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Increase in Mitochondrial Mass in Human Fibroblasts under Oxidative Stress and during Replicative Cell Senescence

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Key Words

Oxidative stress · Aging · Senescence · Mitochondria · Nuclear respiratory factor · Mitochondrial DNA

Abstract

Abnormal proliferation of mitochondria generally occurs in muscle of aged individuals and patients with mitochondrial myopathy. An increase in the mitochondrial DNA (mtDNA) copy number has also been observed in aging human tissues. However, the molecular mechanism underlying the increase in mitochondrial mass and mtDNA is still unclear. In a previous study, we demonstrated that sublethal levels of oxidative stress caused an increase in mitochondrial mass in human lung cells. In this communication, we report our recent findings that the mitochondrial mass in human lung fibroblasts (MRC-5) in a later proliferation stage is significantly increased compared to that in the early stages of proliferation. The extent of the increase in mitochondrial mass in the senescent cells was similar to that in cells in the early stages of proliferation that had been treated with low concentrations ($\leq 180\ \mu M$) of hydrogen peroxide (H_2O_2). Moreover, we found that the rate of reactive oxygen species (ROS) production was higher in cells in the later proliferation stage compared to cells in the early proliferation stages. A similar phenomenon was also observed in cells in the early proliferation stages under low levels of oxidative stress. On the other hand, the mRNA levels of many nuclear DNA-encoded proteins involved in mitochondrial biogenesis, particularly nuclear respiratory factor-1, were found to increase in cells in later proliferation stages and in cells in early proliferation stages that had been treated with 180 μM H_2O_2. Interestingly, the increase in mitochondrial mass in the cells under oxidative stress could be repressed by treatment with cycloheximide or *m*-chlorocarbonyl cyanide phenylhydrazone but not by chloramphenicol. Furthermore, the mitochondrial mass of mtDNA-less ρ^o cells was also significantly increased by exposure to low concentrations (e.g. 180 μM) of H_2O_2. These results suggest that the increase in mitochondrial mass in replicative senescent cells may result from an increase in ROS production, and that it is dependent on both de novo synthesis of nuclear DNA-encoded proteins and their import into mitochondria, dictated by the membrane potential of mitochondria.

Introduction

Mitochondria are the intracellular organelles responsible for the supply of ATP and are also the main intracellular source and target of reactive oxygen species (ROS),

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which are continually generated as byproducts of aerobic metabolism in mammalian cells [8, 13, 23]. The respiratory function of mitochondria has been demonstrated to decline with age in various human tissues [1, 13, 19, 34]. This is thought to result, at least partly, from oxidative damage and mutation of mitochondrial DNA (mtDNA) in somatic tissues of aged individuals [1, 13, 19, 23, 34]. Moreover, defects in the respiratory chain can lead to enhanced production of ROS and free radicals in mitochondria [8, 23].

Each mammalian cell contains several hundred to more than a thousand mitochondria, and each organelle harbors 2–10 copies of mtDNA [24]. Although different tissues have a variable number of mtDNA molecules, the mtDNA copy number in a specific cell type is usually maintained precisely. On the other hand, the mitochondrial mass and the copy number of mtDNA in mammalian cells are subject to change during cell growth and differentiation, hormone treatment and exercise [22, 28, 37–39]. It was thought that proliferation of mitochondria and replication of mtDNA in the human cell take place throughout different phases of the cell cycle and are not limited to the S phase [6]. Moreover, expression of both nuclear DNA-encoded and mtDNA-encoded mitochondrial proteins has been demonstrated to respond in a complex manner to a variety of physiological and developmental stimuli including growth activation [16], neoplastic transformation [29, 32], muscle contraction [38, 39], tissue cell differentiation and hormone action [37]. However, it is still not known how the mtDNA copy number and the mitochondrial mass are regulated under different physiological and developmental conditions.

It has been observed that the mtDNA copy number per cell is increased in the lung tissues of elderly human subjects [12]. Consistent findings were also reported in the tissues of senescent rats [10] and in the brain [4] and skeletal muscle [3, 20] of elderly human subjects. It was suggested that the increase in the mtDNA copy number in tissue cells is the result of a feedback mechanism that compensates for defective mitochondria bearing an impaired respiratory system or mutated mtDNA [23]. However, it remains unclear how this event occurs and how the signals are transmitted from mitochondria to the nucleus or vice versa.

In a previous study, we demonstrated that at sublethal concentrations, hydrogen peroxide (H_2O_2) causes an increase in the mitochondrial mass and the mtDNA copy number in the human lung cell [14]. We have further hypothesized that the age-dependent increase in the production of superoxide anions and H_2O_2 from defective mitochondria is one of the factors contributing to the increase in mitochondrial mass to compensate for the decline in respiratory function during the aging process.

Replicative senescence is well established for normal human diploid fibroblasts grown under culture conditions and has been proposed as a model for cellular aging in vivo. We tested our hypothesis by using human lung fibroblasts as a Hayflick-type model of replicative cell senescence. We measured the change in mitochondrial mass in MRC-5 cells, a human lung fibroblast cell line, under oxidative stress and during replicative senescence. On the basis of the results thus obtained, we have proposed a molecular mechanism to explain the increase in mitochondrial mass elicited by oxidative stress.

Materials and Methods

Cell Culture

Human lung fibroblasts from the MRC-5 cell line were cultured at 37°C in humidified 5% CO_2-95% air in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Bethesda, Md., USA) supplemented with 10% fetal bovine serum (FBS), 50 units/ml penicillin G and 50 mg/ml streptomycin sulfate. Cells were subjected to a 1:3 split every 4 days.

An mtDNA-less human cell line (ρ^o cells) was established by long-term treatment of osteosarcoma 143B TK$^-$ cells with 50 ng/ml ethidium bromide [35]. The ρ^o and 143B cells were grown in DMEM supplemented with 5% FBS, 100 μg/ml pyruvate, 50 μg/ml uridine, 50 units/ml penicillin G and 50 mg/ml streptomycin sulfate.

Oxidative Stress Treatment

Oxidative stress treatment was performed on cells cultured at the same density. Typically, an aliquot of 2.5×10^5 cells was plated in triplicate in 25-cm^2 flasks 24 h before the experiment. For H_2O_2 treatment, a suitable aliquot of 30% H_2O_2 solution was freshly diluted into the culture medium immediately before the experiment. Cells were exposed to 90–180 μ*M* H_2O_2 for a fixed period of time under normal culture conditions as described above. The treated cells were then washed once with Hanks' balanced salt medium (Gibco) and were harvested by trypsinization at the indicated time for further analysis.

Determination of Mitochondrial Mass

We used the fluorescent dye 10-*n*-nonyl-acridine orange (NAO; Molecular Probes, Eugene, Oreg., USA), which binds specifically to the negatively charged cardiolipin (diphosphatidylglycerol) in the inner mitochondrial membrane independently of the membrane potential, to monitor the mitochondrial mass of the MRC-5 cells [14, 17]. Cells were trypsinized and resuspended in 0.5 ml of Medium 199 (Gibco) containing 10 μ*M* NAO. After incubation for 10 min at 25°C in the dark, cells were immediately transferred to a tube on ice for analysis by flow cytometry.

Dichlorofluorescin Staining

Production of H_2O_2 by cultured cells was measured using the probe 2′,7′-dichlorofluorescin diacetate (DCFH-DA; Molecular

Table 1. The sequences of the oligonucleotide primers used in this study

Genes	Primers	Sequences (5′ to 3′)
GAPDH	F78	CGGAGTCAACGGATTTGGTC
	R593	ACTGTGGTCATGAGTCCTTC
c-fos	F2	TGATGTTCTCGGGCTTCAAC
	R475	GCTTGGAGTGTATCAGTCAG
c-jun	F2036	GCATGAGGAACCGCATCGCTGCCTCCAAGT
	R2445	GCGACCAAGTCCTTCCCACTCGTGCACACT
NRF-1	F139	CCAAACCGAACATATGGCTAC
	R611	CCAGGATCATGCTCTTGTAC
mtTFA	F379	AGAATTGCCCAGCGTTGGAG
	R821	AGATCCTTTCGTCCAACTTC
PGC-1	F	TCCTCTGACCCCAGAGTCAC
	R	TAGAGTCTTGGAGCTCCTG
NRF-2α	F814	TAGACCTCACCACACTCAAC
	R1441	GTGACCAAACGGTTCAACTC
NRF-2βγ	F255	GAGCTCCCTTTACTACAGAC
	R717	AACTGTGGTGTTGCAGCATG

Probes) [14, 17, 25]. This probe is accumulated by cells and hydrolyzed by cytoplasmic esterases to become 2′,7′-dichlorofluorescin, which then reacts with H_2O_2 to generate the fluorescent product 2′,7′-dichlorofluorescein (DCF) [17]. Cells were incubated with 5 μ*M* DCFH-DA in the culture medium for 30 min at 37 °C and then washed, resuspended in 0.5 ml PBS and subjected to analysis by flow cytometry.

Flow Cytometry

A FACScan flow cytometer (Becton Dickinson, Bedford, Mass., USA) equipped with a 488-nm argon laser was used for analysis of cellular function by flow cytometry. Forward and side scatters were used to establish size gates and exclude cellular debris from the analysis. The excitation wavelength was set at 488 nm. The observation wavelength of 530 nm was chosen for green fluorescence and 585 nm for red fluorescence, and the intensities of emitted fluorescence were collected on FL1 and FL2 channels, respectively. In each measurement, a minimum of 20,000 cells were analyzed. Data were acquired and analyzed using Cell Quest software (Becton Dickinson). The relative change in the mean fluorescence intensity was calculated as the ratio of the mean fluorescence intensity in the channel of the treated cells to that of control.

Reverse Transcription-Polymerase Chain Reaction

Total RNA was extracted from the cells using TRIzol reagent (Gibco). The cDNA was synthesized in 50 μl of reaction mixture containing 5 μg of RNA, 0.5 μg of oligo $(dT)_{12-18}$, 200 μ*M* of each dNTP, 10 m*M* Tris-HCl (pH 9.0), 60 m*M* KCl, 1.5 m*M* $MgCl_2$, 40 U of ribonuclease inhibitor and 200 U of M-MuLV reverse transcriptase (Promega, Madison, Wisc., USA). The reaction was performed at 42 °C for 45 min, and then incubated at 95 °C for 5 min to inactivate the reverse transcriptase.

Polymerase chain reaction (PCR) was performed on a Perkin-Elmer/Cetus DNA thermal cycler. The reaction was carried out in a 50-μl mixture containing 200 ng of DNA, 200 μ*M* of each dNTP, 5 μCi of α-^{33}P-dATP, 40 pmol of each primer (table 1), 1.0 unit of *Taq* DNA polymerase, 50 m*M* KCl, 1.5 m*M* $MgCl_2$, 10 m*M* Tris-HCl (pH 9.0), 0.1% Triton X-100 and 0.01% (w/v) gelatin. The PCR cycles consisted of 15 s of denaturation at 94 °C, 15 s of annealing at 58 °C and 40 s of primer extension at 72 °C. The PCR products were separated on a 1.5% agarose gel at 100 V for 40 min and were detected under UV transillumination after ethidium bromide staining. After drying, Kodak X-ray film was exposed to the gel for 4–6 h. The band intensity of the PCR products of the target genes was analyzed by a scanning densitometer.

Statistics

Data are presented as mean ± SD of the measured values except where otherwise indicated. Comparisons among multiple groups were made by one-way analysis of variance followed by Fisher's protected least significant difference post hoc test. Differences between groups with a p value of <0.05 were considered significant.

Results

Normal human fibroblasts serially subcultured in vitro lose the capacity to divide after reaching a limited number of population doublings (PDs). In our laboratory, MRC-5 cells were able to attain 48 PDs before they lost proliferative potential and became senescent. Morphologically, the cells became large and flattened and could no longer display the array characteristics of young cells (data not shown).

In an attempt to determine whether there is a change in mitochondrial mass in MRC-5 cells during replicative senescence, we used the fluorescent dye NAO to label the mitochondria. The relative NAO intensity in the cells after 48 PDs was found to be 2.5 times that of the cells at 28 PDs ($p < 0.05$) (fig. 1). Moreover, after treatment with

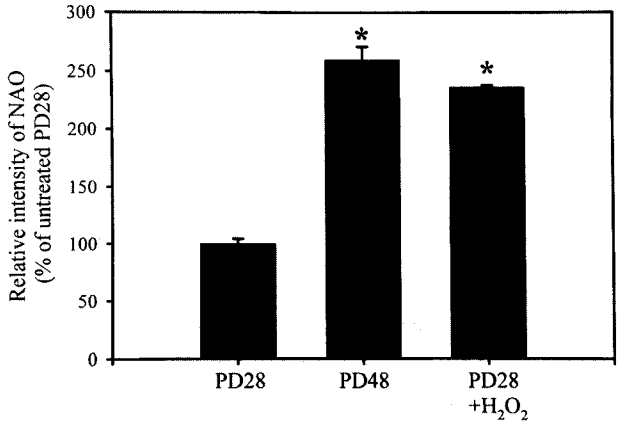


Fig. 1. Increase in mitochondrial mass in MRC-5 cells in the replicative senescent stage or upon treatment with H_2O_2. MRC-5 cells grown by serial passage until the replicative senescent stage (48 PDs; PD48) or MRC-5 cells at 28 PDs (PD28) were treated with 180 μM H_2O_2 for 48 h. The relative intensity of NAO in the cells was analyzed by flow cytometry. It is expressed graphically as a percentage of the fluorescence intensity of the untreated control cells at the stage of 28 PDs. Values are means ± SD of results from three independent experiments. Statistical analysis showed that the differences in the NAO intensity between the untreated cells and H_2O_2-treated or senescent cells were statistically significant (* $p < 0.05$ for treated vs. untreated cells at 28 PDs).

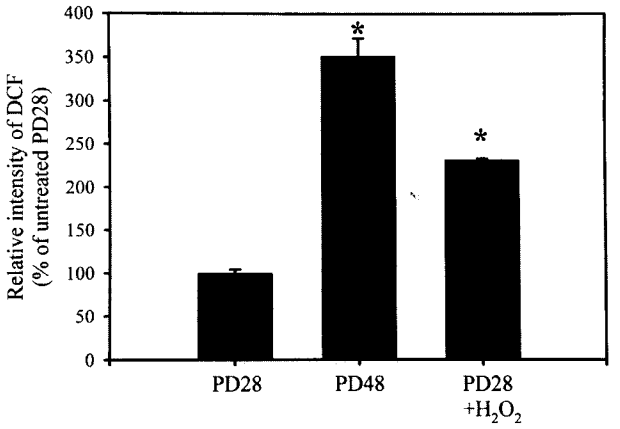


Fig. 2. Increase in ROS production in MRC-5 cells in the replicative senescent stage or upon treatment with H_2O_2. MRC-5 cells grown by serial passage until the replicative senescent stage (48 PDs; PD48) or MRC-5 cells at 28 PDs (PD28) were treated with 180 μM H_2O_2 for 48 h. The production of ROS in the cells, monitored by the DCF fluorescence of the cells, was analyzed by flow cytometry. It is expressed graphically as a percentage of the fluorescence intensity of the untreated cells at 28 PDs. Values are means ± SD of results from three independent experiments in triplicate. Statistical analysis showed that the differences in the DCF intensity between the untreated cells and H_2O_2-treated or senescent cells were statistically significant (* $p < 0.05$ vs. untreated cells at 28 PDs).

180 μM H_2O_2 for 48 h, the mitochondrial mass of the cells at 28 PDs was also significantly increased. These results suggest that the increase in mitochondrial mass in replicative senescent cells is similar to that induced by low oxidative stress in cells in an earlier proliferative stage.

To understand whether there was an alteration of the oxidative stress level in the replicative senescent cells, we used DCFH-DA to monitor the intracellular ROS levels. The relative DCF intensity of cells after 48 PDs was found to be 3.5 times that of cells at 28 PDs ($p < 0.05$) (fig. 2). Moreover, after treatment with 180 μM H_2O_2 for 48 h, the relative DCF intensity in the cells at 28 PDs was also significantly higher than that of the untreated cells. These results clearly indicate that there was a higher level of ROS in both the replicative senescent cells and the H_2O_2-treated cells at an early proliferation stage. In addition, the extent of the increase in ROS production in the replicative senescent cells (3.5-fold) was higher than that in the H_2O_2-treated cells (2-fold). These observations are in agreement with our central idea that increased ROS production in the human cell during replicative senescence results in an increase in mitochondrial mass, which in turn causes an even higher level of ROS production in the senescent cells.

In order to understand the mechanism by which oxidative stress induced by low concentrations of H_2O_2 leads to an increase in the mitochondrial mass of the cells, we further determined the alterations in the mRNA transcripts of the genes that are involved in mitochondrial biogenesis by using the reverse transcription-PCR (RT-PCR) technique. We found that the mRNA levels of nuclear respiratory factor (NRF)-1 and peroxisome proliferator-activated receptor-γ coactivator-1 (PGC-1) were increased in the replicative senescent cells (fig. 3A). Moreover, the mRNA levels of NRF-1, *c-fos* and *c-jun* were increased in the cells after treatment with 180 μM H_2O_2 for 30 min (fig. 3B, C). These results indicate that the mRNA expression levels of the genes involved in mitochondrial biogenesis are induced in replicative senescent cells and in cells exposed to mild oxidative stress.

To further investigate the molecular mechanisms by which the mitochondrial mass is increased in MRC-5 cells under low levels of oxidative stress, we treated cells in the early proliferation stage with low concentrations of H_2O_2

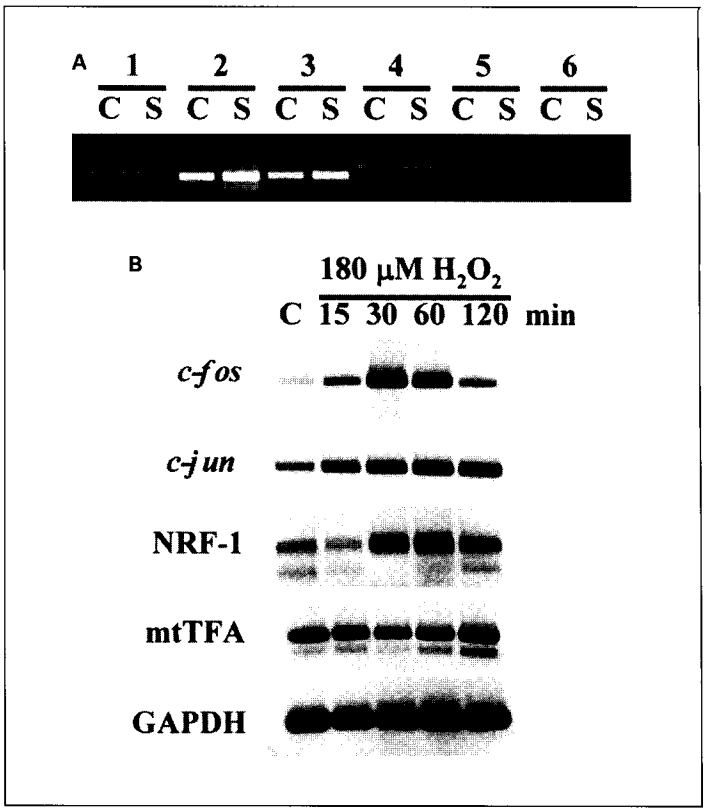
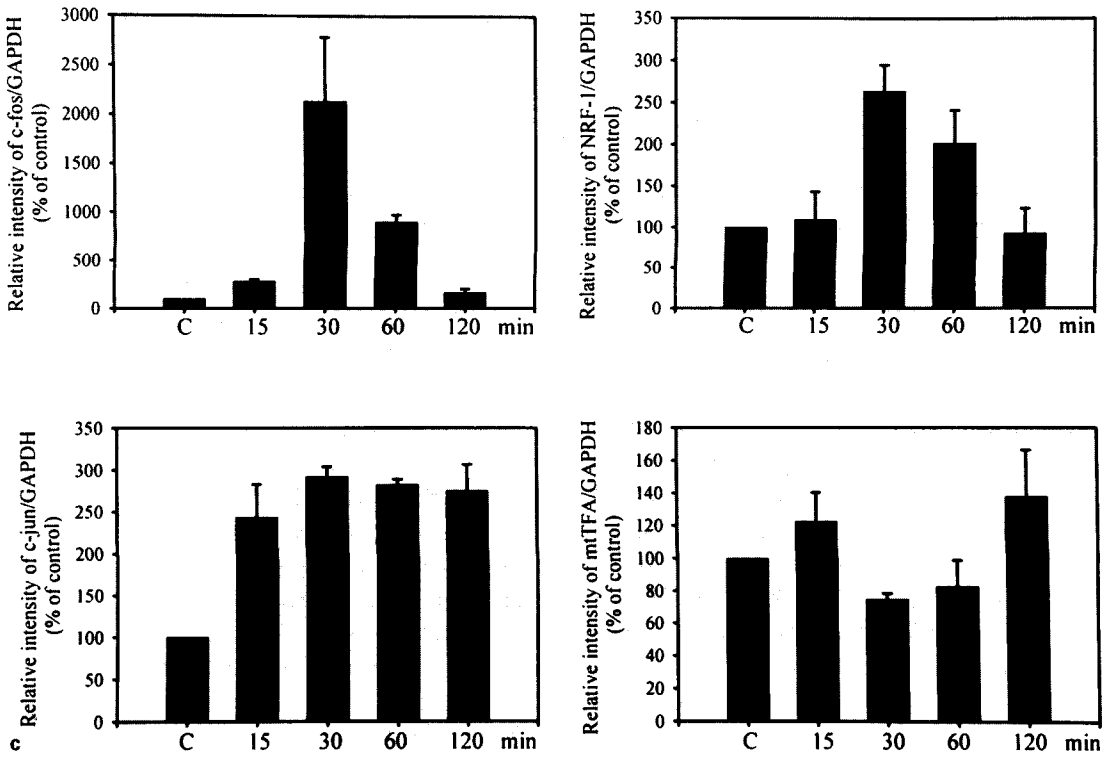


Fig. 3 Increased expression of nuclear genes involved in mitochondrial biogenesis in MRC-5 cells in the replicative senescent stage or upon treatment with H_2O_2. **A** MRC-5 cells were serially subcultured until the replicative senescent stage (48 PDs). The mRNA levels of (1) GAPDH, (2) PGC-1, (3) NRF-1, (4) NRF-2α, (5) NRF-2βγ and (6) mtTFA in senescent cells (48 PDs; S) and in cells at an earlier passage (28 PDs; C) were analyzed by RT-PCR as described in Materials and Methods. **B** MRC-5 cells at 28 PDs were treated with 180 μ*M* H_2O_2. After treatment for 15, 30, 60 or 120 min, the mRNA levels of *c-fos*, *c-jun*, NRF-1, mtTFA and GAPDH were analyzed by the RT-PCR method as described in Materials and Methods. **C** The band intensities were analyzed by scanning densitometry, and the mRNA level of the target gene was corrected for variation in sample loading by normalization with the band intensity of the GAPDH gene. It is expressed graphically as a percentage of the intensity of the target gene in the untreated cells. Values are means ± SD of the results from three independent experiments.



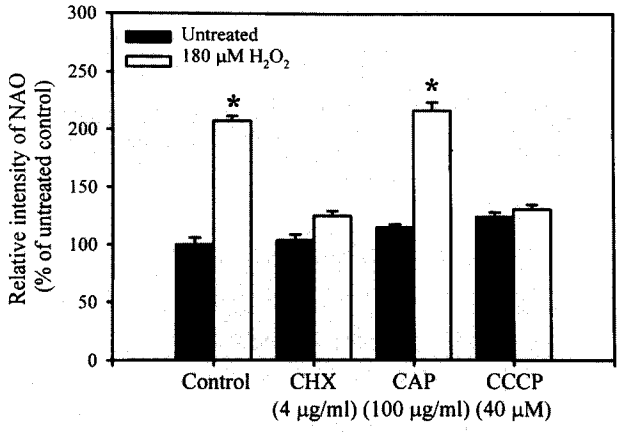


Fig. 4. Cycloheximide and CCCP blocked the increase in mitochondrial mass in MRC-5 cells treated with H_2O_2. After pretreatment with 4 μg/ml cycloheximide (CHX), 100 μg/ml chloramphenicol (CAP) or 40 μ*M* CCCP, MRC-5 cells were cotreated with 180 μ*M* H_2O_2. After 48 h of treatment, the mitochondrial mass of the cells was analyzed by flow cytometry after NAO staining. It is expressed graphically as a percentage of the fluorescence intensity of the control cells. Values are means ± SD of results from three independent experiments in triplicate. Statistical analysis showed that the differences in the NAO intensity between the untreated and H_2O_2-treated cells were statistically significant (* $p < 0.05$ vs. control).

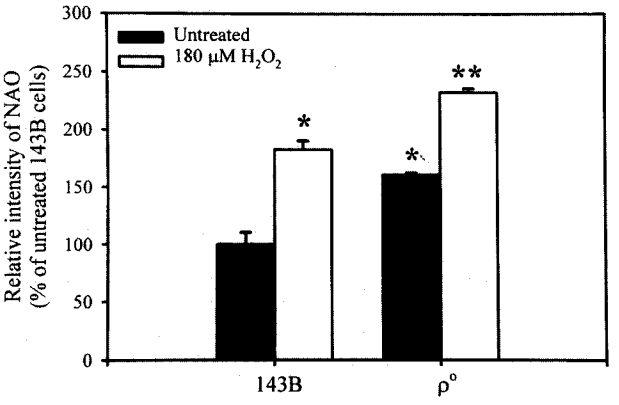


Fig. 5. Increase in mitochondrial mass in mtDNA-less (ρ°) cells treated with H_2O_2. ρ° and 143B cells were grown in DMEM supplemented with 5% FBS, 100 μg/ml pyruvate and 50 μg/ml uridine. Cells were treated with 180 μ*M* H_2O_2 for 48 h. The relative NAO intensity of the treated cells was analyzed by flow cytometry. It is expressed graphically as a percentage of the NAO fluorescence intensity of the untreated 143B cells. Values are means ± SD of results from three independent experiments. Statistical analysis showed that the differences in the NAO intensity between the untreated and H_2O_2-treated cells were statistically significant (* $p < 0.05$ for treated vs. untreated 143B cells; ** $p < 0.05$ for treated vs. untreated ρ^0 cells).

and cycloheximide, a cytosolic protein synthesis inhibitor. In addition, the mitochondrial protein synthesis inhibitor chloramphenicol and the uncoupler of mitochondrial respiration *m*-chlorocarbonyl cyanide phenylhydrazone (CCCP) were also used to treat MRC-5 cells under oxidative stress. We found that the increase in mitochondrial mass of the cells induced by 180 μ*M* H_2O_2 was inhibited by 4 μ*M* cycloheximide, but not by 100 μg/ml chloramphenicol (fig. 4). The results indicate that de novo protein synthesis in the extramitochondrial compartment is involved in the increase in mitochondrial mass in cells under low levels of oxidative stress. Moreover, after treatment with 180 μ*M* H_2O_2 together with 40 μ*M* CCCP for 48 h, we observed that the increase in the mitochondrial mass of the cells under low levels of oxidative stress was repressed (fig. 4). This finding indicates that the increase in the mitochondrial mass of cells under low levels of oxidative stress is dependent on the mitochondrial inner membrane potential.

Furthermore, we also determined changes in the mitochondrial mass of mtDNA-less ρ° cells under oxidative stress. Interestingly, we found that the mitochondrial mass of ρ° cells was higher than that of the parental 143B cells (fig. 5). In addition, after treatment with 180 μ*M* H_2O_2 for 48 h, the mitochondrial mass in the ρ° cells was significantly increased (fig. 5). These results suggest that the increase in the mitochondrial mass of the MRC-5 cells under mild oxidative stress is not dependent on the presence or function of mtDNA.

Discussion

Human mitochondria contain multiple circular copies of their own DNA molecules, which encode 2 rRNAs, 22 tRNAs and 13 polypeptides [2]. Most of the mitochondrial proteins, however, are nuclear gene products. An efficient collaboration between nuclear DNA and mtDNA is essential for the normal functioning of mitochondria [21, 26]. In the past decade, a number of factors have been identified to be involved in the replication of the mitochondrial genome [26]. However, the mechanisms involved in the regulation of mitochondrial biogenesis are still unknown, particularly during the aging process.

In this study, we have shown that the mitochondrial mass of a human lung fibroblast cell line, MRC-5, is increased in the replicative senescence stage (fig. 1). This finding is consistent with our previous observation that the copy number of mtDNA per cell is increased in the lung tissues of elderly human subjects [12]. Interestingly, it has also been demonstrated that fibroblasts from elderly donors displayed significantly higher copy numbers of mtDNA per cell compared with cells from young donors [29]. Moreover, the average number of mtDNA genomes per cell was reported to increase at late passages of various diploid human cells [29]. These observations suggest that both the mitochondrial mass and the mtDNA copy number in fibroblasts are increased in the aging process in vivo and during replicative senescence in vitro.

In the present study, we found that the intracellular level of ROS is higher in human cells at late proliferation passages (fig. 2). The increase in ROS production is thought to play a critical role in the increase in mitochondrial mass and mtDNA molecules in tissue cells during the aging process. In aging tissues, H_2O_2 generated by defective mitochondria might act as a secondary messenger that reaches the nucleus and activates mitochondrial biogenesis. This notion was supported by our previous finding [14] that at nonlethal concentrations, H_2O_2 caused an increase in the mitochondrial mass and the mtDNA copy number of human cells.

We also demonstrated in the present study that mRNA transcripts of nuclear genes involved in mitochondrial biogenesis, especially NRF-1, were induced by H_2O_2, and that a similar increase occurred in replicative senescent cells (fig. 3). Increased expression of mitochondrial transcription factor A (mtTFA) and NRF-1 has also been demonstrated in human cells with impaired mitochondrial electron transfer [31] and in the skeletal muscle of elderly human subjects [15]. It is established that control of mitochondrial biogenesis requires the coordinated expression of hundreds of genes, including structural and regulatory genes [18]. PGC-1 and NRF-1 and NRF-2 have been demonstrated as transcriptional regulators for genes coding for subunits of the oxidative phosphorylation system and for many other genes involved in mtDNA replication [26]. PGC-1 and NRFs are thought to be involved in the control of mitochondrial biogenesis and act as a link between external stimuli and modulation of the mtDNA copy number and mitochondrial mass in animal and human cells [18]. Moreover, H_2O_2 has been proposed to play a role as a secondary messenger in signal transduction pathways of some types of mammalian cells [11, 27]. The increase in intracellular ROS and enhanced oxidative stress stimulate expression of early growth-related genes such as *c-fos* and *c-jun* [7]. Our findings in this study and those of another report [31] clearly indicate that mild oxidative stress leads to increased expression of PGC-1 and NRFs and thereby increases mitochondrial biogenesis. Taken together, these results suggest that an age-dependent increase in ROS production is one of the factors involved in the mechanism leading to the increase in mitochondrial mass and mtDNA copy number in aging tissue cells (fig. 6).

On the other hand, the increase in ROS production in replicative senescent cells might result from increased proliferation of mitochondria. After treatment with sublethal concentrations of H_2O_2 for 48 h, the cells were found to contain higher levels of ROS and have more mitochondria (fig. 2). Since ROS and free radicals, including ubisemiquinone and flavosemiquinone, are continually generated and maintained at a relatively high steady-state level in mitochondria under normal physiological conditions [8, 36], mitochondria are exposed to certain levels of oxidative stress in cells. Moreover, it has also been demonstrated that the rate of production of superoxide anions and H_2O_2 in mitochondria increases with age in mammalian tissues [23, 30]. Recently, H_2O_2 was proposed to be involved in the communication between mitochondria and the nucleus [21]. These observations suggest that the age-dependent increase in the production of superoxide anions and H_2O_2 from defective mitochondria is involved in the increase in mitochondria and mtDNA copy number in aging tissues (fig. 6). Increased ROS production might further cause oxidative damage to cellular constituents, including DNA, RNA, proteins and lipids [34, 35], thereby driving the cell to enter a senescent stage [5, 9].

On the other hand, we further showed that cycloheximide inhibited protein synthesis in the cytosol and blocked the increase in mitochondrial mass in response to mild oxidative stress (fig. 4). Moreover, a mitochondrial uncoupler, CCCP, was shown to inhibit the increase in mitochondrial mass in response to oxidative stress (fig. 4). These results indicate that both de novo biosynthesis of proteins encoded by the nuclear genes and the mitochondrial membrane potential-dependent protein import systems are involved in the mechanism of the increase in mitochondrial mass in response to oxidative stress (fig. 6). However, we found that chloramphenicol, which inhibits protein synthesis in mitochondria, has no effect on the increase in mitochondrial mass in response to oxidative stress (fig. 4). This indicates that the protein expression in the mitochondrial matrix is unlikely to be a major determinant of the increase in mitochondrial mass in response

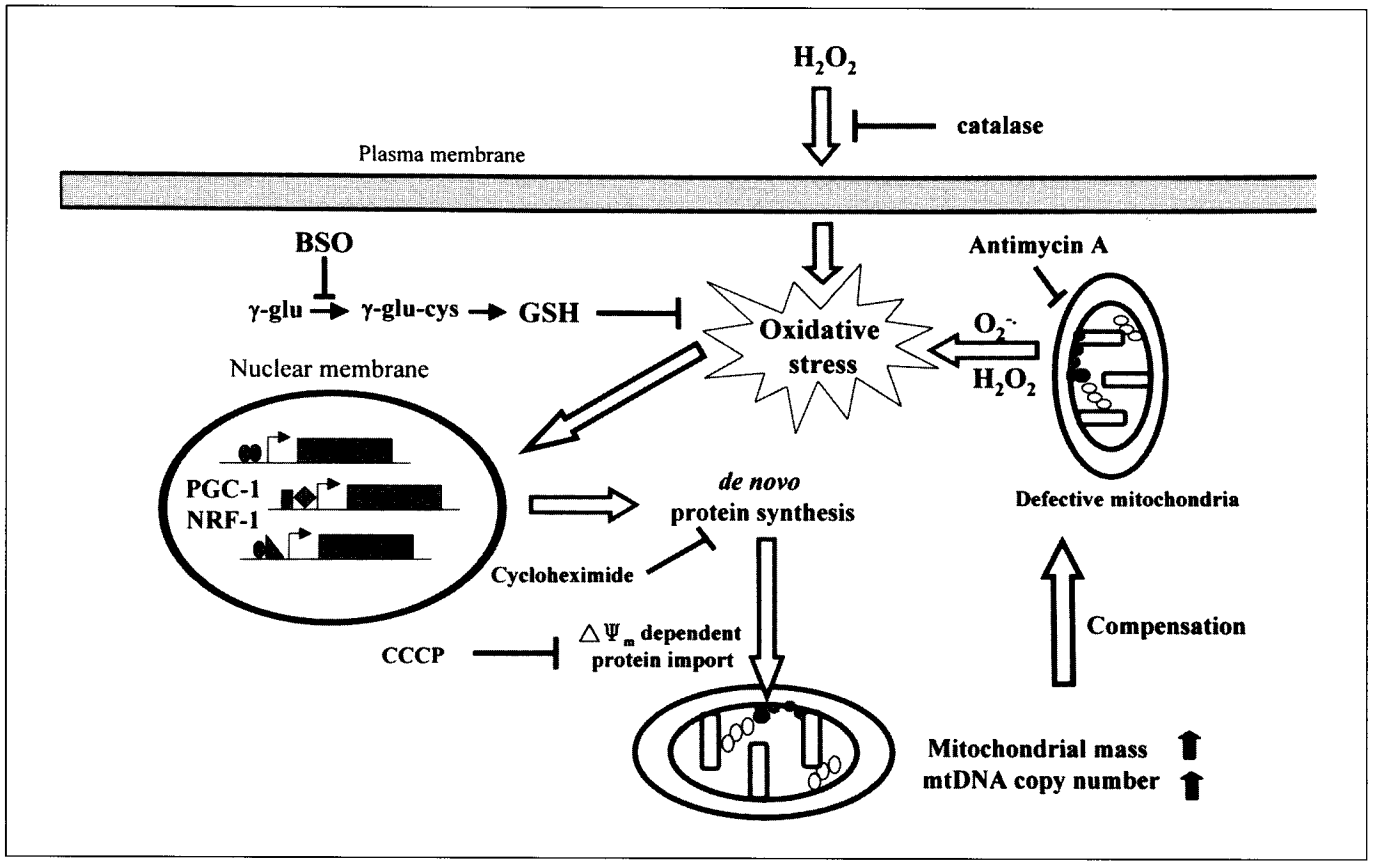


Fig. 6. The possible mechanisms of the increase in mitochondrial mass and mtDNA in human cells under oxidative stress and during replicative cell senescence. Based on our observations in this study and previous findings [14, 35], we suggest that the age-dependent increase in the production of superoxide anions and H_2O_2 from defective mitochondria and environmental oxidative insults leads to activation of ROS-sensitive factors. The increased expression of these nuclear genes (e.g. PGC-1 and NRF-1) is involved in mitochondrial biogenesis. This in turn initiates the coordinated expression of regulatory and structural genes, de novo protein synthesis and mitochondrial membrane potential-dependent import of proteins into mitochondria. This may explain why the increase in mitochondrial mass and mtDNA copy number compensates for the decline in mitochondrial respiratory function during the aging process and replicative cell senescence. The oxidative stress induced by reduction of the intracellular glutathione (GSH) level with buthionine sulfoximine (BSO) treatment or by the inhibition of mitochondrial respiration with antimycin A treatment was found to increase the number of mitochondria in treated cells, but catalase was found to block the increase in mitochondria induced by treatment of cells with sublethal concentrations of H_2O_2 [14]. $\Delta\Psi_m$ = Mitochondrial membrane potential.

to oxidative stress. To further confirm this observation, we tested the effects of oxidative stress on mtDNA-less ρ^o cells. As with MRC-5 cells, the mitochondrial mass of ρ^o cells was increased in response to oxidative stress (fig. 5). Clinically, overproliferation of mitochondria and an enhanced synthesis of mitochondrial enzymes are often manifested as so-called 'ragged-red fibers' in the affected tissues of patients with mitochondrial myopathies and even in the tissues of patients with mtDNA depletion [33]. This increase in mitochondrial biogenesis in the tissues of patients with mtDNA depletion is not accompanied by an increase in mtDNA copy number. Therefore, these results together suggest that the proteins encoded by the nuclear genes are the major determinant of the increase in mitochondrial mass in human cells in response to oxidative stress.

Based on our observations in this study and previous findings [14, 35], we suggest that the age-dependent increase in the production of superoxide anions and H_2O_2 from defective mitochondria and environmental oxidative insults lead to increased expression and activation of some nuclear genes that are involved in mitochondrial biogenesis. This will in turn initiate the coordinated expression of regulatory and structural genes, de novo

protein synthesis and mitochondrial membrane potential-dependent import of proteins into mitochondria, thereby causing an increase in mitochondrial mass and mtDNA copy number (fig. 6).

We believe that the increase in mitochondria and mtDNA molecules in response to oxidative stress could supply the affected cells with more energy to cope with endogenous and exogenous stresses, in order to compensate for the decline in mitochondrial respiratory function during the aging process and replicative senescence of the cells. However, at the same time, more ROS would also be generated from the increased number of mitochondria in the affected cells. This will then cause much more oxidative damage to mitochondria and other intracellular constituents, including DNA, RNA, proteins and lipids, and consequently drive the cell to enter a senescent stage or cell death. Therefore, this kind of oxidative stress response plays two roles in the affected cells. On the one hand, it stimulates mitochondrial proliferation to supply energy to meet the need for cell survival, including repairing DNA damage and synthesizing new proteins. On the other hand, it causes excess ROS production and severe oxidative damage, thereby initiating the cascade leading to cell aging or cell death. The outcome of the events leading to the increase in mitochondrial mass and mtDNA copy number is dependent on the level of oxidative stress, the capacity of the intracellular antioxidant scavenger system and the quality of mitochondria and mtDNA. When both the intracellular capacity of the antioxidant scavenger system and the quality of the parental mitochondria and mtDNA are high, the mechanism in response to mild oxidative stress will lead to the proliferation of mitochondria and mtDNA. This can be seen in the increased energy supply from the increase in mitochondria induced by regular exercise training of skeletal muscle [38, 39]. On the other hand, when the capacity of the antioxidant scavenger system is compromised, defective mitochondria and mutated mtDNA are accumulated in tissue cells. The response to higher oxidative stress in this case leads to an increase in defective mitochondria and mutated mtDNA, and thus increases ROS production and causes severe oxidative damage. Such a 'vicious cycle' leads to ever-increasing deterioration of cellular functions with time, which may well explain the increase in mitochondrial mass and mtDNA in the affected tissues of elderly subjects or patients with chronic diseases such as myopathy and cardiac arrhythmia.

In summary, we have demonstrated that mitochondrial mass is increased in replicative senescent cells in response to increased intracellular ROS production or enhanced environmental oxidative stress. Moreover, the increase in the mitochondrial mass induced by oxidative stress is mainly dependent on de novo synthesis of nuclear DNA-encoded proteins and their import into mitochondria. Based on these observations, we suggest that the increase in mitochondrial mass and ROS production in tissue cells is an important early molecular event accompanying the aging process in humans and animals.

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