



## Mechanism of protection of bystander cells by exogenous carbon monoxide: Impaired response to damage signal of radiation-induced bystander effect

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### ABSTRACT

A protective effect of exogenous carbon monoxide (CO), generated by CO releasing molecule tetrakis(carboxylate)dicarbonylruthenium (II) dimer (CORM-2), on the bystander cells from the toxicity of radiation-induced bystander effect (RIBE) was revealed in our previous study. In the present work, a possible mechanism of this CO effect was investigated. The results from medium transfer experiments showed that  $\alpha$ -particle irradiated Chinese hamster ovary (CHO) cells would release nitric oxide (NO), which was detected with specific NO fluorescence probe, to induce p53 binding protein 1 (BP1) formation in the cell population receiving the medium, and the release peak was found to be at 1 h post irradiation. Treating the irradiated or bystander cells separately with CO (CORM-2) demonstrated that CO was effective in the bystander cells but not the irradiated cells. Measurements of NO production and release with a specific NO fluorescence probe also showed that CO treatment did not affect the production and release of NO by irradiated cells. Protection of CO on cells to peroxynitrite, an oxidizing free radical from NO, suggested that CO might protect bystander cells via impaired response of bystander cells to NO, a RIBE signal in our research system.

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### 1. Introduction

Radiation-induced bystander effect (RIBE), which is defined as the response of cells to their irradiated neighbors, has become progressively more studied in the past decade for its cytotoxic and genotoxic changes beyond the irradiated area [1]. Radiation leads to production of RIBE signal(s) in the irradiated cells, and the released signal(s) diffused into the medium or transferred *via* gap junction intercellular communication can attack the bystander cells and cause DNA damage, chromosomal aberration, gene mutation, malignant transformation and tumor formation [1,2]. Studies on the mechanisms of RIBE have revealed that tumor growth factor (TGF)- $\beta$ , tumor necrosis factor (TNF)- $\alpha$ , nitric oxide (NO), interleukin 1 and interleukin 8, etc. might be produced and released by

the irradiated cells to act as intercellular RIBE signals to “attack” the bystander cells [1,2]. The RIBE phenomenon has challenged the conventional dogma of radiation protection. The presence of RIBE potentially can modify the cancer risk of environmental low-dose irradiation to human beings [2,3], and it also can potentially modify the cancer risk of normal tissues after the radiotherapy since the response of the normal tissues surrounding the targeted tumor might be modified by the bystander effect signal(s) released by the irradiated tumor tissues. As such, minimizing the RIBE in normal tissues has important implications for current and future radiotherapy strategy and procedures.

The physiological and pharmacological functions of carbon monoxide (CO) at low concentrations in circulatory, nervous, respiratory, reproductive, gastrointestinal systems and various organs have hinted its potential applications in therapy [4]. Manipulating heme oxygenase-1 (HO-1) gene to produce endogenous CO, inhaling exogenous CO or employing CO releasing molecule (CORM) have been demonstrated to be effective in animal models against inflammation, hypertension, rejection of organ transplantation, vascular injury and ventilator-induced lung injury; and gas inhalation strategies can benefit therapies for inflammatory diseases of the human lung [4]. The application of exogenous CO produces similar effects as HO in many models [4]. Numerous studies reported that low concentration CO has biological functions such as protection against cell death, anti-inflammatory effects, protection

**Abbreviations:** 53BP1, p53 binding protein 1; CHO, Chinese hamster ovary; CO, carbon monoxide; CORM, CO releasing molecule; COX-2, cyclooxygenase-2; DAF-FM, 4-amino-5-methylamino-2',7'-difluorescein; DIA, DSB-inducing ability; D-NMMA, N<sup>G</sup>-methyl-D-arginine; DSB, DNA double strand break; FBS, fetal bovine serum; HO, heme oxygenase; L-NMMA, N<sup>G</sup>-methyl-L-arginine; NOS, nitric oxide synthase; NO, nitric oxide; RFU, relative fluorescence unit; O<sub>2</sub><sup>-</sup>, superoxide anion; TGF, tumor growth factor; TNF, tumor necrosis factor; ONOO<sup>-</sup>, peroxynitrite; RIBE, radiation-induced bystander effect; ROS, reactive oxygen species.

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against oxidative injury, inhibition of cell proliferation, neurotransmission, and tolerance of organ transplantation [4]. In relation to these functions of CO, studies on the mechanisms showed that CO could influence cells *via* attenuating the production of many factors, which were also demonstrated to be involved in RIBE signaling pathway in previous studies [2], such as reactive oxygen species (ROS), NO, cyclooxygenase-2 (COX-2) and cytokines, etc.

A protective effect of low concentration CO, released by tetracobalicyanoruthenium (II) dimer ((Ru(CO)<sub>3</sub>Cl<sub>2</sub>)<sub>2</sub>, CORM-2) [5], to bystander cells from the damage (DNA double strand break, DSB, and micronuclei) of RIBE in a mixed co-culture system was first reported in our previous study [6]. In the dose range of 5–20 μM of CORM-2 (corresponding to 3.5–14 μM CO), the protective effect increased with the CO concentration. In this co-culture system, medium-mediated RIBE signal transduction might be dominant, since the irradiated and bystander Chinese hamster ovary (CHO) cells were mixed and cultured together at a low density (~260 cells/mm<sup>2</sup>) in such a way that their physical contact was rare. In this study, we focused on the effect of CO (CORM-2) on the production, release and action of NO, which acted as an intercellular signal for RIBE in our system. The results showed that low concentration CO (14 μM) did not significantly affect the amount of NO production and release, but on the other hand the response of bystander cells to NO/peroxynitrite stress was impaired or blocked by CO.

## 2. Materials and methods

### 2.1. Cell culture

CHO-K1 cells were cultured in MEM (Gibco, Grand Island, NY, USA): F-12 (Gibco, Grand Island, NY, USA) (1:1) mixed medium supplemented with 10% fetal bovine serum (FBS, Gibco, Grand Island, NY, USA), 100 U/ml penicillin and 100 mg/ml streptomycin (Gibco, Grand Island, NY, USA). The cells were maintained in a humidified atmosphere with 5% CO<sub>2</sub> at 37 °C. Cells under 70–80% confluence were trypsinized and 5 × 10<sup>4</sup> cells were seeded into each specially designed ring (made of stainless steel, inner diameter = 9.0 mm) holding a 3.5 μm mylar film bottom. The cells were then incubated for 24 h for irradiation, the time at which the cells were under full confluence condition. In addition, 6 × 10<sup>4</sup> cells were seeded into each well of 6-well culture plate at the same time. The cells were then incubated for 24 h, at which time the number of cells was 2 × 10<sup>5</sup>, to receive the medium.

### 2.2. Alpha-particle irradiation

At the cell layer, the average energy of α particles derived from an <sup>241</sup>Am irradiation source with an activity of 1.86 × 10<sup>5</sup> Bq was 5.16 MeV, and the α particles were delivered at a dose rate of 4.77 mGy/s. The source was purchased from Eckert & Ziegler (catalog number is AF-241-A1). This was a thick source which produced a spectrum. The active circular area had a diameter of 5 mm. The distance between the base of the irradiation dish and the surface of the source was 1 mm. The fluence of the alpha particles from the source was experimentally determined by irradiating polyallyldiglycol carbonate (PADC) films for a specific period of time and by counting under an optical microscope the recorded tracks after chemical etching. The number of cells at ~100% confluence was also counted in a field of view under an optical microscope to determine the area per cell. The dose to the cells was 100 mGy. Based on the calculation, each CHO cell under full confluence was irradiated by one α particle on average.

### 2.3. CO (CORM-2) treatment

CO was generated by Ru(CO)<sub>3</sub>(Cl<sub>2</sub>)<sub>2</sub> (Sigma, St. Louis, MO, USA), which was a classic CO releasing molecule (CORM-2) [5]. This chemical could release CO by being dissolved in the medium and for each mole of CORM-2, 0.7 mole of CO was liberated. The stock solution (50 mM) was freshly prepared by dissolving CORM-2 in dimethyl sulfoxide (DMSO, Sigma, St. Louis, MO, USA).

### 2.4. Immunofluorescence of p53 binding protein 1 (53BP1) and DSB measurement

In this work, we employed fluorescence detection of foci formation using 53BP1 as a marker of DSB [7]. Immunocytochemical staining of cells was performed as described by Aten et al. [8]. Briefly, the cells were fixed in 2% buffered paraformaldehyde (Sigma, St. Louis, MO, USA) at chosen time points after cell mixture for 15 min at room temperature, and were then rinsed three times with PBS again. Prior to immunocytochemical staining, the cells were incubated for 30 min in TNBS solution (PBS supplemented with 0.1% Triton X-100 and 1% FBS) to improve their permeability.

The cells were then incubated with rabbit anti-53BP1 antibody (Abcam, Cambridge, MA, USA) at 1:200 in PBS\* (PBS supplemented with 1% FBS) for 90 min, washed in TNBS for 3 × 5 min, and incubated in PBS\* containing secondary anti-rabbit Alex fluor-488 (Invitrogen, Eugene, OR, USA) for 60 min. After another wash with TNBS for 3 × 5 min, the cells were counterstained with Hoechst 33342 at a concentration of 20 μg/ml for 20 min at room temperature. After a final wash with PBS, the stained cells were mounted by fluoroguard antifade reagent (Bio-Rad, Hercules, CA, USA). At least 500 cells were counted using a fluorescent microscope (Nikon Eclipse, Japan). The cells containing three or more BP1 foci were regarded as positive cells [9].

### 2.5. Determination of intercellular and intracellular NO with fluorescence probe

To investigate whether irradiation could induce production and release of NO in CHO cells and the specific function or role of NO in the signal transduction, 4-amino-5-methylamino-2',7'-difluorescein (DAF-FM), a fluorescence probe for quantifying low concentrations (detection limit, 3 nM) of NO, was used in this study [11]. This chemical is not cell-permeant and so its diacetate, DAF-FM diacetate, has been developed, which is cell-permeant and passively diffuses across the cellular membrane. Once inside cells, it is deacetylated by intracellular esterases to become DAF-FM, which will be "caged" in cells [11].

In the detection of NO production in the irradiated cells with DAF-FM diacetate, at 45 min before irradiation the irradiated cells were incubated with 10 μM DAF-FM diacetate (Invitrogen, Eugene, OR, USA) and just before irradiation the excessive fluorescent probe was removed and the cells were rinsed twice with fresh culture medium. At 1 h post irradiation, the cells were lysed and the fluorescence density of solution was determined.

To detect the intercellular NO induced by irradiation, DAF-FM diacetate (10 μM) was added into medium and cells were incubated for 45 min at 37 °C. After loading with the probe, the cells were washed twice with warm D-Hanks' solution, and were then irradiated. To determine the contents DCF-FM T, the product of DAF-FM react with NO, in the medium and in the cells, 0.02% Tiron X-100 was added immediately after irradiation, and the cells were incubated for 20 min at 37 °C so the DCF-FM T would leave the cells. The medium was then decanted and centrifuged for 5 min at 700 g, and the samples were put on ice prior to fluorescence measurement. The samples were determined with a microplate fluorescence reader (polarstar optima, BMG labtechnologies) with excitation and emission wavelengths of 495 and 515 nm, respectively [10].

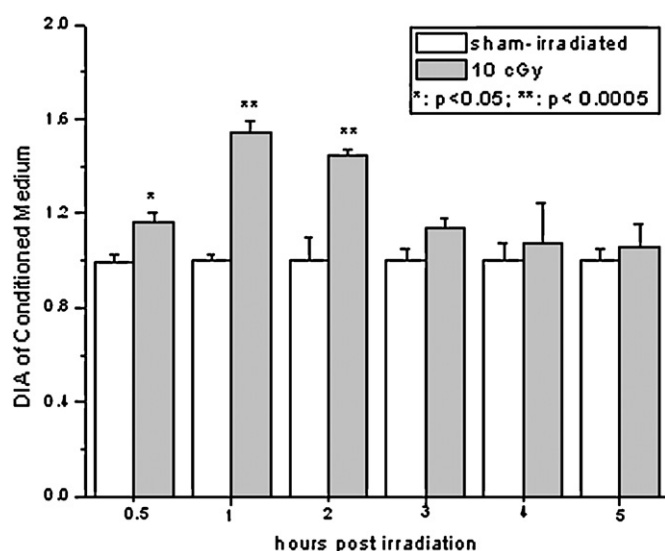
To detect the released NO after irradiation, the culture medium was replaced with D-Hanks' solution containing 2 μM DAF-FM (Invitrogen, Eugene, OR, USA) before irradiation. At 1 h post irradiation, the solution was harvested to measure its fluorescence with a microplate fluorescence reader (polarstar optima, BMG labtechnologies) (excitation/emission = 495/515 nm) [12].

To detect the NO level in the bystander cells which received the medium, the bystander cells were incubated with 2 μM DAF-FM diacetate for 45 min at 37 °C. After the excessive dye was removed, the cells were rinsed with pre-warmed D-Hank's buffer solution twice, and the fluorescence images of at least 150 randomly selected cells per sample was captured using a fluorescent microscope (Nikon Eclipse, Japan). The exposure conditions were the same in each experiment. The fluorescence intensity of bystander cells was measured and analyzed with the software Image J (<http://rsb.info.nih.gov/ij/>) [12].

## 3. Results

### 3.1. Timing of BP1-inducing damage signal released from irradiated cells

Our previous study [6] showed an increased BP1 formation at 5 h post irradiation in the bystander cells, which were co-cultured with cells irradiated with a dose of 100 mGy, and this result indicated that some soluble RIBE signal factor(s) was released into the shared medium in the first several hours after irradiation. To study the possible mechanism of CO protection, the timing of release of DSB (BP1)-inducing damage factor/signal from the irradiated CHO cells was measured. The medium transfer system, which was used often in the study of RIBE signal release [3], was employed to measure the intensity of damage signal. After irradiation, the medium conditioning the irradiated cell population was harvested at various time points (0.5, 1, 2, 3, 4, 5 h) and transferred to the recipient cells cultured in a 6-well plate. The fraction of BP1, one biomarker of DSB, was measured by protein immunofluorescence. The DSB (BP1)-inducing ability (DIA) of the conditioned medium was presented as the fold increase in BP1 positive cells compared to that of the cells which received the conditioned medium from the sham-irradiated cell population. As illustrated in Fig. 1, the DIA of the



**Fig. 1.** DSB (BP1)-inducing ability (DIA) in unirradiated cells of the medium harvested at various time points post irradiation from cells irradiated with a dose of 100 mGy of alpha-particles. The DIA was presented in terms of the fold increase of BP1 positive cells compared to that of cells treated by the conditioned medium from sham-irradiated cells. Data were pooled from three independent experiments. Significance in the differences between the sample and its sham-irradiated control was determined, and  $p < 0.05$  was considered statistically significant.

conditioned medium showed a time-dependent manner and the DIA of the conditioned medium reached the peak at 1 h post irradiation. As such, in this study, 1 h post irradiation was chosen as a representative time point to study the mechanism of the protective effect of CO in a medium transfer system.

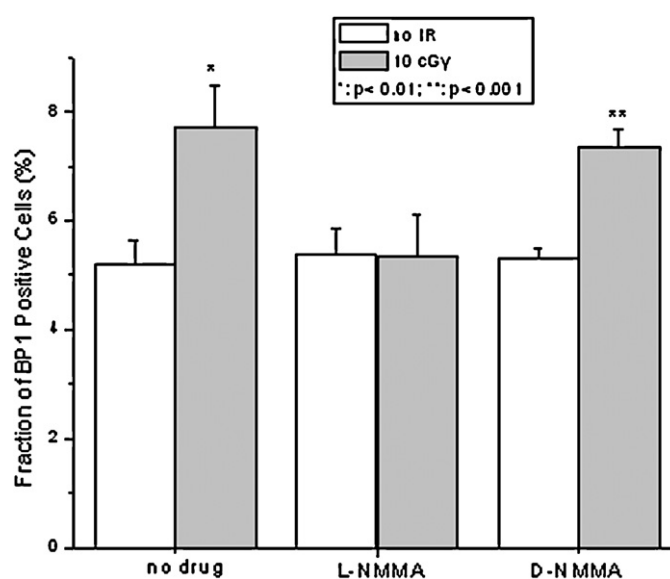
### 3.2. NO-mediated transduction of RIBE

An inhibitor of nitric oxide synthase (NOS),  $N^G$ -methyl-L-arginine (L-NMMA), was used to study the potential involvement of NO [13,14]. The irradiated or bystander cells were treated with L-NMMA (100  $\mu$ M, Invitrogen, Eugene, OR, USA) or its inactive D-enantiomer,  $N^G$ -methyl-D-arginine (D-NMMA, 100  $\mu$ M, Invitrogen, Eugene, OR, USA), at 1 h before irradiation or medium transfer, and the inhibitor was kept in the medium until the samples were fixed for further studies. Fig. 2 showed that, with the treatment of L-NMMA, the DIA of the medium transferred at 1 h after irradiation decreased distinctly to the level of sham-irradiated control. This result indicated that NO, which was an important signal factor, mediated the transduction of RIBE in the medium transfer system.

### 3.3. NO released from irradiated cells acted as intercellular RIBE signal

It was investigated whether irradiation could induce production and release of NO in CHO cells, and the specific function or role of NO in the signal transduction was also examined. 4-amino-5-methylamino-2',7'-difluorescein is a fluorescence probe for quantifying low concentrations (detection limit, 3 nM) of NO [11]. This chemical is not cell-permeant and so its diacetate, DAF-FM diacetate, was developed. DAF-FM diacetate is cell-permeant and passively diffuses across the cellular membrane. Once inside cells, it is deacetylated by intracellular esterases to become DAF-FM, which will be "caged" in cells [11].

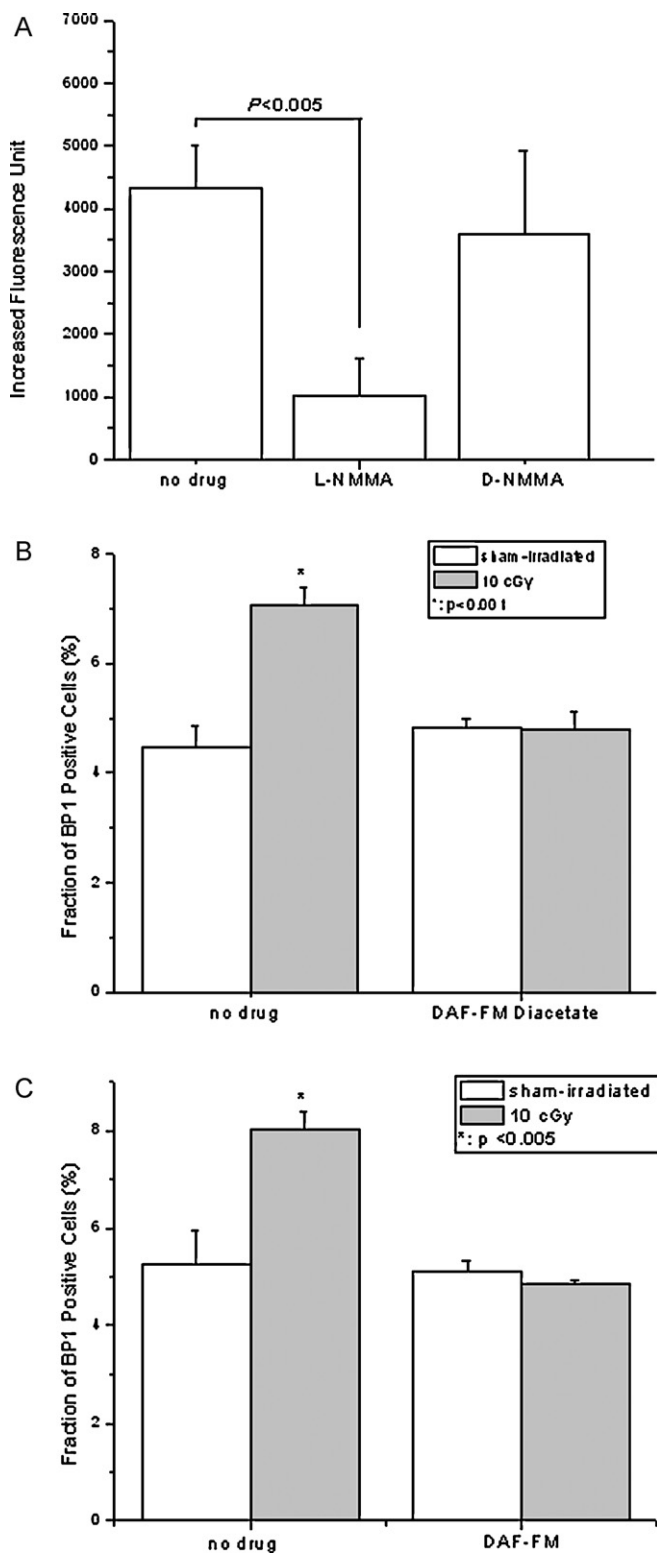
DAF-FM diacetate was used to detect the NO produced in the irradiated cells [10]. At 45 min before irradiation, the irradiated cells were incubated with 10  $\mu$ M DAF-FM diacetate and just before irradiation the excessive fluorescent probe was removed and the



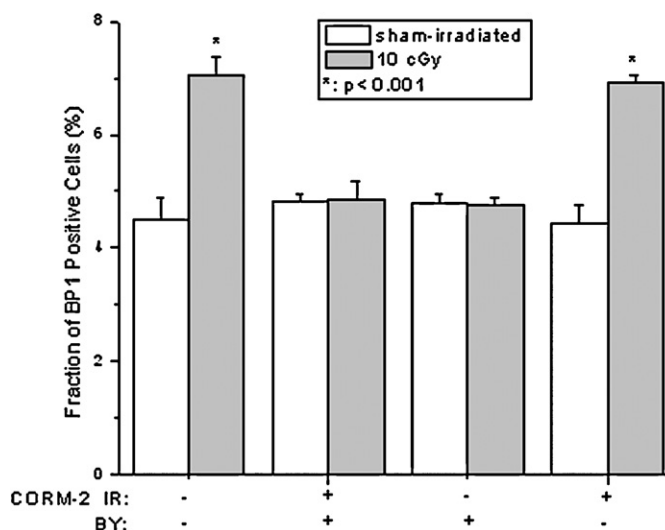
**Fig. 2.** NO-mediated transduction of RIBE in the medium transfer system. The effect of L-NMMA, an inhibitor of NOS, on the DIA of the conditioned medium harvested at 1 h post irradiation (100 mGy) was measured with BP1 immunofluorescence and comparison with inactive D-NMMA. Data were pooled from three independent experiments. Significance in the differences between the sample and its sham-irradiated control was determined, and  $p < 0.05$  was considered statistically significant.

cells were rinsed twice with fresh culture medium. At 1 h post irradiation, the cells were lysed and the fluorescence density of solution was determined. Fig. 3A showed that irradiation could induce NO production distinctly in the first 1 h post irradiation. It was then natural to think about the role of NO generated in the irradiated cells on the transduction of RIBE. The medium from irradiated cells, which had been labeled with 10  $\mu$ M DAF-FM diacetate, was harvested 1 h post irradiation and transferred to test its DIA. Fig. 3B showed that the irradiated cells labeled with DAF-FM diacetate lost their capability in inducing BP1 formation in bystander cells, which was due to the reaction of the NO induced by radiation with the DAF-FM "caged" in the irradiated cells. This indicated the importance of irradiation-induced NO which was necessary for the transduction of RIBE.

In a further study, non-cell-permeant DAF-FM, i.e., not the diacetate form, was used to detect whether the irradiated cells would release NO into medium. Before irradiation, the medium in the irradiated population was replaced with D-Hanks' solution containing 2  $\mu$ M DAF-FM. If NO was released by irradiated cells it would react with DAF-FM to give fluorescence. At 1 h post irradiation the DAF-FM solution was harvested and its fluorescence intensity was detected. A distinct increase ( $2300 \pm 385$  relative fluorescence unit, RFU) of fluorescence intensity was detected. This meant that NO induced by irradiation could be released into medium. NO is cell-membrane permeable and easy to diffuse over a relatively long distance to act as a signal molecule because of its relative chemical stability [15,16]. It is then natural to study the relationship between the released NO and RIBE transduction. DAF-FM (2  $\mu$ M at final concentration) was added into the medium before irradiation and transferred with the conditioned medium at 1 h post irradiation. Fig. 3C showed that the presence of DAF-FM in the conditioned medium distinctly decreased the DIA of the conditioned medium. Furthermore, a significant increase of NO ( $1.135 \pm 0.065$  fold of control,  $p = 0.023$ ) was also detected with the NO probe, DAF-FM diacetate, in the cells having received the conditioned medium harvested at 1 h post irradiation. From these results, it was suggested that NO could be induced by irradiation and the induced NO would



**Fig. 3.** Generation and release of NO induced by irradiated cells after 100 mGy irradiation: (A) detection of NO production in irradiated cells with fluorescence probe, DAF-FM diacetate; (B) the function of NO generated by irradiated cells on the DIA of conditioned medium, where the irradiated cells were treated with or without DAF-FM diacetate before irradiation; (C) the function of NO released by irradiated cells on the DIA of conditioned medium. Data were pooled from three independent experiments. Significance in the differences between the sample and its sham-irradiated control was determined, and  $p < 0.05$  was considered statistically significant.



**Fig. 4.** Effect of treating irradiated (100 mGy) or bystander cells with CORM-2 on the transduction of RIBE. “+” means treatment with CORM-2 and “-” means without CORM-2 treatment; “IR” means irradiated cells and “BY” means bystander cells. Data were pooled from three independent experiments. Significance in the differences between the sample and its sham-irradiated control was determined, and  $p < 0.05$  was considered statistically significant.

be released into medium as a RIBE intercellular signal molecule to induce BP1 formation and NO stress in the bystander cells.

### 3.4. CO protects bystander cells from damage signal(s) in transferred medium

The effect of CO on the release of RIBE signal from the irradiated cell population and the response of recipient cell population were tested, respectively. CORM-2 was added into the medium to treat the irradiated cells at 1 h before irradiation, or to treat the bystander cells (receiving the medium) at 1 h before medium transfer, respectively. Fig. 4 showed that treating both irradiated and bystander cells caused distinct decreases in BP1 induction in the bystander cell population. However, treating only irradiated or bystander cells showed different effects. The treatment of bystander cells with CO showed a significant protective effect from the damage of RIBE, but treatment in irradiated cells did not. This result indicated that CO treatment did not affect the release of RIBE signal from the irradiated cells, and the effect of CO might occur in the bystander cells. In other words, CO might act on the response of bystander cells to RIBE signal.

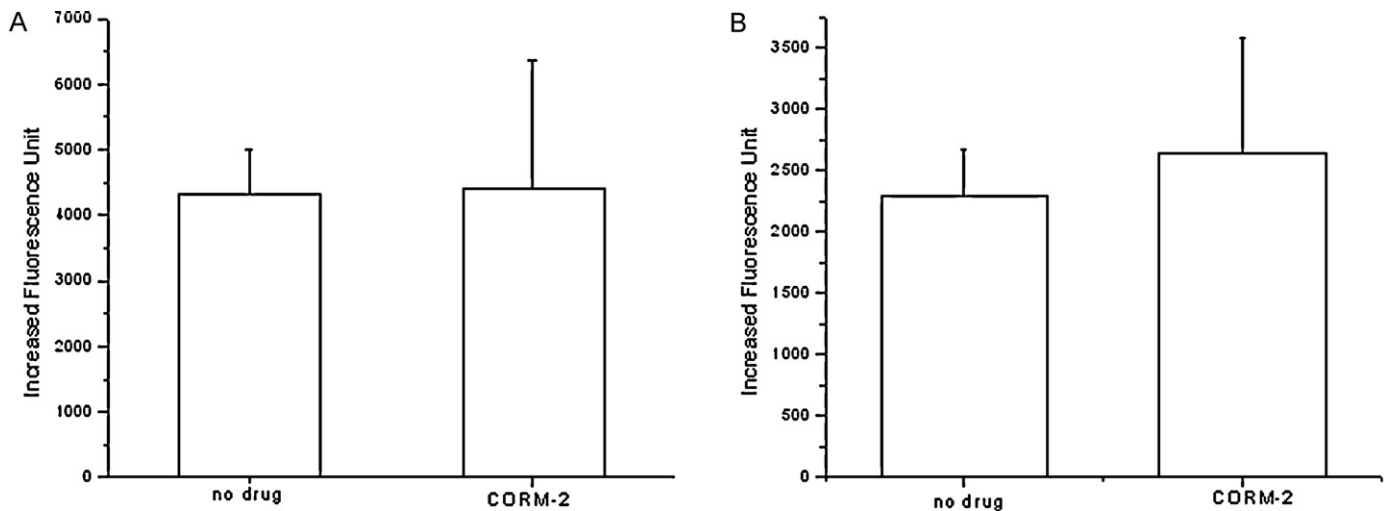
### 3.5. Effect of CO on the production and release of NO in irradiated cells

The effect of CO on the production and release of NO at 1 h post irradiation was assayed. The results in Fig. 5A showed that the NO content in irradiated cells with or without CORM-2 treatment were not significantly different. The released NO after irradiation was determined with DAF-FM. The results in Fig. 5B showed that there was no significant difference between the release of NO from the irradiated cells with or without CORM-2 treatment. These results indicated that treatment with a low concentration (14  $\mu\text{M}$ ) of CO did not affect the generation and release of NO in irradiated cells.

### 3.6. Impairment of response of cells to nitrosative stress induced by CO

An enhanced level of superoxide anion ( $\text{O}_2^-$ ) was detected in the bystander cells in our previous study [6] and other studies [3].





**Fig. 5.** Effect of CORM-2 treatment on (A) the production of NO in the 100 mGy irradiated cells, and (B) the release of NO by the irradiated cells. Data were pooled from three independent experiments. Significance in the differences between the sample and its sham-irradiated control was determined, and  $p < 0.05$  was considered statistically significant.

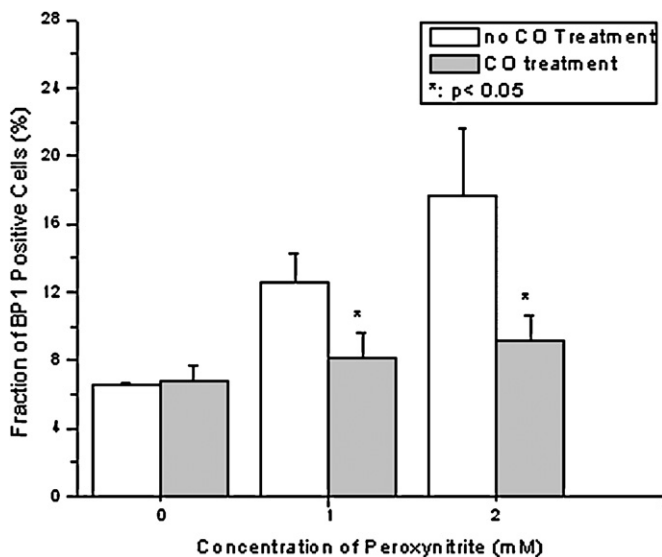
The toxicity of NO is linked to its ability to quickly combine with superoxide anions ( $O_2^-$ ) to form peroxynitrite ( $ONOO^-$ ), an oxidizing free radical which has a relatively longer life and can cause DNA fragmentation and lipid oxidation [17,18]. To investigate whether a low level CO could protect cells from the damage of  $ONOO^-$ , synthesized  $ONOO^-$  [19] was used to treat CHO cells and the cells were then fixed for BP1 immunofluorescence. The results in Fig. 6 showed that CORM-2 (20  $\mu$ M) treatment could distinctly decrease the formation of BP1 upon  $ONOO^-$  treatment. This result implied that pretreatment with CO could impair the response of cells to nitrosative stress induced by  $ONOO^-$  and protect the cells from the damage of nitric stress.

#### 4. Discussion

In the present work, a possible mechanism of protection of bystander CHO cells by low concentrations of CO from the damage of RIBE was explored. The results indicated that  $\alpha$ -particle irradiation

could induce production of NO in the irradiated cells, and the generated NO could be released into the medium as an intercellular signal for RIBE to cause nitrite stress and DNA damage (revealed by BP1 formation) in the bystander cells. Low concentrations of CO did not affect the generation and release of NO, but CO significantly impaired the response of bystander cells to NO stress to avoid the damage of RIBE.

Studies on the mechanisms of RIBE revealed that some chemical or biological factors such as NO, TGF- $\beta$ 1, TNF- $\alpha$ , etc., might act as intercellular signal molecules for RIBE to mediate the transduction of RIBE [1] in various specific research systems. Many previous studies reported an important role of NO in mediating the transduction of RIBE [2,20]. Excessive NO was directly measured in the bystander cells [21–23] or in the medium harvested from a AG 1522 cell population irradiated by low dose  $\alpha$ -particles [24] as a signaling molecule to mediate RIBE. The toxicity of NO is linked to its ability to combine with  $O_2^-$  rapidly to form peroxynitrite ( $ONOO^-$ ), an oxidizing free radical that can cause DNA fragmentation and lipid peroxidation, protein nitration and cell death [18]. The present study also revealed that, at 1 h post irradiation, the irradiated cells could release NO to induce NO stress in bystander CHO cells. Our previous study reported that irradiated fully confluent AG 1522 cells secreted NO into the medium to induce RIBE at an early time of RIBE transduction [24]. Matsumoto et al. [25] also detected a significantly increased amount of nitrite, a main product of NO, in the culture medium several hours after X-ray irradiation. The point in the NO-mediating RIBE signaling pathway at which the low-concentration CO (CORM-2) functioned was further investigated. The results on the effect of CO (CORM-2) treatment on the DIA of conditioned medium showed that only CO (CORM-2) treatment on irradiated cells did not affect the DIA, while only CO (CORM-2) treatment on the bystander cells attenuated the DIA of the conditioned medium received by these bystander cells. This implied that the critical function point of CO (CORM-2) protection was likely in the bystander cells but not in the irradiated cells. Detection of the intercellular signal molecule NO for RIBE, generated in the irradiated cells and released into the medium, with its fluorescence probe, DAF-FM or DAF-FM diacetate, also showed that CO (CORM-2) treatment did not affect the generation and release of NO. In other words, the action point of CO (CORM-2) treatment was on the section of effect but not that of initiation and mediation in the whole transduction of RIBE signal in the present research system.



**Fig. 6.** Protective effect of CORM-2 treatment on peroxynitrite-induced production of BP1. Data were pooled from three independent experiments. Significance in the differences between the sample and its sham-irradiated control was determined, and  $p < 0.05$  was considered statistically significant.

Although CO was revealed to have the potential to reduce the activity or expression of inducible NOS [4], it seemed that the concentration (14  $\mu$ M) of CO used in this study was much less than the concentration which was effective in manipulating the NOS system [26]. The direct measurement of generated NO with the fluorescence probe (Fig. 5A) also proved that the expression or activity of NOS in the irradiated cells were not affected by CO. CO was reported to be able to protect cells from the cytotoxicity of NO/peoxynitrite. Li et al. [27] reported that CO rescued PC12 cells from NO/peoxynitrite stress *via* inducing an increase in glutamate-cysteine ligase, the rate-limiting enzyme in GSH biosynthesis, to elevate the level of important antioxidant GSH to maintain the intracellular redox balance. In our previous study CO could modulate the function of NADPH oxidase, which has been revealed to be the main source of  $O_2^-$  in the bystander cells [1,2], to attenuate the production of  $O_2^-$  *via* binding on the heme structure of this oxidase to inhibit its function [6], and the attenuation of superoxide anion level might be helpful to protect bystander cells from the toxicity of enhanced NO. CO was also reported to protect PC12 cells against peroxynitrite-induced apoptotic death through preventing the depolarization of mitochondrial transmembrane potential to control the release of cytochrome c [28].

As regards other reported intercellular signal molecules, such as TGF- $\beta$ 1 and TNF- $\alpha$ , although Mothersill's group and other groups did not detect significantly increased release of TGF- $\beta$ 1 and TNF- $\alpha$  in the medium harvested at early time (1–4 h) after irradiation, these two cytokines possibly played downstream roles in RIBE transduction [29]. TGF- $\beta$ 1 was reported as a key mediator to induce intracellular ROS production and cell proliferation in the bystander population [9,30]. It was been reported that low-concentration CO could restrain TGF- $\beta$ 1-induced cell proliferation, while TGF- $\beta$ 1 induced HO-1 expression to increase CO production, and then HO-1 up-regulation and CO production might serve as a negative feedback mechanism to function at cells in a physiological range [31]. TNF- $\alpha$  was reported to activate the nuclear factor-kappaB/COX-2 signal pathway to induce inflammation-type effect in bystander cells [2]. Various studies revealed that CO might not only inhibit the production of proinflammatory cytokine TNF- $\alpha$  but also protected cells from the DNA damage, cytotoxicity and apoptosis induced by TNF- $\alpha$  [4].

### Conflict of interest

The authors declare no conflict of interest.

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