

## Heat Shock Response in Human Epithelial Colorectal Adenocarcinoma Caco-2 Cells

Sachiye INOUE, Michiko OHNO, Hidetomo KIKUCHI and Reiko AKAGI

ヒト結腸癌由来 Caco - 2 細胞における熱ショック応答

井上 幸江・太野 路子・菊地 秀与・赤木 玲子

### Abstract

In this study, we investigated the heat shock response using human epithelial colorectal adenocarcinoma Caco-2 cells, which have been widely used for *in vitro* epithelial model system to study intestinal epithelial function. Our results show that the expression of heat shock protein (Hsp) 70, a typical heat-inducible Hsp, is induced time- and temperature-dependently, according to the activation of heat shock transcription factor-1. Hsp110, Hsp40, Hsp27, but not Hsp90 and Hsp60, are also induced in the same manner as Hsp70. Although heme oxygenase-1 (HO-1), which is also known as Hsp32, is not thought to be heat-inducible in human, significant induction of HO-1 was observed in Caco-2 cells.

### Introduction

Heat stroke is a systemic disorder that is characterized by multi-organ injury<sup>1</sup>. Intestinal barrier disruption followed by endotoxemia is thought to contribute to the pathogenesis of heat stroke<sup>2</sup>. Heat shock proteins (Hsps) induced by various type of stress loading including heat stroke, are known to play important role to protect cells from injury<sup>3</sup>, however, the details of heat shock responses in intestinal injury are still remained unknown. The heat shock response is a universal and evolutionary conserved process that is regulated by the heat shock transcription factor (HSF) family. The HSFs control the transcription of various heat shock genes including Hsps<sup>4</sup>. The significance of heat shock response is the rapid accumulation of Hsps, a conserved family of chaperones that facilitate folding and assembly of nascent proteins, and refold of stress-damaged proteins. In mammals, the expression of classical Hsps regulated by HSF1 among four HSF family members<sup>4</sup>. When cells are heat-shocked, HSF1 is highly phosphorylated, converted to a trimer that can bind to a heat-shock element (HSE) and activates the transcription of Hsp genes.

We previously showed that protective role of heme oxygenase-1 (HO-1) in the intestinal tissue injury in an experimental model of sepsis<sup>5</sup>. HO-1 is the inducible isoform of the rate limiting enzyme in heme catabolism and is also known as the heat shock protein 32 (Hsp32)<sup>6</sup>.

However, the induced expression of HO-1 gene in response to hyperthermia is controversial, since the expression differs among species and cell lines. Much evidence has suggested that up-regulation of HO-1 plays an important role in the cytoprotective defense against oxidative stress and inflammatory stimuli<sup>7</sup>. Primary structure analysis of the 5' flanking region of HO-1 genes of mammals revealed a motif similar to the consensus heat shock element (HSE) necessary for up-regulation of heat shock genes including Hsps<sup>8</sup>. The rat HO-1 was induced by heat-shock as like as Hsps<sup>6</sup>, whereas human and mouse HO-1 are apparently not like Hsps<sup>9,10</sup>, except for some cell lines<sup>11,12</sup>.

Human epithelial colorectal adenocarcinoma Caco-2 cells (Caco-2) have been well validated as a useful in vitro epithelial model system to study intestinal functions<sup>13</sup>. In this study, we precisely investigated the heat shock response in Caco-2 in order to further analysis of molecular mechanism understanding the protective effect of the HSPs including HO-1 induction upon heat stroke.

### Materials and Methods

*Cell culture and Treatments.* Human hepatoma cell line (Hep G2) was obtained from American Type Culture Collection, Rockville, MD, and Caco-2 cells were kindly provided by Tooru Taniguchi, Nagoya City University Graduate School of Medical Sciences. Hep G2 and Caco-2 cells were maintained at 37°C 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<sup>13</sup>. Cells were treated with heat shock at different temperature for 1 h and recovered at 37°C for indicated times.

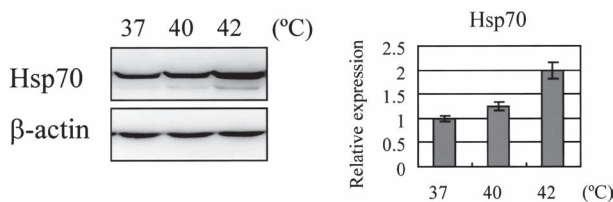
*Western blot analysis.* Whole cell extracts were prepared from cultured cells in NP-40 lysis buffer (150 mM NaCl, 1% Nonidet P-40, 50 mM Tris (pH8.0), 1 mM phenylmethylsulfonyl fluoride, 1 µg/ml leupeptin, 1 µg/ml pepstatin, 1 mM dithiothreitol<sup>14</sup>) and subjected to Western blotting using a rabbit antiserum for HSF1 (α-mHSFj), Hsp110 (Hsp110b), Hsp90 (Hsp90d), Hsp60 (Hsp60-1), Hsp40 (Hsp40-1), Hsp27 (Hsp27a)<sup>15-17</sup>. 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), were obtained commercially. Signals were detected using ECL Western blotting detection reagents (General Electric, Fairfield, CT). Chemiluminescence was detected LAS-4000 IR multi color Image reader (Fujifilm, Japan) and analyzed Adobe Photoshop CS3 (Adobe Systems Inc., CA). Quantification was performed by densitometric analysis of specific bands using NIH Image program. Statistical evaluation was performed with Student's *t* test.

### Results

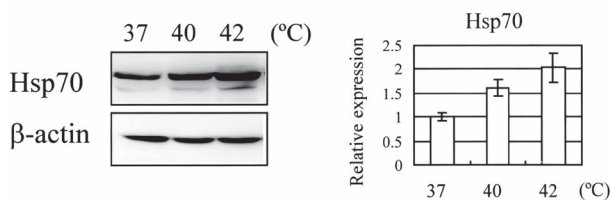
We first examined the heat shock response in Caco-2 and Hep G2 cells. Both cells were heat shocked for 1h at 40°C or 42°C, and recovered for 4 h at 37°C. Expression of Hsp70 or β-actin was determined by Western bolt analysis using a specific antibody (Fig. 1A and B, left). Hsp70 and β-actin protein expression were quantified by densitometric analysis and the level of Hsp70 versus

$\beta$ -actin is shown as relative value to the expression level in control cells (Fig. 1A and B, right). In both cells, Hsp70 expression were induced by heat shock at 40°C and 42°C (Fig. 1). The magnitude of Hsp70 induction was about 2-fold in both cells heat-shocked at 42°C. The results showed that Caco-2 cells as well as HepG2 cells induce Hsp70 expression upon heat shock as generally observed in most cells<sup>4</sup>).

#### A) Caco-2 cells

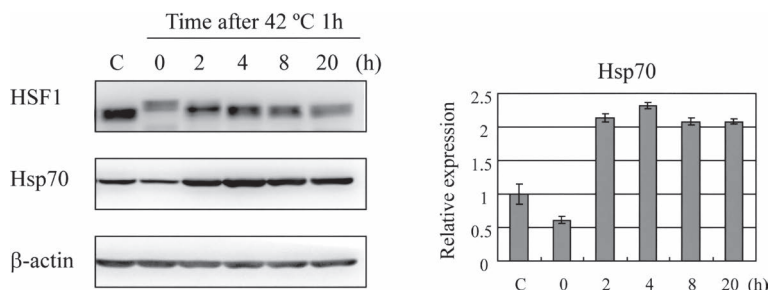


#### B) Hep G2 cells



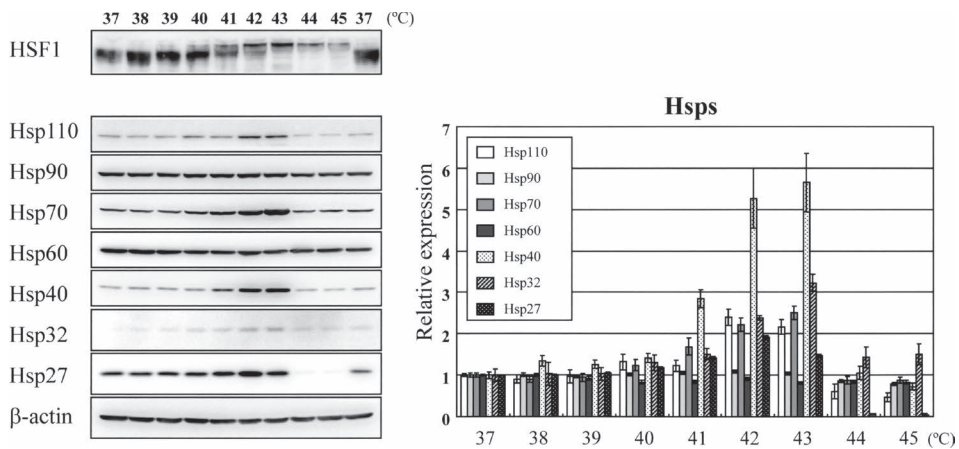
**Figure 1. Heat shock response in Caco-2 and Hep G2 cells.** Caco-2 (A) and Hep G2 (B) cells were heat-shocked for 1 h at 40°C or 42°C, and recovered for 4 h at 37°C. Western blot analysis was performed (left) and relative expression of Hsp70 to  $\beta$ -actin protein is shown (right). Data represent means  $\pm$  SE from three independent experiments ( $n = 3$ ).

We next examined the time course changes in Hsp70 expression after heat shock at 42°C for 1 h, by using Caco-2 cells (Fig. 2). Immediately after 1 h heat stress loading, highly phosphorylated HSF1 was detected, and was reduced during recovery in a time-dependent manner. Induction of Hsp70 was detected at 2 h after heat stress, and was maintained for 20 h during recover time (Fig. 2).



**Figure 2. Time course changes of heat shock response in Caco-2 cells.** Cells were heat-shocked at 42°C for 1 h and recovered for 0, 2, 4, 8, or 20 h at 37°C. Western blot analysis was performed (left) and relative expression of Hsp70 to  $\beta$ -actin protein is shown (right). Data represent means  $\pm$  SE from three independent experiments ( $n = 3$ ).

Since Caco-2 cells significantly induced Hsp70 after heat shock at 42°C for 1 h, we examined the effect of temperature on the expression levels of various Hsps. Heat shock temperature was varied from 37°C to 45°C, and the expression of HSF1 and Hsps were determined by Western blot analysis using specific antibodies. Phosphorylation of HSF1 was detected at 40°C and increased upon the elevation of temperature. According to the activation of HSF1, expression of inducible Hsps (Hsp110, Hsp70, Hsp40, Hsp27) were increased depending on the elevation of temperature up to 43°C. Expression of Hsp32 (HO-1) was also increased in the same manner as other inducible Hsps. The highest expression of HO-1 was observed at 43°C, and relative expression was about 3-fold increase. Expression of Hsp90 and Hsp60 were not changed in the different temperature, suggesting that they were constitutively expressed. Extremely high temperature (44°C or 45°C) may be toxic for cells, since all the inducible Hsps expression examined were decreased.



**Figure 3. Effect of temperature differences on HSF1 and Hsps expression in Caco-2 cells.** Cells were heat-shocked at 37–45°C for 1 h and recovered for 4 h at 37°C. Western blot analysis was performed using extracts prepared from cells just after heat shock (for the analysis of HSF1) or recovered for 4 h (for the analysis of Hsps) (left), and relative expression of Hsps to action protein is shown (right). Data represent means  $\pm$  SE from three independent experiments (n=3).

## Discussion

We investigated the heat shock response in human epithelial colorectal adenocarcinoma Caco-2 cells. Our results showed for the first time that most Hsps were heat-induced in Caco-2 cells accompanied with the activation of HSF1, as similar to the fact observed in most cells. Unexpectedly, HO-1 (Hsp32) was also heat-induced in a human cell line Caco-2. In general, HO-1 is not likely heat-inducible in human and mouse<sup>9,18,19</sup>, the exception is the HO-1 induction in Hep3B human hepatoma cells<sup>12</sup>. The reason of these species- and cell lines-dependent variation is not known at present. However, previous report showed that HO-1 expression could be increased by exposure of Caco-2 cells to stress loading<sup>20</sup>. We observed that HO-1 induction levels in intestine are negatively related with damage observed in experimental endotoxemia rats<sup>5</sup>. Taken together, our present results suggest that HO-1 may be induced by heat stroke in the case of sepsis

to play an important protective role from tissue injury. Further investigation to show functional effects are in progress in our laboratory.

Hsp expression is regulated primarily at the level of gene transcription, a process controlled by the HSF family of sequence-specific DNA binding proteins. There are two typical HSE in the upstream region at around -300 to -400 bp of human HO-1 gene<sup>9</sup>). HSF1 in Caco-2 cells was activated under the heat stress conditions in this study, as shown that HSF1 was highly phosphorylated and most HSPs were heat induced. One possibility of the variation of HO-1 induction might be the chromatin structure of the regulatory sequences. Our previous study indicates that HSF1 recruits CREB-binding protein (CBP) and an ATPase subunit of SWI/SNF nucleosome remodeling complex (BRG1), and partially opens the chromatin structure of the IL-6 promoter for an activator or a repressor to bind to it<sup>14</sup>). Further study is required to understand the molecular mechanism of heat-induction of human HO-1 gene.

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