Korean J. Zool. 31:157-164, 1988

Induction of Stress Proteins in the SCK Tumor Cells

Man-Sik Kang and Kyung-Hee Kim

Dept. of Zoology, Seoul National University, Seoul 151-742, Korea

SCK tumor cells were exposed to heat shock or several sulfhydryl-reacting agents such as iodoacetamide(IAA), zinc chloride(Zn), and 2-mercaptoethanol(ME). Stress proteins induced by these agents were examined and the relationship between the induction of stress proteins and the production of abnormal proteins was discussed.

Based on the present experiments, two classes of intracellular pathways for the induction of stress proteins were defined; one dependent on and the other independent of protein synthesis. The presence of cycloheximide during the induction period blocked the formation of stress proteins in the cells exposed to Zn or ME, but not in those exposed to heat shock or IAA. Therefore, stress protein seems to be induced either by denaturation of pre-existing mature proteins (e.g., heat shock or IAA) or by newly synthesized abnormal proteins(e.g., Zn or ME).

In conclusion, it is likely that the production of abnormal proteins by stresses triggers stress protein induction. In addition, it was found that the cells exposed to HSP and GRP inducers simultaneously responded to more strong stress among several stresses encountered.

KEY WORDS: SCK tumor cells, Heat shock, SH-reagents

Cells respond to a variety of adverse conditions by increasing the synthesis of a relatively small group of proteins (Nover *et al.*, 1984; Schlesinger *et al.*, 1982). These are frequently termed as stress protein and are grouped into two distinct classes, heat shock proteins(HSPs) and glucose-regulated proteins(GRPs).

In the chicken embryo cells, the list of inducers of the heat shock proteins include heat (Kelley and Schlesinger, 1987), amino acid analogs (Kelley and Schlesinger, 1987; Hightower, 1980), sulfhydryl-reactive chemicals, and certain metal ions (Johnston *et al.*, 1980; Levinson *et al.*, 1980a, b). The major HSPs fall into several size classes with molecular weight of 20~30K, 65~70K and 80~110K.

The treatment that elicits the synthesis of the GRPs in cultured cells includes glucose starvation, glucosamine, 2-deoxyglucose (Pouyssegur *et al.*, 1977), tunicamycin (Olden *et al.*, 1979), and calcium ionophore (Wu *et al.*, 1981; Welch *et al.*, 1983). Paramixovirus infection also induces both HSPs and GRPs in chick embryo cells (Collins and Hightower, 1982; Peluso *et al.*, 1978). The major GRPs fall into two classes with molecular weight of 75~83K and 95~100K.

In mammalian systems, HSPs and GRPs can be induced simultaneously (Hightower and White, 1982; Welch *et al.*, 1983), separately, or reciprocally. Sciandra and Subjeck (1983) reported that the addition of glucose to glucose-deprived cells, which induced GRPs (97 and 76K), resulted in the induction of HSPs. In addition, they (1984) suggested that the transition of cells from atmospheric environment to an anaerobic state transiently induce the major HSPs (68 and 89K). As the period of anaerobiosis increased, these HSPs disappeared and GRPs (76 and 97K) were induced. From these results, they suggested that the

*This work was supported by S.N.U. Daewoo Research Fund in 1987.

GRP-induced state represents a sustained response to a glucose-deprived environment or an anoxia, while the HSP-induced state represents a temporary response to a release from such an environment.

Whelan and Hightower (1985) reported that low extracellular pH and 2-mercaptoethanol stimulate chicken embryo cells to synthesize GRPs, whereas high extracellular pH stimulates to synthesize HSPs. They also proposed that an oxidizing environment within the cell might lead to HSP synthesis, whereas a reducing environment might lead to the induction of GRPs. Thus, regardless of the nature of effective stimulus, the same proteins are being synthesized. It suggests a common final molecular pathway functions for the response to stresses (Hammond *et al.*, 1982).

Sulfhydryl-group has been recognized as a potential target for oxidation by several inducers of HSPs including diamide, sodium arsenite, iodoacetamide, and ions of Cu, Zn, and Cd (Levinson *et al.*, 1980b). Abnormal proteins serve as signals and trigger activation of the heat shock genes in eukaryotic cells (Ananthan *et al.*, 1986). This result supports the finding that cycloheximide inhibits the HSP synthesis in amino acid analog-treated cells (Kelley and Schlesinger, 1978).

Recently, Munro and Pelham (1986) reported that GRP_{78} is about 60% homologous to HSP_{70}. It shows the possibility that GRPs perform the same function as HSPs.

In the present study, SCK cells were exposed to heat or several sulfhydryl-reacting agents and the pattern of stress protein synthesis was then analyzed. On the basis of these data, the possibility whether the production of abnormal proteins is involved in the induction of stress protein was discussed.

Materials and Methods

Cell Culture

SCK tumor cells and their culture method were the same as described previously (Kang *et al.*, 1980).

Exposure to Heat Shock

SCK tumor cells were seeded at a density of 2 x 10^5 cells per 35 mm tissue culture dish containing 1.5 ml of RPMI 1640 medium supplemented with 10% calf serum at pH 7.4. After 2-day culture, culture dishes inserted in the plastic shelves were immersed horizontally in a constant temperature-circulating waterbath.

Chemical Treatment

SCK cells in monolayer culture were treated with 5 x 10^{-5}M $ZnCl_2$(Zn), 5 x 10^{-5}M iodoacetamide(IAA) or 0.1% 2-mercaptoethanol (ME) in RPMI 1640 medium supplemented with 10% calf serum at 37°C for 3 hr, rinsed and labelled with ^{35}S-methionine for 2 hr at appropriate times.

Cell Survival Studies

SCK cells were seeded onto 60 mm tissue culture dish at a density of 1 x 10^3 or 5 x 10^4 cells and incubated at 37°C for 11-12 hr prior to chemical treatment. After the chemical treatment, cells were rinsed and incubated for 2 hr, and exposed to 45°C for 30 min. After incubation for 7-10 days, colonies were stained with crystal violet and counted.

Incorporation of ^{35}S-methionine into Proteins

Cells were labelled with 5 μCi/ml of ^{35}S-methionine in methionine-free RPMI 1640 for 2 hr at 37°C. At the end of this period, the medium was removed and the cells were washed three times with cold phosphate buffered saline (PBS) and harvested by adding 200 μl of lysis buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol) and were scrapped off with a rubber pliceman. Samples were stored at −20°C until analyzed.

Gel Electrophoresis

Labelled samples were dissociated by heating in boiling water for 5-6 min. The amount of protein was determined by the method of Lowry *et al.* (1951). Equal amount or cpm of protein was directly loaded on 7.5% SDS-polyacrylamide slab gel by employing the method of Laemmli (1970).

Autoradiography and Fluorography

After electrophoresis the gels were either autoradiographed or fluorographed routinely.

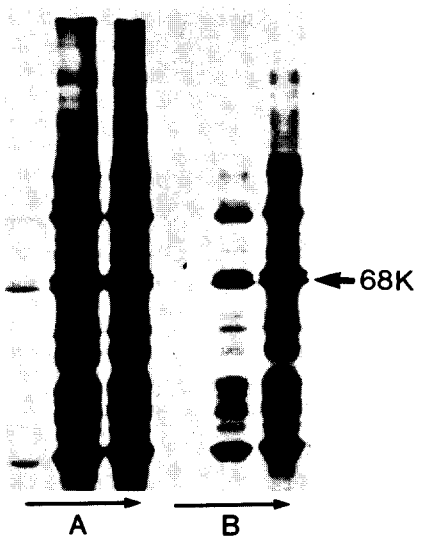

Fig. 1. Stress protein induction in the cells exposed to hyperthermia at 42°C for 1 hr alone(A) or with ME(B). Labelling was performed immediately, 2 hr or 4 hr following the treatment.

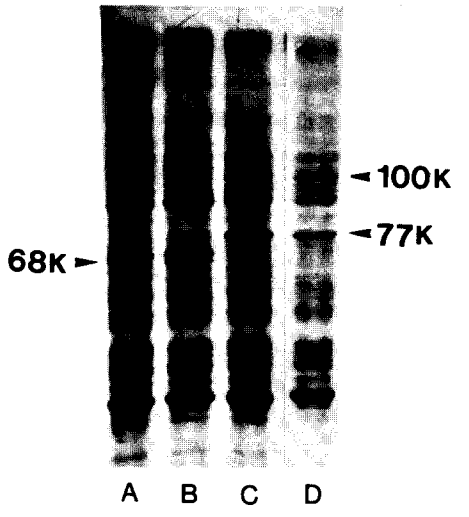

Fig. 2. Stress protein induction by Zn and ME. Cells were exposed to inducer for 3 hr, rinsed and labelled for 2 hr. A, control; B, Zn; C, Zn plus ME; D, ME.

Rate of Protein Degradation

Rate of protein degradation was determined by measuring the ratio of radioactivity of TCA-soluble products to that of TCA-insoluble materials (S/I ratio). The cells were incubated with ^{35}S-methionine at a final concentration of 1 μ Ci/ml for 15 hr. After removing the medium, the cells were washed three times with RPMI 1640 and were then exposed to heat shock or chemicals for 1 or 3 hr, washed three times, and incubated at 37°C. Release of radioactive material was monitored for 20 hr. At various times during incubation, the medium was withdrawn and 10% TCA was added to dish and then cells were harvested. TCA-harvested cell extracts were centrifuged at 12,000 rpm for 5 min, the supernatant was collected and mixed with withdrawn medium and the radioactivity of this mixutre was measured (acid-soluble products). The remaining TCA-insoluble materials were dissolved in 100% TCA and their radioactivity was measured (acid-insoluble materials).

Results

Stress Protein Induction

When the cells were exposed to stress protein inducers, Zn and IAA induced *de novo* synthesis of HSP_{68}, whereas ME induced high level of GRP_{77}.

Figs. 1-3 illustrate the stress protein induction pattern when the cells were exposed to HSP and GRP inducers simultaneously. Simultaneous treatment of heat shock and ME, Zn and ME, and IAA and ME were adopted. Although the cells exposed to heat and ME induced preferentially HSP_{68}, those exposed to Zn and ME or IAA and ME favorably induced GRP_{77}. These results suggest that there exists a certain priority in the action of inducers. Thus, it is likely that cells respond to the strongest stress among the stresses encountered. In certain chemical treatment, it is possible that cells respond to a chemical of highest concentration.

In Fig. 4 are shown the stress protein induction patterns in the cells exposed initially to IAA for 3 hr, rinsed and then exposed to ME for 3 hr. For comparison, those in the cells exposed to IAA or ME alone are also shown. As is evident from Fig.

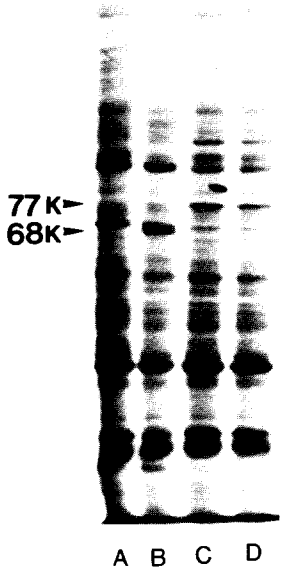

Fig. 3. Stress protein induction by IAA and ME. Cells were exposed to inducer for 3 hr, rinsed and labelled for 2 hr. A, control; B, IAA; C, IAA plus ME: D, ME.

4, the last-treated ME exerted no effect on the HSP induced by first-treated IAA and GRPs were not induced by the last-treated ME. In Fig. 6 is shown a parallel experiment, in which the last-treated IAA was found to give no effect on GRPs induced by first-treated ME. Interestingly, however, HSPs were induced by the last-treatment of IAA, and the HSPs induced by the last-treated IAA were not suppressed by first-treated ME.

In a parallel experiment, Zn and ME were adopted for further investigation (Fig. 6). The change in the induction of HSP or GRP was not noticeable at the combined treatment of first Zn/ the last ME.

Effect of Cycloheximide on the Stress Protein Induction

Fig. 7 shows the effect of cycloheximide on the stress protein induction by various agents. The cells were exposed to cycloheximide (20 μg/ml) for 0.5, 1, 1.5 or 2 hr before inducer treatment, then to the inducer together with cycloheximide. In the cells exposed to Zn or ME, stress protein

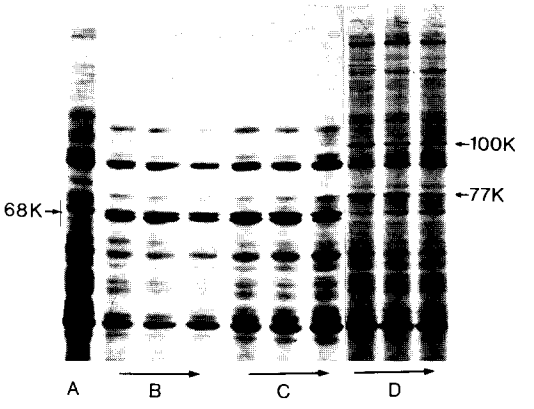

Fig. 4. Cells were exposed first to LAA for 3 hr, rinsed and the last exposed to ME for 3 hr, rinsed and incubated for varying times, then labelled for 2 hr. Comparison was made for IAA or ME alone. A, control; B, experimental; C, IAA; D, ME.

was not induced, whereas those exposed to IAA induced stress protein.

Effect of Glycerol on the Stress Protein Induction

The effect of macromolecular stabilizer, glycerol at a concentration of 1 M, on the HSP induction by heat shock or Zn is shown in Fig. 8. Glycerol blocked HSP synthesis in the cells exposed to heat shock but not in those exposed to Zn. This result suggests an idea that the pathway which leads to HSP synthesis might be different in the cells exposed to heat or Zn.

Determination of Protein Degradation

The rate of degradation for the prelabelled protein by various stress protein inducers is shown in Fig. 9. In the heat-shocked cells protein was degraded to TCA-soluble peptides and amino acids more rapidly than the control, as well as Zn or ME-treated cells. Thus, the protein degradation was not a common phenomenon following exposure to stress or in stress protein induction. In this experiment, IAA was excluded because of its strong cytocidal effect.

Discussion

Prokaryotic and eukaryotic cells respond to heat

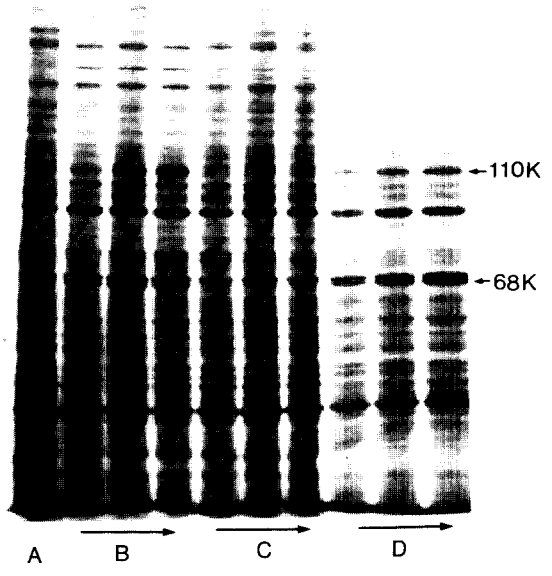

Fig. 5. Cells were first exposed to ME for 3 hr, rinsed and the last exposed to IAA for 3 hr, rinsed and incubated for varying times, then labelled for 2 hr. Comparison was made for ME or IAA alone. A, control; B, experimental; C, ME; D, IAA.

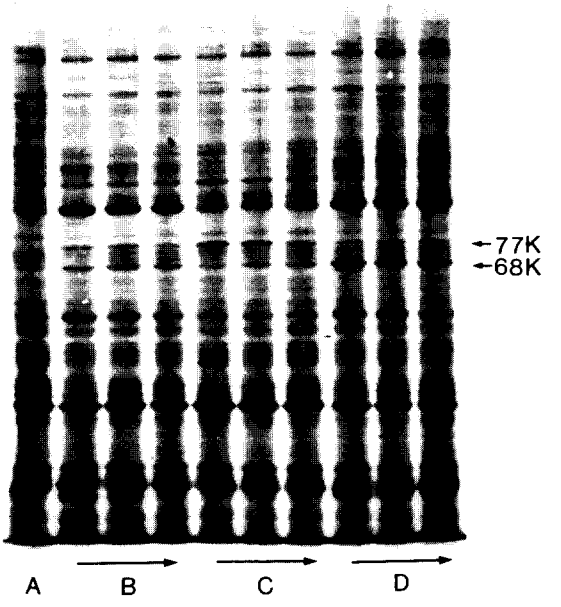

Fig. 6. Cells were exposed first to Zn for 3 hr, rinsed and the last exposed to ME for 3 hr, rinsed and incubated for varying times, then labelled for 2 hr. Comparison was made for Zn or ME alone. A, control; B, experimental; C, ME; D, Zn.

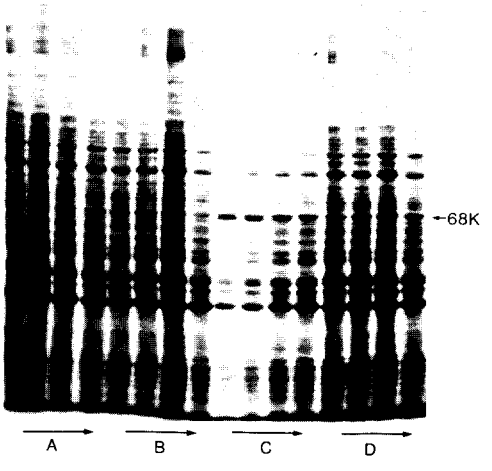

Fig. 7. Effect of cycloheximide on the stress protein induction. Cells were exposed to cycloheximide (20 μg/ml) for 0.5 to 2 hr before inducer treatment, then exposed to indicated inducer together with cycloheximide, rinsed and labelled. A, control; B, Zn; C, IAA; D, ME.

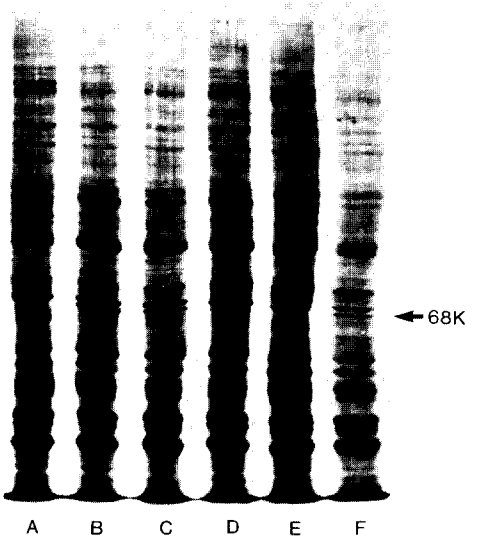

Fig. 8. Differential induction of stress proteins by glycerol. Cells were exposed to inducer and labelled for 2 hr. A, control; B, Zn; C, Zn plus glycerol; D, heat; E, heat plus glycerol; F, heat plus glycerol and Zn.

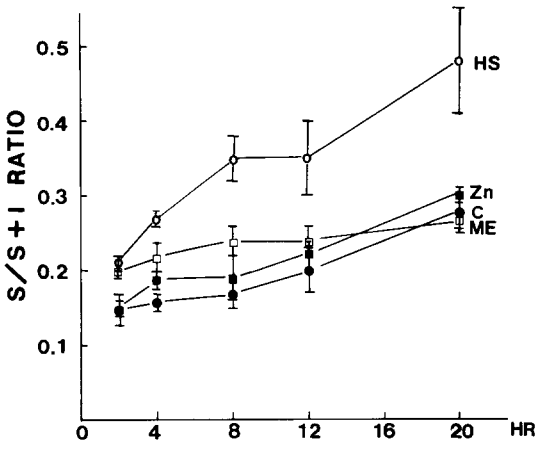

Fig. 9. Rate of degradation of prelabelled proteins by several stress protein inducers. Rate of degradation is expressed as the ratio of TCA-soluble to that of TCA-insoluble and soluble fractions.

shock and certain other environmental abuses by synthesizing a small set of stress proteins, such as HSPs and GRPs. To examine the mechanism for the induction of stress proteins by various inducers, iodoacetamide and $ZnCl_2$ as HSP inducer and 2-mercaptoethanol as GRP inducer were treated to SCK cells in the present experiment.

In the SCK cells HSP_{68}, HSP_{70} and HSP_{110} as HPSs and GRP_{77} GRP_{100} as GRPs were preferentially induced. Among these, HPS_{68} was found to be synthesized *de novo* and GRP_{77} was produced in greater amount, so these two stress proteins were extensively concerned with. The presence of actinomycin D during the induction period blocked the increased synthesis of stress proteins in all cases, while that of cycloheximide during the same period blocked the formation of these proteins in the cells exposed to Zn or ME, but not in those exposed to heat shock or IAA.

This result is not consistent with the finding (Whelan and Hightower, 1985) that even if cycloheximide was treated during the induction period, high concentration of ME (e.g., 0.25%) induced GRP synthesis. These disagreement might be explained as follows: Since high concentration of ME might disrupt disulfide bonds in mature proteins, it is predicted that there are at least two cellular targets, for which stress protein inducers might act on. The more sensitive one, perturbed at low concentration of ME (e.g., 0.1% as used in the present study), involves protein synthesis, whereas the other affected at high concentration of ME is not dependent on protein synthesis to generate an induction signal. The present study suggests that Zn and ME trigger their signals through a sensitive target that involves protein synthesis.

As Ananthan *et al.* (1986) suggested, Zn and ME may affect nascent proteins by reacting to sulfhydryl groups and producing conformational changes in proteins. However, heat and IAA may disrupt normal structure or normal function of mature proteins by irreversibly binding to sulfhydryl groups (IAA) or by increasing the unfolding of proteins (heat).

Glycerol was found to block HSP synthesis in heat-shocked cells but not in Zn-treated cells. Glycerol is generally recognized to stabilize proteins from heat shock (Henle *et al.*, 1982). Thus, glycerol is likely to block HSP synthesis in heat-shocked cells by protecting the heat sensitive proteins. In contrast, in the cells exposed to Zn, glycerol did not block HSP synthesis. Since Zn is thought to induce HSP synthesis by reacting to sulfhydryl groups, glycerol is not likely to give any effect on the reaction.

It seems to be that several stresses that induce stress proteins produce abnormal proteins via different pathways and the production of abnormal proteins as a common signal results in the stress protein induction. Protein degradation, however, was not observed as a common phenomenon following exposure to stress. Ananthan *et al.* (1986) have reported that abnormal proteins serve as eukaryotic stress signals and trigger the activation of heat shock genes. In addition, they proposed a model that transcriptional regulation of the heat shock gene is based on competition for degradation between abnormal intracellular proteins and a labile regulatory factor.

Examination of the relationship between HSP and GRP inductions revealed that the HSP and GRP inductions were correlated with each other in the cells exposed to Zn and ME. Thus, HSP induction by first Zn treatment was affected by the last ME treatment, and GRP induction by first ME treatment was affected by the last Zn treatment. Similarly, HSPs or GRPs induced by the last Zn or

ME treatment was affected by first ME or Zn treatment. It is thought that there are competition between HSP and GRP signals, because Zn and ME may affect nascent proteins and produce different signals for HSP or GRP induction. However, in the cells exposed to IAA and ME, HSP induction by first IAA treatment was not affected by the last ME treatment, and likewise GRP induction by first ME treatment was not affected by the last IAA treatment. In other words, HSP induction by IAA treatment was not affected by first or the last ME treatment. As IAA is an irreversible binding agent to sulfhydryl groups, its attack to nascent proteins or mature proteins might produce irreversibly denatured proteins. On this account, the last ME treatment following first IAA treatment is likely to be not effective. It is also possible that the remnant IAA serves as a powerful oxidizing agent, inhibiting the signal triggered by ME. In contrast, the GRP induction by first ME treatment was not blocked by the last IAA treatment. The triggered signal by the first ME treatment was efficient enough to generate GRP induction. Thus, the last IAA treatment did not affect the initially triggered signal.

References

Ananthan, J., A. L. Goldberg and R. Voellmy, 1986. Abnormal proteins serve as eukaryotic stress signals and trigger the activation of heat shock genes. *Science* **232**:522-524.

Collins, P. L. and L. E. Hightower, 1982. Newcastle disease virus stimulates the cellular accumulation of stress(heat shock) mRNAs and proteins. *J. Virol.* **33:** 703-707.

Hammond, G. L., Y. K. Lai and C. L. Markert, 1982. Diverse forms of stress lead to new patterns of gene expression through a common and essential metabolic pathway. *Proc. Natl. Acad. Sci. USA* **79**:3485-3488.

Hightower, L. E., 1980. Cultured animal cells exposed to amino acid analogs or puromycin rapidly synthesize several polypeptides. *J. Cell Physiol.* **102**:407-427.

Hightower, L. E. and F. P. White, 1982. Preferential synthesis of rat heat-shock and glucose-regulated proteins in stressed cardiovascular cells. *In:* Heat Shock: From Bacteria to Man. Cold Spring Harbor Laboratory, Cold Spring Harbor, N. Y., 369-378.

Johnston, D., H. Opperman, J. Jackson and W. Levison, 1980. Induction of four proteins in chick embryo cells by sodium arsenite. *J. Biol. Chem.* **255**:6975-6980.

Kang, M. S., C. W. Song and S. H. Levitt, 1980. Role of vascular function in response of tumors in vivo to hyperthermia. *Cancer Res.* **40:**1130-1135.

Kelley, P. M. and M. J. Schlesinger, 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. *Nature* **227**:680-685.

Levinson, W., J. Idriss and J. Jackson, 1980. Metal binding drugs induce synthesis of four proteins in normal cells. *Biol. Trace Element Res.* **1**:15-23.

Levinson, W., H. Oppermann and J. Jackson, 1980b. Transition series metals and sulfhydryl reagents induce the synthesis of four proteins in eukaryotic cells. *Biochim. Biophys. Acta* **606**:170-180.

Lowry, O. H., N. J. Rosenbrough, A. L. Farr and R. J. Randall, 1951. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* **193**:263-275.

Munro, S. and H. R. Pelham, 1986. An HSP_{70}-like protein in the ER; Identity with the 78kd glucose-regulated protein and immunoglobulin heavy chain binding protein. *Cell* **46**:291-300.

Nover, L., D. Hellmund, D. Neumann, K. Scharf, and E. Serfling, 1984. The heat shock response of ekaryotic cells. *Biol. Zblatt.* **103**:357-435.

Peluso, R. W., R. A. Lamb, and P. W. Choppin, 1978. Infection with paramixoviruses stimulates synthesis of cellular polypeptides that are also stimulated in cells transformed by Rous Sarcoma virus or deprived of glucose. *Proc. Natl. Acad. Sci. USA* **75**:6120-6124.

Sciandra, J. J. and J. R. Subjeck, 1983. The effects of glucose on protein synthesis and thermosensitivity in Chinese hamster ovary cells. *J. Biol. Chem.* **258:** 12091-12093.

Sciandra, J. J., J. R. Subjeck and C. S. Hughes, 1984. Induction of glucose-regulated proteins during anaerobic exposure and of heat-shock proteins after reoxygenation. *Proc. Natl. Acad. Aci. USA* **81**:4843-4847.

Welch, W. J., N. I. Garrels, G. P. Thomas, J. J. C. Lin and J. R. Feramisco, 1983. Biochemical characterization of the mammalian stress proteins and identification of two stress proteins as glucose and Ca^{2+} ionophore-regulated proteins. *J. Biol. Chem.* **258**:7102-7111.

Whelan, S. A. and L. E. Hightower, 1985. Differential induction of glucose-regulated and heat shock proteins: Effect of pH and sulfhydryl reducing agents on chicken embryo cells. *J. Cell Biol.* **125**:251-258.

(Accepted June 14, 1988)

SCK 종양 세포에서 Stress Protein의 합성유도

강만식 · 김성희 (서울대학교 자연과학대학 동물학과)

SCK 종양세포에 온열처리와 여러가지 sulfhydryl-reacting agents를 처리하여 stress protein의 합성을 유도하고, 그 양상을 검토해 봄으로써 stress protein의 합성유도와 denatured protein의 생성과의 관계를 고찰하였다.

세포에 cycloheximide와 더불어 Zn 또는 ME를 처리한 경우에는 stress protein의 합성이 일어나지 않았으나, 온열처리 또는 IAA를 처리한 경우에는 stress protein의 합성이 유도되었다. 이 결과로 미루어 볼 때, stress protein의 유도 경로에는 두가지가 있어서 새로운 단백질의 합성이 필요한 경로와 새로운 단백질의 합성과는 무관한 경로가 있는 것으로 추정할 수 있었다.

결국, 본 실험에서 사용한 stress들이 기존의 mature protein을 denature시키거나 (온열처리 또는 IAA), 새로 합성된 immature protein을 denature시키는 것 (Zn 또는 ME)으로 알려져 있으므로, stress에 의한 abnormal protein의 출현이 stress protein의 합성을 유도하는 trigger의 구실을 하는 것으로 생각된다. 이밖에 여러가지 stress가 동시에 작용할 경우, 세포는 보다 강한 stress에 대해서 stress protein을 합성하여 대처하게 되는 것으로 생각된다.