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Cellular Localization of Stress Proteins in Mouse Hepatoma Cell Line Hepa 1-6

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マウス肝癌由来細胞Hepa 1-6におけるストレスタンパク質の細胞内局在

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Abstract

Both the heat shock protein 70 (Hsp70) and the heme oxygenase-1 (HO-1) are stress proteins induced by various chemical, biological, and physical stimuli. In this study, we examined the induced expression and subcellular localization of these stress proteins, Hsp70 and HO-1, after treatment of heat shock or heme, respectively, in mouse hepatoma cell line Hepa 1-6 by Western blot analysis and microscopic analysis. Our results showed that heat-induced expression of Hsp70 was observed, and the maximal level of expression was detected during 6~9 h after heat shock. At 6 h later, Hsp70 was localized both cytoplasm and nucleus. Unexpectedly, cells expressing high levels of Hsp70 were not uniform. The heme-induced high level of HO-1 expression was observed during 6-12 h after addition of heme. At 6 h later, HO-1 was mainly located around nuclei, possibly endoplasmic reticulum. Some cells showed that HO-1 was also expressed in nuclei.

These results indicate that although both Hsp70 and HO-1 are members of stress proteins, the subcellular localizations of these proteins are not the same. It suggests that their physiological functions are different.

keywords：heat shock protein, stress response, oxidative stress, heme oxygenase-1

Introduction

Heat shock proteins (Hsps) were originally identified in *Drosophila melanogaster* cells exposed to elevated temperatures and were, therefore, called Hsps[1,2]. Hsps are found in virtually all living organisms, from bacteria to humans. These proteins are defined by the presence of heat shock elements (HSE) in their promoters and induced by transcriptional regulatory proteins called Heat Shock Factors (HSFs)[3]. The high levels of Hsps can be produced by different kinds of stresses, such as UV light, heavy metals, hypoxia, inflammation, and infection. Then, the Hsps are referred to as stress proteins[4]. Many members of stress proteins perform

chaperone function by stabilizing new proteins to ensure correct folding or by helping to refold proteins that were damaged by stresses[5]. One of the most prominent and best characterized stress proteins is the Hsp70 family. In human, the family encompasses at least 11 genes which encode a group of highly conserved proteins[6]. The Hsp70 members have three major functional domains; N-terminal ATPase domain, substrate binding domain, and C-terminal domain. The Hsp70 family includes both costitutive and highly inducible proteins. The constitutive members are present within all the intracellular compartments, while the inducible ones appear to be predominantly cytoplasmic or nuclear location. Although the precise function of each of the Hsp70 members is still unclear, the major inducible Hsp70 has been implicated in the processes of protection and repair of stress-induced protein damage. In addition to the protein folding function of Hsp70, Hsp70 has been shown to involve in apoptosis, immune pathway, and a number of diseases including cancers[7,8].

Heme oxygenase (HO) is an enzyme that metabolizes pro-oxidant heme to carbon monoxide, ferrous ion and biliverdin. CO activates cGMP to influence vascular tone, and biliverdin is converted to bilirubin which can serve as an anti-oxidant[9,10]. Both of them may contribute to the protective role of HO in hemorrhage. However, ferrous ion can produce pro-oxidant hydroxyl radicals which may involve in the harmful role of HO in hemorrhage. The enzymatic reaction requires three mol of oxygen and seven electrons supplied by NADPH cytochrome P450 reductase (CRP). There are two relevant isoforms. The inducible expression of HO-1 (33 kDa) is predominant in spleen and liver to degrade heme derived from hemoglobin and hemoproteins[9]. The constitutive HO-2 (36 kDa) isoform is mainly found in brain and testis that is expressed under homeostatic conditions. The expression of HO-1 is induced by not only the substrate heme but also a variety of cellular stress, such as hyperthermia, heavy metals, hydrogen peroxide, and inflammatory cytokines. Recent works indicate that cells use multiple transcription factors to regulate the HO-1 expression, however, the precise molecular mechanism of transcriptional regulation of HO-1 gene has not been clarified yet[11,12]. Its 5' -regulatory region contains several potential heat shock regulatory elements (HSEs). In rat cells, HO-1 mRNA expression and activity are increased by heat shock[13,14]. Thus, HO-1 is referred as Hsp32. However, heat-induced expression of HO-1 differs between species and cell types[15-18]. HO-1 is an integral protein of the smooth endoplasmic reticulum (ER) by a single transmembrane segment (TMS) located at the C terminus. Recent study indicates that it can be localized to other compartments including nucleus[19].

We recently reported stress response in hepatoma cell lines derived from human, mouse, and rat[20]. Our results showed that the expressions of Hsp70 and HO-1/Hsp32 were induced by heat shock or heme treatment in all three cell lines, respectively. In this study, we investigated the cellular localization of stress proteins, Hsp70 and HO-1/Hsp32 by microscopic analysis to understand the physiological significance.

Material and Methods

Cell culture and treatments. Mouse hepatoma Hepa 1-6 cell line was obtained from National Institute of Biomedical Innovation JCRB cell bank, Riken BioResource Center, Japan. Cells were maintained at 37℃ in a culture medium composed of Dulbecco's modified Eagle medium with 50 U/ml penicillin, 50 U/ml streptomycin, and supplemented with heat-inactivated 10% FBS[21]. Cells were typically passaged every 7 days to obtain 10% confluent culture. Cells were treated by heat shock at 42℃ for 1 h and recovered at 37℃ for indicated time. Heme arginate, a water-soluble and stable reaction product of hemin and L-arginate, was used for heme treatment at a final concentration of 100μM for indicated time.

Western blot analysis. Whole cell extracts were prepared from cultured cells in NP-40 lysis buffer (150mM NaCl, 1% Nonidet P-40, 50mM Tris (pH8.0), 1mM phenylmethylsulfonyl fluoride, 1μg/ml leupeptin, 1μg/ml pepstatin, 1mM dithiothreitol[22]) and subjected to Western blotting using a rabbit polyclonal antibody for heme-oxygenase-1 (Hsp32) (SPA-896; Stressgen), a mouse monoclonal IgG for HSP70 (W27; Santa Cruz, CA), and β-actin (AC-15; Sigma-Aldrich). Signals were detected using ECL Western blotting detection reagents (General Electric, Fairfield, CT). Chemiluminescence was detected by LAS-4000 IR multi-color image reader (Fujifilm, Japan) and analyzed using Adobe Photoshop CS3 (Adobe Systems Inc., CA).

Immunostaining of cultured cells. Hepa1-6 cells were spread on cover slips in a 12-well plate at a cell density of 0.2×10^6 cells per well and cultured for 2 days. Cells were then treated with 100 μM hemin or heat shock (1 h at 42℃). Following to further 6 h incubation at 37℃, samples were fixed with 3.7% paraformaldehyde in PBS for 20min, permealized with 0.2% (w/v) Triton X-100 in PBS for 10 min, and blocked with 3% (w/v) BSA in PBS. Primary antibodies used in immunostaining were mouse monoclonal anti-Hsp70 antibody (W27; Santa Cruz, CA), and rabbit polyclonal anti-heme oxygenase 1 (SPA-895; Stressgen). Secondary antibodies used for cell staining were goat polyclonal anti-mouse IgG Alexa568-conjugate (Molecular Probes, A-11004), goat polyclonal anti-rabbit IgG Alexa568-conjugate (Molecular Probes, A-11011). All antibodies were used at a dilution of 1:200. Samples were mounted with DAPI fluoromount G (SouthernBiotech, 0100-20). Confocal images were taken using Olympus Fluoview FV1000 and processed on Image J.

Results and Discussion

We examined the effect of heat-shock on the expression of Hsp70 in Hepa 1-6 cells by Western blot analysis (Fig. 1A). Without any treatment, Hsp70 was hardly detectable. After heat-shock (HS) at 42℃ for 1 h, cells were recovered at 37℃ for 0-24 h. Hsp70 was induced 3 h after HS, and the induced expression was observed until 24 h later. At 6 h after HS, the maximal expression of Hsp70 was observed. We analyzed the subcellular localization of Hsp70 (Fig. 1B). Hsp70 was not detected significantly in the cells without any treatment (control) and just after heat-shock (post-HS, 0 h). At 6 h after HS (post-HS, 6 h), high level of Hsp70 positive cells were

observed, but not all cells in the field. The reason why Hsp70 was not induced in all cells was unknown. Cell cycle may involve in the induced expression of Hsp70. The stronger signals of Hsp70 were localized to mainly cytoplasm, and less intensive signals were detected in the nucleus. The cytoplasmic Hsp70 may function as a molecular chaperon with other Hsps to protect cells from heat-damaged proteins by folding and refolding[23]. During heat shock stress, nuclear transport pathways mediated by importin β-family are downregulated, whereas a novel nuclear import pathway by Hikeshi is upregulated[24,25]. Hikeshi-mediated nuclear import is coupled with the ATPase cycle of Hsp70. Thus, Hsp70 involved in heat shock-induced damage by acting inside of the nucleus. In addition, Hsp70s were associated with centrioles, nuclear speckles, and nucleolus following thermal stress[26]. These results suggest involvement of Hsp70s in transcriptional recovery.

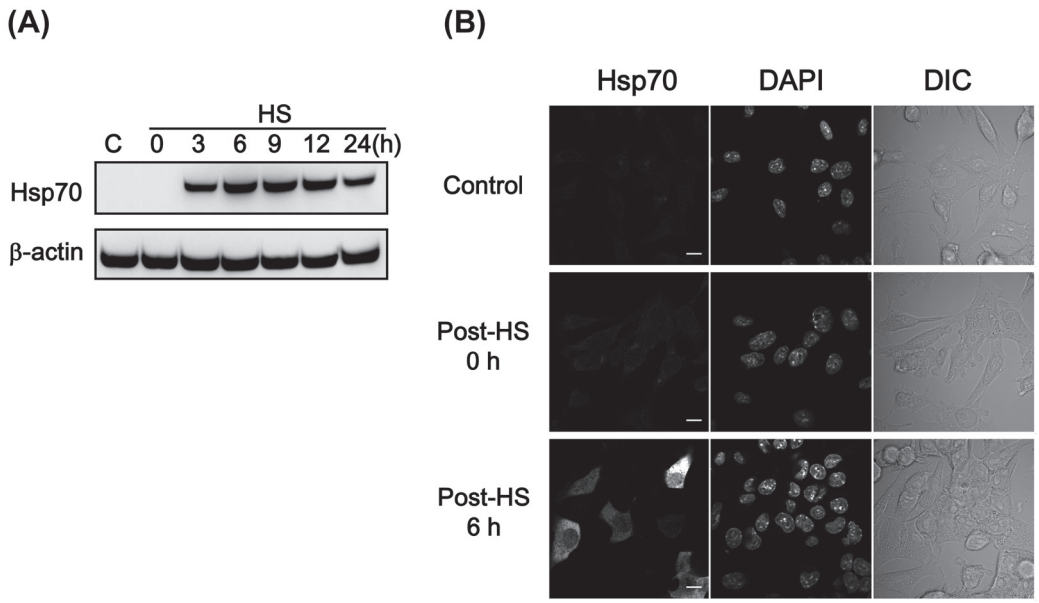

Figure 1. Heat shock-induced upregulation of Hsp70 in Hepa1-6 cells. (A) Western blot analysis of Hsp70 after heat shock. Subconfluent culture of Hepa1-6 was untreated (C) or treated with heat shock (HS) for 1 h at 42℃, and maintained at 37 ℃ for indicated time (0 to 24 h). Cells were harvested, and Western blot analysis with specific antibody was performed. A typical result was shown among three independent experiments. (B) Microscopic analysis of Hsp70 after heat shock. Cells were fixed either immediately or 6 h after heat shock (HS). HSP70 expression was accessed by immunostaining using anti-Hsp70 antibody. Nuclei were stained using DAPI. Cell morphology was imaged by Differential interference contrast (DIC) microscopy. Bar; 10 μm.

We next examined heme-induced expression of HO-1 (Fig. 2A). After treatment of cells with 100μM HA, HO-1 expression was induced after 3 h to 24 h. At 6 h after treatment of HA, cellular localization of HO-1 was analyzed (Fig. 2B). Without any treatment (control), HO-1 was hardly detected in all cells. At 6 h after, HO-1 expression was observed mainly in cytoplasm, especially around nuclei, possibly ER. In some cells, HO-1 was also detected in nuclei. The

beneficial effect of HO-1 in cytoprotection is attributed to its enzymatic action in heme degradation and also to the catalytic products. HO-1 is anchored to the ER and the enzymatic active site is faced to cytoplasm. Thus, HO-1 has been thought one of the anti-oxidant proteins. Recently, HO-1 can be proteolytically cleaved from the ER to allow nuclear translocation[19,27]. The truncated molecule (28 kDa) is an enzymatically inactive form. Nuclear HO-1 was implicated as a regulator of DNA repair activities important to carcinogenesis and tumor progression. Furthermore, nuclear HO-1 interacts with nuclear factor erythroid 2-related factor 2 (Nrf2) and stabilized it from glycogen synthase kinase 3β (GSK3β)-mediated phosphorylation coupled with ubiquitin-proteasomal degradation[19]. However, the truncated HO-1 (28 kDa) was not detected by Western blot analysis in our study. Thus, nuclear-located HO-1 is supposed to be a full length of the protein. There must be other physiological functions of HO-1 in the nucleus.

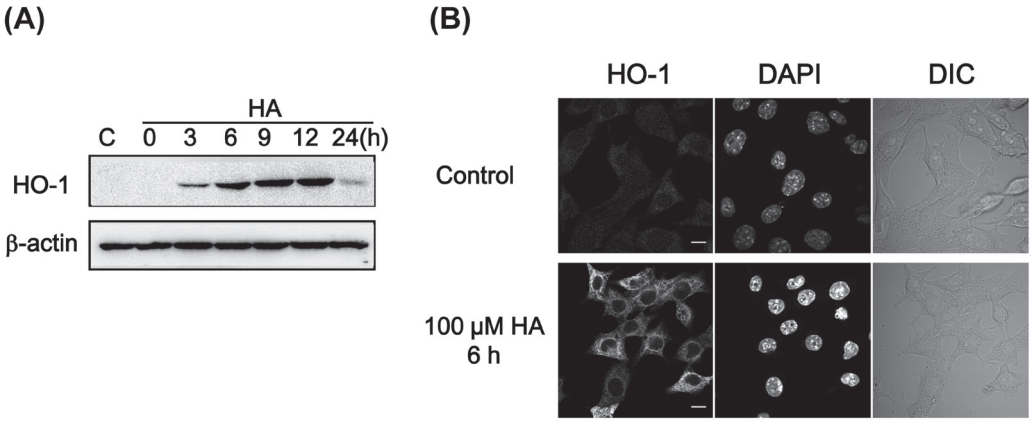

Figure 2. Hemin-induced upregulation of HO-1 in Hepa1-6 cells. (A) Western blot analysis of HO-1 after hemin treatment. Subconfluent Hepa1-6 cells were untreated (C) or treated with 100 μM hemin (HA) for indicated time (0 to 24 h). Cells were harvested, and Western blot analysis with specific antibody was performed. A typical result was shown among three independent experiments. (B) Microscopic analysis of HO-1 after hemin treatment. Cells were fixed either untreated (Control) or treated with 100 μM hemin (HA) for 6 h. HO-1 expression was accessed by immunostaining using anti-HO-1 antibody. Nuclei were stained using DAPI. Cell morphology was imaged by Differential interference contrast (DIC) microscopy. Bar; 10μm.

In conclusion, our study showed that stress-induced Hsp70 and HO-1/Hsp32 were localized to different subcellular compartments in order to protect cells from stress-induced damage under the optimum conditions.

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