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Eukaryotic initiation factor 2α kinase is a nitric oxide-responsive mercury sensor enzyme: Potent inhibition of catalysis by the mercury cation and reversal by nitric oxide

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Abstract The activity of one of the eukaryotic initiation factor 2α kinases, heme-regulated inhibitor (HRI), is modulated by heme binding. Here, we demonstrate for the first time that Hg^{2+} strongly inhibits the function of HRI (IC_{50} = 0.6 μM), and nitric oxide fully reverses this inhibition. Other divalent metal cations, such as Fe^{2+}, Cu^{2+}, Cd^{2+}, Zn^{2+} and Pb^{2+}, also significantly inhibit kinase activity with IC_{50} values of 1.9–8.5 μM. Notably, inhibition by cations other than Hg^{2+} is not reversed by nitric oxide. Our present data support dual roles of Hg^{2+} and nitric oxide in the regulation of protein synthesis during cell emergency states.

Keywords: Protein synthesis; Translation; eIF2α kinase; Nitric oxide; Mercury; Metal cations

1. Introduction

There are four known types of eukaryotic initiation factor 2α (eIF2α) kinases, designated GCN2, PERK, PKR, and heme-regulated inhibitor (HRI)[1] [1–4]. These kinases have a common substrate, and phosphorylate the Ser51 residue of the α subunit of eukaryotic initiation factor 2 (eIF2), leading to the termination of protein synthesis under emergency conditions. However, the sensing systems of these eIF2α kinases in response to cell stress or emergency are different. For example, GCN2 mediates the termination of translation under conditions of amino acid shortage [1] and UV exposure [2], PERK is activated during accumulation of denatured proteins [2], PKR activity is stimulated upon viral infection [3], and HRI acts when cells face a shortage of heme [4–6].

HRI senses the heme concentration in erythroid or red blood cells. Under normal conditions, heme blocks the kinase active site, whereas at reduced heme concentrations, the active site is exposed, allowing phosphorylation of eIF2α [4–6]. While HRI reportedly acts specifically in erythroid or red blood cells, the protein is expressed in almost all tissues, including brain, lung, heart, liver, spleen, kidney, thymus, and stomach [7–9]. It is proposed that HRI additionally regulates protein synthesis in response to factors other than heme. It is thus important to identify the metal cations that effectively inhibit HRI activity, similar to the heme iron.

Nitric oxide (NO) protects cells under conditions of stress and impairs biological systems [10]. Earlier studies using the purified enzyme in a reconstituted system in vivo disclose that NO stimulates HRI activity subsequent to suppression by heme [6].

Here, we examine the effects of metal cations and NO on HRI activity. Our results demonstrate that HRI is significantly inhibited by metal cations, in particular, Hg^{2+} with an IC_{50} value of 0.6 μM. Interestingly, this marked inhibition of HRI catalytic activity by Hg^{2+}, but not other metal ions, is effectively reversed by NO. Additionally, the N-terminal domain of HRI is essential for restoration of activity by NO. Based on these results, we suggest that HRI is a novel NO-responsive mercury sensor enzyme.

2. Materials and methods

2.1. Materials

Restriction and modification enzymes were acquired from Takara Bio (Otsu, Japan), Toyobo (Osaka, Japan), New England Biolabs (Beverly, MA, USA), and Nippon Roche (Tokyo, Japan). Other chemicals of the highest guaranteed grade were purchased from Wako Pure Chemicals (Osaka, Japan), and used without further purification.

2.2. Plasmid construction

A plasmid encoding $(His)_6$-tagged wild-type HRI comprising residues 1–619 (His_6 HRI) from mouse liver cDNA was originally constructed using the pET28 vector (Novagen, Madison, WI, USA), as described previously [6,11]. We introduced a PreScission™ protease (Amersham Biosciences, Piscataway, NJ, USA) recognition site in the expression vector to remove the $(His)_6$-tag. The resulting protein contains extra Gly-Pro-His residues upstream of the Met residue.

2.3. Protein expression and purification

$(His)_6$-tagged HRI was expressed in *Escherichia coli* BL21 (DE3) Codon Plus RIL (Stratagene, La Jolla, CA, USA) harboring pET28a-PreScission/HRI, and purified using a previous protocol [6,11]. The $(His)_6$ tag was cut with PreScission™ protease, according to the manufacturer's protocol. Purified $(His)_6$-free HRI proteins were

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Abbreviations: HRI, heme-regulated eIF2α kinase or heme-regulated inhibitor; eIF2α, α-subunit of eukaryotic initiation factor 2; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Fe^{3+}-hemin, Fe^{3+}-protoporphyrin IX complex; Fe^{2+}-heme, Fe^{2+}-protoporphyrin IX complex; GCN2, general control non-derepressible 2; PKR, pancreatic eIF2α kinase; PERK, PKR-like ER kinase; TBST, 20 mM Tris–HCl, pH 7.7, 137 mM NaCl containing 1% Tween 20

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more than 95% homogenous, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE; 7.5% gel) followed by staining with Coomassie Brilliant Blue R250. The final yields were 0.21 and 0.51 mg/L of culture for full-length and N-terminal truncated (amino acids 146-619) HRI enzymes, respectively.

2.4. Optical absorption spectra

Optical absorption spectra were collected under both anaerobic and aerobic conditions using Shimadzu UV-2500 and Multi Spec 1500 spectrophotometers (Kyoto, Japan) maintained at 25 °C, respectively. The reaction mixture was incubated for 10 min prior to spectroscopic measurements to ensure stable solution temperature. Experiments were performed at least three times for each complex.

2.5. In vitro protein kinase assay

The in vitro protein kinase assay was conducted as described previously [6,11], with some modifications. Briefly, the kinase reaction mixture (20 μl) containing 20 mM Tris–HCl, pH 7.7, 2 mM magnesium acetate, 60 mM KCl, 2 μg of $(His)_6$-tagged eIF2α, 0.35 μM tag-free HRI (full-length and N-terminal truncated proteins), and 50 μM ATP was incubated at 15 °C for 10 or 15 min under aerobic conditions. However, experiments with NO were performed under anaerobic conditions. The reaction was terminated by adding Laemmli sample buffer (62.5 mM Tris–HCl, pH 6.8, 10% glycerol, 2% 2-mercaptoethanol, 2% sodium dodecyl sulfate, and 0.002% bromophenol blue), heated for 10 min at 95 °C, and subjected to 10% SDS–PAGE. Proteins were transferred to polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA, USA). Phosphorylated proteins were detected by immunoblotting with an anti-phosphorylated eIF2α antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) [11].

Primary mouse anti-$(His)_6$ monoclonal IgM (H-3), goat anti-HRI IgG (S-16), rabbit anti-eIF2α IgG (FL-315), and goat anti-phosphorylated eIF2α IgG (Ser52) antibodies were purchased from Santa Cruz Biotechnology, Inc. For immunoblotting, the membrane was blocked for 1 h with 5% bovine serum albumin in Tris-buffered saline (20 mM Tris–HCl, pH 7.7, 137 mM NaCl) containing 1% Tween 20 (TBST), and incubated overnight at room temperature with primary antibody diluted in TBST. After washing with TBST, the membrane was treated with horseradish peroxidase-conjugated sheep anti-mouse IgG, donkey anti-rabbit IgG (Amersham Biosciences) or donkey anti-goat IgG (Santa Cruz Biotechnology) for 1 h. Immunoreactive protein bands were visualized with ECL reagents (Amersham Biosciences), and detected using a Fuji Film Chemiluminescence Reader LAS-3000 IDX6 (Tokyo, Japan). Band intensities were calculated using Image J 1.32j (National Institutes of Health, Bethesda, MD, USA) software.

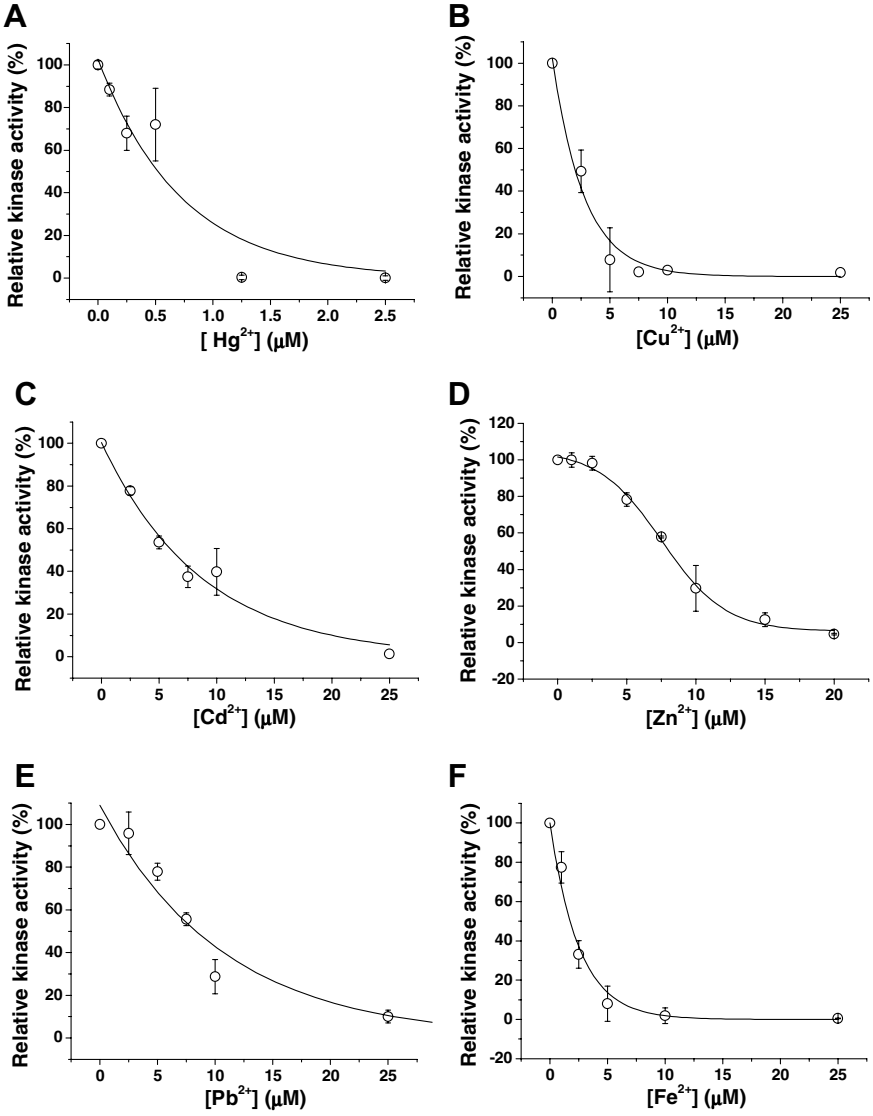

Fig. 1. Effects of Hg^{2+} (A), Cu^{2+} (B), Cd^{2+} (C), Zn^{2+} (D), Pb^{2+} (E), and Fe^{2+} (F) on the activity of full-length wild-type HRI. Inhibitory effects of these metal cations on the N-terminal truncated (first 145 amino acids) mutant were similar to those observed for the full-length enzyme (not shown).