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Proline Analogs, L-Azetidine-2-Carboxylic Acid and 3,4-Dehydro-L-Proline, Induce Stress Response in *Drosophila* Kc Cells

Sung Joon Moon and Ching Tack Han*
Department of Life Science, Sogang University, Seoul, Korea 121-742

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Amino acid analogs, like other inducers of stress response, induce the synthesis of stress proteins in mammalian cells. In this study, *Drosophila* Kc cells, in which translation is tightly controlled during stress response, was treated with proline analogs, L-azetidine-2-carboxylic acid (AzC) and 3,4-dehydro-L-proline (dh-P). Kc cells exposed to AzC or dh-P induced the synthesis of several proteins which had the same molecular weights as known heat shock proteins. However, in Kc cells, normal protein synthesis still continued in the presence of amino acids analogs unlike in heat-shocked cells. For the induction of stress response, the incorporation of dh-P into the protein was not essential, but the incorporation of AzC was. The stress protein synthesis was regulated mainly at the transcriptional level by AzC, whereas it was regulated by dh-P at the transcription level and possibly post-transcription level. During recovery, the stress protein synthesis stopped sooner in analog-treated cells than in heat-shocked cells even though the accumulated amount of Hsp70 was much less in proline analogs-treated cells. It could be concluded that the proline analogs, AzC and dh-P, induced stress response through a different mechanism from heat shock.

Keywords: *Drosophila*, Hsp47, Proline analogs, Stress response.

Introduction

All organisms studied to date respond to temperature elevations of 10°C or more above their normal physiological temperature, with the rapid and preferential synthesis of a small number of highly conserved proteins, the heat shock proteins (Lidquist, 1986; Beckmann, *et al.*, 1990).

A heat-shock like response could also be induced by exposure to a wide variety of other environmental insults, such as amino acid analogs, heavy metals, ethanol, and metabolic poisons (Ashburner and Bonner, 1979; Johnson *et al.*, 1980a; Johnson *et al.*, 1980b; Thomas *et al.*, 1982). Consequently, the response is often referred to generally as the stress response, and the induced proteins are referred to as the stress proteins (Mizzen and Welch, 1988). The stress proteins appear to function in the protection and the enhanced survival of the cells experiencing stress (Schlesinger, 1990). This stress response is a ubiquitous phenomenon in all organisms, even though the detailed regulatory mechanism of stress protein synthesis and the effect of stress agents may vary depending on the cell type or organisms.

In mammalian cells, amino acid analogs induce a full set of stress proteins (Kelly and Schlesinger, 1978; Thomas and Mathews, 1984). In particular, the proline analog, L-azetidine-2-carboxylic acid (AzC), is a well-known inducer of stress protein synthesis. When incoporated into newly synthesized proteins, AzC had an inhibitory effect on cell growth (Fowden and Tristram, 1963; Fowden *et al.*, 1963; Fowden *et al.*, 1967) because the structure and electrical properties of AzC are similar to normal proline (Fowden *et al.*, 1967; Baum *et al.*, 1973). Stress response by exposure to 3,4-dehydro-L-proline (dh-P), another proline analog, has not yet been reported, but it was expected to induce stress protein synthesis since the molecular structure of dh-P (Fig. 1) was close enough to proline so as to be incorporated into newly synthesized

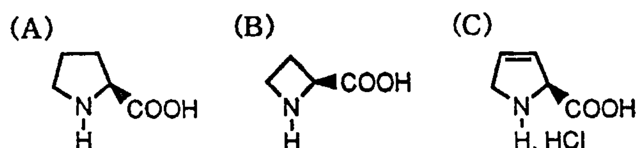

Fig 1. The chemical structure of L-proline (A), AzC (B), and dh-P (C).

* To whom correspondence should be addressed.
Tel: 82-2-705-8454; Fax: 82-2-704-3601
E-mail: cthan@ccs.sogang.ac.kr

proteins resulting in formation of abnormal proteins (Fowden *et al.,* 1967). However, it was expected that such change of protein structure by incorporation of dh-P would be less severe than that of an incorporation of AzC.

In this study, we examined the effect of prolin analogs on protein synthesis in *Drosophila* Kc cells, by comparing the type of inducible proteins, recovery patterns, regulatory level of protein synthesis, and the distribution pattern of induced proteins to elucidate the various pathways of the stress response induction by different stress modes.

Materials and Methods

Cell culture and protein labeling *Drosophila melanogaster* Kc cells were grown in D22 medium supplemented with 10% fetal bovine serum at 25°C as described previously (Schneider and Blumenthal, 1978; Sanders, 1981). Kc cells were diluted with fresh medium the day before each experiment was performed to give an equal density (1×10^7 cells/ml) of active cells. For the labeling of newly synthesized proteins during or after stress treatment, treated cells were rapidly rinsed with fresh medium and incubated in D22 medium without methionine and 40 μCi/ml of [^{35}S]-Met (Amersham, Arlington Height, USA). Cells were generally labeled with [^{35}S]-Met for 1 h. For the protein labeling during recovery, heat-shocked cells were returned to 25°C and the cells exposed to AzC or dh-P were transferred to fresh medium and incubated at 25°C. Aliquots of equal numbers of cells were taken at 30-min intervals and pulse-labeled with 150 μCi/ml of [^{35}S]-Met for 5 min.

Treatment with prolin analogs and heat shock Cells were treated with either various concentrations of AzC (Calbiochem Biochemical Co., St Louis, USA) or dh-P (synthesized and purity-tested in the Department of Chemistry, Sogang Univ. by Dr. Lee, Won Koo) at 25°C for indicated periods. For heat shock treatment, cells were incubated at 37 ± 0.2°C for 40 min in a waterbath (Fisher Scientific, Pittsburgh, USA). For the inhibition of protein synthesis, cycloheximide (Sigma Chemicals, St. Louis, USA) was added to the medium to a final concentration of 0.3 μg/ml as indicated before and after the treatment of AzC or dh-P.

SDS-polyacrylamide gel electrophoresis The same number of labeled cells were rinsed with phosphate buffered saline (PBS: 137 mM NaCl, 3 mM KCl, 10 mM KH_2PO_4, pH 6.9) three times and dissolved in an equal volume of Laemmli buffer (Laemmli, 1970). Dissolved cells were vortexed, sonicated for 3 min, and then boiled for 3 min. An equal amount of lysate was loaded into each lane and separated on 8–15% gradient SDS-PAGE. After electrophoresis, the gel was stained in Coomassie Blue solution, dried, and exposed directly to X-ray film (X-OmatTM, Kodak, Windsor, USA). Autoradiographs were developed by standard procedures (Hames, 1981).

Western blotting For Western blot analysis, proteins were separated on 8% SDS-PAGE and transferred to nitrocellulose membrane (Schreicher & Schuell, Dassel, Germany). The membrane was probed with monoclonal anti-Hsp70 antibody against human Hsp70/Hsc70 raised in mice (StressGen, Victoria, Canada), and visualized by color development using alkaline-phosphatase linked secondary antibodies color development.

RNA preparation and *in vitro* translation Total RNA was extracted from Kc cells by the single-step method (Chomczynsky and Sacchi, 1976), and translated *in vitro* using rabbit reticulocyte lysates (Promega, Madison, USA) for 2 h at 30°C, as previously described (Pelham and Jackson, 1976). In order to determine the proper incubation period for translation, a same amount of total RNA was translated. Equal amounts of each sample were taken at 30-min intervals and precipitated with 10% TCA. The amount of incorporated [^{35}S]-Met was counted in a scintilation counter (Wallac, Turku, Finland). RNA from amino acid analog-treated cells and heat-shocked cells showed the same translation efficiency in the *in vitro* translation system.

Cell fractionation After the treatment, cells were rapidly rinsed and pulse-labeled with [^{35}S]-Met. The labeled cells were harvested and throughly vortexed for 2 min with lysis buffer [20 mM Tris-Cl, pH 7.4, 140 mM KOAc, 5 mM $Mg(OAc)_2$, 0.1 mM EDTA, 0.5 mM DTT, 0.3 M sucrose, 0.2% Triton X-100]. The lysates were diluted in lysis buffer without Triton X-100 and fractionated into the nuclear fraction, mitochondrial fraction, microsomal fraction, and cytosolic fraction by differential centrifugation at 4°C. Each fraction was examined under the microscope to confirm organelles.

Results

AzC and dh-P induced stress protein synthesis in *Drosophila* Kc cells Protein synthesis patterns were changed by AzC or dh-P treatment in Kc cells (Fig. 2 and Fig. 3). Cells were exposed to 1–10 mM of AzC or 1–10 mM of dh-P which induced the synthesis of 70 kDa and 83 kDa proteins. Also, some small proteins, 26 kDa and 23 kDa, with the same molecular weights of small heat shock proteins were induced, implying that the amino acid analogs acted as stress inducers in *Drosophila* Kc cells in the same way as in HeLa cells (Thomas and Mathews, 1984). However, the amino acid analogs did not shut down the normal protein synthesis unlike heat shock response. In particular, the *de novo* synthesis of a 83 kDa protein in amino acid analogs-treated cells was greater than that in heat-shocked cells (lane A6 and A9 of Fig. 2). The amount of 70 kDa and 83 kDa proteins synthesized correlated positively with the concentration and duration to exposure of AzC (Fig. 2, lane A6 and A9) or dh-P (Fig. 3). These two proline analogs showed different dose effects on stress protein synthesis. The concentration of dh-P used for the induction of the same amount of 70 kDa protein synthesis was 1/500th that of the AzC treatment (Fig. 2, lane A6, and Fig. 3, lane P_6). Because dh-P started to show the induction of 83 kDa and 70 kDa protein synthesis at relatively low concentrations, the triggering period for the induction of 70 kDa protein synthesis by dh-P treatment was examined. The results show that induction occurred even at treatment with 10 μM dh-p for 5 min only (Fig. 4, lane P).

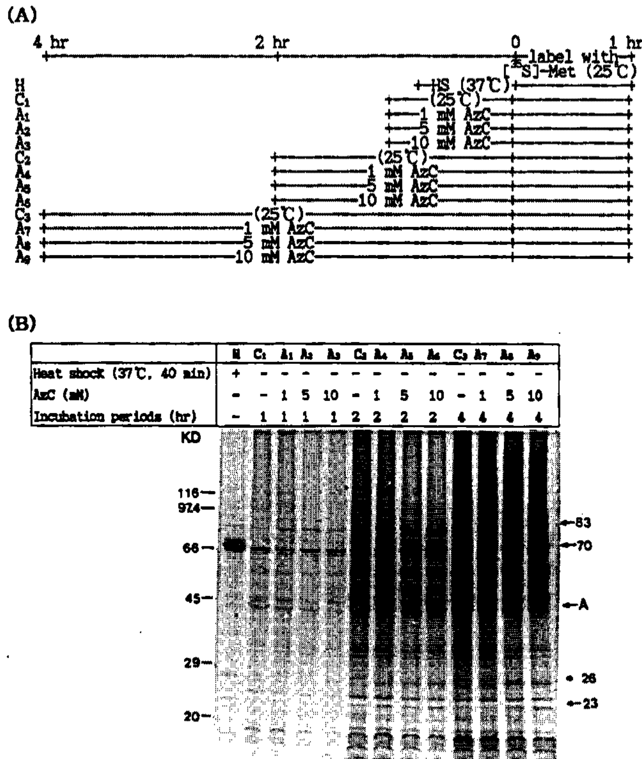

Fig 2. Induction of stress proteins in Kc cells by AzC treatment. Kc cells were treated with AzC of various concentrations and periods as indicated. At the end of the each treatment, cultures were rapidly rinced and labeled with 40 μCi/ml [^{35}S]-Met for 1 h. The lysates of same numbers of cells were loaded and separated on 8–15% gradient SDS-PAGE. The pattern of protein synthesis is shown on the autoradiogram. The bands of stress proteins (SPs)and actin (A) are indicated at the right side of the autoradiogram. **(A)**: time table of treatment; **(B)**: autoradiogram.

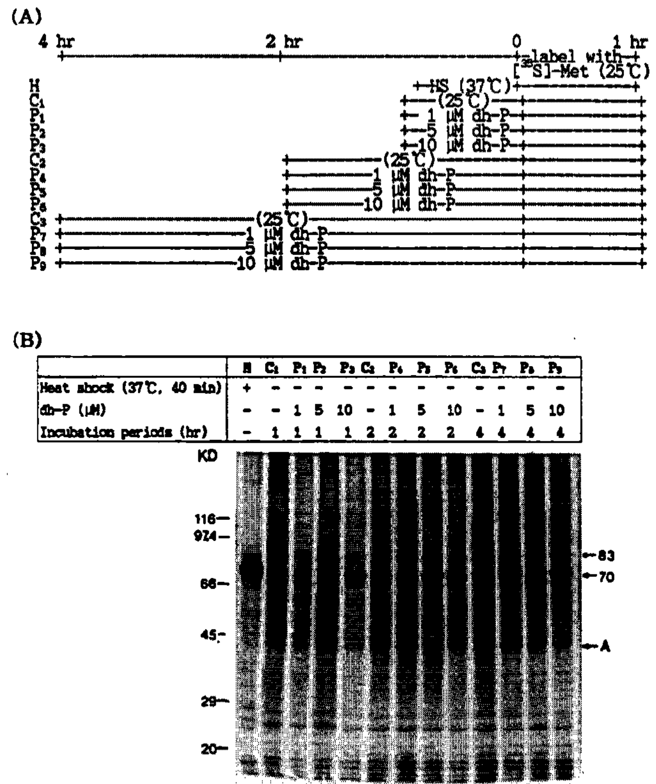

Fig 3. Induction of stress proteins in Kc cells by dh-P treatment. Kc cells were treated with dh-P of various concentrations and periods as indicated. At the end of each treatment, cultures were rapidly rinced and labeled with 40 μCi/ml [^{35}S]-Met for 1 h. The lysates of same numbers of cells were loaded and separated on 8–15% gradient SDS-PAGE. The pattern of protein synthesis is shown on the autoradiogram. The bands of SPs and actin (A) are indicated at the right side of the autoradiogram. **(A)**: time table of treatment; **(B)**: autoradiogram.

To investigate whether the AzC or dh-p induced 70 kDa protein was related to Hsp70, cross-reactivity of the protein with monoclonal antibodies against Hsp70 was tested by Western blotting (Fig. 5). The 70 kDa protein cross-reacted with the antibody implying that the protein was the same or related to the Hsp70 that is induced by heat shock in *Drosophila* Kc cells.

Incorporation of AzC into protein was a prerequisite for the induction of stress response, whereas dh-P induced stress protein synthesis without incorporation into proteins In order to identify whether or not the incorporation of AzC or dh-P into proteins was required for the induction of stress response, cells were incubated with cycloheximide, a specific inhibitor of protein synthesis, at various points of amino acid analog treatment. When cells were incubated with cycloheximide during AzC treatment, no stress protein synthesis was induced, whereas cells incubated with AzC 1 h prior to cycloheximide addition showed stress response (Fig. 6, lanes AX_2, AX_3). This clearly showed that AzC was being incorporated into proteins thus forming abnormal proteins that might act as the stress inducers.

On the other hand, the synthesis of stress proteins was induced by dh-P even though dh-P was not being incorporated into protein synthesis because of the presence of cycloheximide (Fig. 7, lane PX_1-PX_3 and lane PX_4-PX_6). This concluded that the incorporation of dh-P into protein was not essential for the induction of stress protein synthesis implying that dh-P causes stress response by some other mechanism. Cycloheximide itself did not cause any stress induction.

The synthesis of stress protein messages were induced by AzC or dh-P treatment To investigate the level of regulation of stress response to proline analogs, total RNAs from AzC or dh-P treated cells were isolated and translated *in vitro* in reticulocyte lysates. RNAs from stressed and control cells showed the same translation efficiency (Fig. 8A). The same amount of total RNAs was translated

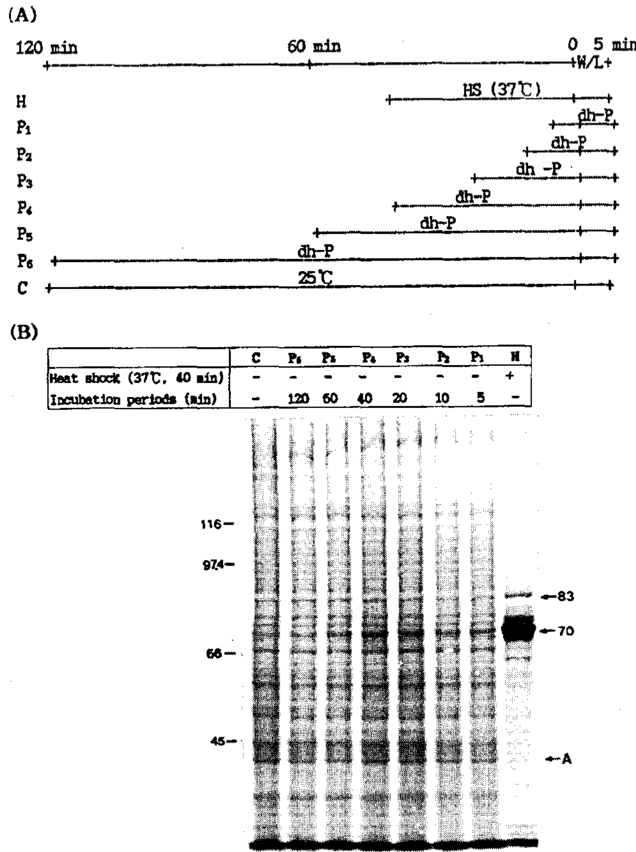

Fig 4. Induction of stress proteins in Kc cells exposed to dh-P treatment for short periods. Kc cells were exposed to 10 μm dh-P for various periods as described in Materials and Methods. At the end of each treatment, cultures were rapidly rinsed to remove dh-P and labeled with 150 μCi/ml [^{35}S]-Met for 5 min. The lysates of same numbers of cells were loaded and separated on 8–15% gradient SDS-PAGE. The pattern of protein synthesis is shown on the autoradiogram. The bands of SPs and actin (A) are indicated at the right side of the autoradiogram. **(A)**: time table of treatment; HS, W, L indicate heat shock at 37°C, washing cells to remove dh-P, and labeling period with [^{35}S]-Met, respectively. **(B)**: autoradiogram.

in vitro for 2 h in the presence of [^{35}S]-Met, and the products were analyzed on the gel. Messages of 70 kDa and 83 kDa proteins were the major RNAs induced by AzC treatment (Fig. 8B, lane AzC), which matched to the increments of 70 kDa and 83 kDa protein *in vivo* (Fig. 2, lane A_6). This implies that the induction of stress protein synthesis by AzC treatment is mainly controlled at the transcription level. On the other hand, the major species of mRNAs in the dh-P treated Kc cells were mRNAs of 90 kDa, 73 kDa, and 47 kDa proteins (Fig. 8B, lane dh-P). These mRNA products were different from the 70 kDa and 83 kDa proteins induced *in vivo* by dh-P (Fig. 3, lane P_6). It could be suggested that in addition to transcription, there is an extra regulation step in the induction of stress proteins.

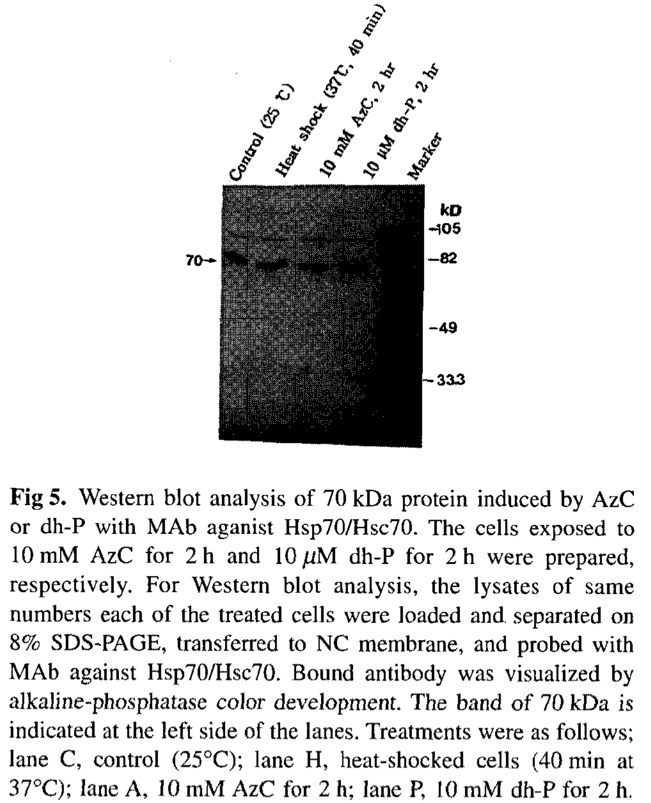

Fig 5. Western blot analysis of 70 kDa protein induced by AzC or dh-P with MAb aganist Hsp70/Hsc70. The cells exposed to 10 mM AzC for 2 h and 10 μM dh-P for 2 h were prepared, respectively. For Western blot analysis, the lysates of same numbers each of the treated cells were loaded and separated on 8% SDS-PAGE, transferred to NC membrane, and probed with MAb against Hsp70/Hsc70. Bound antibody was visualized by alkaline-phosphatase color development. The band of 70 kDa is indicated at the left side of the lanes. Treatments were as follows; lane C, control (25°C); lane H, heat-shocked cells (40 min at 37°C); lane A, 10 mM AzC for 2 h; lane P, 10 mM dh-P for 2 h.

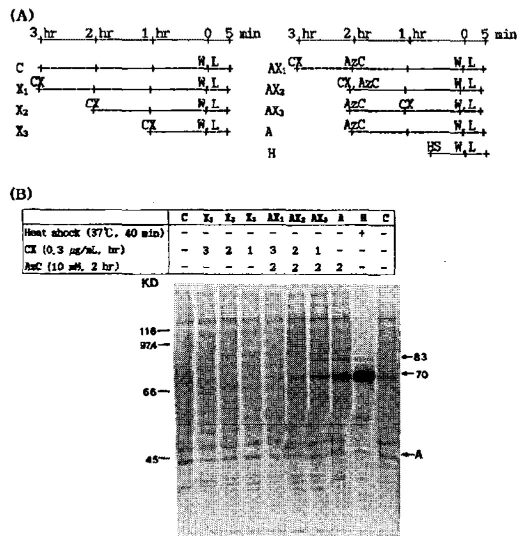

Fig 6. The effect of cycloheximide (CX) on stress protein induction by AzC treatment. Kc cells were exposed to 10 mM AzC with or without 0.3 μg/ml CX for various periods as described. At the end of each treatment, cultures were rapidly rinsed to remove CX and AzC and then labeled with 150 μCi/ml [^{35}S]-Met for 5 min. The lysates of same numbers of cells were loaded and separated on 8–15% gradient SDS-PAGE. The pattern of protein synthesis is shown on autoradiogram. The bands of SPs and actin (A) are indicated at the right side of the autoradiogram. **(A)**: time table of treatment; HS, W, L indicate heat shock at 37°C, washing cells to remove CX and AzC, and labeling period with [^{35}S]-Met, respectively. **(B)**: autoradiogram.

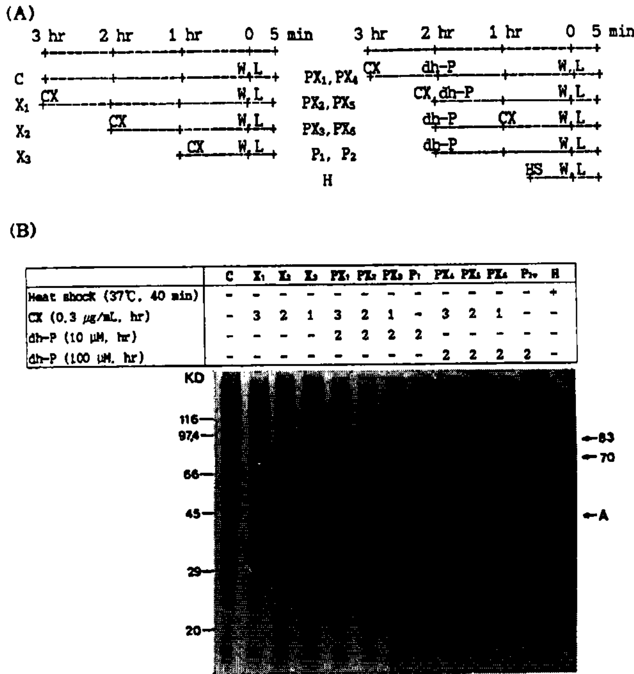

Fig 7. The effect of cycloheximide (CX) on stress protein induction by dh-P treatment. Kc cells were exposed to 10 μM or 100 μM dh-P, with or without 0.3 μg/ml CX, for various periods as described. At the end of each treatment, cultures were rapidly rinsed to remove CX and dh-P and then labeled with 150 μCi/ml [^{35}S]-Met for 5 min. The lysates of same numbers of cells were loaded and separated on 8–15% gradient SDS-PAGE. The pattern of protein synthesis is shown on the autoradiogram. The bands of SPs and actin (A) are indicated at the right side of the autoradiogram. **(A)**: time table of treatment; HS, W, L indicate heat shock at 37°C, washing cells to remove CX and dh-P, and labeling period with [^{35}S]-Met, respectively. **(B)**: autoradiogram.

Kc cells recovered from stress by AzC or dh-P treatment sooner than from heat shock The profiles of protein synthesis observed during recovery from the treatments with the AzC or dh-P was compared with that of heat-shocked cells. The results showed that the stress protein synthesis gradually decreased during recovery from the treatments of AzC or dh-P, but the kinetics of recovery was faster than that in heat-shocked cells.

Heat-shocked Kc cells synthesized 70 kDa proteins upto 2 h after transfer to 25°C, while stress induced protein synthesis was almost stopped in 60 min after removal of AzC (Fig. 9B, lane R_{60}), and in 30 min after removal of dh-P (Fig. 9C, lane R_{30}). The synthesis of normal protein inhibited by heat shock resumed 2 h after being transferred to 25°C, while that in the proline analog-treated cells recovered within 1 h. Also, a 47 kDa protein was synthesized both in the presence of the proline analogs as well as after the removal of the drugs (Fig. 9B and C). This implied that translation of pre-existing messages was not severely affected by amino acid analogs and the function of the protein synthesis machinery was not altered.

The intracellular distribution pattern of 70 kDa proteins was not altered by AzC or dh-P treatment Because of the differences in the recovery patterns observed and the species of stress protein messages induced by heat shock and by AzC or dh-P treatment, it was questioned whether there might be a difference in the intracellular distribution of 70 kDa proteins brought about by the different roles of the proteins. Thus, the proline analog-treated cell lysates were fractionated by differential centrifugation (Fig. 10). As in heat-shocked cells, most of the 70 kDa and 83 kDa proteins were found to exist in the cytoplasmic fractions in both proline analog-treated cells, and a minor portion of the 70 kDa proteins were located in intracellular organelles. Even though the identity of the 70 kDa proteins in different fractions was not tested and elucidated to be Hsp70 and Hsc70, the distribution patterns were nevertheless similar in all stressed cells.

Discussion

A heat-shock like response can also be induced by exposure to a wide variety of other environmental insults.

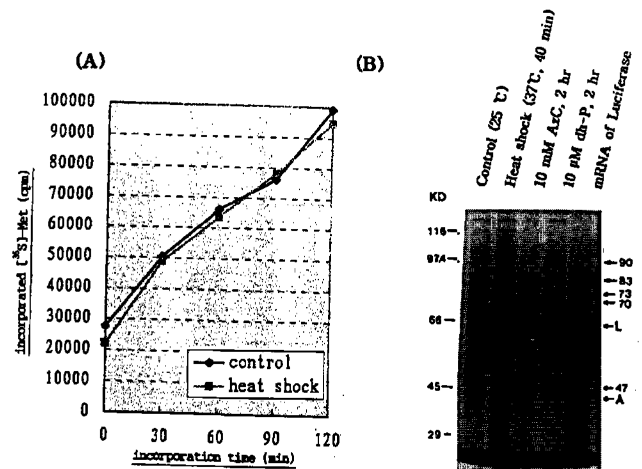

Fig 8. *In vitro* translation products of RNAs from Kc cells. (A) Standard curve of *in vitro* protein synthesis. Total RNA was extracted from control Kc cells and heat-shocked cells for 40 min at 37°C, respectively. Then, the same amount of total RNA was translated *in vitro* up to 2 h at 30°C. Equal amounts of each sample were taken at 30-min intervals and precipitated with 10% TCA and the amount of incorporated [^{35}S]-Met was quantified by liquid scintillation counter. (B) *In vitro* translation products of RNAs from Kc cells. Total RNA was extracted from treated cells and translated *in vitro* using nuclease-treated rabbit reticulocyte lysates for 2 h at 30°C. The same amount of *in vitro* translation lysates was loaded and separated on 8% SDS-PAGE. The pattern of protein synthesis is shown on the autoradiogram. The bands of SPs, luciferase (L), and actin (A) are indicated at the right side of the autoradiogram.

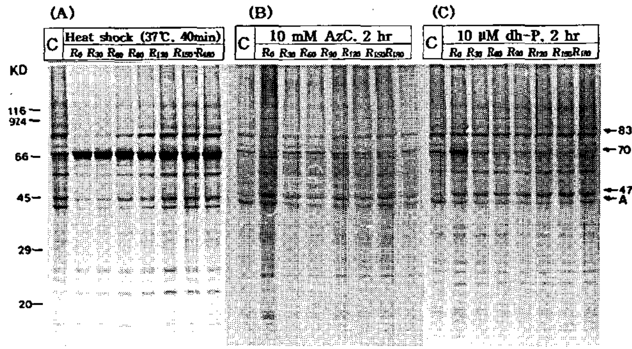

Fig 9. Protein synthesis during recovery from treatment of heat shock (A), AzC (B), and dh-P(C). Cells were either heat-shocked for 40 min at 37°C and then returned to 25°C (A), exposed to 10 mM AzC for 2 h and then returned into normal culture medium (B), or exposed to 10 μM dh-P for 2 h, respectively. Equal numbers of cells were then taken at 30-min intervals and rapidly rinsed and labeled with 150 μCi/ml [^{35}S]-Met for 5 min. The lysates of same numbers of cells were loaded and separated on 8–15% gradient SDS-PAGE. The pattern of protein synthesis is shown on the autoradiogram. The bands of SPs and actin (A) are indicated at the right side of the autoradiogram. Treatments were as follows: lane C, control (25°C); lane R_0, no recovery after stress; ; lane R_{30}, recovery for 30 min; lane R_{60}, recovery for 60 min; lane R_{90}, recovery for 90 min; lane R_{120}, recovery for 120 min; lane R_{150}, recovery for 150 min; lane R_{180}, recovery for 180 min.

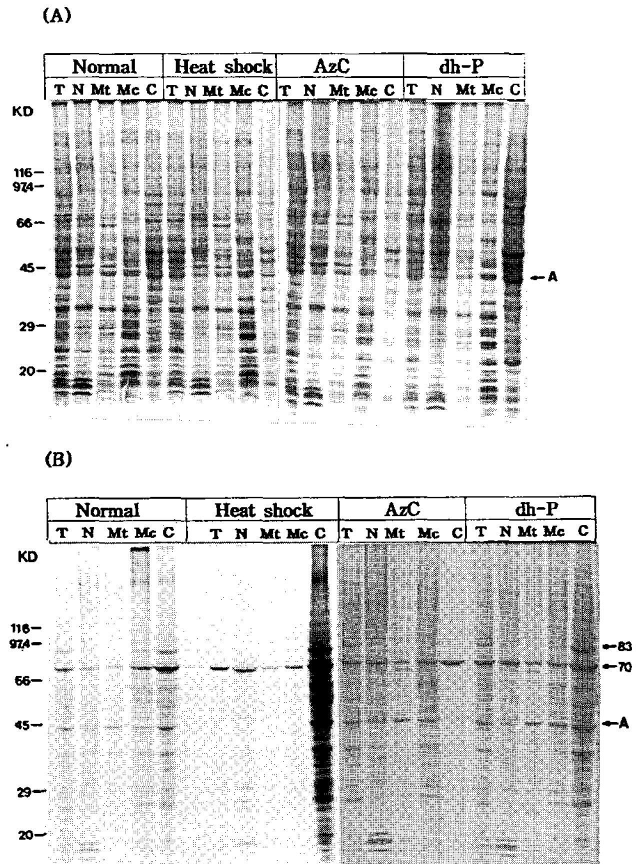

Fig 10. Intracellular distribution pattern of total proteins (A) and newly synthesized proteins (B) in normal, heat-shocked, and AzC- and dh-P-treated cells. Cells heat-shocked for 40 min at 37°C or exposed to 10 mM AzC or 10 μM dh-P for 2 h were prepared. After treatment the cells were rapidly rinsed and labeled with 150 μCi/ml [^{35}S]-Met for 5 min. The labeled cells were harvested and vortexed for 2 min with lysis buffer. After vortexing, the lysates were fractionated by centifugation at 4°C. For each lane, the fractions from the lysates of the same number of cells were loaded and separated on 8–15% gradient SDS-PAGE. The pattern of total proteins and of newly synthesized proteins are shown on Coomassie blue stained gel (A) and autoradiogram (B), respectively. The bands of SPs and actin (A) are indicated at the right side of the autoradiogram. The fractions are as follows; T, total lysates; N, nuclear fraction (600 × g for 10 min); Mt, mitochondrial fraction (10,000 × g for 30 min); Mc, microsomal fraction (105,000 × g for 60 min, precipitant); C, cytosol fraction (105,000 × g for 60 min, supernatant).

It has been suggested that there might be several signaling pathways through which various stresses relay signals to the common responding part of cells, such as transcription factors and factors of protein synthesis (Burdon, 1993). AzC is a well-known inducer of stress protein synthesis in mammalian cells (Thomas and Mathews, 1984). It is incorporated into newly synthesized proteins resulting in abnormal proteins that might trigger the signaling pathways. The deformed collagen proteins from AzC incorporation have been demonstrated by Zagari *et al.* (1994) Also, another protein analog, dh-P, was expected to induce stress protein synthesis but with less toxicity because the ring structure of dh-P is very similar to proline (Fowden *et al.*, 1967). We tested with AzC and dh-P to see whether these compounds could also act as stress inducers in *Drosophila* cells to regulate protein synthesis very tightly at the translational level, unlike mammalian cells. As in HeLa cells, AzC and dh-P also induced stress response in *Drosophilla* Kc cells. The stress response was similar to the heat-shock response where the induction of stress protein synthesis was concerned, but normal protein synthesis was reduced only slightly in proline analog-treated cells, unlike heat-shocked cells in which normal protein synthesis was shut down 100%. Similar results have been reported by Welch and Suhan (1986) that toxic amino acids produced less translation inhibition in mammalian cells. Heat-shocked *Drosophila* cells preferentially translated heat shock protein messages, even though normal protein messages existed (Ballinger and Pardue, 1981). Thus there might be some discriminatory factors involved in protein synthesis. However, the proline analog-treated cells did not seem to have such a regulation.

The amount of synthesized stress protein correspondended with the concentration and duration of AzC or dh-P, which is similar to the effect of toxic amino acids in mammalian cells. However, unlike the prediction

by Fowden *et al.* (1967), dh-P had a stronger inhibitory effect on protein synthesis. The concentration for the induction of the same level of 70 kDa protein synthesis by dh-P treatment was relatively lower than that of AzC treatment. Likewise, the triggering period for induction of stress protein synthesis by dh-P treatment was relatively shorter than that of AzC treatment. One hundred micromolar dh-P abolished protein synthesis in Kc cells (data not shown), while the same concentration of AzC induced stress protein synthesis. This seemed to be contrary to the report that AzC is more toxic than dh-P in the development of the organism (Fowden *et al.*, 1967).

To examine whether the abnormal protein was required for the induction of stress response, cells were incubated with 0.3 μg/ml cycloheximide before, during, or after amino acid analog treatment. The results showed that incorporation of AzC into protein was required for the induction of stress protein synthesis (Thomas and Mathews, 1984), but the incorporation of dh-P into protein was not essential for induction of stress protein synthesis implying that dh-P itself could cause stress response (Beckman *et al.*, 1990). This was why dh-P induced stress response at a lower concentration and in a shorter time than AzC. In other words, dh-P itself was toxic to cell metabolism, possibly by binding to enzymes in a short time thereby inducing stress response. The mechanism of how AzC induces the stress response has been suggested as follows. Abnormal proteins made with AzC might act as stress to cells and trigger the heat shock factors. To confirm the signaling pathways and reacting factors with these toxic molecules, it would be necessary to use radioactive AzC or dh-P.

By the analysis of the change of mRNA pools in AzC or dh-P-treated cells and the synthesized stress proteins *in vivo*, we could get a hint of the control level of stress response in the proline analog-treated cells. The induction of stress response by AzC treatment was mainly controlled at the transcriptional level, which means that stress protein messages were preferentially transcribed in AzC-treated cells by activating heat shock factors, and the messages were translated with other pre-existing normal protein messages. However, there might be an extra regulation step in addition to the transcription in dh-P-treated cells which means that even dh-P-treated cells preferentially transcribe some set of messages, although all the transcribed messages were not used for translation *in vivo*. One common phenomenon found in AzC-treated or dh-P-treated cells was that the splicing step was not blocked. This could be inferred by the continuous synthesis of Hsp83 even at a high concentration of proline analogs. Since the Hsp83 gene contains introns unlike other heat shock genes, at high temperature the blocking of Hsp83 synthesis could be observed as being due to an alteration of splicing.

From comparisons of the pattern of protein synthesis during recovery from the treatments of AzC or dh-P with that of heat shock, it was found that protein synthesis during recovery from AzC treatment and from heat shock might be controlled by different mechanisms. It has been known that *Drosophila* cells autoregulate the recovery from heat shock by the accumulation of Hsp70. If *Drosophila* cells could not synthesize enough amount of Hsp70 during heat shock, the cell's recovery was blocked until a certain amount of Hsp70 was accumulated. In this experiment, heat-shocked cells did not resume the normal protein synthesis until 2 h after the transfer to normal temperature, and the amount of Hsp70 was more than 20 times that of Hsp70 produced in proline analog-treated cells which already resumed 100% of normal protein synthesis in 30 min. These imply that the factors involved in the regulation of recovery could be different.

In addition to the differences described above, the increment of 47 kDa messages was detected in dh-P treated cells (Fig. 8) and the 47 kDa protein synthesis continued during recovery from AzC or dh-P treatment. The protein might be the Hsp47 reported in mammalian cells which was induced by inhibition of the secretion of abnormal collagen due to AzC incorporation (Nagata, 1996). Hsp47 functions in the processing and secretion of collagen as charperone. It will have to be examined whether the 47 kDa protein induced by AzC treatment was related to the Hsp47 of mammalian cells.

Our study on stress response in *Drosophila* Kc cells by amino acids analogs revealed that other stress agents share only a part of the signaling pathways used in heat shock.

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References

Ashburner, M. and Bonner, J. J. (1979) The induction of gene activity in *Drosophila* by heat shock. *Cell* **17**, 241–254.

Ballinger, D. G. and Pardue, M. L. (1983) The control of protein synthesis during heat shock in *Drosophila* cells involves altered polypeptide elongation rates. *Cell* **33**, 103–114.

Baum, B. J., Troxler, R. F., Kagan, H. M., Grasso, J. A., Faris, B., and Ranzblau, C. (1973) Incorporation of L-azetidine-2-carboxylic acid into hemoglobin in rabbit reticulocytes. *Biochem. Biophys. Res. Commun.* **53**, 1350–1356.

Beckmann, R. P., Mizzen, L. A., and Welch, W. J. (1990) Interaction of Hsp70 with newly synthesized proteins. Implications of protein folding and assembly. *Science* **248**, 850–854.

Burdon, R. H. (1993) The heat shock proteins: The molecular charperones. *Mol. Aspects. Med.* **14**, 111–119.

Chomczynsky, P. and Sacchi, N. (1987) Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**, 156–159.

Craig, E. A. (1985) The heat shock response. *CRC Crit. Rev. Biochem.* **18**, 239–280.

Fowden, L., Lewis, D., and Tristram, H. (1967) Toxic amino acids: their action as antimetabolites. *Adv. Enzymol.* **29**, 89–163.

Fowden, L., Neale, S., and Tristram, H. (1963) Effect of 3,4-dehydro-DL-proline on growth and protein synthesis. *Nature* **199**, 35–38.

Fowden, L. and Richmond, M. H. (1963) Replacement of proline by azetidine-2-carboxylic acid during biosynthesis of protein. *Biochem. Biophys. Acta* **71**, 459–461.

Hames, B. D. (1981) One-dimensional polyacrylamide gel electrophoresis; in *Gel Electrophoresis of Proteins: A Practical Approach*, Hames B.D. and Lindquist, D. R. (eds), IRL Press,Oxford, Washinton DC.

Johnson, D., Oppermann, H., Jackson, J., and Levinson, W. (1980a) Induction of four proteins in chick embryo cells by sodium arsenite. *J. Biol. Chem.* **255**, 6975–6980.

Johnson, D., Oppermann, H., Jackson, J., and Levinson, W. (1980b) Transition series metals and sulfhydryl reagents induce the synthesis of four proteins in eukaryotic cells. *Biochim. Biophysica Acta.* **606**, 170–180.

Kelly, P. M. and Schlesinger, M. J. (1978) The effect of amino acid analogs and heat shock on gene expression in chicken embryo fibroblast. *Cell* **15**, 1277–1286.

Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.

Lindquist, S. (1986) The heat-shock response. *Annu. Rev. Biochem.* **55**, 1151–1191.

Lindquist, S. (1993) Autoregulation of heat shock response; in *Translational Regulation of Gene Expression 2.* 2nd ed., Ilan, J. (ed), pp. 279–320, Plenum Press, New York.

Mizzen, L. A. and Welch, W. J. (1988) Characterization of the thermotolerant cell. I. Effects on protein synthesis activity and the regulation of heat-shock protein 70 expression. *J. Cell Biol.* **106**, 1105–1116.

Nagata, K. (1996) Hsp47: A collagen-specific molecular charperone. *TIBS* **21**, 23–26.

Pelham, H. R. B. and Jackson, R. J. (1976) An efficient mRNA dependent translation system from reticulocytes. *Eur. J. Biochem.* **67**, 247–251.

Sanders, M. M. (1981) Identification of histon H2b as a heat-shock protein in *Drosophila. J. Cell. Biol.* **91**, 579–583.

Schlesinger, M. J. (1990) Heat shock proteins. *J. Biol. Chem.* **265**, 12111–12114.

Schneider, I. and Blumenthal, A. B. (1978) *Drosophila* cell and tissue culture; in *The Genetics and Biology of Drosophila.* Vol. 2a, Ashburner, M. and Wright, T. R. F. (eds), pp. 265–315, Academic Press, New York and London.

Thomas, G. P. and Mathews, M. B. (1984) Alteration of transcription and translation in HeLa cells exposed to amino acid analogs. *Mol. Cell. Biol.* **4**, 1063–1072.

Thomas, G. P., Welch, W. J., Mathews, M. B., and Feramisco, J. R. (1982) Molecular and cellular effects of heat shock and related treatments of mammalian tissue culture cells. *Cold Spring Harbor Symp. Quant. Biol.* **64**, 985–996.

Welch, W. J. and Suhan, J. P. (1986) Cellular and biochemical events in mammalian cells during and after recovery from physiological stress. *J. Cell Biol.* **103**, 2035–2052.

Zagari, A., Palmer,K., Gibson, K., Nemethy, G., and Scheraga, H. (1994) The effect of L-azetidine-2-carboxylic acid residue on protein conformation. IV. Local substitutions in the collagen triple helix. *Biopolymers* **34**, 51–60.