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A mechanism of glycine and alanine cytoprotective action: Stimulation of stress-induced HSP70 mRNA

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A mechanism of glycine and alanine cytoprotective action: Stimulation of stress-induced HSP70 mRNA. Studies done both in vitro and in vivo have shown that glycine and alanine protect kidney cells from stress injury. However, the mechanism(s) of this cytoprotection is unknown. Our aim was to test the hypothesis that the cytoprotective action is in part due to stimulation of gene(s) expression encoding stress protein synthesis. Experiments were carried out using heat shock as a model for stress in the opossum kidney cell line (OK cells). The induction of HSP70 mRNA was evaluated in cell monolayers exposed to 45°C for 15 minutes followed by a recovery period at 37°C for either 0.5, 1, 2, 3, 4, 6 or 24 hours. The results demonstrate that the maximum level of HSP70 mRNA occurred at ≈ three hours after heat treatment. Although the mRNA levels declined thereafter, appreciable amounts were still seen even 24 hours after heat-shock. To examine the effect of glycine or alanine on HSP70 mRNA levels and on the synthesis of stress protein, cultures were preincubated for 30 minutes with Krebs-Henseleit buffer, pH 7.4, supplemented with either 1, 2, 5 or 10 mM glycine or alanine, or with no added amino acids. Comparative studies were performed with 10 mM glutamate, aspartate, arginine or leucine. Following preincubation, cultures were heat-shocked (45°C for 15 min) and then reincubated at 37°C for three hours. Both glycine and alanine enhanced the level of HSP70 mRNA and the synthesis of 72,73 kDa stress proteins, but neither amino acid induced HSP70 mRNA without concomitant heat treatment. Glutamate, aspartate, leucine and arginine had no enhancing effect, however, their inclusion in the incubation medium induced heat-shock-like response without heat treatment. The increased level of HSP70 mRNA and the synthesis of stress protein in the presence of glycine or alanine were associated with decreased cellular LDH release, suggesting greater thermotolerance of the cultured cells. Intracellular ATP levels declined following heat shock in all experiments. Supplementation of the medium with glycine or alanine did not alter this stress-induced reduction of intracellular ATP, supporting a previous suggestion that the cytoprotective action of glycine and alanine is independent of cellular ATP levels. The current data suggest a functional role for glycine and alanine in the stimulation of gene(s) expression encoding for stress protein(s) synthesis, and in protecting cells against stress damage. This characteristic is not shared by other amino acids, such as glutamate, asparate, arginine or leucine.

Studies from various laboratories have demonstrated that provision of glycine and alanine protects renal tubule cells from otherwise lethal injury of diverse cause including ischemia, anoxia, hypoxia, ouabain toxicity and chemotherapy-induced nephrotoxicity [1–6]. However, the mechanism(s) of this protection is unknown. In the current study we have explored the hypothesis that glycine and alanine stimulate the expression of the heat-shock genes encoding HSP70 mRNA and, thus, enhance the synthesis of stress proteins as a part of a defense mechanism against various insults.

The induction of stress or heat-shock proteins (HSPs), which has been demonstrated both in vivo and in vitro, constitutes an important cellular defense mechanism that may confer tolerance against various insults, such as, increased temperature, anoxia, ischemia, hypoxia, nutrient deprivation, drugs, metal ion toxicity and cancer therapy [7–11]. Transcription of a specific set of heat shock gene(s) which occurs rapidly in response to stress [8–10] results in the synthesis of HSPs [8, 9]. The most common class of stress protein, the HSPs 70 family, is encountered in almost all cells, where it is abundant in both the cytoplasmic and nuclear compartments [7–11].

The present report describes the use of heat shock as a model for stress to activate HSP70 mRNA gene expression in the renal epithelial cell line derived from the kidney of the American opossum (OK cells). This cell line has been widely used in studies of renal physiology, biochemistry and transport [12]. The current studies demonstrate that both glycine and alanine stimulate the heat-activated transcription of HSP70 mRNA genes with augmentation of stress protein synthesis and of the thermotolerance of OK cells in culture.

Methods

Materials

The OK cell line used in this study, passage 73, was the gift of Dr. Joseph Handler. A mouse HSP68 cDNA probe (PMP 6009) was a gift from Dr. Larry A. Moran [13], University of Toronto. Anti-HSP 72,73 mouse, IgG_1, monoclonal antibody (MAb) and purified 72,73 kDa stress protein were purchased from Stress Gen Biotechnologies Corp., Canada. The culture medium was purchased from Flow Laboratories (McLean, Virginia, USA). All other reagents and enzymes (Sigma Chemical Co., St. Louis, Missouri, USA) were of the highest available grade.

Methods

Experiments were performed using OK cells between passages 79-82. Cells were grown in T75 flasks with α-Minimal Eagle's Medium supplemented with 4 mM glutamine and 3% serum (complete α-MEM). Transfer of cells to new flasks was

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carried out as previously described for NRK cells [14]. Cells were incubated at 37°C under compressed air: CO_2 (95%:5%). At the point of confluence (that is, 6 to 7 days) and 24 hours prior to onset of the experiments, the cells were re-fed with complete α-MEM (steady-state medium).

Time course of HSP70 mRNA induction

The first series of experiments was designed to delineate the time course and the relative magnitude of gene expression for HSP70 mRNA. To this end, the steady state medium from cells grown in T75 flasks was removed and replaced with fresh, complete α-MEM. These flasks were exposed to heat-shock in a water bath at 45°C for 15 minutes, and then incubated at 37°C in air: CO_2 (95:5%) for the times indicated (**Results**) that is, between 0.5 to 24 hours.

Effect of glycine and alanine on HSP70 mRNA induction

Following the demonstration that HSP70 mRNA was expressed in OK cells, a second series of experiments was designed to explore the effects of glycine and alanine on the HSP70 mRNA level and the synthesis of stress proteins. The steady state medium was removed from the flasks containing the cell monolayer and was replaced with 15 ml of Krebs-Henseleit buffer pH 7.4 (KHB), pregassed with 95% O_2:5% CO_2 and supplemented with either glycine or alanine at a concentration of 2, 5 or 10 mM. In addition, a separate series of experiments was carried out with 10 mM glycine or alanine plus 100 μg/ml cycloheximide (CHX), an inhibitor of protein synthesis [16]. Cell monolayers were preincubated for 30 minutes at 37°C, and then exposed to 45°C for 15 minutes and reincubated at 37°C for three hours without a change of medium. Comparative experiments were carried out in the presence of 10 mM glutamate, aspartate, leucine or arginine.

HSP70 mRNA induction—Relationship to ATP levels

Since proteins of the HSP70 family have been shown to bind ATP [15–17], transcription and translation of HSP70 genes should be associated with diminished free intracellular [ATP]. Furthermore, previous studies have indicated that the cytoprotective action of glycine and alanine is independent of cellular ATP level [1, 6]. To further explore the relationship between cell injury, the induction of HSP70 and cellular ATP level, experiments were carried out to determine the ATP level following preincubation with various concentrations of glycine or alanine as indicated above, and then exposed to 45°C for 15 minutes and reincubation at 37°C for three hours. A separate series of experiments was carried out in the presence of 100 μg/ml CHX as indicated above. At the end of the three hour incubation the medium was removed and the cell monolayers were washed three times with cold phosphate-buffered saline (PBS). Cell lysis was accomplished by addition of 5 ml of cold 5% perchloric acid to each T75 flask. The cell monolayers were scraped with a rubber spatula, and flasks were frozen and thawed to assure complete cell lysis. After neutralization with KOH, the supernatants were utilized for determinations of ATP and intracellular amino acid concentrations. ATP was assayed as previously described [18] and amino acids were determined using HPLC [19, 20].

HSP70 mRNA induction—Relationship to thermotolerance

To delineate a possible relationship between the synthesis of HSP70 family and the thermotolerance of OK cells in the presence and absence of either glycine or alanine, an additional series of experiments was performed to determine the percent efflux of lactate dehydrogenase (% LDH efflux) into the incubation medium. Cellular LDH release has been closely correlated to cell injury [1, 6]. OK cells were subjected to heat shock after preincubation for 30 minutes in the presence and absence of various concentrations of amino acids and CHX, as described above. Following three hours recovery at 37°C, 200 μl of medium was removed for measurement of LDH activity. Cell lysis was achieved by addition of 1% Triton-X100 (final concentration) and the monolayer was scraped and transferred to test tubes. The medium and the cell lysate were centrifuged and 200 μl of the resulting supernatant was utilized for determination of LDH activity, as previously described [21]. Percent LDH efflux was calculated for each experiment as: LDH activity of medium/total LDH activity (media + cell) × 100. Incubation of OK cells with KHB, without heat shock, served as a control in experiments involving ATP levels and % LDH efflux.

Preparation of RNA and northern blot analysis

Total RNA was isolated from OK cells according to the procedure of Chomczynski and Sacchi [22]. Northern blotting was carried out as previously described [23]. Briefly, RNA samples (15 μg/lane) were separated by electrophoresis on 1% agarose formaldehyde gels (containing 0.5 μg/ml ethidium bromide). After electrophoresis, the ultraviolet light-exposed gel was photographed to locate the 18S and 28S messenger RNA bands. Following blotting of the RNA to nitrocellulose paper and prehybridization, hybridization was performed with a randomly primed ^{32}P-cDNA probe. The blots were washed three times at room temperature with 2× SSC (1× SSC = 0.15 M NaCl, 0.015 M Na-citrate, pH 7.01) containing 0.1% (wt/vol) SDS and then washed twice (30 min each) at 65°C in a solution with the same composition. The vacuum dried nitrocellulose blots were then exposed to Kodak XAR5 film at −70°C for the time indicated in the Figure legends. HSP70 mRNA levels (radioactivity of the blots) were quantitated by scanning densitometry (Phosphor Imager, Molecular Dynamics).

SDS-PAGE and immunoblot analysis

Cell pellets were solubilized in 100 μl solubilizer mixture containing 2% CHAPS (Bio-Rad) and 0.2 mM phenylmethylsulfonyl fluoride (PMSF). For Western blotting, protein samples (80 μg/lane) were separated using a Bio-Rad protein IIXi cell and 7.5 to 15% acrylamide-PDA (piperazine diacrylamide) gel prepared with XP077 exponential gradient maker (Hoefer Scientific). After completion of electrophoresis run, proteins were transferred from the gel onto a PVDF membrane (Bio-Rad), using a Bio-Rad semi-dry transblot apparatus and Bjerrum and Schafer-Nielson transfer buffer, pH 9.02 [24]. Upon completion of the transfer, the membrane was blocked overnight at 4°C using Gibco BRL blocking solution (cat #99525A) in Tris-buffered saline (TBS) pH 7.5, and then washed and incubated for 30 minutes with primary antibody solution consisting of 0.3 μg/ml anti-HSP 72,73 MAb, IgG_1, in TBS containing blocker

and supplemented with 0.05% Tween 20 (TBST). The membrane was washed twice and then incubated for 30 minutes with biotinylated goat anti-mouse, IgG alkaline phosphatase conjugate (Gibco BRL). After washing the membrane as above, an additional incubation for 30 minutes with streptavidin-alkaline phosphatase (Gibco BRL) was performed for signal amplification. Finally, the membrane was washed twice in TBS and incubated with alkaline phosphatase color development system (Bio-Rad Kit, Cat. #170-6432).

Results

Time course of HSP70 mRNA transcription

Figure 1 illustrates the levels of HSP70 mRNA transcripts following exposure of OK cells to heat and reincubation at physiologic temperature (37°C) for up to 24 hours. The data demonstrate that the level of inducible heat-shock mRNA is time dependent, being apparent at 30 minutes, and reaching maximal message at approximately three hours following heat shock (Figs. 1B and 1C). Although previous studies have shown rapid decrease in levels of HSP70 mRNA [9], the current observations indicate significant expression even at 24 hours following a thermal stress. This observation may be related to the observation that transcription of HSP70 mRNA is induced more slowly and is more sustained in cultured cells [9].

Two distinct mRNA transcripts were recognized by the cDNA probe. A minor transcript appears at approximately 3.2 kb and a major transcript at approximately 2.8 kb. It is clear that the magnitude of both transcripts varies with the duration at physiologic temperature following heat shock. In our parallel studies using a neuronal cell line and the same cDNA probe only a single transcript at 2.8 kb was observed (manuscript in preparation). Hence, the transcript at 3.2 kb may be specific to OK cells. Similarly, previous studies in various tissues of heat stressed rats have demonstrated three distinct HSP70 mRNA species at 2.3 kb, 2.8 kb and 3.1 kb using a cDNA probe isolated from a Chinese hamster ovary cell line [25].

Stimulation of HSP70 mRNA induction by glycine or alanine

Figure 2B depicts the effect of glycine and alanine on the transcription of HSP70 mRNA. There was little effect on the level of the HSP70 mRNA in the presence of 5 mM alanine or glycine in the incubation medium compared with control without added amino acid (Fig. 2). Similarly, addition of 1 mM or 2 mM glycine or alanine as well as 10 mM of glutamate or aspartate had little effect on HSP70 mRNA level compared with post-heat-shock in KHB (data not shown). However, in the presence of 10 mM glycine there was approximately a 2.5-fold increase in the level of HSP70 mRNA three hours following heat shock. Similarly, addition of 10 mM alanine sharply increased HSP70 mRNA levels (Figs. 2B and 2C). Control flasks containing 10 mM glycine or alanine, without heat-shock, did not induce the HSP70 mRNA (Fig. 2B). Therefore, heat shock itself is the signal activating a heat-shock factor (HSF) and, hence, the transcription of HSP70 mRNA in OK cells. Glycine and alanine support and stimulate the induction of HSP70 mRNA. Similarly, previous studies with cultured Drosophila K_c cells [26] and our studies with cultured OK cells [27] have demonstrated that glutamine enhances the level of HSP70 mRNA following thermal stress.

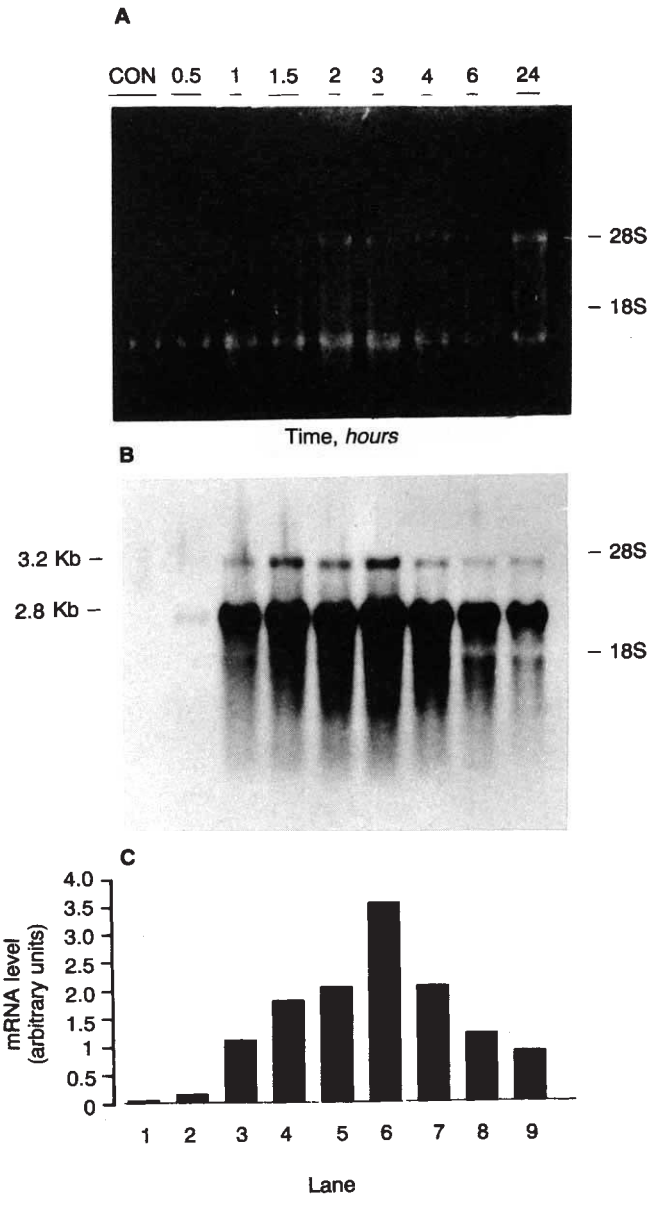

Fig. 1. *Time course of HSP70 mRNA induction.* Flasks containing a monolayer of OK cells in fresh complete α-MEM (described in **Methods**) were subjected to physiologic temperature (37°C, control; Lane 1), or heat-shock (45° for 15 min) and then reincubated at 37°C for the times indicated across the top of the photograph (Lanes 2–9). **A.** A photograph of the ultraviolet exposed ethidium bromide staining of RNA (15 μg/lane), electrophoretically separated by 1% agarose gel electrophoresis. **B.** The radiograph of the Northern blot of the gel in Panel A, hybridized with an HSP70 ^{32}P-cDNA probe. After washing, the blot was exposed to Kodak XAR5 film for 4 hours. **C.** The level of HSP70 mRNA (transcript 2.8 Kb) of the autoradiograph in panel B, quantitated by scanning densitometry using a Phosphor Imager (Molecular Dynamics).

The stimulation of HSP70 mRNA transcription was also associated with enhanced synthesis of stress proteins. The immunoblot of Figure 3 demonstrates an increased production of 72,73 kDa protein in the presence of glycine or alanine. The formation of stress-induced proteins (72,73 kDa) was enhanced with increased levels of those amino acid from 2 mM to 10 mM

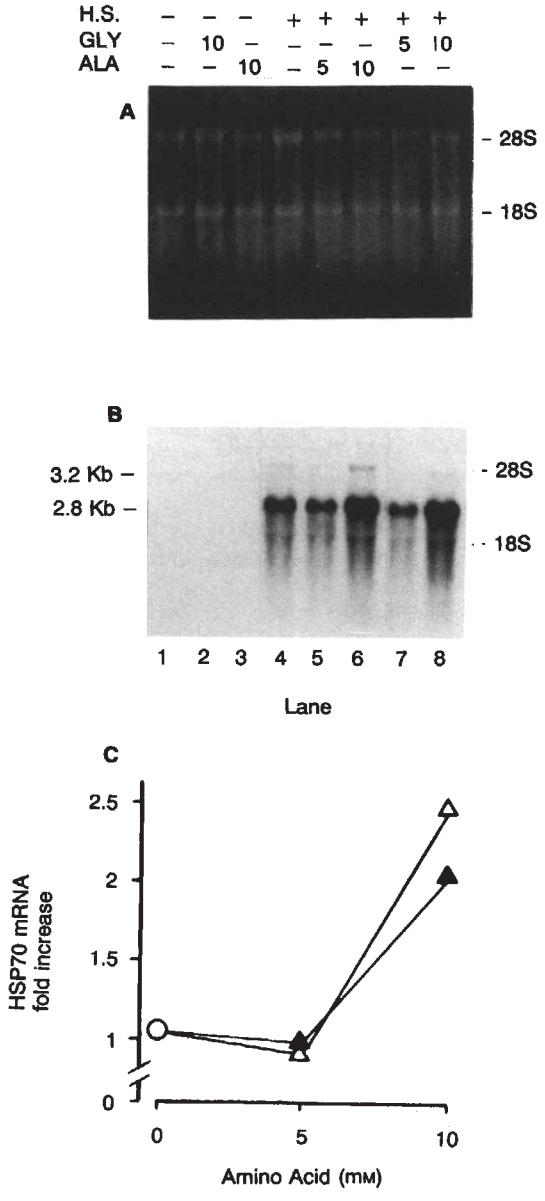

Fig. 2. *Stimulation of HSP70 mRNA induction by glycine or alanine.* OK cells were preincubated at 37°C for 30 minutes with KHB supplemented with either alanine or glycine at the concentrations (mM) indicated across the top of the photograph. Following preincubation, the flasks treated with heat shock (Lanes 4–8) were exposed to 45°C for 15 min and then reincubated in the unchanged medium for 3 hours at 37°C. Lane 1 represents incubation of OK cells in KHB without heat shock and Lanes 2 and 3 represent incubation in the presence of 10 mM glycine or alanine, without heat shock. **A.** The ethidium bromide staining of RNA (15 μg/lane) separated by gel electrophoresis. **B.** The Northern blot of the gel in panel A. The blot was exposed to Kodak XAR5 film for 16 hours. **C.** The level of HSP70 mRNA as determined by Phosphor Imager (see details in the legend of Fig. 1). Symbols are: (▲) ALA; (△) GLY. Fold increase was calculated by dividing individual levels obtained in experiments with either glycine or alanine by the mean level obtained in experiments without addition of amino acids, that is, control (0).

(Fig. 3A). Thus, although low (1 to 5 mM) concentrations of alanine and glycine had no effect on the level of HSP70 mRNA (Fig. 2), the formation of 72,73 kDa proteins was enhanced, especially in experiments with alanine (Fig. 3A). The addition of 100 μg/ml CHX inhibited the heat-induced 72,73 kDa proteins, even in the presence of 10 mM amino acid (Fig. 3A). Furthermore, without heat shock the addition of glycine and alanine did not induce the synthesis of 72,73 kDa proteins. However, it appears that a protein of approximately 75 kDa, present in control monolayers, was enhanced in the presence of amino acid without heat shock. Furthermore, proteins (66 and 75 kDa) which are present in control decreased following heat shock regardless of the addition of either amino acid (Fig. 3). These observations are in agreement with previous data [8–11, 28], indicating that following heat shock there is a rapid transcription of HSP70 mRNA for the synthesis of HSP70, whereas the expression of non heat shock genes is suppressed [8, 9]. This commitment to heat shock protein synthesis is reversible after cells are returned to their normal growth temperature as indicated by the time course of HSP70 mRNA level (Fig. 1).

To determine the effect of other amino acids on the expression of genes encoding for 72,73 kDa proteins, a parallel series of experiments was carried out in the presence of 10 mM glutamate, aspartate, leucine or arginine. Figures 3A and 3B demonstrate the specificity of glycine and alanine in enhancing the synthesis of stress proteins following heat-shock. However, other amino acids in 10 mM concentration resulted in heat-shock-like responses and in formation of stress protein, even without heat-shock. This observation is perhaps due to isotonic stress produced by this concentration of amino acids.

It is of special importance to note that the intracellular glycine level was 37.9 ± 8.1 nmol/mg protein after three hours incubation with KHB at 37°C, without heat-shock treatment. This value was significantly lower, 19.9 ± 6.9 nmol/mg protein, three hours following heat shock treatment ($N = 6$; mean ± SD; $P < 0.001$). Similarly, alanine concentration was 29.9 ± 4.1 nmol/mg protein in control cultures and dropped to 3.3 ± 0.8 nmol/mg protein, three hours following heat shock ($P < 0.001$). However, in the presence of 10 mM alanine or glycine in the incubation medium, the intracellular glycine rose to 308.2 ± 74.5 and alanine to 224.9 ± 49.9 nmol/mg protein ($N = 6$; mean ± SD). Thus, the elevations of HSP70 mRNA levels were obtained when intracellular levels of glycine and alanine were increased by approximately 8-fold and 11-fold from basal level, respectively. In comparison, protection from cisplatin nephrotoxicity in rat was achieved when plasma glycine concentration increased 40-fold from basal values [2]. Protection against injury to isolated tubules has been demonstrated when the intracellular level of glycine was increased by approximately 10-fold [1]. Therefore, comparable to previous data regarding the relationship between the cytoprotective action and the concentration of glycine and alanine [1, 2, 3, 6], the current data may indicate that the stimulation of heat shock genes requires adequate amounts of glycine or alanine. The observations that little change occurred in the level of HSP70 mRNA in the presence of glycine and alanine at and below 5 mM (Fig. 2), while production of 72,73 kDa proteins was increased (Fig. 3A), suggest that at high intracellular concentrations of glycine or alanine there was little or no degradation of HSP70 mRNA, and thereby, increased HSP70 mRNA level.

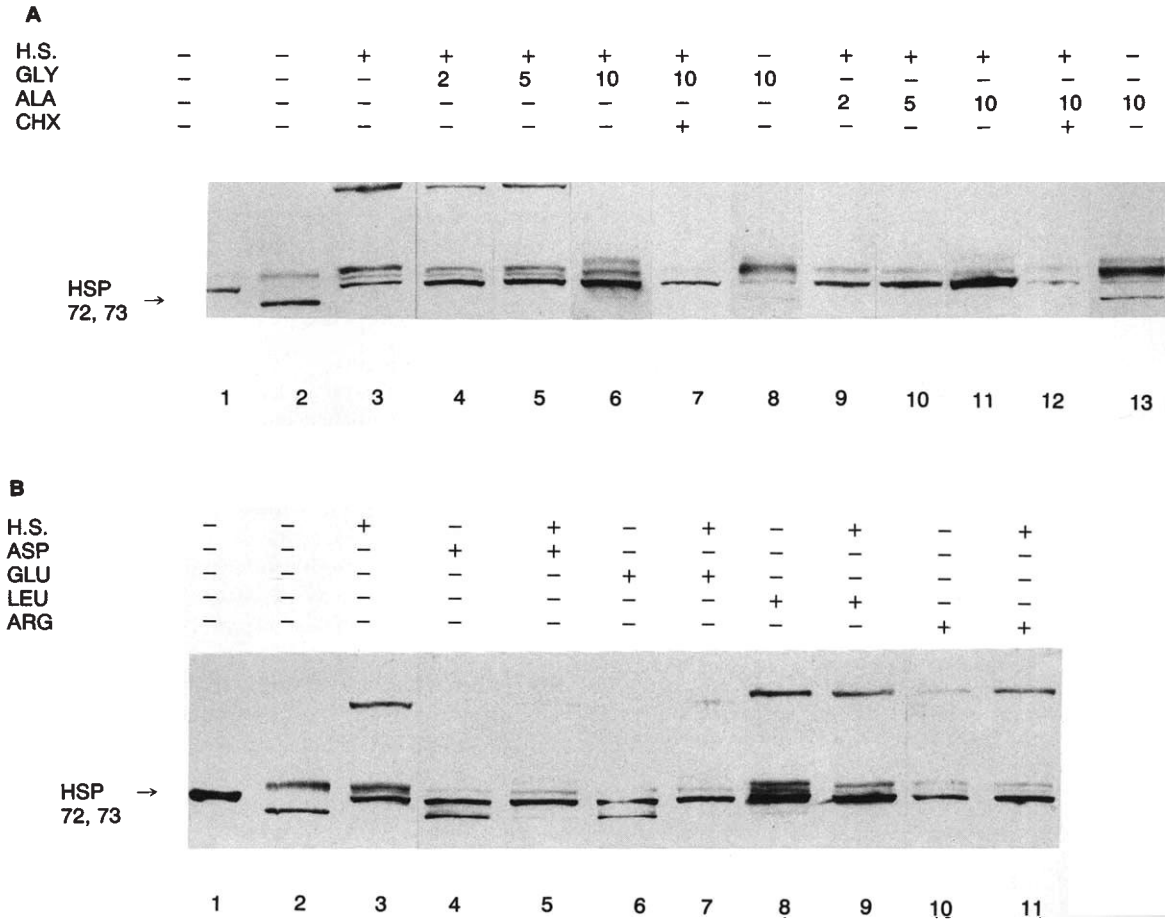

Fig. 3. *Immunoblot analysis of stress proteins.* **A.** Experiments with glycine and alanine. OK cells were preincubated at 37°C for 30 minutes with KHB (Lanes 2 and 3) or KHB supplemented with 2 mM, 5 mM, or 10 mM glycine with and without heat shock (Lanes 4–8) or 10 mM glycine + 100 μg/ml cycloheximide (CHX, Lane 7). Similar experiments were performed with 2 mM, 5 mM or 10 mM alanine (Lanes 9–13), or 10 mM alanine + 100 μg/ml CHX (Lane 12). Following preincubation, the experimental flasks containing a monolayer of OK cells (Lanes 3–7, 9–12) were exposed to 45°C for 15 minutes and then all flasks (Lanes 2–13) were incubated in unchanged medium for 3 hours at 37°C. **B.** Comparative studies with and without heat shock (as indicated above) in the presence of 10 mM aspartate (Lanes 4, 5), glutamate (Lanes 6, 7), leucine (Lanes 8, 9) and arginine (Lanes 10, 11). For immunoblotting, equal amounts of cell extracted protein (80 μg) were resolved by SDS-PAGE, transferred to a PVDF membrane and probed with anti-HSP 72,73 followed by biotinylated goat anti-mouse alkaline phosphatase conjugate and streptavidin-alkaline phosphatase. Bound antibody was visualized by alkaline phosphatase color development (**Methods**). Lane 1 represents the purified HSP 72,73 utilized as a marker for identification of 72,73 stress inducible protein indicated by arrow.

HSP70 induction—Thermotolerance relationship

Does the stimulation of HSP70 gene expression by glycine or alanine increase thermotolerance and decrease cellular injury? To answer this question, we determined the efflux of LDH in cultured OK cells before and after heat-shock and in the presence and the absence of various concentrations of amino acid. Figure 4A demonstrates that when OK cells are exposed to 45°C for 15 minutes a progressive and substantial release of LDH into the medium occurred during the three hour recovery period at 37°C compared with control cells. Preincubation with 10 mM glutamate, aspartate, arginine or leucine had little effect on the percent of LDH efflux (data not shown), compared with incubations in KHB alone (Fig. 4A). However, incubations with addition of glycine or alanine significantly reduced the efflux of intracellular LDH into the medium (Fig. 4A). The presence of CHX had little effect on the release of LDH in both control and following heat-shock treatment. However, LDH efflux was significantly higher upon addition of CHX compared with experiments in the presence of 10 mM alanine or glycine (Fig. 4A). In experiments with complete medium (Fig. 1), the percent of LDH efflux was approximately 3-fold lower than in experiments with Krebs buffer alone (Fig. 4A). This result may relate to the presence of various amino acids, including glycine and alanine as well as glucose and other nutrients in the incubation medium.

A comparison of the data of Figure 4A with Figures 2C and 3A indicates that when the level of HSP70 mRNA and its translation into stress protein were increased in the presence of amino acid (10 mM) there was an approximate 4-fold or 2.5-fold decrease in the percent of efflux of LDH upon supplementation of glycine or alanine, respectively. Furthermore, data of Figures 3A and 4A indicate a decreased percent of LDH efflux with increased amino acid concentrations, particularly in the presence of glycine. The current observation is comparable to

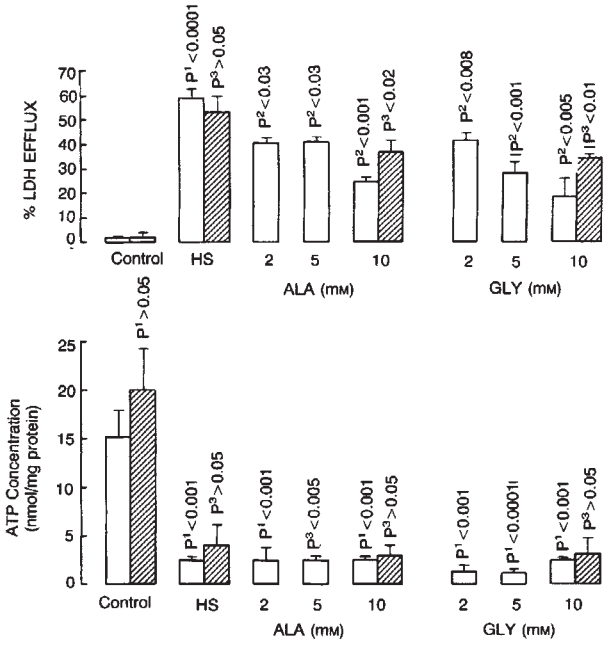

Fig. 4. *Effect of glycine or alanine on the percent of LDH efflux and ATP level.* OK cells were preincubated for 30 minutes with KHB supplemented with glycine or alanine at varying concentrations as indicated and in the presence or the absence of 100 μg/ml cycloheximide. Following preincubation, the cells were heat shocked (45°C for 15 min) and reincubated in the same medium at 37°C for 3 hours. **A.** The percent of LDH efflux calculated as described in **Methods. B.** The intracellular ATP concentration normalized to total cellular protein. HS and control represent experiments with and without heat shock, respectively. Dashed histogram represents experiments in the presence of cycloheximide. Each bar represents mean ± SD of 3 to 5 experiments. P^1 represents the significant difference compared with control; P^2 compared with HS without the addition of amino acid; and P^3 compared with the same experiment in the absence of cycloheximide.

previous reports demonstrating decreased percent of LDH efflux in the presence of glycine or alanine in cultured kidney cells [29] and isolated proximal tubules [1, 6].

Relationship between ATP and HSP70 mRNA

Irrespective of the magnitude of HSP70 mRNA transcription and stress protein synthesis, intracellular ATP levels dropped sharply following heat shock (Fig. 4B), reaching approximately 10 to 18% of non-heated control values under all experimental conditions. Neither the presence of glycine nor alanine prevented the depletion of ATP following heat shock. Consistent with previous findings [1, 3, 6], the data in Figures 4A and 4B demonstrate that glycine and alanine reduced the percent of LDH efflux despite a remarkable decrease in the intracellular ATP level. Thus, protection against heat damage occurred independent of intracellular ATP level. Furthermore, the presence of CHX had little effect on ATP levels (Fig. 4B), indicating that in addition to ATP requirement for the function of HSP70 [9, 16, 17] other factors may contribute to the depletion of ATP levels.

Discussion

It has been demonstrated by both in vivo and in vitro experiments that the neutral amino acids, glycine and alanine, protect kidney cells against various forms of stress and toxic injury [1–6, 29]. Our earlier studies with ^{13}C labeled glycine have indicated that protection occurs without substantial metabolism of the amino acid [1], and the protective effect appears to depend on the structure of the amino acid [4]. However, the mechanism of this protection remains unknown. The current data demonstrate that both glycine and alanine stimulate the expression of genes encoding stress protein, a property which is not shared by the other amino acids, glutamate, aspartate, arginine and leucine (Fig. 3A and 3B).

The presence of glycine or alanine in the incubation medium was associated with: (a) increased levels of HSP70 mRNA, (b) enhanced synthesis of a 72,73 kDa stress protein, and (c) decreased efflux of cellular LDH. Hence, these observations clearly indicate diminished cellular injury and enhanced thermotolerance when glycine or alanine was added to the incubation medium prior to heat shock. Interestingly, CHX did not completely attenuate the protection provided by glycine or alanine (Fig. 4). This observation may relate to partial inhibition of newly synthesized stress proteins as shown in current Figure 3A, as well as previously reported [16]. The cytoprotective effect also may be mediated in part via a mechanism independent of HSPs synthesis.

We propose that the cytoprotective action of glycine and alanine may mediate via two processes. The first site of protection is presumably at the plasma membrane which probably involves acceptor-ligand interaction, depending on the structure of the amino acid as previously indicated [4]. This protective effect probably obtains even with concentration below 2 mM as indicated by the dose dependent manner of LDH release (Fig. 4A), and previously published data [1, 3, 6, 29]. The second cytoprotective process, which probably occurs simultaneously, is a stimulation of gene(s) expression encoding stress protein(s) synthesis (Figs. 2 and 3), thereby conferring additional protection against stress injury. This mechanism may be based on the following observations: (a) our studies using OK cells and ^{31}P-NMR analyses (Nissim et al, unpublished data) indicated that heat shock leads to remarkable increase in membrane phospholipid degradation with elevation of glycerol-3-P, glycerol-P-ethanolamine and glycerol-P-choline, whereas membrane phospholipid content, especially sphingomyelin, was depleted. However, 10 mM glycine or alanine ameliorated this heat-induced damage to cellular membranes. This observation is consistent with a previous study indicating that heat shock stimulated the activity of phospholipase A_2 and release of arachidonic acid [30]; (b) our current data demonstrate that HSP70 mRNA and the formation of HSP70 are time dependent, therefore, as previously indicated [7–11], cellular tolerance against stress will depend upon the time course of stress protein accumulation; (c) other amino acids, such as, glutamate, aspartate, leucine and arginine with structures differing from glycine and alanine, did not protect from thermal injury (current data) or chemical injury [1, 3, 4, 6]. On the contrary, the current studies indicate that these amino acids induced heat-shock-like response and increased the percent of LDH efflux, even without heat shock treatment.

How do small neutral amino acids such as glycine or alanine enhance the steady state level of HSP70 mRNA? The processes by which environmental stresses activate HSF and, hence, regulate heat-shock gene expression are not yet clear. One

thought is that environmental stress mediates the conversion of HSF, a transcriptional activator protein, from an inactive to an active form that binds to the heat shock element (HSE) located at the promoter region of heat shock genes [8, 11, 28, 31–33]. The HSF-HSE complex then activates the transcription of HSP70 mRNA [28, 31–33]. Previous studies in diverse systems have indicated that amino acid analogs induce the synthesis of HSPs [8–10, 34]. In the current studies, no induction of HSP70 mRNA was observed in the presence of alanine or glycine without heat shock (Fig. 2). Hence, thermal stress was the initial signal to activate the pre-existing heat-shock transcription factor in OK cells, a process that is amplified by addition of glycine or alanine. Glycine or alanine may increase either the affinity or accessibility of HSF for its HSE-binding site. Alternatively, these amino acids may act as secondary inducers by binding pre-existing intracellular "repressors" which may block the activation of heat shock genes by the HSF-HSE complex [28, 35]. A third possibility is that these amino acids may minimize or prevent "degradation" of HSP70 mRNA. Further investigation is required to elucidate these possibilities.

The protective effects of glycine and alanine are independent of the intracellular ATP level, which dropped sharply following heat shock with or without the presence of these amino acids (Fig. 4B). The depletion of intracellular ATP and the prevention of LDH release in the presence of these amino acids may be explained by the hypothesis that HSPs 70 averts cell injury and death by binding and refolding of "damaged" protein in a process requiring ATP hydrolysis [7, 15–17]. However, the observation that CHX had little effect on ATP levels under all experimental conditions (Fig. 4B) suggests that an additional factor other than HSPs-mediated consumption of ATP contributes to depletion of this high-energy phosphate compound. Our experiments with ^{13}C labeled alanine and analysis by both GC-MS and NMR (data in preparation) have indicated a diminution in oxidative metabolism and TCA-cycle activity following heat-shock. Therefore, both the enhanced utilization of ATP by HSP and the inhibition of the TCA-cycle following heat shock may explain the fall in ATP levels.

In summary, the current investigation indicates that the cytoprotective action of glycine and alanine, a phenomenon demonstrated both in vivo [2] and in vitro [1–6, 29], is in part due to stimulation of the expression of gene(s) encoding HSP70 mRNA. Enhanced synthesis of stress proteins is of potential clinical importance. Glycine or alanine are small, non-toxic neutral amino acids, and they could be used to augment cellular tolerance and survival following exposure to various stressors, including hypoxia, ischemia, oxidant injury and cancer therapy. Further in vivo and in vitro investigations should be done to explore the regulatory effect of glycine or alanine on gene expression and their possible use as therapeutic agents.

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