RADIATION AND FREE RADICAL EXPOSURE AND REGULATION OF PROTEIN SYNTHESIS BY THE HEME-REGULATED EUKARYOTIC INITIATION FACTOR 2α KINASE

Abhijeet P. Kulkarni and Jayanta K. Pal
Department of Biotechnology, University of Pune
and
T. P. A. Devasagayam
Radiation Biology and Health Sciences Division

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ABSTRACT

The Heme Regulated Inhibitor (HRI) is a member of the eIF-2α kinase family and is a potent regulator of protein synthesis. During a variety of cytoplasmic stresses such as, heme deficiency, heat shock, drug toxicity and lead toxicity, HRI undergoes activation and inhibits protein synthesis. (Investigations from our lab have earlier demonstrated that HRI can be used as a molecular marker of drug induced anemia. Further, over expression and activation of HRI leading to regulation of protein synthesis has been reported during lead exposure and heat shock in human cells *in vitro.)* However, very little information is available about oxidative stress and HRI-mediated regulation of protein synthesis.

In the present investigation, two oxidants namely, hydrogen peroxide and 2, 2′-azobis (2-methyl propionamidine) dihydrochloride (=2.2′-Azobis (2-amidinopropane) dihydrochloride) (AAPH) were used, to induce oxidative stress in K562 (human erythroleukaemia) cell line as a model. Effect of various concentrations of these compounds on cell proliferation, antioxidant enzyme activity, lipid peroxidation, HRI activity and expression at mRNA level has been assessed. The results suggest that oxidative stress exerted by H_2O_2 and peroxyl radicals generated by thermal decomposition of AAPH, inhibits cell proliferation, affecting cell viability in K562 cells in a concentration-dependent manner. In case of H_2O_2, at a dose of 150 mM, concurrent with decreased cell proliferation, there was an induced eIF-2 kinase activity and HRI expression indicating inhibition of protein synthesis. Similarly, peroxyl radical induced lipid peroxidation and eIF-2α kinase activity, in a concentration-dependent manner.

Therefore, H_2O_2 and AAPH-induced oxidative stress, inhibits cell proliferation and protein synthesis in K562 cells by inducing activity and expression of HRI.

Introduction

Protein synthesis is regulated both at the levels of transcription and translation. However, regulation of translation appears to be a predominant mode of protein synthesis regulation, in response to environmental stimuli. Several studies have suggested, that regulation of translation in cells is exercised mostly at the initiation step, by modifications, primarily phosphorylation of components of translational

machinery. Phosphorylation of the alpha (α) subunit of eukaryotic initiation factor 2 (eIF-2) is one of the well known mechanisms, in regulating the overall rate of protein synthesis in eukaryotes. There is a family of eIF-2α specific Ser/Thr protein kinases, each member of which can phosphorylate the alpha subunit of eIF-2. Different members of this family undergo activation during different stress stimuli. Among these, contribution of the heme regulated eIF-2α kinase (which is also called as the heme regulated inhibitor, HRI) in regulating protein synthesis in erythroid precursors has been well established. During heme deficiency, heavy metal toxicity, heat shock, drug toxicity etc., HRI gets activated. Upon activation through autophosphorylation, it phosphorylates the 38 kDa α subunit of eIF-2 at Ser51 residue. Phosphorylated eIF-2 [eIF-2a(P)] sequesters eIF-2B, which is a guanine nucleotide exchange factor (also called the reversing factor), because of formation of a stable complex. Since eIF-2B is rate limiting, unavailability of free eIF-2B inhibits the GTP-GDP exchange, which is essential for reactivation of eIF-2 for recycling. Inhibition of this exchange leads to inhibition of protein synthesis.

Among all the conditions and stress stimuli that induce HRI activity and inhibit protein synthesis, oxidative stress is of interest here. Reactive oxygen species (ROS) and Reactive Nitrogen Species (RNS) are very common and of great importance in biological systems. Free radicals, such as superoxide ($O_2^{\cdot -}$), hydroxyl ($^{\cdot}OH$) and peroxyl ($ROO^{\cdot}$) radicals, besides non-radical species like hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$) and hypochlorous acid (HOCl), are produced during

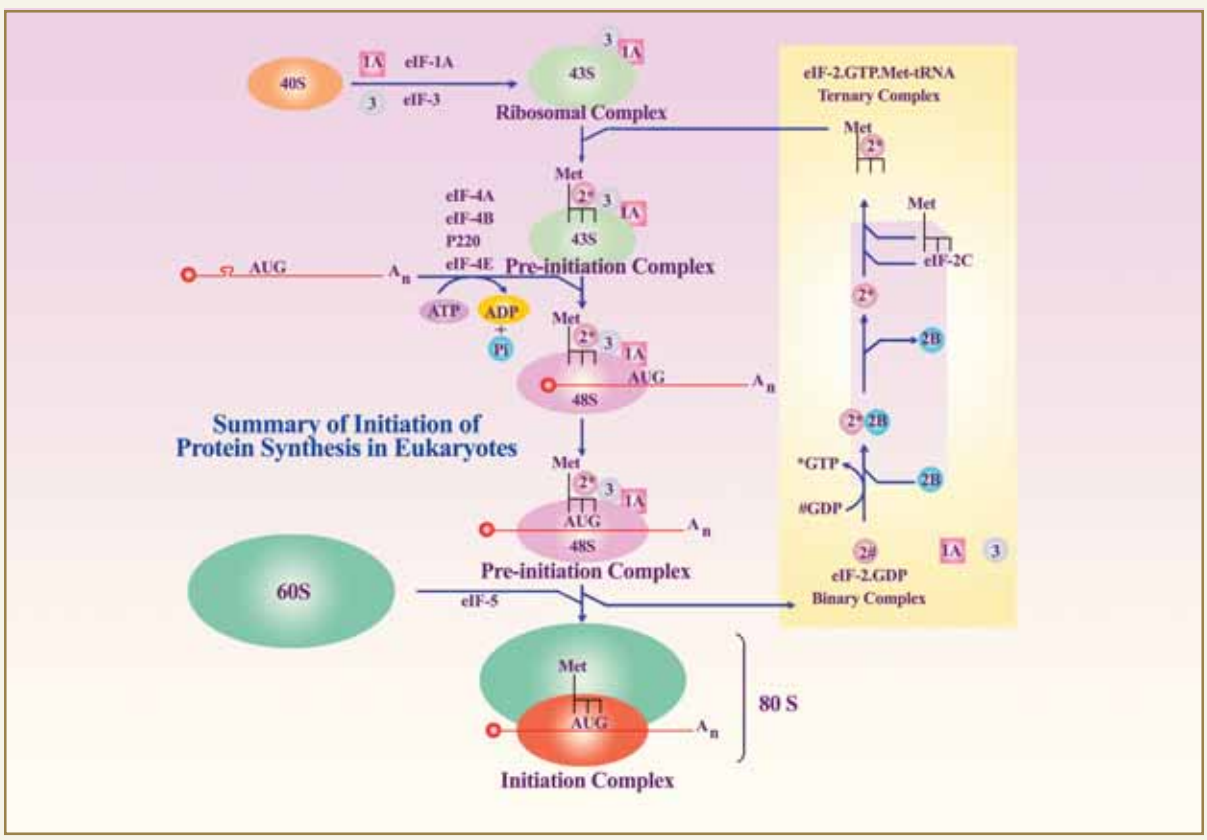


Fig. 1: Schematic diagram showing the summary of initiation of protein synthesis in eukaryotes

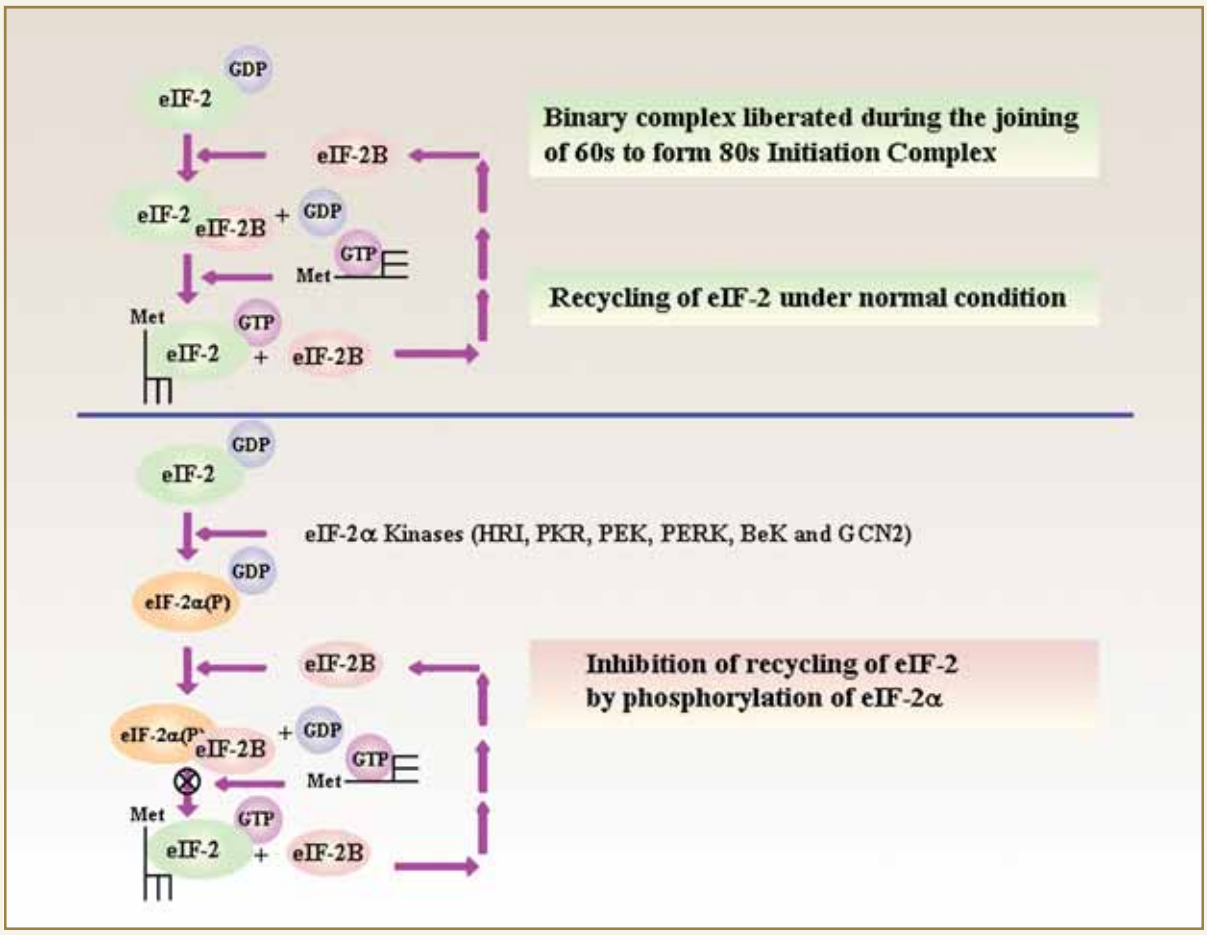


Fig. 2: Schematic diagram showing the role of eIF-2α kinases in initiation of protein synthesis in eukaryotes

normal and altered physiological processes of a cell. For example, H_2O_2 is produced in mitochondria and cytosol by the activity of intracellular oxidases and superoxide dismutases (SOD). Similarly, superoxide radicals are produced due to the auto-oxidation reactions of biologically important molecules as well as during mitochondrial electron transport. Radiation is one of the major factors, that increase the generation of reactive oxygen species in living organisms. Both H_2O_2 and peroxyl radicals are key components of radiation induced oxidative stress. Hence studying their effects on protein synthesis regulation indirectly is linked to radiation-induced modulation of such regulation.

Role of free radicals in mediating various cellular events has been studied in detail. Involvement of free radicals in the regulation of growth and differentiation, gene expression, cell signaling, proliferation and apoptosis indicate their importance in biological systems. On the other hand, their elevated levels are linked with a number of diseases and disorders, indicating their potential to cause cell injury leading to cell death. Protein synthesis also is one of the vital processes which needs to be extensively regulated, in response to oxidative stress. Therefore, it was important to investigate the role of HRI in regulation of protein synthesis during oxidative stress. We have addressed this question by designing experiments using human

K562 cells. Our results, for the first time indicate, that under conditions of oxidative stress generated by two potent oxidants, namely, H_2O_2 and peroxyl radicals generated by 2, 2'-azobis (2-amidinopropane) dihydrochloride (AAPH), there is a significant increase in the expression as well as eIF-2α kinase activity of HRI. Thus our data suggests that inhibition of protein synthesis under oxidative stress may be the combined effect of induced activity and expression of HRI.

Materials and Methods

All the cell culture reagents, namely, Dulbecco's Modified Eagle's Medium (DMEM), Fetal Bovine Serum (FBS), Antibiotic-antimycotic solution (100X) and most of the other molecular biology reagents used in the present study, were purchased from Sigma Chemical Co. (USA). Custom made HRI- and b-Actin cDNA specific primers, TRI reagent, DNase I (amplification grade), Enhanced Avian Hs RT-PCR kit were also purchased from Sigma Chemical Co. (USA). AAPH was purchased from Aldrich chemicals (USA). Anti-phospho-eIF2α (Ser51) and anti-eIF-2α polyclonal antibodies were purchased from Cell Signaling Technology (USA). BM Chemiluminescence Western Blotting kit (Mouse/Rabbit) was purchased from Roche Molecular Biochemicals (Germany). Human erythroid K562 cell lines were obtained from the cell repository at the National Centre for Cell Science, Pune, India.

Human K562 cells were maintained as continuous culture under standard conditions in DMEM. To generate oxidative stress, cells were exposed either to various concentrations of H_2O_2 or AAPH for 1 h at 37^0C. Cell viability and rate of cell proliferation in control and treated groups were determined by trypan blue staining and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasodium bromide) assay, using standard protocols. Effect of AAPH treatment on lipid peroxidation in K562 cells was determined, by estimating total lipid hydroperoxides (LOOH) using FOX II reagent H_2O_2 was used as standard hydroperoxide for calibration.

Effect of various concentrations of H_2O_2 on catalase activity was determined by Native-PAGE activity staining of gels, using protocol described earlier. Total eIF-2α kinase activity in control and treated cells was indirectly determined by detecting the extent of eIF-2α phosphorylation using western blotting. In brief, equal amount of protein from control and treated cells was separated, using SDS-PAGE. Following SDS-PAGE, proteins were electrophoretically transferred to nitrocellulose membranes which were subsequently used for immunoblotting with anti- eIF-2α and anti- eIF-2α(P) antibodies. Blots were developed using the chemiluminescence detection method. The results were analyzed using quantity one software on gel documentation system.

Level of HRI expression in control and treated cells was determined by RT-PCR. In brief, RNA extraction from control K562 cells and cells exposed to oxidative stress was done, using TRI reagent as per the manufacturer's protocol. RNA quantification was done spectrophotometrically. Prior to cDNA synthesis, RNA samples were treated with DNase I (amplification grade) to remove genomic DNA contaminations. 1 μg of RNA was reverse transcribed to cDNA using eAMV-reverse transcriptase provided with Enhanced Avian Hs RT-PCR kit. Equal amount of cDNA was PCR amplified, using HRI- and β-Actin specific primers as described earlier. PCR products were analyzed on a 1.2% agarose gel followed by ethidium bromide staining. The results were analyzed using quantity one software on gel documentation system.

Results

Loss of cell viability and inhibition of cell proliferation in K562 cells exposed to oxidative stress

In order to determine the effect of oxidative stress on K562 cell viability and proliferation, a series of preliminary experiments using increasing concentrations of H_2O_2 and AAPH were carried out. Results obtained from these experiments indicate that,

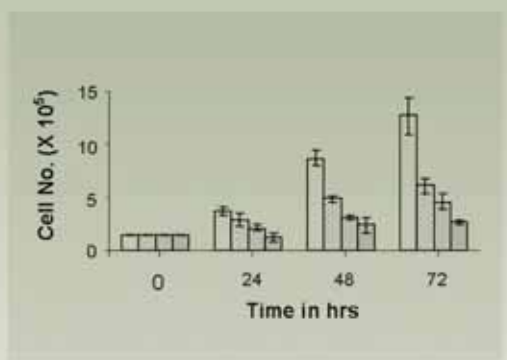
both the oxidants caused loss of cell viability in K562 cells, as a function of increasing concentrations (Figs. 3A and 3B). Results of experiments with MTT assay done after AAPH treatment revealed, that peroxyl radicals were inhibiting K562 cell proliferation. This inhibition was gradual up to 5 mM. However, at higher concentrations (10 mM to 100 mM) there was a further increase in inhibition of cell proliferation (Fig. 3C). All these experiments were performed three times and the results are presented as mean ± standard deviation.

AAPH-induced lipid peroxidation in K562 cells

Our results on lipid peroxidation, as measured by total lipid hydroperoxides produced after AAPH treatment to K562 cells, indicated that peroxyl radicals generated due to thermal decomposition of AAPH were inducing lipid peroxidation in a concentration-dependent manner (Fig. 4). In lower concentrations of AAPH (0.1 mM to 5 mM) there was practically no increase in lipid peroxidation. However, at higher concentrations (10 mM to 100 mM), a significantly high level of lipid peroxidation was observed.

Regulation of eIF-2a kinase activity during oxidative stress

We determined modulation of total eIF-2α kinase activity during H_2O_2 and AAPH-induced oxidative stress, by measuring the eIF-2α phosphorylation by western blot, using a specific antibody that recognizes Ser51 phosphorylated form of eIF-2α. After H_2O_2 exposure, there was approximately a two-fold increase in eIF-2α phosphorylation with 150 μM H_2O_2 as compared to



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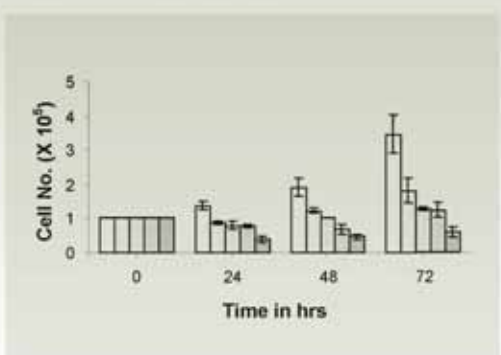

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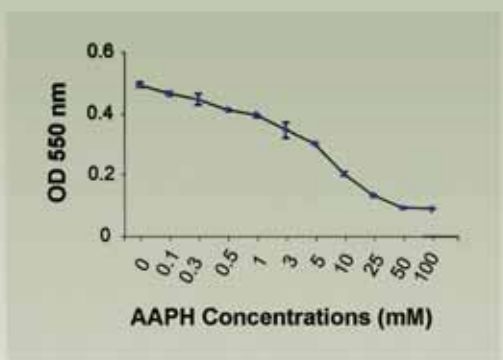

c

Fig. 3: Effect of H_2O_2 and AAPH-induced oxidative stress on K562 cell viability and cell proliferation. K562 cells were grown in DMEM with 10 % FBS for 24 h and were then treated with various concentrations of H_2O_2 (A) or AAPH (B) for 1 h. Viable cell counts were taken by trypan blue staining at every 24 h for 72 h and the data were analyzed. (a) Bar1-Control; 2, 3 and 4- 100, 150 and 200 μM H_2O_2 treated K562 cells, respectively. (b) Bar1-Control; 2, 3, 4 and 5- 1, 5, 10 and 100 mM AAPH treated K562 cells, respectively. (c) Inhibition of cell proliferation in K562 cells treated with different concentrations of AAPH as determined by MTT assay.

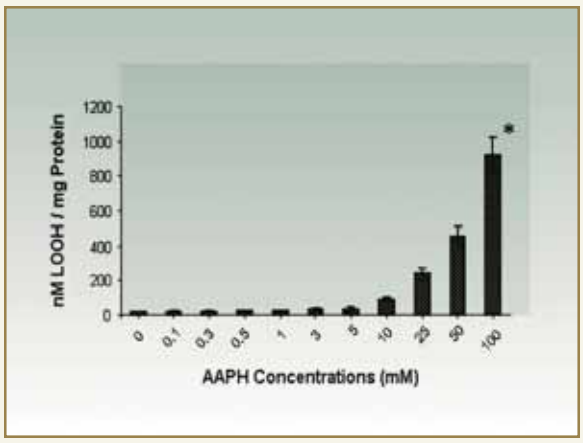

Fig. 4: AAPH-induced lipid peroxidation in K562 cells. K562 cells grown in culture were treated with different concentrations of AAPH. The effect of AAPH treatment on lipid peroxidation in these cells was determined by estimating total lipid hydroperoxides using LOOH assay. The results are expressed as nM LOOH/mg Protein. The asterisk indicates statistically significant difference in lipid peroxidation between control and 100 mM AAPH treated K562 cells (Student's *t* test $p < 0.05$)

the control (Fig. 5A; lane 3 vs lane 1). However, after AAPH treatment (Fig. 6), phosphorylated eIF-2α amount was increased in a concentration dependent manner. Maximum phosphorylation, about 2-fold over control, was observed with 3 mM AAPH (Fig. 6A lane 6 vs lane 1). There was a reduction in eIF-2α phosphorylation in concentrations beyond 3 mM, indicating the toxic effects of higher concentrations. The total amount of eIF-2α was determined by another antibody which recognizes total eIF-2α irrespective of any modification (Figs. 5B, 6B). These results indicated that due to the increased eIF-2α kinase activity during oxidative stress, global protein synthesis was getting inhibited.

Effect of H_2O_2-induced oxidative stress on HRI expression in K562 cells

To determine the effect of oxidative stress on HRI expression, K562 cells were exposed to different concentrations of H_2O_2 (100, 150 and 200 μM) for a duration of 1 h. Total RNA from control and treated cells were extracted and used for RT-PCR experiments.

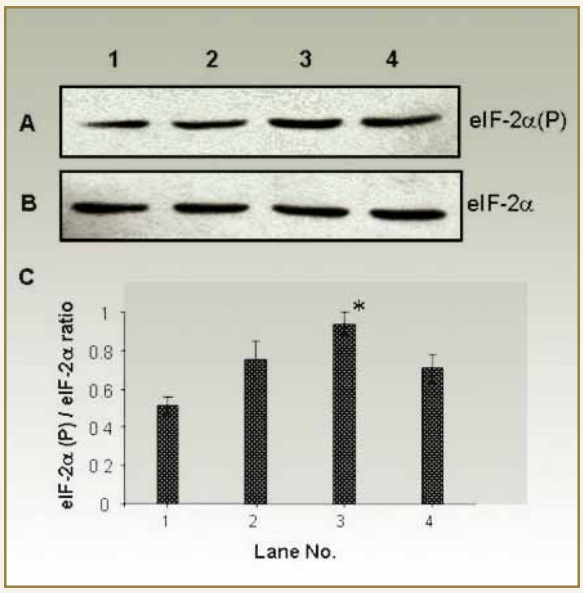

Fig. 5: H_2O_2 induces eIF2-α kinase activity in K562 cells. (A) and (B) are western blots of soluble extracts of cell sample reacted with anti-eIF-2a (P) and anti-eIF-2a antibodies, respectively. Samples loaded in various lanes are Control (lane1), 100, 150 and 200 μM H_2O_2 treated (lanes 2 to 4, respectively). (C) is quantification profile of (A) and (B) expressed as eIF-2α (P) / eIF-2α optical density ratio. The asterisk indicates statistically significant difference in eIF-2a phosphorylation between sample 1 and 3 (Student's *t* test $p < 0.05$)

Results obtained from these experiments indicated, that H_2O_2 could induce HRI expression. At 150 μM concentration, there was maximum HRI expression and it was almost 1.5 fold over control (Fig. 7A, lane 3 vs lane 1). These experiments were carried out keeping levels of β-Actin expression as internal control. Thus these results indicate, that induced HRI activity as well as expression is instrumental, in regulating protein synthesis during oxidative stress in K562 cells.

Effect of H_2O_2 on catalase activity

Catalase activity is a marker of H_2O_2-induced oxidative stress. So to make sure that H_2O_2 concentrations selected for this work are able to exert the oxidative stress and protein synthesis, related parameters can be analyzed at these concentrations, in gel catalase

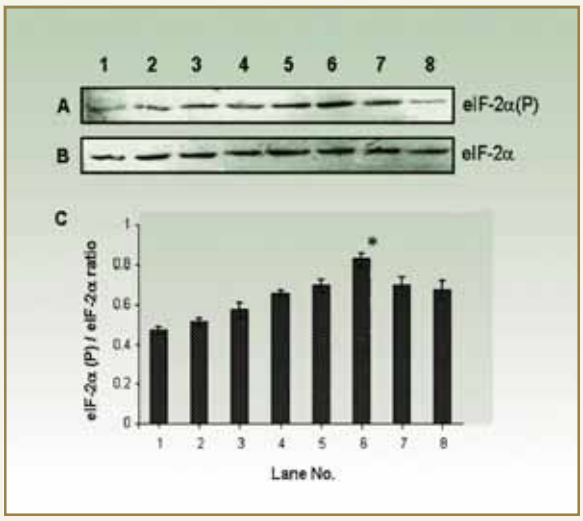

Fig. 6: Effect of AAPH on total eIF-2α kinase activity in K562 cells. (A) and (B) are western blots of soluble extracts of cell sample reacted with anti-eIF-2α (P) and anti-eIF-2α antibodies, respectively. Samples loaded in various lanes are control (lane1), 0.1, 0.3, 0.5, 1, 3, 5 and 10 mM AAPH treated (Lanes 2 to 8, respectively). (C) is quantification profile of (A) and (B) expressed as eIF-2α (P) / eIF-2α optical density ratio. The asterisk indicates statistically significant difference in eIF-2α phosphorylation between sample 1 and 6 (Student's *t* test $p < 0.05$)

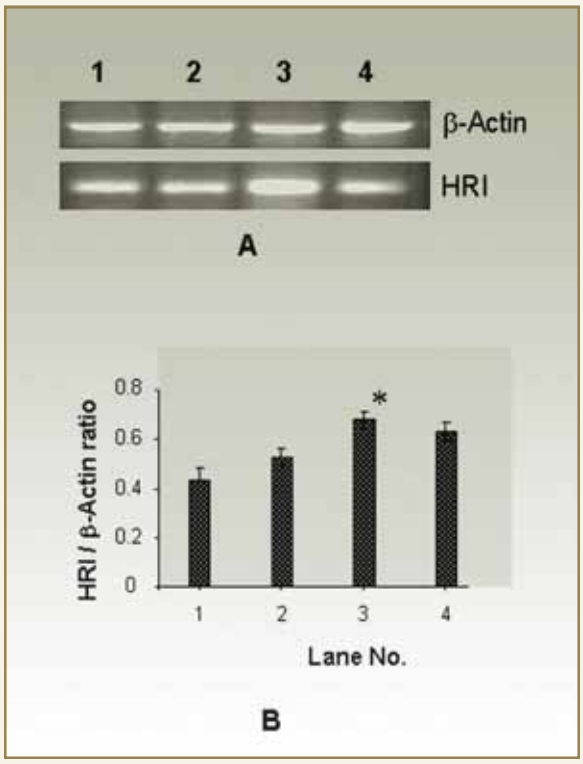

Fig. 7: Oxidative stress upregulates HRI expression in K562 cells. (A) and (B). RT-PCR was carried out using 1 μg RNA. PCR products were analyzed on a 1.2 % agarose gels. In (A), samples loaded in various lanes are, control (lane 1), 100, 150 and 200 μM H_2O_2 treated (lanes 2, 3 and 4, respectively). (B) Quantification profiles of (A) expressed as HRI / b-Actin optical density ratio. The asterisk indicates statistically significant difference in HRI expression between samples. (Student's *t* test $p < 0.05$)

activity. There was increased catalase activity and the highest activity was observed at 150 μM of H_2O_2. (Fig. 8)

Discussion and Conclusion

In the present study, for the first time, we demonstrate the contribution of HRI in the regulation of protein synthesis during H_2O_2- and AAPH-induced oxidative stress, in cultured human K562 cells. Although, there are a few reports indicating oxidative stress-induced eIF-2α phosphorylation and inhibition of protein synthesis, there are no reports on the effect of oxidative stress on HRI expression, it's eIF-2α kinase activity, and regulation of protein synthesis *in vivo*. Our results indicate that during oxidative stress, inhibition of protein synthesis in K562 cells is caused because of induced activity as well as expression of HRI.

Experiments carried out to determine the effect of H_2O_2 and AAPH on K562 cell viability and proliferation indicated that, oxidative stress induces loss of cell viability and inhibits cell proliferation in a concentration dependent manner. There are reports available stating that, AAPH and H_2O_2 induce apoptosis, which also can be true in case of K562 cells. However, further studies will be required to verify this possibility. Increased lipid peroxidation leading to loss of membrane integrity, induced apoptosis and inhibition of protein synthesis due to induced activity and expression of HRI, might be responsible for loss of viability in K562 cells.

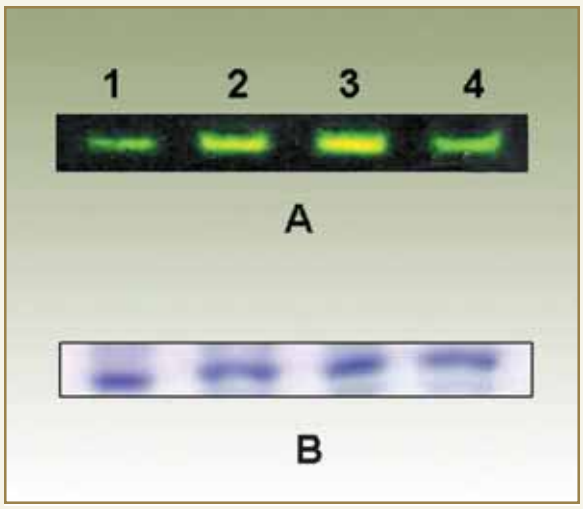

Fig. 8: Oxidative stress induces catalase activity in K562 cells. (A) Catalase activity (Native-PAGE activity staining *in situ* on gel) in the extracts of K562 cells treated with various concentrations of H_2O_2. Samples loaded in various lanes are, control (lane 1), 100 μM H_2O_2 (lane 2), 150 μM H_2O_2 (lane 3) and 200 M H_2O_2 (B)- CBB stained gel profile of the samples as in (A).

Our data indicates that AAPH and H_2O_2 in the exposure time of 1 h, induce eIF-2α kinase activity in a dose dependent manner. The transduction of an oxidant signal into a biological response can be mediated in several ways, but one principal mechanism involves the oxidation of thiols (–SH), present on side chain of amino acids. Lipid peroxides are known to induce HRI activity by oxidation of thiol (–SH) groups of this protein. Therefore, in the present study, AAPH-induced lipid hydroperoxides might be responsible for HRI activation, through thiol oxidation in K562 cells.

RT-PCR analysis indicated that oxidative stress caused by H_2O_2 induces HRI expression in K562 cells, indicating the possibility of activation of some redox sensitive transcription factor during H_2O_2 exposure.

Considering these results together, we conclude that

1) induced lipid peroxidation and HRI-mediated inhibition of protein synthesis, are responsible for inhibition of cell proliferation in human K562 cells,

2) oxidative stress-induced inhibition of protein synthesis in K562 cells is a combined effect of induced activity and expression of HRI and

3) lipid peroxides produced during oxidative stress might be playing a role in HRI activation, by altering the oxidation status of –SH groups of this protein.

ABOUT THE AUTHOR

Dr. T. P. A. Devasagayam joined BARC in 1975, after completing the BARC Training School programme in Biology and Radiobiology subsequent to his Masters in Zoology from the American College, Madurai. At present he is Scientific Officer (H) and Head, Radiation Biochemistry Section in the Radiation Biology and Health Sciences Division of BARC. His major area of research relates to role of free radicals in human health and radiation biology and potential uses of natural antioxidants. He has done his Post-Doctoral research in Germany and USA and has over 130 publications.