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Radiation-induced oxidative stress regulates protein synthesis by modulating the expression of heme-regulated eIF2α kinase in human K562 cells

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Inhibition of protein synthesis executed at the initiation step is the overall response of cells during stress. Here, we evaluated the effect of gamma radiation induced oxidative stress on protein synthesis in human K562 cells. In erythroid cells such as K562, the heme-regulated eukaryotic initiation factor 2α (eIF2α) kinase, also called the heme-regulated inhibitor (HRI), is abundant and is instrumental in regulating protein synthesis. We, therefore, examined the modulation of expression and activity of HRI in K562 cells at various time points following their exposure to 6 Gy of gamma radiation. Radiation-induced oxidative stress was reflected by a dose-dependent increase in the intracellular reactive oxygen species with time up to 6 h. Further, cell membrane damage in terms of lipid peroxidation and lipid hydroperoxide formation was also observed. Interestingly, radiation induced oxidative stress led to a significant decrease in the rate of protein synthesis caused due to induced activation as well as expression of HRI within 1 h. Furthermore, radiation exposure also caused increased expression of heat shock protein 90 (Hsp90) in 1 h. These results have demonstrated shutdown of global protein synthesis in K562 cells during radiation induced oxidative stress, mediated by overexpression and activation of HRI possibly caused by Hsp90.

Keywords: eIF2α kinase, Gamma radiation, Heme-regulated inhibitor (HRI), Hsp90, Human K562 cells, Protein synthesis

During adverse conditions, cells must be able to maintain their intracellular homeostasis. Typically, they respond by invoking complex regulatory mechanisms, including global inhibition of translation. Continued gene expression during potentially error-prone conditions inside the cells leads to alterations in protein synthesis[1]. During oxidative stress, cell allows turnover of existing mRNAs and proteins, while gene expression is reprogrammed to deal with the stress. Regulation of gene expression occurs at both the levels of transcription and translation. However, translation regulation appears to be a predominant mode of regulation of gene expression/protein synthesis in response to environmental stimuli[2]. Regulation of translation, occurring at the initiation step is most common, as it is a highly complex process and it requires many factors. It is well established that regulation of protein synthesis at the level of initiation is crucial in response to various cellular stresses such as heme deficiency, nutrient deficiency, viral infection, heat shock, etc.[3,4].

One of the well-known events, involved in the global inhibition of translation initiation in eukaryotes, is phosphorylation of the alpha subunit of the eukaryotic initiation factor 2 (eIF2) which is rapidly followed by a decline in the overall rate of protein synthesis[2]. In mammals, phosphorylation of eIF2α is caused by one of the 4 Ser/Thr kinases belonging to the eIF2α kinase family[5,6]. The 4 different members of this eIF2α kinase family are, the heme-regulated eIF2α kinase, also called the heme-regulated inhibitor (HRI), the double-stranded RNA-dependent protein kinase (PKR), the PKR like endoplasmic reticulum kinase (PERK) and the General control nonderepressible 2 (GCN2) kinase[7-10]. All these enzymes are regulated independently in response to different cellular stresses; and upon activation during a particular stress they phosphorylate the α subunit of eIF2 at Ser51 residue[5,11]. Towards the end of initiation, before the formation of 80S initiation complex, eIF2.GTP gets hydrolyzed and is liberated from the initiation complex as eIF2.GDP. The inactive eIF2.GDP binary complex needs to be reactivated as a functional eIF2.GTP complex by a GDP-GTP exchange reaction carried out by eukaryotic initiation factor 2B (eIF2B), which is a guanine nucleotide exchange factor. However, when eIF2α gets phosphorylated by an eIF2α

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kinase at Ser51 residue [eIF2α (P)], it tightly binds to eIF2B and sequesters it. Since eIF2B is rate limiting, this sequestration leads to a failure of GTP-GDP exchange, and thus it results in inhibition of protein synthesis[11]. A few recent studies have also established eIF2α kinase as a new anticancer target, thus indicating its importance in cancer therapy[12-16].

Various reactive oxygen species (ROS) such as superoxide ($O_2^{\cdot-}$), hydroxyl ($^{\cdot}OH$) and peroxyl ($ROO^{\cdot}$) radicals, besides non-radical species such as hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$) and hypochlorous acid (HOCl), are produced during normal and altered physiological processes in a cell, and their elevated levels are linked to cell injury leading to cell death[13]. Free radicals are important in biological systems as they are involved in different cellular processes *viz*. regulation of growth and differentiation, gene expression, cell signaling, proliferation, and apoptosis[17,18]. Radiation is one of the major factors that increases ROS generation in living organisms[19,20]. Although information on the effect of radiation on protein synthesis regulation by cell cycle mediating proteins is available[21], the role of eIF2α kinases during this condition is still not clear.

Changes in the levels of expression and activity of HRI during heme deficiency[22], heavy metal exposure[11] and drug toxicity[23] are well studied. However, only little is known about radiation exposure and HRI-mediated regulation of protein synthesis. Here, we studied the effects of ionizing radiation on the regulation of protein synthesis and also on the expression and activity of HRI in human K562 cells in which HRI is abundant.

Materials and Methods

Materials

Dulbecco's Modified Eagle's Medium (DMEM), Foetal bovine serum (FBS) and penicillin/streptomycin solution (100X) were purchased from HiMedia (HiMedia, Pvt. India). Custom-made HRI- and β-Actin cDNA specific primers, TRI reagent, DNase I (amplification grade), enhanced Avian HsRT-PCR kit and monoclonal antibodies, namely Hsp90 and β- Actin antibodies were also purchased from Sigma Chemical Co. (USA). Anti-phospho-eIF2α [eIF2α (P)], anti-eIF2α and anti-HRI polyclonal antibodies were purchased from Abcam (USA). BM Chemiluminescence Western blotting kit (Mouse/Rabbit) was purchased from Roche Molecular Biochemicals (Germany). Radioisotope ^{35}S-methionine was purchased from Board of Radiation and Isotope Technology (BRIT), India. Human erythroid K562 cell line was obtained from the cell repository at the National Centre for Cell Science (Pune, India).

Maintenance of K562 cells and gamma-irradiation

Human K562 cells were maintained as a continuous culture in DMEM containing 100 U/mL penicillin, 100 µg/mL streptomycin and 10% FBS, at 37°C in 5% CO_2 atmosphere in a humidified chamber. For all radiation exposures, a ^{60}Co blood irradiator was used with a dose rate of 1.78 Gy/min.

Effect of gamma radiation on K562 cell

To determine the suitable radiation dose, K562 cells were exposed to different doses of gamma radiation (0, 2, 4, 6, 8 and 10 Gy) in serum free medium followed by MTT [3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. Immediately after completion of radiation exposure, FBS was added to the medium to make a final concentration of 10% and 1×10^4 cells/200 µL were seeded in each well of a 96-well plate. Cells were allowed to grow for 72 h at 37°C in CO_2 incubator. MTT solution (10 µL of 5 mg/mL) was added 4 h before completing 72 h of incubation. Formazan crystals formed were dissolved by adding 100 µL solubilisation buffer (10% SDS containing 0.01N HCl) and incubated overnight at 37°C. On the following day, developed colour was measured at 550 nm using a micro plate reader.

To monitor radiation induced cell death in K562 cells, propidium iodide staining was done and cells were analyzed by a flow cytometer. In brief, 1×10^6 K562 cells/2mL were exposed to different doses of gamma radiation (0, 2, 4, 6, 8 and 10 Gy) inserum- free medium. After radiation exposure FBS was added in the tubes to get a final concentration of 10%. Cells were allowed to grow for 72 h at 37°C in 5% CO_2 atmosphere. These cells were washed with 1 X PBS and stained with PI-staining solution (0.5 µg/mL propidium iodide, 0.1% sodium citrate and 0.1% Triton X-100) overnight. A total of 20000 cells were acquired in Partec flow cytometer and analyzed using Flow max software (Partec). The sub-G1 population (<2n DNA content) represented the apoptotic cells/dead cells, and cells having 2n-4n DNA were considered as live cells.

Measurement of radiation-induced Intracellular Reactive Oxygen Species (ROS)

To measure radiation induced intracellular ROS, 2',7'-dichloro dihydro fluorescein diacetate (H_2DCFDA)

dye was used[24]. In brief, cells (1×10^5/mL) were pre-incubated with 20 μM H_2DCFDA for 20 min at 37°C. Excess of the dye was removed by washing with the medium. These dye-loaded cells were exposed to different doses of gamma-radiation (0, 2, 4, 6, 8 and 10 Gy). Changes in fluorescence intensity was measured at the excitation wave length of 485 nm and emission wave length of 520 nm at different time points of post-irradiation recovery, such as 15 min, 1, 2, and 6 h by a microplate reader.

Measurement of radiation-induced membrane damage

Effect of gamma radiation on lipid peroxidation in K562 cells was measured in terms of n moles of malondialdehyde equivalents formed. Briefly, gamma irradiated and control samples of K562 cells (2×10^6 cells) were incubated with TBA reagent (20% TCA, 0.5% TBA, 2.5 N HCl and 6 mM EDTA) for 20 min in a boiling water bath. After cooling, the pink colour representative of thiobarbituric acid reactive substances (TBARS) was measured at 532 nm and was expressed as nmoles of TBARS formed per mg protein[22]. As standard calibration, 1, 1, 3, 3-tetra methoxypropane (TMOP) was used. Cell damage caused by gamma radiation in K562 cells was also determined by estimating total lipid hydro peroxides (LOOH) formed using FOXII reagent[25]. Cells were suspended in 1 X PBS and proteins were quantified from intact cells present in the suspension. The suspension volume corresponding to 1 mg of protein was used for LOOH assay. Results were expressed as nmoles of LOOH/mg protein. H_2O_2 was used as standard peroxide for calibration. For both the assays, post irradiation time points of 15 min, 1, 2, 6 and 24 h were taken.

Determination of protein synthesis by metabolic ^{35}S-methionine labelling

Rate of protein synthesis in control and irradiated cells was determined by incorporation of ^{35}S-methionine into proteins, followed by liquid scintillation counting. In brief, cells were exposed to 6 Gy gamma radiation and 1×10^6 cells were collected at different time points (1, 2, 6 and 24 h) by centrifugation. Cells were suspended in a methionine-free DMEM and were incubated at 37°C for 1 h prior to ^{35}S-methionine labelling. Such cells were seeded in DMEM containing 10 % FBS and 0.5 mCi/mL of ^{35}S-methionine and incubated at 37°C for 4 h. After completion of incubation, cells were harvested by centrifugation and washed 4 times by 1 X PBS followed by extraction of total soluble proteins. Equal volume of protein was TCA precipitated by spotting it on Whatman no. 1 filter paper discs and incubated with 10% ice cold TCA. Paper discs were washed sequentially with 5% ice cold TCA, 2% ice cold TCA, followed by washing with absolute ethanol to facilitate drying. Dried filter discs were taken into scintillation cocktail solution in vials and then the vials were used for liquid scintillation counting.

Protein extraction and SDS-PAGE

Proteins were extracted from control and gamma irradiated cells by lysing them in lysis buffer (50 mM Tris-HCl, pH 8.0; 5 mM EDTA, 0.1% (v/v) Triton X-100, 1 mM PMSF, protease inhibitor cocktail). Total soluble proteins were collected by centrifuging the cell lysate at 14000 × g.Total proteins in the supernatant were quantified by Bradford's micro estimation method[26]. Equal quantities of proteins (120 μg/well) from all groups were separated by 10% SDS-PAGE[27].

Western blot analysis

Following SDS-PAGE, proteins were electrophoretically transferred to nitrocellulose membranes[28] which were subsequently used for immunoblotting with various antibodies, namely anti-eIF2α, anti-eIF2α (P), anti-HRI, anti-Hsp90 and anti-β-Actin antibodies. In brief, blots were saturated with blocking reagent for 1h, and incubated overnight with primary antibody in 5% (w/v) bovine serum albumin (BSA) or 1% blocking reagent containing non-fat milk at 4°C and then with anti-mouse/rabbit IgG-Horse Radish Peroxidase (HRP)-conjugated secondary antibody for 1h at room temperature. Following each antibody incubation, blots were washed thrice (5 min each) in 1 X TBST (Tris-buffered saline with Tween 20). Blots were developed using the chemiluminescence detection kit (Roche, Germany). The results were analyzed by using Bio-Rad gel documentation system (Bio-Rad, USA).

RNA extraction, RT-PCR and PCR

Total RNA from K562 cells exposed to gamma radiation was extracted using TRI reagent as per the manufacturer's protocol and quantified spectro photometrically. Prior to cDNA synthesis, RNA samples were treated with DNase I to remove genomic DNA contaminations, and 5 μg of RNA was reverse transcribed to cDNA using eAMV-reverse transcriptase. An equal amount of cDNA was PCR amplified using HRI, Hsp90 and β-Actin specific

primers. PCR products were analysed on a 1.5% agarose gel followed by ethidium bromide staining.

Statistical analysis

All the experiments were performed at least thrice and the data are expressed as mean ± standard error of mean. Student's t-test was performed to determine significant differences between treatment and control. Differences at $P<0.05$ level were considered statistically significant.

Results and Discussion

Optimization of dose of gamma radiation on human K562 cells

In order to determine the effect of radiation on human K562 cells, it was important to optimize the dose of gamma radiation. Therefore, the cells cultured *in vitro* were exposed to various doses of radiation and the effect was monitored by determining cell viability and apoptosis. Results of MTT assay and propidium iodide staining experiments indicated that there is a dose-dependent inhibition of K562 cell proliferation and an increase in cell death after radiation exposure (Fig. 1A). The LD50 value was around 6 Gy. Furthermore, flow cytometric analysis showed a significant increase in sub-G1 population in irradiated cells as compared to the untreated control cells. Almost a 50 % cell death was observed at the 6 Gy radiation dose when the cells were exposed for a period of 72h (Fig. 1 B & C). Since in both the above experiments, about 50% cell death was observed at the dose of 6 Gy, this dose was considered as the suboptimal dose for further studies.

Irradiation causes an increase in intracellular ROS level

To ascertain the effectivity of the radiation dose further, we determined the intracellular ROS levels in K562 cells irradiated with gamma radiation at 2, 6 and 8 Gy using H_2DCFDA dye. A dose- and time-dependent increase in the intracellular ROS levels was observed in K562 cells which were also found to be significant over control at both 6 and 8 Gy at 6 h after irradiation (Fig. 2). These results, thus indicated that the dose of gamma radiation chosen, *i.e.*, 6 Gy causes a significant level of intracellular ROS production.

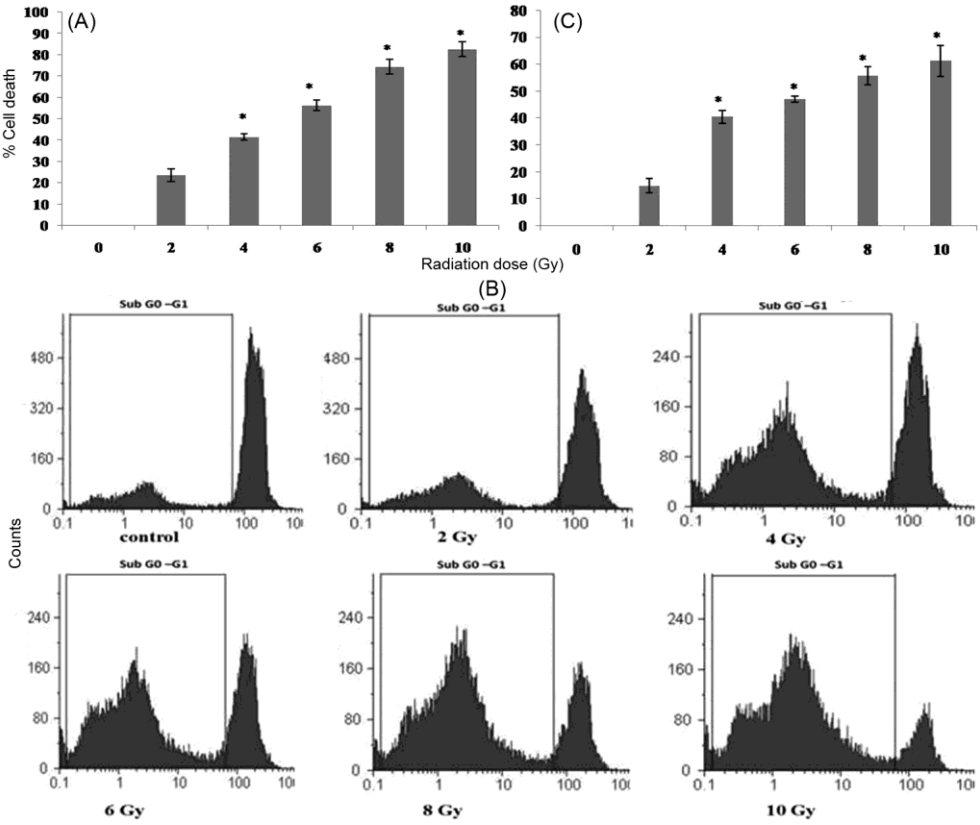

Fig. 1—Effect of gamma radiation on cell death as determined by MTT assay and propidium iodide staining. (A) Cell death as monitored by MTT assay; (B) Flow cytometric data of propidium iodide stained cells for live and dead cells analysis; and (C) Percentage cell death (sub-G1 population) at different radiation doses calculated from the flow cytometer data. [The results are mean ± SEM; * $P \leq 0.05$ compared to control]

Irradiation induces cell membrane damage

We also determined the effect of radiation (6 Gy) on cell membrane in K562 cells. As seen in Fig. 3, the 6 Gy gamma radiation significantly increased the membrane damage as monitored by lipid peroxidation and lipid hydroperoxide formation. In non-irradiated control K562 cells, 0.061 n moles of TBARS/mg protein were formed which increased to 0.158 n moles of TBARS/mg protein after 6 h of radiation exposure. Further, this increase in lipid peroxidation took place in an incremental fashion till 6 h and then it decreased at 24 h (Fig. 3A). Attempts were also made to verify the possibility of occurrence of lipid peroxidation by estimating total lipid hydroperoxides in K562 cells irradiated at 6 Gy. It was observed that with time, there were significant increases in the production of lipid hydroperoxides over control up to 6 h and then it decreased to control level at 24 h (Fig. 3B). Thus, the results of lipid hydroperoxide estimation (LOOH assay) corroborated well with that of lipid peroxidation (TBARS) assay[29].

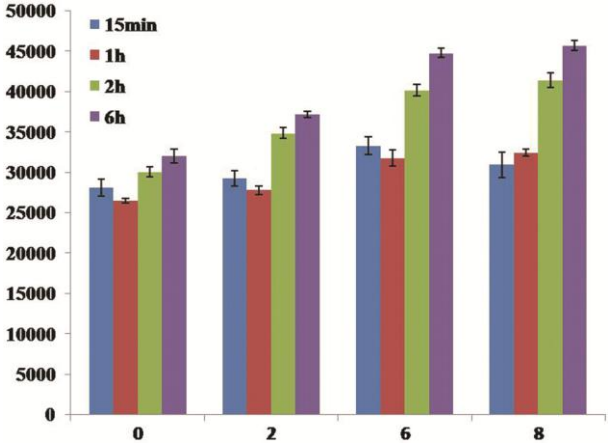

Fig. 2 — Measurement of intracellular ROS levels using DCFH-DA fluorescent dye. Cells were irradiated with different doses of radiation (Gy) and levels of ROS (Fluorescence in arbitrary units, AU) were measured at different time points during post-irradiation recovery

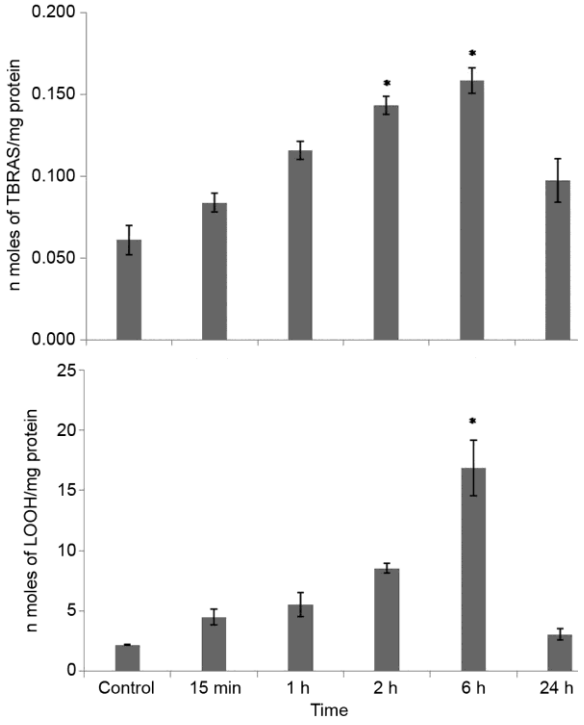

Fig. 3—Effect of gamma radiation on membrane damage in K562 cells. Cells were irradiated with 6 Gy radiation dose and membrane damages were measured at different time points. (A) Estimation of total lipid peroxidation products using TBARS assay. The results are expressed as nmoles of TBRAS/mg protein; and (B) Estimation of total lipid hydroperoxides using LOOH assay. The results are expressed as n moles of LOOH/mg protein. [The results are mean ± SEM; * $P \leq 0.05$ compared to control]

Radiation-induced oxidative stress inhibits protein synthesis

It is known that protein synthesis, being an important cytoplasmic process, is often the target of oxidative stress. We, therefore, determined the effect of radiation induced oxidative stress on the rate of global protein synthesis in K562 cells by metabolic ^{35}S-methionine labelling of proteins. In our experiment, we found that after irradiation, there is an inhibition of protein synthesis at 1 h and thereafter there is a gradual increase in protein synthesis up to 6 h. However, after this time point, the rate of protein synthesis gradually declines and it comes back to that of the control cells at 24 h (Fig. 4).

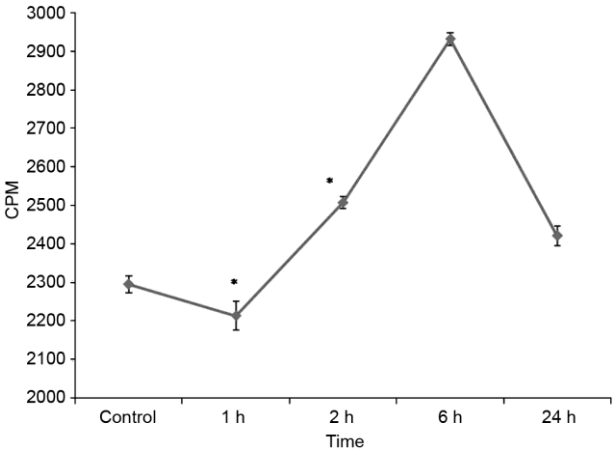

Fig. 4 —Oxidative stress decreases the initial rate of global protein synthesis in K562 cells. Cells were maintained in methionine-free DMEM at 37°C for 1 h prior to ^{35}S-methionine labeling. Equal number of such cells (1×10^6) was seeded in 1 mL complete medium containing 0.5 mCi/mL of ^{35}S-methionine in control and irradiated K562 cells and incubated at 37°C for indicated time points. Total soluble proteins were extracted from the samples and 15 µL of which was used for liquid scintillation counting. [The data presented are counts per minute (CPM) (mean ± standard deviation) of three independent estimations (value * $P \leq 0.05$ considered statistically significant)]

Initial inhibition of protein synthesis at 1 h is an important regulatory event for cell survival. The increase in protein synthesis after initial inhibition at 1h following radiation exposure/shock serves as a protective response. Indeed, it was previously proposed that increased protein synthesis following ionizing radiation might serve as a protective stress response[30]. The above results put together indicate that at the 24 h time point of post irradiation, it is not only protein synthesis but also cell membrane damage move toward normal level (Fig. 3).

Oxidative stress induced protein synthesis inhibition is caused at the initiation step

One of the primary mechanisms of regulation of initiation of protein synthesis is modulation of eIF2α phosphorylation by eIF2α kinases. Hence, we determined the modulation of eIF2α kinase activity during oxidative stress by measuring the eIF2α phosphorylation by Western blot analysis using a specific antibody that recognizes the phosphorylated (Ser51) form of eIF2α. The phosphorylated eIF2α amount increased at 15 min followed by a gradual decrease till 6 h (the last time point monitored). However, the decrease from 2 to 6 h was minimal (Fig. 5). The amount of total eIF2α was determined by another antibody which recognizes total eIF2α irrespective of its modification (phosphorylation). The

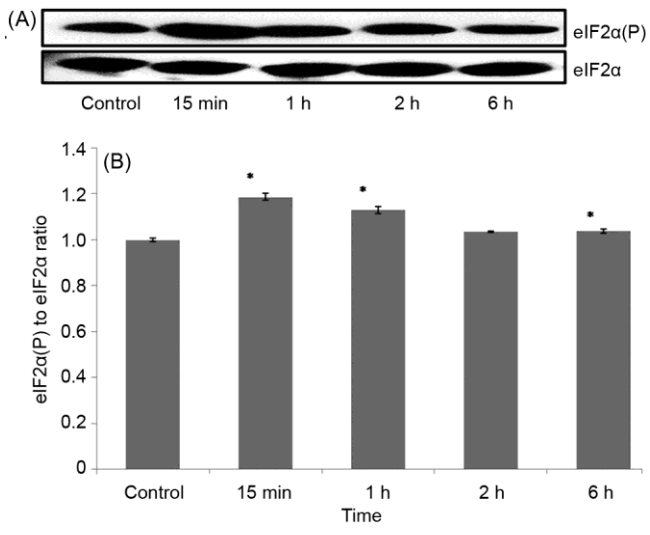

Fig. 5—Radiation induced oxidative stress causes phosphorylation of eIF2α. Western blots of soluble extracts of K562 cell samples irradiated at a dose of 6 Gy at different time points. The samples (as indicated) were probed with anti-eIF2α (P) antibody, and anti-eIF2α antibody. (A) Western blot images of eIF2α (P) and eIF2α proteins; and (B) Ratio of eIF2α (P) to eIF2α from the Western blot images. [The results are mean ± standard deviation of triplicate samples (* $P \leq 0.05$)]

data on eIF2α phosphorylation is presented in the form of eIF2α (P)/total eIF2α ratio (Fig. 5B) Thus, this data on eIF2α phosphorylation correlates well with that of the inhibition of protein synthesis (Fig. 4). These results, therefore, indicate that oxidative stress mediated inhibition of protein synthesis is exercised at the translation initiation step by induced phosphorylation of eIF2α after irradiation.

Ionizing radiation induces expression of HRI in K562 cells

As eIF2α kinases including HRI, are known to regulate protein synthesis during various stresses such as heat-shock, heavy metal toxicity, free radical exposure, etc., they are also overexpressed and activated. Therefore, it was important to determine the effect of radiation on HRI expression both at the levels of protein and mRNA and also its activation. K562 cells were irradiated with 6 Gy, and proteins were extracted at different time points of post-irradiation. The levels of HRI at various time points were measured by Western blot analysis using anti-HRI polyclonal antibody. As seen in Fig. 6 A & B, the quantity of HRI increased over control significantly at 15 min and 1 h time points followed by a gradual decrease till 6 h. It is interesting to note that HRI migrates as a doublet representing two forms of HRI: the slow migrating upper band represents hyper-phosphorylated form while the lower faster-migrating band represents the non-phosphorylated form of HRI. At the time points of 2 and 6 h, HRI level was more or less equal as compared to that in 1 h. Thus, it appears that the expression of HRI followed the same pattern as that of eIF2α phosphorylation (P). These results together indicate that HRI regulates inhibition of protein synthesis at the initiation step by phosphorylating eIF2α during radiation exposure of K562 cells. However, the role of other eIF2α kinases in this process remains to be determined.

Radiation-induced oxidative stress increases expression of Hsp90

As Hsp90 expression is known to be induced in response to various cellular stresses, we determined the effect of radiation induced oxidative stress on expression of Hsp90. The results obtained from Western blotting experiments indicated that the expression of Hsp90 protein increased after exposure to radiation from 15 min to 1 h and it decreased thereafter (Fig. 6 A & C). The pattern of expression of Hsp90 corroborated with that of HRI, and this result, thus supports our earlier observation that Hsp90 interacts with HRI and acts as a chaperon during various stress stimuli[10].

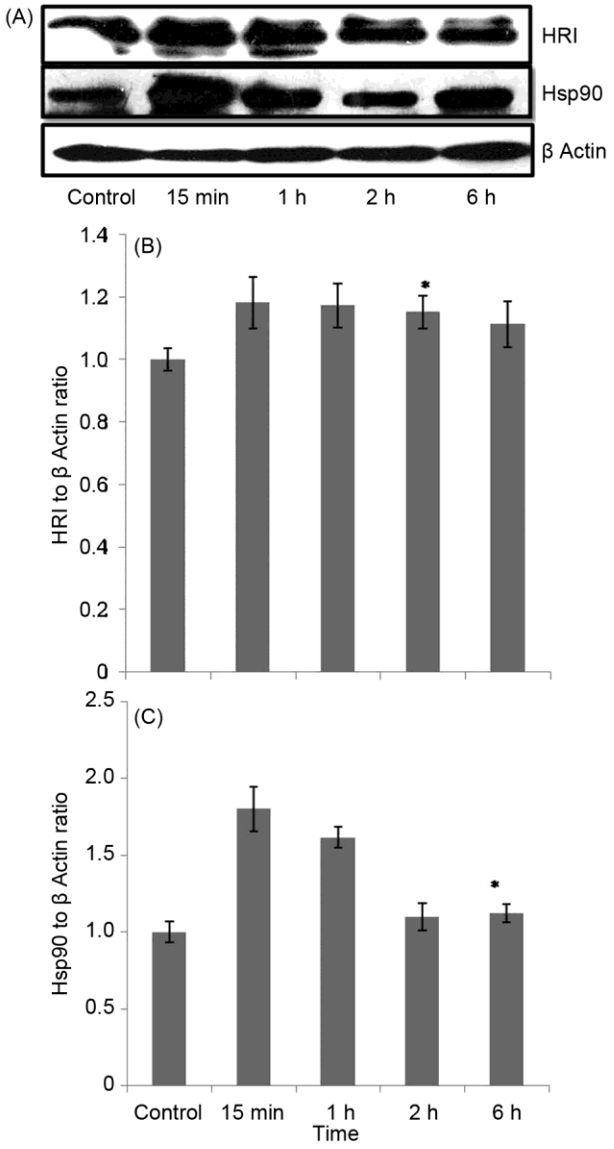

Fig. 6—Radiation induced oxidative stress causes increase in expression of HRI and Hsp90 at the protein level. Western blots of soluble extracts of K562 cell samples, irradiated at a dose of 6 Gy and protein was extracted at different time intervals. (A) The samples (as indicated) were probed with anti-HRI, anti-Hsp90 and anti-β-Actin antibodies; β-Actin was taken as the loading control; and (B & C) graphs indicate expression levels of HRI and Hsp90, respectively as protein/β-Actin ratio. [The results are mean ± standard deviation of triplicate samples (* $P \leq 0.05$)]

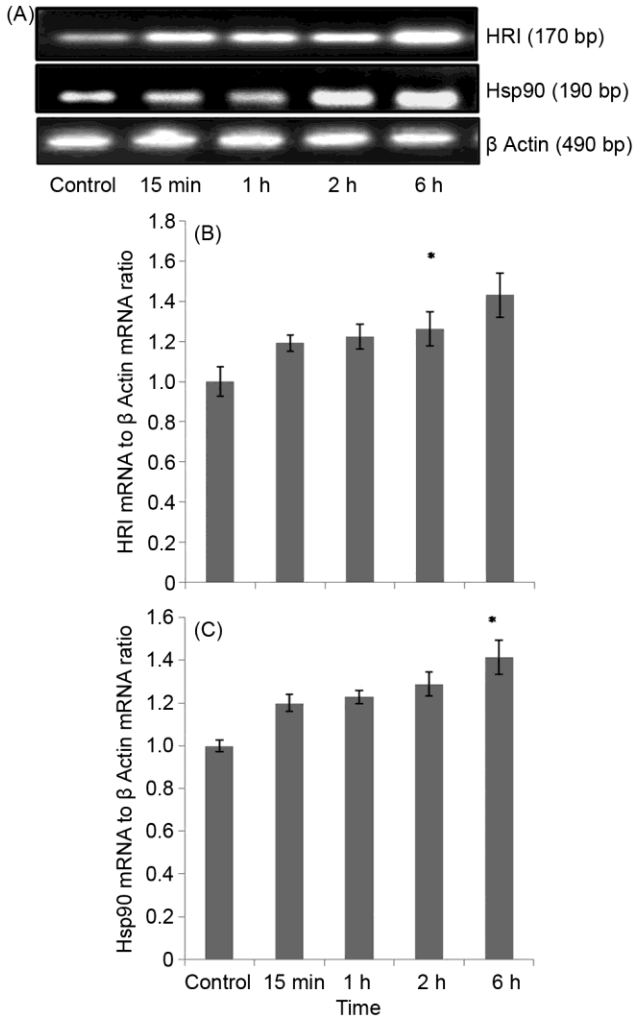

Fig.7—Radiation induced oxidative stress causes increase in expression of HRI and Hsp90 at the mRNA level. Total cDNA prepared from RNA extracted from K562 cells at different time intervals after irradiation, at a dose of 6 Gy was subjected to PCR using different primers specific for HRI, Hsp90 and β-Actin (internal control). (A) RT-PCR profiles of HRI, Hsp90 and β-Actin; and (B & C) graphs indicate mRNA expression levels of HRI and Hsp90, respectively. [The results are mean ± standard deviation of triplicate samples (* $P \leq 0.05$)]

Ionizing radiation induces HRI and Hsp90 expression at mRNA level

To determine the effect of oxidative stress on HRI and Hsp90 gene expression, K562 cells were irradiated at 6 Gy. Total RNA from control and treated cells at different time intervals were extracted and used for RT-PCR using HRI- and Hsp90 specific primers; amplification of β Actin using β Actin-specific primers was used as an internal control. As seen in Fig. 7, immediately after irradiation, the expression of HRI and Hsp90 increased from 15 min and reached its peak at 6 h. Expression of both the genes then decreased to normal level at 24 h (data not shown). These data, therefore, indicated that oxidative stress-induced inhibition of protein synthesis is due to both activation and induced expression of HRI.

We have investigated the effect of radiation-induced oxidative stress on cell damage and inhibition of protein synthesis. ^{60}Co-gamma radiation was used to irradiate human K562cells *in vitro* and all the

parameters linked to cell physiology and protein synthesis were investigated at different post irradiation time points. Firstly, a suitable radiation dose was determined which can be used in further experiments on human K562 cells. By MTT assay, radiation dose of 6 Gy was found to be suitable as it caused approximately 50% inhibition of cell growth at 72 h. To confirm the LD_{50} dose of gamma radiation for K562 cells, flow cytometric analysis was done, in which apoptotic cell death was determined by measuring the number of cells in Sub G1 phase of cell cycle after gamma radiation exposure. Approximately 50% cell death in human K562 cell line was observed at 6 Gy radiation dose after 72 h. Hence, for all further experiments, 6 Gy was used considering it as the LD_{50} dose.

Our results indicate that exposure of K562 cells to gamma radiation increases the oxidative stress in a dose- and time-dependent manner and affects cells at: (i) membrane level by inducing lipid peroxidation leading to membrane damage by generating intracellularfree radicals; (ii) cytoplasmic level by inhibiting initiation of protein synthesis mediated by both activation and over-expression of the heme-regulated eIF2α kinase (HRI) and Hsp90. Further, chemically generated free radicals and activation of HRI has been reported by us previously[31]. Therefore, as observed in the present study, radiation induced lipid peroxidation and activation/induced expression of HRI appear to be through generation of free radicals.

Free radicals generated due to gamma radiation treatment react with lipid producing lipid peroxides, hydroperoxides, etc., and membrane lipids are highly susceptible to free radical attack. Lipids, on reaction with free radicals undergo a highly damaging chain reaction of lipid peroxidation leading to loss of membrane integrity. Lipid peroxidation mediated membrane damage affects cell proliferation both directly and indirectly. Lipid peroxidation is the most important consequence after radiation induced damage to cell. Malonaldehyde and other aldehydes have been identified as products of lipid peroxidation that react with thiobarbituric acid (TBA)[29]. Our study shows that exposure of cells to radiation results in a time-dependent increase in membrane damage as monitored in terms of TBARS and hydroperoxides (Fig. 3 A & B). Both the assays well corroborated with each other.

The effect of stress at the cytoplasmic level includes inhibition of global protein synthesis. Since protein synthesis is an important cytoplasmic process, in this study, we have determined the effect of oxidative stress, generated by ionizing radiation, on the rate of global protein synthesis by metabolic ^{35}S-methionine labeling of proteins. Results obtained from these experiments (Fig. 4) indicated that incorporation of ^{35}S-methionine was reduced in cells exposed to ionizing radiation as compared to the unexposed control cells at 1 h of post-irradiation. Thus, these results indicate that radiation-induced oxidative stress inhibits global protein synthesis in K562 cells. After post-irradiation times of 2 and 6 h again protein synthesis increased and at 24 h protein synthesis rate reached to the normal level. These data further indicate that after 1 h and up to 6 h, radioprotective proteins, in particular, may be overexpressed, thereby contributing to an increased rate of protein synthesis. This modulation of the rate of global protein synthesis during radiation exposure takes place primarily at the initiation step of protein synthesis as indicated by modulation of eIF2α kinase activity during irradiation (Fig. 5). The induced phosphorylation of eIF2α during initial exposure of 15 min to 1 h is contributed by eIF2α kinases. Among the 4 eIF2α kinases in mammals, the role of the heme-regulated eIF2α kinase (HRI) during oxidative stress is well established[6]. Therefore, we investigated on the expression and activity of HRI, and our results indicated its involvement in regulating protein synthesis during radiation-induced oxidative stress in K562 cells. The transduction of an oxidant signal into a biological response can be mediated in several ways. However, one principal mechanism involves the oxidation of thiols (-SH) present on side chains of amino acids. Lipid peroxides are known to induce HRI activation *in vitro* by oxidation of thiol (-SH) groups of this protein[31]. Therefore, oxidative stress induced lipid hydroperoxides might be responsible for HRI activation through thiol oxidation in K562 cells. Oxidative stress induced lipid bilayer damage and HRI-mediated inhibition of protein synthesis caused a reduced rate of cell proliferation. In conclusion, our results demonstrated the involvement of HRI-mediated inhibition of protein synthesis and cell cycle arrest in affecting cell proliferation during radiation induced oxidative stress.

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