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The catalytic subunit of DNA-dependent protein kinase is downstream of ATM and feeds forward oxidative stress in the selenium-induced senescence response☆

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Abstract

Selenium induces a senescence response in cells through induction of ataxia–telangiectasia mutated (ATM) and reactive oxygen species (ROS). Although a role of the catalytic subunit of DNA-dependent protein kinase (DNA-PK_{cs}) in DNA double-strand break repair is established, it is unclear how these proteins function in response to selenium-induced oxidative stress and senescence induction. In this study, we demonstrated that pretreating normal human diploid fibroblasts with DNA-PK kinase inhibitor NU 7026 suppressed selenium-induced senescence response. Selenium treatment induced phosphorylation of DNA-PK_{cs} on Thr-2647 and Ser-2056, the extent of which was decreased in the presence of ATM kinase inhibitor KU 55933 or the antioxidants *N*-acetylcysteine or 2,2,6,6-tetramethylpiperidine-1-oxyl. In contrast, the selenium-induced phosphorylation of ATM on Ser-1981 was not affected by NU 7026. Cells deficient in DNA-PK_{cs} or pretreated with NU 7026 or *N*-acetylcysteine were defective in selenite-induced ROS formation. Taken together, these results indicate a distinct role of DNA-PK_{cs}, in which this kinase can respond to and feed forward selenium-induced ROS formation and is placed downstream of ATM in the resultant senescence response.
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Keywords: Selenium; Senescence; ATM; DNA-PK

1. Introduction

Selenium is a micronutrient essential for optimal health. A strong body of geographical, animal and clinical evidence points to a role for selenium in counteracting tumorigenesis (for details, see Ref. [1]). In particular, the Nutritional Prevention of Cancer Trial concluded that dietary supplementation of selenium three- to four-fold higher than nutritional need, in the form of selenium-enriched yeast containing 65%–80% selenomethionine, reduced mortality from all cancers and decreased the incidence of lung, colorectal and prostate cancers [2]. In contrast, the Selenium and Vitamin E Cancer Prevention Trial failed to demonstrate that selenomethionine or vitamin E, alone or in combination, prevented the incidence of prostate cancer in a population of relatively healthy men [3]. Together with results from animal studies [4,5], selenomethionine is unlikely the best active selenium compound to counteract tumorigenesis. Whatever the effective selenium speciation, the proposed mechanisms of selenium chemoprevention include induction of apoptosis by reactive oxygen species (ROS) [6–9], activation of cell cycle arrest [6,10,11], an enhancement of DNA repair [12,13], an increase in mitochondrial dysfunction [14], limiting endoplasmic reticulum stress [15], as well as decreasing angiogenesis [16–19] in cancerous cells. We have recently shown a different perspective, that selenium compounds can activate a senescence response in noncancerous, but not in cancerous, cells with doses $\leq LD_{50}$ [20]. Thus, selenium compounds, in principle, could activate early barriers of tumorigenesis and prevent the cells from progressing to the malignant stage.

DNA damage response, an early barrier of tumorigenesis [21], can be induced by the catalytic subunit of DNA-dependent protein kinase (DNA-PK_{cs}) and ataxia–telangiectasia mutated (ATM). The only human *PRKDC* (*DNA-PK_{cs}*) mutation known to date is the L3062R missense mutation found in a severe combined immunodeficiency patient [22]; however, the human glioma cell line M059J lacks DNA-PK_{cs} protein and expresses low levels of ATM protein [23]. *ATM* is

Abbreviations: ATM, ataxia–telangiectasia mutated; CM-H_2DCFDA, 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester; DAPI, 4,6-diamidino-2-phenylindole; DNA-PK_{cs}, the catalytic subunit of DNA-dependent protein kinase; DSBs, double-strand breaks; MEFs, mouse embryonic fibroblasts; MSeA, methylseleninic acid; NAC, *N*-acetylcysteine; Na_2SeO_3, sodium selenite; NHEJ, nonhomologous end-joining; PBS, phosphate-buffered saline; *p*ATM Ser-1981, phospho-ATM at Ser-1981; *p*DNA-PK_{cs} Ser-2056, phospho-DNA-PK_{cs} at Ser-2056; *p*DNA-PK_{cs} Thr-2647, phospho-DNA-PK_{cs} at Thr-2647; γH2A.X, phospho-H2AX at Ser-139; ROS, reactive oxygen species; SA-β-gal, senescence-associated -β-galactosidase; Tempo, 2,2,6,6-tetramethylpiperidine-1-oxyl.

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mutated in the genome instability syndrome ataxia–telangiectasia which is characterized by neuronal degeneration, immune defects, premature aging and cancer predisposition [24]. The ATM pathway is activated by interactions with the MRE11-RAD50-NBS1 complex and by autophosphorylation on the Ser-1981 (*p*ATM Ser-1981) residue [25–28]. DNA-PK_{cs}, Ku70 and Ku80 form the holoenzyme DNA-PK that repairs DNA double-strand breaks (DSBs) by the nonhomologous end-joining (NHEJ) pathway [29,30]. The Thr-2647 residue of DNA-PK_{cs} is phosphorylated (*p*DNA-PK_{cs} Thr-2647) *in vivo* in an ATM-dependent manner after ionizing radiation [31]. DNA-PK_{cs} autophosphorylation on Ser-2056 (*p*DNA-PK_{cs} Ser-2056) is induced by DNA DSBs and is required for proper NHEJ repair [32,33].

Originally known as major kinases in the signaling and repair of DNA DSBs, emerging lines of recent evidence link DNA-PK and ATM to redox regulation. Low levels of oxidative stress can activate DNA-PK during mitochondrial respiration [34]. Moreover, ATM protein can be activated by direct oxidative stress or in selenium-treated cells [20,35,36], and neurons deficient in ATM show increased oxidative stress [37,38]. Because ATM kinase is not the only kinase attributed to H2A.X phosphorylation on Ser-139 (γH2A.X) [20] and both ATM and DNA-PK_{cs} can function redundantly in the cellular exposure to ionizing radiation [31,37,39], we hypothesized that DNA-PK_{cs} participates in the selenium-induced DNA damage and senescence responses. We show here that DNA-PK_{cs} is placed downstream of ATM, relays oxidative stress and is required for the senescence response in normal diploid fibroblasts treated with selenium compounds.

2. Method and materials

2.1. Cell culture and chemicals

The human pulmonary MRC-5 and colorectal CCD 841 CoN normal fibroblasts and wild-type and DNA-$PK_{cs}{}^{-/-}$ mouse embryonic fibroblasts (MEFs) were cultured as described previously [20,40]. MRC-5 and CCD 841 CoN cells were chosen to recapitulate the selenium-induced senescence response based on our previous results [20]. The DNA-$PK_{cs}{}^{-/-}$ and wild-type MEFs have been extensively employed for DNA damage response studies [40]. Sodium selenite (Na_2SeO_3, 2 μmol/L, 24 h), methylseleninic acid (MSeA, 2 μmol/L, 24 h), *N*-acetylcysteine (NAC, a thiol-containing derivative of L-cysteine, 5–10 mmol/L, 24 h) and 2,2,6,6-tetramethylpiperidine-1-oxyl (Tempo, a membrane permeable nitroxide compound, 0.5–1 mmol/L, 24 h) were obtained from Sigma-Aldrich (St. Louis, MO, USA) and were dissolved in phosphate-buffered saline (PBS). NU 7026 and KU 55933 (10 μmol/L, 24 h) were purchased from Tocris (Ellisville, MO, USA) and were dissolved in dimethyl sulfoxide.

2.2. Detection of ROS

Intracellular ROS were detected using 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA) (Invitrogen, Carlsbad, CA, USA), which is nonfluorescent until the removal of the acetate group by intracellular esterases upon increased oxidative stress. After Na_2SeO_3 treatment, the cells were rinsed once with PBS and then incubated with 10 μmol/L CM-H_2DCFDA in PBS for 45 min at 37°C. The intracellular ROS levels were detected by using the filter set: Ex., 540 nm; Em., 490 nm, which is equipped in a fluorescence microplate reader (FLUOstar OPTIMA, BMG Labtech, Cary, NC, USA). The fluorescent intensity was measured and normalized to cells without any treatment. In preparation for ROS analysis by means of flow cytometry, the cells were trypsinized and washed before incubation with CM-H_2DCFDA. After the cells were spun down, the resuspended cells were analyzed by recording the mean FITC-A signals of each sample using the BD FACSCanto II flow cytometer. The data were analyzed using FlowJo version 7.6.4 (Tree Star Inc., Ashland, OR, USA). For ROS analysis by fluorescence microscope, cells were incubated with 10 μmol/L CM-H_2DCFDA in 4% paraformaldehyde for 30 min at 37°C in the dark [41]. After being rinsed gently with PBS, cells were imaged immediately under a Zeiss Axio Observer Z1m fluorescent microscope (Zeiss, Thornwood, NY, USA) using the software Axiovision. All samples were imaged using the GFP 488-nm excitation spectra setting, and corresponding bright-field pictures were taken.

2.3. Immunofluorescence

Immunofluorescence analysis was performed as described previously with modifications [20,42]. Briefly, cells were fixed in 4% paraformaldehyde for 15 min, permeabilized with ice-cold methanol for 10 min at −20°C and then with 0.3% Triton X-100 for 10 min, and blocked in 10 % normal goat serum in PBS containing glycine at 0.3 mol/L for 1 h. The coverslips were incubated with the following antibodies overnight at 4°C: *p*DNA-PK_{cs} Thr-2647 (lot 903801, 1:300, Abcam, Cambridge, MA, USA), *p*DNA-PK_{cs} Ser-2056 (lot 696143, 1:300, Abcam), DNA-PK_{cs} (lot 715104, 1:300, Abcam), ATM (lot YF-10-17-02, 1:500, Epitomics, Burlingame, CA, USA) and *p*ATM Ser-1981 (lot 20772, 1:500, Rockland, Gilbertsville, PA, USA). The slides were then washed in PBS and incubated with Alexa secondary antibodies (Alexa Fluor 488 and 594, 1:200, Invitrogen) for 1 h at room temperature in the dark. Cells were then washed in PBS and mounted onto slides containing a drop of 4,6-diamidino-2-phenylindole (DAPI) (Invitrogen). All the images were obtained using the same parameters of brightness, contrast and exposure time by using a Zeiss Axio Observer Z1m fluorescence microscope, and the images were processed using deconvolution with the software AxioVision Release 4.7.2.0. Ten nuclei were randomly chosen and outlined using the spline function of the software, and the densometric intensity of each of the proteins (DsRed and GFP) was obtained and normalized by that of DAPI as described previously [43,44].

2.4. Cell survival assay

Cells were trypsinized and counted using a hemocytometer. The number of untreated cells was set as 100%. For apoptotic analysis, cell pellets were resuspended in 500 μl binding buffer containing Annexin V-FITC and Sytox green dye according to manufacturer's instruction (Apoptosis Detection Kit Plus, K201-400; Biovision Inc., Mountain View, CA, USA). The cells were then analyzed (Ex., 488 nm; Em., 530 nm in the FL1 channel) by using a BD FACSCanto II flow cytometer.

2.5. Senescence assays

We detected expression of senescence associated-β-galactosidase (SA-β-gal) by using a Senescence Detection Kit (MBL Co. Ltd., Woburn, MA, USA) according to the manufacturer's instructions and our previous publication [20].

2.6. Statistics

All the experiments were independently performed and repeated three times. The data were analyzed using GraphPad Prism software version 5.04 (GraphPad Software Inc., La Jolla, CA, USA). One-tailed Student's *t*-test was applied to determine statistical significance ($P<.05$) between the treatments.

3. Results

3.1. DNA-PK_{cs} is involved in the selenium-induced senescence response

We first tested the hypothesis that DNA-PK_{cs} is involved in the selenium-induced senescence response. Pretreatment of normal MRC-5 and CCD 841 CoN human fibroblasts with NU 7026, a DNA-PK kinase inhibitor [45–51], completely suppressed the SA-β-gal expression induced by Na_2SeO_3 (Fig. 1A and C) and MSeA (Fig. 1B and D). Consistent with our previous results [20], pretreatment of MRC-5 cells with the antioxidants NAC or Tempo significantly decreased cellular expression of SA-β-gal after the selenium treatment. Compared to MRC-5 cells, CCD 841 CoN cells showed greater SA-β-gal expression before and after selenium treatment. Representative pictures are shown in Supplemental Figs. 1 and 2. Thus, DNA-PK_{cs} is involved in selenium-induced senescence response in normal diploid fibroblasts.

3.2. DNA-PK_{cs} is phosphorylated on Ser-2056 and Thr-2647 after Na_2SeO_3 treatment and is downstream of ATM and ROS-dependent

We next determined whether and how selenium treatment activates DNA-PK_{cs} and ATM. Analyses of immunofluorescence results indicated that Na_2SeO_3 treatment in MRC-5 cells induced the formation of *p*DNA-PK_{cs} Ser-2056 (Fig. 2A and Supplemental Fig. 3) and Thr-2647 (Fig. 2B and Supplemental Fig. 4). Because selenite-treated MRC-5 cells show induction of *p*ATM Ser-1981 [20], we next determined the sequential events of DNA-PK_{cs} and ATM phosphorylation after selenium treatment. The selenite-induced *p*DNA-PK_{cs} Ser-2056 focus formation was completely prevented by pretreatment either with KU 55933 (10 μmol/L, 24 h) or NU 7026 (10 μmol/L, 24 h) (Fig. 2A). Furthermore, the selenite-induced *p*DNA-PK_{cs} Thr-2647 focus formation was significantly decreased by pretreatment with KU 55933, but not by NU

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