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Stress Response in Hepatoma Cell Lines Derived From Different Species

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肝癌由来細胞株におけるストレス応答の動物種における違い

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Abstract

In this study, we investigated the heat shock response using three hepatoma cell lines derived from human, mouse and rat. There are well conserved heat shock elements (HSEs) in the regulatory regions of heat shock protein 70 (Hsp70) and Hsp32 (heme oxygenase-1/HO-1) genes of all three species. Our results showed that the expression of Hsp70 was induced in all three cells treated with heat-shock. However, the expression of Hsp32 /HO-1 was not induced in human Hep 3B cells and was slightly induced in mouse Hepa 1-6 cells after heat-shock treatment. On the other hand, significant induction of Hsp32/HO-1 was observed after heat-shock treatment in rat H-4-II-E cells. Heme treatment induced the expression of Hsp32/HO-1 in all three cell lines. These results indicate that although both Hsp70 and Hsp32/HO-1 are members of heat shock proteins, the mode of Hsp32/HO-1 expression induced by heat shock varies among cell lines derived from different species, independent of the HSE sequence.

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

Introduction

The heat shock response was discovered in 1962 by Ritossa [1, 2], who observed a pattern of *Drosophila* salivary gland chromosome puff that were induced in response to transient exposure to elevated temperature. The heat shock response is a ubiquitous and highly conserved system for biological defense mechanisms that is induced by proteotoxic insults such as heat, oxidative stress, heavy metals, toxins, and bacterial and viral infections [3, 4].

A key part of the heat shock response is the up-regulation of heat shock proteins (Hsps), which are found in virtually all living organisms, from bacteria to humans [5, 6]. Heat shock proteins are named according to their molecular weight. For example, Hsp40, Hsp70, Hsp90, and Hsp110 refer to families of heat shock proteins on the order of 40, 70, 90, and 110 kilodaltons in size. The primary function of the family of Hsps is to serve as molecular chaperones to protect thermally damaged proteins from aggregation, unfold aggregated proteins, and

refold damaged proteins or target them for efficient degradation [7]. Their general role as molecular chaperones is now central to many areas of active research including aging, neurodegeneration, signaling, immunology and cancer [8-11].

The induced expression of Hsps during heat shock is generally controlled by a single transcription factor; in mammals this regulation is performed by heat shock factor 1 (HSF1) [3, 12]. HSF1 exists as an inactive monomer with a complex with Hsp40, Hsp70 and Hsp90 in cytosol under unstressed conditions. Upon stress, such as elevated temperature, HSF1 is released from the chaperone complex and trimerizes. HSF1 is then transported into the nucleus where it is hyper-phosphorylated. HSF1 binds to *cis*-acting element that are composed of inverted nGAAn pentamers collectively called heat shock elements (HSEs) [13].

Among Hsps, Hsp32 is a unique protein which has an enzymatic activity, known as heme oxygenase-1 (HO-1) [14-17]. HO-1 catalyzes the degradation of heme into carbon monoxide, ferrous iron, and biliverdin which is subsequently converted to bilirubin by biliverdin reductase [18-20]. HO-1 is responsible for the regulation of cellular levels of heme and for the recycling of iron. The expression of HO-1 is high in spleen and liver to degrade heme derived from hemoglobin and hemoproteins [20]. Because bilirubin (the reduced form of biliverdin) and carbon monoxide have antioxidant and anti-inflammatory properties, respectively, HO-1 is considered to function cytoprotectively [21]. However, because HO-1 produces ferrous iron, it may catalyze deleterious cellular reactions. Thus, HO-1 can serve both pro- and antioxidant roles [22] and it is of interest to study the regulation mechanism of HO-1 expression.

The expression of HO-1 is activated 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 [23, 24]. Its 5'-regulatory region contains several potential HSEs, however, heat-induced expression of HO-1 differ with species and cell types [25-28]. In general, the heat-mediated induction of HO-1 was observed in rat [29]. However, in human its induction was observed only in certain cell lines [30].

In this study, we examined the heat shock response of three hepatoma cell lines derived from human, mouse, and rat in order to compare the difference of heat shock response.

Materials and Methods

Cell culture and treatments. Human hepatoma Hep 3B cell line, mouse hepatoma Hepa 1-6 cell line, and rat hepatoma H-4-II-E cell line were 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 [31]. Cells were treated by heat shock at 42℃ for 1h and recovered at 37℃ for 6 h. Heme arginate, a water-soluble and stable reaction product of hemin and L-arginate, was used for heme

treatment at a final concentration of 100 μM for 6 h. Morphology of growing cells were observed using an OLYMPUS CKX31 microscope.

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, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1mM dithiothreitol [32] 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). Quantification was performed by densitometric analysis of specific bands using NIH Image program[31].

Nucleotide sequences. Nucleotide sequences of upstream regions of human, mouse, and rat Hsp70A1 gene and HO-1 gene (HMOX1) were depicted from Ensemble genome browser.

Results

General models for Hsp70 and HO-1 gene regulation are shown (Fig. 1).

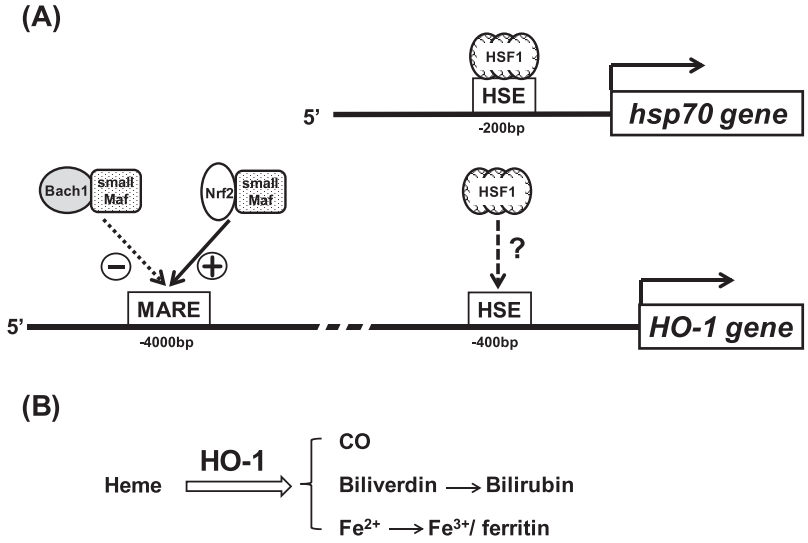

Figure 1. Regulatory regions of Hsp70 and HO-1 genes and enzymatic activity of HO-1. (A) There is a HSE upstream at -200bp from the promoter of hsp70 gene. Heat-activated HSF1 trimer binds to the HSE and activates the hsp70 transcription. There are two regulatory regions in the upstream region of HO-1 gene, Maf recognition element (MARE) at -4000bp and HSE at -400bp. A heterodimer of Bach1 and small Maf binds to MARE and represses the HO-1 expression. A heterodimer of Nrf2 and small Maf binds to MARE and activates the HO-1 expression. The function of HSF1 on the HO-1 expression has not been clarified yet. (B) HO-1 is an enzyme that catalyzes the degradation of heme to carbon monoxide (CO), biliverdin, and Fe^{2+}. Biliverdin is then converted to bilirubin by biliverdin reductase. Fe^{2+} is immediately sequestered by associated increase in ferritin.

The heat-activated HSF1 trimer binds to HSE at -200bp upstream from Hsp70 gene, and activates its expression [3, 4]. In non-stressed cells, Bach1 and small Maf heterodimer binds to MARE at -4000bp upstream from HO-1 gene, and represses its expression. Cellular stimulation such as heme-treatment promotes dissociation of Bach1/small Maf from MARE, followed by the binding of Nrf2 and small Maf heterodimer to MARE, as a result, activates its expression [23, 24]. Although HO-1 gene was reported to have HSEs in promoter region at -400bp, the function of HSF1 on HO-1 expression has not been clarified yet. The catalytic activity of HO-1 was summarized [18-20].

We compared the nucleotide sequences of HSE in promoter regions of Hsp70 and HO-1 genes in human, mouse, and rat (Fig. 2). The HSE consensus sequence (GAAnnTTC) n is well conserved in the promoter regions of both Hsp70 and HO-1 genes from all three species.

HSE consensus sequence (GAAnnTTC)

(A) *Hsp70A1 gene*

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Human1  GGAGGCGAAACCCCTGGAATATTCCCGACCTGGC
Mouse1  AGACGCGAAACTGCTGGAAGATTCCTGGCCCCAA
Rat1    AGACCCGAAACTGCTGGAAGATTCTTGGCCCCAA
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(B) *HO-1 gene*

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Human1  GCTTTCTGGAACCTTCTGGGACGCCT
Mouse1  TAGTTCTGAAACCTTCAAGATTCCTG
Rat1    TAGTTCTGGAACCTTCCAGATTCCTG

Human2  AGGGGGGGCTCTGGAAGGAGCAAAAT
Mouse2  GTAGAGGGTTCTGGAAAGGACCCAAA
Rat2    GTAGAGGGTTCTGGAAAGGACCCAAA
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Figure 2. Comparison of HSE sequences of Hsp70 and HO-1 genes. HSE consensus sequence is shown. (A) Comparison of HSE of Hsp70 gene from human, mouse, and rat. Conserved sequences are highlighted. (B) Comparison of HSEs of HO-1 gene from human, mouse, and rat. Two consensus regions are shown. Conserved sequences are highlighted.

We observed the morphology of growing cells of human Hep 3B, mouse Hepa1-6, and rat H-4-II-E cell lines (Fig. 3A). Although the shape and the size of cells were slightly different among cell lines, they grow well in these culture conditions.

We examined the effects of heat-shock and heme-treatment in three cell lines (Fig. 3B). Hsp70 protein was induced significantly in all three cells, 6h after heat-shock (HS) at 42°C for 1h and recovered at 37°C. On the other hand, the expression of Hsp70 was not changed after heme-treatment (HA) at a concentration of 100 μM for 6 h. However, heat-induced expression of HO-1 was not detected in Hep 3B. Five-fold induction of HO-1 was observed in Hepa 1-6 cells, and more than fifteen-fold induction of HO-1 that was similar extent to heme-induced

level, was observed in H-4-II-E cells. Instead, heme-treatment induced the expression of HO-1 more than 10-fold in all three cells.

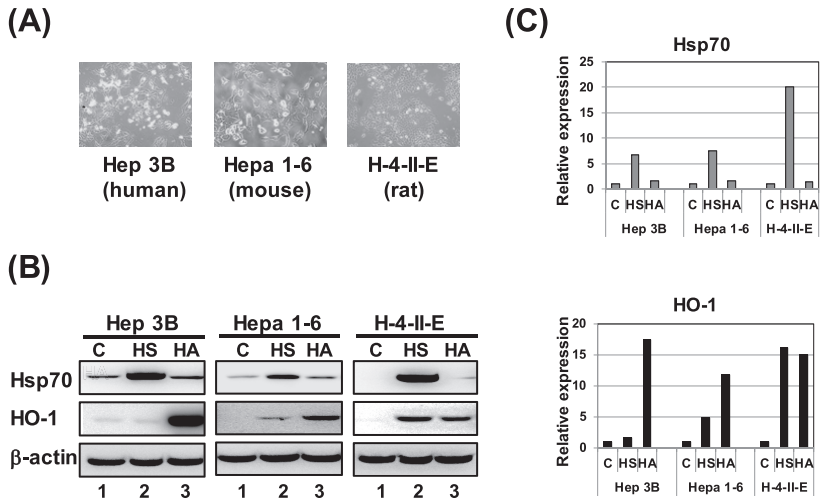

Figure 3. Heat- and heme-induced Hsp70 and HO-1 expression in three hepatoma cell lines. (A) A typical photograph of Hep 3B, Hepa1-6, and H-4-II-E cells growing in culture dishes are shown. (B) Cells were untreated (C), heat-shocked at 42℃ for 1 h and recovered for 6h (HS), or treated with 100 μM hemin for 6 h (HA). Western blot analysis using with specific antibodies was performed. A typical result was shown with three cell lines. (C) Relative protein levels of Hsp70 to β-actin and HO-1 to β-actin were shown.

Discussion

We investigated the heat shock response in hepatoma cells derived from human, mouse, and rat. Our results showed that the expression of Hsp70 was clearly induced by heat shock in three cell lines. However, the heat-induced expression of Hsp32/HO-1 was quite different in cell lines. The expression in human Hep 3B was not induced at all by heat-shock loading. In mouse Hepa 1-6, a little induction of Hsp32/HO-1 was observed. In rat H-4-II-E cells, heat-shock treatment caused a significant induction of Hsp32/HO-1 (Fig.3). These results indicate that the effects of heat-activated HSF1 on Hsp32/HO-1 are not the same in different cell lines. The mechanisms that bring the different reactivity are not clear at present. The heat-induced expression of Hsp70 indicated that there are heat-activated functional HSF1 in three cell lines. In addition, the HSE consensus sequence which is required for HSF1 binding is well conserved in these cells we used in the present experiments. On the other hand, heme-induced expression of Hsp32/HO-1 was observed in these cells, indicating that Bach1 and Nrf2 regulation on Hsp32/HO-1 gene expression is functional.

We previously reported that most Hsps including Hsp32/HO-1 were heat induced in human epithelial colorectal adenocarcinoma Caco-2 cells [31]. Thus, even human cell line does show the heat-induced expression of HO-1. There are reports showing that heat shock induced HO-1

mRNA in human hepatoma cells Hep 3B but not Hep G2 [28, 30]. Another report suggests the possible role of HSF1 in repression of HO-1 expression in human Hep 3B cells [33]. In fact, recent studies indicated that HSF1 negatively regulates TNF α and IL-1 β [34, 35]. These results are conflict from our results for in Hep 3B cells. The distinct observation may reflect differences in the conditions of culture or heat-shock treatments. One possible reason for the variation of heat-induced HO-1 induction among cell lines and species might be dependent on the chromatin structure around the HSE regulatory sequences. Our previous study indicates that HSF1 recruits nucleosome remodeling complex and partially opens the chromatin structure of the IL-6 promoter [32].

Since Hsp32/HO-1 can serve both pro- and antioxidant roles [22], the expression should be regulated strictly. There are many *cis* elements in addition to MARE and HSE in the regulatory region of HO-1 gene [23, 24], indicating the presence of complex regulatory system of transcription factors. HO-1 plays a critical role in human pathogenesis, including inflammatory diseases, cardiovascular diseases, and ischemia-perfusion injury [18]. Therefore, it should be very helpful to understand pharmacological aspects of HO-1 to elucidate the molecular mechanism of the regulation of HO-1 gene expression. Our results in this study might provide a window into the understanding of molecular mechanisms and physiological significances of heat-induced expression of Hsp32/HO-1.

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