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Oxidative Stress and Upregulation of Mitochondrial Biogenesis Genes in Mitochondrial DNA-Depleted HeLa Cells

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The signaling mechanism through which deficitary mitochondrial function would activate nuclear genes required for mitochondrial biogenesis, has not been established. To explore the hypothesis that reactive oxygen species (ROS), a mitochondrial product, constitute part of the mitochondria-nuclei signaling pathway, we obtained HeLa cells depleted of mitochondrial DNA (ρ^0 cells) through exposure to ethidium bromide. We found evidences of oxidative stress in ρ^0 cells, employing a fluorescent probe and measuring NF-κB activation. Nuclear Respiratory Factor-1 (NRF-1) and Mitochondrial Transcription Factor A (Tfam) mRNA were measured by RT-PCR. For both transcription factors, ρ^0 cells revealed significantly higher levels of mRNA. These results support several hypothesis: that endogenous ROS enhance the expression of nuclear mitochondrial biogenesis genes NRF-1 and Tfam; that DNA deprived mitochondria lead to cellular oxidative stress, probably because of incomplete biogenesis of the mitochondrial electron transport chain, and consequently, that ROS are part of a mitochondria-nuclei regulatory signaling pathway.

The mitochondrial (mt) genome encodes for only 13 of the more than hundred mitochondrial proteins, the rest are encoded by the nucleus (1). The coordination between the nuclear and mitochondrial genomes is achieved by two transcription factors: Nuclear Respiratory Factor 1 (NRF-1) and mt transcription factor A (Tfam). NRF-1 regulates the transcription of nuclear encoded mt genes and the expression of Tfam, while Tfam regulates the transcription of the mt genome (2). Thus, a regulatory pathways from the nucleus to the mitochondria, has been recognized in mammalian cells, yet a signaling pathways from the mitochondria to the nucleus has still not been identified.

Mitochondria are the main source of intracellular reactive oxygen species (ROS). These ROS are generated in the electron transport chain, mainly at the ubiquinone site (3). We and others (2, 4) have proposed that ROS mediate the retrograde signaling from mitochondria to nuclei. In a previous communication (5) we showed that NRF-1 and Tfam are up-regulated in HeLa cells exposed to menadione, which increases intracellular levels of superoxide. These findings are in agreement with the putative role of ROS in the coordination of the mitochondrial genome and the expression of nuclear encoded mitochondrial genes.

In order to further characterize the role of ROS in the mitochondria-nuclei signaling we derived HeLa cells lacking mtDNA (ρ^0 cells), by exposing normal HeLa cells to ethidium bromide (EB) for several generations (6) with the aim of developing a cellular model characterized by endogenous oxidative stress. The lack of mtDNA results in mitochondria with an incomplete electron transport chain, where only complex II, totally encoded by the nucleus, is completely functional, while the following electron acceptors of the chain, complex III and IV, are not functional (7). We predicted that ρ^0 cells would generate an increased amount of ROS due to the leakage of electrons transferred mainly from complex II to ubiquinone, since there are no further acceptors in the electron transport chain. The increase in intracellular ROS generated by the blockage of the electron transport chain by antimicyn A, is in agreement with our prediction (8).

In this report, we first confirm that ρ^0 cells do have an increased amount of intracellular ROS, and that under these conditions, NRF-1 and Tfam are up-

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Abbreviations used: BHT, butylated hydroxytoluene; EB, ethidium bromide; DCF, 2′,7′-dichlorofluorescein; $DiOC_6(3)$, dihexyloxacarbocyanine iodine; mtDNA, mitochondrial DNA; NRF-1, nuclear respiratory factor 1; Tfam, mitochondrial transcription factor A; ROS, reactive oxygen species; DMEM, Dulbecco's modified Eagle's medium; COX III, subunit III of human cytochrome c oxidase.

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regulated, further supporting the postulate role of ROS in mt signaling to the nucleus.

MATERIALS AND METHODS

Cell culture and incubation procedure. HeLa cells (ATCC, CCL 2, Rockville, MD) were grown under 5% CO_2 in DMEM containing 1 mg/ml glucose and 10% fetal bovine serum (Life Technologies, Inc.). To deplete the cells of mtDNA (ρ^0 cell line) normal HeLa cells (ρ^+) were cultured during 70 passages, or more, in presence of 10 ng/ml of EB, under 10% CO_2 in DMEM supplemented with 4.5 mg/ml of glucose, 50 μg/ml uridine and 100 μg/ml pyruvate, to compensate for the respiratory metabolism deficit. Reagents were purchased from Sigma (St. Louis, MO) unless otherwise stated.

Mitochondrial DNA determination. Total DNA was isolated from cells using DNAzol reagent (Life Technologies, Inc.) according to the manufacturer instructions. For Southern blot analysis DNA samples were digested with XbaI and samples containing 0.5 μg of total DNA were loaded on 0.8% agarose gel for electrophoresis. Hybridization after blotting, was performed with a [α-^{32}P] dCTP (NEN-Dupont Boston, MA) radiolabeled mtDNA probe, for subunit III of human cytochrome *c* oxidase (COX III).

RNA isolation, reverse transcription and polymerase chain reaction (RT-PCR). Total RNA was extracted from cells using Trizol reagent (Life Technologies, Inc.) according to the manufacturer instructions. RT was performed with 5 μg of total RNA, 125 ng/ml of random primers, and 5 U/μl of Super Script II reverse transcriptase (Life Technologies, Inc.) in a final volume of 20 μl. PCR amplification of human NRF-1 and Tfam cDNA fragments was performed in a volume of 25 μl, containing 0.5 units of *Taq* polimerase (Life Technologies, Inc.) and 50 pmol of each primer: NRF-1, upstream 5′-GGAGTGATGTCCGCACAGAA-3′, downstream 5′-CGCTGTTAAGCGCCATAGTG-3′ (643 bp) and Tfam, upstream 5′-TATCAAGATGCTTATAGGGC-3′, downstream 5′-ACTCCTCAGCACCATATTTT-3′ (441 pb). Amplification of a 300 bp fragment of human β-actin gene was performed to enable semi-quantitative normalization using the oligonucleotides: upstream 5′-CGACATGGAGAAAATCTGGC-3′ and downstream 5′-AGGTCCAGACGCAGGATGG-3′. PCR products were analyzed on a 1.2% agarose gel. In previous experiments, we demonstrated that the product amplification by PCR was linear at the number of cycles and for the primers used for each gene (not shown). Cycle number corresponding to the exponential phase of the reaction was determined to be 34 cycles at 58°C annealing temperature for NRF-1 and 26 cycles at 55°C annealing temperature for Tfam. For human β-actin primers, optimal product yield was obtained at 20 cycles at 56°C annealing temperature. To avoid unspecific annealing between different probes and to minimize the background, amplification was performed separately for each gene.

Immunocytochemical detection of NF-κB. Cells were seeded and grown on Permanox-chamber slide (Lab-Tek, Nalge Nunc). The cells were washed briefly in cold PBS, fixed with 4% formaldehyde and permeabilized with phosphate-buffered saline (PBS) containing 0.3% Triton X100. Non-specific binding was blocked with 1% (w/v) BSA in PBS containing 0.05% Tween-20 (30 min, room temperature). Antibodies were diluted in 1% BSA in PBS before use and cells were washed three times in PBS after each stage of the immunostaining procedure. NFκB p65 were detected with a specific affinity-purified (C-20) (Santa Cruz Biotechnology, Santa Cruz, CA) goat policlonal primary antibody (1:200 dilution, 1 h at room temperature) and with anti-goat IgG linked to FITC secondary antibody (1:250 dilution, 1 h at 37°C). The cells were washed three times with PBS containing 0.05% Tween-20 and the slides were mounted, viewed and photographed in a Nikon fluorescence microscope.

Fluorescence microscopy of mitochondria. For labeling of mitochondria, rhodamine 123, prediluted in methanol, was added to the cell culture for 5 min at 37°C, at a final concentration of 1 μg/ml. The cells were then washed three times in PBS and examined by fluorescence microscopy. For vital staining of mitochondria the cells were fixed and labeled as reported (9, 10). Briefly, the fixative (0.25% glutaraldehyde in 0.1 M cacodylate, 0.1 M sacarose buffer pH 7.4) was added dropwise on cells cultured on coverslips and then incubated for 30 min in 0.1 M cacodylate buffer containing 0.2 μg of the dihexyloxacarbocyanine iodine ($DiOC_6(3)$). Then, the cells were rinsed several times and mounted with fresh cacodylate buffer. Cells preparations were immediately observed by fluorescence microscopy as mentioned above. This cationic fluorescent dye is a membrane-potential sensitive probe, which selectively accumulates in mitochondria (10).

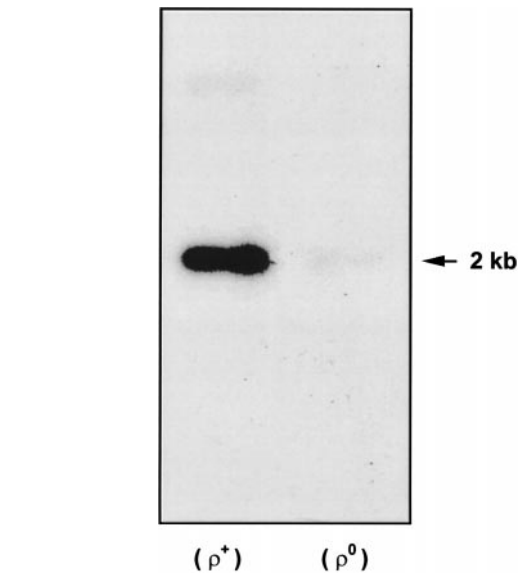

FIG. 1. Southern blot analysis of DNA from parental HeLa cell line (ρ^+) and derivative cell line depleted of mitochondrial DNA (ρ^0) by growth in presence of ethidium bromide. Total DNA was digested with XbaI, and equal amount were used for agarose gel electrophoresis. Hybridization was performed with a labeled mtDNA probe for subunit III of human cytochrome *c* oxidase (COX III). The arrowhead indicates the position of the predicted 2 kb band which is present only in ρ^+ cells.

Detection of reactive oxygen species (ROS). ROS generation in mtDNA depleted HeLa cells was detected with 2,7-dichlorofluorescein (DCF) which produces a green fluorescence when oxidized (11). Cells incubated with 5 μM DCF for 15 min at 37°C, were rinsed three times with PBS, and visualized immediately by epifluorescence microscopy (excitation, 450-490 nm; emission, 515-550 nm) and photographed in an image acquisition time of 30 sec and 4 min, using HeLa cells as control.

Statistical analysis. Results are expressed as mean ± S.D. for 3 independent experiments or as representative separate experiments performed at least three times. Statistical analysis was performed using Student's *t* test for paired values.

RESULTS

In order to obtain a cell line selectively depleted of mtDNA, we exposed HeLa cells to low concentrations of EB (10 ng/ml) for up to 70 generations (6). A Southern blot hybridization analysis of total DNA from parental and derivative cell lines, designated ρ^+ and ρ^0, respectively, for the mitochondrial encoded subunit III

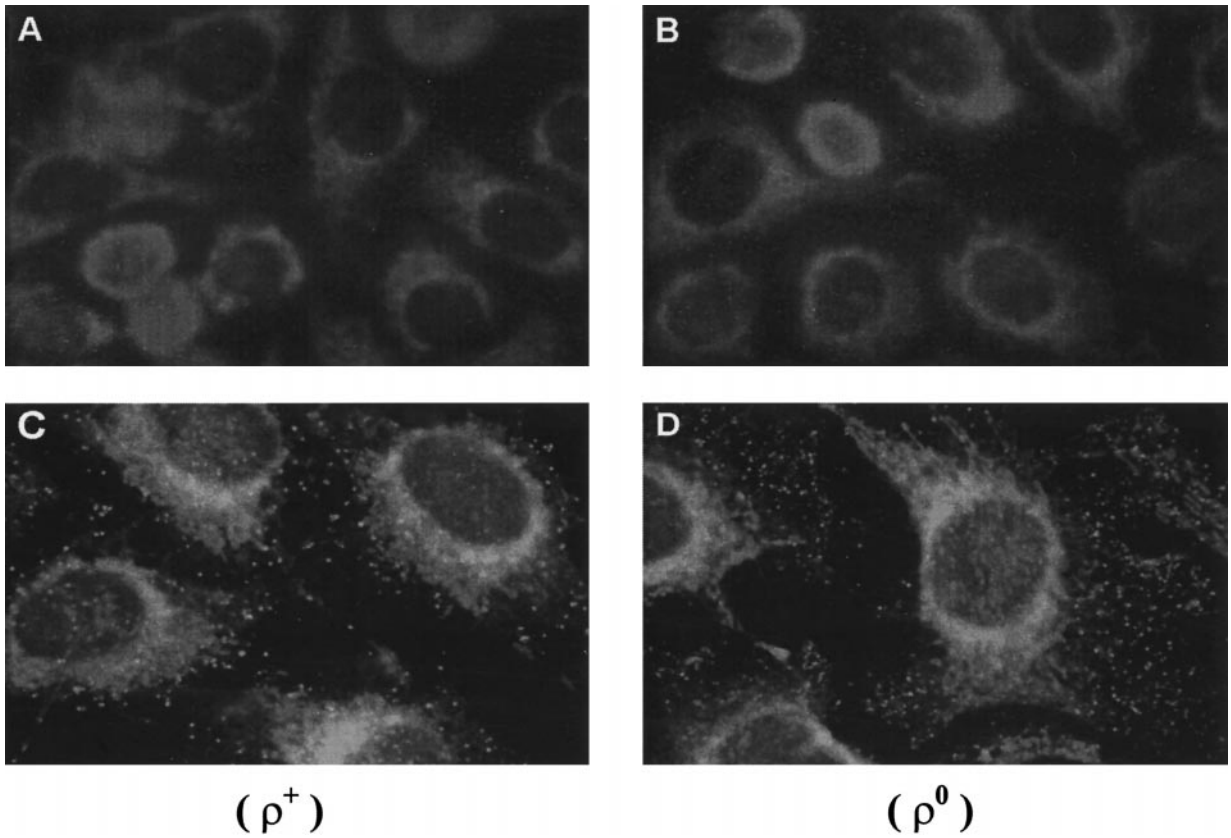

FIG. 2. Fluorescence microscopy characterization of mitochondria in living cells, using Rhodamine 123 and $DiOC_6(3)$ staining. ρ^0 (A and C) and ρ^+ (B and D) cells were stained with Rhodamine-123 (A and B) or $DiOC_6(3)$ (C and D) and the respective fluorescence results are shown.

of cytochrome *c* oxidase was performed. The hybridization reveals an almost complete depletion of mtDNA in the ρ^0 cells, with an approximate 98% reduction of the mtDNA content, respect the ρ^+ cells (Fig. 1). As previously shown by others (6, 12) ρ^0 cells grow slowly, depend on glycolysis for their energy requirements, and require pyruvate and uridine in the growth medium.

To explore if mtDNA depletion leads to a modification in mitochondrial content and function, fluorescence techniques were used (Fig. 2). Mitochondrial membrane potential, detected through uptake and retention of rhodamine-123, reveals that ρ^0 do not significantly differ from the ρ^+ cells (Fig. 2, A and B). Both the intensity and the perinuclear distribution pattern of the fluoresce staining were indistinguishable in these cell lines. We next analyzed the staining pattern of $DiOC_6(3)$, a mitochondrial specific dye (9, 10), to determine if the EB treatment alters the characteristic mitochondrial staining in these cell line. Our results shown that the apparent number and distribution of mitochondria do not change after prolonged treatment with EB (Fig. 2, C and D).

The effect of mtDNA depletion on intracellular ROS generation was assessed using DCF fluorescent probe. Compared with the parental cell line (ρ^+), ρ^0 cells showed an early and significant increase in fluorescence, revealing the presence of increased free radicals production (Fig. 3). The increase in ROS generation, in presence of mtDNA depletion, suggests that the dysfunction in the electron transport chain was associated with an increase in oxidative stress in ρ^0 cells.

The mtDNA depletion also was associated with increased nuclear localization of the transcription factor NF-κB (Fig. 4, A and B). NF-κB nuclear translocation was inhibited by the treatment of ρ^0 with the antioxidant BHT (25 μM) for 2 hr (Fig. 4, C). Additionally, incubation of ρ^+ with antimicyn A, an inhibitor of complex III, also induced translocation of NFκB, to a similar pattern observed in ρ^0 cells (data not shown).

Finally, to asses changes in the expression of nuclear genes that control mitochondrial biogenesis, we examined the relative abundance of mRNA transcripts encoding for transcription factors NRF-1 and Tfam in ρ^+ and ρ^0 cells. Figure 5 shows the expression of NRF-1, Tfam and β-actin mRNA by RT-PCR. A significant 2-fold increase was observed for both mitochondrial biogenesis genes when compared ρ^0 with ρ^+ cells (Fig. 5, bottom).

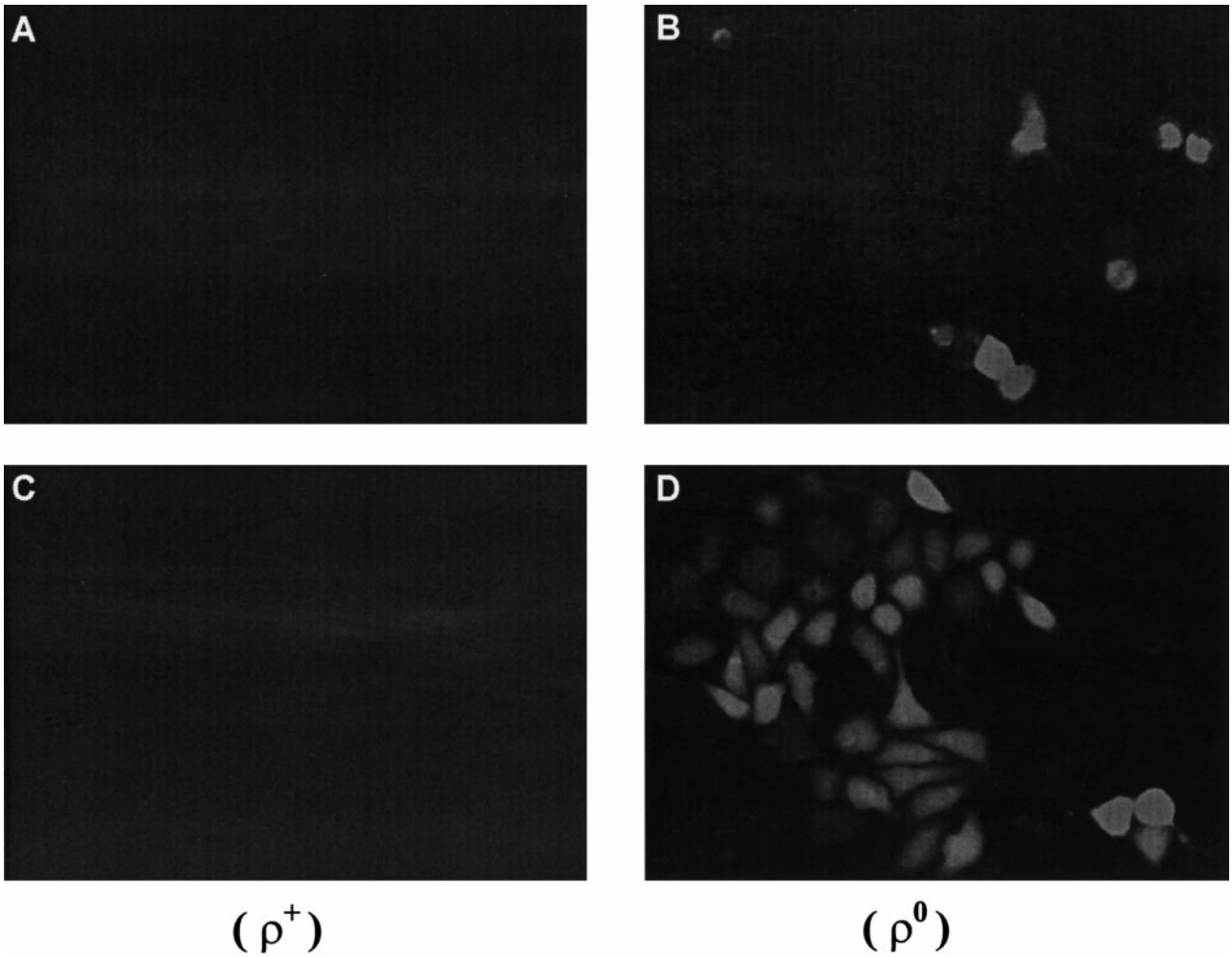

FIG. 3. Detection of ROS production, in ρ^0 and ρ^+ cells lines. Confluent culture cells were labeled with 5 μM of fluorescent agent DCF for 15 min at 37°C. At 0 min (A and B) the ρ^0 cells emit low levels of ROS, while many cells reach a maximum at 4 min (C and D). The ρ^+ cells did not emit fluorescence light during the time evaluated. A representative experiment is shown.

DISCUSSION

Mitochondria have been recognized as important site where reactive oxygen species are generated in cells (3, 13). Studies using electron transport inhibitors have provided evidence that mitochondria is the source of ROS (14). Inhibition with rotenone (site I) that block electron supply to ubiquinol, attenuate the ROS generation, whereas antimicyn A, an inhibitor of complex III, accelerates oxidant production, demonstrating that mitochondria function as a source of ROS generation in cells (14). Accumulating evidence exists that oxygen radicals may provide a cellular control mechanism and play important role to gene regulation (14, 15, 16) although the mechanisms responsible for intracellular oxidant generation involved in the activation of those pathways are still unclear.

Mitochondria are semi-autonomous organelles containing a resident genome. However the replication and transcription of the mtDNA depend entirely of the nuclear-encoded proteins, such as NRF-1 and Tfam (2). The mechanism of the coordinated expression of nuclear and mitochondrial genomes are not yet dilucidated. The present study postulate a role of ROS in mt signaling to the nucleus. The results of the present study demonstrate that deficiency of electron transport chain, produced by elimination of mtDNA, induce an increase of intracellular ROS, and that under these conditions the transcription factors, NRF-1 and Tfam, that control mt biogenesis, are up-regulated. Mitochondrial DNA depleted cell line (ρ°) serve as a model to investigate the potential role of mt generated ROS in regulating nuclear-encoded genes involved in mt biogenesis. We found increased ROS levels in our ρ° cells (Fig. 3), in concordance with an increased leakage of electrons by ubiquinone, loaded mainly by complex II of the electron transport chain. We also observed, in agreement with previous studies (12, 17), that ρ° cells are viable, maintain an electrochemical gradient

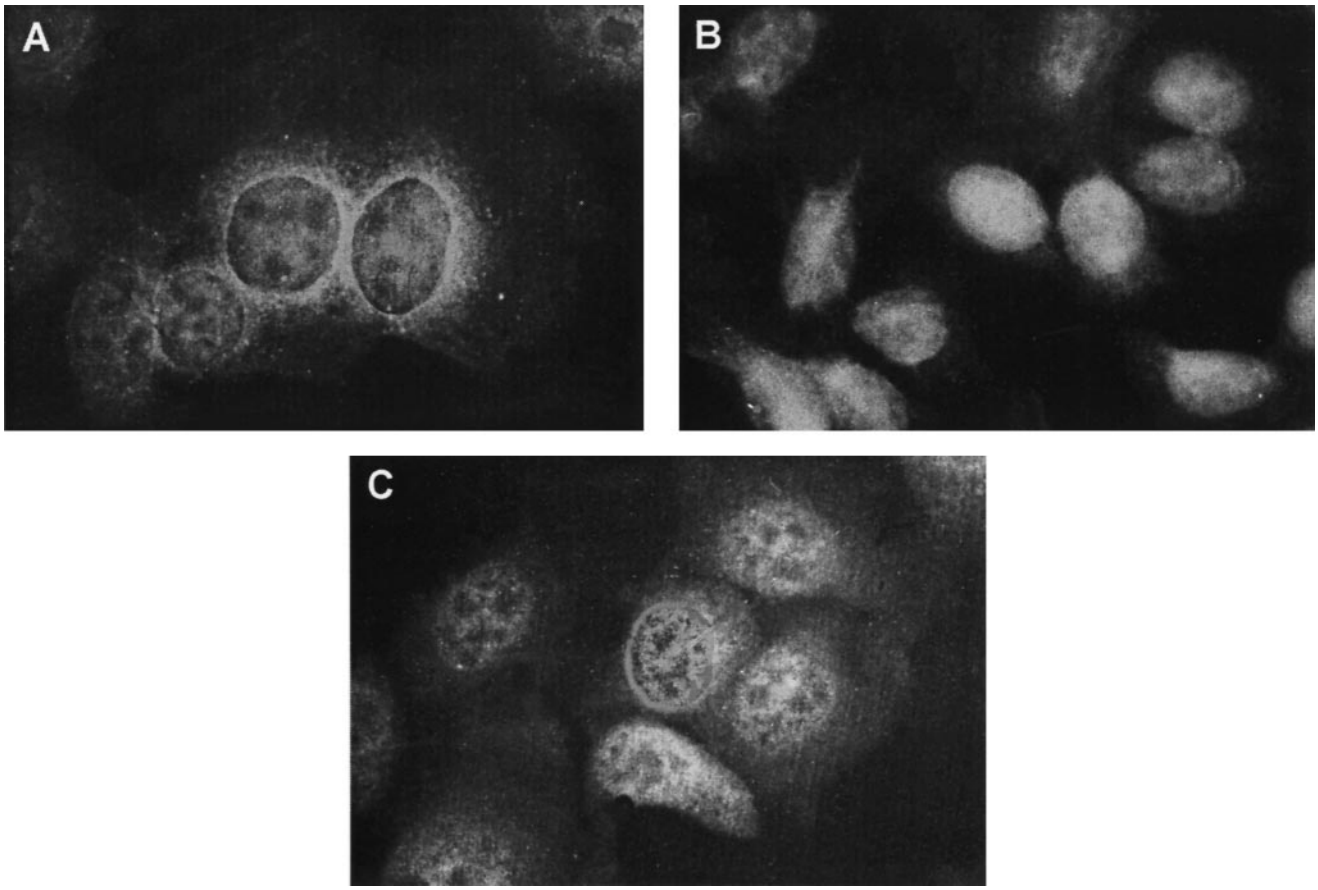

FIG. 4. Effect of mtDNA depletion in nuclear translocation of the transcription factor NFκB. NFκB was detected by immunofluorescence with an antibody against the 65-kDa subunit. NFκB immunoreactivity was located in the cytoplasm of ρ^+ cells (A) but in the nuclei of ρ^0 cells (B). The translocation was completely inhibit in the ρ^0 cells treated with an antioxidant, BHT (C). The figure illustrates a representative result of three experiments.

across the mitochondrial membranes by uptake of rhodamine 123 (Fig. 2, A and B) and present a relatively normal distribution of mt in the cytoplasm (Fig. 2, C and D). Concomitantly, under oxidative stress conditions generated by mt impairment, the expression of nuclear genes encoding transcription factors, NRF-1 and Tfam, was up-regulated (Fig. 5). A similar effect was observed by Li *et al.* (17) showing an increased expression of nuclear components of enzymes of the electron transport chain, in absence of mt genome. This is not an unspecific effect of cellular stress condition, since immediate early genes or heat shock proteins are not induced in ρ° cells (2, 17). In addition, recently has been demonstrated an up-regulation in nuclear respiratory genes under increased ROS production by blocking the electron transport chain with antimicyn A (3). To distinguish the relative importance of ROS in the cell response we investigate the activation of NFκB, a transcription factor that is rapidly stimulated by the intracellular generation of ROS (18). In ρ° cell line NFκB was activated, however the treatment of these cells with an antioxidant restores the basal conditions found in ρ^+ cells (Fig. 4), confirming that redox state was responsible of the activation of NFκB signaling system.

While NRF-1 expression was to our knowledge not previously investigated in ρ° cells, redox regulation was previously demonstrated for NRF-2 (2, 19) a transcription factor structurally unrelated to NRF-1, that regulates the expression of respiratory genes among others. Previously, Tfam expression was found low in ρ° cells derived from other cell type (20) or by transient mtDNA depletion (21). These studies show a decrease in cellular Tfam protein content while Tfam mRNA levels seem to remain unchanged, as assayed by Northern Blot (20). These results suggest that the increase in Tfam mRNA detected by the highly sensible RT-PCR technique in the present report does not result in an increase of Tfam protein, probably as a consequence of the loss in Tfam protein stability in absence of mtDNA, as was demonstrate by Davis *et al.* (21). Thus, under mtDNA depletion condition the redox signaling system that normally leads to changes in cellular mt content would be impaired. Indeed, by simple visual inspection

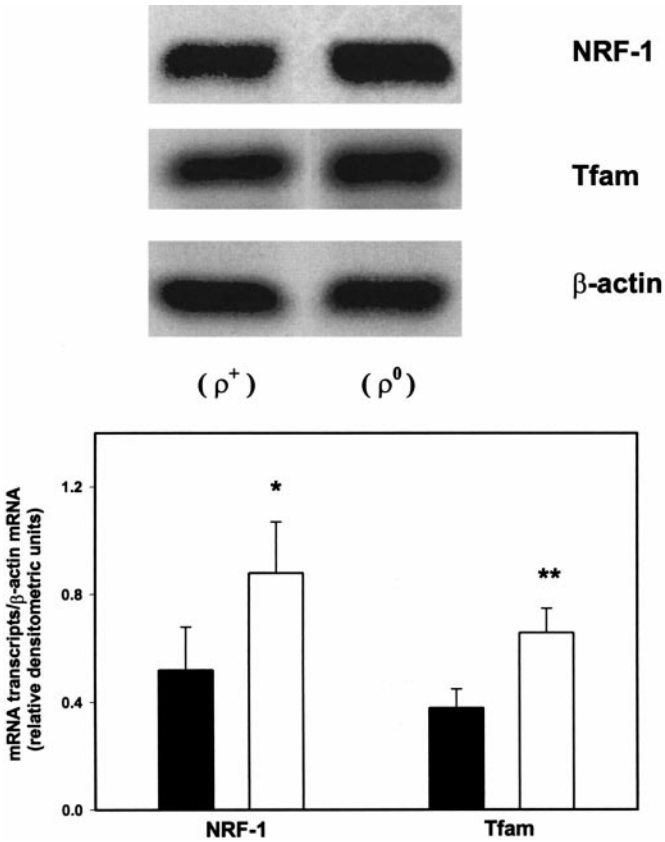

FIG. 5. Effect of mitochondrial DNA depletion on regulation of mitochondrial biogenesis. Top: Detection of NRF-1 and Tfam and β-actin by RT-PCR in ρ^+ and ρ^0 cells (representative results are shown). Bottom: Quantitative analysis of relative densitometric data. Results were plotted as the optical density ratio of each transcripts to β-actin, used as an internal control, in ρ^+ (black bars) and ρ^0 (white bars) cells. Bars shown mean ± SD. *$p < 0.05$ and **$p < 0.01$ vs control (ρ^+) value of the same transcript.

of mt staining (Fig. 2), there is no apparent change in mt mass between control and $\rho°$ cells.

The retrograde signal between mt status and nuclear genome was also proposed to be the ATP/ADP ratio (22), we can not rule out this possibility, because since $\rho°$ cells have impaired oxidative phosphorylation, the ATP concentration should be lower than in ρ^+ cells. In this regard it is important to note that we further increased ROS concentration in $\rho°$ cells by treatment with menadione (not shown), finding a further increase in NRF-1 and Tfam transcripts, showing that the system still reacts to increased oxidative stress, which further suggest a role of ROS in this signaling system.

In conclusion, the deficiency of mitochondrial respiration is associated to an increase in the production of ROS that could act as second messengers in the regulation of nuclear transcription factors responsible for mitochondrial biogenesis.

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