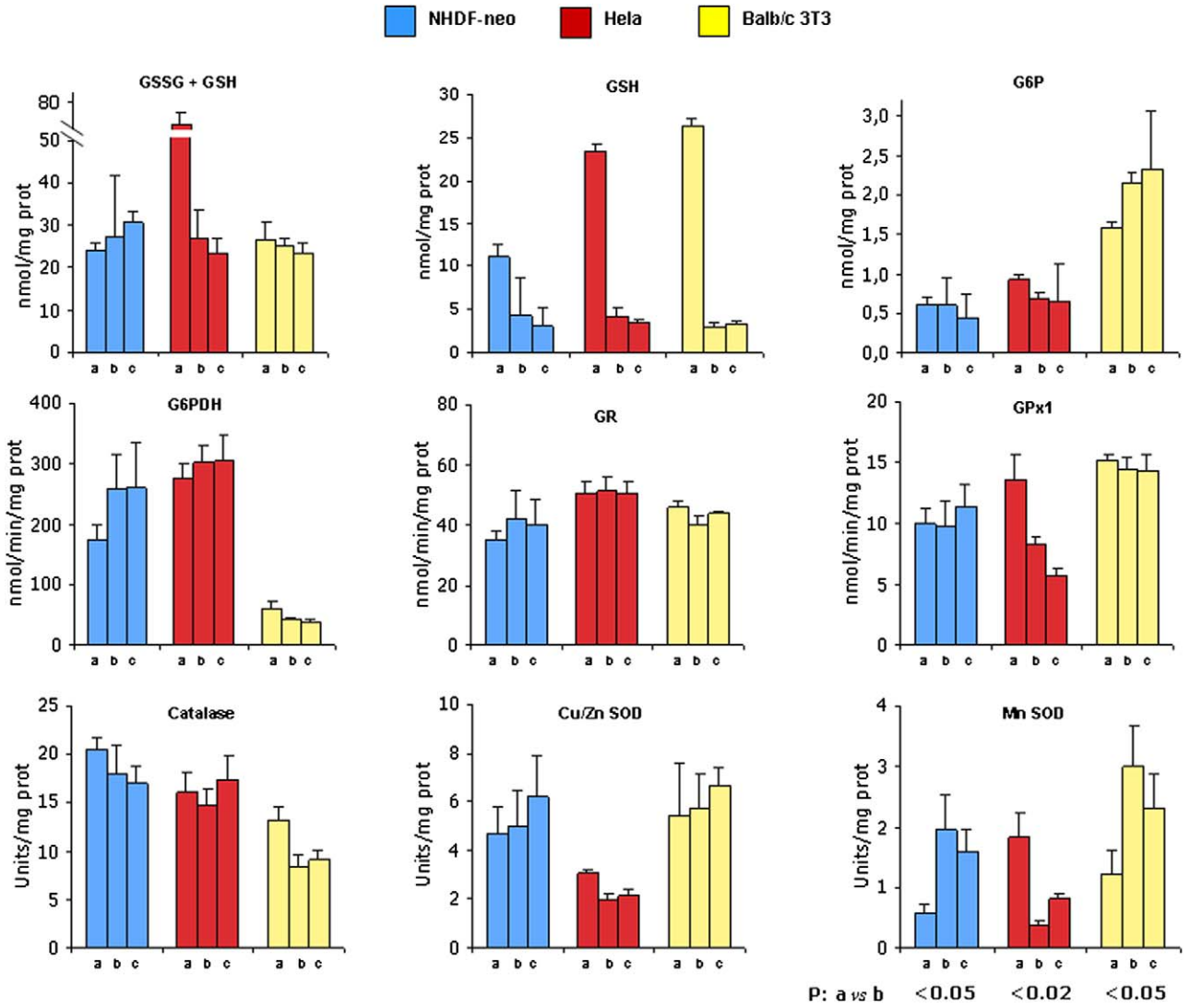

Fig. 2. Pattern of ROS scavenger systems in human and murine cell cultures. Total cellular content of glutathione (GSH + GSSG), reduced glutathione (GSH), glucose-6-phosphate (G6P), activity of glucose-6-phosphate dehydrogenase (G6PDH), glutathione reductase (GR), glutathione-peroxidase-1 (GPX-1), catalase, Cu/Zn-superoxide dismutase (Cu/Zn SOD) and Mn-superoxidedismutase (Mn SOD) were determined in NHDF-neo, Hela cells and Balb/C 3T3 grown in the exponential phase (a), under 48 h serum-limitation (b) and under 48 h serum-limitation followed by 60 min treatment with 100 μM dibutyryl cAMP. For details see Section 2.

3.2. Serum-limitation-induced ROS production is not associated with depression of ROS-scavenger enzymes

RT-PCR analysis has shown some depression in the transcript levels of ROS scavenger enzymes in serum starved Hela cells [10]. We have now carried out a systematic study of the impact of serum-limitation and cAMP on the levels of total and reduced glutathione, and the functional activity of scavenger enzymes in three different cell lines. Serum-limitation had no effect on the total glutathione cellular content, except Hela cells which showed a significant decrease. The accumulation of ROS was associated with an extensive oxidation of glutathione, which was not reversed upon subsequent treatment of the three cell lines with cAMP (Fig. 2). Serum-limitation and subsequent treatment with cAMP had no significant effect on the level of glucose-6-phosphate and glucose-6-phosphate dehydrogenase activity, a major producer of NADPH, which is utilised by glutathione reductase (Fig. 2) [11]. Also, no significant change was produced by serum-limitation and cAMP treatment in the activity of glutathione reductase, glutathione peroxidase and catalase. Thus the ROS accumulation induced by serum-limitation and the ROS – removing effect of cAMP were not due to depression and reactivation of these scavenger systems respectively. They appear, rather, to be associated with modulation of ROS production.

The distribution of ROS in/around mitochondria shows the primary role of mitochondrial redox enzymes in their production. In keeping with this, it was found that whilst the activity of the cytosolic Cu/Zn-SOD did not show any change, the mitochondrial Mn-SOD increased in the serum-limited cells, with the exception of Hela cells. The expression of Mn-SOD has been shown to be up-regulated by oxidative stress [12] and in patients with complex I deficiency, which also present increased ROS levels [13]. No significant further change in the activity of Mn-SOD of serum-limited cells was induced by cAMP (Fig. 2).

3.3. cAMP-abrogation of ROS production correlates with re-activation of mitochondrial complex I

Analysis of the impact of cell serum-limitation and cAMP on the activity of complex I in the mitoplast fraction revealed an inverse relationship between the observed changes in cellular ROS and the forward NADH-ubiquinone oxido-reductase activity of the complex. In the three cell lines tested, serum-limitation was associated with depression of the V_{max} of the NADH-ubiquinone oxido-reductase activity, which was largely rescued by the addition of dibutyryl-cAMP (see also [10]) (Fig. 3). In other cell lines, like mouse C2C12 myoblasts, cAMP stimulation of complex I activity was also observed in the exponential growth phase [9]. No significant changes in the activity of complex IV were, on the other hand, produced by serum-limitation of cell growth and subsequent treatment with cAMP (Fig. 3).

Genetic deficiency in the activity of complex I represents the most frequent cause of inborn mitochondrial disease [14]. In two cases of autosomal recessive genetic defects of complex I, in children affected by fatal neurological disorder, we have investigated the pathogenic mechanism. One, consisting of a non-sense G44A mutation in the nuclear NDUFS4 gene-coding for a 18 kDa subunit (the complex is constituted by 46 subunits; see Ref. [15]), was associated with defective assembly and suppression of the NADH ubiquinone oxidoreductase activity of the complex [16,17]. The other, C1564A mutation in the nuclear NDUFS1 gene (Gln 522 Lys replacement) coding for a 75 kDa FeS subunit, was associated with severe but not complete depression of the NADH ubiquinone oxidoreductase activity (Fig. 3) [18]. In the fibroblasts of the patient with the NDUFS4 mutation, H_2O_2 and $O_2^{\cdot-}$ levels were very low. In the NDUFS1 mutant cells large levels of H_2O_2 and $O_2^{\cdot-}$ were detected. The depression of complex I activity and the accumulation of ROS observed in the NDUFS1 mutant cells were largely reversed by cAMP (Fig. 3A and B; cf. Ref.

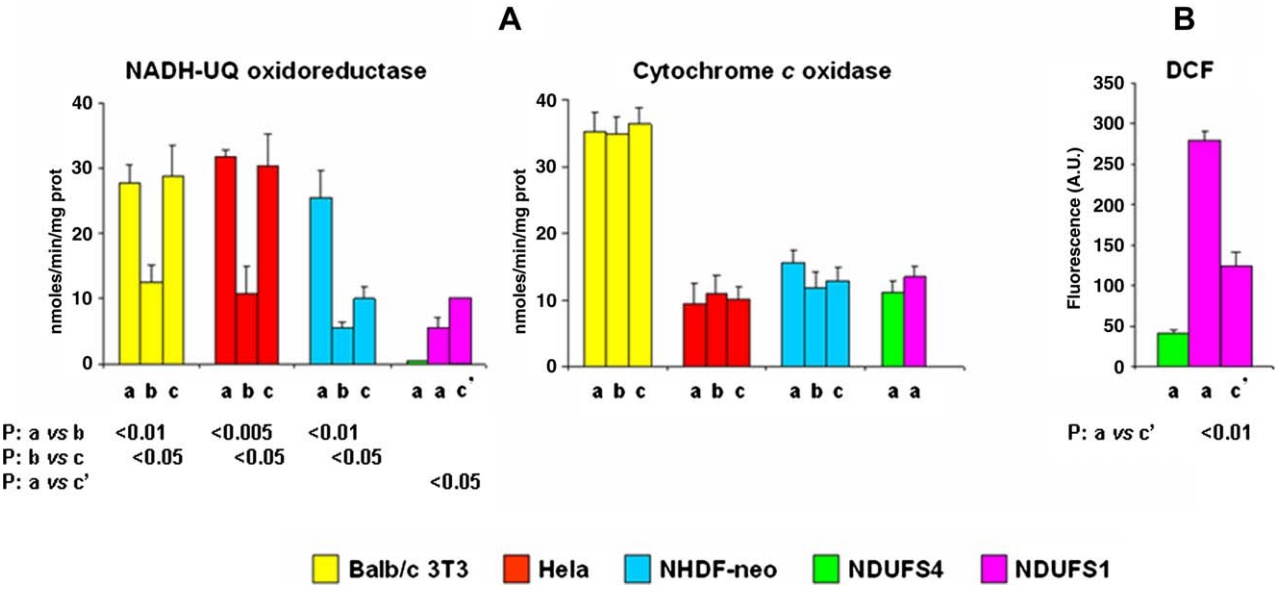

Fig. 3. Complex I and complex IV activities in human and murine cell cultures and ROS levels in patient's fibroblasts. (A). Complex I and complex IV activities represent the means ± S.E.M. of three or more determinations in mitoplasts from human (NHDF-neo, Hela cells), murine (Balb/c 3T3) and fibroblasts from human patients with mutations in the NDUFS1 and NDUFS4 genes respectively. (B). Semi-quantitative analysis of H_2O_2 levels (means of three determinations) in patient's fibroblasts, using the DCF fluorescent probe as described in Fig. 1C. For the experimental conditions in (a), (b) and (c) see legend to Fig. 2; c′ refers to NDUFS1 mutant fibroblasts grown in the exponential phase and exposed to 100 μM dibutyryl-cAMP for 60 min.

[18]). In the case of the NDUFS1-mutation, the inhibition of the NADH-ubiquinone oxidoreductase can be attributed to altered function of the 75 kDa Fe–S protein encoded by this gene. The Gln 522 Lys substitution can promote direct oxidation by molecular oxygen of the Fe-S center of this subunit, once reduced by the electrons delivered to the enzyme by NADH (see also [19]).

All the above shows that the changes in the cellular ROS balance in mammalian cells, observed in the physio-pathological conditions examined, are correlated with modulation/defect of the redox activity of complex I.

The reduction of ROS level and activation of complex I promoted by cAMP involve post-translational protein phosphorylation. The cAMP effects can be directly associated with phosphorylation of complex I subunits [9,20–22] and or PKA-mediated changes in the redox [23]/nitrosylation [24] state of complex I subunits.

The cAMP effects described here can play a general role in the physiological control of free-radical balance and the course of oxidative stress-associated human pathophysiology.

Acknowledgements: We thank Dr Massimo Zeviani., Neurological Besta Institute, Milan for providing fibroblasts of the patient with the NDUFS1 mutation and Michele Minuto for contribution to glutathione assay. Supported by Grants from: MIUR-PRIN ''Enzyme complexes of oxidative phosphorylation: functional mechanisms, biogenesis and physiopathology'', Project No. 2003057323_001; MIUR Research Grant No. 157, D.1416 28.05.2004; Center of Excellence on ''Comparative Genomics'' University of Bari and Research Project Fondazione Cassa di Risparmio di Puglia.

References

[1] Saraste, M. (1999) Oxidative phosphorylation at the fin de siecle. Science 283, 1488–1493.
[2] Finkel, T. and Holbrook, N.J. (2000) Oxidants, oxidative stress and the biology of ageing. Nature 408, 239–247.
[3] Cadenas, E. and Davies, K.J. (2000) Mitochondrial free radical generation, oxidative stress, and aging. Free Radic. Biol. Med. 29, 222–230.
[4] Chen, Q., Vazquez, E.J., Moghaddas, S., Hoppel, C.L. and Lesnefsky, E.J. (2003) Production of reactive oxygen species by mitochondria: central role of complex III. J. Biol. Chem. 278, 36027–36031.
[5] Papa, S., Guerrieri, F. and Capitanio, N. (1997) A possible role of slips in cytochrome *C* oxidase in the antioxygen defense system of the cell. Biosci. Rep. 17, 23–31.
[6] Winyard, P.G., Moody, C.J. and Jacob, C. (2005) Oxidative activation of antioxidant defence. Trends Biochem. Sci. 30, 453–461.
[7] McKnight, G.S., Cummings, D.E., Amieux, P.S., Sikorski, M.A., Brandon, E.P., Planas, J.V., Motamed, K. and Idzerda, R.L. (1998) Cyclic AMP, PKA, and the physiological regulation of adiposity. Recent Prog. Horm. Res. 53, 139–160.
[8] Zippin, J.H., Chen, Y., Nahirney, P., Kamenetsky, M., Wuttke, M.S., Fischman, D.A., Levin, L.R. and Buck, J. (2003) Compartmentalization of bicarbonate-sensitive adenylyl cyclase in distinct signaling microdomains. FASEB J. 17, 82–84.
[9] Technikova-Dobrova, Z., Sardanelli, A.M., Speranza, F., Scacco, S., Signorile, A., Lorusso, V. and Papa, S. (2001) Cyclic adenosine monophosphate-dependent phosphorylation of mammalian mitochondrial proteins: enzyme and substrate characterization and functional role. Biochemistry 40, 13941–13947.
[10] Bellomo, F., Piccoli, C., Cocco, T., Scacco, S., Gaballo, A., Papa, F., Boffoli, D., Signorile, A., D'Aprile, A., Scrima, R, Sardanelli, A.M., Capitanio, N., Papa, S. Regulation by the cAMP cascade of oxygen free radical balance in Mammalian cell. Antioxidants & Redox Signaling, in press.
[11] Tian, W.N., Braunstein, L.D., Apse, K., Pang, J., Rose, M., Tian, X. and Stanton, R.C. (1999) Importance of glucose-6-phosphate dehydrogenase activity in cell death. Am. J. Physiol. 276, C1121–C1131.
[12] Abid, M.R., Schoots, I.G., Spokes, K.C., Wu, S.Q., Mawhinney, C. and Aird, W.C. (2004) Vascular endothelial growth factor-mediated induction of manganese superoxide dismutase occurs through redox-dependent regulation of forkhead and IkappaB/NF-kappaB. J. Biol. Chem. 279, 44030–44038.
[13] Robinson, B.H. (1998) Human complex I deficiency: clinical spectrum and involvement of oxygen free radicals in the pathogenicity of the defect. Biochim. Biophys. Acta. 1364, 271–286.
[14] Smeitink, J., van den Heuvel, L. and DiMauro, S. (2001) The genetics and pathology of oxidative phosphorylation. Nat. Rev. Genet. 2, 342–352.
[15] Hirst, J., Carroll, J., Fearnley, I.M., Shannon, R.J. and Walker, J.E. (2003) The nuclear encoded subunits of complex I from bovine heart mitochondria. Biochim. Biophys. Acta. 1604, 135–150.
[16] Petruzzella, V., Vergari, R., Puzziferri, I., Boffoli, D., Lamantea, E., Zeviani, M. and Papa, S. (2001) A nonsense mutation in the NDUFS4 gene encoding the 18 kDa (AQDQ) subunit of complex I abolishes assembly and activity of the complex in a patient with Leigh-like syndrome. Hum. Mol. Genet. 10, 529–535.
[17] Scacco, S., Petruzzella, V., Budde, S., Vergari, R., Tamborra, R., Panelli, D., van den Heuvel, L.P., Smeitink, J.A. and Papa, S. (2003) Pathological mutations of the human NDUFS4 gene of the 18-kDa (AQDQ) subunit of complex I affect the expression of the protein and the assembly and function of the complex. J. Biol. Chem. 278, 44161–44167.
[18] Iuso, A., Scacco, S., Piccoli, C., Bellomo, F., Petruzzella, V., Trentadue, R., Minuto, M., Ripoli, M., Capitanio, N., Zeviani, M. and Papa, S. (2006) Dysfunctions of cellular oxidative metabolism in patients with mutations in the NDUFS1 and NDUFS4 genes of complex I. J. Biol. Chem. 281, 10374–10380.
[19] Vinogradov, A.D. and Grivennikova, V.G. (2005) Generation of superoxide-radical by the NADH:ubiquinone oxidoreductase of heart mitochondria. Biochemistry (Moscow) 70, 120–127.
[20] Scacco, S., Vergari, R., Scarpulla, R.C., Technikova-Dobrova, Z., Sardanelli, A., Lambo, R., Lorusso, V. and Papa, S. (2000) cAMP-dependent phosphorylation of the nuclear encoded 18-kDa (IP) subunit of respiratory complex I and activation of the complex in serum-starved mouse fibroblast cultures. J. Biol. Chem. 275, 17578–17582.
[21] Papa, S., Scacco, S., Sardanelli, A.M., Vergari, R., Papa, F., Budde, S., van den Heuvel, L. and Smeitink, J. (2001) Mutation in the NDUFS4 gene of complex I abolishes cAMP-dependent activation of the complex in a child with fatal neurological syndrome. FEBS Lett. 489, 259–262.
[22] Pasdois, P., Deveaud, C., Voisin, P., Bouchaud, V., Rigoulet, M. and Beauvoit, B. (2003) Contribution of the phosphorylable complex I in the growth phase-dependent respiration of C6 glioma cells in vitro. J. Bioenerg. Biomembr. 35, 439–450.
[23] Taylor, E.R., Hurrell, F., Shannon, R.J., Lin, T.K., Hirst, J. and Murphy, M.P. (2003) Reversible glutathionylation of complex I increases mitochondrial superoxide formation. J. Biol. Chem. 278, 19603–19610.
[24] Clementi, E., Brown, G.C., Feelisch, M. and Moncada, S. (1998) Persistent inhibition of cell respiration by nitric oxide: crucial role of *S*-nitrosylation of mitochondrial complex I and protective action of glutathione. Proc. Natl. Acad. Sci. USA 95, 7631–7636.
[25] Bergmeyer, H.U., Grassl, M., Walter, H.E. (1983) Methods in Enzymatic Analysis (Bergmeyer HU ed), 3rd ed., Vol. 2.
[26] Noltmann, E.A., Gubler, C.J. and Kuby, S.A. (1961) Glucose 6-phosphate dehydrogenase (Zwischenferment), I. Isolation of the crystalline enzyme from yeast. J. Biolo. Chem. 236, 1225–1230.
[27] Wheeler, C.R., Salzman, J.A., Elsayed, N.M., Omaye, S.T. and Korte, D.W. (1990) Automated assays for superoxide dismutase, catalase, glutathione peroxidase, and glutathione reductase activity. Anal. Biochem. 184, 193–199.