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Oxidative stress perturbs cell proliferation in human K562 cells by modulating protein synthesis and cell cycle

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Abstract

Oxidative stress leads to perturbation of a variety of cellular processes resulting in inhibition of cell proliferation. This study has determined the effect of oxidative stress on protein synthesis in human K562 cells using a hydrophilic peroxyl radical initiator, AAPH and H_2O_2. The results indicated that oxidative stress leads to a significant decrease in the rate of protein synthesis caused due to induced activation as well as expression of the erythroid cell-specific eIF-2α kinase, called the Heme Regulated Inhibitor (HRI). Elevated levels of HRI expression and activity were accompanied by increased lipid peroxidation and decreased cell proliferation. Further, oxidative stress also caused inactivation of $p34^{cdc2}$ kinase, thereby arresting cell division leading to apoptosis. Thus, the data provides the mechanism of inhibition of protein synthesis and perturbation of a cell cycle regulatory protein leading to inhibition of cell proliferation in K562 cells during oxidative stress.

Keywords: *HRI, expression, kinase activity, cell proliferation, AAPH, H_2O_2*

Abbreviations: *AAPH, 2, 2′-azobis (2-amidinopropane) dihydrochloride; DCFH-DA, 2′, 7′-dichlorodihydrofluorescein diacetate; eIF-2B, eukaryotic initiation factor-2B; eIF-2α, eukaryotic initiation factor-2alpha; H_2O_2, hydrogen peroxide; HRI, heme regulated inhibitor; LOOH, lipid hydroperoxide; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; $p34^{cdc2}$, cyclin dependent protein kinase 1; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis*

Introduction

Protein synthesis, a vital cellular process, plays an important role in cell proliferation and differentiation [1,2]. Deregulation of protein synthesis is associated with a variety of human diseases including certain cancers and metabolic disorders. In abnormal situations, cellular signalling cascades which control cell growth and proliferation are also known to affect components of translation machinery [3]. Therefore, deregulation of proliferation pathways and protein synthesis are strongly involved in cancer and metastasis. Cells regulate protein synthesis 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 [4].

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) sub-unit of eukaryotic initiation factor 2 (eIF-2) is one of the well known mechanisms in regulating the overall rate of protein synthesis in eukaryotes [4]. There is a family of eIF-2α-specific Ser/Thr protein kinases, each member of which can phosphorylate the α sub-unit of eIF-2. Different members of this family undergo activation during a particular stress stimulus and phosphorylate the α

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sub-unit of eIF-2 at Ser51 residue [5]. Prior to formation of 80S initiation complex, eIF-2 is liberated as eIF-2-GDP inactive binary complex which requires to be reactivated by GDP-GTP exchange. The exchange of GDP by GTP is carried out by eIF-2B, which is a guanine nucleotide exchange factor. When eIF-2α is phosphorylated at Ser51 residue [eIF-2α(P)], it tightly binds to eIF-2B and sequesters it. Since eIF-2B is rate limiting, unavailability of free eIF-2B inhibits the GTP-GDP exchange, leading to inhibition of protein synthesis [6–8].

Reactive Oxygen Species (ROS) are very common and are of great importance in biological systems. Free radicals, such as superoxide ($O_2^{\bullet -}$), hydroxyl ($^{\bullet}OH$) and peroxyl ($ROO^{\bullet}$) radicals, besides non-radical species like hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$) and hypochlorous acid (HOCl), are produced during normal and altered physiological processes in a cell [9]. The role of free radicals in mediating various cellular events has been well studied. Involvement of free radicals in the regulation of growth and differentiation, gene expression [10], cell signalling [11], proliferation [12] and apoptosis [13] indicate their importance in biological systems. On the other hand, their elevated levels are linked with cell injury leading to cell death [9].

Protein synthesis is one of the vital processes which is extensively regulated in response to various insults to cells including oxidative stress [14–21]. However, a detailed mechanism of oxidative stress-induced inhibition of protein synthesis and cell proliferation is yet to be understood. In the present study, we investigated the effect of a hydrophilic peroxyl radical initiator, 2, 2′-azobis (2-amidinopropane) dihydrochloride (AAPH) on the regulation of protein synthesis in human erythroleukaemic K562 cells. We took advantage of the availability of AAPH because it is water-soluble with a half-life of 175 h and generation of peroxyl radicals is virtually constant and directly proportional to the concentration [13]. Free radicals produced from AAPH react with lipid stably producing lipid peroxides. However, we have also used H_2O_2 as standard peroxide wherever necessary. We report for the first time that under conditions of oxidative stress, in addition to increased eIF-2α kinase activity of HRI, there is a significant increase in its transcript level. Oxidative stress induced activity and expression of HRI leads to inhibition of protein synthesis at the initiation step. Stress-induced lipid peroxidation, protein synthesis inhibition and inactivation of $p34^{cdc2}$ causing G_2/M arrest mediates inhibition of K562 cell proliferation leading to apoptosis.

Materials and methods

Materials

All the cell culture reagents, namely Dulbecco's Modified Eagle's Medium (DMEM), Foetal Bovine Serum (FBS), Antibiotic-antimycotic solution ($100\times$) and most of the other molecular biology reagents were purchased from Sigma Chemical Co. (USA). Custom-made HRI- and β-Actin cDNA specific primers, TRI reagent, DNase I (amplification grade), Enhanced Avian Hs RT-PCR kit and monoclonal antibodies namely, anti-$p34^{cdc2}$ and Actin antibodies were also purchased from Sigma Chemical Co. (USA). AAPH was purchased from Aldrich chemicals (USA). Anti-phospho-eIF2α (Ser51), anti-eIF-2α and anti-phospho-cdc2 (Tyr15) polyclonal antibodies were purchased from Cell Signaling Technology (USA). BM Chemiluminescence Western Blotting kit (Mouse/Rabbit) and DIG high prime DNA labelling and detection starter Kit II 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).

In vitro *cell culture and stress challenge to cells*

Human K562 cells were maintained as continuous culture in DMEM containing 10% FBS at 37°C and 5% CO_2 with antibiotic-antimycotic (1X) solution. To generate oxidative stress, K562 cells were exposed either to various concentrations of H_2O_2 or AAPH for 1 h at 37°C.

Detection of lipid peroxidation

Effect of AAPH treatment on lipid peroxidation in K562 cells was determined by estimating total lipid hydroperoxides (LOOH) using FOX II reagent [22]. Cells were suspended in 1X PBS and proteins were quantified from intact cells present in the suspension. The suspension volume corresponding to 150 µg of protein was used for LOOH assay. Results were expressed as nM LOOH/mg protein. H_2O_2 was used as standard peroxide for calibration.

Determination of intracellular ROS levels

Intracellular ROS levels in control and treated cells were determined using a cell-permeable probe 2′, 7′-dichlorodihydrofluorescein diacetate (DCFH-DA) as described elsewhere [23]. The effects were monitored by measuring changes in fluorescence intensity resulting from differential oxidation of intracellular probe. In brief, an equal number (10^5) of cells were incubated in 10 µM DCFH-DA for 30 min at 37°C. Such cells were then subjected to treatment with different concentrations of AAPH for 1 h at 37°C. Changes in fluorescence intensity were measured at an excitation wavelength of 485 nm and emission wavelength of 520 nm.

Metabolic ^{35}S-methionine labelling

Rate of protein synthesis in control and treated cells was determined by incorporation of ^{35}S-methionine into proteins followed by scintillation counting of incorporated label in TCA precipitated proteins [24]. In brief, cells were maintained in methionine-free DMEM at 37°C for 30 min prior to labelling. Such cells were seeded at 10^6 cells/ml seeding density in DMEM containing 10% FBS and 100 μCi/ml of ^{35}S-methionine in the presence or absence of H_2O_2/AAPH and incubated at 37°C for different time points. Cells were harvested by centrifugation and total soluble proteins were extracted. Equal volume of protein was TCA precipitated by spotting on Whatman no. 1 filter paper discs and incubation with 10% ice cold TCA. Paper discs were washed twice with 5% ice cold TCA followed by washing with absolute ethanol to facilitate drying. Dried filter discs were used for liquid scintillation counting.

Protein extraction and SDS-PAGE

Protein extraction from treated and control cells was done using lysis buffer (50 mM Tris-HCl, pH 8.0; 5 mM EDTA, 0.1% (v/v) Triton X-100, 1 mM PMSF) supplemented with protease inhibitor cocktail (Roche). Total soluble proteins in the extracts were quantified by Bradford's micro-estimation method [25]. Proteins (40 μg) were separated by SDS-PAGE [26].

Western blot analysis

Following SDS-PAGE, proteins were electrophoretically transferred to nitrocellulose membranes [27] which were subsequently used for immunoblotting with various antibodies. In brief, blots were saturated with blocking reagent (provided in the kit) for 1 h and incubated overnight with primary antibody in Tris-buffered saline containing 0.1% (v/v) Tween-20 (TBST, pH 7.5) and 5% (w/v) bovine serum albumin (BSA) and then with anti-mouse/rabbit IgG-Horse Radish Peroxidase (HRP)-conjugated secondary antibody for 1 h at room temperature. Following each antibody incubation, blots were washed three times (5 min each) in TBST. Blots were developed using the chemiluminescence detection kit. The results were analysed densitometrically using quantity one software and Bio-rad gel documentation system.

RNA extraction, RT-PCR and Northern blot analysis

Total RNA from K562 cells exposed to oxidative stress was extracted using TRI reagent as per the manufacturer's protocol and quantified spectrophotometrically. Prior to cDNA synthesis, RNA samples were treated with DNase I to remove genomic DNA contaminations; and 1 μg of RNA was reverse transcribed to cDNA using eAMV-reverse transcriptase. An equal amount of cDNA was PCR amplified using HRI- and β-Actin-specific primers as described earlier [14,15]. PCR products were analysed on a 1.2% agarose gel followed by ethidium bromide staining.

Northern blot analysis was performed as per the protocol described elsewhere [28] with slight modifications. In brief, 20 μg of total RNA extracted from control and treated K562 cells was resolved on 1.2% denaturing formaldehyde agarose gels and transferred to positively charged nylon membranes by capillary transfer method. Membranes were used for hybridization with DIG labelled, 1.9 Kb HRI cDNA probe. Detection was done by chemiluminescence method using anti-DIG-ALP antibody and CSPD substrate. The RT-PCR and Northern blot results were analysed densitometrically.

Determination of cell viability and cell proliferation

K562 cells were cultured with a seeding density of 1 or 1.5×10^5 cells/ml. Cells were allowed to grow for 24 h for recovery. Such cells were then treated with AAPH or H_2O_2. Viable cells in control and treated groups were counted by trypan blue staining using a haemocytometer after every 24 h for a period of 72 h.

The effect of AAPH treatment on K562 cell proliferation was determined by MTT assay. In brief, cells treated with various concentrations of AAPH were seeded at a density of 1×10^4 cells/well/100 μl complete medium in 96 well plates. Cells were allowed to grow for 48 h when 10 μl/well of 5 mg/ml MTT solution prepared in 1X Phosphate buffered saline (PBS) was added. Plates were further incubated for 4 h. To each well, 100 μl of solubilization buffer (10% w/v SDS in 0.01 N HCl) was then added and incubated overnight. Optical density was taken at 550 nm the next day.

Cell cycle analysis

The control and treated cells were washed with 1X PBS containing 2% FBS and fixed at 4°C for 2 h using 70% ethanol. After fixation, cells were rinsed with 1X PBS and passed through a nylon mesh for getting single cell suspension. Such cells were then treated with RNase A (100 μg/ml) at 37°C for 30 min followed by staining with 50 μg/ml propidium iodide [29]. The stained cells were then analysed with a FACSVantage fluorescence activated cell sorter using cell quest software (Becton Dickinson, USA). The cell cycle profile was obtained by analysing 10 000 cells per sample.

DNA fragmentation assay

Fragmentation of chromatin into units of single or multiple nucleosomes is characteristic of apoptosis.

Therefore, DNA fragmentation analysis is the most commonly used technique to distinguish between apoptosis and toxic necrosis. In order to check the possibility of oxidative stress-induced apoptosis, DNA fragmentation assay was performed using a procedure described elsewhere [30]. In brief, cells were harvested by centrifugation and washed once with 1X PBS. Such cells were incubated in lysis buffer (20 mM EDTA, 50 mM Tris-HCl, pH 7.5 containing 1% NP40). The preparations were centrifuged, supernatant was collected and extraction was repeated with the same amount of lysis buffer. SDS (1%) was added to the supernatants and treated with RNase A (5 μg/μl) followed by digestion with Proteinase K (2.5 μg/μl). Following DNA precipitation, pellets were dissolved in $T_{10}E_1$ buffer. Preparations were analysed on a 2% agarose gel followed by ethidium bromide staining.

Statistical analysis

All the experiments were performed at least three times and the data are expressed as mean ± standard deviation. 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

Oxidative stress induces membrane lipid bilayer damage through lipid peroxidation

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 proliferation both directly and indirectly. We verified the possibility of occurrence of lipid peroxidation by estimating total lipid hydroperoxides in K562 cells treated with increasing concentrations of AAPH. The assay results indicated that peroxyl radicals generated by thermal decomposition of AAPH caused lipid peroxidation in a concentration-dependent manner (Figure 1A). However, significant increase in production of lipid hydroperoxides over control was observed from 3 mM and higher AAPH concentrations (Student's *t*-test, $p < 0.05$).

We also determined intracellular ROS levels in cells treated with increasing concentrations of AAPH using DCFH-DA assay. A dose-dependent increase in intracellular ROS levels was observed in cells treated with various concentrations of AAPH (Figure 1B) which was also found to be significant over control from 3 mM and higher AAPH concentrations (Student's *t*-test, $p < 0.005$). These results thus indicated that the effect of AAPH is not only restricted to membrane level but also spans to cytoplasm causing increased production of intracellular ROS.

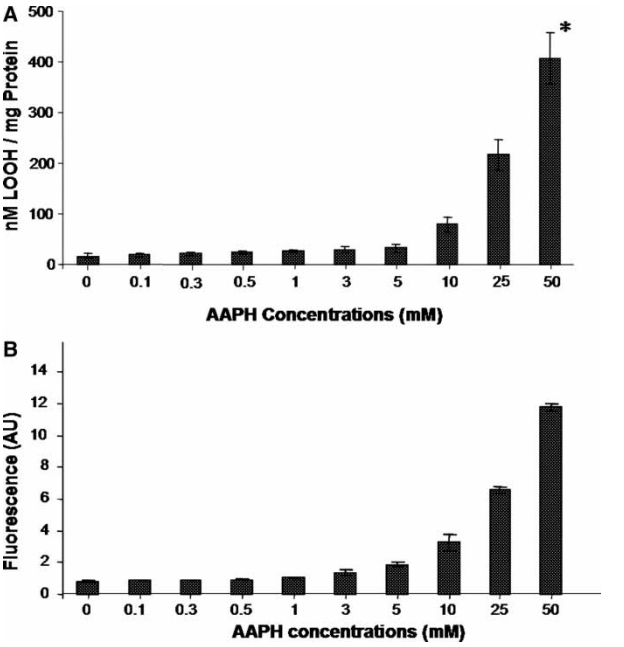

Figure 1. AAPH induces lipid peroxidation and increases intracellular ROS levels in K562 cells. (A) Cells were treated with indicated concentrations of AAPH for 1 h. The effect of AAPH treatment on lipid peroxidation in these cells was determined by estimating total lipid hydroperoxides using LOOH assay. The results are expressed as nM LOOH/mg protein. The asterisk indicates statistically significant difference in lipid peroxidation between control and 50 mM AAPH treated cells (Student's *t*-test, $p < 0.05$). (B) Intracellular ROS levels estimated using DCFH-DA assay. Cells treated with increasing concentrations of AAPH as indicated for 1 h were used for the assay.

Oxidative stress causes reduction in the rate of global protein synthesis

Lipid peroxidation and intracellular ROS determination experiments indicated that AAPH at a minimum of 3 mM concentration could significantly affect these parameters. These experiments also indicated that oxidative stress is affecting cells at the cytoplasmic level. Protein synthesis being an important cytoplasmic process, we determined the effect of oxidative stress, generated by using 3 mM AAPH, on the rate of global protein synthesis by metabolic ^{35}S-methionine labelling of proteins. For these experiments, we have also used H_2O_2 as standard peroxide at 150 μM concentration. This concentration has already been used to study its effect on protein synthesis in primary culture of neurons [19] and yeast cells [21]. Results obtained from these experiments (Figure 2) indicated that incorporation of ^{35}S-methionine was significantly reduced in cells exposed to AAPH as compared to the unexposed control cells. Similarly, cells exposed to H_2O_2 also showed a time-dependent reduction in ^{35}S-methionine incorporation as expected. Thus, these

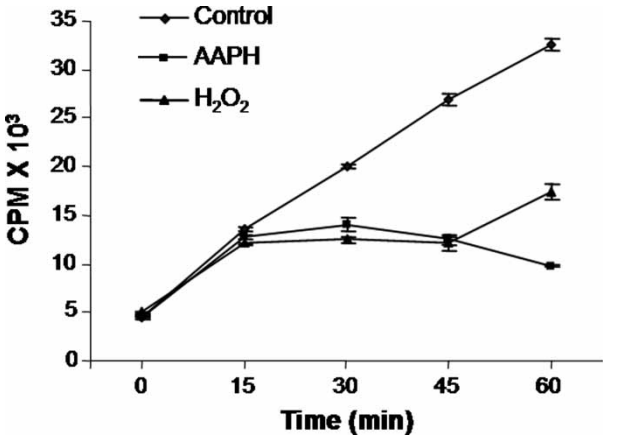

Figure 2. Oxidative stress decreases the rate of global protein synthesis in K562 cells. Cells were maintained in methionine free DMEM at 37°C for 30 min prior to labelling. Equal number of such cells (10^6) were seeded in 1 ml complete medium containing 100 μCi/ml of ^{35}S-methionine in the presence or absence of 3 mM AAPH/150 μM H_2O_2 and incubated at 37°C for indicated time points. Total soluble proteins were extracted and equal volume of protein was TCA precipitated on Whatman no. 1 filter paper discs. Dry filter paper discs were used for liquid scintillation counting. The data presented are counts per minute (CPM) (mean ± standard deviation) of three estimates.

results indicate that oxidative stress inhibits global protein synthesis in K562 cells.

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

One of the major mechanisms of regulation of initiation of protein synthesis is modulation of eIF-2 phosphorylation by eIF-2α kinases. We thus determined modulation of total eIF-2α kinase activity during oxidative stress by measuring the eIF-2α phosphorylation by Western blot analysis using a specific antibody that recognizes Ser51 phosphorylated form of eIF-2α. Phosphorylated eIF-2α amount increased in a concentration-dependent manner after AAPH treatment (Figure 3A). Maximum phosphorylation, ~2-fold over control, was observed with 3 mM AAPH (Figure 3A). Time course studies of eIF-2α phosphorylation after exposure of K562 cells to 3 mM AAPH indicated that exposure time of 1 h induced maximum eIF-2α phosphorylation (data not shown). Similarly, after H_2O_2 exposure, there was ~2-fold increase in eIF-2α phosphorylation with 150 μM H_2O_2 as compared to the control (Figure 3D). The total amount of eIF-2α was determined by another antibody which recognizes total eIF-2α irrespective of any modification (Figure 3B and E). These results thus indicate that oxidative stress mediated inhibition of protein synthesis is exercised at the translation initiation step by induced phosphorylation of eIF-2α.

Oxidative stress induced eIF-2α phosphorylation is mediated by the heme regulated eIF-2α kinase

In order to determine if HRI is the predominant contributor of the eIF-2α phosphorylation, we studied the effect of heme on the modulation of total eIF-2α kinase activity during oxidative stress in K562 cells. Western blot analysis indicated that ~95% of oxidative stress induced eIF-2α phosphorylation was heme sensitive. When the cells were cultured in the presence of 25 μM hemin for 24 h, prior to exposure with these oxidants, there was a significant reduction in eIF-2α phosphorylation. Hemin-suppressed eIF-2α phosphorylation was almost similar to basal level phosphorylation in control cells compromising the effect of oxidative stress (Figure 4A and D). The total amount of eIF-2α was fairly constant (Figure 4B and E). These results thus indicate that the increased total eIF-2α kinase activity during oxidative stress is heme-sensitive and therefore the induced phosphorylation of eIF-2α is most likely contributed by the heme-regulated eIF-2α kinase (HRI).

Oxidative stress induces HRI expression

To determine the effect of oxidative stress on HRI expression, K562 cells were exposed to either 3 mM AAPH or different concentrations of H_2O_2 (100, 150 and 200 μM) for 1 h. Total RNA from control and treated cells was extracted and used for RT-PCR and Northern blot experiments. Results obtained from these experiments indicated that both the oxidants could induce HRI expression in all the selected concentrations. AAPH at 3 mM concentration, induced ~2-fold increase in HRI expression over control (Figure 5A and B). Similarly, H_2O_2, at 150 μM concentration, was found to induce maximum HRI expression and it was almost 2-fold over control (Figure 5C and D). RT-PCR experiments were carried out keeping levels of β-Actin expression as internal control (Figure 5A and C). For Northern blot experiments, ethidium bromide stained RNA gel profile showing equal intensity of 28S and 18S ribosomal RNAs was kept as loading control (Figure 5B and D). Therefore, oxidative stress-induced inhibition of protein synthesis is due to both activation and induced expression of HRI.

Oxidative stress causes loss of cell viability and inhibition of cell proliferation

In order to determine whether oxidative stress-induced lipid peroxidation and inhibition of protein synthesis are affecting cell viability and proliferation, trypan blue exclusion and MTT assay experiments were carried out using increasing concentrations of AAPH and H_2O_2. Results obtained from these experiments indicated that both the oxidants caused loss of cell viability in K562 cells, as a function

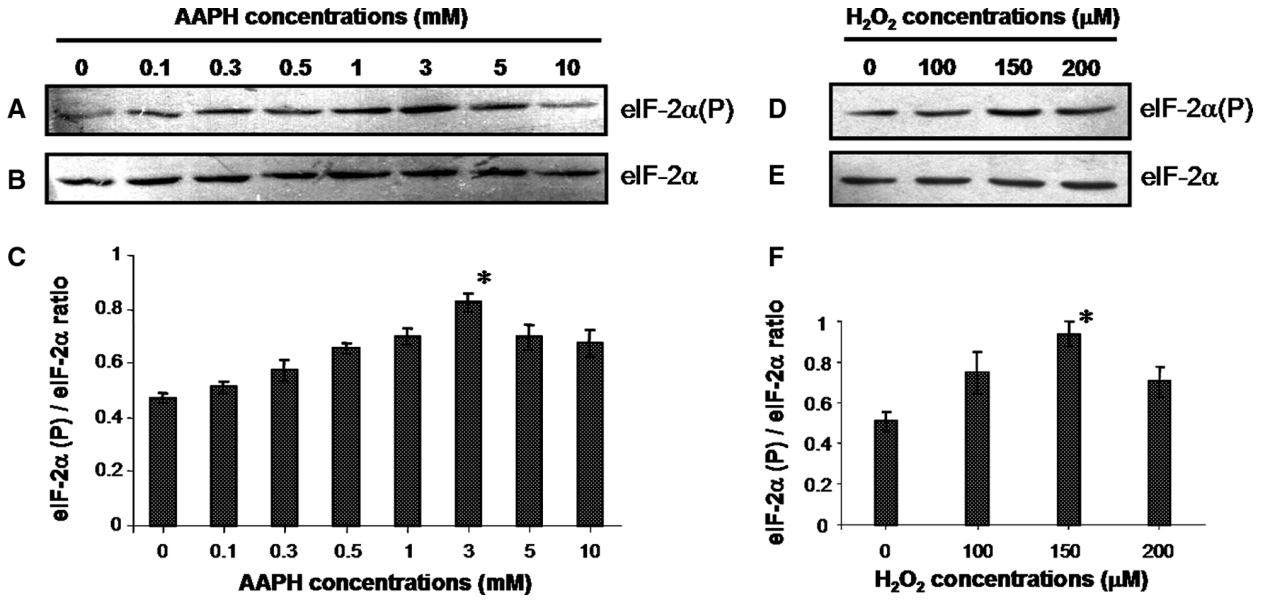

Figure 3. AAPH and H_2O_2 induces eIF-2α kinase activity in K562 cells. (A, D) and (B, E) are Western blots of soluble extracts of cell sample reacted with anti-eIF-2α (P) and anti-eIF-2α antibodies, respectively. In (A) and (B), extracts loaded in various lanes are from untreated control cells and cells treated with indicated concentrations of AAPH (1 h at 37°C). In (D) and (E), extracts loaded in various lanes are control and cells treated with indicated concentrations of H_2O_2 (1 h at 37°C). (C) and (F) are quantification profile of (A, B) and (D, E), respectively, expressed as eIF-2α (P)/eIF-2α optical density ratio. The asterisks indicate statistically significant difference in eIF-2α phosphorylation between control and respective treatment (Student's t-test, $p < 0.05$).

of increasing concentrations (Figure 6A and C). MTT assay done after AAPH treatment revealed that peroxyl radicals were inhibiting K562 cell proliferation in a concentration-dependent manner (Figure 6B).

AAPH induced oxidative stress causes G_2-M arrest leading to apoptosis in K562 cells

Oxidative stress-induced inhibition of cell proliferation was an indication that the stress is affecting cell

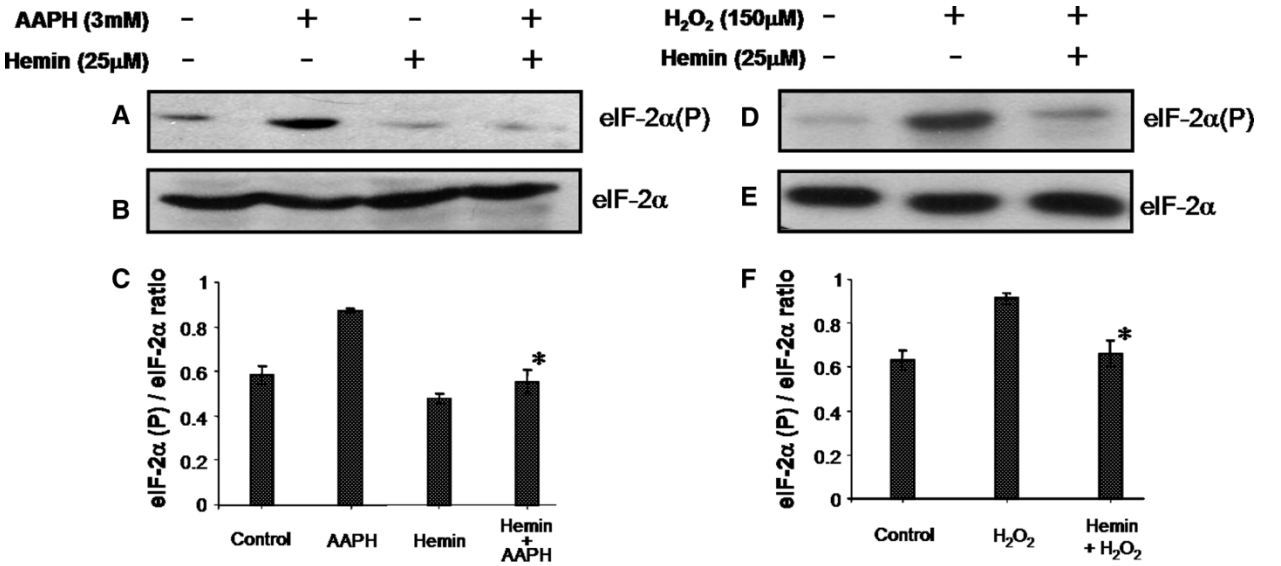

Figure 4. Oxidative stress-induced phosphorylation of eIF-2α is mediated by the hemin sensitive kinase. (A, D) and (B, E) are Western blots of soluble extracts of cell samples reacted with anti-eIF-2α (P) and anti-eIF-2α antibodies, respectively. In (A) and (B), samples loaded in various lanes are control, 1 h AAPH treated, 24 h hemin treated and 24 h hemin followed by 1 h AAPH treated with indicated concentrations. In (D) and (E), samples loaded in various lanes are control, 1 h H_2O_2 treated and 24 h hemin followed by 1 h H_2O_2 treated with indicated concentrations. (C) and (F) are quantification profiles of (A, B) and (D, E), respectively, expressed as eIF-2α (P)/eIF-2α optical density ratio. In (C), the asterisk indicates statistically significant difference in eIF-2α phosphorylation between alone AAPH and hemin followed by AAPH (indicated as hemin+AAPH) treated cell samples. In (F), the asterisk indicates a statistically significant difference in eIF-2α phosphorylation between alone H_2O_2 and hemin followed by H_2O_2 (indicated as hemin+H_2O_2) treated cell samples (Student's t-test, $p < 0.05$).

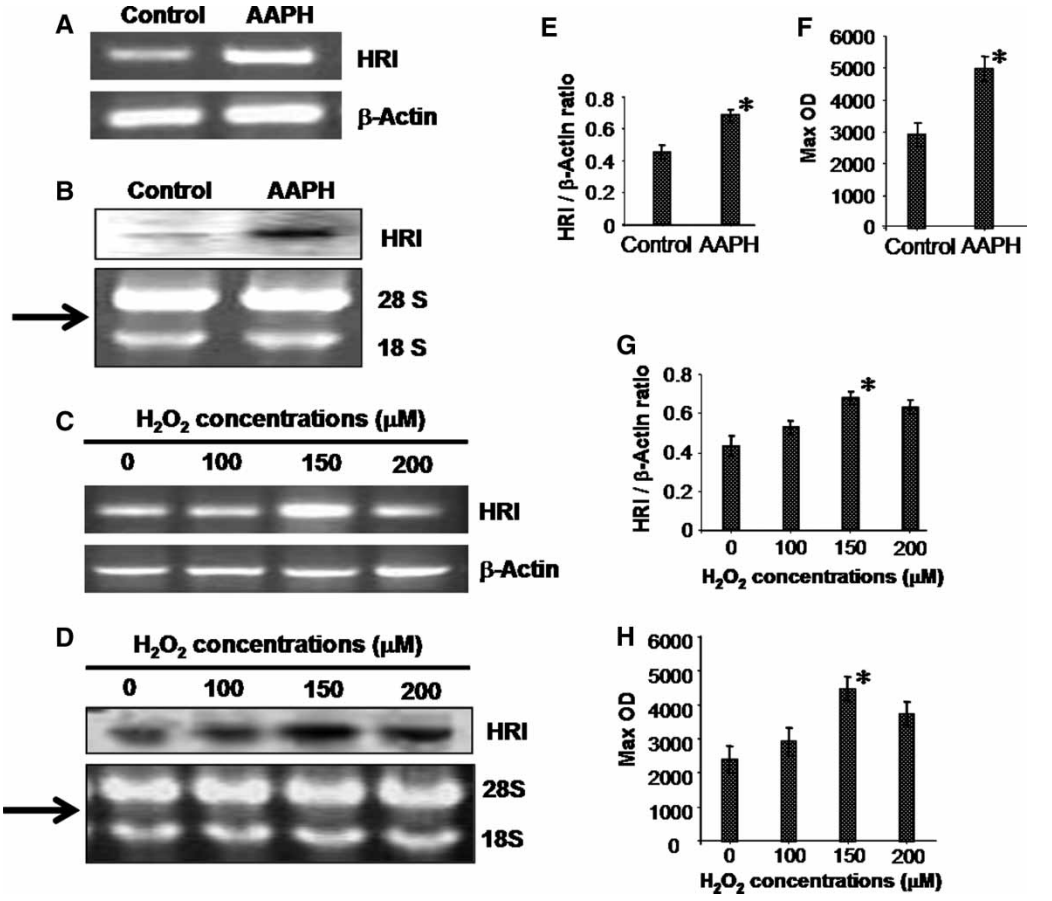

Figure 5. Oxidative stress upregulates HRI expression in K562 cells. (A, C), RT-PCR analysis using 1 μg RNA. In (A), samples loaded in various lanes are control and 3 mM AAPH treated for 1 h. In (C), samples loaded in various lanes are control and 1 h H_2O_2 treated with indicated concentrations. (B) and (D), Confirmation of RT-PCR results as in (A) and (C), respectively, by Northern blot analysis. Treatment parameters were similar as in (A) and (C); 20 μg of total RNA extracted from control and treated cells was used for blotting. Hybridization was performed with DIG labelled, 1.9 Kb HRI cDNA probe. Detection was done by chemiluminescence method using anti-DIG-ALP antibody and CSPD substrate. Ethidium bromide stained RNA gel profile showing equal intensity of 28S and 18S ribosomal RNAs is shown as loading control in (B) and (D). The arrow indicates the relative position of HRI mRNA signal with respect to 28S and 18S ribosomal RNA positions. (E, F, G and H), densitometric quantification profiles of (A–D), respectively. The asterisk indicates statistically significant difference in HRI expression between samples and the respective control (Student's *t*-test, $p < 0.05$).

cycle progression. In order to verify such a possibility, and to determine the particular phase of cell cycle that is affected, cell cycle analysis was done. Longer exposure (24 h) with 3 mM AAPH was used for these experiments. Control and treated cells were then subjected to PI staining and FACS analysis. Results of these experiments indicated that there was a significant increase in sub-G_0 and G_2-M cell population in AAPH treated cells as compared to control (Figure 7A and B). Induction of apoptosis following treatment with increasing concentrations of AAPH was also determined by DNA fragmentation analysis. There was a dose-dependent increase in fragmented DNA in AAPH treated cells (Figure 7C).

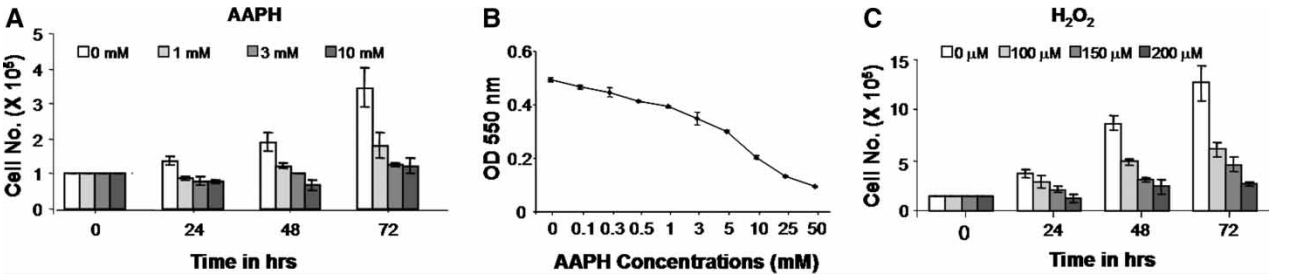

Figure 6. Oxidative stress causes loss of K562 cell viability and inhibits cell proliferation. Cells cultured for 24 h were treated with various concentrations of AAPH (A) or H_2O_2 (C) for 1 h at 37°C. Viable cell counts were taken by trypan blue staining at every 24 h until 72 h and the data were analysed. (A) Effect of indicated concentrations of AAPH on cell viability. (B) Inhibition of proliferation in cells treated with indicated concentrations of AAPH for 1 h as determined by MTT assay. (C) Effect of indicated concentrations of H_2O_2 on cell viability.

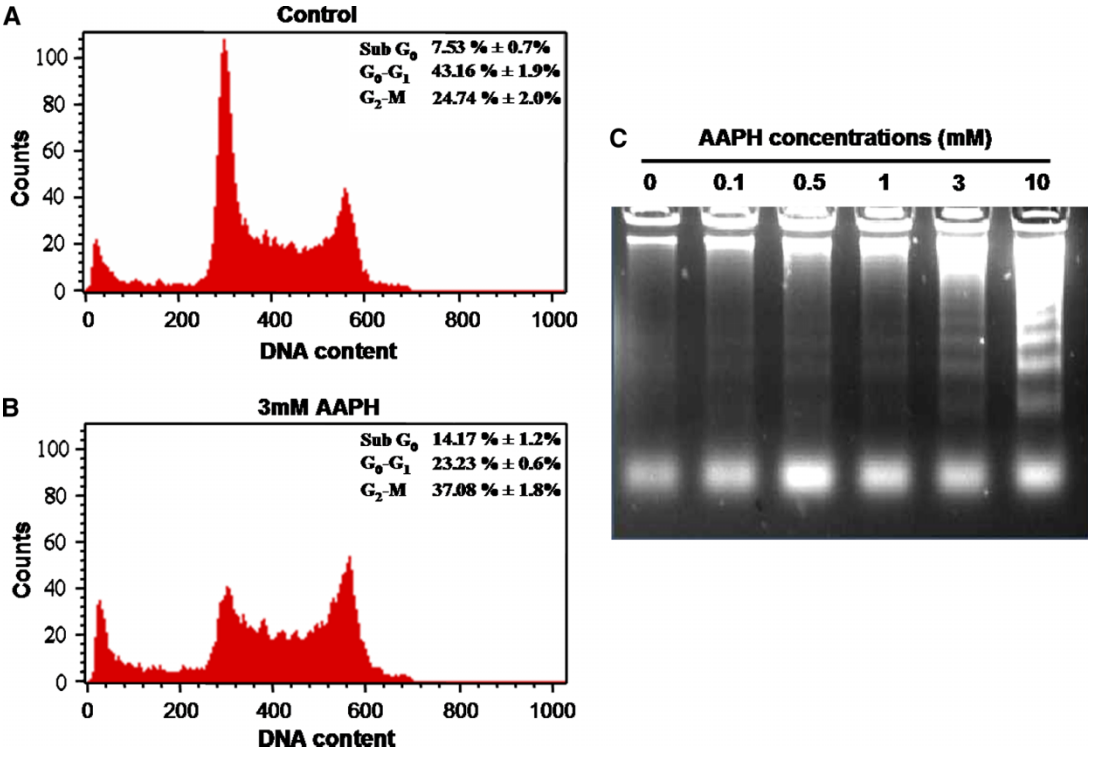

Figure 7. Oxidative stress causes G_2-M arrest and subsequently induces apoptosis in K562 cells. (A) Control and (B) AAPH (3 mM for 24 h) treated cells were subjected to cell cycle analysis. The percentage of Sub G_0, G_0–G_1 and G_2–M cells were evaluated by PI staining followed by flow cytometric analysis. Presented are the mean of percentage cell numbers ($\pm$ standard deviation) in different phases of cell cycle and a representative cell cycle profile of control and treated cells. (C) Cells were treated for 24 h with increasing concentrations of AAPH as indicated. Such cells were then subjected to DNA fragmentation analysis. An ethidium bromide stained 2% agarose gel profile showing induced DNA fragmentation after AAPH treatment is presented. DNA samples loaded in various lanes are from untreated control cells and 0.1, 0.5, 1, 3 and 10 mM AAPH treated cell samples. M is 100 bp DNA ladder.

Thus, these results suggest that oxidative stress generated by AAPH is causing G_2-M arrest affecting cell proliferation followed by induction of apoptosis in K562 cells.

AAPH induced oxidative stress inactivates a cell cycle regulatory protein p34^{cdc2} in K562 cells

Since AAPH induced peroxyl radicals were causing stress response leading to inhibition of K562 cell proliferation and protein synthesis in a dose-dependent manner, it was of interest to determine the effect of AAPH on changes in the levels of a cell cycle regulatory protein, p34^{cdc2}. The stress was causing G_2-M arrest; therefore, we investigated Tyr15 phosphorylation status of p34^{cdc2}, as Tyr15 phosphorylation normally functions to delay M phase by preventing p34^{cdc2} activation [31]. The results obtained from Western blot experiments indicated that p34^{cdc2} levels in K562 extracts treated with various concentrations of AAPH increased in a dose-dependent manner (Figure 8A). Further, Tyr15 phosphorylation of cdc2 also increased in a concentration-dependent manner until 1 mM AAPH (Figure 8B). β-Actin levels in the extracts were kept as internal control for these experiments (Figure 8C). Thus, these results indicate that oxidative stress induced G_2-M arrest leading to inhibition of cell proliferation in K562 cells is caused due to inactivation of p34^{cdc2} through induced Tyr15 phosphorylation.

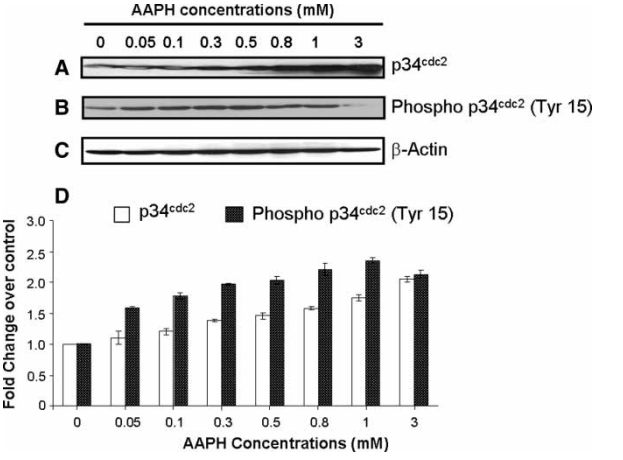

Figure 8. Effect of AAPH-induced oxidative stress on p34^{cdc2} kinase. (A, B and C) are Western blots of soluble extracts of cell samples reacted with anti-p34^{cdc2}, anti-Phospho-p34^{cdc2} (Tyr15) and anti-β-Actin antibodies, respectively. Samples loaded in various lanes are control (untreated), 0.05, 0.1, 0.3, 0.5, 0.8, 1, 3 mM AAPH treated (1 h at 37°C) cell extracts. (D) Densitometric quantification profile indicating fold change in the levels of respective proteins over control.

Discussion

In this report, we have investigated the effect of oxidative stress on cell proliferation mediated through the inhibition of protein synthesis. We have used a hydrophilic peroxyl radical initiator, AAPH, because it is water-soluble with a half-life of 175 h and generation of radicals is virtually constant and directly proportional to the concentration [13]. Although we have used millimolar concentrations of AAPH, its slow decomposition rate produces radical concentration of physiological relevance [32,33]. Free radicals produced from AAPH react with lipid stably producing lipid peroxides. However, we have also used H_2O_2 as the standard peroxide as effects of H_2O_2 on protein synthesis are known [19,21].

Our results indicate that oxidative stress affects cells at the (1) membrane level by lipid peroxidation leading to membrane damage, (2) cytoplasmic level by (i) inhibiting initiation of protein synthesis mediated by both activation and over-expression of the heme regulated eIF-2α kinase and (ii) perturbing cell cycle through inactivating $p34^{cdc2}$ caused due to Tyr15 phosphorylation leading to inhibition of cell proliferation and (3) nuclear level, leading to apoptotic DNA fragmentation.

The effect of stress at the cytoplasmic level was inhibition of global protein synthesis. Oxidative stress in the exposure time of 1 h induced eIF-2α kinase activity indicating inhibition of protein synthesis at the translation initiation step. Interestingly, $\sim$95% of stress-induced eIF-2α kinase activity was found to be heme-sensitive as it was compromised by hemin pre-incubation. Therefore, the induced phosphorylation of eIF-2α is most likely contributed by the heme-regulated eIF-2α kinase (HRI). 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 chain of amino acids. Lipid peroxides are known to induce HRI activation *in vitro* by oxidation of thiol (–SH) groups of this protein [34]. Therefore, AAPH induced lipid hydroperoxides might be responsible for HRI activation through thiol oxidation in K562 cells. A working model based on our data is presented in Figure 9.

Transcriptional upregulation of HRI gene during various cytoplasmic stresses is known [14,15]. Therefore, we determined the effect of these oxidants on HRI transcript level. Expression of HRI was induced in cells exposed to oxidative stress. This induced expression was not due to stabilization of HRI mRNA (data not shown). The role of Elk-1 and Mzf-1 transcription factors in regulation of human HRI expression during heavy metal stress has been reported [35]. Therefore, involvement of these transcription factors in upregulating HRI expression in a similar manner during oxidative stress is highly possible.

Oxidative stress-induced lipid bilayer damage and HRI-mediated inhibition of protein synthesis caused a reduced rate of cell proliferation. Therefore, we determined the changes of a cell cycle regulatory protein $p34^{cdc2}$. There was a concentration-dependent increase in $p34^{cdc2}$ levels after AAPH treatment in K562 cells. We also determined Tyr15 phosphorylation status of $p34^{cdc2}$ using such site-specific antibodies. Phosphorylation of Tyr15 residue in $p34^{cdc2}$ normally functions to delay M phase by preventing $p34^{cdc2}$ activation [31]. Interestingly, Tyr15

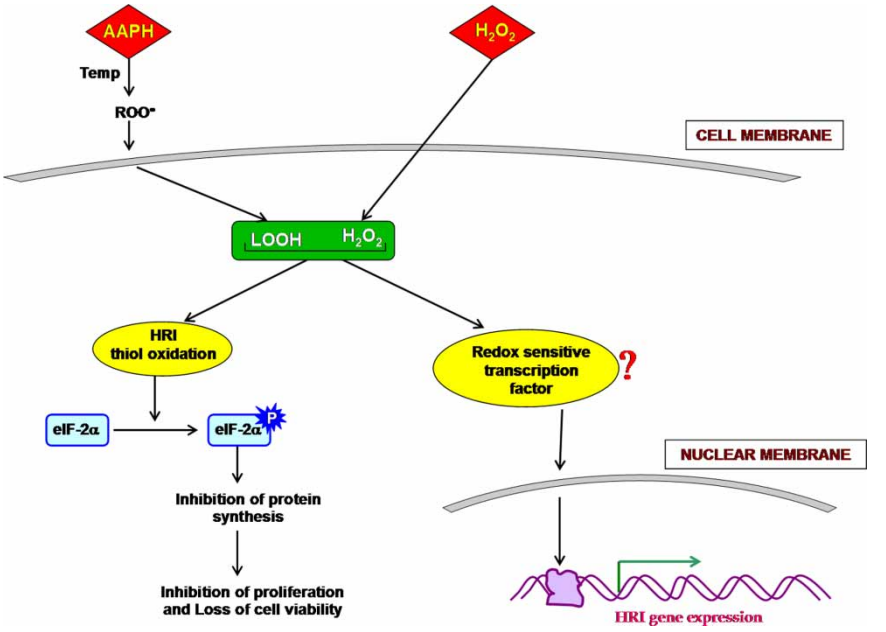

Figure 9. Schematic representation of the proposed mechanisms of HRI activation and expression by AAPH and H_2O_2 induced oxidative stress.

phosphorylation of cdc2 was increased in a concentration-dependent manner. Therefore, inactivation of $p34^{cdc2}$ through Tyr15 phosphorylation is instrumental in oxidative stress-induced inhibition of cell proliferation in K562 cells. It is also known that induced activity and expression of $p34^{cdc2}$ is a crucial mechanism of mediating apoptosis during stress [36,37]. Therefore, during AAPH-induced oxidative stress, increased $p34^{cdc2}$ level and its inactivation through Tyr15 phosphorylation caused G_2-M arrest leading to apoptosis. However, the involvement of other cell cycle regulatory proteins in oxidative stress-induced inhibition of cell cycle progression cannot be ruled out. In conclusion, our results demonstrated the involvement of HRI-mediated inhibition of protein synthesis and cell cycle arrest in affecting cell proliferation during oxidative stress.

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