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Radioprotection by short-term oxidative preconditioning: Role of manganese superoxide dismutase

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ABSTRACT

Manganese superoxide dismutase (MnSOD) is vital to the protection of mitochondria and cells against oxidative stress. Earlier, we demonstrated that catalytically active homo-tetramer of MnSOD can be stabilized by oxidative cross-linking. Here we report that this effect may be translated into increased radioresistance of mouse embryonic cells (MECs) by pre-exposure to oxidative stress. Pre-treatment of MECs with antimycin A, rotenone or H_2O_2 increased their survival after irradiation. Using MnSOD siRNA, we show that MECs with decreased MnSOD levels displayed a lowered ability to preconditioning. Thus oxidative preconditioning may be used for targeted regulation of MnSOD.

Structured summary:
MINT-7288408: *MnSOD* (uniprotkb:P04179) and *MnSOD* (uniprotkb:P04179) *physically interact* (MI:0915) by *zymography* (MI:0512)

1. Introduction

Mitochondrial damage realized through superoxide dependent pathways plays a critical role in radiation injury to cells and tissues [1]. These effects of superoxide and other superoxide-derived reactive oxygen species on mitochondria may be realized immediately due to irradiation (IR) induced radiolysis of water, as well as at later time during the mitochondrial stage of apoptotic response [2]. Manganese superoxide dismutase (MnSOD) provides the first line of defense against superoxide overproduced in the mitochondria [3]. Post-irradiation generation of reactive oxygen and nitrogen species, likely associated with the formation of peroxynitrite, may lead to inactivation of MnSOD [4]. Notably, MnSOD confined to mitochondria – but not MnSOD genetically manipulated to be in the cytosol – is radioprotective [5]. Moreover, treatment with MnSOD plasmid has been shown to improve survival of mice exposed to total body irradiation (9.5 Gy) [6].

Adaptive response, or preconditioning, allowing to avoid lethal effects of radiation exposures, has been observed in cell-survival studies from yeast to mammalian and human cells in vitro as well as animal models in vivo [7]. The precise mechanisms by which preconditioning occurs are unknown. Recently, we have shown that treatment of recombinant human (rh)MnSOD with oxidants (hydrogen peroxide (H_2O_2) plus horseradish peroxidase or Cu^{2+}) induced formation of dityrosine cross-linked high molecular weight oligomers with intact activity and increased resistance to unfolding, degradation, and peroxynitrite mediated inactivation [8]. Based on this, we hypothesized that oxidative modification of MnSOD represents a novel radioprotective strategy that may be realized in a preconditioning paradigm. Here we show that short-term pre-treatment of cells with pro-oxidant inhibitors of mitochondrial complexes I and III – rotenone or antimycin A (AA) – caused protection against IR-induced cell death. We further

Abbreviations: H_2O_2, hydrogen peroxide; PI, propidium iodide; PS, phosphatidylserine; AA, antimycin A; MnSOD, manganese superoxide dismutase; IR, ionizing radiation; MECs, mouse embryonic cells; SC, scrambled control

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demonstrate the involvement of MnSOD oxidative modification in the protective effect.

2. Materials and methods

2.1. Reagents

Annexin-V apoptosis detection kit was purchased from BioVision (Mountain View, CA). Caspase-3/7 activity kit was obtained from Promega (Madison, WI). siRNA and transfection reagents were from Invitrogen (Carlsbad, CA). Other reagents were purchased from Sigma (St. Louis, MO).

2.2. Cell culture

Mouse embryonic cells (MECs, courtesy of Dr. X. Wang, University of Texas, Dallas) were cultured in DMEM media supplemented with 15% fetal bovine serum, 25 mM of HEPES, 50 mg/L of uridine, 110 mg/L of pyruvate, 2 mM of glutamine, 1 × non-essential amino acids, 0.05 mM of β-mercaptoethanol, 0.5×10^6 U/L of mouse leukemia inhibitory factor, 100 U/L of penicillin, and 100 μg/L of streptomycin.

2.3. Treatment and irradiation

Cells ($0.5–2 \times 10^5$) were seeded in 35 or 60 mm cell culture dishes and left to attach overnight. Cells were incubated with AA, rotenone or H_2O_2 in complete culture medium before IR (30 min) and were γ-irradiated using a Shepherd model 143-45A irradiator (J.L. Shepherd & Associates, San Fernando, CA) at a dose rate of 4 Gy/min. Then cells were washed twice and medium was replaced with fresh one.

2.4. Caspase activity

Caspase activity in cell lysates was measured with a Caspase-Glo-3/7 assay kit. Chemiluminescence was determined at 25 °C with ML1000 plate reader (Dynatech Laboratories, Chantilly, VA) and activity was expressed as luminescence intensity per milligram protein produced within 1 h incubation.

2.5. Cell viability

Cell viability was determined with propidium iodide (PI). For assessments of phosphatidylserine (PS) externalization Annexin V-FITC (plus PI) was employed. Both measurements were performed on FACScan flow cytometer (Beckton-Dickinson, Franklin Lakes, NJ). PS externalization was assessed as the sum of Annexin-V(+) cells with PI(−) and PI(+) cells.

2.6. Cell morphology

MECs were grown on collagen-coated glass cover slips prior to AA treatment and IR. At 48 h after irradiation, cells were fixed with paraformaldehyde (4%), stained with Hoechst3343 (2 μg/ml) for 15 min followed by fluorescence and visible light microscopy to visualize the morphological characteristics associated with apoptotic cells, including blebs, nuclear breakdown and heterochromatin aggregation [9].

2.7. MnSOD activity

Prior to measurements, cells were ruptured by sonication in PBS containing a protease inhibitor cocktail. MnSOD activity was measured using cytochrome *c* reduction method of McCord and Fridovich [10]. Samples were pre-incubated with 5 mM KCN for 45 min at RT to deactivate Cu,Zn-SOD.

2.8. MnSOD zymography and Western blotting

Cells were collected and lysed in RIPA buffer with protease inhibitors on ice for 45 min, centrifuged at 10 000×*g* for 5 min and the supernatants were collected for electrophoresis. For zymography, samples were incubated in Laemmli buffer containing 1.5% SDS and 100 mM DTT for 15 min, and 30 μg of protein

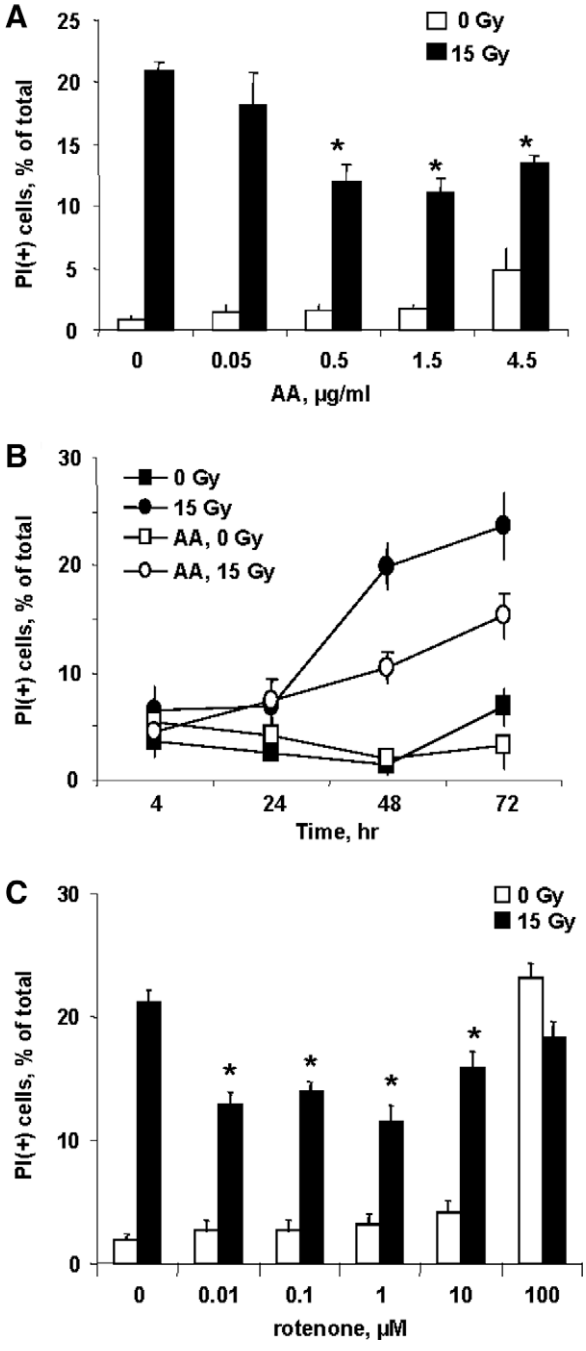

Fig. 1. Protection of MECs against IR-induced death by pretreatment (30 min prior to IR) with inhibitors of mitochondrial respiratory complexes – AA (A and B) and rotenone (C). (A) Dependence of preconditioning effect on AA concentration (0.05–4.5 μg/ml). The number of PI(+) cells was measured at 48 h after IR. (B) Time-course of IR-induced cell death (AA, 1.5 μg/ml). (C) Protective effect of preconditioning with rotenone (0.01–100 μM, other conditions as in A).