MITOCHONDRIAL DNA DELETION AND IMPAIRMENT OF MITOCHONDRIAL BIOGENESIS ARE MEDIATED BY REACTIVE OXYGEN SPECIES IN IONIZING RADIATION-INDUCED PREMATURE SENESCENCE

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Mitochondrial DNA (mtDNA) deletion is a well-known marker for oxidative stress and aging, and contributes to harmful effects in cultured cells and animal tissues. mtDNA biogenesis genes (NRF-1, TFAM) are essential for the maintenance of mtDNA, as well as the transcription and replication of mitochondrial genomes. Considering that oxidative stress is known to affect mitochondrial biogenesis, we hypothesized that ionizing radiation (IR)-induced reactive oxygen species (ROS) causes mtDNA deletion by modulating the mitochondrial biogenesis, thereby leading to cellular senescence. Therefore, we examined the effects of IR on ROS levels, cellular senescence, mitochondrial biogenesis, and mtDNA deletion in IMR-90 human lung fibroblast cells. Young IMR-90 cells at population doubling (PD) 39 were irradiated at 4 or 8 Gy. Old cells at PD55, and H2O2-treated young cells at PD 39, were compared as a positive control. The IR increased the intracellular ROS level, senescence-associated β-galactosidase (SA-β-gal) activity, and mtDNA common deletion (4977 bp), and it decreased the mRNA expression of NRF-1 and TFAM in IMR-90 cells. Similar results were also observed in old cells (PD 55) and H_2O_2-treated young cells. To confirm that a increase in ROS level is essential for mtDNA deletion and changes of mitochondrial biogenesis in irradiated cells, the effects of N-acetylcysteine (NAC) were examined. In irradiated and H_2O_2–treated cells, 5 mM NAC significantly attenuated the increases of ROS, mtDNA deletion, and SA-β-gal activity, and recovered from decreased expressions of NRF-1 and TFAM mRNA. These results suggest that ROS is a key cause of IR-induced mtDNA deletion, and the suppression of the mitochondrial biogenesis gene may mediate this process.

Keywords: Reactive oxygen species, Mitochondrial DNA deletion, Mitochondrial biogenesis, Nuclear Respiratory Factor-1, Mitochondrial transcription factor A, Ionizing radiation

1. INTRODUCTION

Many reactive oxygen species (ROS) are formed by several external and endogenous factors. ROS have an affect on lipids, proteins, and DNA, and can cause damage to cells and tissues. The accumulation of damage from oxidative stress leads to a change of biological outcome, including inflammation, cancer, and diabetes [1]. Namely, the immoderate generation of ROS, or a deficiency in ROS scavenging, causes body impairment [2]. Also, a recent study reported that ROS are an important cause of cellular senescence and aging [3]. The immoderate generation of ROS by external stress and the accumulation of generated ROS induce aging by degree.

Human mitochondria consist of 16569 bp double-stranded DNA of a circular form [4]. Since mitochondria lack histone protein, and are involved electron transport complexes that generate ROS, mtDNA is more easily injured than nuclear DNA [5]. Increased ROS from various types of stress can mediate mitochondrial DNA damage and the alteration of mitochondrial gene expression [6,7]. Also, mtDNA common deletion, which is one of the occurring events in aged cells, causes an accumulation of ROS [8,9]. ROS can affect the steady

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mass and DNA copy number of mitochondria needed for maintenance of mitochondrial function in cells. The mass and DNA copy number of mitochondria are regulated through mitochondrial biogenesis, a response that depends on oxidative stress from diverse factors [10, 11]. In mitochondrial biogenesis, mitochondrial transcription factor A (TFAM) and nuclear respiratory factor-1 (NRF-1) are the main regulators. TFAM is an essential nuclear encoded protein for the maintenance of copy number, transcription of several mitochondrial-encoded genes, and replication in mitochondrial genomes [12]. NRF-1 regulates the transcription and expression of TFAM genes and other nuclear genes involved in mitochondrial function [13,14].

IR induces damage to DNA, cells, and organs through oxidative stress, and induces premature senescence in various cell lines [15,16]. It is also well known that IR can induce mtDNA common deletions in cultured cells and in human or animal tissues [17-19]. However, the underlying mechanism of IR-induced mtDNA deletion is unclear. Therefore, we postulated that the impairment of these transcription factors by oxidative stress (ROS) such as IR may induce mitochondrial dysfunction through mtDNA mutations.

The aim of this study was to determine whether an increase of ROS level in cellular senescence induced by IR could mediate mtDNA deletion via impairment of mitochondria biogenesis in IMR-90 human lung fibroblast cells. Our results showed that IR induced cellular senescence, intracellular ROS, and mtDNA deletion, and in particular, suppressed the expression of mitochondrial biogenesis genes (NRF-1, TFAM). Furthermore, these IR-induced events were abolished using a potent antioxidant, NAC, which suggests that ROS is a key cause of mtDNA deletion in IR-induced cellular senescence, and that the alteration of mitochondrial biogenesis may mediate these processes.

2. MATERIALS AND METHODS

Cell line and culture Human lung fibroblast (IMR-90) cells at population doubling (PD) 24 were obtained from ATCC (USA) and cultured in a Minimum Essential Medium (MEM; Sigma-Aldirich, USA) supplemented with 10% (v/v) fetal bovine serum (GibcoBRL, USA), penicillin (100 U/ml), and streptomycin (100 μg/ml) at 37°C in a 5% CO_2-humidified atmosphere. Cells were subcultured every 4 days. In every subculture, the PD number was added by ΔPD, which was calculated as follows: ΔPD = the log of the ratio of the final count (N) to the starting (baseline) count (Xo), divided by the log of 2; that is ΔPD = [log (N / Xo)] / log 2.

Irradiation and treatment of cells For irradiation, cells in a culture dish were exposed to ^{137}Cs γ-rays (1.1 Gy/min) using a Gamma Cell 40 Exactor (Nordion International Inc., Canada) and the culture medium was replaced with a fresh one within 30 min. For the treatment of H_2O_2, the cells were incubated in the presence of 100 μM H_2O_2 for 2 hr, and the culture medium was then replaced with a fresh medium. At 2 hr prior to the irradiation or treatment of H_2O_2, N-acetyl-L-cysteine (NAC; Sigma-Adrich, USA) was added to the culture medium at a final concentration of 5 mM, and the culture medium was replaced with a fresh medium containing 5 mM NAC within 30 minutes after irradiation.

Cell growth assay Young IMR-90 cells at PD39 were plated in 96-well plates at 3×10^3 cells/well. At 24 hr after culture initiation, the cells were irradiated at 4 and 8 Gy. At 1, 4, and 7 days after irradiation, CCK-8 (Cell Counting Kit-8; Dojindo, Japan) was added to the cells for 1 hr, and the absorbance was measured at 450nm using a microplate reader (Molecular Devices, USA).

Measurement of intracellular ROS Intracellular ROS were assessed using 2′,7′-dichlorofluorescein diacetate (DCFH-DA; Sigma-Aldrich, USA). After detachment with trypsin-EDTA, the cells were incubated in a culture medium containing 20 μM DCFH-DA at 37°C for 30 min, and the fluorescence was then measured using flow cytometric analysis with a Cytomic FC 500 (Beckman Coulter, USA).

Assay of senescence-associated β-galactosidase activity by FDG Senescence-associated β-galactosidase (SA-β-gal) activity was detected through a fluorometric method with di-β-$_D$-galactopyranoside (FDG; Sigma-Aldrich, USA) as previously described [20]. The cells (7×10^3 cells/well) were cultured in a 24-well plate overnight for attachment, washed, and then fixed in 3% formaldehyde in PBS. After twice washing in PBS, an aliquot (500 μl) of a 20 ml reaction buffer containing 3.7 ml 0.2 M citric acid, 6.3 ml 0.4 M Na_2HPO_4 (citrate–phosphate buffer, pH 6.0), 1 ml 100 mM potassium ferricyanide, 1 ml 100 mM potassium ferrocyanide, 0.6 ml 5M NaCl, 0.2 ml 0.2 M $MgCl_2$, and 7.2 ml distilled water was added into each well. Then, 15 μl of 2 mM FDG was added per well, and the plate was incubated in the dark at 37°C for 48 h without CO_2 supply. After incubation at 37°C for 48 h, 100 μl of the supernatant was transferred to a 96-well plate for fluorescent measurement in

triplicate. The fluorescein fluorescence was measured using a fluorometer (Mithras LB940; Berthold Technologies, Germany) with an excitation at 485nm and an emission at 535 nm. One well containing the reaction mixture without cells was used as a blank for subtracting the background fluorescence.

Total DNA isolation The harvested cells were suspended in a 600 μl digestion buffer (100 mM NaCl, 10 mM Tris-Cl, pH 8.0, 25 mM EDTA, pH 8.0, 0.5% SDS, 0.1 mg/ml proteinase K) at 50°C for 18 hr. Total DNA was purified by phenol-chloroform-isoamyl alcohol extraction, dissolved in a Tris-EDTA buffer solution, and then used for the detection of mtDNA and its deletion through nested PCR.

Detection of mtDNA deletion by nested PCR The mtDNA common deletion (4977 bp) was detected as described previously [21]. The presence of mtDNA was detected through PCR amplification of a 247 bp constant region of mtDNA using H1/L1(5′-atgcttgtaggacataataa-3′/5′-agtgggaggggaaaataata-3′) primer [22]. To confirm the deletion, nested PCR was carried out using H2/L2(5′-ccgggggtatactacggtca-3′/5′-ggggaagcgaggttgacctg-3′) and H3/L3(5′-cagtttcatgcccatcgtc-3′/5′-gatgagagtaatagatagg-3′) primer [23]. PCR was performed with 1 μl of DNA, 1U Taq polymerase (Neurotic, Korea), a 1×Taq buffer (Neurotic, Korea), 200 μM dNTP, and 1 μM of primer. After initial denaturation (94°C, 10 minutes), PCR conditions of the primers were as follows: [H1/L1] denaturation, 94°C, 1 minute; annealing, 58°C, 1 minute; elongation, 72°C, 45 seconds; 30 cycles; [H2/L2] denaturation, 94°C, 30 seconds; annealing, 55°C, 35 seconds; elongation, 72°C, 1 minute; 40 cycles; [H3/L3] denaturation, 94°C, 30 seconds; annealing, 52°C, 45 seconds; elongation, 72°C, 1 minute; 25 cycles. Final elongation (72°C, 10 minutes) was the last step of each of these PCRs. PCR products were detected using ethidium bromide after 1.2% agarose gel electrophoresis. The first set (H2/L2) of primers for the deletion provided a 524 bp fragment, and the set (H3/L3) of nested primers provided a 404 bp fragment. Nested PCR was carried out with 0.5 μl of the 30 μl mixture obtained with the H2/L2 primers [21].

Total RNA isolation and RT-PCR Total RNA was isolated using an easy-BLUE total RNA extraction kit (iNtRON Biotechnology, Korea). Quantification and purity of the RNA was estimated based on an absorbance rate of A260/A280. 5 μg aliquots of total RNA were reverse transcribed into cDNA using Moloney-murine leukemia virus (M-MLV) reverse transcriptase (Promega, USA). cDNAs were amplified from 1 μl RT product per PCR using Taq DNA polymerase (Promega, USA). The primers were as follows [24]: NRF-1, 5'-GGAGT GATGTCCGCACAGAA -3' and 5'-GCTGTTAA-GCG CCATAGTG-3', 495 bp; TFAM, 5'-TATCAAGATGC TTATAGGGC-3' and 5'-ACTCCTCAGCACCATATTTT - 3'. 440 bp; β-actin, 5'-GTGGGGCGCCCCAGGCA-CCA-3' and 5'-CTCCTTAATGTCACGCACGATTTC-3', 500 bp. Amplification of the β-actin (22cycle), NRF-1 (30cycle), and TFAM (28cycle) were performed for 60 s at 95°C, 30 s at 60°C, and 60 s at 72°C in a thermal cycler (UNO II; Biometra; Germany). PCR products were resolved on 1.2% agarose gel with ethidium bromide using electophoresis and were quantitated using a Digital Gel Image system (EDAS 290; Kodak, USA).

3. RESULTS AND DISCUSSION

Cell growth inhibition by ionizing radiation IMR-90 cells were exposed to 4 or 8 Gy of ionizing radiation (IR), and the cell growth was measured at 1, 4 and 7 days after the IR. As shown in Fig. 1, growth inhibition from the IR was not observed at 1 day, but there were significant growth inhibitions at 4 and 7 days after IR. The 4 and 8 Gy IR doses showed similar growth inhibitions. Based on these results, further experiments were performed within 3 days after IR at a dose of 4 Gy to minimize the effects of growth inhibition.

Inhibition of ionizing radiation and hydrogen peroxide-induced ROS by NAC Next, we examined the effects of IR and H_2O_2 on the intracellular ROS level. Young IMR-90 cells at PD 39 were exposed to 4 Gy

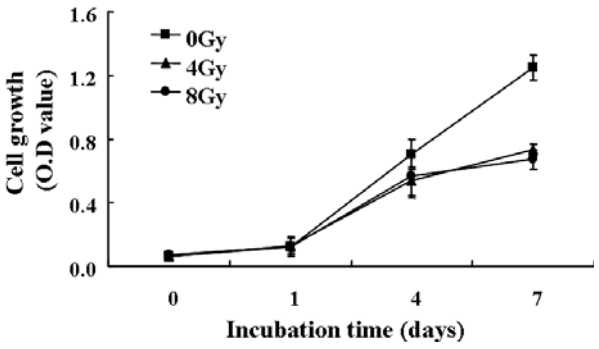

Fig. 1. Effects of ionizing radiation on cell growth in IMR-90 cells. The cell growth of IMR-90 cells was determined using a cell counting kit (CCK-8). The cells were exposed to ionizing radiation at 0, 4, and 8 Gy and incubated at 37°C for 1, 4, and 7 days. 10 μl of CCK-8 solution was then added to each well and, after incubation for 3hr, the absorbance at 450nm was read using a microplate reader. The population doubling (PD) number of the cells used for the experiment was PD 39. The results represent the mean ±SD from three separate experiments.

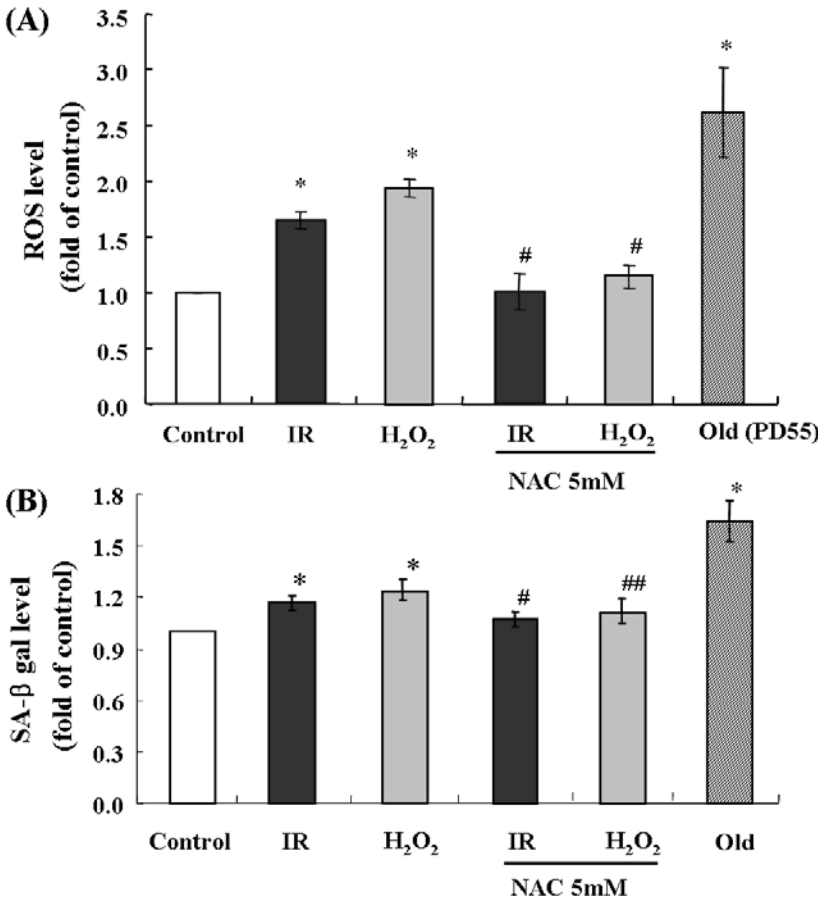

Fig. 2. Effects of ionizing radiation on ROS level and senescence-associated β-galactosidase activity in IMR-90 cells. (A) The intracellular ROS level was detected using a DCFH-DA. At 24 hr after plating, the cells were treated with 5 mM NAC for 2 hr, and then exposed to ionizing radiation at 4 Gy or H_2O_2 at 100 μM for 2 hr. They were incubated at 37°C for 24 hr and detached with trypsin-EDTA. After treatment with the DCFH-DA solution (20 μM) for 30 minute, the mean fluorescence was measured using a flow cytometer. (B) The SA-β-gal activity was measured using the FDG. After exposure to ionizing radiation at 4 Gy or H_2O_2 at 100 μM, they were incubated at 37°C for 72 hr and 2 mM FDG for SA-β-gal were treated for 2 days at 37°C without CO_2. SA-β-gal activity was determined using FDG as described in the Materials and Methods section. The population doubling (PD) number of cells for the radiation groups was PD 39, and that of the old group was PD 55. The results represent the mean ±SD from three separate experiments. *$p < 0.05$ vs control, $^{\#}p < 0.05$ vs IR alone, and $^{\#\#}p < 0.01$ vs H_2O_2 alone.

of IR or 100 μM H_2O_2, and the intracellular ROS levels were then measured using DCF fluorescence. As shown in Fig. 2A, ROS levels were significantly increased by IR (1.6 fold) and H_2O_2 (1.9 fold). Similar ROS increases were observed in old IMR-90 cells (2.6 fold) at PD 55. These increases of ROS level by IR and H_2O_2 were reduced by NAC, a well-known antioxidant compound.

Inhibition of ROS-induced senescence-associated β-galactosidase activity by NAC To confirm the induction of cellular senescence by IR and H_2O_2, SA-β-gal activity was determined as a cellular senescence marker [25]. Young IMR-90 cells at PD 39 were exposed to IR and H_2O_2, and the SA-β-gal activity level was measured using FDG. As shown in Fig. 2B, SA-β-gal activity was increased marginally yet significantly by the use of IR (1.17±0.01 fold) and H_2O_2 (1.25±0.15 fold). The increase of SA-β-gal activity was observed in old IMR-90 cells at PD 55 (1.65±0.12 fold). In accordance with the results of ROS levels, NAC sufficiently reduced the IR and H_2O_2-induced SA-β-gal activities to normal control levels. This result suggests that increased intracellular ROS levels mediate cellular senescence induced by IR and H_2O_2.

Inhibition of ROS-induced mitochondrial DNA common deletion by NAC Next, we investigated whether ROS plays a role of an inducer of mtDNA common deletion, which is one of the aging markers [8, 9]. As expected, IR and H_2O_2-treated cells, and old non-treated cells, showed higher levels of mtDNA deletion compared to young non-treated cells. The treatment of NAC again abated the increased mtDNA deletion in IR and H_2O_2-treated cells, indicating that ROS is an inducer of mtDNA common deletion in IMR-90 cells.

Effects of ROS on mitochondrial biogenesis gene expression To identify the effects of IR and H_2O_2-induced ROS on mitochondrial biogenesis, we examined the changes in mRNA transcription of the genes involved in mitochondrial biogenesis. The relative abundances of mRNA transcript encoding for nuclear respiratory factor -1 (NRF-1) and mitochondrial transcription factor A (TFAM) were examined using RT-PCR. The expressions of NRF-1 and TFAM were markedly lower in IR and H_2O_2-treated cells and old cells (Fig. 4A and B). The NRF-1 mRNA expression was decreased to 0.60±0.05 by IR, and 0.63±0.08 fold by H_2O_2, and to 0.6±0.04 in old cells compared to non-treated young cells. TFAM mRNA expressions were reduced to 0.8±0.06 by IR and 0.7±0.07 by H_2O_2 and to 0.8±0.06 in old cells compared to non-treated young cells. Decreased mRNA expressions of NRF-1 and TFAM using IR and H_2O_2 were recovered to the level of young control cells through the use of NAC treatment. The above results demonstrate that the expression of mitochondrial biogenesis genes was affected by IR and H_2O_2-induced intracellular ROS.

ROS have been reported to be the key mediator of cellular senescence and aging in humans and animals [26]. The treatment of H_2O_2 triggers senescence-related cell cycle arrest, and oxidative damage correlates with the senescence of human fibroblast cells [27,28]. ROS induce cellular senescence not only through DNA damage but also by mitochondria injury. Many researchers have reported that the accumulation of mtDNA mutation is a common event during normal aging, and that 4977bp deletion, which is called common deletion in mtDNA, is characterized as a mutation for a marker of aging in human cells [8,9]. The accumulation of common deletion in mtDNA has been observed in normally aged and normal UVA-irradiated human skin fibroblasts, and ROS are involved in the induction of common deletion from UV irradiation [17,18]. Another research group reported that γ-rays increase mtDNA common deletion in normal fibroblast cell lines and that γ-ray-induced common deletion is mediated by oxidative stress [19]. In this study, we also demonstrated that IR and H_2O_2 induced cellular senescence and mtDNA deletion, which were reversed by NAC treatment (Fig. 2B, Fig 4). These results suggest that ROS plays a role in the accumulation of common deletion in mtDNA. Although one study reported that mtDNA common deletion may be induced through replication errors from ROS [17], it largely remains unclear how IR-induced ROS can mediate mtDNA deletion.

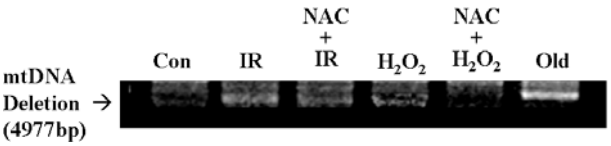

Fig. 3. Effects of ionizing radiation on mtDNA deletion in IMR-90 cells. The mtDNA deletion was detected using a nested PCR assay. At 24 hr after plating, the cells were treated with 5mM NAC for 2hr, and then exposed to ionizing radiation at 4 Gy or H_2O_2 at 100 μM for 2 hr. They were incubated at 37 °C for 72 hr. After harvesting the cells, the total DNA was isolated from the cells and PCR was performed as described in the Materials and Methods section. The mtDNA common deletion was detected using a 404 bp product. The population doubling (PD) number of cells for radiation groups was PD 39, while that of the old group was PD 55.

Mitochondrial biogenesis is involved in the regulation of metabolism, redox reaction, and signal transduction in cells, and is a response that depends on physiological and pathogenic elements [5]. Thus, mitochondrial biogenesis is an important reaction for maintenance of a cell's energetic and metabolic balance. Impairment of mitochondrial biogenesis is often reported in metabolic diseases, including diabetes [29], and is related with cellular energetic imbalance, induction of oxidative stress, and dysfunction of endothelial cells [30]. This mitochondrial biogenesis is modulated by two key regulators, NRF-1 and TFAM. TFAM is necessary for the maintenance of mtDNA copy number, transcription, and replication of the mitochondrial genome, while NRF-1 regulates the expression of TFAM and the transcription of nuclear-encoded mitochondria-related genes [6]. Several studies have reported that abnormalities in mitochondrial biogenesis are induced under oxidative stress conditions [31,32]. Transient oxidative stress (200 μM H_2O_2, 10 min) augments the induction of mitochondrial ROS in β-cells, thereby leading to a decrease of TFAM expression [31]. Also, the change of mitochondrial biogenesis is induced by a high-fat diet, which is likely to be an oxidative stress condition. Cardiomyocytes from mice fed a high-fat diet appeared to increase the ROS levels and reduce the expression levels of NRF-1 and TFAM [32].

Because mitochondrial biogenesis genes regulate the transcription and replication of the mitochondrial genome, ROS-induced abnormalities of mitochondrial biogenesis may affect mtDNA mutations, including common deletion via errors in mtDNA replication. Thus, changes in mitochondrial biogenesis-related factors by ROS may lead to a common deletion of mtDNA. We examined whether ROS can affect the expression of NRF-1 and TFAM mRNA, and mtDNA deletion. Our study showed that IR or H_2O_2-treated cells exhibited decreased mRNA expressions of NRF-1 and TFAM (Fig.

Fig. 4(A)

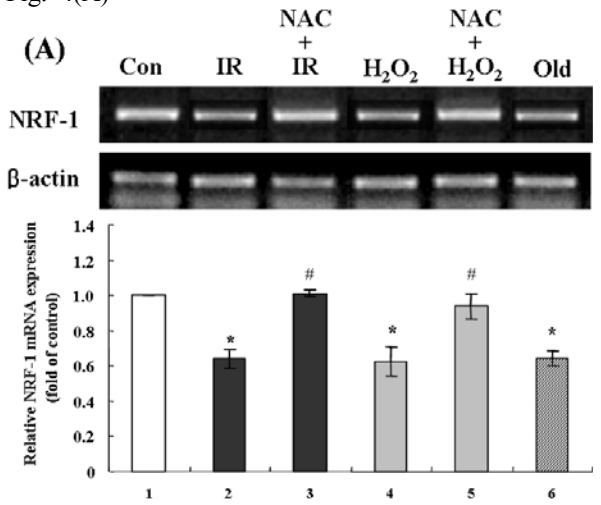

Fig. 4(B)

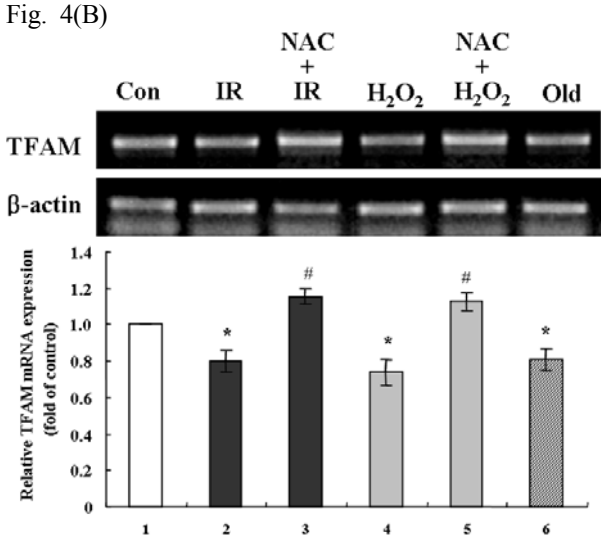

Fig. 4. Effects of ionizing radiation on mitochondrial biogenesis genes in IMR-90 cells. At 24 hr after plating, the cells were treated with 5 mM NAC for 2 hr, and then exposed to ionizing radiation at 4 Gy or H_2O_2 at 100 μM for 2 hr. They were incubated at 37℃ for 72hr. After harvesting the cells, the total RNA was isolated and the mRNA levels of NRF-1 and TFAM were detected using RT-PCR. Densitometric quantification for each gene is shown as a relative change of NRF-1 and TFAM mRNA expression compared to the control after normalizing with β-actin. The results represent the mean ±SD from three separate experiments. *$p < 0.05$ vs control and #$p < 0.05$ vs IR and H_2O_2 alone.

3), and increased mtDNA deletion (Fig. 4), the effects of which were ameliorated by NAC (Fig. 3, Fig. 4). These results suggest that impairment of these transcription factors by oxidative damage may induce mtDNA deletion.

4. CONCLUSION

Our results cannot yet clearly confirm the correlation between mtDNA deletion and mitochondrial biogenesis genes. Accordingly, future studies will investigate the influence of down- and up-regulations of mitochondrial biogenesis genes on mtDNA common deletion.

In conclusion, our results suggest that ROS is a key inducer of mtDNA deletion in cellular senescence induced by IR, which may be mediated by the regulation of mitochondria biogenesis genes, and that a change of mitochondrial biogenesis genes may mediate this process. This study will contribute toward research on the mechanisms of mtDNA deletion and its related degenerative disorders, such as Alzheimer's and Parkinson's diseases, and further more aging process.

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