Hsp90 mediates activation of the heme regulated eIF-2α kinase during oxidative stress

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The heme-regulated inhibitor (HRI), a member of the eIF-2α kinase family is crucial for regulating protein synthesis during stress. In addition to heme, stress proteins Hsp90 and Hsp70 are known to regulate HRI. The present study aims to determine the physical association of these Hsps in the regulation of HRI activation during oxidative stress using human K562 cells as a model. Extracts from the stress-induced cells were used for determining HRI kinase activity by measuring eIF-2α phosphorylation, and Hsp-HRI interaction by immunoprecipitation and immunoblot analyses. The results indicate a significant increase in both Hsp70 and Hsp90 expression during AAPH (2, 2'-azobis (2-amidinopropane) dihydrochloride)-induced oxidative stress. Further, their interaction with HRI, which correlates well with its increased HRI kinase activity leads to inhibition of protein synthesis. Thus, we demonstrate that Hsps play an important role in the regulation of initiation of protein synthesis during oxidative stress.

Keywords: eIF-2α Kinase, Hsp70, Hsp90, Protein synthesis, Oxidative stress

Protein synthesis is a highly regulated process and its deregulation is associated with a variety of diseases, including certain types of cancer. The regulation of protein synthesis in cells is exercised mostly at the translation initiation step by covalent modifications, such as phosphorylation of protein factors of the translational machinery. In eukaryotes, phosphorylation of the α subunit of the eukaryotic initiation factor 2 (eIF-2) is one of the well known mechanisms in regulating the overall rate of protein synthesis. There is a family of eIF-2α-specific Ser/Thr protein kinases, each member of which can phosphorylate the α subunit of eIF-2. Different members of this family undergo activation during a particular stress stimulus and phosphorylate the α subunit of eIF-2 at Ser51 residue. Phosphorylated eIF-2α being inactive cannot participate in the initiation process, resulting in inhibition of protein synthesis.

The heme-regulated eIF-2α kinase (HRI), a member of the eIF-2α kinase family is activated under a variety of conditions, including heme deficiency, heat-shock and heavy metal toxicity. Upon activation through autophosphorylation, it phosphorylates the α subunit of eIF-2 and inhibits protein synthesis. Thus, HRI is a potent regulator of protein synthesis during cytoplasmic stresses, in particular. Members of heat shock protein family (Hsps) are also expressed/overexpressed during diverse forms of stress, such as heat shock, exposure to heavy metals and amino acid analogs. However, Hsps are also expressed in cells for normal physiological processes. For example, Hsp70 is expressed during cell cycle, response to serum stimulation, early stages of mouse embryogenesis, mouse spermatogenesis, and differentiation of HL60 promyelocytic leukemia cells. Similarly, Hsps are important for maturation and transformation event of HRI, leading to regulation of protein synthesis. The details are described below.

In rabbit reticulocytes and their lysates (RRL), HRI is synthesized as an inactive kinase (early HRI folding intermediates). The transformation of newly synthesized HRI to a mature competent (activable) form requires the Hsp90 chaperone machinery. Mature competent HRI is unstable and its subsequent transformation to an active kinase (transformed HRI) requires its continued interaction with the Hsp90 chaperone machinery and autophosphorylation events. Transformed HRI is stable, heme responsive and no
longer dependent upon Hsp90\textsuperscript{15,16}. Activation of transformed HRI is accompanied by further phosphorylation events. In absence of heme, activation of transformed HRI is accompanied by its hyperautophosphorylation at additional sites\textsuperscript{6}. Therefore, hyperphosphorylation of HRI appears to increase its catalytic activity and decrease its sensitivity to suppression by hemin\textsuperscript{17}. Further, activation of transformed HRI may also involve its cystine sulphydryl group oxidation or sulphydryl/disulfide bond rearrangements\textsuperscript{18}. Beside Hsp90, HRI is known to interact with several other hsps in RRL, including Hsp70 and their associated cohorts FKBP52 and p23\textsuperscript{16}. Thus, Hsp70 in collaboration with Hsp90 plays an important role in de novo folding, maturation, maintenance, and transformation of HRI.

Increased production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) is one of the important cytoplasmic stresses, called as oxidative stress (OS). The eIF-2\(\alpha\) kinase-mediated regulation of protein synthesis is known even in response to OS. The present study aims to determine the role of Hsp70 and Hsp90 in HRI-mediated regulation of protein synthesis during OS. To generate OS, we have used a hydrophilic peroxyl radical initiator, 2, 2'-azobis (2-amidinopropane) dihydrochloride (AAPH) and human erythroleukemic K562 cells as a model. Here, we report interaction of HRI with Hsps to execute regulation of protein synthesis in response to oxidative stress.

**Materials and Methods**

**Materials**

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 general and molecular biology reagents, monoclonal antibodies, anti-Hsp70, anti-Hsp90 and anti-\(\beta\)-actin antibodies and custom made rabbit polyclonal HRI antibody were purchased from Sigma Chemical Co., USA. 2, 2'-azobis (2-amidinopropane) dihydrochloride (AAPH) was purchased from Aldrich Chemicals, 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.

**In vitro cell culture and stress challenge to cells**

Human K562 cells were maintained as continuous culture in DMEM containing 10\% FBS at 37\(^\circ\)C and 5\% CO\(_2\) with antibiotic-antimycotic (1X) solution. To generate oxidative stress, K562 cells were treated with AAPH for 1 h at 37\(^\circ\)C. Hemin preincubation was performed for 24 h using 25 \(\mu\)M hemin.

**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. Total soluble proteins in the extracts were quantified by Bradford’s method\textsuperscript{19}. Proteins (40 \(\mu\)g) were separated by 12\% SDS-PAGE\textsuperscript{20}.

**Western blot analysis**

Following SDS-PAGE, proteins were electrophoretically transferred to nitrocellulose membranes\textsuperscript{21} 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 thrice (5 min each) in TBST. Blots were developed using the chemiluminescence detection kit. The results were analyzed densitometrically using quantity one software and Bio-rad gel documentation system.

**In vitro eIF-2\(\alpha\) kinase assay**

Protein kinase assay mixture (20 mM Tris-HCl, 40 mM KCl, 2 mM (CH\(_2\)COO)\(_2\) Mg pH 7.6, 0.5 mM ATP) containing 10 \(\mu\)Ci of \(\gamma\textsuperscript{32P}\)-ATP was added with 40 \(\mu\)g of protein extracted from control and treated K562 cells and the kinase assay was carried out at 30\(^\circ\)C for 20 min in presence of exogenously added purified recombinant human eIF-2\(\alpha\) (1 \(\mu\)g) as the substrate\textsuperscript{22}. Assay mixture with eIF-2\(\alpha\) substrate and control K562 protein extract, incubated in presence of 5 mM NEM was used as the positive control. Reactions were stopped by the addition of 5X Laemmli buffer (sample buffer) and by boiling the samples for 5 min. Proteins were then resolved on 12\% SDS-PAGE and gels were exposed to X-ray film overnight at -70\(^\circ\)C.

**Co-immunoprecipitations**

Co-immunoprecipitations were done using the protocol described earlier with modifications\textsuperscript{23}. Control
and treated cell lysates were prepared and cleared by centrifugation. Soluble proteins were incubated with anti-mouse IgG at 4°C, followed by a short incubation with protein A/G agarose beads. After centrifugation, supernatants were transferred to another tube and pellets were washed 3-times and were taken as preimmune (control) complexes. The supernatants from the previous steps were then incubated with either anti-Hsp70 or Hsp90 antibodies overnight at 4°C, followed by 4 h incubation with protein A/G agarose beads. The immune complexes were harvested by centrifugation and washed 3-times with lysis buffer. Cell lysates or immunoprecipitates were separated by 12% SDS-PAGE and transferred to nitrocellulose membranes, which were then incubated with anti-HRI antibody. The immune complexes were visualized by chemiluminescence.

**Benzidine staining**

In order to determine percent cell differentiation during various treatments, benzidine staining was done using the protocol described elsewhere. Benzidine dihydrochloride (2 mg/ml) was prepared in 3% acetic acid and H$_2$O$_2$ (1%) was added immediately before use. Control and treated cell suspensions were mixed with benzidine solution in 1:1 ratio and incubated for 5 min. Preparations were either counted in hemocytometer or photographed using bright field microscope on a plane glass slide. To determine percent differentiation, at least 500 cells were counted per sample. Blue cells were considered to be positive for hemoglobin production.

**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. The effects were monitored by measuring changes in fluorescence intensity resulted from differential oxidation of intracellular probe. In brief, equal number (10$^5$) of control or 24 h hemin-treated cells were incubated in 10 µM DCFH-DA for 30 min at 37°C and cells were then subjected to treatment with 3 mM 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.

**Results**

**Oxidative stress increases Hsp70, Hsp90 levels**

As Hsps are induced in response to various stresses to maintain homeostasis, we determined the effect of AAPH-induced OS on quantitative changes in the levels of the two important Hsps, Hsp70 and Hsp90. The results obtained from Western blot experiments indicated that Hsp70 and Hsp90 levels in K562 extracts treated with various concentrations of AAPH increased in a dose-dependent manner (Fig. 1). The levels were maximum at 3 mM AAPH concentration, beyond which the levels did not increase (Fig. 2). Therefore, further experiments were carried out using 3 mM AAPH concentration.

**AAPH-induced oxidative stress increases phosphorylated form of HRI**

As the eIF-2α kinases (including HRI) are known to regulate protein synthesis during various cytoplasmic stresses including OS, therefore, it was important to determine the effect of AAPH-induced OS on HRI protein level, as well as its autophosphorylation and activation status. Hemin is known to regulate HRI negatively, inhibiting its autokinase and eIF-2α kinase activity. Similarly, hemin downregulates HRI promoter activity, affecting its transcription and protein production. Therefore,

![Fig. 1.—AAPH-induced oxidative stress leads to increase in the levels of Hsp70 and Hsp90 (A, B and C are Western blots of soluble extracts of cell samples reacted with anti-Hsp70, anti-Hsp90 and anti-β-actin antibodies, respectively. Samples loaded in various lanes were: control (untreated), 0.05, 0.1, 0.3, 0.5, 0.8, 1, 3 mM AAPH treated (1 h at 37°C) K562 cell extracts. Fold changes over control in the levels of respective proteins determined by densitometric analysis are indicated. β-Actin is shown as loading control)](image)

![Fig. 2.—Hsp70 and Hsp90 protein levels attain saturation at 3 mM AAPH-induced oxidative stress (A, B and C are Western blots of soluble extracts of cell samples reacted with anti-Hsp70, anti-Hsp90 and anti-β-actin antibodies, respectively. Samples loaded in various lanes were: control (untreated), 0.5, 1.0, 3.0, 5.0, 10.0 mM AAPH treated (1 h at 37°C) K562 cell extracts. β-Actin is shown as loading control)](image)
hemin preincubation was used as the negative control in these experiments. The results obtained using anti-HRI polyclonal antibody indicated the presence of three forms of HRI in control, as well as treated cells (Fig. 3A). Of the two slow migrating bands, the upper and the lower represented the hyperphosphorylated and phosphorylated forms of HRI, respectively, while the lower fast migrating band represented the unphosphorylated form.

The two slow migrating phosphorylated forms of HRI increased significantly during AAPH treatment compared to control, indicating activation of HRI. Hemin preincubation alone or followed by AAPH treatment could reduce the slow migrating forms of HRI, indicating its inactivation as expected. β-Actin was used as an internal control for these experiments (Fig. 3B).

**Oxidative stress induces autophosphorylation of HRI and its eIF-2α kinase activity**

*In vitro* eIF-2α kinase assay was performed in order to monitor autophosphorylation status of HRI and its effect on the eIF-2α phosphorylation during AAPH-induced OS. N-ethylmaleimide (NEM), a thiol alkylating agent, is known to activate HRI, inducing its autokinase and eIF-2α kinase activity. Therefore, hemin and NEM treatments were used as negative and positive controls, respectively in kinase assay. As seen in the autoradiograph (Fig. 4), HRI was detected as two bands, representing its phosphorylated and hyperphosphorylated forms. These two bands correspond to the upper two slow migrating forms of HRI detected by polyclonal anti-HRI antibody in Western blot experiments (Fig. 3). Autophosphorylation of HRI and eIF-2α phosphorylation (shown by arrows) increased significantly during AAPH treatment, as compared to the control (Fig. 4). Hemin preincubation alone or hemin preincubation, followed by AAPH exposure significantly inhibited both the autokinase and eIF-2α kinase activity of HRI as expected (Fig. 4). Similar activation was observed in the sample incubated with NEM as the positive control (Fig. 4). These results thus indicated that during AAPH-induced OS, HRI was activated through autophosphorylation and that it phosphorylated eIF-2α, which is known to inhibit protein synthesis1.

**Increased association of HRI with Hsp90 during oxidative stress**

Hsp90 and Hsp70 are known to interact with HRI and act as chaperone and co-chaperones, respectively.
during various stress stimuli\textsuperscript{16}. In the present study, as shown earlier (Fig. 1), these Hsps were induced during AAPH-generated OS. Therefore, it was important to determine their role in HRI-mediated regulation of protein synthesis during OS. Co-immunoprecipitation experiments performed with control and AAPH-treated cell samples using anti-Hsp90 and anti-Hsp70 antibodies indicated that both the Hsps interacted with HRI and form complexes (Fig. 5). Interestingly, however, the intensity of the Hsp90-HRI complex was significantly higher during AAPH-induced OS (Fig. 5B), while the Hsp70-HRI complex did not increase significantly (Fig. 5A). Increase in Hsp90 association was equal with unphosphorylated, phosphorylated and hyperphosphorylated forms of HRI in AAPH-treated cells (Fig. 5B). Hsp90, Hsp70 and HRI levels determined by Western blots in control and AAPH-treated K562 cell extracts which were used for co-immunoprecipitation are shown in Fig. 5C. Thus, these results indicated that interaction of these Hsps, in particular Hsp90 with HRI was instrumental in HRI activation and inhibition of protein synthesis during OS.

**Fig. 5—Association of HRI with Hsps during oxidative stress** [Proteins were extracted from control and AAPH-treated cells. Co-immunoprecipitation was done using anti-hsp70 (A) and anti-hsp90 (B) antibody. Immunoprecipitates were separated by SDS-PAGE, transferred to nitrocellulose membranes (blots) and the blots were probed with anti-HRI antibody. (C): Equal amount of sample used from control and treated cells]

**Oxidative stress and short-term hemin treatment did not induce differentiation of K562 cells**

Exogenous hemin as well as ROS generated by OS are known to induce differentiation in K562 cells\textsuperscript{24,27}. Benzidine staining experiments were performed to determine whether hemin and AAPH exposure induced differentiation in K562 cells. The results indicated that AAPH (3 mM) exposure for 1 h at 37°C did not induce differentiation (Fig. 6), whereas cells pre-incubated in hemin (25 µM) for 24 h alone or followed by 1 h treatment with AAPH (3 mM) showed a marginal increase (2%) in differentiated population. On the other hand, among cells exposed to hemin (25 µM) for continuous 7 days (two sub-cultures), 60% showed differentiated phenotype (Fig. 6). Thus, these results indicated that transient exposure (1 h) of AAPH alone or with hemin pre-incubation (24 h) did not induce differentiation in K562 cells.

**Effect of hemin treatment on intracellular ROS levels in K562 cells**

In order to determine intracellular ROS levels in K562 cells treated with AAPH and hemin alone or in combination, DCFH-DA assay was performed. The results indicated that intracellular ROS levels remained unaltered in cells treated with 3 mM AAPH alone and in cells pre-incubated with 25 µM hemin, followed by AAPH treatment. Similarly, ROS levels in control cells and in cells pre-incubated in hemin alone were unchanged (Fig. 7). Thus, in the present study, 25 µM hemin during the incubation period of 24 h did not significantly affect the intracellular ROS levels in K562 cells.

**Discussion**

OS damages various biological macromolecules affecting vital physiological processes of cell, leading to a number of pathological conditions\textsuperscript{28}. Hsps are the major endogenous cytoprotective molecules that act against deleterious stresses, including OS\textsuperscript{28-31}. Protein synthesis, one such vital process is often a target of OS. Generally, protein synthesis is highly regulated in adverse conditions by inhibiting production of malformed proteins and stimulating specific proteins which mitigate cell injury\textsuperscript{32}.
Role of Hsps in maturation and activation of HRI, a master regulator of protein synthesis in cells of erythroid lineage is known. The present study was planned to determine the role of Hsps, namely Hsp70 and Hsp90 in HRI-mediated regulation of protein synthesis under OS in K562 human erythroleukaemia cells. To generate OS, we used a hydrophilic peroxyl radical generator, 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 of AAPH.

Results obtained from Western blot experiments using anti-Hsp70 and anti-Hsp90 antibodies indicated a dose-dependent increase in the levels of these proteins during OS. It is well known that these proteins are induced in cells during heat shock as well as many other types of stresses like hydrostatic pressure, ultraviolet radiation, viral or bacterial infections and different chemical exposures, and they provide protection against stress and injury. Therefore, our observation on the induction of Hsp70 and Hsp90 during AAPH-induced OS was in agreement with earlier reports.

We also determined the level of HRI protein and its autophosphorylation and thus activation status of HRI and Hsp90 in HRI-mediated regulation of protein synthesis under OS in K562 human erythroleukaemia cells. To generate OS, we used a hydrophilic peroxyl radical generator, 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 of AAPH.

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We also determined the level of HRI protein and its autophosphorylation and thus activation status of HRI during OS. Using anti-HRI polyclonal antibody, HRI was detected as three bands because of their differential electrophoretic mobility. The slow migrating bands were found capable of incorporating radioactive phosphate during in vitro eIF-2α kinase assay, confirming their phosphorylated status. The presence of multiple differentially phosphorylated forms of mouse HRI detected as multiple bands in

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**Fig. 6**—AAPH- and short-term hemin treatment do not induce differentiation in K562 cells [Cells were either treated with 3 mM AAPH (1 h at 37°C), and 25 µM hemin (1 day) alone or along with 3 mM AAPH for 1 h as indicated. Cells exposed to hemin (25 µM) for continuous 7 days (two sub-cultures) were used as a positive control in the assay. Control and treated cells were then subjected to benzidine staining and photographed. Blue cells were considered to be positive for hemoglobin production (A). To determine percent differentiation, preparations were counted in hemocytometer. At least 500 cells were counted per sample. The data presented are percent benzidine positive cells (mean ± SD) of 3 independent counts (B)].

**Fig. 7**—Hemin treatment does not affect intracellular ROS levels in K562 cells [DCFH-DA assay was performed with control and cells treated as follows. Cells were cultured in four experimental sets and treated as, control (untreated), AAPH (3 mM) for 1 h, hemin (25 µM) for 24 h alone or along with AAPH (3 mM) for 1 h. The mean of fluorescence intensities (AU) ± SD from 3 experiments are presented. The asterisk and hash indicates statistically significant and non-significant difference respectively in intracellular ROS levels between the compared treatments (Student’s t-test, p < 0.05)].
Western blots has been reported previously. Considering these, it appeared that during AAPH-induced OS, HRI was activated through autophosphorylation and it phosphorylated eIF-2α, leading to inhibition of protein synthesis.

We observed increased interaction of HRI with Hsp90 during AAPH-induced OS. HRI-Hsp70 interaction was also observed, however, there was no significant change in the interaction during OS. This could be particularly so, because Hsp70 is a component of HRI co-chaperone machinery and basically performs function in de novo folding and maturation of HRI. Hsp90 on the other hand, has pleiotropic functions which include basic chaperoning, protecting HRI from stress-induced aggregation as well as activation of HRI, leading to inhibition of protein synthesis. Such inhibition leads to reduced production of newly synthesised polypeptides and thus reduce load on chaperones involved in their folding. Earlier, similar increase in HRI activation in RRL has been reported. Expression and activation of HRI is modulated upon induction of erythroid differentiation in K562 and MEL cells. Exogenous hemin as well as ROS generated by OS are known to induce differentiation in K562 cells. However, in the present study, OS and hemin exposure parameters used during the experiments did not induce differentiation in K562 cells. Similarly, hemin pre-incubation used did not affect intracellular ROS levels. Therefore, the observed changes in autophosphorylation and activation status of HRI were actually induced by AAPH or hemin and were not linked to differentiation events.

Our data conclude that Hsp90 and HRI functionally interact during the regulation of protein synthesis in a cell under stressed conditions.

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