Metformin alleviates ionizing radiation-induced senescence by restoring BARD1-mediated DNA repair in human aortic endothelial cells

Jeong-Woo Park, Ji-Eun Park, So-Ra Kim, Myeong-Kyu Sim, Chang-Mo Kang, Kwang Seok Kim

Divisions of Radiation Biomedical Research, Korea Institute of Radiological and Medical Sciences, Seoul 01812, Republic of Korea

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ABSTRACT

Metformin is one of the most effective therapies for treating type 2 diabetes and has been shown to also attenuate aging and age-related disorders. In this study, we explored the relationship between metformin and DNA damage repair in ionizing radiation (IR)-induced damage of human aortic endothelial cells (HAECs). Metformin treatment suppressed IR-induced senescence phenotypes, such as increased senescent-associated β-galactosidase (SA-β-gal) activity and decreased tube formation and proliferation. Moreover, metformin increased BRCAl-associated RING domain protein 1 (BARD1) and RAD51 expression in both aging and IR-exposed cells. Metformin-treated cells exhibited higher levels of the BRCAl-BARD1-RAD51 complex during irradiation, even in the presence of compound C, an AMP-activated protein kinase inhibitor. BARD1 knockdown confirmed its critical role in metformin-mediated inhibition of endothelial senescence. Metformin increased blood vessel sprouting and decreased SA-β-gal activity in mouse aortas. Collectively, our findings provide new insights into how metformin can prevent endothelial cell senescence by promoting BARD1-related DNA damage repair, suggesting that metformin may be an effective anti-aging agent and a promising therapeutic for protecting against radiation-induced cardiotoxicity.

1. Introduction

Metformin has been one of the most effective drugs used for treating patients with type 2 diabetes during the last five decades (Bailey and Day, 1989; Inzucchi et al., 2015). Interestingly, metformin has also been shown to be a potential therapeutic for aging, inflammation, cancer, and neurodegenerative disease (Martin-Montalvo et al., 2013; Pearce et al., 2009; Wallace, 2005). The mechanism of action of metformin has been divided into two different pathways in the anti-aging process. The most well-known involves stimulation of adenosine monophosphate AMP-activated protein kinase (AMPK) by increasing the AMP:ATP ratio (Hardie, 2007; Shaw et al., 2005). The other pathway relies on reactive oxygen species (ROS) induced by the accumulation of DNA damage (Halicka et al., 2012; Kasznicki et al., 2014). The AMPK-independent metformin pathway regulates downstream ROS pathways, such as insulin-like growth factor 1, AKT, and p38, which are targets of aging (Anisimov and Bartke, 2013; Yoon et al., 2002; Zhang et al., 2020).

Recent studies have demonstrated that metformin reduces DNA double-strand break (DSB) repair mediated by paraquat (Aligire et al., 2012) and promotes DNA base excision repair (BER) (Dogan Turacli et al., 2018a; Dogan Turacli et al., 2018b). These effects are related to the expression of the p53-dependent DNA damage response (DDR) (Aligire et al., 2012; Dogan Turacli et al., 2018a). Indeed, metformin increases ATM and checkpoint kinase 2 activity through the AMPK-independent pathway and promotes DDR (Vazquez-Martin et al., 2011). However, the mechanism of how metformin promotes ATM activity is unknown.

Many environmental factors, such as toxic chemical agents, ROS, ionizing radiation (IR), and ultraviolet (UV) radiation, trigger genomic instability, and maintaining genomic stability is an important factor in achieving a prolonged lifespan (Herskowitz, 1946; Swanson, 1948). DDR is crucial for avoiding loss of genetic information, maintaining genomic stability, and determining cell fate (Harper and Elledge, 2007). Cells have special systems, such as DNA repair, cell cycle checkpoints, and apoptosis, to maintain genomic stability. Cells respond to DNA
damage via at least four pathways, namely DDR, BER, mismatch repair (MMR), homologous recombination (HR), and non-homologous end-joining (NHEJ). NHEJ leads to ligation that is independent of DNA sequence homology ends in G1-S phase cells and requires many factors such as Ku70/80, DNA-PKc, and Artemis (Ceccaldi et al., 2016). On the other hand, HR is a more accurate repair process than NHEJ and occurs mainly during the S-G2 phases. During HR, the sticky end of damaged chromatin extends to the undamaged sister chromatin and is restored using the sister DNA sequence. HR is driven by ATM activation and the BARD1-BRCA1 complex (Ceccaldi et al., 2016; Chapman et al., 2012).

In this study, we demonstrate that metformin suppresses IR-induced cellular senescence through BARD1-related DNA damage repair. These results suggest that metformin may have an anti-aging effect, as well as a potential role in radioprotection during radiotherapy, by maintaining genomic stability.

2. Materials and methods

2.1. Cell culture and animal care

Human aortic endothelial cells (HAECs) were purchased from PromoCell (San Diego, CA, USA) and cultured in Endothelial Growth Media-2 Medium (PromoCell, Walkersville, MD, USA) in a humidified incubator at 37 °C and 5% carbon dioxide. When the subcultures reached 80%-90% confluence, serial passage was performed by trypsinization. Cells were used at passages 4–8 (population doubling < 30; young) and 12–14 (population doubling > 50; old). For IR treatment, cells were γ-irradiated from a 137 Cs γ-ray source (Atomic Energy of Canada, Ltd., Canada) administered at a dose of 3.5 Gy/min. To inhibit cell damage, HAECs were treated with 10 mM metformin (Calbiochem, East Brunswick, NJ, USA).

For in vivo experiments, 12-week-old female BALB/c nude mice were obtained from Orient Biotech Co., Ltd. (Gyeonggi-do, Korea). Mice were housed in a pathogen-free facility and fed a normal diet along with autoclaved water ad libitum. All mouse procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the Korea Institute of Radiological and Medical Sciences (IACUC permit number: KIRAMS2017-0013).

2.2. Cell growth assay

Cells were cultured in 100-mm dishes. After sub-culturing, cells were cultured in 96-well plates and 35-mm dishes according to the date, and the absorbance was measured spectrophotometrically at 570 nm using a 1.25 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (Sigma-Aldrich, St. Louis, MO, USA). Cells were detached and counted to confirm cell proliferation.

2.3. Tube formation assay

Cells (4 × 10⁵) were seeded onto a Matrigel (Corning Life Sciences, Lowell, MA, USA)-coated 24-well plate and incubated for 12 h. Tube formation was observed using an optical microscope. Tube length was measured using NIH ImageJ software (National Institutes of Health, Bethesda, MD).

2.4. RNA extraction and real-time polymerase chain reaction analysis (RT-PCR)

RNA was isolated from cells using TRIzol solution (Bioline, London, England, UK) according to the manufacturer’s instructions. Complementary DNA was synthesized using the SensiFAST™ cDNA Synthesis kit (Bioline, London, UK) from the isolated RNA. RT-PCR was performed using specific primers (Table 1) with the Qualitative PCR SYBR 2× Master Mix Kit (Mbiotech, Gyeonggi-do, Korea).

Table 1

| Gene  | Forward | Reverse |
|-------|---------|---------|
| BARD1 | GAGGAGCTTTCATCCAGAG | CAGGTGTAAGGAAGCAAC |
| RADS1 | AGTGGAGCTGAAAGAAGG | TAAAGGAGCCTGGTCGTTG |

2.5. Immunoblotting

HAECs were seeded into 100-mm dishes and incubated for 24 h in Endothelial Growth Media-2 Microvascular (MV) Medium. Cells were washed with ice-cold phosphate-buffered saline (PBS) and then lysed with 80 μL of radioimmunoprecipitation assay (RIPA) buffer (ELPIS Biotech, Daejeon, Korea). For western blot analysis, proteins (30 μg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes (GE Healthcare, Chicago, IL, USA). The membranes were incubated at 4 °C with specific primary antibodies for p-p53(s15) (#9284), AMPK (#2532), and p-AMPK (#2535; Cell Signaling Technology, Danvers, MA, USA), Vimentin (AP2739a; Abgent, San Diego, CA, USA), Lamin B (sc-6216), p21 (sc-397), BRCA1 (sc-6954), BARD1 (sc-74559; Santa Cruz Biotechnology, Santa Cruz, CA, USA), and RAD51 (ab133534; Abcam, Cambridge, England, UK) at a dilution 1:1000 overnight. Horseradish peroxidase-conjugated anti-mouse (#7076F2) and anti-rabbit (#7074F2; Cell Signaling Technology, Danvers, MA, USA) secondary antibodies were incubated at a dilution of 1:3000 at room temperature for 3 h. and followed three washes with Tris-buffered saline 0.1% Tween20. Protein bands were visualized by enhanced chemiluminescence using an Amersham Imager 600. The membranes were then re-probed with antibody against β-actin (Santa Cruz Biotechnology), which was used as a protein-loading control.

2.6. Immunoprecipitation

Equal amounts (1 mg/mL) of protein samples were pre-cleared with normal rabbit IgG and protein A/G agarose beads (Amersham Biosciences, Upsala, Sweden) for 1 h at 4 °C. The supernatants were added to 1 μL BARD1 antibody and incubated in presence of 50 μL of A/G agarose beads overnight at 4 °C on a rotator. After incubation, samples were centrifuged for 1 min at 13,000 rpm. The pellets were washed four times with the lysis buffer (150 mM NaCl, 50 mM Tris, pH 8.0, 1% Triton X-100) and resuspended in 50 μL of SDS sample buffer. Immune complexes were resolved by SDS-PAGE and proteins were transferred to a nitrocellulose membrane for further analysis by western blot.

2.7. DNA damage response-related foci analysis

HAECs were seeded onto glass coverslips placed on 8-well slides and incubated for 24 h. Cells were fixed with a 3.7% formaldehyde solution for 10 min at room temperature, permeabilized in ice-cold PBS with 0.25% Triton X-100 for 10 min at 4 °C, and blocked with 5% donkey serum in PBS for 1 h at room temperature. Cells were incubated overnight at 4 °C with primary antibodies against γ-H2AX (#2577; Cell Signaling Technology, Danvers, MA, USA), phospho-ATM (ab36810; Abcam, Cambridge, England, UK), and 53BP1 (sc-22760; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Cells were washed and incubated at room temperature with Alexa Fluor 488-conjugated goat anti-mouse IgG and Alexa Fluor 555-conjugated donkey anti-rabbit IgG (Life Technologies Corporation, Carlsbad, CA, USA) for 1 h. Glass coverslips were attached to the glass slides with a mounting solution ( Vectashield; Vector Laboratories, Burlingame, CA, USA) containing 6-diamidino-2-phenylindole.
2.8. Senescence-associated (SA) β-gal activity assay

Senescence-associated beta-galactosidase staining experiments were performed according to a previous study (Heo et al., 2016).

2.9. Aortic ring assay

Aortic rings (1 mm in length) were sectioned and extensively rinsed twice with PBS. Mouse aorta rings were embedded in Matrigel with same volume of PromoCell MV media. The cultures were kept at 37 °C in a
humidified incubator for 7 days. Sprouting vessels were observed by optical microscopy.

2.10. Statistical analysis

Data were analyzed by one-way analysis of variance using the SPSS software package (IBM, Armonk, NY, USA). A $P$-value $< 0.05$ was considered statistically significant. Duncan’s multiple-range test was performed if the differences between the groups were identified as $\alpha = 0.05$.

3. Results

3.1. Metformin inhibits IR-induced cellular senescence in human aortic endothelial cells (HAECs) through an AMPK-independent pathway

To determine whether metformin treatment reduces IR-induced cellular senescence through an AMPK-dependent pathway, cells were treated with metformin during IR exposure. First of all, the effect of metformin on endothelial cell proliferation was investigated using a MTT assay. Metformin treatment had no effect on cell proliferation up to 10 mM concentration, and treatment of 10 mM metformin was shown to inhibit IR-induced damage in HAECs (Fig. 1A). As shown in Fig. 1B, even though metformin is known as an AMPK activator that induces the phosphorylation of AMPK, as AMPK was activated by irradiation, metformin did not affect AMPK phosphorylation in IR-treated HAECs. Moreover, the level of IR-mediated Vimentin, phospho-p53 and p21 was decreased and the level of Lamin B, known as a cellular senescence marker, was restored by metformin treatment (Fig. 1B). In order to confirm the involvement of AMPK in metformin effects during irradiation, compound C, an inhibitor of AMPK, was treated in IR-treated cells. It was shown that AMPK was inactivated by compound C (Fig. 1C). Next, we performed cell growth, tube formation, and SA-β-gal assays to confirm whether metformin inhibits IR-induced cellular senescence. Our data demonstrate that metformin promoted cell growth (Fig. 1D) and tube formation (Fig. 1E) compared to IR treatment. However, cell growth and tube formation in IR-treated cells were not significantly inhibited in the presence of compound C. Nevertheless, metformin reduced SA-β-gal activity in IR-treated cells (Fig. 1F).

To further investigate whether metformin treatment promotes DNA damage repair in IR-treated cells, we performed DNA damage response-related foci analysis. As shown in Fig. 1G, the number of γ-H2AX foci stained was increased by IR exposure for 1 h but reduced with metformin treatment. Collectively, these results suggest that metformin treatment alleviates the progression of cellular senescence and regulates IR-induced DNA damage.

3.2. Metformin regulates senescence in old HAECs

To investigate whether metformin treatment inhibits replicated senescence in endothelial cells, we examined senescence-associated (SA) phenotypes, such as cell growth, tube formation, and SA-β-gal staining in...
the absence or presence of metformin in old HAECs. As expected, metformin treatment promoted tube formation (Fig. 2A), reduced SA β-gal activity (Fig. 2B), and increased cell proliferation (Fig. 2C) in these cells. Metformin treatment increases ATM activity. Thus, in order to explore the relevance between metformin and homologous DNA repair, we measured the expression level of BARD1 and RAD51-composed complexes in the progression of HR in metformin-treated cells. BARD1 and RAD51 expression remained unchanged in young HAECs but decreased in old cells. This decrease was reversed by metformin treatment (Fig. 2D). These data suggest that metformin treatment alleviates replicative cellular senescence and increases the expression of HR-related genes in endothelial cells.

Fig. 3. Metformin promotes DNA damage response in HAECs.
(A) Cells were treated with IR for 1 h in the absence or presence of metformin or compound C. Total RNA was extracted and then RT-PCR was performed using specific primers for BARD1, RAD51, and GAPDH. Data are expressed as the mean ± standard deviation of three independent experiments. **P < 0.01. (B) Cell lysates were immunoprecipitated with anti-BARD1 antibody followed by immunoblotting with anti-BRCA1 and anti-RAD51 antibodies. (C) Confocal immunofluorescent images of 53BP1 (red) and phospho-ATM (green) co-localization in cells. DAPI staining (blue) was used to visualize nuclei. The number of protein foci was quantified using the quantification algorithm of nuclear foci in NIH ImageJ software. ***P < 0.001. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
Fig. 4. Downregulation of BARD1 promotes cellular senescence induced by IR.
HAECs were treated with metformin and transfected with BARD1 siRNA in 4 Gy radiation-treated cells. (A) Depletion of BARD1 expression with BARD1 siRNA was confirmed by immunoblot analysis. (B) Cell number was analyzed to assess cell proliferation. Cells were plated at $2 \times 10^4$ and cultured for 1, 2, or 4 days and then the cells were counted. (C) Effect of metformin and BARD1 siRNA on tube formation, which was visualized by optical microscopy (top). Quantitation of tube length is shown on the bottom. (D) The number of SA β-gal-positive cells was measured. Data are expressed as the mean ± standard deviation of three independent experiments. ***$P < 0.001$.

Fig. 5. Metformin inhibits IR-induced aging in mouse aorta.
(A) The effect of metformin on vessel formation in mouse aortas was assessed by the aortic ring assay. Photomicrographs of mouse aortic rings that were incubated with media in a humidified incubator at 37 °C and 5% CO₂ were shown. (B) Representative photographs and densitometric analysis of SA β-gal-stained aortas ($n = 5$ per group). Aortas from control and metformin-treated animals either unexposed or exposed to 8 Gy radiation were examined by the SA β-gal activity assay. The amount of blue and green staining was measured by the algorithm of cell counter in ImageJ software and the intensity score was calculated. **$P < 0.01$ and ***$P < 0.001$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
3.3. Metformin promotes repair of IR-induced DNA damage in HAECs

Since metformin inhibited the formation of IR-induced γH2AX foci and can promote repair of IR-induced DSBs, we measured the expression of DSB repair genes in metformin-treated cells during gamma radiation exposure. Our results demonstrate that the expression levels of BARD1 and RAD51 were increased by metformin (Fig. 3A). Thus, we hypothesized that metformin-mediated BARD1 expression is involved in repairing IR-induced DSB and inhibiting IR-induced senescence. Because BARD1 and RAD51 form a complex with BRCA1 during HR, we examined the interaction of BARD1 and BRCA1 in metformin-treated cells exposed to gamma radiation. Immunoprecipitation experiments show that, in the metformin-treated group, more BRCA1-BARD1-RAD51 complex was formed due to elevated expression levels of the individual components at 1 h after IR treatment (Fig. 3B). According to 53BP1 and phospho-ATM foci detection analysis, phospho-ATM foci were rapidly reduced in metformin treatment compared to the IR only group regardless of the presence or absence of compound C (Fig. 3C). As soon as DDR occurred by IR-induced DSB, metformin induced the formation of the BRCA1-BARD1-RAD51 complex and enhanced HR repair at DSB, resulting in fewer phospho-ATM foci. Although metformin promoted DDR, there was no significant difference in the number of 53BP1 foci in metformin-treated cells during irradiation, indicating that metformin-mediated repair of DSB was not associated with activation of 53BP1 in HAECs. Taken together, these results suggest that metformin promotes HR by increasing the expression of BARD1, BRCA1, and RAD51 via an AMPK-independent pathway.

3.4. Metformin regulates a BARD1-dependent pathway

To confirm the role of BARD1 in metformin-mediated inhibition of cellular senescence, we investigated whether BARD1 knockdown inhibits the effects of metformin by using an SA phenotype assay. BARD1 siRNA was transfected in HAECs and as a result BARD1 expression was reduced by more than 70% (Fig. 4A). Proliferation of metformin-treated cells during irradiation was reduced by BARD1 siRNA transfection, similar to the reduction in cells treated with IR only (Fig. 4B). Tube formation in BARD1-depleted cells also was not ameliorated despite metformin treatment during irradiation (Fig. 4C). Next, we confirmed that SA β-gal activity was not affected by metformin treatment in BARD1 knockdown cells during irradiation (Fig. 4D). These data suggest that metformin alleviates IR-induced cellular senescence through a BARD1-dependent pathway.

3.5. Metformin inhibits IR-induced aging in the mouse aorta

Since metformin alleviated the effects of radiation in aortic endothelial cells, we hypothesized that metformin can reduce radiation-induced senescence and promote vessel sprouting in the mouse aorta. As shown in Fig. 5A, a metformin-treated aorta during IR exposure was more spread out in contrast to a mouse aorta treated with IR only. Similar to endothelial cells, metformin decreased IR-induced SA β-gal activity in the mouse aorta (Fig. 5B). Taken together, these data indicate that metformin promotes angiogenesis and inhibits radiation-induced senescence in vivo.

4. Discussion

Radiotherapy is used to control and eliminate malignant cells as part of treating cancer. Patients who receive radiotherapy exhibit a prolonged their life span compared to those who do not (Packer et al., 1994). However, radiation therapy has a fatal disadvantage in that it causes a lot of damage to blood vessels, especially those in the aorta of breast cancer patients (Castaneda and Strasser, 2017; Viallard and Larivée, 2017). Through in vitro and in vivo experiments, we provided knowledge of the molecular mechanisms and consequences of radiation-induced damage to aortic endothelial cells (Park et al., 2016).

The present study demonstrates that metformin alleviates IR-induced senescence phenotypes, such as decreasing cell proliferation and tube formation and increasing SA β-gal staining via an AMPK-independent pathway (Fig. 2). Our study also shows that metformin reduces SA markers induced by replicative senescence (Fig. 1) and promotes the expression of HR-related genes such as BARD1 and RAD51 (Figs. 1D and 3A). Taken together, these results suggest that metformin promotes replication senescence as well as IR-induced senescence through induction of HR-related genes.

This study focused on BARD1 and the effect of metformin on HR repair of IR-induced DSBs. BARD1, the heterodimeric partner of BRCA1, participates in homology-directed repair of DSBs (Lauffer et al., 2007; Westermark et al., 2003). Association of the BRCA1-BARD1 complex with RAD51 enhances DNA invasion and formation of the nascent DNA joint in the D-loop reaction (Zhao et al., 2017), resulting in the promotion of HR. BARD1 depletion leads to DNA damage sensitivity, HR deficiency, and genome destabilization (McCarth et al., 2003; Zhao et al., 2017). Mutations in BARD1 have been found in some cancer patients (Ghimenti et al., 2002; Ishidobi et al., 2003). Our data demonstrate that metformin treatment induces formation of the BRCA1-BARD1-RAD51 complex (Fig. 3) and inhibits cellular senescence through a BARD1-dependent pathway (Fig. 4).

Finally, our study demonstrates that metformin treatment prevents IR-induced cardiotoxicity. The aortas of metformin-injected mice sprout more vessels than the aortas of IR-treated mice (Fig. 5A). Moreover, by measuring IR-induced SA β-gal activity in the aorta, we show that metformin prevents IR-induced cardiovascular aging in vivo. Chemotherapy and radiotherapy increase cardiac dysfunction, a major cause of morbidity and mortality among cancer patients (Lenneman and Sawyer, 2016). Thus, there is a need to enhance treatment of the cardiotoxicity that develops as a consequence of radiotherapy. Since inhibition of oxidative DNA damage induced by doxorubicin and IR is one approach to alleviate cardiotoxicity, metformin can also exert cardioprotective effects comparable to those of pitavastatin (Yoshida et al., 2009).

In conclusion, based on the data presented here, metformin is potentially an effective anti-aging agent for treating cardiovascular diseases in the elderly and a possible radioprotector in radiotherapy.

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CRediT authorship contribution statement

J.W. Park: Formal analysis, Data Curation, Visualization, and Writing-Original Draft. M.K. Sim: investigation and validation. C.M. Kang: Methodology and Resources. K.S. Kim: Supervision, Project administration, and Writing-Review and Editing.

Declaration of competing interest

The authors declare that they have no competing interests.

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