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cAMP controls oxygen metabolism in mammalian cells

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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].

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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.

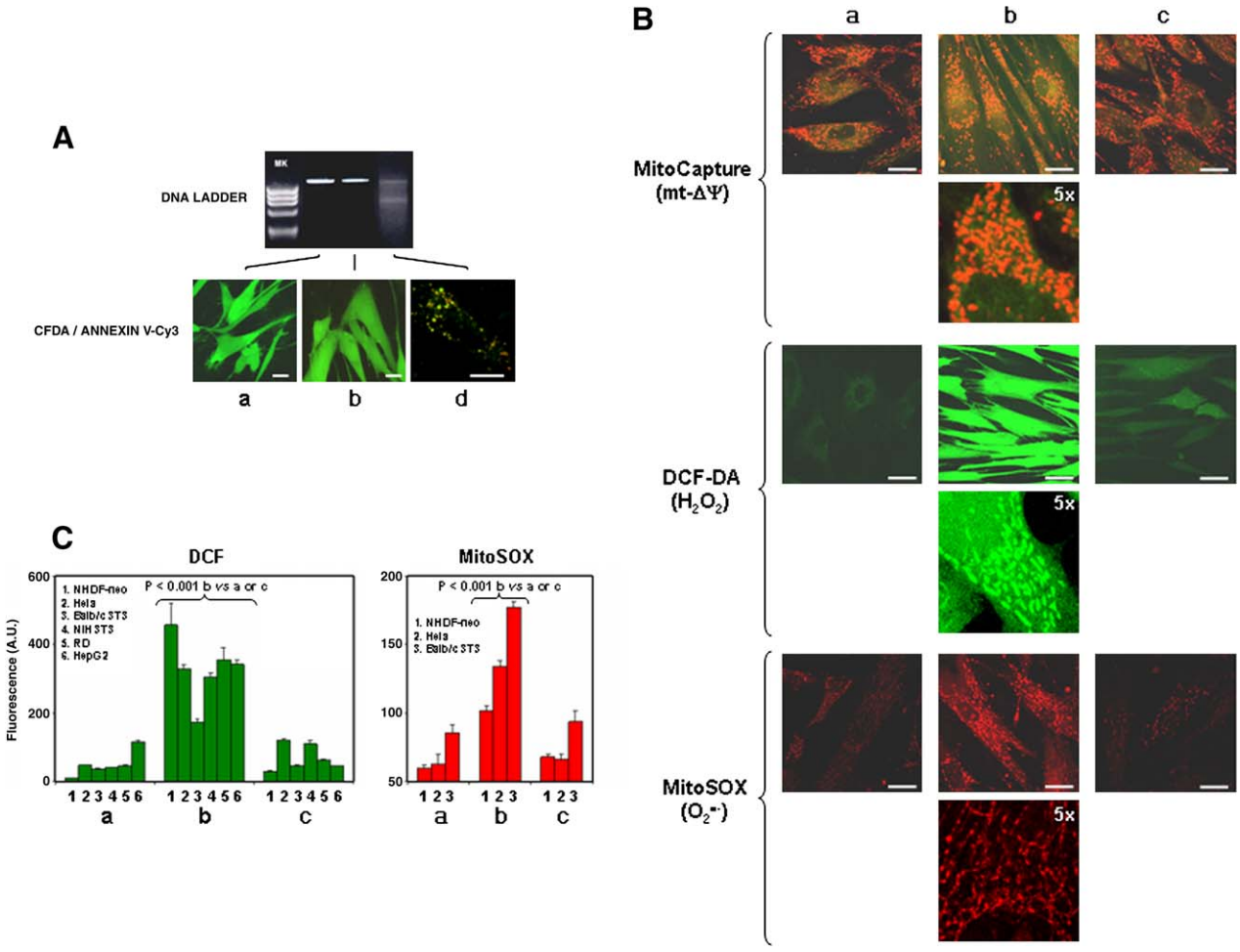

Fig. 1. Cellular ROS pattern in human and murine cell cultures. (A) Apoptosis assays. Neonatal normal human dermal fibroblasts (NHDF-neo) were grown in the exponential phase (a) (10% fetal bovine serum in DMEM), followed, where indicated, by 48 h cultivation under serum-limitation (0.5% FBS in DMEM) (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.