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Suppression of Thermotolerance Development through Cycloheximide-Induced Negative Control of Stress Protein Gene Expression[1]

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Expression of a luciferase reporter gene by Chinese hamster ovary cells under the control of the human heat shock protein (hsp) 70 gene promoter was suppressed by incubation at 37°C after treatment with cycloheximide (CHX) during 42°C heat shock exposure. The CHX-induced suppression of hsp gene expression induced no development of thermotolerance. However, 42°C heat shock treatment without CHX followed by CHX inhibition of protein synthesis during recovery incubation at 37°C induced thermotolerance expression by inducing enhanced synthesis of hsps including hsp70 in subsequent heat challenge incubation at 43°C. The results demonstrated a direct correlation between suppression (induction) of stress protein gene expression and non-expression (expression) of thermotolerance. Kinetic analysis showed that the CHX suppression of hsp gene induction was greater than the CHX inhibition of protein synthesis, and that it depended on the severity of heat stress: it decreased with increasing heat stress doses. Moreover, prior feeding of the proline analog L-azetidine 2-carboxylic acid abrogated the CHX-induced suppression of hsp gene expression. In addition, CHX treatment during heat cell-killing at 43°C induced protection of cells. These results were well explained by the proposed model of negative or positive control of stress protein gene expression depending on the level of free hsp70, which may be modulated by both the rate of protein synthesis and the severity of heat stress.

Key words: L-azetidine 2-carboxylic acid, cycloheximide, stress protein, stress protein gene control, thermotolerance.

When exposed to nonlethal heat shock, a variety of organisms and cells aquire transient resistance to subsequent exposure to elevated temperature (*1, 2*). This phenomenon has been termed thermotolerance (*3, 4*). The mechanism of thermotolerance is not well understood. However, conditions which produce thermotolerance in cells also induce a rapid and transient increase in the synthesis of a family of so-called heat-shock proteins (hsp) (*4*). The protein hsp70 is a major member of hsps. It is synthesized by cells from a wide variety of organisms in response to heat shock or other environmental stresses and assumed to play an important role in protection and recovery of cells from stress-induced damage. A direct link between induced thermotolerance and hsp synthesis is found in the procaryote *Escherichia coli*, where a strain with a mutation of the heat-shock induction regulatory gene (*hin*) fails to synthesize hsps and is unable to acquire thermotolerance (*5*). In mammalian cells, a few reports contradict the general trend and suggest that synthesis of hsp70 is not required for thermotolerance acquisition (*6*), but other experiments provide a compelling argument for the importance of hsp70 in induced thermotolerance (*7-12*).

In recent years, evidence has revealed that hsps have essential functions as molecular chaperons involved in protein folding, translocation, assembly to higher structures, and protein degradation (*13-15*). In particular, hsp70 has been reported to play a crucial role in folding of newly synthesized polypeptides (*16, 17*) and in regulation of hsp gene expression (*18, 19*).

Understanding the mechanisms of hsp gene regulation as well as thermotolerance is important not only for molecular biology but also for developing ways to modulate hsp gene expression in diseases induced by various forms of physiological stress (*20-22*) and to control thermotolerance development in hyperthermic treatment of cancer (*1*).

We previously reported that stress protein gene expression induced by heat shock or sodium arsenite treatment was selectively suppressed depending on a limited stress dose by treatment with the protein synthesis inhibitor cycloheximide (CHX) during stress treatment (*23*). We here report that the suppression of heat-induced stress protein gene expression by CHX is accompanied by failure of cells to acquire thermotolerance. However, CHX treatment between the first heat shock and the second heat challenge was ineffective in suppressing thermotolerance development by inducing an enhanced hsp70 synthesis in the heat challenge incubation. We also report that the prior feeding of an amino acid analog, L-azetidine 2-carboxylic acid, abrogated the suppression of stress protein gene

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induction by CHX. These results are discussed in terms of free hsp70 levels controlling stress protein gene expression.

MATERIALS AND METHODS

Cell Culture—A Chinese hamster ovary cell line, CHO(HS-luc), which stably expresses the human hsp70B promoter-directed luciferase reporter gene depending on heat shock or other stress treatments (*23*), was cultured in Ham's F12 medium (Wako Junyaku) supplemented with 10% fetal bovine serum (Flow Laboratories) in a CO_2 incubator (5% CO_2 and 95% air).

Luciferase (Luc) Assay—Luc activity was assayed by using a PIKAGENE assay kit (Toyo Ink) according to the manufacturer's procedure. The luminofluorescense generated by oxidation of luciferine by Luc in cell lysate was quantitated as relative light units (rlu) by using a luminophotometer (LUMIPHOTOMETER TD-4000, Futaba Medical).

Induction of Expression of hsp70B-Luc Reporter Gene by Heat Shock Treatment—CHO(HS-luc) cells (3.8×10^5) were cultured in 1.5 ml of medium in 35-mm plastic dishes at 37°C for 3 days (100% confluent growth). The cultured cells were treated with or without CHX (2.5 μM) in fresh medium for 10 min at 37°C, then heat-shocked with or without CHX for 30, 60, or 90 min by transferring the cell cultures into a CO_2 incubator maintained at 42°C. After heat shock treatment, CHX was removed by washing cells twice with 0.75 ml of warmed medium, then cells were cultured in 1.5 ml of fresh medium at 37°C for 5 h, unless otherwise specified, to express the hsp70B-Luc reporter gene. Duplicate dishes were used to determine each data point.

Assay of Heat Protection and Thermotolerance—CHO-(HS-luc) cells (3.8×10^5) were cultured in 35-mm plastic dishes at 37°C overnight. The cultures (about 30% confluent growth) were treated for 10 min at 37°C with or without CHX (2.5 μM), then exposed to heat at 43°C for various times up to 150 min by transferring the cells into a CO_2 incubator maintained at 43°C, washed twice with 0.75 ml of warmed medium, then cultured at 37°C for 40 h to allow cell growth (heat protection assay). Cells were similarly treated with or without CHX, then heat-shocked for 60 min at 42°C , washed free of CHX, incubated at 37°C for 5 h for synthesizing heat shock proteins, then subjected to heat challenge at 43°C for various times up to 150 min, and cultured at 37°C for 40 h to allow cell growth (thermotolerance assay). After trypsinization, the number of cells in cultures was determined with a Coulter counter (Coulter Electronics). Duplicate dishes were used to determine each data point.

Analysis of Protein Synthesis—Protein synthesis during heat shock treatment was measured by counting radioactivity incorporated into trichloroacetic acid (TCA)-insoluble materials as follows. CHO(HS-luc) cells were cultured for 3 days, washed with methionine-free warmed growth medium, preincubated for 10 min at 37°C with or without CHX (0.1 to 2.5 μM), then subjected to labeling for 30, 60, and 90 min at 42°C by adding L-[^{35}S]methionine to the medium. Labeled cells were lysed with a RIPA buffer (50 mM Tris-HCl, pH 7.2, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 1% SDS, and 1 mM EDTA), and the lysate was centrifuged at 15,000 rpm for 10 min. Ten percent TCA-insoluble materials of the supernatant were collected on GF/C filters (Whatman), washed with 10% TCA and 98% ethanol, and radioactivity of the filters was determined. In other experiments, cells were treated with mild heat shock at 42°C for 60 min, then labeled with L-[^{35}S]methionine for 2 h at 37°C. Alternatively, following the 42°C heat treatment, cells were incubated at 37°C for 5 h with or without CHX (25 μM), then washed twice with methionine-free warmed growth medium, and labeled with L-[^{35}S]methionine for 2 h by transferring cell culture into a CO_2 incubator maintained at 43°C. After labeling, supernatant of cell lysate was prepared, and an aliquot of the supernatant was mixed with an equal volume of loading buffer [20% glycerol, 4.6% SDS, 10% β-mercaptoethanol, 0.004% bromophenol blue, and 83 mM Tris-buffer (pH 6.8)]. L-[^{35}S]Methionine-labeled proteins synthesized after 42°C heat shock treatment or during 43°C heat challenge were analyzed by SDS-9% polyacrylamide gel electrophoresis (SDS/PAGE) and autoradiography. The radioactivity of β-actin band (measured with a Bio-Image Analyzer BAS 2000, Fujix) was adjusted to the same level for each lane. Two aliquots of the supernatant solution of each sample were treated overnight with the monoclonal antibodies against human hsp70 (inducible form) or Chinese hamster cell hsc70 (constitutive form) proteins (Stress Gen Biotechnologies). The solutions were further treated with second rabbit IgG antibodies against the mouse and rat IgG monoclonal antibodies for 30 min, then adsorbed on protein A-Sepharose beads. The immune complexes on beads were collected by centrifugation, washed with RIPA buffer five times, mixed with an equal volume of twofold concentrated loading buffer, and analyzed. The synthesis of hsp70 and hsc70 was estimated by determining protein band intensities with the Image Analyzer Bass 2000.

RESULTS

Suppression of hsp70B-Luc Reporter Gene Expression by Cycloheximide Treatment during Mild Heat Shock Treatment—When CHO(HS-luc) cells were heat-shocked at 42°C for 60 min, they linearly expressed hsp70B-Luc reporter gene in the subsequent incubation for 5 h at 37°C (Fig. 1). However, the reporter gene expression was completely suppressed when cells were treated with CHX (2.5 μM) 10 min prior to and during the heat shock treatment at 42°C for 60 min. The treatment with CHX (25 μM) during 37°C incubation following the 42°C heat shock treatment also inhibited the reporter gene expression. To examine if the reporter gene suppression by CHX may be due to protein synthesis inhibition by CHX that was not completely removed by washing, cells were treated with CHX (2.5 μM) for 70 min at 37°C, similarly washed, then heat-shocked at 42°C and incubated at 37°C to express Luc reporter gene. Luc reporter activity increased linearly at the same rate as that of control cells, indicating that CHX was sufficiently removed by the washing, and the suppression of induction of Luc reporter gene expression was caused by the effect of CHX only during the heat shock treatment at 42°C.

Lack of Thermotolerance Acquisition by Cycloheximide-Induced Suppression of hsp Gene Expression and hsp

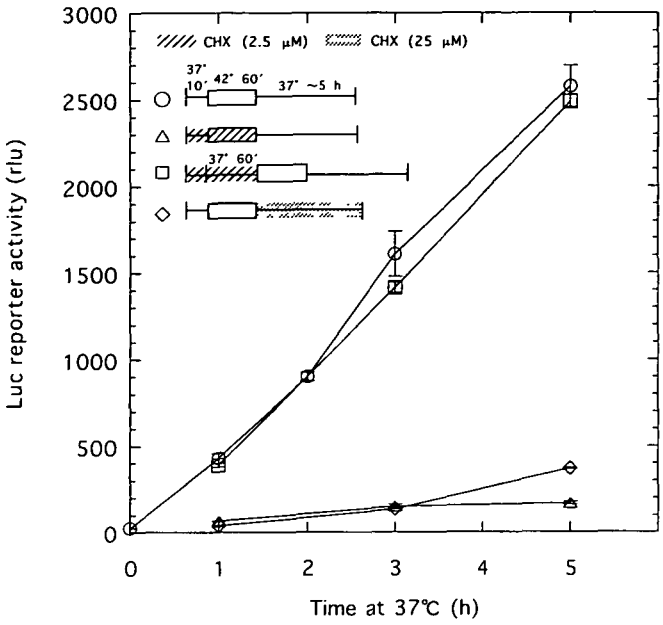

Fig. 1. **Suppression of hsp70B-luciferase reporter gene expression by cycloheximide treatment before and during mild heat shock treatment.** CHO(HS-luc) cells were treated with (△, □) or without (○, ◇) 2.5 μM cycloheximide (CHX) at 37°C for 10 (○, △) or 70 min (□), heat-shocked with (△) or without (□) CHX at 42°C for 60 min, washed free of CHX, then incubated at 37°C for 5 h to express luciferase reporter gene in fresh medium with (◇) or without (○, △, □) 25 μM CHX.

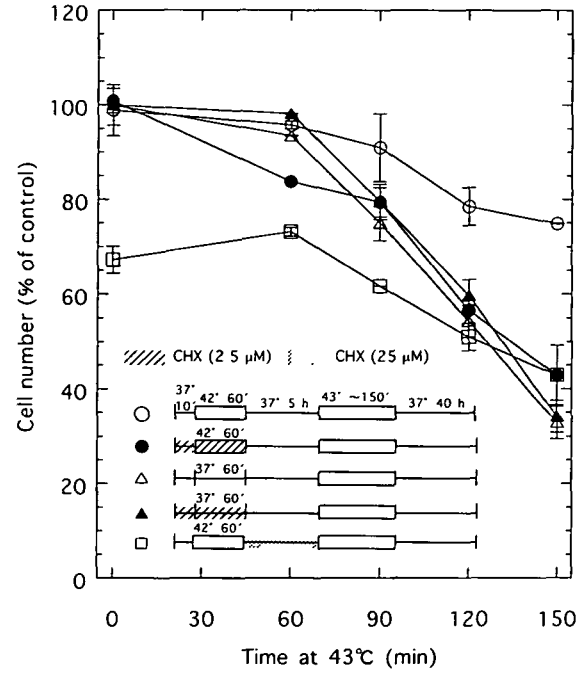

Fig. 2. **Inhibition of thermotolerance development by cycloheximide during mild heat shock treatment and lack of inhibition by cycloheximide treatment between heat shock and heat challenge.** CHO(HS-luc) cells were treated for 10 min at 37°C with (●, ▲) or without (○, △, □) 2.5 μM cycloheximide (CHX), heat-shocked at 42°C for 60 min (○, ●, □) or incubated at 37°C for 60 min (△, ▲), washed free of CHX and further incubated at 37°C for 5 h in a fresh medium with (□) or without (○, ●, △, ▲) 25 μM CHX. Cells were then washed, heat-challenged at 43°C for the various times, and cultured to allow cell growth at 37°C for 40 h.

Synthesis—We next examined if thermotolerance is induced in cells whose hsp gene induction is suppressed by CHX. Cells were treated with CHX (2.5 μM) for 60 min at 42°C, washed free of CHX, and incubated in a fresh medium for 5 h at 37°C. These cells were heat challenged at 43°C for various times up to 150 min then cultured at 37°C to allow cell growth. Cells were counted after 40 h of culture to estimate the thermotolerance expressed against the 43°C heat challenge. The 42°C heat shock treatment without CHX induced significant thermotolerance (Fig. 2). In contrast, the heat shock treatment with CHX, which suppressed heat-induced hsp gene expression (Fig. 1), did not induce thermotolerance; the heat-response cell survival curve was similar to that of controls that were not heat-shocked but treated with or without CHX (Fig. 2).

We previously showed that the 42°C heat shock treatment for 30 min with CHX (0.5 μM) selectively suppressed the induction of hsp synthesis (*23*). Furthermore, hsp synthesis was also suppressed under the experimental conditions used for examining thermotolerance in Fig. 2, namely, 42°C heat shock with CHX (2.5 μM), then 37°C incubation and 43°C heat challenge (Fig. 3, lane 6), while hsp synthesis was induced in the thermotolerance-positive control (lane 7). These results clearly indicate a lack of thermotolerance induction in cells where heat-induced hsp gene expression and thereby hsp synthesis were suppressed by CHX.

Thermotolerance Development after Cycloheximide Treatment between Heat Shock and Heat Challenge Treatments—Failure to acquire thermotolerance may also be expected when hsp synthesis is inhibited by CHX treatment at 37°C between 42°C heat shock and 43°C heat challenge, since Luc reporter activity was not expressed when protein synthesis in cells was inhibited by 25 μM CHX during the interval (Fig. 1). However, such cells expressed similar thermotolerance to the 42°C heat-shocked cells when heat-challenged at 43°C (Fig. 2, □). Therefore, we analyzed hsp synthesis in the 43°C heat challenge by the 42°C heat- and CHX-treated cells. Synthesis of hsp70, hsp90, and hsp110 was greatly enhanced in the 43°C heating as well as the incubation at 37°C (Fig. 3, lanes 4 and 5). The synthesis of hsp70 in the cells was much greater than in the cells immediately labeled at 37°C after 42°C heat-treatment (Fig. 3, lane 2). The results strongly suggest the accumulation and/or stabilization of hsp gene transcripts during the recovery incubation at 37°C with CHX. On the other hand, induction of hsp synthesis by 43°C incubation of un-preheated cells was low (Fig. 3, lanes 3 and 8). These results, together with the data of prevention of thermotolerance acquisition by suppressing heat-induced hsp gene expression and hsp synthesis, demonstrate a direct correlation between induction of stress protein synthesis and thermotolerance induction. The synthesis of the constitutive form of hsc70 was also induced by heat shock treatment (Fig. 3E, lane 7), suggesting a role of hsc70 synthesis as well as hsp70 synthesis in thermotolerance development. The synthesis of hsc70 was also inhibited by CHX treatment during the heat shock (lane 6, and see Fig. 3 legend).

Role of Free hsp70 in CHX-Induced Negative Control of

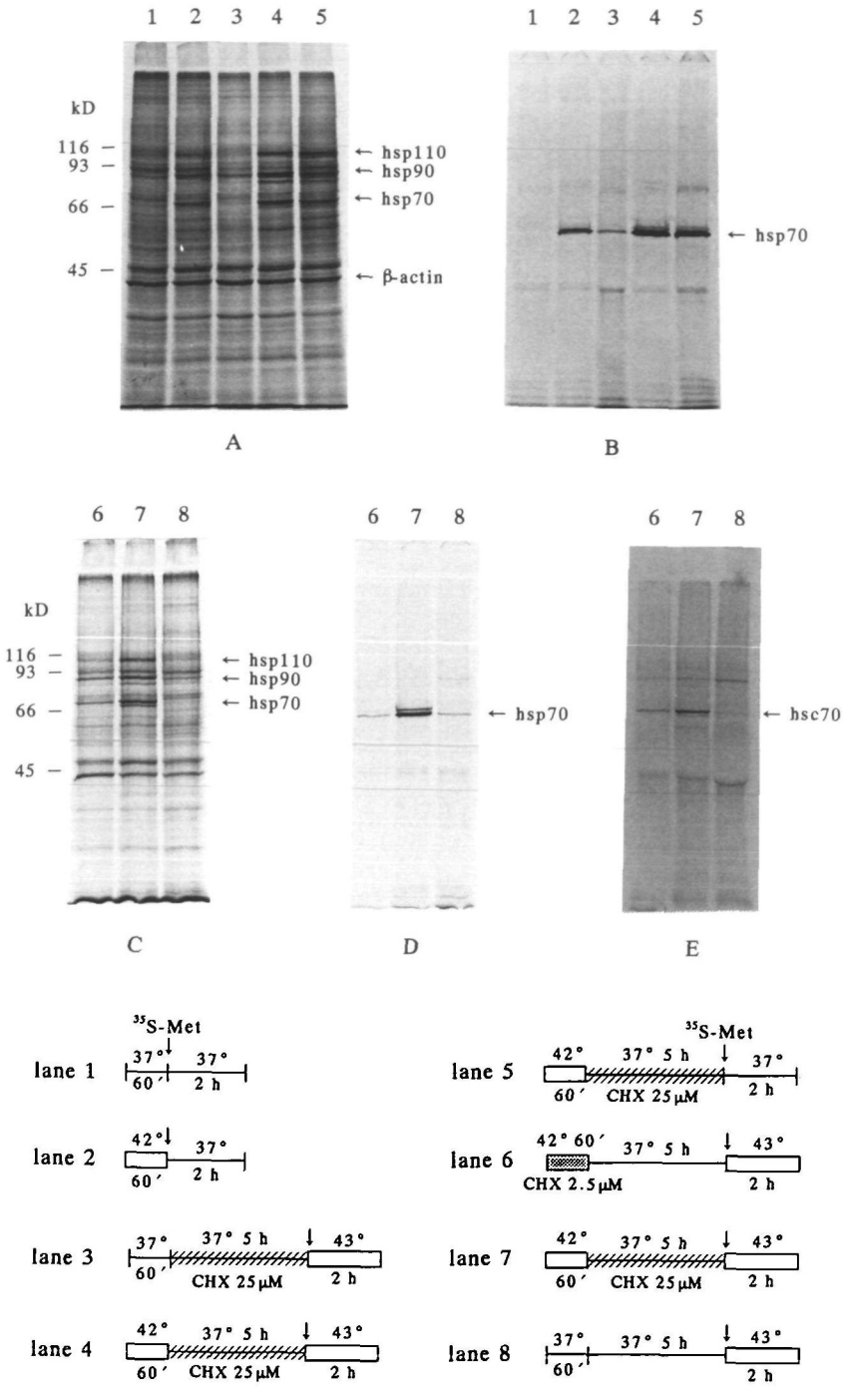

Fig. 3. **Heat shock protein synthesis after 42°C heat shock with or without cycloheximide, or after cycloheximide treatment between 42°C heat shock and 43°C heat challenge.** Cells were heat-shocked for 60 min at 42°C with (lane 6) or without CHX (lanes 2, 4, 5, and 7), or incubated at 37°C (lanes 1, 3, and 8), then labeled with L-[^{35}S]methionine for 2 h at 37°C (lanes 1 and 2), or further incubated with lanes (3, 4, 5, and 7) or without lanes (6 and 8) 25 μM CHX for 5 h at 37°C, washed free of CHX, and labeled with L-[^{35}S]methionine for 2 h at 43°C (lanes 3, 4, 6, 7, and 8) or 37°C (lane 5). Synthesis of total proteins (A and C), hsp70 (B and D), and hsc70 (E) was analysed by autoradiography on SDS-9% polyacrylamide gel electrophoresis. The relative ratios for hsp70 synthesis (D) were 0.11 (lane 6), 1.0 (lane 7), and 0.08 (lane 8), and for hsc70 synthesis (E) 0.31 (lane 6), 1.0 (lane 7), and 0.30 (lane 8).

hsp Gene Expression—We previously reported that the CHX-induced suppression of Luc reporter gene expression was hsp gene promoter-specific (*23*). We next studied the kinetics of suppression by CHX of Luc reporter gene expression under different heat shock doses as well as CHX inhibition of protein synthesis during different heating times. Cells were treated with various concentrations of CHX at 42°C for 30, 60, and 90 min. The effect of CHX on suppression of the reporter gene expression varied with heating time (Fig. 4); it was the most effective in 30 min heat shock time (0.1 μM for 50% suppression), intermediately effective in 60 min (0.3 μM for 50% suppression) and the least effective in 90 min (4 μM for 50% suppression). However, the CHX inhibition of protein synthesis (2 μM for 50% inhibition) was independent of the heating time, whether 30, 60, or 90 min, demonstrating that the induction of reporter gene expression by the modest and mild heat shocks was more sensitive to CHX inhibition than was protein synthesis. The data demonstrated that, in addition to the reduction in rate of protein synthesis during heat shock treatment, severity of heat stress influenced the suppression of hsp gene expression. A model of positive and negative control of hsp gene expression by free hsp70 levels has previously been proposed

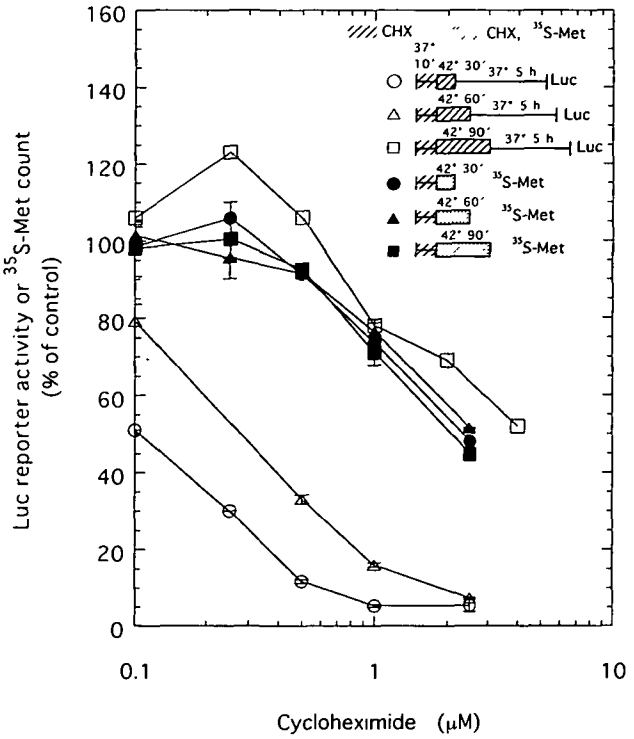

Fig. 4. **Heat shock dose-dependent suppression of hsp70B-luciferase gene expression and inhibition of protein synthesis by cycloheximide.** Cells were treated with the various concentrations of cycloheximide (CHX) for 10 min at 37°C, heat-shocked at 42°C for 30 (○), 60 (△), or 90 min (□) with CHX, then incubated at 37°C for 5 h to express Luc reporter gene in fresh medium after removal of CHX. To determine protein synthesis, cells were treated with CHX for 10 min at 37°C, then labeled with L-[^{35}S]methionine following heat-shock treatment with CHX at 42°C for 30 (●), 60 (▲), and 90 min (■).

(*19*), and our results may be explained well by the model. Briefly, the decrease in free hsp70 caused by heat-induced denaturation of proteins positively controlled hsp gene expression. When protein synthesis was inhibited by CHX, the level of free hsp70 was increased due to the decrease in nascent proteins to which hsp70 bound, and this resulted in negative control of hsp gene expression. Thus the negative control induced by protein synthesis inhibition balanced the positive control caused by limited heat stress doses, but was overcome by severe heat stress.

It has been demonstrated in HeLa cells that hsp70 remained bound as long as 2 h to newly synthesized proteins which incorporated the proline analog L-azetidine 2-carboxylic acid (Azc) (*16*). In terms of the hsp gene control model, the level of free hsp70 may be expected not to increase in the Azc-incorporated cells by CHX treatment, and therefore the heat-induced reporter gene expression would not be suppressed by CHX. To examine this, cells were incubated with added Azc (20 mM) at 37°C for 2 h, heat-shocked at 42°C for 60 min with CHX in fresh medium, then incubated again at 37°C for up to 3 h to express Luc reporter gene. The Azc-incorporated cells indeed expressed a greater amount of Luc reporter activity after heat shock treatment both with and without CHX (Fig. 5, ○ and △, *vs.* □). Luc reporter gene expression of Azc-free control cells was suppressed by CHX (▲ *vs.* ●).

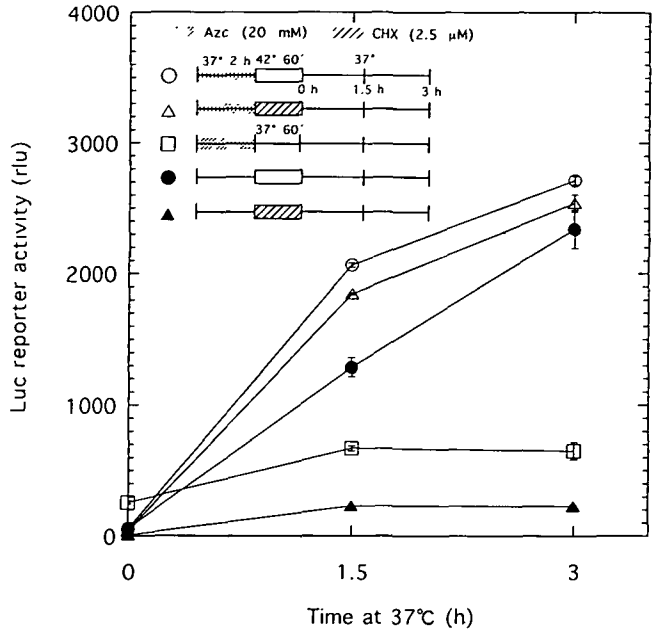

Fig. 5. **No suppression of heat-induced luciferase reporter gene expression by cycloheximide in L-azetidine 2-carboxylic acid (Azc)-incorporated cells.** Cells were incubated at 37°C for 2 h with (○, △, □) or without (●, ▲) Azc (20 mM), washed free of Azc, then heat-shocked at 42°C for 60 min with (△, ▲) or without (○, ●) 2.5 μM cycloheximide (CHX) or incubated at 37°C for 60 min without CHX (□), then washed free of CHX and further incubated in a fresh medium at 37°C for 0, 1.5, or 3 h to express Luc reporter gene.

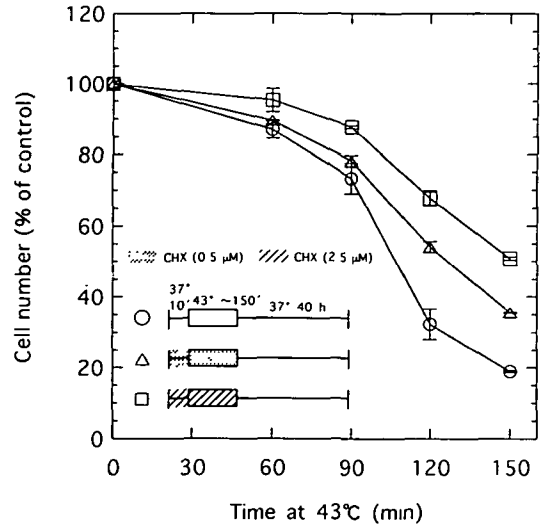

Fig. 6. **Cycloheximide-induced heat protection against cell killing at 43°C.** Cells were treated for 10 min at 37°C with 0.5 μM (△), 2.5 μM (□), or without (○) cycloheximide, heated at 43°C for the indicated times, then washed free of cycloheximide, and cultured for cell growth in a fresh medium at 37°C for 40 h.

The incorporation of the amino acid analog itself induced only a low level of Luc reporter gene expression (□), indicating that Azc acted only as a very weak stressor in terms of inducing hsp gene under the conditions used. These results may be explained by noting that a certain amount of hsp70 is fixed in Azc-incorporated cells, binding

to Azc-incorporated nascent proteins, and free hsp70 increase through CHX inhibition of protein synthesis may be inhibited.

It has also been demonstrated that hsp70 has a protective effect against cell death by heat. Therefore CHX treatment may be expected to modify heat sensitivity of cells through modulating the status of hsp70 by inhibiting the synthesis of nascent proteins to which hsp70 would bind. Cells exposed to heat at 43°C for up to 2.5 h in the presence of CHX (0.5 and 2.5 μM) exhibited heat-resistant cell survival curves depending on the CHX doses compared with the survival curve of untreated cells (Fig. 6), demonstrating the protection from killing by heat.

DISCUSSION

Experiments with mammalian cells provide a compelling argument for the importance of enhanced synthesis of hsp70 in acquired thermotolerance (*7-12*). In the present study, by using our experimental system, we examined if the suppression of heat-induced hsp gene expression by CHX would inhibit the acquisition of thermotolerance. Our data indeed demonstrated clearly a lack of thermotolerance acquisition by the CHX-induced suppression of hsp gene expression and hsp synthesis (Figs. 1, 2, and 3). Quercetin was also reported to inhibit the synthesis of hsps through inhibiting the heat shock factor activation (*24*) and thereby inhibiting the development of thermotolerance in human colon carcinoma cells (*25*). In those experiments, however, cell viability was greatly decreased to about 20% of control by quercetin treatment. Therefore, the inhibition of thermotolerance may have resulted from the toxic effect of pleiotropic actions of the compound, unrelated to the inhibition of hsp synthesis. In contrast, the present study demonstrated that CHX treatment combined with heat shock did not reduce cell viability, except for about a 30% decrease in viable cells due to CHX treatment after heat shock treatment (see cell counts at time zero in Fig. 2). Thus it may be argued that CHX specifically inhibited a process essential for and directly connected with the acquisition of thermotolerance. Some other inhibitors of protein synthesis such as puromycin, anisomycin, and emetine were reported to exhibit the same suppressive effect on hsp gene expression as CHX (*19*).

In addition, CHX treatment between heat shock and heat challenge treatments did not inhibit the acquisition of thermotolerance (Fig. 2). This result contrasts with reports by others that CHX treatment during the recovery incubation period after heat shock treatment or sodium arsenite treatment inhibited the development of thermotolerance (*26, 27*). We analyzed heat shock protein synthesis during the 43°C heat challenge following CHX treatment during the recovery incubation after the first heat shock treatment. We found that hsp synthesis was stimulated in the 43°C heat challenge as well as in the incubation at 37°C (Fig. 3, lanes 4 and 5 *vs.* lane 2). The significantly higher rate of hsp synthesis may reflect stabilization and increased accumulation of hsp gene transcripts by the CHX inhibition of protein synthesis during the recovery incubation, as has also been reported in rat fibroblasts cells (*28*). Under our experimental conditions, it took several minutes for the temperature of cell culture to reach the heat challenge temperature of 43°C from the recovery incubation temperature of 37°C. Therefore, it could be expected that hsp was rapidly synthesized from the accumulated hsp mRNA during the lag period between 37 and 43°C. Several studies have reported that CHX inhibited heat shock-induced hsp synthesis during the recovery incubation but did not block the development of thermotolerance against the subsequent heat challenge at 44 or 45°C in Chinese hamster V79 and HA1 cells (*29, 30*), human colon carcinoma cells (*25*), or rat embryonic fibroblasts cells (*28*). In those studies, however, hsp synthesis in heat challenge incubation was not examined, especially during the lag times of elevating temperature from 37°C to 44 or 45°C. At present, however, we can not exclude the previously proposed alternative mechanism of thermotolerance expression that is independent of induction of hsp synthesis (*30*).

In the present study, we also demonstrated clearly that the CHX-induced suppression of hsp gene expression was dependent on the severity of the heat stress as well as the reduced rate of protein synthesis (Fig. 4); the suppression decreased with increasing heat stress dose and increased with increasing CHX dose. The data indicated that heat-induced hsp gene expression was balanced between a reduced rate of nascent protein synthesis and an increase in heat-induced damage, *e.g.*, amounts of heat-denatured proteins. Baler *et al.* (*19*) previously studied the suppression of hsp gene expression by CHX in HeLa cells, although they did not analyze the kinetics of suppression in detail. They proposed a model of autoregulation of hsp gene expression by levels of free hsp70. In the model an increase (decrease) in free hsp70 may negatively (positively) regulate hsp gene expression, and the level of free hsp70 is increased by a reduction in protein synthesis and decreased by heat stress event. Our kinetic data on stress dose-dependent hsp gene suppression by CHX may be explained well by the model: the negative control of hsp gene expression induced by CHX inhibition of protein synthesis may balance the positive effect caused by a range of heat stress treatment, but may be overcome by the much greater positive effects of severe heat stresses.

It has been reported that in HeLa cells exposed to the proline analog L-azetidine 2-carboxylic acid (Azc), newly synthesized proteins were still bound to hsp70 as long as 2 h after synthesis, since proteins probably containing the proline analog cannot fold correctly and therefore the bound hsp70 is not released (*16*). It may be expected, therefore, that free hsp70 is not increased by CHX treatment and that CHX may not suppress hsp gene expression in Azc-incorporated cells. We obtained the expected results that Luc reporter gene expression was fully activated in the Azc-incorporated cells after heat shock treatment with CHX (Fig. 5), further supporting the model control mechanism of the stress protein gene for which the change in level of free hsp70 may be a crucial factor determining induction of the gene expression. In addition, the result that CHX treatment protected cells from heat damage (Fig. 6) may be related to change in the equilibrium between free and bound levels of hsp70 by the inhibition of synthesis of nascent proteins to which hsp70 binds. Studies have demonstrated the protective effect of hsp70 against thermal stress (*7-12*). Similar protection by CHX or puromycin against heat killing was reported and the protection was not related with the synthesis of particular proteins (*31*).

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