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Intrinsic attenuation of post-irradiation calcium and ER stress imparts significant radioprotection to lepidopteran insect cells

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ABSTRACT

Sf9 lepidopteran insect cells are 100–200 times more radioresistant than mammalian cells. This distinctive feature thus makes them suitable for studies exploring radioprotective molecular mechanisms. It has been established from previous studies of our group that downstream mitochondrial apoptotic signaling pathways in Sf9 cells are quite similar to mammalian cells, implicating the upstream signaling pathways in their extensive radioresistance. In the present study, intracellular and mitochondrial calcium levels remained unaltered in Sf9 cells in response to radiation, in sharp contrast to human (HEK293T) cells. The isolated mitochondria from Sf9 cells exhibited nearly 1.5 times greater calcium retention capacity than mammalian cells, highlighting their inherent stress resilience. Importantly, UPR/ER stress marker proteins (*p*-eIF2α, GRP4 and SERCA) remained unaltered by radiation and suggested highly attenuated ER and calcium stress. Lack of SERCA induction further corroborates the lack of radiation-induced calcium mobilization in these cells. The expression of CaMKII, an important effector molecule of calcium signaling, did not alter in response to radiation. Inhibiting CaMKII by KN-93 or suppressing CaM by siRNA failed to alter Sf9 cells response to radiation and suggests CaM-CaMKII independent radiation signaling. Therefore, this study suggests that attenuated calcium signaling/ER stress is an important determinant of lepidopteran cell radioresistance.

1. Introduction

Lepidopteran insect cells are an excellent higher eukaryotic model system due to their exemplary radioresistance as evidenced in various studies since the middle of 1970s [1–3]. These cells display over 100–200 times higher resistance to ionizing radiation as compared to human cells. Understandably therefore, identifying cellular factors and mechanisms contributing in their radioresistance could have significant implications for designing better strategies for biological radioprotection, especially as Lepidopteran cells carry numerous homologies with the mammalian system [4]. Previous studies have suggested potential role of multiple factors in the exemplary stress resistance displayed by these cells, i.e., more efficient protection against DNA and cytogenetic damage [3] contributed by stronger antioxidant system [5,6], DNA repair machinery [2,7] and DNA-histone interactions [8]. A highly attenuated NOS signaling [9] as well as certain alternate pathways regulating Lepidopteran apoptosis [10] seem to further protect these model cells from radiation-induced death. Interestingly, these cells have very well conserved mitochondria-mediated mechanisms of stress-induced apoptosis [11], suggesting a more critical role of upstream signaling [12].

Calcium signaling constitutes a major upstream cellular stress response mechanism, and is significantly regulated via endoplasmic reticulum (ER) as it is a major calcium store of the cell. The ER plays primary role in relaying/mediating the intracellular signals of calcium by changing its spatial-temporal pattern in the form of oscillations, spikes and puffs. These signals or patterns in turn activate various kinases, phosphatases and transcription factors in response to external stimuli including ionizing radiation, and thereby play significant role in determining the fate of a cell [13]. One of the major calcium-sensing kinases is calmodulin-dependent protein kinase-II (CaMKII), which is activated by transient or prolonged increase in the intracellular Ca^{2+} concentration and its subsequent binding to calmodulin or CaM. CaMKII activates various cellular pathways by decoding the calcium signals it receives, and has even been termed the linchpin of ER stress-induced apoptosis as it is a major protein kinase activating various downstream mitochondrial apoptotic pathways [14]. Effects of modulating other

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calcium signaling proteins have also been assessed during variety of stresses. For example, overexpression of calcium binding proteins such as calbindin 28 K has been shown to reduce radiation-induced ROS/RNS generation [15] while overexpression of ER-resident calcium binding protein calreticulin has been shown to protect cells from H_2O_2 induced cellular damage [16]. Intracellular calcium chelator BAPTA-AM (1,2-bis(*o*-aminophenoxy)ethane-N,N,N′,N'-tetraacetic acid) has been demonstrated to protect cells against toxic calcium overload [17]. These different studies point to the significant role of calcium dynamics in maintaining cellular homoeostasis as well as in countering extraneous stress.

Several studies have reported that calcium may play an important role in regulating various cellular processes in response to ionizing radiation. These include ionizing radiation induced DNA repair [18], cell cycle arrest [19] and cell death [20]. Further calcium mobilization has also been associated with mobilizing the lethal effects of X-irradiation [21]. Apart from calcium disturbances, ionizing radiation has also been associated with the induction of 'unfolded protein response' (UPR) due to accumulation of unfolded and misfolded proteins in ER [22]. In our recent study [23], both these facets of ER stress, i.e., UPR induction and calcium disturbances were found to be uniquely attenuated in the model Lepidopteran system (Sf9 cells) in response to various chemical ER stress inducers. In the present study, we further investigate whether similar alterations in calcium signaling and ER stress response play a role in insect cell radioresistance. We report an unusually strong attenuation of both these radiation-inducible responses despite the presence of very well conserved pathways. Since calcium and ER disturbances are strong determinants of cell fate, these findings may have important implications for improving biological radioprotection.

2. Material and methods

2.1. Cell culture

Sf9, a spheroidal semi-adherent insect cell line originally derived from the ovaries of *Spodoptera frugiperda*, the Fall armyworm (order Lepidoptera), was maintained as monolayer in 25-cm^2 culture flasks at 28 °C in Grace's insect cell culture medium (Cat. No. G9771, Sigma USA) as described in Ref. [3]. The Human Embryonic Kidney (HEK) cell line was maintained by passaging twice a week at 37 °C in high glucose DMEM (Cat. Number: D5648, Sigma, USA) supplemented with antibiotics (as mentioned above) and 10% FBS (Cat. Number: F2442 Sigma, USA).

2.2. Irradiation and treatments

Exponentially growing cells were irradiated using ^{60}Co gamma chamber (Gamma Chamber 5000, Board of Radiation and Isotope Technology, Department of Atomic Energy, Mumbai, India) at a dose rate of 19.16 Gy/min. Irradiation was carried out at room temperature. Axiovert-200 Zeiss inverted DIC microscope (Carl Zeiss) was used for routine morphological observations. KN-93 (1 μM or 5 μM) was added 1 h prior to irradiation. 5 μM KN-93 was used for further experiments and morphological observations were taken at 24 h post irradiation. siRNA against calmodulin (Eurofins MMG operon, Bangalore, India); 0.25 μg/ml) was added 24 h prior to irradiation whereas morphological observations were taken after 24 h irradiation.

2.3. Cell death analysis

For morphological discrimination of apoptotic, necrotic and intact cell population, cells treated with various stress inducing agents were embedded in agarose and were stained with PI (Cat number P4170, Sigma USA) and fluorescein isothiocyanate (Cat number F7250, Sigma USA) as described earlier [24].

2.4. Analysis of intracellular calcium distribution using flow cytometry

Fluo-3 AM loading was done by incubating cells with 4 μM Fluo-3 AM (Cat Number: 73881, Sigma, USA) as described in Ref. [23]. Fluorescence changes were measured over time by excitation at 488 nm using *FACSCalibur flow cytometer* (Becton Dickinson, USA) at various time points as indicated. Indo-1-am labeling and flow cytometry acquisitions were done as detailed earlier [23].

2.5. Analysis of mitochondrial calcium distribution using flow cytometry

Rhod-2 AM labeling was performed by incubating cells at with 10 μM Rhod-2 AM (Cat Number: R1245, Molecular probes, Oregon, USA) as described in Ref. [23]. Fluorescence changes were measured using *FACSCalibur flow cytometer* (Becton Dickinson, USA).

2.6. Immuno-blotting of proteins

Immunoblotting was done as described in Ref. [23] The primary antibodies used were: *p*-eIF2α, *p*-CaMKII (Cell signaling Technology); GRP94 (Santa Cruz Biotechnology, USA), and SERCA (Sigma USA). Anti-β-actin antibody (Santa Cruz) was used as loading control.

2.7. Statistical analysis

Differences between the mean values were analyzed for significance using the paired two tailed student's 't' test for independent samples using Microsoft Excel, with p value ≤ 0.05 considered as statistically significant.

3. Results

3.1. Sf9 cells exhibit negligible increase in cytosolic and mitochondrial calcium levels in response to radiation

Using fluo-3-AM, which detects cytosolic calcium, radiation-induced alterations in calcium levels were investigated in Sf9 cells and compared with mammalian cell responses. Radiation-induced increase in cytosolic calcium was observed in mammalian (HEK) cells from as early as 30 min and continued till 24 h. In sharp contrast, Sf9 cells failed to show any significant calcium accumulation (except a transient calcium spike at 30 min) even when followed up to 24 h post-irradiation at 10Gy (Fig. 1a). We further used rhod-2-AM for studying radiation-induced accumulation of mitochondrial calcium. Radiation-induced increase in mitochondrial calcium was observed in mammalian cells starting from 4 h up to 24 h whereas negligible changes could be observed in mitochondrial calcium in Sf9 cells up to 24 h post-irradiation at 10Gy (Fig. 1b). Incidentally, the basal constitutive level of cytosolic as well as mitochondrial calcium was significantly higher (nearly 2 and 1.5 times, respectively) in Sf9 cells as compared to mammalian cells (Fig. 1a and b; insets in lowest line graph).

3.2. Sf9 cells undergo significantly lower radiation-induced UPR/ER stress

Further, alterations in two well-characterized markers/