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Forum Rapid Letter

Regulation by the cAMP Cascade of Oxygen Free Radical Balance in Mammalian Cells

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ABSTRACT

A study is presented of the effect of the cAMP cascade on oxygen metabolism in mammalian cell cultures. Serum-starvation of the cell cultures resulted in depression of the forward NADH-ubiquinone oxidoreductase activity of complex I, decreased content of glutathione, and enhancement of the cellular level of H_2O_2. Depressed transcription of cytosolic Cu/Zn-SOD 1, mitochondrial glutathione peroxidase and catalase was also observed. Activation of the cAMP cascade reversed the depression of the activity of complex I and the accumulation of H_2O_2. The effect of cAMP involved the cAMP-dependent protein kinase. *Antioxid. Redox Signal.* 8, 495–502.

INTRODUCTION

MAMMALIAN CELLS utilize large amount of oxygen in the production of ATP coupled to oxidation of respiratory substrates in mitochondria. In addition to complete reduction to H_2O some oxygen can be partially reduced with generation of free radical species (5, 8). Cellular ROS production, which generally accounts for about 1% of the overall oxygen consumption, under particular physiopathological conditions can significantly increase (21, 23). Mitochondria are the primary source of cellular ROS (2, 4, 5, 18). Oxygen superoxide is produced in the mitochondrial matrix by complex I (NADH-ubiquinone oxidoreductase) and complex III (ubiquinone-cytochrome *c* oxidoreductase) (3), the latter also produces oxygen superoxide in the intermembrane mitochondrial space (6). Complex IV (cytochrome *c* oxidase), due to its high catalytic capacity of complete reduction of O_2 to H_2O, does not produce ROS, rather prevents their formation by maintaining a low oxygen concentration in cells (14).

In addition to mitochondrial oxidoreductases, other enzymes generate ROS in different cell compartments (5, 8, 10). These include the xanthine dehydrogenase/xanthine oxidase, peroxisomal fatty acid metabolism, prostaglandin intermediates, the plasma membrane NAD(P)H oxidase, this particularly active in the respiratory burst of neutrophiles (10). Oxygen superoxide, with a p*K* of 4.8, is at physiological pH's essentially in the anionic form, which is *per se* membrane impermeable, is converted by the mitochondrial and the cytosolic superoxide dismutase to H_2O_2. H_2O_2 diffuses freely across cellular membranes (7) and can be neutralized by a set of endogenous scavenger systems which include ubiquinone, glutathione, glutathione reductase, glutathione peroxidase, thioredoxins, glutaredoxins, catalase, etc. (12), as well as by nutritional antioxidants (tocopherols, β carotenoids, vitamin C, and flavonoids (12, 13).

In mammalian cells the metabolic energy supply has to adapt itself to the continuously changing energy demand (19). A key feature of cellular respiration is represented by

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regulation of the functional capacity of the respiratory chain enzymes at the level of gene expression, membrane assembly, and flux control processes. In mammals, regulation of metabolic energy supply is effected by hormones and neurotransmitters, in particular those that utilize the cAMP cascade and cAMP-dependent reversible protein phosphorylation (15). The cAMP cascade modulates utilization of glycogen and lipid energy stores and energy requiring processes such as cell growth and differentiation and neuronal activity. Recently it has been found that cAMP exerts in mammalian cell cultures a stimulatory effect on mitochondrial respiration, particularly at the level of complex I (16, 17, 22). The activation of complex I activity appears to be associated with reversible cAMP-dependent phosphorylation of mitochondrial proteins, including 18 kDa subunit(s) of complex I (17, 22).

The present study shows that serum starvation of mammalian cell cultures results in a depression of the forward NADH-ubiquinone oxidoreductase activity of complex I and the appearance of a significant level of H_2O_2. Both the inhibition of complex I and the accumulation of H_2O_2 are reversed by cAMP.

MATERIALS AND METHODS

Materials

Murine Balb/c 3T3 and NIH 3T3 fibroblasts and human HeLa and RD cell lines were from ATCC-LGC Promochem (Milan, Italy). DMEM, PBS, trypsin (0.05%)-EDTA (0.02%), penicillin (10,000 U/ml)–streptomycin (10,000 μg/ml), calf serum, and fetal bovine serum from EuroClone (Milan, Italy); cholera toxin, dibutyryl cyclic AMP, and diphenylene iodonium (DPI) were from Sigma-Aldrich (Milan, Italy); NADH from Boehringer; decylubiquinone and 3-isobutyl-1-methylxanthine (IBMX) from Calbiochem (Milan, Italy); all other chemicals were of the highest purity available. 2′,7′-Dichlorofluorescein diacetate (DCF-DA), MitoCapture, and TOPRO-1 were from Molecular Probes (Milan, Italy). Apoptotic DNA Ladder detection assay was from Roche (Basel, Switzerland). Trizol and SuperScript Reverse Transcriptase were from Invitrogen (Milan, Italy).

Cell culture preparation

Mouse fibroblasts and human cell lines were maintained in culture with DMEM with 10% calf serum. To obtain the contact inhibited cell cultures, cells were allowed to grow to full confluence before harvesting. For the serum starvation experiments, once cultures were at 70% confluence the medium DMEM was replaced with 0.5% fetal bovine serum. To induce intracellular increase of cAMP cell cultures were supplemented with 1 μg/ml cholera toxin + 100 μ*M* IBMX. Fibroblasts were harvested from 150 mm Petri dishes with 2 ml of trypsin-EDTA and washed in 20 ml phosphate buffer pH 7.4 (PBS) with 5% calf serum, centrifuged at 500 *g*, resuspended in 200 μl PBS, counted and immediately used.

Laser scanning confocal microscopy analysis

Cells were seeded at low density onto fibronectin coated 35 mm glass bottom dishes. After adhesion, living cells were directly incubated for 20 min at 37°C with MitoCapture (1/1000 dilution, for mitochondria staining) or 10 μ*M* DCF-DA (for intracellular ROS detection). For nuclei staining, cells were permeabilized with 0.02% Triton X-100 for 5 min and incubated with 1 μ*M* TOPRO-1 for a further 5 min at room temperature. Then cells were washed twice with PBS and examined by a microscope [TE 20000, Nikon, Florence, Italy; images collected using a 60× objective (1.4NA)] coupled to a Radiance 2100 dual laser (four-lines Helium-Neon, single-line Argon-Krypton) scanning confocal microscopy system (Bio-Rad, Hercules, CA, USA). The images were collected using a 60× objective (1.4 NA). Confocal planes (0.2 μm thickness) were examined along the z-axes, going from the top to the bottom of the cells. Acquisition, storage, and analysis of data made by using LaserSharp and LaserPix software from Bio-Rad.

DNA ladder detection

Handling of samples for DNA preparation was performed accordingly to the protocol indicated by the manufacturer of the Apoptotic DNA Ladder detection assay (Roche) which also provided, as positive control, samples of apoptotic fragmented DNA.

Spectrophotometric assay

All the assays were performed at 37°C with a double beam Johnson Foundation spectrophotometer. The NADH–UQ oxidoreductase activity was measured in 700 μl 50 m*M* potassium phosphate buffer, $MgCl_2$ 5 m*M*, pH 7.4, with 2×10^5 cells, in the presence of 0.2 m*M* decylubiquinone and 3 m*M* KCN. The reaction was started with different concentrations of NADH (2–25 μ*M*). Absorbance decrease was followed at 360–374 nm ($\Delta\epsilon = 2.01\ mM^{-1}$) and corrected for the reaction in the presence of rotenone (1 μg/ml). From the activity measurements Lineweaver-Burk plots were drawn to calculate V_{max} and K_m of Complex I activity.

Reverse transcription-polymerase chain reaction

Two micrograms of total cellular RNA isolated by Trizol reagent was reverse transcripted to cDNA with specific antisense primers (50 pmoles each) by SuperScript Reverse Transcriptase. Samples of 5 μl of RT reaction were PCR-amplified in 50 μl with 50 pmoles each of sense and antisense primers by 35 cycles of denaturation at 94°C (1 min), annealing (1 min), and extension at 72°C (2 min), followed by a further 10 min extension.

Measurement of cell total glutathione

3T3 Balb/c mouse fibroblasts were scraped with ice-cold PBS and centrifuged at 500 *g* for 5 min at 4°C. Cell pellet was resuspended and lysed in 0.25 *M* sucrose, 10 m*M* Tris, 1 m*M* EGTA, 0.25 m*M* PMSF. An aliquot of cell lysate was assayed for protein content determination by the Bradford method, using bovine serum albumin as standard. After acidification with 2% sulfosalicylic acid, the precipitated proteins were removed by centrifugation at 13000 *g* for 5 min and the supernatant was used for glutathione determination (9). Total glutathione was measured spectrophotometrically as in Ref. 25, using glutathione reductase and 5,5′-dithiobis(2-nitrobenzoate) (DTNB).

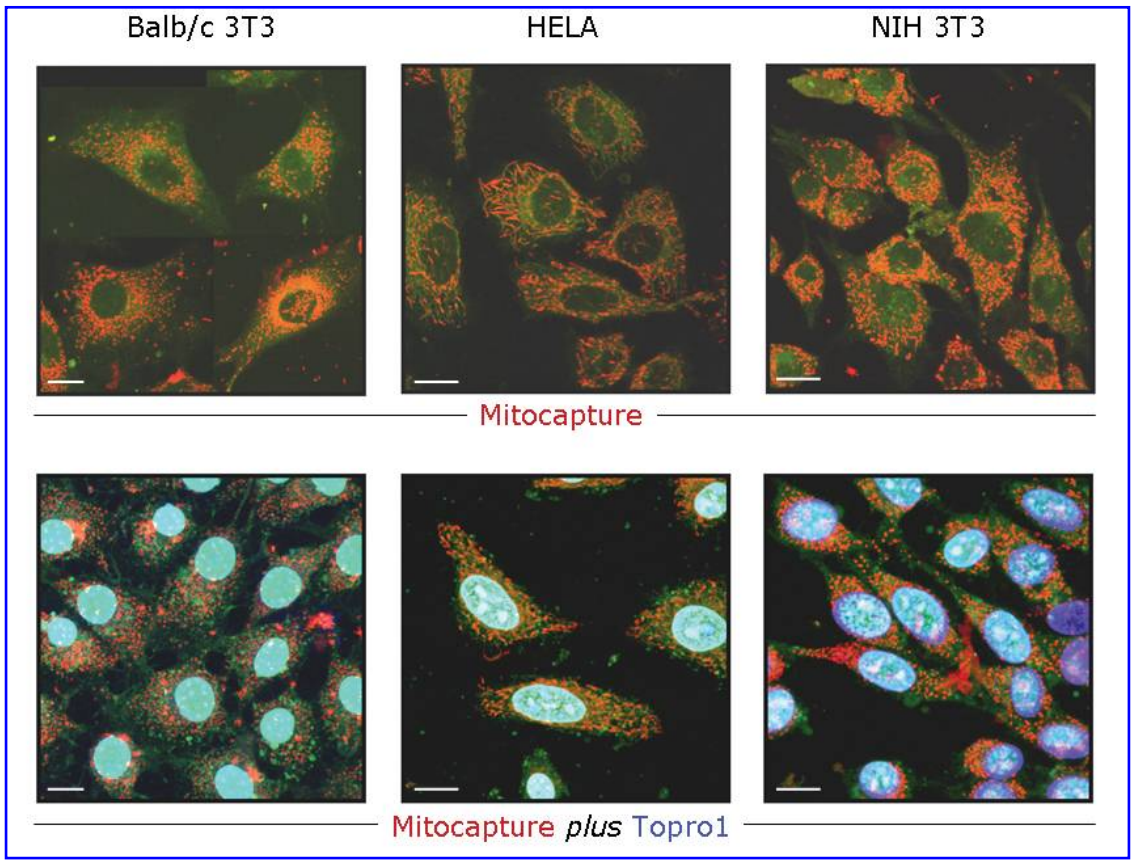

FIG. 1. Laser scanning confocal microscopy analysis of cultured Balb/c 3T3 fibroblasts, HeLa cells, and NIH 3T3 fibroblasts. *Top panels*: imaging of intracellular distribution and content of mitochondria stained by the specific accumulation of the fluorescent dual-emitter dye MitoCapture (Ar-Kr, λex = 488 nm; He-Ne λex = 543 nm). *Bottom panels*: double staining of mitochondria by MitoCapture and nuclei by Topro 1 (Ar-Kr, λex = 514 nm). The images shown are superimpositions of confocal planes (bars 20 μm) and are representative of at least three different preparations for each cell type.

Statistical analysis

Two-tailed Student's *t* test was applied to evaluate the significance of differences measured throughout the data-sets reported.

RESULTS

Serum starvation brings mammalian cell cultures in a quiescent state and induces apoptosis (1, 11). Examination in mouse fibroblast and HeLa cell cultures of the surviving quiescent cells, harvested from the glass-stacked layer, shows that 48 h serum starvation had no detrimental effect on the nucleus integrity and capacity of mitochondria of energy-linked, $\Delta\psi$ driven capture of a positively charged probe in these cells (Fig. 1). Also no sign of DNA fragmentation was observed in the glass-stacked cells indicating that they had not yet undergone apoptosis (Fig. 2).

Forty-eight hours of serum starvation of mouse fibroblasts resulted in significant depression of the specific activity of complex I (Fig. 3). A similar depression of the activity was observed when fibroblast replication was arrested at confluence. Thirty-minute treatment of quiescent fibroblasts with

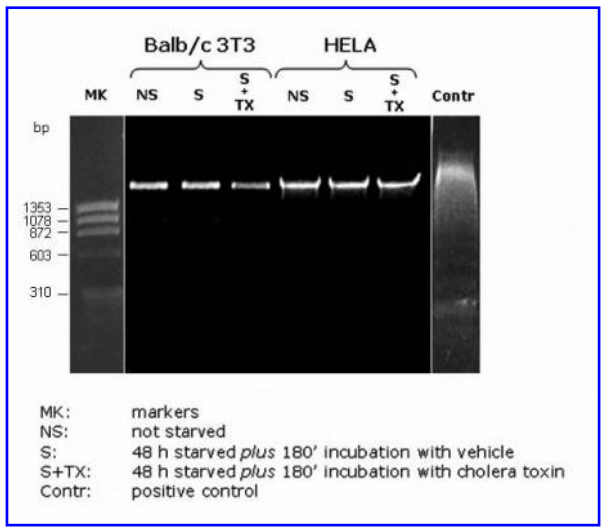

FIG. 2. Effect of serum starvation on DNA fragmentation. Apoptotic DNA fragmentation was analyzed by gel electrophoresis followed by ethidium bromide staining of DNA extracted from Balb/c 3T3 fibroblasts and HeLa cells. For cell culturing conditions, cholera toxin treatment, and further experimental details, see Materials and Methods.

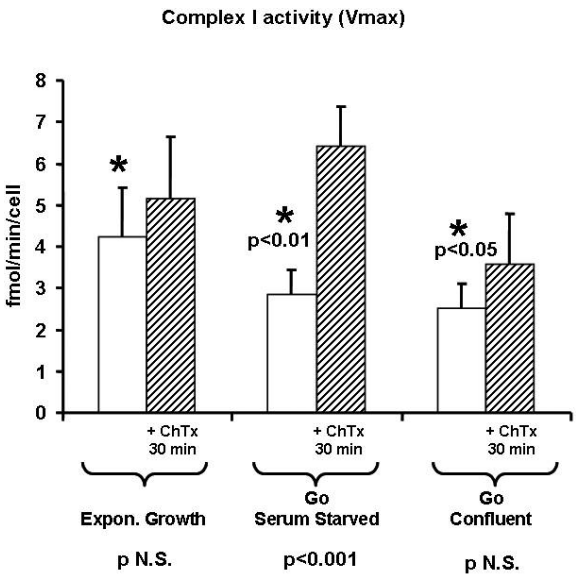

FIG. 3. Effect of cholera toxin treatment on rotenone sensitive complex I activity in Balb-c/3T3 mouse fibroblasts in exponentially growing, G_0 serum starved and G_0 confluent fibroblast cultures. The values reported as are mean V_{max} ± SEM, from three separate experiments, of rotenone-sensitive NADH-UQ oxidoreductase activity in untreated cells (*white bars*) and cholera toxin treated cells (*dashed bars*). Student's *t* test analysis showed a significant stimulation by cholera toxin treatment in serum starved cells ($p < 0.001$) and a significant decrease of complex I activity of untreated cells in both serum starved ($p < 0.01$) and confluent cultures ($p < 0.05$). For other experimental details, see Materials and Methods.

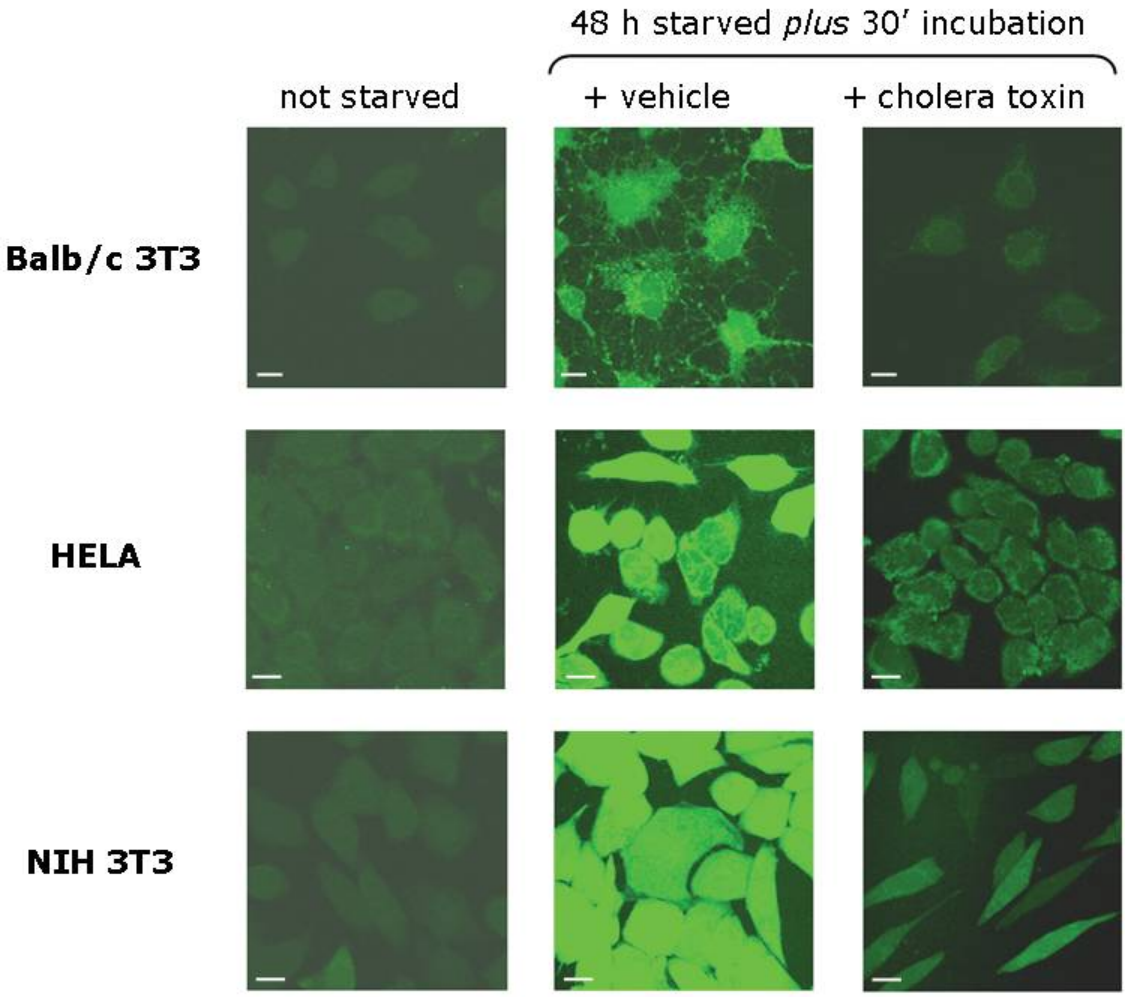

FIG. 4. Laser scanning confocal microscopy analysis of intracellular ROS production in Balb/c 3T3 fibroblasts, HeLa cells, and NIH 3T3 fibroblasts. DCF-DA added to living cells is hydrolyzed by intracellular esterases and converted to the ROS-reacting product (DCF). The green fluorescence of oxidized DCF was analyzed by exciting the sample with the Argon-Krypton laser beam (λex 488 nm). For cell culture conditions, cholera toxin treatment, and other experimental details, see Materials and Methods. The images shown are superimpositions of confocal planes (bars 20 μm) and are representative of at least three different preparations for each cell type.

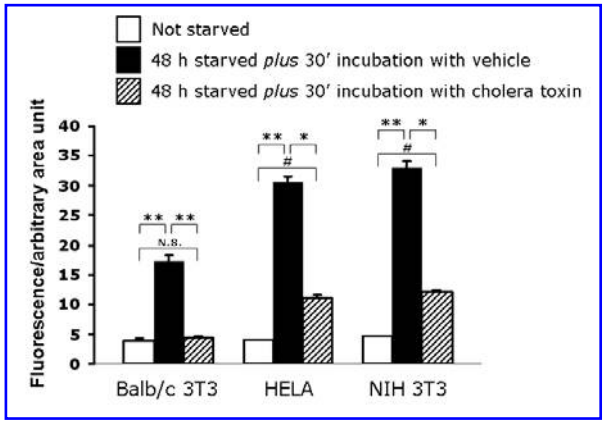

FIG. 5. Effect of serum starvation and cholera toxin treatment on intracellular ROS production. The data shown refer to the CLSM analysis illustrated in Figure 4. The fluorescence intensity of the DCF-staining is relative to selected intracellular areas (circles of 5–10 μm diameter) quantified in arbitrary units by the software provided by the confocal microscope manufacturer. At least 20 spots were randomly selected for each cell sample under indicated conditions and statistically analyzed. The bars are averages ± SEM. Results of Student's *t* test analysis: (*), $p < 0.0005$; (**), $p < 0.0001$; (#), $p < 0.001$; N.S., not significant.

cholera toxin, which is known to produce a lasting enhancement of the intracellular concentration of cAMP (11), resulted in a stimulation of complex I activity, which was particularly significant in the serum-starved fibroblasts (Fig. 3).

Confocal microscopic monitoring of the level of H_2O_2 in the cell cultures by the green fluorescent probe 2′,7′-dichlorofluorescin diacetate (DCF-DA) showed that in mouse fibroblasts and HeLa cells (as well as in other cell cultures, see below) 48 h starvation resulted in the appearance of a significant level of H_2O_2 (Fig. 4 and Fig. 5). H_2O_2, which appeared to accumulate in/around mitochondria (Fig. 6), practically disappeared upon 30-min treatment of the cell cultures with cholera toxin (Figs. 4, 5, and 7).

Semiquantitative analysis of the DCF-DA fluorescence in a human myoblast cell line (RD) showed that inhibition of the plasma membrane NADPH oxidase by DPI did not prevent the production of H_2O_2 induced by serum starvation (Fig. 7). H89, a permeant inhibitor of cAMP-dependent protein kinase, depressed, on the other hand, the H_2O_2-removal was affected by cholera toxin (Fig. 7).

RT-PCR analysis of the transcripts of cellular ROS-scavenger enzymes showed that serum starvation resulted in depression the expression of cytosolic Cu/Zn-SOD 1, and mitochondrial glutathione peroxidase and catalase. Some depression of mitochondrial Mn-SOD 2 was also observed. Cytosolic glutathione peroxidase was, apparently unaffected (Fig. 8). Serum starvation caused also a decrease of cellular glutathione content, which was not affected by the addition of dibutyryl cyclic AMP (Fig. 9). The 30-min cholera toxin treatment had no effect on the transcript levels of scavenger enzymes (Fig. 8) and the total content of glutathione (Fig. 9) in the serum-starved cells.

DISCUSSION

The present results show that serum starvation of mammalian cell cultures results in a depression of the forward NADH-ubiquinone oxidoreductase activity of complex I and appearance of H_2O_2 around mitochondria (Fig. 6). Enhancement of the cellular level of H_2O_2 affected by serum starvation can be due to increased production of ROS, depression of scavenger systems, or both. RT-PCR analysis shows, in fact, that serum starvation resulted in depressed transcription of mitochondrial glutathione peroxidase, cytosolic Cu/Zn-SOD 1, and catalase (Fig. 8). Expression of mitochondrial Mn-SOD 2 was, on the other hand, not significantly depressed in the serum-starved cells. Starvation resulted also in a marked decrease of the cellular content of glutathione (Fig. 9). It is conceivable that in serum-starved cells, oxygen superoxide, produced in the mitochondrial matrix by complex I and complex III, is converted by Mn-SOD 2 to H_2O_2. Due to the shortage of glutathione and the depressed activity of mitochon-

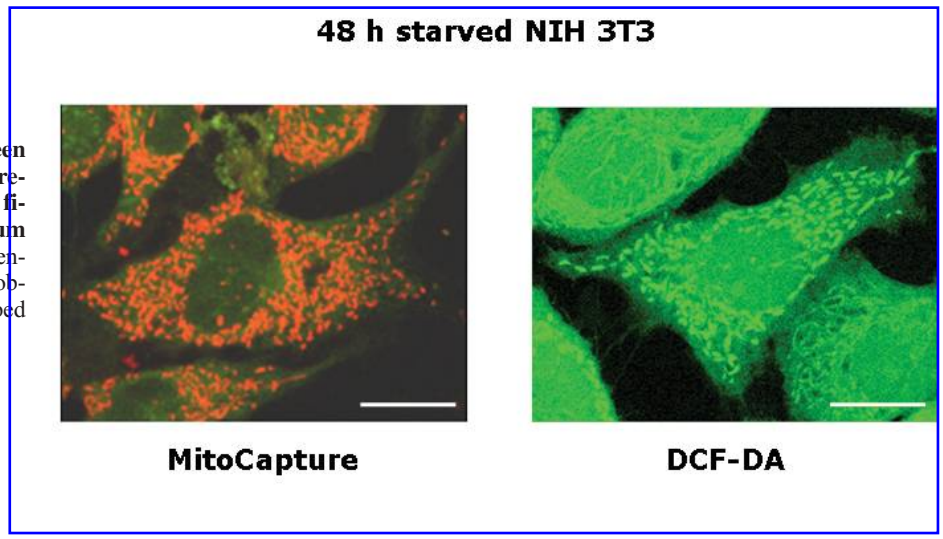

FIG. 6. Comparison between CLSM images of MitoCapture- and DCF-treated NIH 3T3 fibroblasts following 48 h serum starvation. The pictures are enlargements of CLSM images obtained in the experiments described in Figures 1 and 4 (bars 20 μm).

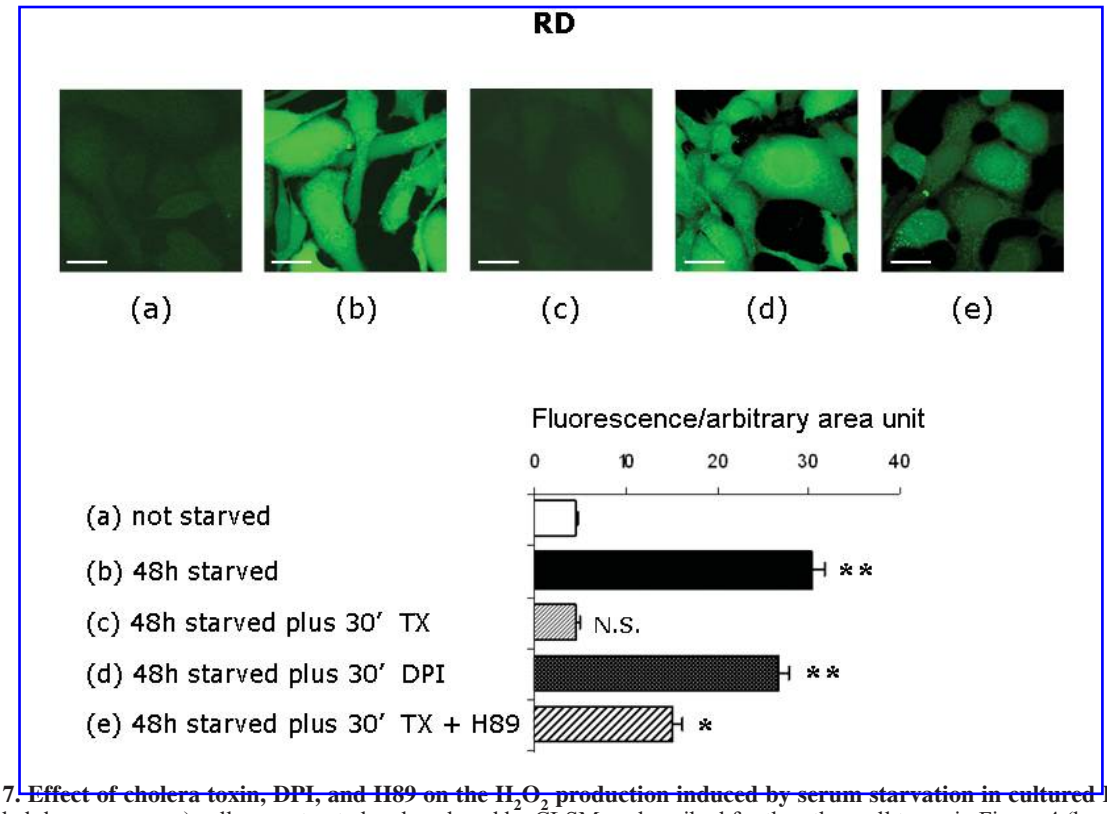

FIG. 7. ~~Effect of cholera toxin, DPI, and H89 on the H_2O_2 production induced by serum starvation in cultured~~ RD cells. RD (rhabdomyosarcoma) cells were treated and analyzed by CLSM as described for the other cell types in Figure 4 (bars 20 μm). Where indicated 10 μ*M* DPI and 5 μ*M* H89 were used. Quantification of the H_2O_2-oxidized DCF probe was carried out as detailed in Figure 5. The bars are averages ± SEM. Results of Student's *t* test analysis with respect to not starved cells: (*), $p < 0.005$; (**), $p < 0.001$; N.S., not significant.

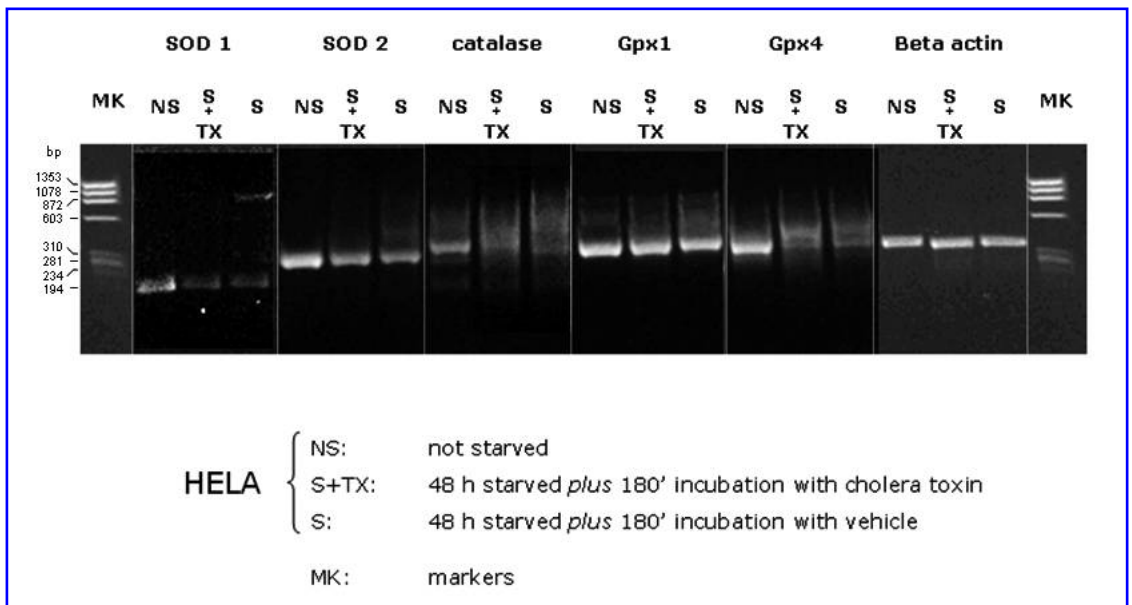

FIG. 8. ~~Effect of serum starvation and cholera toxin treatment on antioxidant enzymes expression in HeLa cells analyzed~~ by Reverse Transcription-Polymerase Chain Reaction. See under Materials and Methods for details; primers used: cytosolic Cu-Zn superoxide dismutase (SOD 1), f-5′-TGAAGAGAGGCATGTTGGAG-3′, r-5′-TCTTCATTTCCACCTTTGCC-3′ (T_a: 55°C); mitochondrial Mn superoxide dismutase (SOD 2), f-5′-GACAAACCTCAGCCCTAAC-3′, r-5′-TGCTCCCACACATCAATCC-3′ (T_a: 57°C); catalase, f-5′-TCCATTCGATCTCACCAAGG-3′, r-5′-GTAGTAATTTGGAGCACCACC-3′ (T_a: 58°C); cytosolic glutathione peroxidase (Gpx1), f-5′-AATTCCCTCAAGTACGTCCG-3′, r-5′-CTCGATGTCAATGGTCTGGAA-3′ (T_a: 57°C); mitochondrial glutathione peroxidase (Gpx4), f-5′-AACTACACTCAGCTCGTCG-3′, r-5′-GCAGGTCCTTCTCTATCACC-3′ (T_a: 58°C); β-actin, f-5′-ACCAACTGGGACGACATGGAG-3′, r-5′-CGTGAGGATCTTCATGAGGTACTC-3′ (T_a: 58°C).

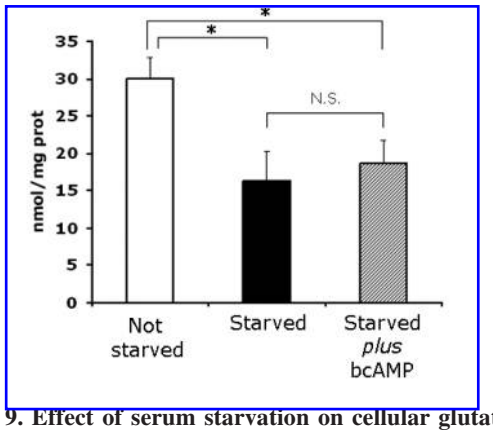

FIG. 9. Effect of serum starvation on cellular glutathione content in Balb/c 3T3 fibroblasts. Concentration of total GSH in cell lysate was estimated spectrophotometrically as described under Material and Methods. *Open bar*: cells collected at 70% confluence; *solid bar*: cells incubated 48 h under serum-starved condition; *hatched bar*: cells starved for 48 h and subsequently treated for 60 min with 100 μ*M* dibutyryl cyclic AMP plus 100 μ*M* IBMX. The values reported are means ± SEM. Results of Student's *t* test analysis: (*), $p < 0.05$; N.S., not significant.

drial glutathione peroxidase, H_2O_2 accumulates in mitochondria and diffuses out of the organelles. Oxygen superoxide can also be formed at the external surface of the inner mitochondrial membrane by complex III (6). There might be a reciprocal relationship between the depression of the rotenone-sensitive forward NADH-ubiquinone oxidoreductase activity of complex I and ROS accumulation, that is, the first process could be associated with enhanced production of oxygen superoxide which in turn can further depress normal forward activity and enhance oxygen superoxide production by complex I (20, 24).

A novel important feature of the regulation of cellular oxygen metabolism is revealed by the finding that cAMP restores the forward NADH-ubiquinone oxidoreductase activity (see also Refs. 17, 22) and produces disappearance of H_2O_2 in starved cells. These effects of cAMP exhibit the same time course, reaching completion in about 30 min. Furthermore, both effects are suppressed by H89, a permeant inhibitor of cAMP-dependent protein kinase. This indicates that both effects involve modulation of critical enzymes by cAMP-dependent phosphorylation. Previous observations have shown that in cell cultures *in vivo* cAMP promotes reversible phosphorylation of nuclear encoded subunits of complex I, associated with activation of the forward NADH-ubiquinone oxidoreductase activity of complex I (17, 22). This activation of complex I could be accompanied by depression of $O_2^{\cdot -}$ production by the complex. In cell cultures *in vivo*, the stimulation of complex I activity by cAMP could, however, also result from modulation of the level of metabolic positive or negative effectors of the complex.

Activation of scavenger enzymes can also be involved in the cAMP-induced removal of ROS. Possible mutual relationship between effects of cAMP-dependent phosphorylation on forward electron transfer activity and superoxide anion production in complex I and activity of ROS scavenger enzymes is a matter of further exploration. In this respect, it might be possible that the effects of cAMP can also involve changes in the redox state of subunits of the complex (24).

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ABBREVIATIONS

cAMP, cyclic AMP; CLSM, confocal laser scanning microscopy; DCF-DA, 2′,7′-dichlorofluorescein diacetate; DMEM, Dulbecco's modified Eagle medium; DPI, diphenylene iodonium; DTNB, 5,5′-dithiobis(2-nitrobenzoate); Gpx1, cytosolic glutathione peroxidase; Gpx4, mitochondrial glutathione peroxidase; GSH, glutathione; IBMX, 3-isobutyl-1-methylxanthine; PBS, phosphate-buffered saline; ROS, reactive oxygen species; SOD-1, CU/Zn superoxide dismutase; SOD-2, mitochondrial Mn-superoxide dismutase.

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