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Low-dose radiation activates Nrf1/2 through reactive species and the Ca^{2+}/ERK1/2 signaling pathway in human skin fibroblast cells

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In the current study, we explored the effect of LDR on the activation of Nrfs transcription factor involved in cellular redox events. Experiments were carried out utilizing 0.05 and 0.5 Gy X-ray irradiated normal human skin fibroblast HS27 cells. The results showed LDR induced Nrf1 and Nrf2 activation and expression of antioxidant genes HO-1, Mn-SOD, and NQO1. In particular, 0.05 Gy-irradiation increased only Nrf1 activation, but 0.5 Gy induced both Nrf1 and Nrf2 activation. LDR-mediated Nrf1/2 activation was accompanied by reactive species (RS) generation and Ca^{2+} flux. This effect was abolished in the presence of N-acetyl-cysteine and BAPTA- AM. Furthermore, Nrf1/2 activation by LDR was suppressed by PD98059, an inhibitor of ERK1/2. In conclusion, LDR induces Nrf1 and Nrf2 activation and expression of Nrf-regulated antioxidant defense genes through RS and Ca^{2+}/ERK1/2 pathways, suggesting new insights into the molecular mechanism underlying the beneficial role of LDR in HS27 cells. [BMB Reports 2013; 46(5): 258-263]

INTRODUCTION

The biological effect of exposure to low doses of ionizing radiation has been paid a great deal of attention, although exposure at high doses would cause cancer and degenerative diseases. Radiation hormesis refers to a process in which exposure to low-dose radiation (LDR), in doses just above the range of natural background levels, induces beneficial effects on a cell or organism (1). The beneficial effects of LDR include growth rate stimulation (2), survival enhancement after lethal high-dose radiation (HDR) (3), life span prolongation (4, 5), immune function activation (6), and disease prevention and cure (7, 8). The possible mechanisms of these effects may be involved in activation of the adaptive stress responses involved in transcriptional regulation of nuclear factor-κB (NF-κB) (9), activator protein 1 (AP1) (10), and forkhead box transcription factors (FOXO) (4). For example, LDR plays a role as an anti-inflammatory, via modulation of NF- κ B activity in human endothelial cells (9). Increased intracellular glutathione by LDR is mediated by AP-1 in mouse macrophage-like cells (10). FOXO is required for the extension of *Drosophila melanogaster* lifespan induced by LDR (4).

Nrf2 is a basic leucine zipper (bZip) transcription factor, with a Cap 'n' Collar (CNC) structure that also includes Nrf1, Nrf3, and p45NFE2, and is expressed abundantly in the liver, intestine, lung, and kidney, where detoxification reactions routinely occur. Nrf2 is a central transcription factor that initiates the expression of encoding antioxidant genes (heme oxygenase-1 (HO-1)) and phase II detoxification enzymes (NADPH dehydrogenase, quinine 1 (NQO-1)), which are important in the protection of cells against oxidative damage caused by reactive oxidants and xenobiotics (11, 12). Under normal conditions, Nrf2 is constantly degraded via the ubiquitin- proteasome pathway, in a Kelch-like ECH-associated protein 1 (Keap1)-dependent manner. In the presence of oxidative damage, Nrf2 is released from a complex formed with Keap1, and moves from the cytoplasm to the nucleus of the cell, and induces the expression of genes that have an antioxidant response element (ARE) in their regulatory regions (13). More interestingly, the plausible involvement of Nrf2 in response to LDR has been demonstrated in the immune system. For example, low-dose γ-rays activate Nrf2 through the extracellular signal-regulated kinase (ERK)1/2-dependent pathway in Raw 264.7 cells (14). However, the molecular mechanisms underlying the antioxidant system of the Nrf family induced by X-ray LDR are not yet known.

Thus, the aim of the present study was to determine whether LDR (0.05 and 0.5 Gy) lead to activation of the entire Nrf family, and expression of Nrf-dependent antioxidant genes in normal human fibroblast cells. We also examined whether activated Nrfs are affected by intracellular signaling pathways, which are likely to be involved in reactive species (RS) generation, and

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Ca^{2+} flux in X-ray irradiated HS27 cells.

RESULTS

Nrf1/2 activation by LDR in HS27 cells

To determine whether LDR exposure would activate Nrf family members in human normal fibroblast cells, we first checked the expression of three isoforms, Nrf1, Nrf2 and Nrf3 over time, after 0.5 Gy X-irradiation in cytosolic- and nuclear fraction. Fig. 1A shows that Nrf1 was translocated into the nucleus at 24 h, and translocation of Nrf2 was increased in a time-dependent manner into the nucleus, after X-ray exposure. In cytosolic fraction, Nrf1 expression didn't show a significant change, but Nrf2 expression was decreased in a time-dependent manner. On the other hand, expression of Nrf3 in the nucleus showed a decreased pattern, but there was no significance. In the next study, therefore, we focused on Nrf1 and Nrf2, in response to irradiation.

Next, we determined Nrf1/2 expression in response to three doses of radiation (0.05, 0.5 and 5 Gy), at 2 and 24 h. The results showed the exposure of the minimum radiation dose of 0.05 Gy significantly increased the expression of Nrf1 in the nucleus at 2 h (Fig. 1B, top). The irradiation of 0.5 Gy increased not only Nrf1 expression in the nucleus at 24 h, but also caused a significant Nrf2 expression at 24 h (Fig. 1B, bottom). In addition, the maximum dose of 5 Gy of irradiation also faintly increased weak Nrf2 expression at 24 h. To further investigate ARE promoter binding activity after LDR, we measured the ARE-driven reporter gene assay in HS27 cells. The results showed that ARE activity of cells exposed to 0.05 and 0.5 Gy increased 1.3 and 1.8 fold, compared with the non-irradiated ARE control, but 5 Gy didn't increase activity.

Effect of LDR on Nrf1/2-regulated genes expression and cell proliferation/survival

To investigate downstream events of Nrf1/2 activation after irradiation, we examined the expression of manganese-superoxide dismutase (Mn-SOD), HO-1, and NQO1 at 24 h after X-irradiation. As shown in Fig. 2, Mn-SOD and HO-1 levels increased significantly at 24 h after LDR exposure. NQO-1 expression was induced weakly, in response to 0.5 Gy of X-ray. Exposure of 5 Gy only increased expression of HO-1. Collectively, the expression of Mn-SOD and HO-1 was induced by two doses of 0.05 and 0.5 Gy, but NQO1 was weakly affected only by 0.5 Gy.

Next, we performed cell proliferation and viability by MTT assay. As shown in Fig. 2B, cell proliferation was slightly enhanced by LDR irradiation (0.05 and 0.5 Gy), at 24 and 48 h. However, it was found that only 0.05 Gy irradiated cells showed a significant increase, compared to control cells, at 24 h. The effect of pre-irradiation with 0.05 and 0.5 Gy of X-ray on the cytotoxicity induced by paraquat (oxidative stress inducer) was investigated. As shown in Fig. 2C, paraquat treatment induced cell death 73.8 ± 1.1%, compared to non-irradiated control cells, but the pre-exposure to LDR (0.05 and 0.5 Gy) significantly inhibited the paraquat-induced cell cytotoxicity, producing 90.7 ± 3.8 and 90.7 ± 1.31%, respectively, compared to paraquat-induced cells. However, 5 Gy didn't affect either cell proliferation, or viability.

Involvement of RS and Ca^{2+} on Nrf1/2 activation following LDR exposure

Cellular response to radiation-induced stress is generally mediated through the production of the second messenger, RS, and influx of Ca^{2+} (15). In addition, it is known that intracellular RS and

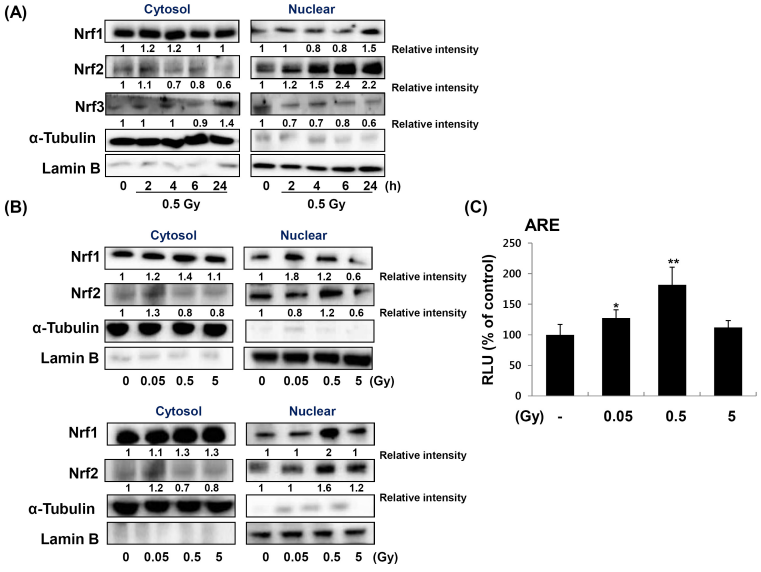

Fig. 1. Activation of Nrf1/2 in HS27 cells upon LDR. (A) HS27 cells were irradiated with 0.5 Gy of X-rays, and incubated for 2 h or 24 h. (B) Cells were irradiated with X-rays (0.05, 0.5 and 5 Gy), and then incubated for 2 h and 24 h, respectively. Expression of Nrf1/2/3 in cytosolic and nuclear fraction was determined by Western blotting. Levels were normalized to α-tubulin and lamin B. One representative result is shown from independent experiments that yield similar results. Relative intensity is shown under each blot. (C) HS27 cells were transfected with 0.5 μg ARE-luciferase reporter plasmid, and irradiated with X-rays (0.05, 0.5 and 5 Gy). After 24 h incubation, cells were harvested, and the luciferase activity was measured. *P < 0.05, **P < 0.01 vs. non-radiated control.

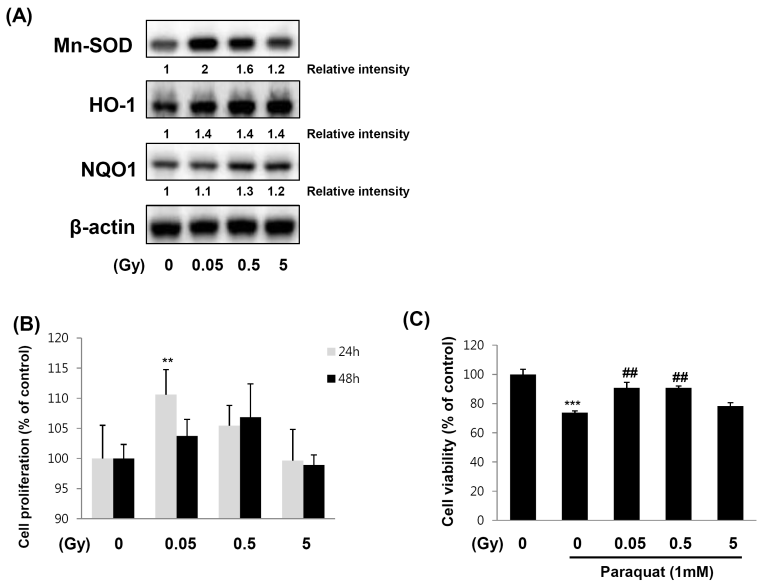

Fig. 2. Change in the Nrf1/2 target genes and cell proliferation/viability following LDR. (A) HS27 cells were irradiated with three doses (0.05, 0.5 and 5 Gy), and then incubated for 24 h. Western blot analysis was performed, to detect expression of Mn-SOD, HO-1 and NQO1 levels in whole cell lysates. Levels were normalized to β-actin. One representative result is shown from independent experiments that yielded similar results. Relative intensity is shown under each blot. (B) Cells were exposed to three doses as indicated, and then incubated for 24 h and 48 h. (C) Cells were exposed to three doses, and were then treated with paraquat (1 mM) for 24 h. $^{**}P < 0.01$, $^{***}P < 0.001$ vs. non-radiated control, $^{\#\#}P < 0.01$ vs. paraquat-treated group.

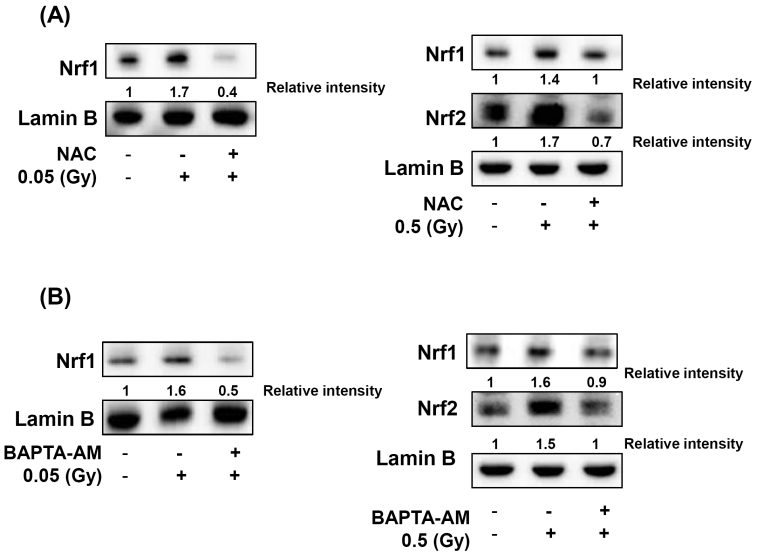

Fig. 3. Effect of RS and Ca^{2+} on LDR-mediated Nrf1/2 activation. HS27 cells were pretreated (1 h) with NAC (500 μM) or BAPTA-AM (2.5 μM), and then irradiated with 0.05 and 0.5 Gy, respectively. After 2 h (A and B, left), and 24 h (A and B, right) incubation, nuclear fraction was extracted. Western blotting was performed for Nrf1 and Nrf2. Levels were normalized to lamin B, and one representative result is shown from independent experiments that yielded similar results. Relative intensity is shown under each blot.

Ca^{2+} levels are an important regulator of Nrf2 activation (13, 16). In this study, we confirmed the effect of intracellular RS and Ca^{2+} on LDR-mediated Nrf1/2 activation, with or without the antioxidant NAC and Ca^{2+} chelator, BAPTA- AM. Pretreatment with NAC or BAPTA-AM significantly reduced Nrf1 activation after 0.05 Gy exposures (Fig. 3A and B, right). In parallel, 0.5 Gy-induced Nrf1 and Nrf2 activity also decreased in the presence of NAC or BAPTA-AM (Fig. 3A and B, left). Thus, in agreement with our previous study, LDR induced both Nrf1 and Nrf2 activity, via mild stress induced by both RS and Ca^{2+}.

Role of ERK on LDR-induced Nrf1/2 activation

Mitogen-activated protein kinases (MAPK) are involved in the regulation of the ARE in an Nrf2-dependent manner (17). To elucidate the upstream signaling events that lead to activation of Nrf1/2, we examined the LDR-induced phosphorylation of MAPK, one of the representative signal transducing kinases. We observed that LDR, 0.05 and 0.5 Gy increased phosphorylation of ERK1/2, whereas the phosphorylation of c-Jun N-terminal kinase (JNK) or P38 was either not changed, or decreased (Fig. 4A), after incubating for 1 h. To confirm the involvement of ERK1/2 in

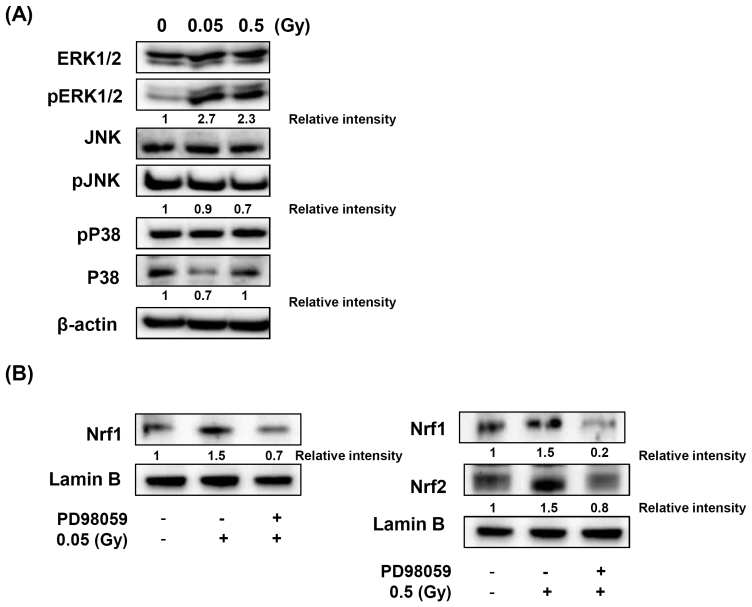

Fig. 4. Association of ERK on LDR-induced Nrf1/2 activation. (A) HS27 cells were irradiated with two dose rates (0.05 and 0.5 Gy), and incubated for 1 h. Western blot analysis was performed, to detect the phosphorylated form of ERK1/2, JNK and P38 in whole cell lysate, and the levels were normalized to ERK1/2, JNK and P38. (B) HS27 cells were pretreated (1 h) with PD98059 (20 μM), and then irradiated with 0.05 and 0.5 Gy, respectively. After 2 h (B, left), and 24 h (B, right) incubation, nuclear fraction was extracted. Western blotting was performed for Nrf1 and Nrf2. Levels were normalized to lamin B, and one representative result is shown from separate experiments that yielded similar results. Relative intensity is shown under each blot.

LDR-induced Nrf1/2 activation, cells were exposed to radiation in the presence or absence of ERK inhibitor, PD98059. Fig. 4B shows that pretreatment with ERK inhibitor completely abolished activation of Nrf1 and Nrf2 after irradiation. Therefore, these results indicate that LDR leads to activation of Nrf1/2 in an ERK1/2-dependent manner.

DISCUSSION

Biphasic radiation response, often called radiation hormesis, is a phenomenon that is beneficial in low dose, but harmful in high dose (1). The transcription factors, Nrf1 and Nrf2, are known to regulate ARE-mediated expression and coordinated induction of a battery of antioxidant and detoxification systems (11). The coordinated induction of these genes is a mechanism of critical importance in cellular defense mechanism. Nrf1 is localized primarily in the endoplasmic reticulum (ER), and is cleaved and translocated from the ER to the nucleus in response to ER stress (18). Nrf2 is retained in the cytosol by its inhibitor Keap1, and oxidative stress leads to nuclear translocation of Nrf2 (13). Therefore, both Nrf1 and Nrf2 serve as cellular sensors of various stresses that contribute to cellular redox homeostasis. However, information on Nrf1 and Nrf2 response to radiation still remains obscure. The present study has investigated Nrf1 and Nrf2 response to radiation, with various doses and exposure times.

We found that Nrf1 and Nrf2 responded to X-ray irradiation. However, Nrf1 response differed from Nrf2 response to irradiation. Nuclear location of Nrf1 was induced by LDR (0.05 and 0.5 Gy), after 2 h and 24 h exposure, respectively. In contrast, Nrf2 was only translocated by 0.5 Gy exposure, from 2 h to 24 h. It is likely that the different effects of 0.05 Gy vs. 0.5 Gy on Nrf expression are due to the relative amount of RS generated and intracellular calcium levels increased after irradiation. Ionizing radiation induced RS generation and increased intracellular calcium levels, leading to modulation of the intracellular signaling cascade (15). Unlike the result of LDR, HDR (5 Gy) showed significant nuclear exclusion of both Nrf1 and Nrf2 at 2 h after radiation, and recovered it at 24 h.

The adaptive response of LDR is associated with induction of antioxidant and detoxification enzymes. For example, low-dose γ-ray irradiation increases glutathione (GSH) and thioredoxin (TRX), leading to the prevention of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) induced brain damage (19). In addition, low-dose X-ray irradiation enhances the activity of SOD in mouse spleen (20). Consistent with other reports regarding induction of antioxidant and detoxification enzyme by LDR, we also found LDR (0.05 and 0.5 Gy) increased Mn-SOD, HO-1, and NQO1 expression. This finding is in accordance with reports that those genes are Nrf1 and Nrf2/ARE-mediated expressions (11, 18).

Our present study showed LDR (0.05 and 0.5 Gy) induced slight increase of cell protection against paraquat-induced cell damage. The maximum preventive effect occurred at 20 h pre-irradiation, with a less preventive effect at 4 h pre-irradiation (data not shown). These results suggest that the optimal doses and exposure times of LDR for inducing cell proliferation and cell survival response may vary in different cell types. Several investigations (21, 22) demonstrated that different cell lines, such as human normal fibroblast, keratinocyte, melanoma, and breast carcinoma, showed variations of the optimal LDR, from 0.01 to 1.0, and even 2 Gy, including the cell-proliferation and cell-survival adaptive responses. Interestingly, HDR also induced significant expression of HO-1, but it is conceivable that other transcription factors may be involved in coordinating and relaying different cellular signals, to turn on the transcriptional machi-

nery of the gene.

The next question is how induction of Nrf1 and Nrf2 activity occurs by LDR exposure. As we mentioned above, a mild increase of RS and intracellular calcium by LDR might initiate signaling for the nuclear import of Nrf1 and Nrf2. To test this hypothesis, we examined Nrf1 and Nrf2 expression in the presence or absence of NAC (antioxidant) or BAPTA-AM (calcium chelator), before LDR exposure. The results showed that the increased expression of Nrf1 and Nrf2 by LDR were attenuated in the presence of NAC or BAPTA-AM. These findings indicate that LDR-induced RS generation and Ca^{2+} influx are needed for the activation of Nrf1 and Nrf2.

MAPK kinases, including ERK1/2, p38 MAPK, and JNK, have been shown to be involved in the activation of Nrf2 (17). Our data also showed that phosphorylated ERK1/2 was remarkably increased with LDR exposure, and these results were confirmed by using the specific ERK1/2 inhibitor, PD98059. Therefore, our findings confirmed that increased Nrf1 and Nrf2 expression by LDR exposure were mediated by the ERK1/2 pathway. It is possible that other kinases (e.g. PKC, PERK and PI3K) (23) might have contributed to the activation of Nrf2, following LDR exposure. However, these kinases were not investigated in the present study.

In conclusion, the present study documents for the first time that low dose X-irradiation (0.05 and 0.5 Gy) elevated Nrf1 and Nrf2 in HS27 cells, and that these changes promote expression of Nrf1/2-regulated antioxidant genes HO-1, Mn-SOD, and NQO1. Our data clearly indicate that low dose 0.05 Gy irradiation may contribute to cellular beneficial effects through Nrf1 activation, but that 0.5 Gy irradiation may participate in activation of cellular defense mechanisms, through induction of Nrf1 and Nrf2. In addition, our findings indicate that under mild oxidative and Ca^{2+} stress, the ERK1/2- dependent signaling pathway may be involved in LDR-induced activation of an antioxidant defense mechanism. Thus, we propose targeting the LDR-mediated Nrf1/2 signaling pathway, as a possible new molecular mechanism, by which to control radiation hormesis.

MATERIALS AND METHODS

Cells culture and X-ray irradiation

HS27 cells (normal human fibroblast cell line) were obtained from ATCC (American Type Culture Collection, Manassas, VA). These cells were grown in Dulbecco's modified Eagle medium (DMEM) (WelGENE Inc., Daegu, Korea) containing 10% fetal bovine serum (HyClone Inc., Logan, UT). Cells were maintained at 37°C in a humidified atmosphere, containing 5% CO_2/95% air. Cells from passage 20 to 30 were used in this study. After the HS 27 cells were seeded in 150-mm dishes for 24 h, the cells were starved by incubation for 24 h in serum-free medium. The cells that reached 60-70% confluence were exposed to IR at room temperature, using an Elekta infinity X-ray generating system (dose rate 400 cGy/min, 6 MV; Elekta AB, Stockholm, Sweden). Because serum with the ability of radical scavengers interferes with water radiolysis (24), the cells were irradiated under serum-free medium.

Reagents

2′,7′-dichlorofluorescin diacetate (H_2DCF-DA), N-acetylcystein (NAC), paraquat and PD98059 were obtained from Sigma Chemical Co. (St. Louis, MO). BAPTA-AM and Fluo-4 NW calcium assay kit were purchased from Invitrogen Corp. (Carlsbad, CA). MTS assay kit was obtained from Promega Corp. (Madison, WI). Antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), or Cell Signaling (New England BioLabs, Hertsfordshire, UK). All the other materials were obtained in the highest available grade.

Western blotting

After irradiation, cells were rinsed with ice-cold PBS, and harvested with a cell scraper, followed by centrifugation. The cell pellets were lysed with NE-PER nuclear and cytoplasmic extraction reagents (Thermo Fisher Scientific Inc., Bremen, Germany). After Bradford protein assay (Bio-Rad Laboratories Inc., Hercules, CA), equal amounts of protein plus loading dye were added per lane on a 10% SDS-polyacrylamide gel, electorphoresed, and transferred to polyvinylidene fluoride (GE Healthcare Ltd., Buckinghamshire HP7 9NA England). The membranes were blocked and probed with primary antibodies (indicated in figures), and secondary anti-rabbit, mouse, goat horseradish peroxidase-conjugated IgG antibodies (Invitrogen Corp.). Bands were detected using Enhanced chemiluminescence (ECL) reagent (AbFrontier Co., Seoul, Korea), with FluorChem (Vilber Lourmat, Nice, France).

Transient transfection and luciferase assay

Cultures were seeded at 2×10^5 cells/6-well plates and incubated for 18 h. The cells were transfected with 0.5 μg of the ARE luciferase plasmid, using lipofectamine 2000 transfection reagent (Promega Corp.), according to the manufacturer's instruction. The pRL-TK plasmid encording renilla luciferase (0.1 μg) was included as an internal control of transfection efficiency. After 24 h, transfected cells were exposed to X-ray at dose of 0.05, 0.5 and 5 Gy, respectively, and incubated for 24 h. The cells were harvested, lysed, and analyzed for luciferase activity using the dual-luciferase reporter assay system (Promega Corp.).

Cell proliferation and viability assay

Cell proliferation and viability assay was measured by MTS assay kit, according to the manufacturer's instructions (Promega Corp.). For cell proliferation assay, cells (9×10^3 cells) were seeded in 96-well plates. After 18 h, cells were exposed to X-ray radiation, and then incubated for 24 and 48 h. After incubation, MTS solution was added to the 96-well plates. For cell viability, cells were seeded in 24-well plates (5×10^4 cells). After 18 h, cells were exposed to X-ray radiation, and incubated for 20 h before exposure to paraquat. After incubation with paraquat (1 mM) for 24 h, MTS solution was added to the 24-well plates.

Statistical analysis

The statistical significance of differences between the groups was determined by one-factor analysis of variance (ANOVA), followed by a Bonferroni multiple comparison test. The results were expressed as mean ± SD (n = 6). Values of $P < 0.05$ were considered statistically significant. The signal intensity of the Western blot bands was acquired, using Bioprofile Software Version 15.01.

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