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γ-Irradiation-induced DNA damage enhances NO production via NF-κB activation in RAW264.7 cells

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Abstract

We investigated the mechanism of augmentation of nitric oxide (NO) production in the murine macrophage cell line RAW264.7 after γ-irradiation. The cells treated with interferon-γ (IFN-γ) or lipopolysaccharide (LPS) showed enhanced NO production by γ-irradiation in a dose-dependent manner, accompanying the induction of inducible nitric oxide synthase (iNOS) expression. Nuclear factor kappa B (NF-κB) activation was induced 1 h after γ-irradiation dose-dependently, which was detected by the degradation of I-κB. Inhibitors of I-κB degradation, MG132 and N^{α}-*p*-tosyl-L-lysine chloromethyl ketone (TLCK), suppressed the further increase by γ-irradiation in IFN-γ-induced NO production, showing that γ-irradiation induced NO production via NF-κB activation. Although NF-κB is known to be a redox-sensitive transcription factor, the antioxidant agents *N*-acetyl-cysteine (NAC) and 6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid (trolox) showed no suppression and treatment with H_2O_2 showed only slight enhancement of IFN-γ-induced NO production. The DNA damaging agents camptothecin and etoposide enhanced IFN-γ-induced NO production and showed I-κB degradation, indicating that the increase in NO production was due to direct DNA damage. Furthermore, 3-aminobenzamide (3AB) and benzamide, inhibitors of poly (ADP-ribose) polymerase (PARP) that are activated upon recognition of DNA strand breaks, suppressed the further increase by γ-irradiation in IFN-γ-induced NO production and the I-κB degradation by γ-irradiation. We concluded that (1) the increase in NO production was due to direct DNA damage by γ-irradiation, and that (2) PARP activation through DNA damage induced NF-κB activation, leading to iNOS expression and NO production.

Keywords: RAW264.7 cell; γ-Irradiation; Nitric oxide; DNA damage; NF-κB; Poly (ADP-ribose) polymerase

1. Introduction

Nitric oxide (NO), a short-lived radical gas, acts as an intercellular messenger in most mammalian organs, participating in vascular homeostasis, neurotransmission and antimicrobial defense [1]. Its formation is regulated by a family of enzymes, known as nitric oxide synthase (NOS), that oxidize the guanidio moiety of L-arginine, resulting in the equimolar production of NO and L-citrulline [2]. At least three distinct but functionally and structurally related isoforms of NOS have been identified in mammalian cells. These are referred to as nNOS and eNOS, constitutively expressed in cells of neuronal and endothelial origin, respectively, and inducible NOS (iNOS), which is induced in response to a wide array of proinflammatory cytokines and bacterial cell wall products in various cell types [1,2]. In the case of macrophages, NO is produced following the induction of iNOS by interferon-γ (IFN-γ) and/or lipopolysaccharide (LPS), and shows antimicrobial and antitumoricidal activities [3].

Macrophages are known to be radioresistant, as demonstrated by experiments that cell functions were not affected by high-dose irradiation [4,5]. Furthermore, there have been several reports that ionizing radiation can enhance the activation of macrophages. Gallin et al. showed that γ-irradiation of J774.1 macrophages with 20 Gy produced morphological and enzymatic changes, along with an increase in cell activation [6]. Lambert and Paulnock reported that 50 Gy of γ-irradiation induced MHC class I antigen expression and sensitivity to LPS in RAW264.7 cells

Abbreviations: NO, nitric oxide; iNOS, inducible nitric oxide synthase; NF-κB, nuclear factor kappa B; I-κB, inhibitor of NF-κB; TLCK, N^{α}-*p*-tosyl-L-lysine chloromethyl ketone; NAC, *N*-acetyl-cysteine; Trolox, 6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid; 3AB, 3-aminobenzamide; PARP, poly (ADP-ribose) polymerase; IFN-γ, interferon-γ; LPS, lipopolysaccharide; TNF-α, tumor necrosis factor α

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or peritoneal exudate macrophages, and irreversibly primed the cells to induce tumor cell killing [7]. Production of some cytokines such as interleukin-1 and expression of their mRNAs were reported after irradiation in human alveolar macrophages, mouse spleen macrophages, etc. [8–10].

In our previous studies, we showed that the cytotoxic activity of mouse resident peritoneal macrophages was enhanced by low dose of in vivo γ-irradiation (4 cGy), which was due to an increase in NO production in activated macrophages [11]. Furthermore, high doses (6–12 Gy) of in vitro γ-irradiation also enhanced the tumoricidal activity by augmentation of NO production [12]. Yoo et al. [13] reported that γ-irradiation (5–40 Gy) enhanced NO production in murine embryonic liver cells due to formation of H_2O_2. Reactive oxygen species (ROS) were also reported to be important in the induction of iNOS by sphingomyelinase in RAW264.7 cells [14]. McKinney et al. [15] reported that γ-irradiation (0.5–10 Gy) potentiates the induction of NO in J774.1 and RAW264.7 cells, which was mediated by the induction of tumor necrosis factor α (TNF-α), but was not attributable to ROS. Although ionizing radiation has been confirmed to potentiate NO production from macrophages, the activation mechanism is not yet clear.

In this study, we examined the mechanism of enhancement of IFN-γ-induced NO production in short periods after γ-irradiation using RAW264.7 cells. Enhanced NO production by γ-irradiation was mainly due to direct DNA damage. DNA damage activated poly (ADP-ribose) polymerase (PARP), which induced nuclear factor kappa B (NF-κB) activation, finally resulting in increased iNOS expression and NO production.

2. Materials and methods

2.1. Materials

Recombinant mouse IFN-γ and LPS were purchased from Genzyme, USA and Difco Laboratories, USA, respectively. *N*-Acetyl-L-cysteine (NAC), 3-aminobenzamide (3AB), benzamide, etoposide, camptothecin and 6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid (trolox) were obtained from Sigma-Aldrich Co., Japan. MG132 and N^{α}-*p*-tosyl-L-lysine chloromethyl ketone (TLCK) were purchased from Calbiochem-Novabiochem. Co., USA and Research Organics, Inc., USA, respectively. Anti-iNOS IgG was obtained from Transduction Laboratories, USA. Anti-I-κB-α IgG (H-4) and anti-actin IgG (C-11) were purchased from Santa Cruz Biotechnology, Inc., USA.

2.2. Cell culture

RAW264.7 cells were maintained in Dulbecco's modified Eagle's medium containing 10% FBS, 2 mM L-glutamine and 100 U/ml penicillin/streptomycin at 37 °C in a humidified atmosphere containing 5% CO_2 in air.

2.3. γ-Irradiation

The cells in 24-well plates or 60-mm dishes were irradiated with several doses of γ-rays (dose rate: 0.3 Gy/min) in an Irradiation Exposure System (Pony Atomic Industry, PS-600SB, ^{137}Cs: 22.2 TBq). The various inhibitors were added 1 h before γ-irradiation and were present up to 6 h after irradiation.

2.4. Measurement of NO production

The production of NO was measured by assaying NO_2^- in the culture supernatants using colorimetric Griess reaction. RAW264.7 cells (5×10^4 cells/24 well/400 μl) cultured for 24 h were irradiated, then cultured for 24 h and further in the presence of IFN-γ and/or LPS at 37 °C for 24 h. Using 96-well plates, aliquots (100 or 50 μl) of culture supernatants were incubated with an equal volume of Griess reagent (1% sulfanilamide/0.1% napthylethylene diamine dihydrochloride/2.5% H_3PO_4) at room temperature for 10 min, and the absorbance at 550 nm was measured with a microplate reader (EL340, Bio-Tec, Inst., Inc., Japan). Absorbance measurements were converted to *n* moles of NO_2^- per well using a standard curve of $NaNO_2$ and further to the value per milligram of protein. Protein was measured using DC Protein Assay Kit (Bio-Rad Lab., USA).

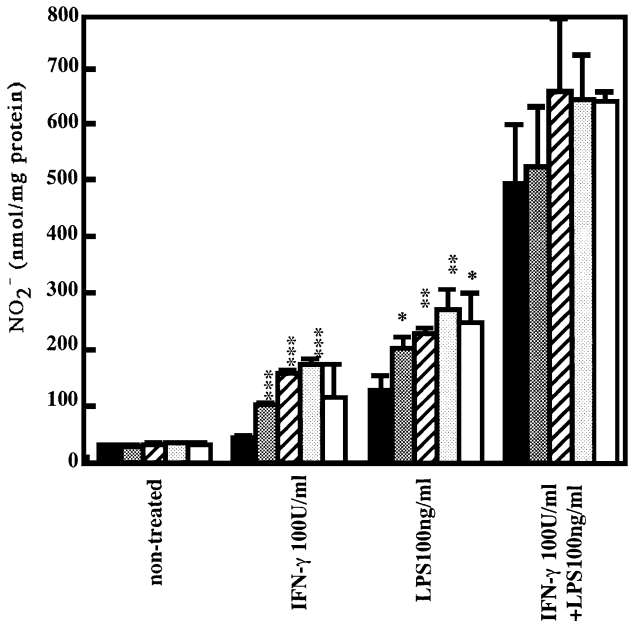

Fig. 1. NO production from RAW264.7 cells irradiated with several doses of γ-rays. RAW264.7 cells were irradiated with several doses of γ-rays and cultured at 37 °C for 24 h. They were further cultured in the presence of IFN-γ and/or LPS for 24 h. The culture supernatant was used for determination of NO_2^- with Griess reagent. ■, 0 Gy; ■, 3 Gy; ▨, 6 Gy; ▨, 10 Gy; □, 20 Gy. The values are means ± S.D. ($n=9$); significantly different from unirradiated controls: $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

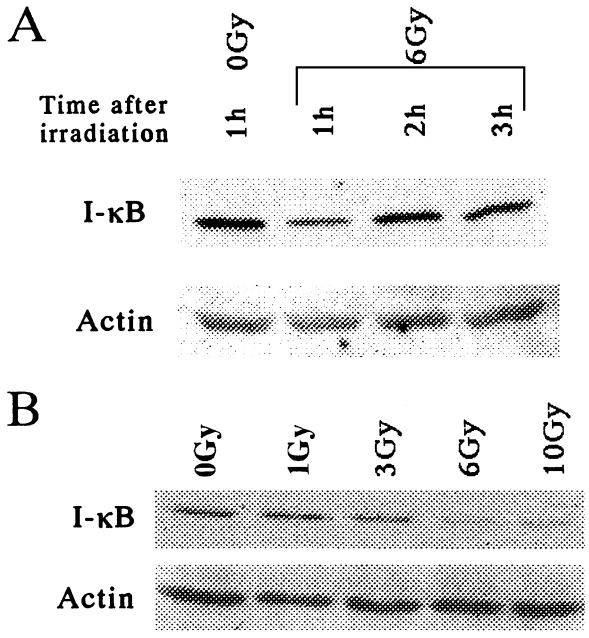

Fig. 2. Degradation of I-κB by γ-irradiation. (A) I-κB degradation after γ-irradiation. RAW.264.7 cells were γ-irradiated at a dose of 6 Gy and cultured for predetermined times. (B) Dose dependency of I-κB degradation. RAW.264.7 cells were γ-irradiated at several doses and cultured for 1 h. The cell lysates were subjected to SDS-PAGE followed by Western blotting using anti-I-κB and anti-actin antibodies.

2.5. Immunoblotting analysis for iNOS expression

The cells (8×10^5 cells/4 ml/60 mm dish) cultured for 24 h were irradiated, then cultured for 24 h and further in the presence of IFN-γ and/or LPS at 37 °C for 12 h. They were washed with PBS and scraped with 100 μl of SDS sample buffer [10% (w/v) glycerol, 2% SDS, 10% (v/v) 2-mercaptoethanol and 0.0625 M Tris–HCl, pH 6.8]. The lysates were boiled for 5 min and separated by SDS-PAGE followed by electroblotting onto PVDF membranes (Immobilon-P, Millipore Co., USA). After blocking of nonspecific binding with 10% skimmed milk, the membranes were incubated with iNOS antibody followed by horseradish peroxidase-conjugated secondary antibody. The secondary antibody was detected with the ECL chemiluminescence detection system (Amersham Pharmacia Biotech, UK).

2.6. Immunoblotting analysis for Iκ-B degradation

To evaluate NF-κB activation, Iκ-B degradation was detected by immunoblotting using cytosolic protein extracts obtained as follows. Following incubation for predetermined times after γ-irradiation, the cells were washed with PBS, suspended in lysis buffer (10 mM HEPES–KOH, pH 7.8, 10 mM KCl, 0.1 mM EDTA, 1 mM DTT, 2 μg/ml aprotinin, 2 μg/ml pepstatin, 2 μg/ml leupeptin and 1 mM sodium orthovanadate), then disrupted using a Dounce homogenizer. Lysates were centrifuged at $10{,}000 \times g$ for 10 min and the supernatants were further centrifuged at $100{,}000 \times g$ for 30 min. The proteins in the supernatants were separated by SDS-PAGE followed by immunoblotting as described above.

2.7. Statistics

All experiments were repeated two or three times. The Student's *t*-test was used to test the significance between the groups.

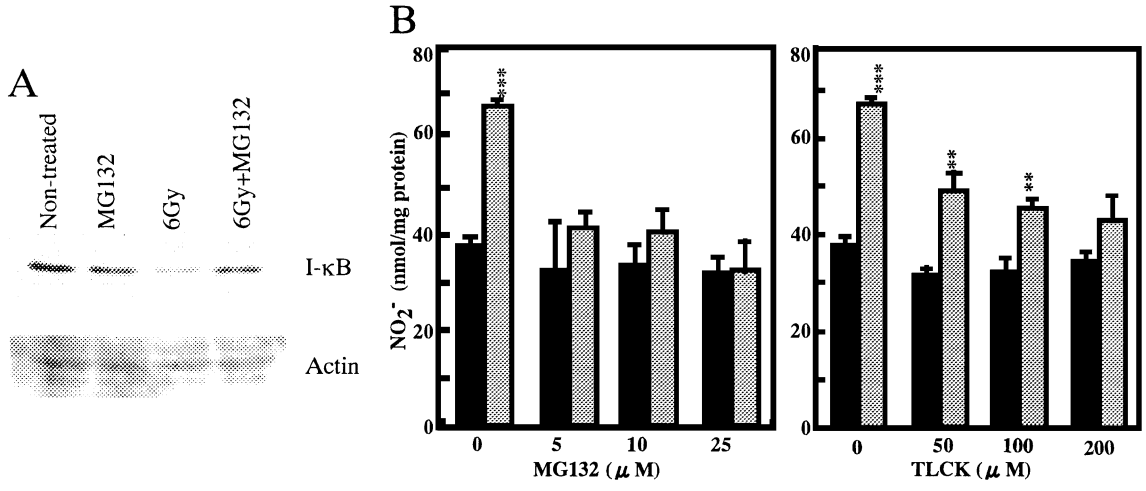

Fig. 3. Participation of NF-κB activation in enhancement of NO production by γ-irradiation. (A) Inhibition of I-κB degradation by MG132. After treatment with MG132 (10 μM) for 1 h, RAW.264.7 cells were γ-irradiated at a dose of 6 Gy and cultured for 1 h. The cell lysates were subjected to SDS-PAGE followed by Western blotting using anti-I-κB and anti-actin antibodies. (B) Effect of NF-κB inhibitors on the enhancement of NO production by γ-irradiation. After treatment with either MG132 or TLCK for 1 h, RAW264.7 cells were γ-irradiated at a dose of 6 Gy and cultured for 6 h. After washing and further culture for 18 h, they were cultured in the presence of IFN-γ (100 U/ml) at 37 °C for 24 h. The culture supernatant was used for determination of NO_2^- with Griess reagent. ■, 0 Gy; ▩, 6 Gy. The values are means ± S.D. ($n=9$). Significantly different from unirradiated controls: $^{**}P<0.01$, $^{***}P<0.001$.

3. Results

3.1. Inhibition of NO production by NF-κB inhibitor

RAW264.7 cells showed further significant increase by 3–10 Gy of γ-irradiation in IFN-γ- or LPS-induced NO production (Fig. 1), which was consistent with the results reported by McKinney et al. [15]. Induction of iNOS was also enhanced by γ-irradiation (data not shown). NF-κB is a redox-sensitive transcription factor [16,17] and is activated by γ- or UV-irradiation [18–20]. NF-κB activation (translocation from the cytosol to the nucleus) is suppressed by the interaction with I-κB [16]. We examined NF-κB activation by detection of Iκ-B degradation. I-κB degradation was induced 1 h after γ-irradiation in a dose-dependent manner (Fig. 2). To examine the relationship of NF-κB activation with the enhancement of IFN-γ-induced NO production by γ-irradiation, the proteasome inhibitor MG132 and the serine protease inhibitor TLCK were present during γ-irradiation. These reagents are potent inhibitors of NF-κB activation through prevention of Iκ-B degradation and 10 μM of MG132 and 100 μM of TLCK were respectively reported to show complete inhibition [21–23]. We also confirmed that MG132 inhibited I-κB degradation as shown in Fig. 3A. Both MG132 and TLCK significantly inhibited the enhancement of NO production (Fig. 3B), showing that the transient activation of NF-κB by γ-irradiation was necessary for enhanced NO induction.

3.2. Effects of ROS on NO production

As mentioned above, NF-κB is redox-sensitive [16,17] and is activated by γ-irradiation [18–20]. To clarify the relationship between ROS and the enhancement of IFN-γ-

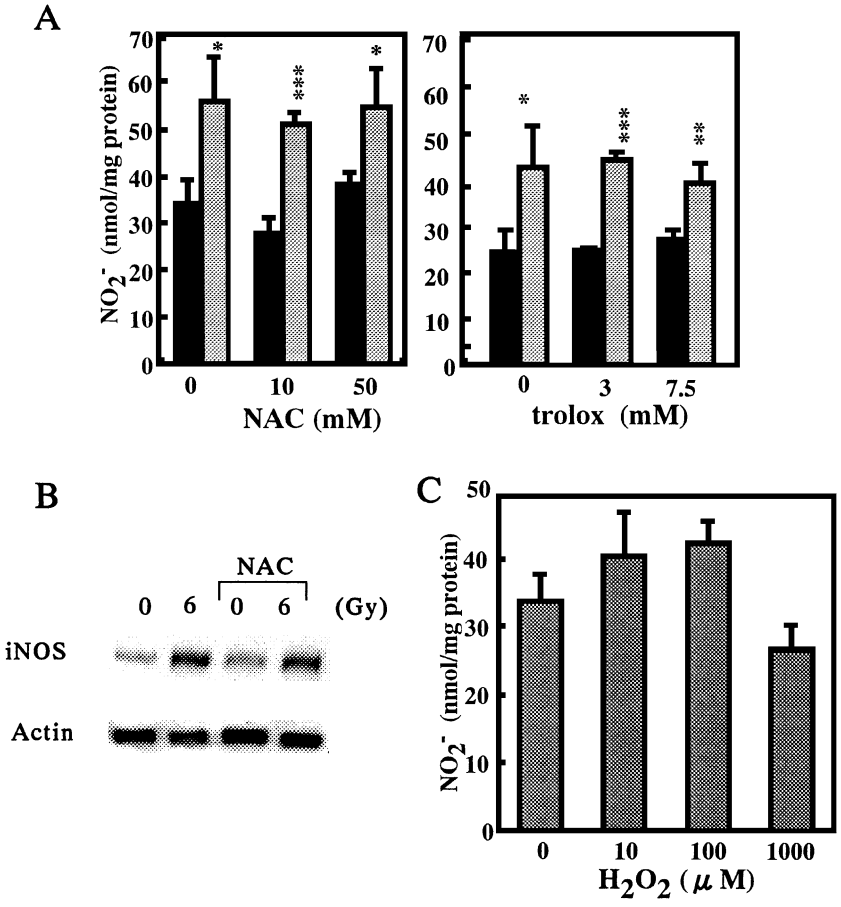

Fig. 4. Participation of ROS in enhancement of NO production by γ-irradiation. (A) Effects of anti-oxidant agents. After treatment with either NAC or trolox for 1 h, RAW264.7 cells were γ-irradiated at a dose of 6 Gy and cultured for 6 h. After washing and further culture for 18 h, they were cultured in the presence of IFN-γ (100 U/ml) at 37 °C for 24 h. The culture supernatant was used for determination of NO_2^- with Griess reagent. ■, 0 Gy; ▩, 6 Gy. (B) Effects of NAC on induction of iNOS by γ-irradiation. After treatment with NAC (50 mM) for 1 h, RAW264.7 cells were γ-irradiated at a dose of 6 Gy and cultured for 6 h. After washing and further culture for 18 h, they were cultured in the presence of IFN-γ (100 U/ml) at 37 °C for 12 h. The cell lysates were subjected to SDS-PAGE followed by Western blotting with anti-iNOS and anti-actin antibodies. (C) NO production after treatment with H_2O_2. After treatment with H_2O_2 for 1 h, RAW264.7 cells were washed, cultured at 37 °C for 24 h, and then further cultured in the presence of IFN-γ (100 U/ml) for 24 h. The culture supernatant was used for determination of NO_2^- with Griess reagent. The values are means ± S.D. ($n=9$). Significantly different from unirradiated controls: $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

induced NO induction by γ-irradiation, the cells were treated with antioxidant reagents during γ-irradiation. Neither NAC (~ 50 mM) nor trolox (~ 7.5 mM) showed any inhibitory effect on NO production (Fig. 4A); however, each lower dose (1 mM NAC [24] and 50 μM trolox [25]) of both agents was reported to show the ROS scavenging effect. iNOS induction was also not suppressed by NAC (Fig. 4B). H_2O_2 treatment slightly enhanced NO production, but to a lesser degree than the enhancement of NO production observed after γ-irradiation (Fig. 4C).

3.3. Effects of DNA damage on NO production

γ-Irradiation is known to induce both direct and indirect DNA damage. From the above results, the indirect DNA damage by ROS was considered to be hardly involved in the enhancement of NO production. To examine whether direct DNA damage was related to enhancement of NO production, etoposide and camptothecin, which are topoisomerase inhibitors and are capable of induction of DNA strand breaks [26–28], were used instead of γ-rays. Both agents enhanced IFN-γ-induced NO production in a dose-dependent manner (Fig. 5A). Cisplatin, which reacts with guanine and forms DNA adducts containing DNA cross-links [29,30], also enhanced NO production (data not shown). On the other hand, hydroxyurea (DNA synthesis inhibitor) and vincristine (microtubule inhibitor), which inhibited mitosis without DNA damage, showed no enhancement (data not shown). These findings indicated that the increase of NO production could be induced by direct DNA damage by γ-irradiation, and not by inhibition of mitosis. Furthermore, Iκ-B degradation was observed following treatment with camptothecin (Fig. 5B).

3.4. Participation of PARP in the increase in NO production

The activity of PARP is known to be activated upon recognition of DNA strand breaks induced by γ-irradiation [31,32]. To examine the participation of PARP in the enhancement of IFN-γ-induced NO production by γ-irradiation, the PARP inhibitors 3AB and benzamide were present during γ-irradiation. Higher doses of 3AB (12 mM) and benzamide (10 mM) suppressed the enhancement of NO production (Fig. 6A and B). On the other hand, lower doses (6 mM 3AB and 5 mM benzamide) showed no effects, although 1 mM of both agents is reported to inhibit about 90% of the PARP activation [33]. The reason why only high dose showed the inhibitory effect could not be clarified. 3AB could inhibit the enhancement of NO production by several doses of γ-irradiation (Fig. 6C). iNOS induction was also completely suppressed by the inhibitor (Fig. 6D). Le Page et al. [34] reported that 10 mM of 3AB impaired iNOS gene transcription via inhibition of NF-κB activation in RAW264.7 cells. Therefore, Iκ-B degradation was analyzed in the presence of 3AB and benzamide during γ-irradiation. Iκ-B degradation was significantly inhibited 1 h after irradiation (Fig. 7A and B).

4. Discussion

We examined the mechanism of the further increase by γ-irradiation in IFN-γ-induced NO production in macrophages. The enhancement of NO production was accompanied by induction of iNOS production. As the promoter of iNOS gene contains binding sites for NF-κB [35,36], a redox-sensitive transcription factor [16,17], which is acti-

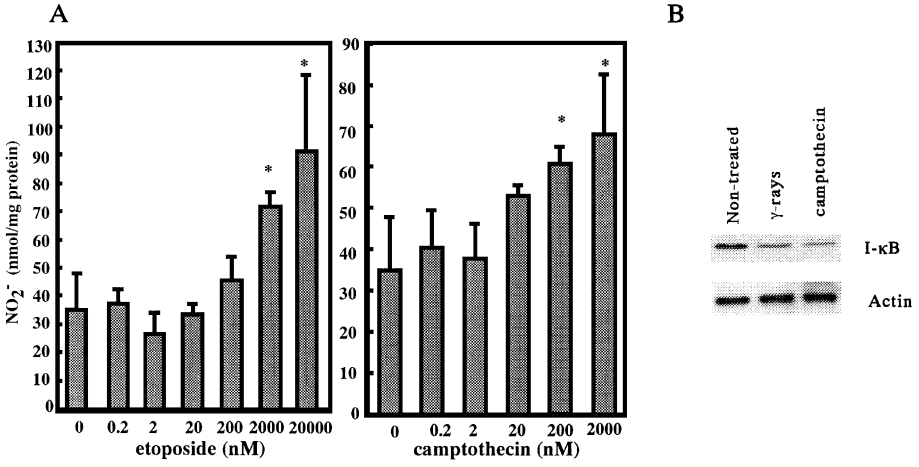

Fig. 5. Participation of DNA damage in enhancement of NO production by γ-irradiation. (A) NO production after treatment with DNA damaging agents. RAW264.7 cells were treated with camptothecin or etoposide for 6 h. After washing and further culture for 18 h, they were cultured in the presence of IFN-γ (100 U/ml) at 37 °C for 24 h. The culture supernatant was used for determination of NO_2^- with Griess reagent. The value are means ± S.D. ($n=9$). Significantly different from unirradiated controls; *$P<0.05$. (B) I-κB degradation by treatment with camptothecin. After treatment with camptothecin (10 μM) for 2 h or γ-irradiation (6 Gy), RAW264.7 cells were lysed and subjected to SDS-PAGE followed by Western blotting with anti-I-κB and anti-actin antibodies.

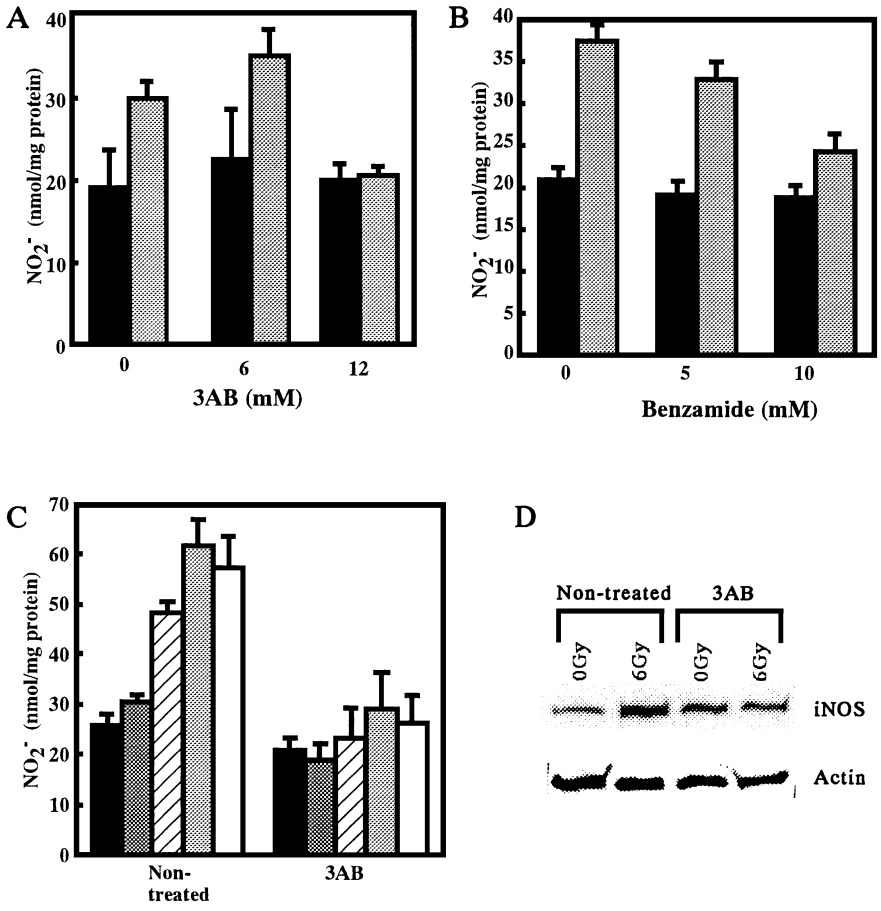

Fig. 6. Participation of PARP activation in enhancement of NO production by γ-irradiation. (A, B) Effects of PARP inhibitors. After treatment with 3AB (A) or benzamide (B) for 1 h, RAW264.7 cells were γ-irradiated at a dose of 6 Gy and cultured for 6 h. After washing and further culture for 18 h, they were cultured in the presence of IFN-γ (100 U/ml) at 37 °C for 24 h. The culture supernatant was used for determination of NO_2^- with Griess reagent. ■, 0 Gy; ▩, 6 Gy. The values are means ± S.D. ($n=9$). (C) Effect of 3AB on irradiation-dose-dependent NO production. After treatment with 3AB (15 mM) for 1 h, RAW264.7 cells were irradiated with several doses of γ-rays. ■, 0 Gy; ▩, 1 Gy; ▨, 3 Gy; ▩, 6 Gy; □, 10 Gy. (D) Effects of PARP inhibitor on induction of iNOS by γ-irradiation. After treatment with 3AB (10 mM) for 1 h, RAW264.7 cells were γ-irradiated at a dose of 6 Gy and cultured for 6 h. After washing and further culture for 18 h, they were cultured in the presence of IFN-γ (100 U/ml) at 37 °C for 12 h. The cell lysates were subjected to SDS-PAGE followed by Western blotting with anti-iNOS and anti-actin antibodies.

vated by several oxidative stresses containing UV [20] and ionizing radiation [18–20], we were interested in the relationship between the activation of NF-κB and the enhancement of NO production. NF-κB is located in the cytoplasm bound to an inhibitor protein, Iκ-B, which prevents the uptake of NF-κB into the nucleus. Treatment of cells with several oxidative stresses induces the phosphorylation and degradation of IκB and results in the translocation of free NF-κB to the nucleus [16,17]. Iκ-B degradation was found 1 h after γ-irradiation in RAW264.7 cells (Fig. 2A). The cells were incubated for 1 h before and 6 h after γ-irradiation in the presence of inhibitors of NF-κB activation, MG132 and TLCK. Both inhibitors were reported to inhibit NF-κB activation through the suppression of I-κBα degradation [21–23]. The enhancement of NO production after γ-irradiation was completely suppressed by treatment with both inhibitors, showing that NF-κB activation by γ-irradiation was attributable to the enhancement of NO production. Although there have been some inconsistent reports that NF-κB was activated without I-κBα degradation by X-irradiation [37] and that tyrosine phosphorylation of Iκ-B induced NF-κB activation without degradation [38], the results of MG132 and TLCK treatment showed the importance of I-κB degradation in the enhancement of NO production by γ-irradiation. NF-κB activation is reported to occur at much higher doses (~ 50 Gy) [18]; however, most significant NO production was observed at lower doses (6–10 Gy) as shown in Fig. 1. These results suggested that the activation of NF-κB was not sufficient for enhancement of NO production by γ-irradiation. Furthermore, as shown in Fig. 2A, NF-κB activation was transient and recovered 3 h after γ-irradiation. We added IFN-γ at 24 h after γ-irradiation. McKinney et al. [15] also reported that the enhancement of NO production was only manifest if the radiation was applied 24 h before the addition of IFN-γ and not 1 or 4 h, which was due to the production of TNF-α during 24 h after irradiation. Some cytokines such as TNF-α interleukin-1 and -6 are known to be induced by irradiation

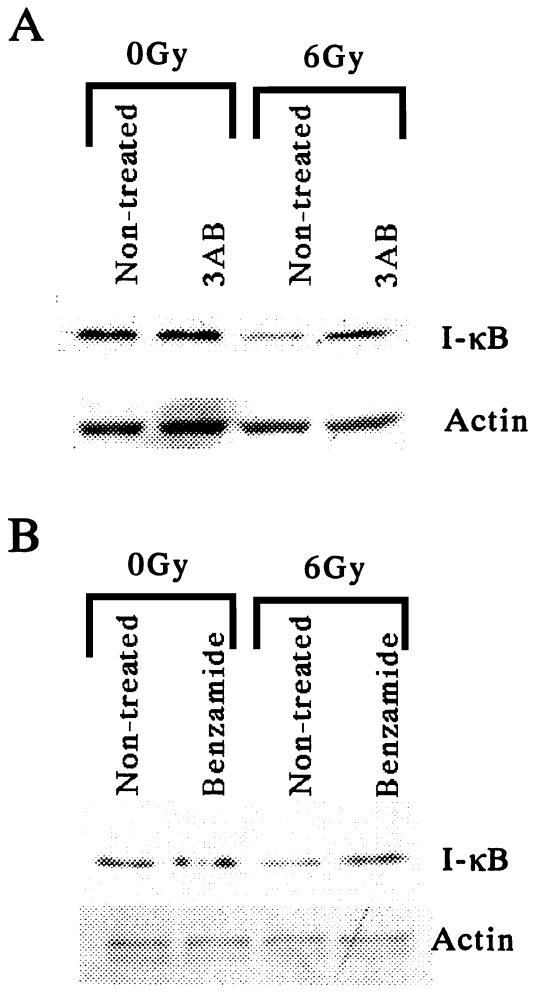

Fig. 7. Participation of PARP activation in I-κB degradation after γ-irradiation. After treatment with 3AB (A) or benzamide (B) (10 μM) for 1 h, RAW264.7 cells were γ-irradiated at a dose of 6 Gy and further cultured for 1 h. Cell lysates were subjected to SDS-PAGE followed by Western blotting with anti-I-κB and anti-actin antibodies.

[8–10,39,40], which were mediated by NF-κB activation [40]. These autocrine factors produced by NF-κB activation might act synergistically with IFN-γ and lead to enhancement of NO production. Therefore, we consider that NF-κB activation at the beginning by γ-irradiation is necessary for the enhancement of NO production, but not sufficient for enhanced NO induction after treatment with IFN-γ.

γ-Irradiation is well known to produce ROS such as hydroxyl radicals and superoxide anion radicals. Antioxidants NAC and pyrrolidine dithiocarbamate were reported to inhibit NF-κB activation [41–43]. However, no suppression of enhancement of NO production by γ-irradiation was observed after treatment with either SH reagent NAC or vitamin E derivative trolox. Enhancement of iNOS induction also did not change in the presence of NAC. These results indicated less participation of ROS induced by γ-irradiation in the enhancement of NO production. Furthermore, H_2O_2 treatment slightly enhanced NO production induced by IFN-γ, but to a lesser extent than the effect of γ-irradiation. We reported previously that H_2O_2 treatment enhanced NO production in resident peritoneal macrophages, the effect of which was also less marked than that of γ-irradiation [12]. On the other hand, Yoo et al. [13] reported that γ-irradiation potentiates iNOS induction in embryonic liver cells via production of H_2O_2. The results of the following experiments using DNA damaging agents suggested that the NO enhancement by H_2O_2 might be mediated via DNA damage.

Topoisomerases are nuclear enzymes that catalyze the formation of various topological isomers of DNA by transiently breaking and rejoining DNA. Camptothecin, an inhibitor of topoisomerase I that catalyzes single strand breakage, and etoposide, an inhibitor of topoisomerase II that catalyzes double strand breakage, induce DNA damage by stabilizing covalent complexes of enzyme and strand-cleaved DNA [26–28]. These agents enhanced NO production induced by IFN-γ in a dose-dependent manner. Cisplatin, which forms a DNA adduct including interstrand and intrastrand DNA cross-links [29,30], also enhanced NO production. Son and Kim [44] reported that cisplatin-exposed macrophages in vivo showed increased NO synthesis. On the other hand, hydroxyurea and vincristine that inhibit mitosis without DNA damage showed no enhancement. These results suggested that DNA strand breakages by γ-irradiation might induce the increase in NO production. Furthermore, treatment with camptothecin induced I-κB degradation, showing that DNA damage induced NO production via NF-κB activation. H_2O_2 was reported to induce NF-κB activation [43]. However, the anti-oxidant agent NAC did not suppress the increases in iNOS and NO production in the present study. This might have been due to the markable contribution of direct DNA damage by γ-irradiation compared with indirect DNA damage by ROS.

As the repair mechanism of DNA damage might be involved in the increase in NO production, we used the PARP inhibitors 3AB and benzamide [32,33]. PARP is known to be activated upon recognition of DNA strand breaks by γ-irradiation and H_2O_2 treatment [31,32]. The further enhancement of IFN-γ-induced NO production by γ-irradiation was suppressed in the presence of 3AB and benzamide. Le Page et al. [34] reported that 3AB inhibited iNOS induction through inhibition of NF-κB activation in RAW264.7 cells. Furthermore, PARP was reported to be important in NF-κB activation using PARP (−/−) murine fibroblast cells [45] and in NO production involving of NF-κB activation using peritoneal macrophages obtained from PARP (−/−) mice [46]. In the present study, I-κB degradation was clearly inhibited by 3AB and banzamide. These results suggested that the activation of DNA damage repair mechanisms after γ-irradiation enhanced NF-κB activity, which increased iNOS expression leading to NO production. Recently, Hassa et al. provided evidence that NF-κB activation required the direct interaction of PARP with NF-κB but not the PARP enzymatic activity and indicated that the inhibition of NF-κB by inhibitors of PARP enzymatic

activity, which was shown in the report by Le Page et al. [34], might be due to the mediation by other proteins, not by inhibition of PARP activity itself [47]. This confusion is not clarified at present.

Held et al. [48] reported that there are two distinct mechanisms of macrophage activation; the activation of signal transduction involving NF-κB and the autocrine activation by TNF-α and interleukin-1 production. In this study, we clarified that signal transduction was affected immediately after γ-irradiation, i.e. γ-irradiation induced DNA breakage, which enhanced PARP activity leading to NF-κB activation and then iNOS induction resulting in NO production. DNA breakage and transient activation of PARP and NF-κB are necessary but not sufficient for enhanced NO induction after treatment with IFN-γ. The transient activation of the pathway might lead to the autocrine activation by production of some cytokines, which could augment the IFN-γ-induced signals for NO production. Further studies are necessary to clarify the mechanism of autocrine activation.

Acknowledgements

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