Low-Dose Gamma-Ray Irradiation Induces Translocation of Nrf2 Into Nuclear in Mouse Macrophage RAW264.7 Cells

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Nrf2/γ-ray/ERK1/2.

The transcription factor nuclear erythroid-derived 2-related factor 2 (Nrf2) regulates expression of genes encoding antioxidant proteins involved in cellular redox homeostasis, while γ-ray irradiation is known to induce reactive oxygen species *in vivo*. Although activation of Nrf2 by various stresses has been studied, it has not yet been determined whether ionizing irradiation induces activation of Nrf2. Therefore, we investigated activation of Nrf2 in response to γ-irradiation in mouse macrophage RAW264.7 cells. Irradiation of cells with γ-rays induced an increase of Nrf2 expression. Even 0.1 Gy of γ-irradiation induced a translocation of Nrf2 from cytoplasm to the nucleus, indicating the activation of Nrf2 by low-dose irradiation. Expression of heme oxygenase-1, which is regulated by Nrf2, was also increased at 24 h after irradiation with more than 0.1 Gy of γ-rays. Furthermore, the activation of Nrf2 was suppressed by U0126, which is an inhibitor of the extracellular signal regulated protein kinase 1/2 (ERK1/2) pathway, suggesting involvement of ERK1/2-dependent pathway in the irradiation-induced activation of Nrf2. Our results indicate that low-dose γ-irradiation induces activation of Nrf2 through ERK1/2-dependent pathways.

INTRODUCTION

Ionizing radiation, including γ rays, induces ionization of water, followed by generation of reactive oxygen species (ROS), such as singlet oxygen ($^1O_2$), superoxide anion radical ($O_2^{-\cdot}$), H_2O_2 and hydroxyl radical ($OH^\cdot$). The hydroxyl radical is a potent oxidant, which attack lipids, proteins, and nucleotides. ROS, including nitric oxide (NO) and H_2O_2, act as second messengers in signaling pathways that regulate various cell functions, and intracellular ROS levels can be increased in response to various stimuli, including growth factors, cytokines, and environmental stress.[1–4] Superoxide anion radical ($O_2^{-\cdot}$) plays a crucial role in ROS-induced damage, because it affords $OH^\cdot$ and highly reactive peroxynitrite ($ONOO^-$) via Fenton reaction with nitric oxide. In phagocytic and non-phagocytic cells, $O_2^{-\cdot}$ is generated by NADPH oxidase complex.[5] Recently, several homologues of NADPH oxidase have been discovered, and these proteins are now designated as the Nox family.[5] Nox1 has been proposed to be involved in ROS induction by high-dose ionizing radiation.[6]

Although ionizing radiation induces DNA damage by both direct energy transfer and generation of ROS,[7,8] low-dose and small-dose irradiation not only induce DNA damage but also activate antioxidative mechanism and modulate immune functions.[9–13] However, the detail mechanism of radiation–induced antioxidative effects is still unknown.

The transcription factor nuclear factor erythroid-derived 2-related factor 2 (Nrf2) is a member of the Cap'n'Collar family of basic region leucine zipper transcription factors, and is expressed abundantly in liver, intestine, lung, and kidney, where detoxification reactions occur routinely.[14,15] Nrf2 is a central transcription factor involved in expression of antioxidant and phase II drug-metabolizing enzymes, such as heme oxygenase-1 (HO-1) and NAD(P)H: quinine oxidoreductase-1 (NQO-1), which are important in the protection of cells against oxidative damage caused by electrophiles and reactive oxidants.[16,17] Nrf2 is activated by its dissociation from Keap1, a cytosolic repressor that acts to sequester the transcription factor in the cytoplasm. Stabilization of Nrf2 protein is important for its activity, and Keap1 also plays a significant role in this respect.[18] Although Nrf2

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activity has been extensively studied, it has been not yet known whether ionizing irradiation induces activation of Nrf2, or whether Nrf2 is involved in the anti-oxidative effects of low-dose irradiation.

In this study, we investigated the activation of Nrf2 in response to γ-ray irradiation in mouse macrophage line RAW264.7 cells. The reason why we used RAW264.7 cells is that we have previously shown that irradiation with small-dose (0.5 Gy) γ-rays induces an increase of glutathione, an antioxidant, in RAW264.7 cells. Here, we observed accumulation of Nrf2 in cytoplasm and translocation to the nucleus in response to γ-ray irradiation, indicating activation of Nrf2. This activation of Nrf2 by γ-irradiation was blocked by pretreatment with an inhibitor of the extracellular signal regulated protein kinase 1/2 (ERK1/2) pathway. Our results indicate that low-dose γ-ray irradiation activates Nrf2 through ERK1/2-dependent pathway, at least in part.

MATERIALS AND METHODS

Reagents and antibodies

SIN-1 was purchased from Dojindo (Kumamoto, Japan). Mitogen-activated protein kinase kinase (MEK) inhibitor U0126 was from Calbiochem (San Diego, CA). The primary antibodies used were anti-Nrf2 (C-20) rabbit polyclonal IgG (sc-722) (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-HO-1 antibody (H4535) (Sigma-Aldrich, St Louis, MO), anti-actin C-2 mouse monoclonal antibody (sc-8432) (Santa Cruz Biotechnology), and anti-histone H3 rabbit monoclonal antibody (#1326-1) (Epitomics, Burlingame, CA). The secondary antibodies used were anti-rabbit IgG HRP-linked antibody (#7074) (Cell Signaling Technology, Inc., Beverly, MA), goat anti-mouse IgG-HRP (sc-2005) (Santa Cruz Biotechnology), and anti-rabbit IgG (whole molecule)-FITC antibody produced in goat (9887) (Sigma-Aldrich, St Louis, MO).

Cell culture and irradiation

Cells of the mouse macrophage-like cell line RAW264.7 were maintained in DMEM supplemented with 10% fetal bovine serum, penicillin and streptomycin at 37°C with 5% CO_2. The cells were then irradiated with γ-rays from a Gammacell 40 (^{137}Cs source) (Nordin International, Inc., Ontario, Canada, 0.88 Gy/min) at room temperature for a suitable time. After irradiation, the cells were incubated at 37°C.

Subcellular fractionation

Cells were collected by centrifugation and washed with PBS. To concentrate the cytoplasm and nuclear fraction, we used Nuclear Extraction Kit from Marligen Biosciences (Urbana Pike, MD), according to the manufacturer's instructions. Briefly, cells were allowed to swell in Complete Hypotonic Cell Lysis Buffer and lysis was facilitated by the addition of Detergent Solution. The cell nuclei were collected by gentle centrifugation, and the cytoplasm was removed and stored at –30°C. The nuclear pellet was washed twice in Complete Nuclear Wash Buffer, then extracted by addition of Complete Extraction Buffer 1 and Complete Extraction Buffer 2, and incubated on ice for 30 minutes. The nuclear extract was clarified by centrifugation, and stored at –30°C.

Immunoblotting

Confluent, resting RAW264.7 cells were washed twice with ice-cold PBS and lysed in PBS containing 10 mM HEPES-NaOH, pH 7.4, 1% Triton X100, 5 mM EDTA, 30 mM sodium pyrophosphate, 50 mM sodium fluoride, 1 mM sodium orthovanadate, 1.04 mM 4-(2-aminoethyl)benzene-sulfonyl fluoride, 0.8 μM aprotinin, 21 μM leupeptin, 36 μM bestatin, 15 μM pepstatin A and 14 μM E-64 for 30 min on ice. Samples were centrifuged (10,000 xg, 10 min) at 4°C and the supernatants were collected. The samples were mixed with $2 \times$ sample buffer and incubated at 95°C for 10 min. Concentration of protein in samples was determined with the Bio-Rad Protein assay reagent (Bio-Rad, Hercules, CA). Aliquots of samples containing protein were analyzed by 10% or 15% SDS-PAGE and bands were transferred to PVDF membranes. The blots were incubated overnight with 1% BSA in TBST (0.1% Tween-20, 10 mM Tris-HCl, 0.1 M NaCl), then further incubated overnight with primary antibody (1:1000) at 4°C. After having been washed with TBST, blots were incubated with appropriate secondary antibody (1:20,000) for 1.5 h at room temperature. The levels of specific proteins were visualized by using ECL Western detection reagents (GE Healthcare, Piscataway, NJ).

Immunofluorescence staining

Cells were fixed with 4% paraformaldehyde for 10 min at room temperature, permeabilized with 0.5% Triton X-100 for 5 min on ice, and incubated in blocking buffer (10% FBS in PBS) for 1 h. Fixed cells were incubated with rabbit polyclonal anti-Nrf2 antibody (1:200) for 20 h at 4°C, and then with FITC-conjugated anti-rabbit IgG antibody (1:200) for 1 h. Counterstaining with propidium iodide (0.5 μg/mL) were used to verify the location and integrity of the nuclei. Fluorescence images were obtained with a confocal laser scanning microscope (DMIRBE; Leica, Deerfield, IL) and scanned at 63× objective combined with a lower zoom to collect enough photons to make the cell just visible.

RESULTS AND DISCUSSION

Ionizing irradiation causes generation of ROS, and also induces anti-oxidative mechanisms, such as glutathione synthesis, in cells for protection from oxidative damage. The Keap1/Nrf2 system is a major regulatory pathway of cytoprotective gene expression in response to stress. Nrf2 regulates the expression of various genes encoding antioxidant proteins involved in cellular redox homeostasis. In the
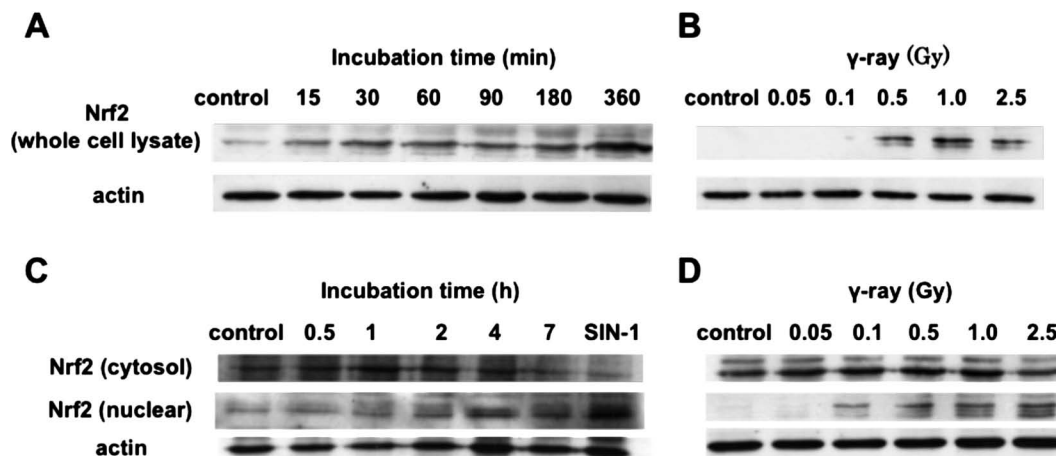
Fig. 1. Activation of Nrf2 by γ-irradiation in RAW264.7 cells. (A) RAW264.7 cells were irradiated with 0.5 Gy of γ-rays and incubated for 15–360 min. (B) Cells were irradiated with γ-rays (0.05–2.5 Gy) and incubated for 4 h. (A, B) Expression of Nrf2 in whole cell lysate was determined by immunoblotting. The blot was stripped and re-probed with anti-actin antibody to confirm equal loading of proteins among the lanes. (C) Cells were irradiated with 0.5 Gy of γ-rays and incubated for 0.5–7 h. As a positive control, RAW264.7 cells were stimulated with 0.1 mM SIN-1 for 7 h. (D) Cells were irradiated with γ-rays (0.05–2.5 Gy) and incubated for 4 h. (C, D) The cells were harvested, and cytosol and nuclear extracts were prepared as described in Materials and methods. Levels of Nrf2 in cytosol and nuclear extracts were analyzed by immunoblotting. The blot was stripped and probed with anti-actin antibody to confirm equal loading of proteins among the lanes.

present study, we investigated whether the Nrf2-dependent anti-oxidative pathway is activated after exposure of cells to γ-irradiation.

We first exposed RAW264.7 cells to various doses of γ-rays. As shown in Fig. 1A and 1B, γ-irradiation caused a time- and dose- dependent increase of Nrf2 expression in whole cell lysate. As a positive control, we also confirmed translocation of Nrf2 from cytoplasm to the nucleus in RAW264.7 cells in response to a peroxynitrite donor SIN-1 (Fig. 1C). Figure 1C and 1D show that γ-irradiation also caused translocation of Nrf2 from cytoplasm to the nucleus in a time- and dose-dependent manner. The level of Nrf2 in cytoplasm increased 1–2 h after γ-irradiation, and accumulation of Nrf2 in the nucleus began at 4 h. The minimum radiation dose inducing translocation of Nrf2 was 0.1 Gy. Further, the increase of nuclear Nrf2 in response to γ-irradiation (0.5 Gy) was confirmed by immunofluorescence staining (Fig. 2). These results indicate that low-dose γ-irradiation induces an increase of Nrf2 expression, together with translocation of Nrf2 from cytoplasm to nucleus in RAW264.7 cells.

To investigate downstream events of Nrf2 activation after irradiation, we examined the induction of HO-1 expression after γ-irradiation in RAW264.7 cells. Heme oxygenase-1 not only plays a central role in heme metabolism, but also has a protective role against cell injury evoked by various oxidative stresses,[19–21] and it is considered to be a marker protein of oxidative stress. Increase of HO-1 level was observed 24 h after γ-irradiation at 0.1–2.5 Gy (Fig. 3). We observed a dose-dependent increase of HO-1 expression
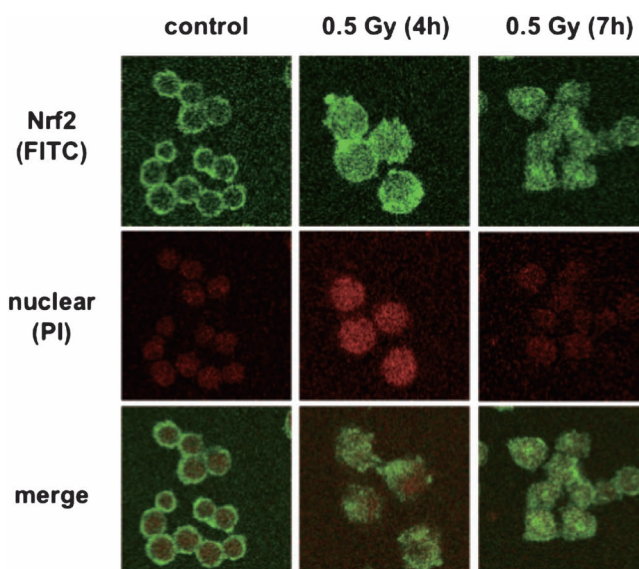
Fig. 2. Nuclear translocation of Nrf2 in response to γ-irradiation in RAW264.7 cells. Cells were incubated for 4 or 7 h after 0.5 Gy of γ-irradiation. Localization of Nrf2 (green) was detected by immunocytochemistry. To confirm the location of the nuclei, PI-counterstaining (red) is shown both separately and merged with Nrf2 immunofluorescence.

after irradiation, and the extent of HO-1 expression was correlated to the extent of translocation of Nrf2 after irradiation. These findings suggested that low-dose γ-irradiation induces accumulation of HO-1, which is an Nrf2-dependent anti-

oxidative protein.

Since it has been reported that hypoxia triggers NADPH oxidase- and ERK1/2-dependent activation of Nrf2,[22] we examined the effect of U0126, which specifically blocks the activation of the MEK1/ERK1/2 kinase pathway, on the irradiation-induced activation of Nrf2. Translocation of Nrf2 was markedly suppressed by pretreatment with U0126 (Fig. 4), suggesting that the ERK1/2-dependent signaling pathway plays an important role in radiation-induced activation of Nrf2. The mechanism involved is unclear, and other signaling molecules might also be involved in the activation of Nrf2. For example, it is known that ionizing radiation induces expression of iNOS and production of NO,[23] and increased NO production by iNOS contributes to Nrf2 activation.[24,25] Recent studies suggest that ROS generation by NADPH oxidase occurs after exposure to ionizing irradiation and Nox1 plays a pivotal role in this ROS generation.[6] Since NADPH oxidase-mediated activation of Nrf2 has been reported,[22] activation of Nox1 by irradiation might mediates activation of Nrf2. Although various signaling pathways are likely to be involved in Nrf2 activation, our results show that activation of ERK1/2 is a contributor to the irradiation-induced activation of Nrf2.
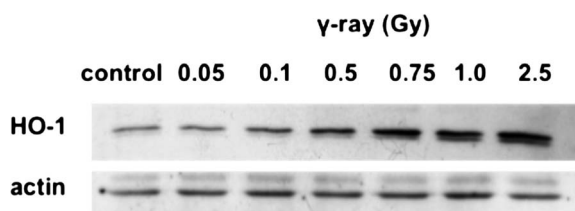
Irradiation with γ-rays induces the expression of antioxi-

Fig. 3. γ-irradiation induces Nrf2-dependent expression of HO-1. RAW264.7 cells were irradiated over the dose range from 0.05 Gy to 2.5 Gy, and incubation was continued for 24 h. The cells were harvested and whole cell lysate was analyzed on SDS-PAGE, followed by immunoblotting for HO-1 protein. The blot was stripped and probed with anti-actin antibody to confirm equal loading of proteins among the lanes.
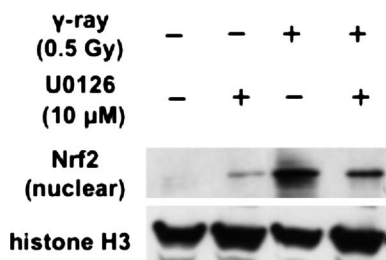
Fig. 4. Inhibitory effects of MEK inhibitor on γ-irradiation-induced translocation of Nrf2. RAW264.7 cells were preincubated with vehicle or U0126 (10 μM) for 1 h prior to irradiation with 0.5 Gy of γ-rays. Cells were incubated for 4 h after irradiation, nuclear extracts were fractionated, and the expression of Nrf2 was analyzed by immunoblotting. Histone H3 were used as loading controls.

dants, such as glutathione, and enhances the activity of enzymes such as catalase, Cu/Zn superoxide dismutase, glutathione peroxidase and glutathione reductase.[26,27] These enzymes act to counter oxidative stress due to excessive ROS *in vivo*. We reported that the immediate induction of endogenous glutathione in living cells exposed to 0.5 Gy of γ-rays is responsible for the appearance of enhanced NK activity.[10] Further, Nrf2 regulates the expression of genes encoding antioxidant proteins involved in cellular redox homeostasis, including HO-1, glutathione S-transferase, quinone oxidoreductase, glucose-6-phosphate dehydrogenase and glutamylcysteine synthetase.[28,29] However, a common anti-oxidative denominator induced by γ-irradiation and by activation of Nrf2 has not been identified. Here, we show that an accumulation of Nrf2 in nuclei is induced after low-dose γ-irradiation, which can cause anti-oxidative event through transactivation of HO-1, NQO-1 and so on.

In conclusion, our results indicate that γ-irradiation induces accumulation of Nrf2 in nuclei through an ERK1/2-dependent pathway. Accumulation of Nrf2 in nuclei would be a novel mechanism choreographing the anti-oxidative effect induced by low-dose ionizing irradiation.

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