Cyclooxygenase-2-mediated upregulation of heme oxygenase 1 mitigates the toxicity of deuterium-tritium fusion radiation

XIAOYAO YANG1,2*, HUI LIU1*, XU JIANG1,2, CHUFENG JIN1, ZHAO XU1, TAOSHENG LI1, ZHIGANG WANG1 and JUN WANG1

1Key Laboratory of High Magnetic Field and Ion Beam Physical Biology, Key Laboratory of Neutronics and Radiation Safety, Chinese Academy of Sciences, Hefei, Anhui 230031; 2University of Science and Technology of China, Hefei, Anhui 230026, P.R. China

Received March 27, 2018; Accepted July 30, 2018

DOI: 10.3892/ijmm.2018.3799

Abstract. Utilizing the energy released from the nuclear fusion of deuterium with tritium (D-T) may be an important method of supplying energy in the future. The ionizing radiation emitted from nuclear fusion is a potential health risk to humans, including scientists who are currently performing nuclear fusion experiments and the employees of fusion nuclear plants, in the future. However, there have been few reports on the biological effects of fusion radiation. In the present study, using the High Intensity D-T Fusion Neutron Generator, the DNA damage and its regulation in normal human fibroblasts exposed to fusion radiation were investigated. Heme oxygenase 1 (HO-1), which is reported to induce anti-inflammatory activity, was upregulated in the irradiated cells. Pretreatment with the HO-1 inhibitor, protoporphyrin IX zinc (II), exacerbated double strand break formation following exposure to fusion radiation. The expression of cyclooxygenase-2 (COX-2) contributed to the upregulation of HO-1, as demonstrated by the result that its inhibitor, NS-398, inhibited the induction of HO-1 in irradiated cells. It was further clarified that the ataxia telangiectasia mutated DNA damage response was activated and it stimulated the phosphorylation of p38 mitogen-activated protein kinase, which was responsible for the upregulation of COX-2 and HO-1. These results provide novel information on fusion radiation-induced biological effects and potential targets for decreasing the associated health risks.

Introduction

Exposure to ionizing irradiation produces DNA damage in cells, including DNA single strand breaks (SSBs) and double strands breaks (DSBs). While the majority of the SSBs can be repaired, DSBs are particularly hazardous to the cell due to the possibility of them resulting in rearrangement of the genome, which is lethal to the cell (1). Radiation induces the upregulation of proteins, including nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB), inducible nitric oxide synthases, tumor necrosis factor-α, interleukin-1 (IL-1) and IL-6, which are associated with inflammatory responses; additionally, this may result in DNA damage and damage to non-targeted tissues, and result in increased risk of carcinogenesis (2).

Heme oxygenase 1 (HO-1), an antioxidant enzyme that exhibits low basal expression levels in the majority of cells and tissues, is notably upregulated by a variety of oxidative stress stimuli. The upregulation of HO-1 is generally considered to be an adaptive cellular response against the toxicity of oxidative stress, and has been recognized to exhibit important anti-inflammatory functions (3-5). Previous studies have demonstrated that the targeted overexpression of HO-1 demonstrated beneficial effects in various experimental animal models of inflammation (6,7). The upregulated gene expression of HO-1 is mediated by a network of signaling pathways, among which mitogen-activated protein kinases (MAPKs) serve a primary role (8). Cyclooxygenase-2 (COX-2), the inducible form of COX, is activated by growth factors and cytokines in order to catalyze the conversion of arachidonic acid to prostaglandins (9). The activity of COX-2 is associated with reactive oxygen species production and inflammatory signs in cells, which are important in mediating radiation-induced biological effects (10). The expression of COX-2 can be activated by ionizing radiation and its upregulation is indicated to be associated with the decreased sensitivity of cells to radiation (11-13). As HO-1 and COX-2 contribute to radiation protection and are involved in the equilibrium of oxidative stress, it is hypothesized that interconnections exist between them in irradiated cells, which requires investigation.

The nuclear fusion of deuterium with tritium (D-T) releases notable energy, and investigations into using fusion for the production of electricity has been pursued for decades.
A number of experimental fusion reactors are under development globally. As fusion reaction produces large quantities of ionizing radiation, including high energy neutron and photon, biological investigations associated with the health risks of fusion radiation are important and necessary for the benefit of scientists and workers (14). In the present study, whether HO-1 and COX-2 are involved in the regulation of damage caused by fusion radiation was investigated, and their upstream regulators were identified.

Materials and methods

Chemicals. KU55933 was purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA), SB203580 was purchased from Selleck Chemicals (Houston, TX, USA), NS-398 was purchased from Abcam (Cambridge, UK) and protoporphyrin IX zinc (II) (ZnPP) was purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). These chemicals were dissolved in 100% dimethyl sulfoxide and stored in small aliquots at 20°C. Protease inhibitor cocktail and protein inhibitor cocktail were purchased from Roche Diagnostics GmbH (Mannheim, Germany) and Sigma-Aldrich (Merck KGaA), respectively.

Normal human lung fibroblasts (NHFLs), an adherently grown human primary lung fibroblast, were purchased from BeNa Culture Collection (Beijing, China). The NHLF cells were cultured in Dulbecco’s modified Eagle’s medium/F12 (GE Healthcare Life Sciences, China). The NHLF cells were cultured in dulbecco’s modified Eagle’s medium/F12 (GE Healthcare Life Sciences, Little Chalfont, UK) with 10% fetal bovine serum (Clark Bioscience, Richmond, VA, USA) and grown in a humidified 5% CO₂ incubator at 37°C. The neutron radiation appliance was a High Intensity D-T Fusion Neutron Generator, which was developed at the Institute of Nuclear Energy Safety Technology, Chinese Academy of Sciences (Hefei, China) by the Fusion Design and Study team. The production and the property of the fusion neutron were described previously by Wu (15). Radiation doses (0.01, 0.012, 0.06, and 1.2 Gy) were adjusted by setting the cells at different linear distances from the neutron source.

Immunofluorescence staining of γ-H₂A histone family member X (γ-H₂AX). The NHFL cells were cultured on 0.17 mm-thick glass-bottom cell culture dishes and exposed to neutron radiation at the desired time points (0, 0.5, 1, 3, 6, and 12 h), the cells were washed with PBS and fixed with 4% paraformaldehyde at room temperature for 30 min. Subsequently, the cells were washed with PBS and permeated with 0.5% Triton X-100 at room temperature for 30 min. Following blocking with PBST (0.1% Triton X-100) containing 1% bovine serum albumin (BSA; Sangon Biotech Co., Ltd., Shanghai, China) and 0.1% Triton X-100 at 37°C for 1 h, the cells were incubated with anti-γ-H₂AX antibody (1:400; cat. no. 2577; Cell Signaling Technology, Inc., Danvers, MA, USA), which was diluted in PBST containing 1% BSA at 4°C overnight with gentle agitation. The dishes were then washed three times with PBST for 5 min. Alexa Fluor-594-conjugated goat anti-rabbit IgG (1:800; cat. no. 115-035-003; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) diluted in PBST containing 1% BSA was added into the samples. Following incubation for 2 h at room temperature, the cells were washed three times with PBST for 15 min and counterstained with Hoechst 33342. Images were captured using a Olympus IX83 microscope with a 40X air-objective (Olympus Corporation, Tokyo, Japan) and analyzed with Image J1.49 software (National Institutes of Health, Bethesda, MD, USA). For γ-H₂AX quantification, images captured in the Hoechst 33342 channel were used to define the nuclear region. The fluorescence intensity of γ-H₂AX in the defined nuclear region was measured to reflect the levels of DNA DSB. At least 500 cells were analyzed for each sample.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. Total RNA (100 ng/µl) was extracted using RNAiso reagent (Takara Bio, Inc, Otsu, Japan). The total RNA was added into the reaction mixture from One-Step SYBR® PrimeScript™ PLUS RT-PCR kit. The reaction mixture was as follows: Reaction volume, 20 µl; 2X One Step SYBR RT-PCR Buffer, 10 µl; Takara Ex Taq HS Mix, 1.2 µl; PrimeScript PLUS RTase Mix, 0.4 µl; Forward Primer (10 µM), 0.8 µl; Reverse Primer (10 µM), 0.8 µl; Rox Reference Dye, 0.4 µl; Total RNA, 2 µl; RNase Free ddH₂O, 4.4 µl. RT-qPCR analysis was performed on a StepOne™ Plus Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to 2ΔΔCt method (16). Reverse transcription was performed at 42°C for 10 min. The PCR conditions were as follows: 95°C for 1 min, 50°C for 30 sec, 72°C for 30 sec, 35 cycles. The primers for HO-1 were: Forward 5'-CTGTGTAACCTCTGTGTTCC-3' and reverse 5'-CCACATACCTGAGTTCTACC-3'. The primers for human β-actin were: Forward 5'-CTGGGCACCCAGACAAAT-3' and reverse 5'-GGGCCGGACTGTCATAC-3'.
Statistical analysis. All data are presented as the mean ± standard deviation of at least three independent experiments performed in triplicate. Statistical significance between two groups was evaluated using Student’s t-test with GraphPad Prism 5 (GraphPad Software, Inc., San Diego, CA, USA). Statistical significance between multiple groups was evaluated using one-way analysis of variance with SPSS 12.0 software (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Induction of DNA DSBs by neutron radiation. The induced levels of DNA DSBs in the irradiated NHLF cells were evaluated to assess the toxicity of D-T neutron radiation. The cells were exposed to 0.01, 0.6 and 1.2 Gy 14.1 MeV neutrons and, 1 h later, an immunofluorescence staining assay was performed to analyze the DSB levels. As shown in Fig. 1A, there was a dose-dependent tendency for the induction of DSB in the dose range used in the experiment. Subsequently, the levels of DSBs at different time intervals following 0.6 Gy neutron radiation were detected (Fig. 1B). A significant increase in the number of DSBs was observed 30 min following radiation exposure, and enhanced DSB formation was sustained to the 6 h time point. At 12 h post-radiation, the DSB levels decreased, reflecting the repair of neutron-induced DNA damage.

Upregulation of HO-1 alleviates neutron-induced DSBs. HO-1 serves important biological roles in maintaining cellular oxidative equilibrium and has been reported to protect cells against stress induced damage; therefore, whether neutron radiation stimulated the expression of HO-1 was determined. Following 6 or 12 h of exposure to 0.6 Gy neutrons, the NHLF cells were collected and the expression of HO-1 in total cell lysates was detected by western blot analysis. As shown in Fig. 2A, upregulation of HO-1 was observed 6 h post-radiation. At the 12-h time point, the expression level of HO-1 was enhanced by 75%, compared with that in the control. Subsequently, the effect of HO-1 on radiation-induced DSB formation was determined. Pretreatment with Znpp increased DSB formation by ~30% in the 0.6 Gy neutron-irradiated cells (Fig. 2B). These results demonstrated that induction of the expression of HO-1 by neutron radiation alleviated DNA damage.

COX-2 mediates the upregulation of HO-1 in irradiated cells. COX-2 has been reported to be upregulated in ionizing irradiated...
cells and the inhibition of COX-2 results in enhanced toxicity; therefore, whether COX-2 affected the expression of HO-1 in neutron irradiated cells was investigated. The expression level of COX-2 was detected at 6 and 12 h following radiation exposure. As shown in Fig. 3A, COX-2 was upregulated by 30% at 6 h compared to 0 h in the irradiated cells. Subsequently, the protein and mRNA levels of HO-1 were detected in irradiated cells treated with COX-2 inhibitor NS-398. The cells were
pre-incubated with NS-398 for 1 h prior to irradiation. As shown in Fig. 3B and C, pretreatment with NS-398 inhibited the induced expression of HO-1 in the neutron-irradiated NHLF cells. The effect of COX-2 on radiation-induced DSB formation was then investigated. Treatment of the NHLF cells with COX-2 inhibitor NS-398 increased neutron-induced DSB formation (Fig. 3D). The DSB formation rate increased by ~15% in the cells pretreated with NS-398 and then exposed to 0.6 Gy neutron irradiation. These results demonstrated that the induction of the expression of COX-2 by neutron radiation alleviated DNA damage.

**COX-2/HO-1 is regulated by p38 in irradiated cells.** p38, a member of the MAPKs, is reported to be involved in ionization radiation-induced biological responses. To determine the upstream regulator of COX-2, phosphorylation was first determined. As shown in Fig. 4A, the phosphorylation of p38 showed a tendency to increase following radiation exposure, to a level of 1.5-fold at the 2 h time point and 2.2-fold at the 8 h time point, compared with the control group. To determine the association between the activation of p38 and the expression of COX-2, the cells were treated with p38 inhibitor SB203580 for 1 h prior to radiation exposure. At 6 h post-radiation exposure, the expression levels of COX-2 and HO-1 in the total cell lysate were detected and are shown in Fig. 4B and C. The results demonstrated that the induction of COX-2 and HO-1 was significantly suppressed by SB203580, indicating that the activation of p38 by radiation exposure contributed to the induced expression of COX-2 and HO-1. Subsequently, the expressions levels of DSB formation in the irradiated cells treated with p38 inhibitor SB203580 were detected. Preincubation with
SB203580 increased the DSB formation rate by ~13% in the 0.6 Gy neutron-irradiated cells (Fig. 4D). These results demonstrated that the induction of the expression of p38 by neutron radiation alleviated DNA damage.

**ATM DNA damage response stimulates p-p38/COX-2/HO-1.**

ATM is a DNA damage response kinase and it is rapidly activated through autophosphorylation in irradiated cells. Following neutron radiation exposure, the phosphorylation of ATM was observed at 30 min and was enhanced up to 3 h post-radiation exposure (Fig. 5A). Subsequently, the ATM inhibitor, KU55933, was used to investigate whether the enhanced expression of COX-2 and HO-1 was due to the activation of ATM. The cells were treated with KU55933 for 0.5 h prior to irradiation, and at 6 h post-radiation, the levels of COX-2 and HO-1 were detected. As shown in Fig. 5B and C, the radiation-induced expression of COX-2 and HO-1 was suppressed, indicating the ATM-regulated DNA damage response contributed to COX-2- and HO-1-associated protection against neutron-induced toxicity. Furthermore, the phosphorylation of p38 following treatment with KU55933 in irradiated cells was identified, which is shown in Fig. 5D. In irradiated cells pretreated with KU55933, the phosphorylation of p38 was significantly inhibited. These results indicated that the activation of p38 was controlled by ATM in neutron-irradiated cells, and also confirmed the role of p38 in upregulating the expression of COX-2 and HO-1.
Discussion

The notable energy release from the process of D-T nuclear fusion has attracted the attention of nuclear physicists due to its possible significant influence on the methods of supplying energy in the future. The 14.1 MeV neutron produced during the reaction of D-T fusion is a potential health risk due to ionization and damaging tissues; therefore, an understanding of the biological effects induced by the 14.1 MeV neutron is important for protection and medicinal treatment against neutron radiation risks. However, current knowledge on the biological effects and the underlying mechanisms remains limited. In the present study, the level of DNA DSBs, which is a typical DNA damage marker for ionizing radiation, was assessed to detect the level of damage induced by fusion radiation in NHLFs. The results demonstrated that the DSB levels were radiation dose-dependent in the range of 0-1.2 Gy. Compared with gamma-rays, the linear energy transfer of neutrons is notably higher, which means neutron deposits increase energy when traversing cells and causes increased ionization and DSBs (17). Broustas et al (18) compared gene expression profiles in a human peripheral blood model to neutron and X-ray radiation. They identified 125 genes that responded significantly to the two types of radiation as a function of dose, with the magnitude of response to neutrons generally being increased, compared with that observed following X-ray exposure (18). The relative biological effects induced by neutrons varied with the neutron energy. Tanaka et al (19) compared the efficacies of neutron with energy from 0.18-2.30 MeV in the induction of comet DNA and chromosome aberrations. They concluded that 0.37 MeV neutron caused of the most DNA damage, whereas the 2.30 MeV neutron caused the least damage (19); however, the biological effects induced by neutrons with energies >10 MeV, for example, the 14.1 MeV fusion neutron, remain to be fully elucidated. The results of the present study demonstrated that the neutron emitted from D-T fusion was effective in causing DNA damage; therefore, its biological effects warrant further examination.

HO-1, an essential enzyme in heme catabolism, cleaves heme to form biliverdin, carbon monoxide (CO) and ferrous iron. The transcription of HO-1 can be mediated by redox-dependent transcription factors, including NF-E2-related factor 2 (NRF2), NF-kB, and activating protein-1 (8). It has been shown to protect cells from various stresses-induced toxicity. HO-1-derived CO is an anti-inflammatory molecule. A previous study indicated that CO exposure facilitated the homologous recombination repair of DNA damage through an ATM/ataxia telangiectasia and Rad3-related protein (ATR)-dependent pathway (20). Chen et al (21) reported that, in low dose α-particle irradiated A549 cells, HO-1 was upregulated in a NRF2-dependent manner, and its increased expression conferred A549 resistance following high dose α-particle radiation. Furthermore, scavenging CO reduced the resistance to radiation (21). Additionally, in a study on radiation-induced bystander effects, Han et al (22) determined that CO (released from CO release molecule 2) treatment resulted in the decreased formation of DSBs in bystander cells. A report by Singh et al (23) demonstrated that treatment with the mixture of podophyllotoxin and rutin reduced the levels of oxidative stress and apoptotic cell death in gamma-ray
irradiated mouse bone marrow and spleen. Additionally, the NRF2-mediated upregulation of HO-1 partially contributed to the protective role of podophyllotoxin and rutin (23). The results of the present study indicated that the inhibition of HO-1 activity exacerbated fusion radiation-induced DNA damage, confirmed the role of HO-1 in radiation protection, and indicated that the targeted upregulation of HO-1 may be a potential method to decrease the risk of fusion radiation to health.

The expression of COX-2 is upregulated in numerous types of cancer. Furthermore, its product prostaglandin H2 is converted by prostaglandin E2 (PGE2) synthase into PGE2, which in turn can stimulate cancer progression. Consequently, COX-2 is a target for the development of drugs that prevent and treat cancer. Previous studies have reported that the targeted inhibition of COX-2 improved the efficacy of cancer radiotherapy, indicating the protective role of COX-2 in irradiated tumor cells (9-13). Hofer et al (24) determined in animal experiments that, following gamma-ray radiation, male COX-2-knockout wild-type mice exhibited attenuated hematopoiesis, compared with wild-type mice. Additionally, it was concluded that the genetic disruption of COX-2 has a positive effect in hematopoiesis under basal conditions, but is detrimental following radiation exposure (24). Ozbilgin et al (25) indicated that the increased expression of COX-1 and COX-2 in the urothelium of mice may prevent bladder damage from acute gamma-ray radiation, and benefits the differentiation and restoration of the urothelium. Zuo et al (26) reported that treatment of mouse epidermal cells with a low concentration of arsenite increased the expression of COX-2, and this induction of COX-2 resulted in decreased levels of apoptosis following ultraviolet B treatment. These data indicated that, although COX-2 is considered to mediate inflammation and promote carcinogenesis, its upregulation is beneficial under a number of stress conditions. To the best of our knowledge, there has been no previous report on the functions of COX-2 in cell cycle arrest and apoptosis (32,33). Acheva et al (36) observed in an in vitro epithelial wounding model that the production of PGE2 was increased in a time-dependent manner via the activation of COX-2, which was stimulated by the phosphorylation of extracellular signal-regulated protein kinase 1/2, another member of the MAPK family (37). The results of the present study demonstrated that COX-2 and its mediated upregulation of HO-1 were regulated by activated p38, and that p38 is critical for the enhanced transcription and expression of COX-2 in gamma-ray-irradiated human epithelial cells (36), which was consistent with the observation by Hung et al (35). Additionally, Hu et al (37) observed in an in vitro epithelial wound model that the production of PGE2 was increased in a time-dependent manner via the activation of COX-2, which was stimulated by the phosphorylation of extracellular signal-regulated protein kinase 1/2, another member of the MAPK family (37). The results of the present study demonstrated that COX-2 and its mediated upregulation of HO-1 were regulated by activated p38, and that p38 is critical for the enhanced transcription and expression of COX-2 in gamma-ray-irradiated human epithelial cells (36), which was consistent with the observation by Hung et al (35). Additionally, Hu et al (37) observed in an in vitro epithelial wound model that the production of PGE2 was increased in a time-dependent manner via the activation of COX-2, which was stimulated by the phosphorylation of extracellular signal-regulated protein kinase 1/2, another member of the MAPK family (37). The results of the present study demonstrated that COX-2 and its mediated upregulation of HO-1 were regulated by activated p38, and that p38 is critical for the enhanced transcription and expression of COX-2 in gamma-ray-irradiated human epithelial cells (36), which was consistent with the observation by Hung et al (35). Additionally, Hu et al (37) observed in an in vitro epithelial wound model that the production of PGE2 was increased in a time-dependent manner via the activation of COX-2, which was stimulated by the phosphorylation of extracellular signal-regulated protein kinase 1/2, another member of the MAPK family (37).
study provide novel information on fusion radiation-induced biological effects and potential targets for decreasing the health risks.

Acknowledgements

The authors would like to thank the other members of the Fusion Design and Study team for their assistance in the present study.

Funding

The present study was supported by the National Magnetic Confinement Fusion Science Program of China (grant no. 2014GB112006), the Natural Science Foundation of Anhui Province of China (grant no. 1508085ME220), the Natural National Science Foundation of China (grant nos. 11575232 and 31370842), the International Partnership Program of Chinese Academy of Sciences (grant no. 116134KYSB20160084) and the Innovative Program of Development Foundation of Hefei Center for Physical Science and Technology (grant no. 2016FXCX005).

Availability of data and materials

The analyzed data sets during the study are available from the corresponding author upon reasonable request.

Authors' contributions

JW, XY and HL designed the experiments. XY, HL and XJ performed the biological experiments. CJ, ZX, TL and ZW performed the deuterium-tritium fusion radiation of biological samples. XY, HL and JW analyzed the results and wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References


This work is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International (CC BY-NC-ND 4.0) License.