Oxidative Stress–Induced Depolymerization of Microtubules and Alteration of Mitochondrial Mass in Human Cells

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ABSTRACT: Mitochondrial biogenesis is a biological process that has been intensively studied over the past few years. However, the detailed molecular mechanism underlying this increase in mitochondria remains unclear. To investigate the mechanism of such a mitochondrial proliferation, we examined alterations in mitochondria of human osteosarcoma 143B cells that had been treated with 100 to 500 μM hydrogen peroxide (H_2O_2) for 48 h. The results showed that mitochondrial mass of the cell was increased with the increase of the concentration of H_2O_2. On the other hand, by using real-time PCR techniques, we observed the changes of mitochondrial DNA (mtDNA) content in the cells exposed to oxidative stress. The copy number of mtDNA was increased by treatment with a low dose of H_2O_2 but was drastically decreased after treatment with H_2O_2 higher than 300 μM. Transmission electron microscopic images revealed that mitochondria were abnormally proliferated in cells exposed to oxidative stress. Moreover, we found that the percentage of 143B cells arrested at the G_2/M phase increased upon treatment with H_2O_2. Immunostaining and microtubule fractionation assay revealed that microtubules were depolymerized in the cells that had been treated with H_2O_2. To understand the effect of microtubules depolymerization on the mitochondrial mass, we treated the cells with several kinds of microtubule-active drugs, which arrest cultured cells at the G_2/M phase. The results showed that mitochondrial mass and mtDNA copy number all were increased after such treatments. Taking these findings together, we suggest that oxidative stress–induced microtubule derangement is one of the molecular events involved in the increase of mitochondrial mass upon treatment of human cells with H_2O_2.

KEYWORDS: oxidative stress; microtubule; mitochondrial mass; osteosarcoma

INTRODUCTION

It has been well documented that abnormal proliferation of mitochondria occurs frequently in affected tissues of patients with mitochondrial myopathy and muscle of aged individuals. This is one of the most prominent clinical hallmarks in mito-

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chondrial diseases such as chronic progressive external ophthalmoplegia (CPEO) and myoclonic epilepsy and ragged-red fiber (MERRF) syndromes. Morphologic features of giant mitochondria were observed in nerve and muscle tissues of elderly subjects and patients with mitochondrial disorders.[1,2] Moreover, giant mitochondria also appeared in mtDNA-depleted ρ^0 cells,[3] and mtDNA depletion caused a reduction in the amount of mitochondrial inner membranes.[4] Therefore, structure and distribution of mitochondria are closely related to the oxidative status of mammalian cells.

Mitochondrial biogenesis has been a subject of intensive study in the past few years. Many factors, such as oxidative stress, deficiency in ATP production, cell cycle stage, and even the structure of microtubules, have been shown to affect the mitochondrial mass of mammalian cells.[5–8] Mitochondria proliferate independently throughout the cell cycle and are inherited by daughter cells upon cell division. They actively move along cytoskeletal tracks and frequently change their shape and size through fission and fusion of the organelles.

Microtubules seem to be the major components of cytoskeletal systems that are involved in the regulation of the distribution of mitochondria in the cells. It has been reported that microtubules in interphase are involved in mitochondrial biogenesis aside from participation in the regulation of mitochondrial distribution in mammalian cells.

In this study, we treated human osteosarcoma 143B cells with sublethal doses of H_2O_2 to increase reactive oxygen species (ROS) in the cells, and we investigated the role that microtubules may play in oxidative stress–induced alteration of mitochondrial mass.

MATERIALS AND METHODS

Cell Culture

Human osteosarcoma 143B TK^- cells were grown at 37ºC in a humidified atmosphere with 5% CO_2/ 95% air in Dulbecco's modified Eagle's medium (Life Technologies, Grand Island, NY) containing 100 µg/mL pyruvate and 50 µg/mL uridine and supplemented with 5% fetal bovine serum.

Determination of Mitochondrial Mass

The fluorescent dye 10-*n*-nonyl-acridine orange (NAO; Molecular Probes, Eugene, OR), which binds specifically to cardiolipin at the inner mitochondrial membrane independently of membrane potential ($\Delta\Psi_m$), was used to monitor the mitochondrial mass.[9] Cells at subconfluent stage were trypsinized and resuspended in 0.5 mL of PBS containing 2.5 µM of NAO. After incubation for 10 min at 25ºC in the dark, cells were immediately transferred to a tube for analysis with an EPICS XL-MCL flow cytometer (Beckman-Coulter, Miami, FL).

Determination of the Relative Content of mtDNA

A LightCycler-FastStar DNA Master SYBR Green I kit (Roche Diagnostics, Mannheim, Germany) was used to perform quantitative PCR on the LightCycler

PCR machine (Roche Diagnostics). The relative content of mtDNA was determined by amplification of a DNA fragment each of the ND1 gene and β-actin gene (as internal standard) with specific primer pairs (5′-GGAGTAATCCAGGTCGGT-3′ and 5′-TGGGTACAATGAGGAGTAGG-3′; 5′-CATGTGCAAGGCCGGCTTC-3′ and 5′-CTGGGTCATCTTCTCGCGGT-3′), respectively. PCR was usually performed under the following conditions: initial 6-min denaturation at 95°C followed by 31 cycles of 5 s at 95°C, 10 s at 62°C, and 20 s at 72°C. The relative content of mtDNA in the cell was then calculated by the RelQuant software.

Transmission Electron Microscope

Human osteosarcoma 143B TK^- cells were fixed for 2 h in 2% paraformaldehyde and 2.5% glutaraldehyde in 0.2 M cacodylate buffer, washed in 0.1 M cacodylate buffer containing 0.2 M sucrose three times, and postfixed for 2 h in 1% osmium tetroxide. Dehydration was achieved by 35, 50, 75, 95, and 100% ethanol, respectively. Samples then were infiltrated in a mixture of ethanol and spurr (Electron Microscopy Sciences, Fort Washington, PA) and were embedded in spurr. Ultrathin sections were cut on a Leica AG ultramicrotome, placed on 200-mesh copper grids, stained with uranyl acetate and lead citrate, and photographed on a Hitachi T-600 electron microscope.

Cell Cycle Analysis

Cells were trypsinized and fixed with 70% ethanol. After washing twice with PBS buffer, cells were resuspended in 1 mL of cell cycle assay buffer (1% Triton X-100, 0.1 mg/mL RNase A and 4 μg/mL propidium iodide in PBS buffer) and further incubated for 30 min. Samples were stored in the dark at 4°C until cell cycle analysis, which was performed with a flow cytometer (Beckman-Coulter, Miami, FL).

Microtubule Fractionation and Quantitative Immunoblotting

Polymerized and monomeric fractions of tubulin were isolated by the method described by Banan *et al.*[10] Cells were pelleted and subsequently resuspended in microtubule stabilization buffer containing 0.1 M PIPES, pH 6.9, 30% glycerol, 5% dimethyl sulfoxide, 1 mM $MgSO_4$, 10 μg/mL anti-protease cocktail (Roche Diagnostics, Mannheim, Germany), 1 mM EGTA, and 1% Triton X-100 at room temperature for 20 min. Cell lysate was centrifuged at $105{,}000 \times g$ for 45 min at 4°C. The supernatant containing the soluble monomeric tubulin was gently removed. The polymerized tubulin fraction then was resuspended in calcium-containing microtubule depolymerization buffer containing 0.1 M PIPES, pH 6.9, 1 mM $MgSO_4$, 10 μg/mL anti-protease cocktail (Roche Diagnostics), and 10 mM $CaCl_2$, at 4°C for 1 h. Subsequently, the samples were centrifuged at $48{,}000 \times g$ for 15 min at 4°C, and the supernatant was removed. The remaining pellet was treated with the calcium-containing microtubule depolymerization buffer twice by resuspension and centrifugation. Polymerized and monomeric fractions of tubulin were recovered by incubating with 10 μM Taxol and 1 mM GTP at 37°C for 1 h to promote polymerization. Microtubules were recovered by centrifugation and resuspended in the microtubule stabilization buffer. An aliquot of 1 μg of protein was subjected to electrophoresis on a 10% polyacrylamide gel and blotted onto a piece of Hybond-P^+ membrane

TABLE 1. Cell biological features of 143B cells after treatment with H_2O_2 for 48 h

H_2O_2 (μM)	Viability of the cells (%) ($N = 3$)	Depolarization of mitochondrial membrane	Relative amount of mitochondrial mass (%) ($N = 5$)	Relative amount of mitochondrial DNA (%) ($N = 4$)	Percentage of the cells at the indicated cell cycle (%)		
					G_0/G_1	S	G_2/M
0	89.1 ± 1.8	8.1 ± 3.2	100.0 ± 2.9	100.0 ± 4.3	55.0 ± 3.7	20.3 ± 2.2	24.5 ± 5.2
100	89.2 ± 1.5	3.3 ± 1.7	131.7 ± 7.3**	138.3 ± 20.5*	38.5 ± 13.0	19.2 ± 1.7	48.1 ± 2.5**
200	87.3 ± 0.9	6.7 ± 2.8	140.9 ± 7.5**	175.9 ± 33.5**	31.5 ± 2.6**	21.0 ± 1.8	45.5 ± 2.8**
300	86.6 ± 2.4	11.4 ± 6.0	144.1 ± 7.2**	136.7 ± 17.4*	31.6 ± 2.8**	20.1 ± 4.5	45.0 ± 5.6**
400	85.3 ± 2.8	11.7 ± 2.0	154.9 ± 10.9**	86.2 ± 13.0	17.9 ± 6.5**	14.9 ± 3.6	65.6 ± 8.8**
500	81.8 ± 3.9*	9.2 ± 4.5	145.8 ± 14.0**	30.4 ± 7.3**	9.2 ± 3.2**	19.1 ± 3.8	70.5 ± 2.5**

*$P < 0.05$ vs. control; **$P < 0.01$ vs. control.

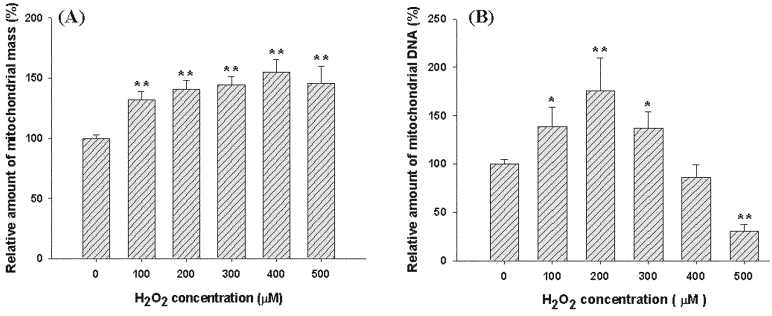

FIGURE 1. Hydrogen peroxide–induced changes of mitochondrial mass and mtDNA content in 143B cells. (**A**) The relative NAO intensity of 143B cells increased after H_2O_2 treatment for 48 h ($n = 5$; $^{**}P < 0.01$ vs. control). (**B**) Relative content of mtDNA was determined by Q-PCR after the cells had been treated with H_2O_2 for 48 h. The results showed that mtDNA content increased in 143B cells that had been treated with 100–300 μM H_2O_2, but decreased in the cells that had been treated with 500 μM H_2O_2 ($n = 4$; $^{*}P < 0.05$ vs. control; $^{**}P < 0.01$ vs. control).

(Amersham Biosciences, Uppsala, Sweden). Western blotting was performed at room temperature with anti–α-tubulin antibody as described previously.[11] To quantify the relative levels of tubulin, we measured the optical density of the bands with a laser densitometer.

RESULTS

We first demonstrated that mitochondrial mass was increased in 143B cells that had been treated with 100 to 500 μM of H_2O_2 for 48 h (FIG. 1A). After treatment with 100–300 μM H_2O_2 , the copy number of mtDNA was increased in 143B cells but decreased in the cells that had been treated with 500 μM H_2O_2 (FIG. 1B). This dual-phase response to oxidative stress was not caused by the possibility that cells were undergoing apoptosis or necrosis (TABLE 1). Moreover, we showed that H_2O_2 treatment may alter the morphology of mitochondria; mitochondria were enlarged and their cristae were disorganized after treatment with varying concentrations of H_2O_2 for 48 h (FIG. 2). Moreover, H_2O_2 treatment cause cell cycle arrest at G_2/M (FIG. 3) and disruption of microtubules in 143B cells (FIG. 4). Quantitative immunoblotting demonstrated that polymerized tubulin was decreased by approximately 30% after 400 μM H_2O_2 treatment; there was no significant decrease in cells treated with 200 μM H_2O_2. On the other hand, the monomeric tubulin was increased by approximately 15% and 25% after treatment with 200 and 400 μM H_2O_2 , respectively. These results suggest that H_2O_2 induced depolymerization of microtubules and resulted in the alteration of mitochondrial mass in human cells. Furthermore, after treatment of 143B cells with Taxol, colchicine, and nocodazole, we found that mitochondrial mass and mtDNA copy number of the cells were all increased (FIG. 5A and B). This finding was consistent with our contention that H_2O_2 may disrupt the organization of microtubules and thus alter the mitochondrial mass in human cells.

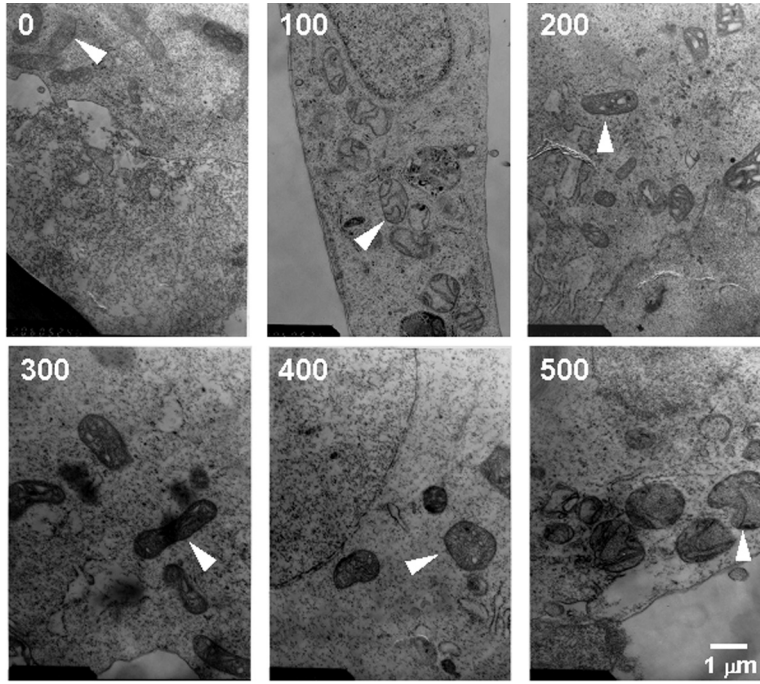

FIGURE 2. The ultrastructure of mitochondria in 143B cells treated with H_2O_2. Under examination by TEM, we found that mitochondria were enlarged and their cristae were disorganized after treatment of 143B cells with varying concentrations (0–500 μM) of H_2O_2 for 48 h. *White arrowheads* indicate the single mitochondrion inside the cell.

DISCUSSION

Human cells regulate their energy production by mitochondrial oxidative phosphorylation (OXPHOS) according to their needs. Depending on the challenge, mitochondria respond to the energy demands by subtle change in the activity of respiratory enzymes, by changing the expression of constituent enzyme subunits, or by increasing the number and size of the organelles. It is known that mitochondrial proliferation occurs in skeletal muscles in response to increased contractile activity, in adipose tissues in response to adaptive thermogenesis, and in cardiac myocytes in response to electrical stimulation or hypothermia. In clinical settings, the proliferation of abnormal mitochondria is generally observed in skeletal muscles of patients with a defective respiratory chain of mitochondria caused by mutation or depletion of mtDNA. It is thought that ROS, generated continuously by the respiratory chain, might play a role in mitochondrial biogenesis. However, solid evidence in support of this hypothesis has not yet been available.

The coordination between mitochondrial and cytoplasmic events leading to the growth and division of mammalian cells is largely unknown. It was reported that the mitochondrial mass was closely related to the cell cycle.[8] Our results confirmed the

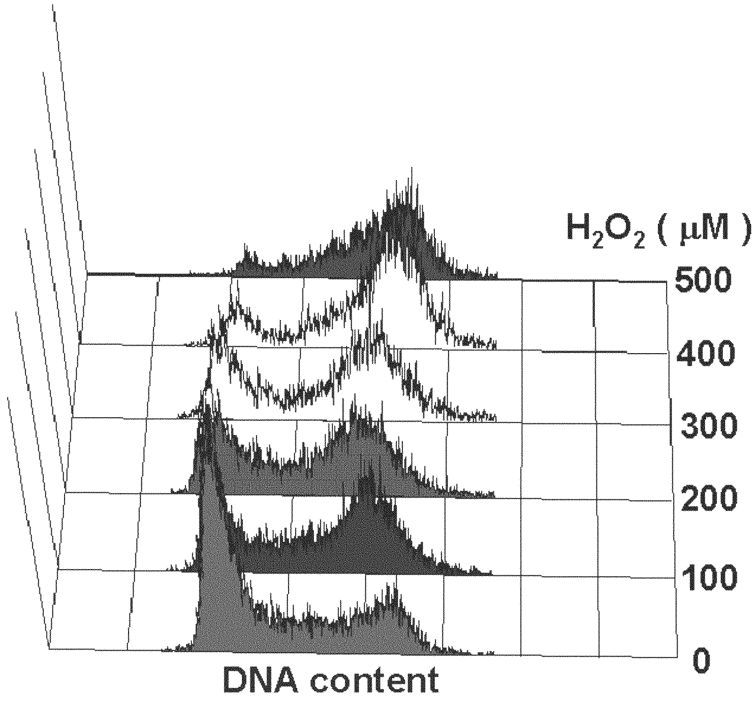

FIGURE 3. Hydrogen peroxide–induced cell cycle arrest at G_2/M in 143B cells. The percentage of 143B cells arrested at G_2/M phase increased with the increase of H_2O_2 concentration. Cells were fixed and stained with propidium iodide. The DNA content of the cell was estimated by use of a flow cytometer.

notion that stress-induced mitochondrial mass increase was correlated to the arrest of the cells at G_2/M phase.

The key elements that regulate cell cycle in mammalian cells are microtubules. Microtubules are the major components of cytoskeletal systems that are responsible for the regulation of the mitochondrial distribution in the cell. Several studies demonstrated that oxidants disassemble the microtubules in human cells.[10] By using immunostaining and microtubule fractionation assay, we showed that microtubules were depolymerized in the cells after H_2O_2 treatment (FIG. 4). This finding suggests that H_2O_2 induces depolymerization of microtubules and results in the alteration of mitochondrial mass in human cells. To substantiate this notion, we treated human osteosarcoma cells and hepatocellular carcinoma cells with microtubule-active drugs, such as Taxol, colchicine, and nocodazole, which stabilize or destabilize microtubules and arrest cultured cells at the G_2/M phase. The results showed that mitochondrial mass and mtDNA copy number of 143B cells were all increased after treatment with these drugs.

The copy number of mtDNA is an important index of mitochondrial biogenesis. Our results showed that mtDNA copy number was increased by treatment with a low concentration of H_2O_2. However, the mtDNA content was drastically decreased after

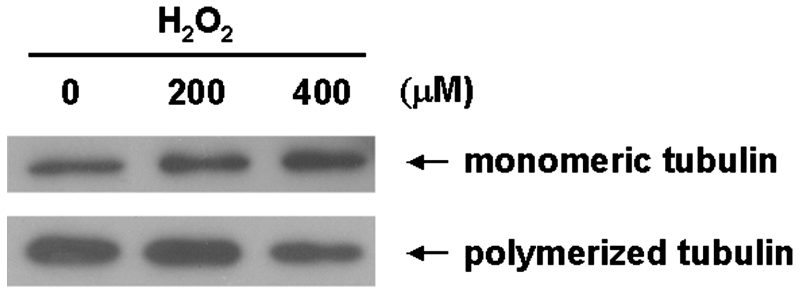

FIGURE 4. Microtubule fractionation and immunoblotting for the assessment of tubulin assembly and disassembly in 143B cells after H_2O_2 treatment. The results showed that the content of monomeric tubulins increased and that of polymerized tubulins was decreased, respectively, in the cells that had been treated with 200 and 400 μM H_2O_2 for 48 h.

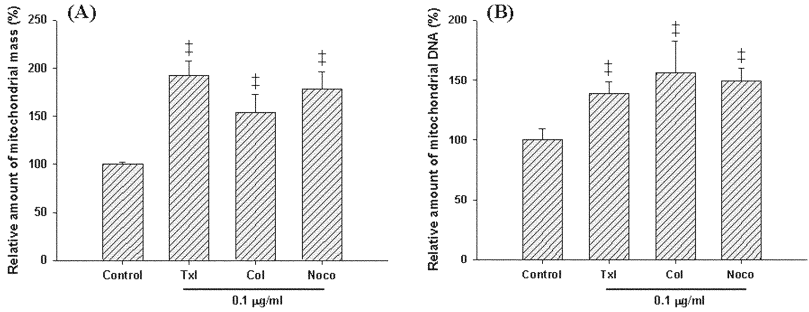

FIGURE 5. Increase of mitochondrial mass and mtDNA content in 143B cells after treatment with microtubule-active drugs. After treatment with 0.1 μg/mL of Taxol, colchicine, and nocodazole, respectively, the 143B cells were arrested at the G_2/M phase. The results showed that mitochondrial mass (**A**) and mtDNA content (**B**) increased after such treatments ($n = 4$; ‡$P < 0.005$ vs. control).

treatment with H_2O_2 at a concentration higher than 300 μM. This phenomenon may be caused by the low fidelity of the mtDNA template with high oxidative damage or caused by low activity of impaired mitochondrial DNA polymerase γ.[12] This dual-phase change of mtDNA copy number is consistent with our previous findings in the cybrids harboring different proportions of mtDNA with a 4,977-bp deletion and in leukocytes from healthy subjects of different ages.[9,13]

It has been reported that Taxol, but not colchicine or nocodazole, promotes the biogenesis of mitochondria in mammalian cells.[5] However, in this study, we demonstrated that the alteration in the stability of microtubules might control the biogenesis of mitochondria and that a H_2O_2-induced mitochondrial mass increase might be a result of the depolymerization of microtubules. It is plausible that H_2O_2 disrupts the organization of microtubules and alters the mitochondrial mass. Further studies are warranted to clarify the mechanism of action of H_2O_2 on the depolymerization of microtubules and on mitochondrial biogenesis in human cells.

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