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Effect of Histidine on Histidinol-Induced Heat Protection in Chinese Hamster Ovary Cells

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The possible mechanism for heat protection by the protein synthesis inhibitor histidinol was investigated in CHO cells. Histidinol (HST, 5 mM), an analogue of the essential amino acid L-histidine, added for 2 hr before and during heating at 43°C, protected cells from killing at 43°C. Treatment with HST produced a 600-fold increase in survival from 3×10^{-4} to 1.8×10^{-1} after 2.5 hr at 43°C. Although the cells were washed after HST treatment, substantial protective effect was still observed during heating at 43°C. This protective effect gradually decreased with increased incubation time after the drug treatment. However, the protective effect was *immediately* reduced by treatment with histidine (HIS, 0.25–5 mM) during heating. The amount of reduction was dependent upon HIS concentration: five millimolar HIS *completely* inhibited HST-induced heat protection. Furthermore, protein synthesis which was inhibited by 95% by 5 mM HST, resumed *immediately* with 5 mM HIS treatment. In addition, when cells were labeled during or after HST treatment, neither preferential accumulation of heat shock protein families nor phosphorylation of 28 kDa protein was observed. Therefore, these results suggest that the cessation of protein synthesis itself is one of the events involved in protection.

Many chemical agents are known to alter the heat sensitivity of cells (Fisher et al., 1982; Henle and Warters, 1982; Kim et al., 1984; Kim et al., 1985; Lee and Dewey, 1986; Lee et al., 1987). It has been shown that a good correlation between the inhibition of protein synthesis and heat resistance exists by treatment of heat protectors (Lee and Dewey, 1986; Lee et al., 1987), although the role of protein synthesis inhibition on drug-induced thermal resistance has yet to be clarified. Lee et al. (1990) suggested that heat protection results from an event(s) which is caused by inhibition of protein synthesis. To investigate this possibility, we have used histidinol, which has been shown to inhibit protein synthesis (Scornik, 1983) and protect CHO cells from hyperthermic killing (Lee et al., 1989). Histidinol, an analogue of the essential amino acid L-histidine, is a competitive inhibitor of histidyl-tRNA synthetase (Vaughan and Hansen, 1973). Therefore, histidinol-induced protein synthesis inhibition can be reversed by treatment with excess histidine even in the presence of histidinol. We also examined the protective effect of histidinol compared with that of cycloheximide since the two drugs inhibited protein synthesis by different mechanisms (Vaughan and Hansen, 1973; Stryer, 1981).

Many studies have shown that stress proteins might play a role in thermal resistance (Subjeck et al., 1982; Li and Werb, 1982; Landry et al., 1982; Tomasovic et al., 1983; Li, 1985; Laszlo and Li, 1985; Lee and Dewey, 1987a,b). Since several researchers (Kelley and Schlesinger, 1978; Hightower, 1980; Hightower and White, 1981) have shown that amino acid analogues, e.g., the arginine analogue canavanine, stimulate stress protein synthesis in chicken embryo fibroblast and mammalian cells, we examined the synthesis of stress proteins after treatment with histidinol. We also studied other possibilities, such as the role of phosphorylation of heat shock protein 28 kDa on drug-induced thermal resistance (Landry, 1989; Crete and Landry, 1990). Our data indicated that the inhibition of protein synthesis itself is the event causing protection rather than phosphorylation or synthesis of new stress proteins.

MATERIALS AND METHODS

Cell culture and survival determination

Exponentially growing Chinese hamster ovary (CHO) cells were cultured in McCoy's 5a medium (Gibco) supplemented with 26 mM sodium bicarbonate and 10% heat-inactivated (56°C for 40 min) fetal calf serum. Three days prior to the experiment, 3×10^5 cells were plated into T-75 flasks. The flasks containing cells were kept in a 37°C humidified incubator with a mixture of 95% air and 5% CO_2. For survival determination after various treatments, cells were trypsinized, counted, and plated at appropriate dilutions. X-irradiated feeder cells (25 Gy) were used to maintain

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the plated cell density at 4,000 cells/cm^2 (Highfield et al., 1984). After 1–2 weeks of incubation at 37°C, colonies were stained and counted.

Drug treatment

Histidinol (HST, M.W. 214.1), histidine (HIS, M.W. 209.6), and cycloheximide (CHM, M.W. 281.3) were obtained from Sigma Chemical Co. Medium with drug was prepared 1 day prior to the experiment. The pH was adjusted to 7.4 using 6 N NaOH. Drug treatment was accomplished by aspirating the medium from the cells and replacing it with medium containing drug. The drug treatments were terminated by aspirating the medium containing drug, rinsing twice with Hanks' balanced salt solution (HBSS) and replacement with drug free medium.

Hyperthermic treatment

T-75 flasks were heated by total immersion in a circulating water bath (Heto, Thomas Scientific), which was maintained within ± 0.02°C of the desired temperature.

Determination of incorporation of [^{3}H]-leucine into protein

Cells in 35-mm petri dishes were pulse-labeled at pH 7.4 with 4 μCi/ml [^{3}H] leucine (sp. act. 55 Ci/mmol, ICN) for 15 min at various times (0–20 hr) after HST pretreatment. After the cells were labeled; they were washed thrice with cold HBSS for 3 min and twice with cold 10% TCA for 5 min. TCA-insoluble material was solubilized with 1 ml of 0.25 N NaOH for 1 hr, and 0.5 ml of the solubilized sample was added to 10 ml of Opti-Fluor (Packard). Radioactivity was determined by liquid scintillation counting. Counting efficiency was 54%. The protein content in the dishes was determined by method of Lowry et al. (1951). The protein synthesis in untreated cells cultured in logarthmic phase at 37°C was always used as the control.

Labeling and gel electrophoresis

To investigate the synthesis of new proteins, cells were labeled with 10 μCi/ml [^{35}S]-methionine (sp. act. 1196 Ci/mmole, ICN) for 3 to 26 hr. After labeling, cells were washed three times with ice-cold HBSS and dissolved in sample buffer. Extracts were further boiled for 5–8 min, and equal amounts of proteins (42 μg) were analyzed on a 10% polyacrylamide gel, in the buffer system of Laemmli (1970). To study the phosphorylation of proteins, cells are labeled with 25 μCi/ml [^{32}P]-H_3PO_4 (carrier free, 285 Ci/mg, ICN) in phosphate-free medium for 2 to 2.5 hr. Cells were rinsed and lysed as described above. Extracts were boiled and equal amounts of radioactivity (94,000 cpm) were analyzed on a 7.5–20% linear gradient SDS polyacrylamide gels (Walker, 1984). For autoradiography, gels were fixed in 30% trichloracetic acid (TCA) for 30 min, stained with Coomassie Brilliant Blue R in 3.5% perchloric acid overnight, destained 2× in 7% acetic acid, rinsed with water, dried with a slab gel dryer (Model 483, Bio-Rad, Richmond, CA), and placed into a cardboard cassette for [^{35}S]-labeled protein or a stainless steel cassette with intensifying screen for [^{32}P]-labeled protein. Gels were autoradiographed on Kodak SB-5 X-ray film. The cardboard cassette or the stainless steel cassette was placed at the room temperature for 1 day or in the −70°C freezer for 2 days, respectively.

To identify heat shock proteins, cells were labeled with 20 μCi/ml [^{3}H] amino acid mixture (sp. act. 215 mCi/mg, ICN) for 12 hr at 37°C after heating at 45.5°C for 10 min. Cells were rinsed and lysed as described above. A volume of lysate giving an equal number of TCA-insoluble cpm (1.3×10^5 cpm) was applied to a gradient polyacrylamide gel. For fluorography, gels were fixed in fixing solution (7% acetic acid, 20% methanol) for 1 hr, and dehydrated by washing for 15 min in each of 25% acetic acid, 50% acetic acid, and glacial acetic acid consecutively. After fixation, the gel was placed in 125 ml of PPO solution (20% (w/v) 2,5-diphenyloxazole in glacial acetic acid) for overnight. The PPO solution was removed, the gel was shaken gently for 2 hr in distilled water, dried and placed into a cassette with Kodak SB-5 X-ray film. The cassette was placed in the −70°C freezer for 8 days. After exposure, fluorograph film was developed with Kodak GBX developer and fixed with Kodak GBX fixer.

RESULTS

Induction of heat resistance by pretreatment with histidinol

Figure 1 shows the effect of histidinol (HST) pretreatment on cell survival at 43°C. Cells were exposed to either 5 mM HST (A) or 50 mM HST (B) for 2 hr and rinsed with HBSS, and then incubated at 37°C for various times (0–26 hr) before heating. Figure 1A shows that there was a 3,000-fold increase in survival from 1×10^{-5} to 3×10^{-2} after 3 hr at 43°C by pretreatment with 5 mM HST (○). The amount of heat resistance decreased with increased incubation time after pretreatment with the drug. Figure 1B also shows induction of heat resistance by pretreatment with 50 mM HST. However, maximum resistance, i.e., a 25,000-fold increase in survival from 1×10^{-5} to 2.5×10^{-1} after 3 hr at 43°C, required 3 hr incubation time after pretreatment with the drug. The amount of heat resistance also decreased with increased incubation time.

Profiles of protein synthesis after histidinol pretreatment

To examine if there was any correlation between induction of heat resistance by pretreatment with HST and the synthesis of heat shock proteins, the cells were labeled with [^{35}S]-methionine for various times (3–26 hr) after the drug pretreatment. Figure 2 shows the autoradiograph of an SDS-polyacrylamide slab gel of [^{35}S] methionine-labeled proteins. HST (5–50 mM) inhibited protein synthesis by 95–98% (data not shown). Gel lanes 3h of Figure 2 show that protein synthesis was still drastically reduced, although the drug treatments were terminated by aspirating the medium containing drug, rinsing twice with HBSS and replacing with drug free medium. Figure 2 also shows that protein synthesis was recovered gradually with increased incubation time. During the recovery period, the profiles of newly synthesized protein were observed in Figure 2. Compared to the heated cells (lane H of Fig. 2), there was no significant synthesis of heat shock proteins (70, 87, and 110 kDa) after the drug pretreat-

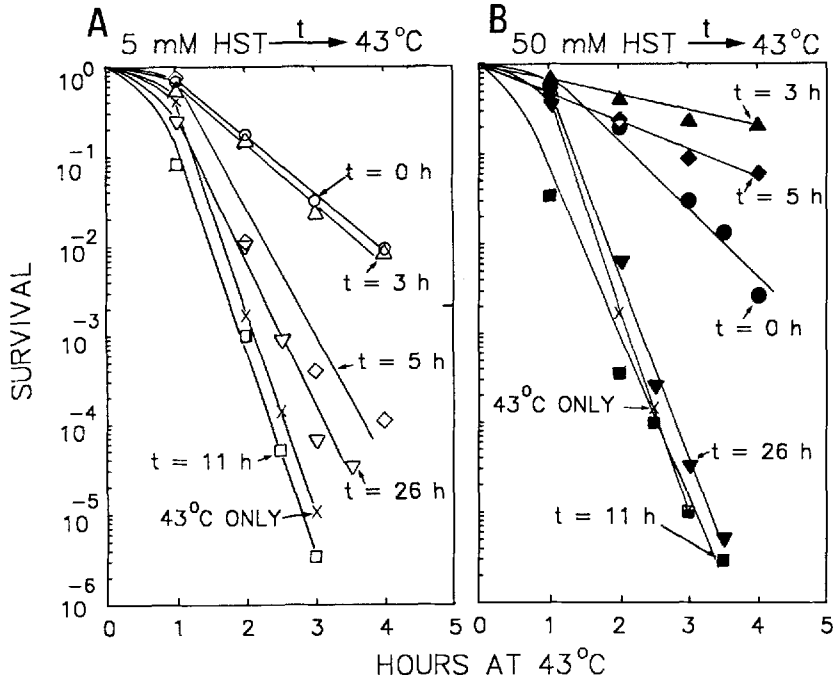

Fig. 1. Induction of heat resistance by pretreatment with 5 mM histidinol (**A**) or 50 mM histidinol (**B**). Cells were treated with histidinol (HST) for 2 hr. After pretreatment, cells were rinsed and then incubated for various times (0–26 hr) before heating at 43°C. The times next to each survival curve correspond to incubation times after histidinol pretreatment. x = survival curve of control cells heated at 43°C.

ment. This observation was confirmed from an autoradiograph of the gel on which lysates from equal number of cpm for each lane were loaded (data not shown). However, during an 11-hr recovery period, 32 kDa protein was preferentially synthesized (Fig. 2), but as shown in Figure 1, HST-induced heat resistance disappeared during this recovery period. Therefore, this protein is unlikely to be involved in drug-induced heat resistance.

Effect of histidine on recovery of protein synthesis after histidinol pretreatment

The kinetics of recovery of protein synthesis and the effect of histidine (HIS: 5 or 50 mM) on the recovery of protein synthesis after HST (5 or 50 mM) pretreatment were systematically studied (Fig. 3). Protein synthesis was still inhibited by 91% or 96% immediately after 5 or 50 mM HST pretreatment, respectively. This suggests that residual amount of drug remained in the cell even after rinsing the cell with HBSS. The addition of 5 or 50 mM HIS resulted in an immediate increase of the rate of protein synthesis by 60% or 37%, respectively, of that of control. Figure 3 also shows that the rate of protein synthesis gradually returned to control rate within 10 hr or 12 hr for 5 mM or 50 mM HST pretreatment, respectively. HIS treatment accelerated the recovery of protein synthesis. However, the maximum protein synthesis of 50 mM HIS treated cells was 80% of that of untreated control cells, because this concentration of the drug itself inhibited protein synthesis by 20% (data not shown).

Effect of histidine on histidinol-induced heat protection

The effect of HIS on HST-induced heat protection is illustrated clearly in Figures 4 and 5. CHO cells were protected from killing at 43°C when the cells were treated with HST (5 mM) 2 hr before heating and during heating (Figs. 4, 5). Figure 4 shows that there was a 600-fold increase in survival from 3×10^{-4} to 1.8×10^{-1} after 2.5 hr at 43°C by treatment with 5 mM HST. However, the protective effect of HST was reduced when HIS was added immediately before heating. HIS treatment was accomplished by aspirating the medium containing 5 mM HST from the cells and immediately replacing it with medium containing various concentrations of HIS (0.25–5 mM) and 5 mM HST. The amount of reduction was dependent on the concentration of HIS. Figure 5 also shows that protective effect of HST (5 mM) was reduced when HIS (5 mM) was added at the same time with HST or added only during heating after pretreatment of HST. There is little sensitization of heat killing by treatment with HIS (5 mM) during heating.

Common mode of action of cycloheximide and histidinol on heat protection

Figure 6 shows a common mode of action of cycloheximide (CHM: 10 μg/ml) and histidinol (HST: 5 mM) on heat protection. There was a 500–600-fold increase in survival after 2.5 hr at 43°C by treatment with CHM and HST. To get heat protection by CHM and HST, the drug was added before heating and left on during heat-

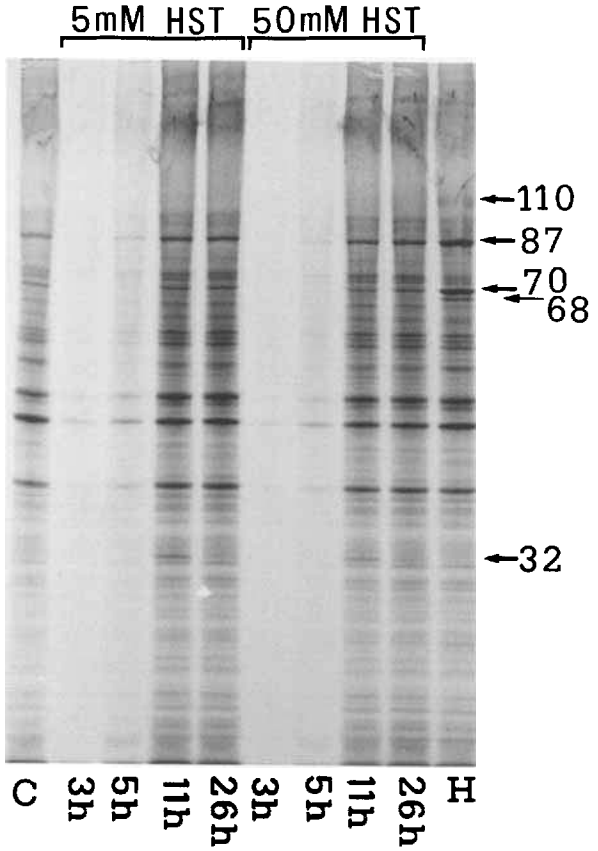

Fig. 2. Autoradiographs of SDS-polyacrylamide slab gels of [^{35}S]-methionine-labeled proteins. Cells were treated with HST (5 mM or 50 mM) for 2 hr. After pretreatment, cells were rinsed and continuously labeled with 10 μCi/ml [^{35}S]-methionine for various periods (3–26 hr). Lysates from an approximately equal amount of protein (42 μg) were analyzed. **C:** Untreated cells. **H:** Cells were heated at 45.5°C for 10 min and labeled for 19 hr. The times below each gel lane correspond to incubation times after histidinol pretreatment. Molecular weight ($\times 10^{-3}$) shown at the right.

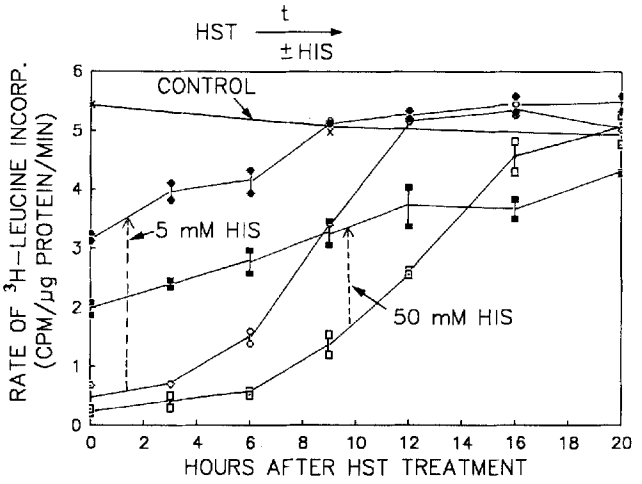

Fig. 3. Effect of histidine (HIS) on recovery of protein synthesis in HST pretreated cells. Cells were treated with 5 mM (○,●) or 50 mM (□,■) HST for 2 hr. After HST treatment, cells were rinsed with HBSS and then incubated at 37°C for various times (0–20 hr) with (●,■) or without (○,□) excess amount of HIS (5 or 50 mM). Dashed arrows designate cells incubated at 37°C with 5 mM (●) or 50 mM (■) HIS. After various periods of incubation, cells were pulse-labeled with 4 μCi/ml [^{3}H]-leucine for 15 min. X = protein synthesis of untreated control cells growing in logarithmic phase.

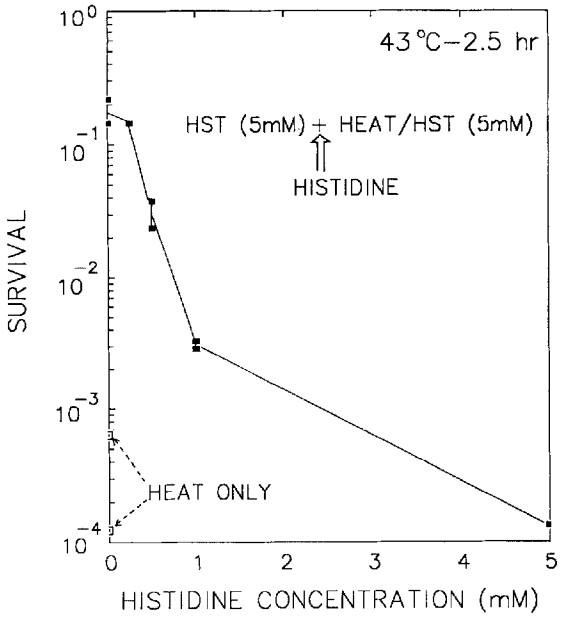

Fig. 4. Effect of excess amount of histidine on HST-induced heat protection. Cells were exposed to 5 mM HST for 2 hr before and a combination of various excess amount of HIS (0–5 mM) and 5 mM HST during heating at 43°C for 2.5 hr (■). There was a 600-fold increase in survival when HST (5 mM) was added before and during heat. This protective effect was reduced by adding HIS (0.25–5 mM) immediately before heating. □ = survival level of control heated cells.

ing (B and C in Fig. 6). There was little or no protection when CHM was added only before heating (E in Fig. 6) or HST was added only during heating (F in Fig. 6). However, heat protection was obtained by treatment with CHM before heating and HST during heating (D in Fig. 6). Therefore, these results suggest that there is a common mode of action for heat protection for CHM and HST, and since both inhibit protein synthesis, the mode might involve protein synthesis.

Effect of histidine on cycloheximide-induced heat protection and heat- or sodium arsenite-induced thermotolerance

To examine if there might be an ubiquitous effect of HIS on drug-induced heat protection or thermotolerance, cells were treated with cycloheximide, heat, or sodium arsenite. To induce heat protection by cycloheximide (CHM: 10 μg/ml), the drug was added 2 hr before heating and left on during heating at 43°C for 2.5 hr (C in Fig. 7). To induce thermotolerance, cells were treated either for 10 min at 45.5°C or for 1 hr with 100 μM sodium arsenite (ARS), and incubated at 37°C for 12 hr or 6 hr, respectively, before heating at 43°C for 2.5 hr (E and G in Fig. 7). Figure 7 shows little or no effect of HIS (5 mM) on CHM-induced heat protection

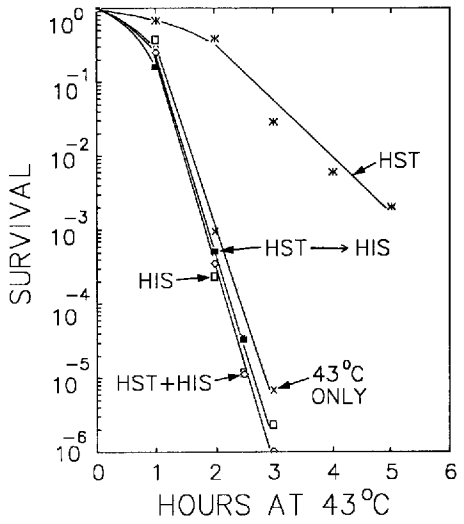

Fig. 5. Effect of HIS on HST-induced heat protection. Cells were treated with 5 mM HST (*, ■) or a combination of 5 mM HST and 5 mM HIS (○) for 2 hr and then heated at 43°C with 5 mM HST (*), 5 mM HIS (■), or a combination of 5 mM HST and 5 mM HIS (○). X = survival curve of untreated control cells heated at 43°C, ⊐ = survival curve of cells treated with 5 mM HIS only during heating.

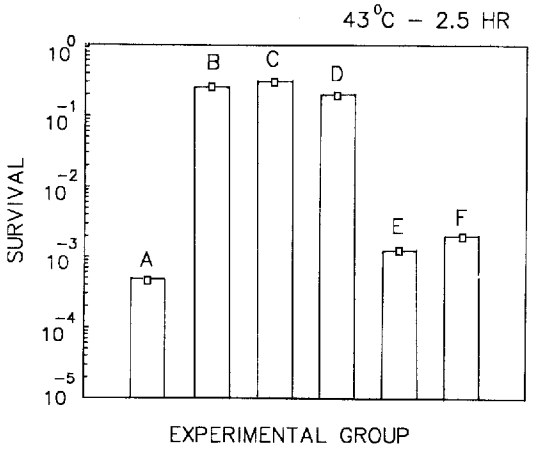

Fig. 6. Effect of cycloheximide (CHM: 10 μg/ml) or HST (5 mM) on cell survival at 43°C for 2.5 hr. Protection from hyperthermic killing was obtained by treatment with CHM **(B)** or HST **(C)** 2 hr before and during heat. Heat protection was not modified when CHM was added 2 hr before heat and HST was added during heat **(D)**. There was little or no protection when CHM was added only before heat **(E)** or HST was added only during heat **(F)**. **A:** Survival level of control heated cells.

and heat- or ARS-induced thermotolerance when the drug was added during heating (D, F, and H in Fig. 7).

Effect of HST and/or HIS on phosphorylation of protein before and during heating

Since phosphorylation of 28 kDa heat shock protein (HSP 28) after CHM treatment might play a role in heat protection (Landry, 1989), the phosphorylation of 28 kDa heat shock protein was investigated after treatment with HST at either 37°C or 43°C (Fig. 8B). A fluorograph of the gel in Figure 8A shows hyperthermia-induced five major heat shock proteins (HSPs: Mr 110,000, 87,000, 70,000, 28,000, and 8,500). Figure 8B shows heat-induced alterations in protein phosphorylation. Figure 8B clearly shows that the 28 kDa protein was phosphorylated during heating at 43°C for 2.5 hr. Nevertheless, the phosphorylation of this protein did not occur before heating or was not enhanced during heating by treatment with HST or the combination of HST and HIS. However, an increased labeling of histone, H2B was observed during heating with treatment with HST.

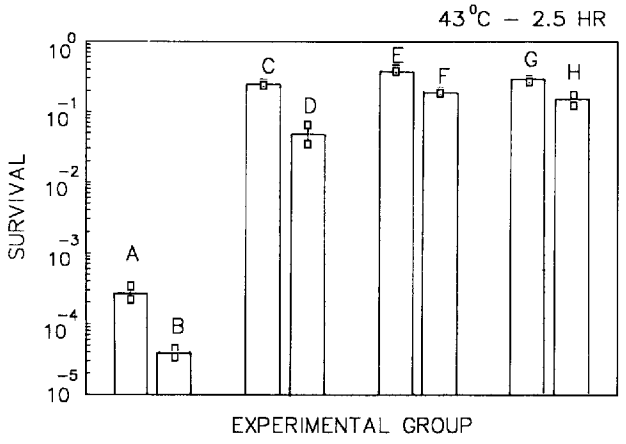

Fig. 7. Effect of HIS (5 mM) on heat protection by cycloheximide (CHM: 10 μg/ml) or thermotolerance. **A:** Survival level of control cells heated at 43°C for 2.5 hr. **B:** Cells were heated with HIS. **C:** Heat protection by CHM was observed when cells were exposed to CHM for 2 hr before and left on during heating at 43°C for 2.5 hr. **D:** CHM-induced heat protection was little affected by treatment of HIS and CHM during heating. **E:** Development of thermotolerance was observed when cells were first heated at 45.5°C for 10 min, incubated at 37°C for 12 hr, and then challenged at 43°C for 2.5 hr. **F:** Heat-induced thermotolerance was not affected by treatment of HIS during the second heating. **G:** Development of thermotolerance was observed when cells were exposed to sodium arsenite (ARS: 100 μM) at 37°C for 1 hr and then incubated at 37°C for 6 hr before heating at 43°C for 2.5 hr. **H:** ARS-induced thermotolerance was not affected by treatment with HIS during heating.

DISCUSSION

Our data clearly demonstrated (Figs. 4, 5) that histidinol-induced heat protection can be reduced by treatment with histidine, even in the presence of histidinol. The degree of thermal resistance reduction depended upon the concentration of histidine (Fig. 4). Furthermore, there was little or no sensitization by treatment with histidine alone (Figs. 5 and 7). These data suggest that histidine interferes with the process of histidinol-induced heat protection.

Both histidinol and cycloheximide, two drugs which inhibit protein synthesis by different mechanisms, protect cells from heat killing. Histidinol inhibits protein synthesis by inactivating histidyl-tRNA synthetase (Vaughan and Hansen, 1973). Cycloheximide inhibits ribosomal protein synthesis by inhibiting the peptidyl transferase activity (Stryer, 1981). Histidine reversed histidinol-induced protein synthesis inhibition at 37°C (Fig. 3) as well as at 43°C but had no effect on cycloheximide-induced protein synthesis inhibition (data not shown). Our experiments (Fig. 6) illustrated that

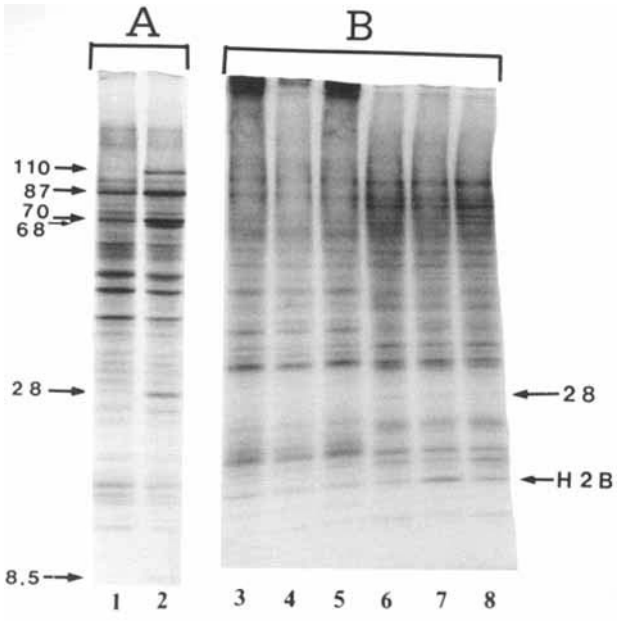

Fig. 8. Effect of HST and/or HIS on phosphorylation of protein. **A:** Fluorographs of gradient SDS polyacrylamide slab gel of [^{3}H]-amino acid mixture labeled proteins. Cells were either heated at 45.5°C for 10 min (**2**) or unheated (**1**), and labeled for 12 hr at 37°C. **B:** Autoradiographs of a gradient SDS polyacrylamide slab gel of [^{32}P]-H_3PO_4 labeled proteins. Cells were labeled for 2 hr at 37°C with 5 mM HST (**4**), 5 mM HST and 5 mM HIS (**5**), or without the drug (**3**). Cells were pretreated for 2 hr at 37°C with 5 mM HST (7,8) or without the drug (**6**). After pretreatment, cells were heated for 2.5 hr at 43°C with 5 mM HST (**7**), 5 mM HST and 5 mM HIS (**8**), or without the drug (**6**). In this case, cells were labeled during heating. Molecular weight ($\times 10^{-3}$) of heat shock protein are shown at the left.

heat protection by treatment with these two drugs may occur through a common mechanism. Furthermore, cycloheximide-induced thermal resistance was not affected by treatment with histidine (Fig. 7). These results indicate that heat protection probably results from an event(s) that is caused by inhibition of protein synthesis.

Results from Figures 2 and 8 indicate that neither preferential synthesis of major heat shock protein families (70, 87, 110 kDa) nor modification of 28 kDa heat shock protein involves in the mechanism of histidinol-induced heat protection. Histidinol, like cycloheximide (Makino et al., 1984; Forsdyke, 1984; Ishihara et al., 1984; Elder et al., 1984), may stimulate the transcription of certain genes by decreasing the concentration of repressor. Preferential synthesis of a 32 kDa protein during an 11-hr recovery period is interesting but can not be correlated with heat protection. Because thermal resistance was not observed during this period (Fig. 1). Clearly, histidinol neither lead to phosphorylation of 28 kDa protein before heating, nor increased the heat-induced phosphorylation of this protein (Fig. 8). Furthermore, histidine did not affect the level of phosphorylation of the protein (Fig. 8). Heat-induced changes in the level of phosphorylation of 28 kDa protein was consistent with the observation from Arrigo and Welch (1987) and Landry et al. (1988). Landry (1989) suggested the possible role of heat-induced phosphorylation of HSP 28 on development of thermoresistance. However, we failed to demonstrate a correlation between histidinol-induced heat protection and enhancement of HSP 28 phosphorylation.

At the present time, only speculations can be made concerning mechanisms. First, since histidinol-induced heat protection and protein synthesis inhibition can be reversed by treatment with excess histidine even in the presence of histidinol, it suggests that the inhibition of protein synthesis itself is the major event causing protection. To obtain protective effect, it is a prerequisite to add histidinol to inhibit protein synthesis before heating. This inhibition of protein synthesis may be important in eliminating nascent polypeptides which may be among the most thermolabile proteins in the cells. Second, histidinol may not only eliminate thermolabile cellular proteins but also protect them from denaturation. For instance, glycerol as a heat protector stabilizes thermolabile proteins (Lepock et al., 1990) and protects cells from heat killing (Henle and Warters, 1982). Recent work from Hightower's laboratory (Edington et al., 1989) showed that the presence of D_2O or glycerol during a pre-heating that would normally induce stress proteins prevented HSP induction during recovery period. Since the presence of abnormal proteins stimulates the synthesis of a set of stress proteins (Hightower, 1980; Goff and Goldberg, 1985; Ananthan et al., 1986), this observation suggested that glycerol and D_2O protected heat-sensitive proteins from thermal denaturation. Obviously, further studies at the cellular and molecular levels are necessary to understand the mechanism of heat protection by protectors.

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