中文摘要

細胞在正常生理下，如果受到熱、重金屬、病毒、藥物等外來因子的刺激後 ，細胞會因因應這些刺激而產生熱休克蛋白(Heat Shock proteins)。而一般熱休克蛋白可因分子量分成四群：1.熱休克蛋白110(HSP110)，2.熱休克蛋白90(HSP90) ，3.熱休克蛋白70(HSP70) ，4.熱休克蛋白70KD以下的熱休克蛋白。熱休克蛋白含有保護子(Chaperone)的功能，可以幫助新合成或受破壞之蛋白質的折疊，使其具有應有正確的結構和功能，當細胞處於逆境環境下，將會誘發細胞中熱休克蛋白表現增加。之前的研究發現，膠達納黴素(geldanamycin，GA)處理下會造成細胞之中蛋白質的折疊不正常，引發細胞之逆境反應而誘導熱休克蛋白的合成增加，特別是熱休克蛋白70s(HSP70s)。

在此論文中我們以H460人類非小型肺癌細胞為材料，探討GA誘發H460人類非小型肺癌細胞中HSP70表現所參與的訊息傳遞路徑。在我們的研究中發現，經過GA處理的H460人類非小型肺癌細胞，其細胞內HSP70蛋白質的表現會隨著GA的濃度以及處理時間的改變而改變，並且利用北方點墨(Northern Blot)實驗發現，GA是影響HSP70的轉錄過程。由於人類熱休克蛋白70含有許多isoforms存在，利用2-D及北方點墨(Northern Blot)的分析讓我們知道GA其所誘發熱休克蛋白70s主要為HSP70A(HSP70-1)熱休克蛋白，另外我們是第一個發現GA所誘導的HSP70D可能會透過PKC等Pathway去誘發。在kinas inhibitor 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H-7)完全抑制HSP70A蛋白質的表現，而HA1043則無明顯的抑制作用，同時，H-7，H-8及HA1043對GA所誘發mRNA的增加也有相同的抑制現象。因此，可以知道在H460人類非小型肺癌細胞，GA所誘發HSP70A表現增加的訊息傳遞路徑中，細胞內的激鋂(Kinase)扮演重要的角色。在另一方面，利用electrophoretic mobility shift assay (EMSA)實驗，可以清楚的了解細胞核中轉錄蛋白和promoter的結合情形。因此經由EMSA的實驗，我們得到在GA處理狀況下轉錄蛋白會和熱休克轉錄序列(Heat shock element)有結合增加的現象。同時轉錄蛋白和HSE結合增加的現象也會被H-7，H-8所抑制，因此，我們更清楚的了解，HSE轉錄序列在GA所誘發HSP70表現增加的訊息傳遞過程裡扮演重要的角色。

The abbreviations used are:

GA: geldanamycin

H460 cells: human non-small cell lung cancer cells;

HSP: heat shock protein;

HSP70: 70 kDa HSP;

HSF: heat shock factor;

HSE: heat shock element;

SDS-PAGE:sodium dodecyl sulfate-polyacrylamide gel electrophoresis;

EMSA: electrophoretic mobility shift assay;

H-7: 1-(5-isoquinolinesulfonyl)-2-methylpiperazine;

H-8:N-(2-[methylamino]ethyl)-5-isoquinoline sulfonamide;

HA1004:N-[2-guanidinoethyl]-5-isoquinolinesulfonamide hydrochloride;

KT5823: Highly specific peameable inhibitor of cGMP-dependent protein kinase (PKG);

ML-7: (5-Iodonaphthalene-1-sulfonyl)homopiperazine;

PD98059:2'-Amino-3'-methoxyflavone;

SB203580:4-(4-Fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole;

ERK: extracellular-regulated protein kinases;

p38: p38 mitogen-activated protein kinase;

ABSTRACT

Geldanamycin (GA) specifically binds to HSP90 and disrupts the interaction of HSP90 and its target proteins. The binding will lead to substrate protein dissociation from HSP90 and may affect their structures and functions. Herein, we showed that exposure of non-small lung cancer H460 cells to 0.5 mM GA leads to enhancement of the synthesis of the 70 kDa heat shock proteins (HSP70s). The induction of HSP70s by GA is concentration- and time-dependent and this process coincides to the accumulation of its mRNA. By using the specific probes for *hsp70-1* and *hsp70b*, we found that HSP70-1 is abundantly involved in GA-induced HSP70s. Furthermore importantly, we demonstrated for the first time that the PKC pathway is significant in this process since it is inhibited by H-7 and H-8 but not affected by HA1043. Similar results can be found in the protein and mRNA level. These data lead us to conclude that PKC pathway plays a major role in the GA-induced HSP70-1 expression in H460 cells. By using the electrophoretic mobility shift assay (EMSA), we showed that proximal Heat shock element (HSE1) was responsible for the inducible expression of HSP70. We found that the nuclear extracts prepared from GA-treated cells exhibited a significant increase in binding activity toward Heat shock element. Moreover, this increase in binding activity toward the Heat shock element is reduced by H-7 and H-8 but not affected by HA1004. We concluded that the PKC pathway is the major pathway involved in GA-induced expression of HSP70-1 in H460 cells.

INTRODUCTION

Geldanamycin (GA), a benzoquinoid antibiotic isolated from *Streptomyces hygroscopicus* is a highly selective binding agent of HSP90,[Smith et al., 1995] [Whitesell and Cook, 1996] [Neckers et al., 1999] which is a ubiquitous molecular chaperone [Pratt, 1997] [Csermely et al., 1998]. GA possess selective tumoricidal activity in preclinical models [Schulte and Neckers, 1998] and is developed to be an antitumor agent [Supko et al., 1995] [Kelland et al., 1999]. The cellular activity of several regulatory and signal transduction proteins such as nuclear hormone receptors [Pratt, 1997], transcription factors[Lees and Whitelaw, 1999] [Zou et al., 1998], and protein kinases [Chavany et al., 1996] [Ochel et al., 1999] [Schulte et al., 1995], which depend on the HSP90 and GRP94 molecular chaperones for folding, is markedly affected by GA. Thus, GA affects a number of essential cell signaling components and pathways.

In previous studies, GA has been further shown to function in regulating expression of a number of specific genes, such as c-myc [Yamaki et al., 1995], transferrin receptor gene [Hirsch and Miskimins, 1996], stress response genes [Lawson and Hendershot, 1998] [Xiao et al., 1999] and the gadd153/CHOP transcription factor [Lawson and Hendershot, 1998]. The activation of stress response genes by GA resulted in the synthesis of the stress proteins, including HSP70, HSP90, and GRP78 and GRP94 [Lawson and Hendershot, 1998]. With regards to the mechanism of HSP70 induction by GA, it has been demonstrated that human HSF1 can be activated by GA which then led to the expression of hsp70 [Zou et al., 1998] [Kim HR et al., 1999].

The human hsp70 family encompasses at least 11 genes which encode a group of

related proteins. These proteins include both constitutive/cognate and inducible members [Tavaria et al., 1996]. Among them, the nuclear-cytosolic resided HSP70s are the HSC70/HSP73 and HSP70s, which are resolved as two protein bands on SDS-PAGE gels with distinct apparent molecular mass [Murakami and Elzinga, 1991]. The situation is complicated by the fact that highly similar or even identical HSP70s can be coded by distinct genes which may be under differently regulatory controls. Thus far, only two of the promoters were reported and, fortunately, the coding regions of these two genes differ enough to be distinguished by specific Northern probes. These two genes are respectively designated as the *hsp70A* (the HSPA1A/HSP70-1 located on chromosome 6q21.3) [Hunt and Moromoto, 1985] [Wu et al.,1986] [Harrison et al.,1987] and the *hsp70B* (the HSPA7 located on chromsome 1q) [Leung et al.,1990] [Amin et al.,1994] [Arai et al.,1999]. In an initial attempt to characterize the GA-induced stress response in human H460 cells, we found that only the expression of HSP90s and the HSP70s are up-regulated. Herein, we found that HSP70-1 is the predominant protein of induced HSP70s in GA-treated H460 cells.

Furthermore, this study also aims to unravel the signaling events which are specifically elicited by GA and lead to the induced synthesis of HSP70-1. In the previous studies, both GA and its analog herbimycin A have been reported to induce hsp70 after been activated by the HSF-1 in mammalian cells [Kim et al.,1999] [Xiao et al.,1999]. However, until now, the mechanism of GA-induced hsp70 signal pathway is not very clear. And the heat shock protein induction pathway in lung cells is also not completely resolved [Wong and Wispe, 1997]. In eukaryotic cells, stress-induced expression of HSPs is regulated by HSFs, which acts through HSEs found in the promoters of hsp genes. The activation of HSF1 and expression of HSP70 involved in

many kinases such as PKC [Holmberg et al.,1998] [Ding et al.,1998] , p38[Hung et al.,1998] [Kim et al.,1997], ERK [Hung et al.,1998] and PKA [Kiang et al.,2000]. A battery of protein kinases including PD98059, SB203580, H7, H8, HA1004, KT5823 and ML-7 were thus employed. And we have found that PKC is involved in GA-induced hsp70-1. Here we showed for the first time that PKC pathway plays a major role in the process of HSP70-1 induction by GA in H460 cells.

EXPERIMENTAL PROCEDURES

Materials—Geldanamycin was purchased from Sigma (St. Louis, MO), dissolved in dimethylsulfoxide at a concentration of 1 mM, and stored in the dark at -20°C. It was diluted to appropriate concentrations with culture medium before use. All cultureware was purchased from Corning (Corning, NY) and culture medium components were purchased from Gibco Laboratories (Grand Island, NY). [^{35}S]methionine (specific activity >800 Ci/mmole), [α-^{32}P]dCTP (3,000 Ci/mmole) and [γ-^{32}P]ATP (5,000 Ci/mmole) were purchased from Amersham (Buckinghamshire, England). Alkaline-phosphatase conjugated antibody with against HSP70 (produced by human hsp70-1 peptide) was purchased from Stressgen Corp. Secondary antibody conjugated with alkaline-phosphatase to actin was purchased from Promega. Alkaline-phosphatase Detection system was purchased from Bio-Rad (Richmond, CA). Synthetic oligonucleotides were ordered from DNAFax. Chemicals for electrophoresis were from Bio-Rad (Richmond, CA). H-7, H-8, HA1004, ML7, KT5823, PD98059 and SB203580 were from Calbiochem (La Jolla, CA). Other chemicals were purchased from Merk (Darmstadt, Germany) or Sigma (St. Louis, MO).

Cells and Drug Treatment—The H460 human non-small cell lung cancer cells from ATCC were maintained in RPMI-1640 minimum essential medium plus 10% fetal bovine serum supplemented with 100 units/ml penicillin G and 100 mg/ml streptomycin in a 37°C incubator under 5% CO_2 and 95% air. Prior to each experiment, stock cells were plated in 25-cm^2 flasks or six-well plates at a density of 4-6 x 10^4 cells per cm^2. Exponentially growing cells at 80-90% confluence were used. To investigate the effects of GA, cells were treated with various concentrations of GA for various durations as indicated at 37°C. For the studies concerning the effects of inhibitors, the cells were

pre-incubated with respective inhibitors, at the specified concentrations for 1 h, followed by treatment with GA in the presence of the inhibitors.

Metabolic Labeling and Gel Electrophoresis — *De novo* protein synthesis was revealed by [^{35}S]methionine labeling at a concentration of 20 *m*Ci/ml. After various treatments, the cells were labeled for 1 h, washed twice with PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.4), and lysed in sample buffer [Laemmli, 1970]. Equal amounts of cell lysates were resolved by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [Laemmli, 1970]. After electrophoresis, the gels were fixed, dried, and processed for autoradiography as descried elsewhere [Lee et al., 1991]. Protein bands of interest were quantified by densitometric scanning (Molecular Dynamics, Inc., Sunnyvale, CA). The relative synthesis rate of HSP70 was presented as sum of pixel values of each band divided by that of actin in the same lane (internal control). 2-D PAGE was performed according to the method of O'Farrell [1975]. Equal amounts of cell lysates were loaded onto the pre-run isoelectrofocusing (IEF) gels and run for 16 h at 400 V and then 1 h at 800 V. Subsequently, the IEF gels were loaded onto 10% SDS-polyacrylamide slab gels with a 4.75% stacking gel for electrophoresis in the second dimension. After electrophoresis, the gels were processed as described above.

Immunoblot Analysis — Whole cell extracts were fractionated by SDS-PAGE and then transferred to nitrocellulose membrane in TBE buffer (50 mM Tris, 50 mM boric acid, 1 mM EDTA, pH 7.0) by using a semidry transfer apparatus according to the manufacturer's protocols (OWL Scientific). After been blocked with 3% nonfat milk in TTBS (0.5% Tween-20, 20 mM Tris-HCl, pH 7.4, 0.5 M NaCl) for 90 min and washed

once with TTBS, the membranes were incubated with a 1:2,000 dilution of respective antibodies against HSP70 and actin at room temperature for 12 h. Subsequently, the membranes were washed three times with TTBS, for 10 min each time, and incubated with 1:5,000 dilution of alkaline phosphotase-conjugated anti-mouse antibodies for 60 min. After been washed three times for 15 min each time with TTBS, the immunocomplexes were detected by the AP system and quantified by densitometric scanning.

Polymerase Chain Reaction (PCR) Genomic DNA was isolated from the H460 cells as described elsewhere [Gross et al., 1973] [Hung et al., 1998]. The PCR primers purchased from (Stressgen Corp.) produce the human *hsp70a* and *hsp70b* probes for Northern hybridization were, with respect to the coding sequences, 5'-TGTTCCGTTTCCAGCCCCCAA-3' (sense) and 5'-GGGCTTGTCTCCGTCGTTGAT-3' (antisense) for *hsp70a* and 5'-CTCCAGCATCCGACAAGAAGC-3' (sense) and 5'-ACGGTGTTGTGGGGGTTCAGG-3' (antisense) for *hsp70b*. The PCR reactions were performed in a volume of 50 ml in a thermal cycler (Hybaid, Teddington, UK). For each reaction, 50 pmole of oligonucleotide primer and 1 mg DNA template were incubated with 1 U of Bio-Thermal polymerase and 200 mM dNTPs according to the method of Saiki et al (Saiki, 1988). The following amplification protocol was used. The first denaturation step was carried out at 95 °C for 10 min. The following cycles consisted of denaturation at 95 °C for 1 min, 30 sec annealing at 50 °C and 30 sec extension at 72 °C. The reaction was allowed to proceed for 35 cycles. The amplified products were electrophoresed on 1 % agarose gels. The gels were stained with ethidium bromide and the DNA fragments with expected length were excised and

further purified by a commercial kit (Qiagen).

RNA Isolation and Northern Blotting— Total RNA was isolated from H460 cells according to the method of Chomczynski and Sacchi [1987] with minor modifications as previously described [Chen et al., 1998]. The RNA samples were subsequently fractionated on 1% agarose gels. After electrophoresis, the gels were incubated in 0.05 N NaOH for 30 min and washed with 2× standard saline citrate (SSC) buffer. The RNA samples were then electrotransferred onto nylon membranes (Hybond-N, Amersham) in 10×SSC buffer for at least 12 h. The membranes were then dried, and the RNA samples were fixed onto the membranes by using an ultraviolet cross-linker (Stratagene, La Jolla, CA). The oligonucleotide probes were labeled with [α -^{32}P]dCTP by Rediprime DNA Labeling System (Amersham). Following prehybridization, hybridization and washing step, the membranes were then dried and processed for autoradiography. Bands on autoradiograms were quantified by densitometric scanning using rRNAs as the internal controls.

Nuclear Extract Preparation—Nuclear extracts were prepared as described by Roy et al. [Roy et al., 1991] with some modifications [Chen et al., 1998]. In brief, T125 cells were trypsinized, collected by centrifugation at 1,000 x g for 8 min at 4 ℃, washed once with PBS, and centrifuged as above. Cells were resuspended in 3 ml NE-1 buffer (250 mM sucrose, 15 mM Tris-HCL, pH7.9, 140 mM NaCl, 2 mM EDTA, 0.5 mM EGTA, 0.15 mM spermidine, 1 mM dithiothreitol, 0.4 mM phenylmethylsulfonyl fluoride, 25 mM KCl and 2 mM $MgCl_2$) and homogenized by a Dounce grinder with 3 strokes. Nonidet P-40 was then added to a final concentration of 0.5% and the mixture

was incubated on ice for 5 min. Following another round of homogenization (6 more strokes), nuclei and cell debris were collected by centrifugation at 1000 x g for 10 min. Nuclei were washed with 5 ml of buffer NE-1 and centrifuged as above. The nuclei were then lysed by incubating the sample on ice for 5 min in 1 "Packed cell volume" (PCV) of NE-2 buffer (NE-1 buffer containing 350 mM KCl), followed by a 25-stroke homogenization. The homogenate was transferred to 1.5-ml microcentrifuge tubes and centrifuged at 18,000 x g for 90 min. The supernatant was dialyzed against dialysis buffer (20 mM Hepes, 100 mM KCl, 0.1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol and 20% glycerol) for 2 hr at 4 °C and then stored frozen at -70°C until use.

Electrophoretic Mobility Shift Assays (EMSA) — The regulatory elements binding activities of the nuclear extracts were determined by EMSA using double-stranded oligonucleotides as probes. The HSE oligonucleotide probes were prepared by annealing HSE-sense: 5'-GGCGAAACCCCTGGAATATTCCCGACCTG-3', and HSE-antisense: 5'-CAGGTCGGGAATATTCCAGGGGTTTCGCC-3', followed by end labeling with T4 polynucleotide kinase. Each gel shift reaction was carried out in a 15-µl volume in the presence of binding buffer (15 mM Hepes, pH 7.9, 100 mM KCl, 3 mM $MgCl_2$, 1 mM EDTA, 0.5 mM dithiothreitol, 10% glycerol and 1 mg of poly (dI-dC)). Nuclear extracts were added to the binding buffer, and the samples were incubated on ice for 5 min. After incubation, the DNA probe (2 x 10^4 cpm for each reaction) was added and incubated on ice for 15 min. The reaction mixtures were loaded onto an 8% nondenaturing polyacrylamide gel which had been pre-run for 1 hr in 0.5 x TBE buffer (50 mM Tris, 50 mM boric acid, 1 mM EDTA, pH 7.0) at 200 V until ampere stays steady. After electrophoresis, the gels were dried and processed for

autoradiography.

RESULTS

Effects of GA on Induction of HSP70 Synthesis—To assess the kinetics of induction of HSP70 synthesis, H460 cells were treated with various concentrations of GA for various incubation durations and the rates of HSP70 synthesis were monitored by metabolic labeling with [^{35}S]methionine. Bands of HSP70 in autoradiography were

quantified by densitometric scanning, and the actin was used as the internal control. For 6 h treatments, the level of HSP70 synthesis was initially enhanced about 6-fold when cells were treated with 0.2 μM GA (Fig.1), and could be further enhanced 8- to 9-fold, and reached its maximal level when GA concentration was increased to 0.5 μM (Fig.1). At higher concentrations, enhanced synthesis of HSP70 was found to level off (Fig. 1A, B). Enhanced synthesis of HSP70 in GA-treated cells was time-dependent. Under 0.5 μM GA, HSP70 synthesis was initially enhanced about 4-fold within 2 h and up to 8- to 9-fold after 6 h treatment, respectively (Fig. 2A, B). On the other hand, GA could also induce the synthesis of a number of stress proteins, including HSC70, HSP90, and HSP70 (Fig. 1). Taken together, the results indicated that GA-induced synthesis of HSP70 is in a concentration- and time-dependent manner. To determine whether HSP70 induction by GA consisted of different isoforms, the cell extracts were resolved by 2-D PAGE and the data indicates that only one major tailing HSP70 was induced . The identity of this protein band was confirmed to be HSP70-1 by western blotting analysis using hsp70 inducible form antibody produced by Stressgen using human HSP70-1 partial peptide(Fig.3).

Time-dependent accumulation of hsp70-1 mRNA in GA-treated H460 cells—We used specific hsp70a (hsp70-1) and hsp70b probes to distinguish the hsp70 genes that may be induced in the previous experiment. The result showed that hsp70a (hsp70-1) is much more responsive than that of hsp70b as shown in the expression profile (Fig.4). We measured the steady-state accumulation of *hsp70-1* mRNA in GA-treated cells by Northern blotting technique. The rRNAs were used as the internal controls (Fig.4). Cells were treated with 0.5 μM GA for various durations up to 24 h before RNA extraction. We found that hsp70-1 mRNA was highly induced with treatment of 0.5 μM

GA for 6 h. The kinetics of hsp70-1 mRNA accumulation and HSP70-1 protein synthesis are similar (Fig. 2).

Inhibitory Effects of H-7, H-8 and HA1004 on HSP70-1 Synthesis and hsp70-1 mRNA accumulation in GA-treated H460 Cells—As a first step to elucidate the possible involvement of signaling pathways in the induction of HSP70-1 under GA treatment, several protein kinase inhibitors involving kinase pathway in the induction of HSP70 including PD98059, SB203580 and H-7 were employed. We found that H-7 almost completely suppressed the GA-induced HSP70 protein synthesis while other inhibitors possessed little effect(data not shown). Since H-7 can affect many kinase such as myosin-light chain kinase (Ki=97 μM), protein kinase C (Ki=6.0 μM), protein kinase G (Ki=5.0 μM) and protein kinase A (Ki=3.0 μM). Therefore we use other kinase inhibitors (such as H-8, HA1004, ML-7, KT5823) to rule out other kinases which may involve in HSP70-1 induction. Among these inhibitors, HA1004 is a strong PKA inhibitor , H-8 has almost the same effect as H-7, ML-7 inhibits myosin-light chain kinase while KT5823 inhibits PKG. In the present study, our results demonstrated that only H-7 as well as H-8 inhibited HSP70-1 gene expression and protein synthesis. In contrast, HA1004, KT5823 and ML-7 did not affect HSP70-1 protein synthesis (Fig 5.). Both H-7 and H-8 abolished the induction of HSP70-1 synthesis and *hsp70-1* mRNA accumulation completely, but the process was not affected by HA1004 (Fig. 6). The total protein of HSP70-1 was also analysed by Western blotting against HSP70-1 and similar results were obtained (Fig. 7). Taken together these results clearly indicated that protein kinase C is involved in the GA-induced HSP70-1 synthesis.

Interaction of nuclear factors extracted from GA-treated H460 cells with the heat

shock element from the promoter region of human hsp70-1—In a first step to characterize the molecular mechanisms underlying the induction of *hsp70-1*, which is transcriptionally regulated in H460 cells, we analyzed the binding activities of nuclear proteins extracted from the GA-treated cells to heat shock element, derived from the promoter region of human *hsp70-1 (*Wu et al.1986). Using EMSA assay, we found that the nuclear extracts from control cells were reacted with the heat shock element to form DNA-protein complexes as indicated (Fig. 8). Significant difference of complex (complex I) binding to the heat shock element were detected in GA-treated cells (Fig. 8). Furthermore, we investigated the effects of H-7 and H-8 on the binding activities of nuclear factors to the heat shock element. Again, both H-7 and H-8, but not HA1004 suppressed the binding activities of nuclear factors to the heat shock element (complex I) (Fig. 9). These results demonstrate that GA activates the binding activities of heat shock element in the treated cells. And the binding activity of heat shock element was reduced to the control level after H-7 and H-8 treatment. In conclusion, the heat shock element and PKC pathway are both involved in the induction of *hsp70-1*.

FIGURE LEGENDS

Fig.1. Concentration-dependent effect of GA on the induction of HSP70 synthesis in H460 cells. Cells were treated with GA at the concentrations as indicated for 5 h and labeled with [^{35}S] methionine for 1 h absent of drug before lysed. Equal amounts of cell lysates were resolved by SDS-PAGE followed by autoradiography (A).Bands of HSP70 and actin in the autoradiograms as shown in A were quantified by densitometric scanning, and the relative synthesis rate of HSP70 was presented as sum of the pixel values of each band divided by that of actin in the same lane (internal control) (B). Data

represent the means ±S.D. of three independent experiments.

Fig.2.Time-dependent effect of GA on the induction of HSP70 synthesis in H460 cells. Cells were treated with 0.5 mM GA for various durations as indicated. The cells were then labeled with [^{35}S] methionine for 1 h before lysed. The cell lysates were processed for autoradiograghy as indicated (A). The relative synthesis rate of HSP70 was determined as described in the legend of Fig. 1. Data represent the means ±S.D. of three independent experiments.

Fig.3. Two-dimensional gel analysis of the differences of the control and GA-induced changes of HSP70. Cells were untreated (A) or treated (B) with 0.5 μM of GA for 5h then labeled with [^{35}S] methionine for 1 h before lysed. The cell lysates were then processed for autoradiogrpghy. Data represent the means ±S.D. of three independent experiments. The direction of electrophoresis was shown at the top of the panels. Under same condition, (C) and (D) showed. The results of Western blotting against HSP70-1.

Fig.4. Time course of *hsp70-1* mRNA accumulation in GA-treated H460 cells. Cells were treated with 0.5 mM GA for various durations as indicated. After treatment, total cytoplasmic RNA was extracted and analyzed for the expression of *hsp70* mRNA by Northern blotting with α-^{32}P-labeled *hsp70a* probe and *hsp70b* probe (A). The relative levels of *hsp70* mRNA were presented as the sum of the pixel values after background subtraction (B). The data are the mean ±S.D. from three independent experiments.

. Fig.5. Inhibitory effects of H-7, H-8 and HA1004 on HSP70-1 induction in GA-treated H460 cells. Cells were respectively pre-incubated for 1 h with 100 μM H-7, 100 μM H-8, 100 μM HA1004, 2 μM ML-7 and 2 μM KT5823. The cells were then treated with 0.5 μM GA for 6 h in the presence of inhibitors. After treatment, the cells were labeled with [^{35}S] methionine for 1 h. The cell lysates were then processed for autoradiography. Shown are the autoradiogram (A) and quantitation of the relative synthesis rate of HSP70 (B). Data represent the means ±S.D. of three independent experiments.

Fig.6. Inhibitory effects of H-7, H-8 and HA1004 on the induction of *hsp70-1* mRNA in GA-treated H460 cells. Cells were pre-incubated for 1 h with 100 μM H-7, 100 μM H-8 and 100 μM HA1004 and then treated with 0.5 μM GA for 6 h in the presence of drugs. After treatment, the same experimental procedures were performed as in Fig. 4. Shown are the Northern blotting (A) and the relative levels of *hsp70* mRNA (B). The data are the mean ±S.D. from three independent experiments.

Fig.7.Effects of H-7, H-8 and HA1004 on HSP70 in GA-treated H460 cells. In Western blot analysis, cells were pre-incubated with 100 μM H-7, 100 μM H-8 and 100 μM HA1004 for 1 h and then treated with 0.5 μM GA in the presence of each inhibitor for 6 h. Total amounts of HSP70 were presented divided by that of actin in the same lane (internal control).

Fig.8. Interaction of nuclear factors extracted from GA-treated H460 cells with the regulatory elements from the promoter of human hsp70-1. Cells were treated with 0.5 μM GA for various durations as indicated. The nuclear extracts were prepared from treated cells (see "Experimental Procedures"). Synthetic HSE oligonucleotides of Human *hsp70-1* were annealed, end-labeled, and mixed with the nuclear extracts and analyzed in the EMSA. Lane 1 contained only labeled DNA. Labeled probes were reacted with nuclear extracts prepared from untreated cells (Lane 2), and cells were treated with GA for 2, 4 and 6 h respectively (Lane 3-5). The autoradiogram is shown. Complexes I, II were marked as indicated. Similar results were observed in three independent experiments.

Fig.9. Effects of H-7, H-8 and HA1004 on the binding activities of nuclear factors to HSE in GA-treated H460 cells. Cells were pre-incubated with 100 μM H-7, 100 μM H-8 and 100 μM HA1004 for 1 h and then treated with 0.5 μM GA for 2 h. Nuclear extracts were prepared from cells after treatment. Synthetic oligonucleotides corresponding to the HSE of human *hsp70-1* were annealed, end-labeled, and mixed with the nuclear extracts. The DNA-protein complexes were resolved by 8% nondenaturing PAGE and visualized by autoradiography. Complex I was marked as indicated.

DISCUSSION

Herein we report for the first time that the PKC pathway is involved in GA-induced stress and this process is crucial for the GA-induced expression of HSP70-1 in H460 cells. In this study, we have shown that GA effect through the PKC pathway results in the transactivation of *hsp70* gene, and this process is abolished by H-7 or H-8 completely, and not affected by HA1004. In resolving which HSP70 isoform, we almost didn't observe *hsp70b* gene expression compared that we surveyed *hsp70-1* gene (Fig 4.). So we thought the HSP70-1 is more responsive in GA-treated H460 cells , and HSP70B is an inducible instead of constitutive form. The reason perhaps GA and its analog HA, similarly resulted in increased expression of the constitutivly expressed stress proteins. [Hegde et al., 1995 and Hung ,unpublished data] So the next experiments, we take much focus on *hsp70-1 gene*.

With regard to the HSP70-1 expression, signaling pathway concerning PKC pathway has been studied in a variety of experiments. [Holmberg et al.,1998] [Lee et al.,1994] [Lee et al.,1994] [Lee et al.,1995] [Ohnishi et al.,1998] [Osaki et al.,1998] [Ding et al.,1996] It has been shown that heat shock activate PKC as well as HSP70 in the heat shock-treated T47-D cells, a human breast cancer cell [Kiang et al.,1998]. Activation of PKC can cause phosphorylation of HSF1, which leads to an enhanced but transient increase in HSP-70 production in human epidermoid A431 cells. [Ding et al.,1997] However, the relationship between the PKC and transactivation of *hsp70-1* gene has not been elaborated upon in these studies. Nevertheless, we pioneered to establish that there is a causal relationship between the PKC and transactivation of the *hsp70-1* gene in GA-treated cells.

Some studies using protein kinase inhibitors have suggested that protein phosphorylation plays an important role in regulation of heat shock protein gene expression. [Erdos and lee, 1994] [Lee et al.,1994] [Ohnishi et al.,1998] [Ohnishi et al.,1999] The synthesis of *hsp70* mRNA and accumulation of HSP70 in human carcinoma cells are suppressed by the protein kinase inhibitors H-7, H-8 and STP [Erdos and lee, 1994] [Lee et al.,1994] [Ohnishi et al.,1998] [Ohnishi, et al.,1999]. However, there have been no reports describing significant effects of protein kinase inhibitors on activation of HSP70-1 by GA. We demonstrated here the HSP70 protein expression, mRNA expression are all suppressed by H-7. H-7 inhibits many kinases such as PKC, PKA, PKG, and myosin light chain kinase. So we use more specific kinases inhibitors to rule out the contribution of other PKs-mediated signal transduction pathways to HSP70-1 induction by GA.(Fig.4) Our results suggested that the PKC is important in HSP70-1 expression in GA-treated H460 cells.

To further study what parts play a important role in human *hsp70-1* promoter. Previously studies have been shown that the HSE is essentially involved in induction of HSP70-1 under heat shock [Stephanou et al.,1999] [Hatayama and Hayakawa, 1999]. However, the mechanism of transactivation of *hsp70* gene under GA treatment has not been fully characterized. In lately research, the protein kinase inhibitor (H-7) suppresses heat induced activation of heat shock transcription factor 1 was described [Ohnishi, 1999]. Herein, we showed that aforementioned element participate in the inducible expression of HSP70-1 in H460 cells. The increased binding activities of nuclear factors toward the HSE is observed in GA-treated cells. One band was presented in the lower place in the control cell (Fig.8). A factor (CHBF), which constitutively binds to the HSE at 37 ^{0}C, functions like a negative regulator and the heat-induced HSFs act as an activator. [Yang et al.,1996] [Kim et al.,1995] So the complex II could act like CHBF as a repressor. Upon GA treatment, following the dissociation of CHBF-HSE, then the other factors (ex:HSFs) can bind to the region to transactivate the *hsps* gene. Subsequently, due to the different inhibition mechanisms of inhibitors H-7, H-8 and HA1004, we have shown that the DNA-protein complexes formation were abolished to complex I by H-7 and H-8 but not by HA1004 which further demonstrated that the protein kinase C is important in the HSP70-1 expression in GA-treated H460 cells.

It is very interesting to know how GA, a kind of tyrosine kinase inhibitor , affect the HSF-1 phosphorylation and further to turn on *hsp70*. Because HSF-1 not phosphorylated in any other tyrosine residues [Cotto, 1996]. This implies that inhibitor

must occur indirect influences on other kinases and phosphatases which direct affect the phosphorylation status of HSF-1. Further work will be needed to elicit HSF-1 phosphorylation by PKC in GA-induced HSP70-1.

In summary, we have shown that the majority of GA-induced HSP70-1 major through PKC pathway. The signaling pathways from the upward and downward to the transactivation of *hsp70-1* gene warrants further investigation. Nevertheless, we provided the first evidence that PKC pathway plays a major role in GA-induced *hsp70-1* expression. Furthermore, we showed that the previous identified HSP70-1 is the major involved in the GA-induced expression of HSP70s, and the HSE plays the most crucial role.

Reference

1. Neckers, L., Schulte, TW., Mimnaugh, E. (1999) Geldanamycin as a potential anti-cancer agent: its molecular target and biochemical activity. *Invest New Drugs.* **17,** 361-73

1. Smith, D. F., Whitesell, L., Nair, S. C., Chen, S., Prapapanich, V., and Rimerman, R. A. (1995) Progesterone receptor structure and function altered by geldanamycin, an hsp90-binding agent. *Mol. Cell. Biol.* **15,** 6804-6812

2. Whitesell, L., and Cook, P. (1996) Stable and specific binding of heat shock protein 90 by geldanamycin disrupts glucocorticoid. *Mo.l Endocrinol.* **10,** 705-712

3. Pratt, W. B. (1997) The role of the *hsp90*-based chaperone system in signal transduction by nuclear receptors and receptors signaling via MAP kinase. *Annu. Rev. Pharmacol. Toxicol.* **37,** 297-326

4. Csermely, P., Schnaider, T., Soti, C., Prohaszka, Z., and Nardai, G. (1998) The 90-

kDa molecular chaperone family: structure, function, and clinical applications. A comprehensive review. *Pharmacol. Ther.* **79,** 129-168

5. Schulte, T.W., and Neckers, L. M. (1998) The benzoquinone ansamycin 17-allylamino-17-demethoxygeldanamycin binds to HSP90 and shares important biologic activities with geldanamycin. *Cancer. Chemother. Pharmacol.* **42,** 273-279

6. Supko, J. G., Hickman, R. L., Grever, M. R., Malspeis, L. (1995) Preclinical pharmacologic evaluation of geldanamycin as an antitumor agent. *Cancer Chemother Pharmacol* **36,** 305-315

6. Kelland, L.R., Sharp, S. Y., Rogers, P.M., Myers, T. G., and Workman, P. (1999) DT-Diaphorase expression and tumor cell sensitivity to 17-allylamino, 17-demethoxygeldanamycin, an inhibitor of heat shock protein 90.*J Natl Cancer Inst.* **91,** 1940-1949

7. Lees, M. J., and Whitelaw, M. L. (1999) Multiple roles of ligand in transforming the dioxin receptor to an active basic helix-loop-helix/PAS transcription factor complex with the nuclear protein Arnt. *Mol. Cell. Biol.* **19,** 5811-5822

8. Zou, J., Guo, Y., Guettouche, T., Smith, D. F., and Voellmy, R. (1998) Repression of heat shock transcription factor HSF1 activation by HSP90 (HSP90 complex) that forms a stress-sensitive complex with HSF1. *Cell.* **94,** 471-480

9. Chavany, C., Mimnaugh,. E., Miller, P., Bitton, R., Nguyen, P., Trepel, J., Whitesell, L., Schnur, R., Moyer, J., and Neckers, L. (1996) p185erbB2 binds to GRP94 in vivo. Dissociation of the p185erbB2/GRP94 heterocomplex by

benzoquinone ansamycins precedes depletion of p185erbB2.*J. Biol. Chem.* **271,** 4974-4977

10. Ochel, H. J., Schulte, T., Nguyen, P., Trepel, J., and Neckers, L. (1999) The benzoquinone ansamycin geldanamycin stimulates proteolytic degradation of focal adhesion kinase.*Mol. Genet. Metab.* **66,** 24-30
11. Schulte, T. W., Blagosklonny, M., Ingui, C., and Neckers, L. (1995) Disruption of the Raf-1-Hsp90 molecular complex results in destabilization of Raf-1 and loss of Raf-1-Ras association.*J. Antibiot. (Tokyo)* **48,** 1021-1026
12. Yamaki, H., Nakajima, M., Seimiya, H., Saya, H., Sugita, M., and Tsuruo, T. (1995) Inhibition of the association with nuclear matrix of pRB, p70 and p40 proteins along with the specific suppression of c-MYC expression by geldanamycin, an inhibitor of Src tyrosine kinase.*J. Antibiot. (Tokyo)* **48,** 1021-1026
13. Hirsch, S., Miskimins, R., and Miskimins, W. K. (1996) Mitogenic activation of the transferrin receptor gene promoter is modulated by inhibitors of tyrosine kinases and tyrosine phosphatases.*Recept. Signal. Transduct.* **6,** 121-129
14. Lawson, B., Brewer, J., and Hendershot, L. M. (1998) Geldanamycin, an hsp90/GRP94-binding drug, induces increased transcription of endoplasmic reticulum (ER) chaperones via the ER stress pathway.*J. Cell. Physiol.* **174,** 170-178
15. Xiao, N., Callaway, C. W., Lipinski, C. A., Hicks, S. D., and DeFranco, D. B. (1999) Geldanamycin provides posttreatment protection against glutamate-induced oxidative toxicity in a mouse hippocampal cell line.*J. Neurochem.* **72,** 95-101
16. Kim, H. R., Kang, H., and Kim, H. D. (1999) Geldanamycin induces heat shock protein expression through activation of HSF1 in K562 erythroleukemic cells.

IUBMB. Life. **48,** 429-433

17. Tavaria, M., Gabriele, T., Kola, I., and Anderson, R. L (1996) A hitchhiker's guide to the human Hsp70 family.*Cell. Stress. Chaperones.* **1**, **23-28**.

18. Murakami, N., Mehta, P., and Elzinga, M. (1991). Studies on the distribution of cellular myosin with antibodies to isoform-specific synthetic peptides.*FEBS Lett* **278**: 23-25.

19. Hunt, C., and Morimoto, R. I. (1985) Conserved features of eukaryotic hsp70 genes revealed by comparison with the nucleotide sequence of human hsp70.*Proc. Natl. Acad. Sci. U. S .A* .**82,** 6455-6459

20. Wu, B. J., Kingston, R. E., and Morimoto, R. I. (1986) Human HSP70 promoter contains at least two distinct regulatory domains.*Proc. Natl. Acad. Sci. U. S. A* .**83,** 629-633

21. Harrison, G. S., Drabkin, H. A., Kao, F. T., Hartz, J., Hart, I. M., Chu, E.H., Wu, B. J., and Morimoto, R. I. (1987) Chromosomal location of human genes encoding major heat-shock protein HSP70.*Somat. Cell. Mol. Genet*.**13,** 119-130

22. Leung, T. K., Rajendran, M. Y., Monfries, C., Hall, C., and Lim, L. (1990) The human heat-shock protein family. Expression of a novel heat-inducible HSP70 (HSP70B') and isolation of its cDNA and genomic DNA.*Biochem. J*.**267,** 125-132

23. Amin, J., Fernandez, M., Ananthan, J., Lis, J. T., and Voellmy, R. (1994) Cooperative binding of heat shock transcription factor to the Hsp70 promoter in vivo and in vitro.*J. Biol. Chem.* **269,** 4804-4811

24. Arai, Y., Kubo, T., Kobayashi, K., Ikeda, T., Takahashi, K., Takigawa, M., Imanishi, J., and Hirasawa, Y. (1999) Control of delivered gene expression in chondrocytes using heat shock protein 70B promoter.*J. Rheumatol.* **26,** 1769-1774

25. Wong, H. R., and Wispe, J. R. (1997) The stress response and the lung.*Am. J. Physiol.* **273,** L1-9

26. Holmberg, C. I., Roos, P. M., Lord, J. M., Eriksson, J. E., and Sistonen, L. (1998) Conventional and novel PKC isoenzymes modify the heat-induced stress response but are not activated by heat shock. *J. Cell. Sci*.**111,** 3357-3365

27. Ding, X. Z., Tsokos, G. C., and Kiang, J. G. (1998) Overexpression of HSP-70 inhibits the phosphorylation of HSF1 by activating protein phosphatase and inhibiting protein kinase C activity.*FASEB. J*.**12,** 451-459

28. Hung, J. J., Cheng, T. J., Lai, Y. K., and Chang, M. D. (1998) Differential activation of p38 mitogen-activated protein kinase and extracellular signal-regulated protein kinases confers cadmium-induced HSP70 expression in 9L rat brain tumor cells.*J. Biol. Chem.* **273,** 31924-31931

29. Kim, J., Nueda, A., Meng, Y. H., Dynan, W. S., and Mivechi, N. F. (1997) Analysis of the phosphorylation of human heat shock transcription factor-1 by MAP kinase family members.*J. Cell. Biochem.* **67,** 43-54

30. Kiang, J. G., Gist, I. D., and Tsokos, G. C. (2000) Regulation of heat shock protein 72 kDa and 90 kDa in human breast cancer MDA-MB-231 cells.*Mol. Cell. Biochem.* **204,** 169-178

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

32. Lee, W. C., Lin, K. Y., Chen, C. M., Chen, Z. T., Liu, H. J., and Lai, Y. K. (1991) Induction of heat-shock response and alterations of protein phosphorylation by a novel topoisomerase II inhibitor, withangulatin A, in 9L rat brain tumor cells.*J. Cell. Physiol.* **49,** 66-76

33. PH, O. F. (1975) High resolution two-dimensional electrophoresis of

proteins.*J. Biol. Chem.* **250,** 4007-4021

34. Gross-Bellard, M., Oudet, P., and Chambon, P. (1973) Isolation of high-molecular-weight DNA from mammalian cells.*Eur. J. Biochem.* **36,** 32-38

35. Saiki, R. K., Gelfand, d. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B. and Erlich, H. A. (1988) Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase.*Science.* **239**, 487-491.

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

37. Chen, K. D., Chen, L. Y., Huang, H. L., Lieu, C. H., Chang, Y. N., Chang, M. D., and Lai, Y. K. (1998) Involvement of p38 mitogen-activated protein kinase signaling pathway in the rapid induction of the 78-kDa glucose-regulated protein in 9L rat brain tumor cells.*J. Biol. Chem.* **273,** 749-755

38. Roy, R. J., Gosselin, P., and Guerin, S. L. (1991) A short protocol for micro-purification of nuclear proteins from whole animal tissue.*BioTechniques.* **11,** 770-777

39. Hegde, R.S., Zuo, J., Voellmy, R., and Welch, W. J. (1995) Short circuiting stress protein expression via a tyrosine kinase inhibitor, herbimycin A.*J Cell Physiol,* **165,** 186-200

40. Lee, Y. J., Berns, C. M., Erdos, G., Borrelli, M. J., Ahn, C. H., and Corry, P. M. (1994) Effect of 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H-7) on HSP70 and HSP28 gene expression and thermotolerance development in human colon carcinoma cells.*Biochem. Pharmacol.* **48,** 2057-2063

41. Lee, Y. J., Berns, C. M., Erdos, G., and Corry, P. M. (1994) Effect of isoquinolinesulfonamides on heat shock gene expression during heating at 41

degrees C in human carcinoma cell lines.*Biochem. Biophys. Res. Commun.* **199,** 714-719

42. Lee, Y. J., Berns, C. M., Galoforo, S., Erdos, G., Cho, J. M., and Corry, P. M. (1995) Differential effect of 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H-7) on alpha B-crystallin and hsp70 gene expression in murine cell lines.*Biochem. Pharmacol.* **50,** 1149-1155

43. Ohnishi, K., Wang, X., Takahashi, A., Matsumoto, H., Aoki, H., and Ohnishi, T. (1998). Effects of protein kinase inhibitors on heat-induced hsp72 gene expression in a human glioblastoma cell line.*Cell. Signal.* **10**: 259-264.

44. Osaki, J., Haneda, T., Kashiwagi, Y., Oi, S., Fukuzawa, J., Sakai, H., and Kikuchi, K. (1998) Pressure-induced expression of heat shock protein 70 mRNA in adult rat heart is coupled both to protein kinase A-dependent and protein kinase C-dependent systems.*J. Hypertens.* **16,** 1193-1200

45. Ding, X. Z., Smallridge, R. C., Galloway, R. J., and Kiang, J. G. (1996) Increases in HSF1 translocation and synthesis in human epidermoid A-431 cells: role of protein kinase C and [Ca2+]i.*J. Invest. Med.* **44,** 144-153

46. Kiang, J. G., Gist, I. D., and Tsokos, G. C. (1998) Cytoprotection and regulation of heat shock proteins induced by heat shock in human breast cancer T47-D cells: role of [Ca2+]i and protein kinases.*FASEB. J.* **12,** 141571-141579

47. Ding, X. Z., Tsokos, G. C., and Kiang, J. G. (1997) Heat shock factor-1 protein in heat shock factor-1 gene-transfected human epidermoid A431 cells requires phosphorylation before inducing heat protein-70 production.*J. Clin. Invest.* **99,** 136-143

48. Erdos, G., and Lee, Y. J. (1994) *Biochem. Biophys. Res. Commun.* **202,** 476-483

49. Ohnishi, K., Wang, X., Takahashi, A., Matsumoto, H., Aoki, H., and Ohnishi, T.

(1998) Effects of protein kinase inhibitors on heat-induced hsp72 gene expression in a human glioblastoma cell line.*Cell. Signal.* **10,** 259-264

50. Ohnishi, K., Wang, X., Takahashi, A., Matsumoto, H., and Ohnishi, T. (1999) The protein kinase inhibitor, H-7, suppresses heat induced activation of heat shock transcription factor 1.*Mol. Cell. Biochem.* **197,** 129-135

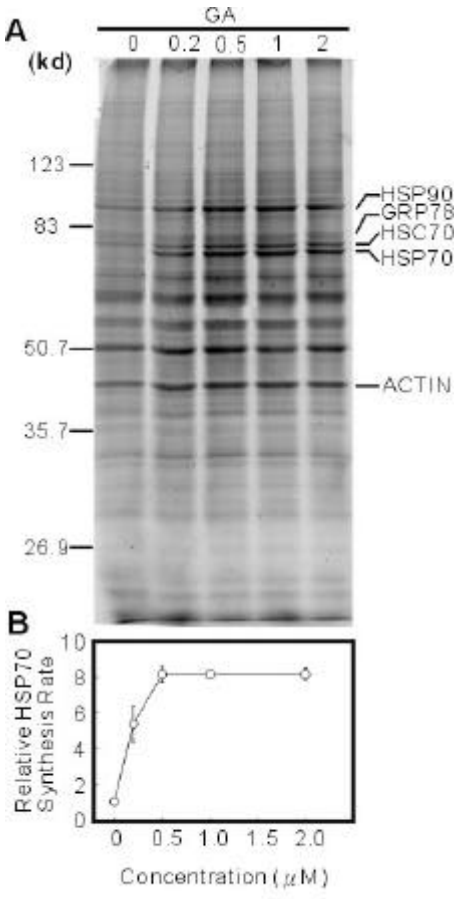
51. Stephanou, A., Isenberg, D. A., Nakajima, K., and Latchman, D. S. (1999) Signal transducer and activator of transcription-1 and heat shock factor-1 interact and activate the transcription of the *Hsp70* and *Hsp90*beta gene promoters.*J. Biol. Chem.* **274,** 1723-1728

52. Hatayama, T., and Hayakawa, M. (1999) Differential temperature dependency of chemical stressors in HSF1-mediated stress response in mammalian cells.*Biochem. Biophys. Res. Commun.* **265,** 763-769

53. Yang, S.H., Nussenzweig, A., Li, L., Kim, D., Ouyang, H., Burgman, P., and Li G. C. (1996) Modulation of thermal induction of hsp70 expression by Ku autoantigen or its individual subunits.*Mol Cell Biol*, **16,** 3799-3806

54. Kim, D., Ouyang, H., Yang, S.H., Nussenzweig, A., Burgman, P., and Li, G. C. **(**1995) A constitutive heat shock element-binding factor is immunologically identical to the Ku autoantigen.*J Biol Chem,* **270,** 15277-15284

55. Cotto, J. J., Kline, M., and Morimoto, R. I. (1996) Stress-induced activation of the heat-shock response: cell and molecular biology of heat-shock factors.*J. Biol. Chem.* **271,** 3355-3358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GA
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(kd)
123
83
50.7
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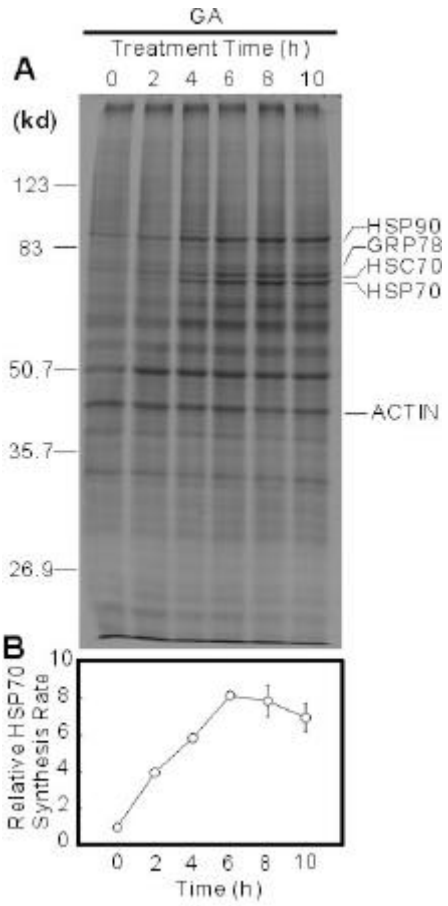
HSP90
GRP78
HSC70
HSP70
ACTIN
B
Relative HSP70 Synthesis Rate
0 2 4 6 8 10
0 0.5 1.0 1.5 2.0
Concentration (μM)

Fig. 1


GA
Treatment Time (h)
A
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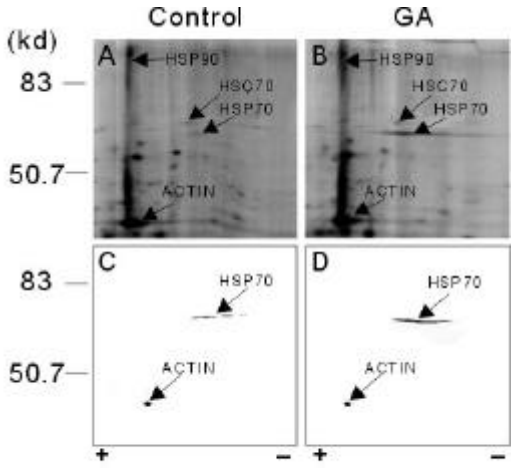
(kd)
123
83
50.7
35.7
26.9
HSP90
GRP78
HSC70
HSP70
ACTIN
B
Relative HSP70 Synthesis Rate
10
8
6
4
2
0
0 2 4 6 8 10
Time (h)

Fig. 2


Control
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(kd)
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HSP90
HSC70
HSP70
ACTIN
B
HSP90
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HSP70
ACTIN
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ACTIN
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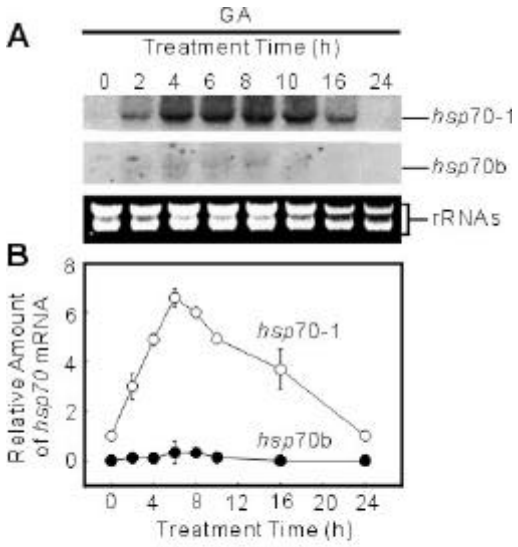
Fig. 3


A
GA
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hsp70b
rRNAs
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hsp70b
0 4 8 12 16 20 24
Treatment Time (h)

Fig. 4

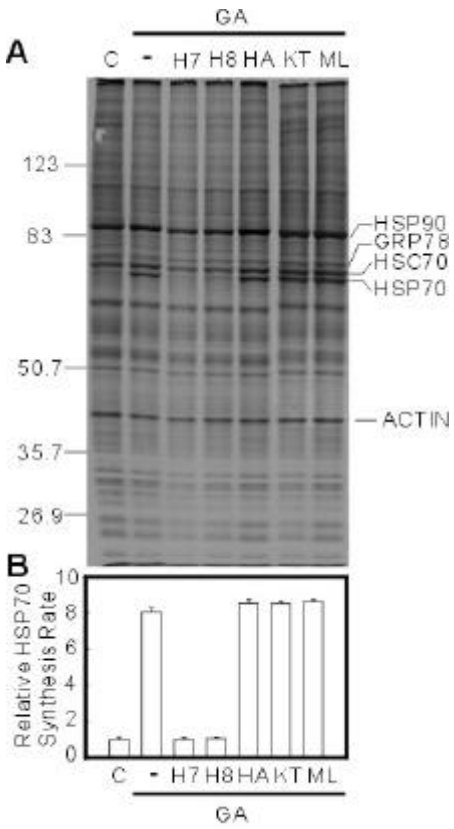
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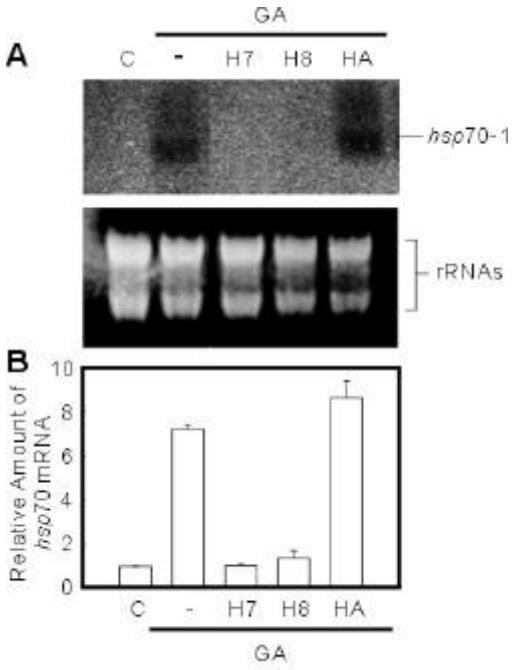
Fig. 5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GA

Fig. 6

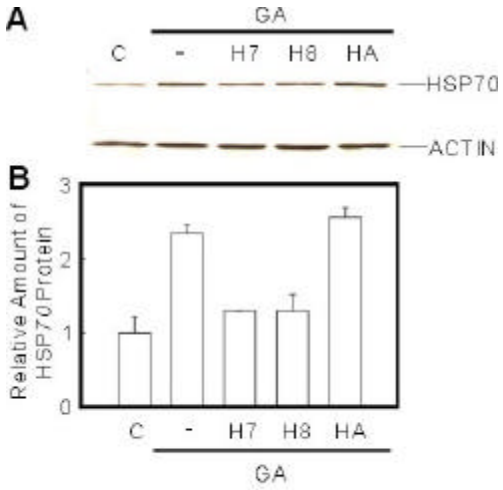
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Fig. 7

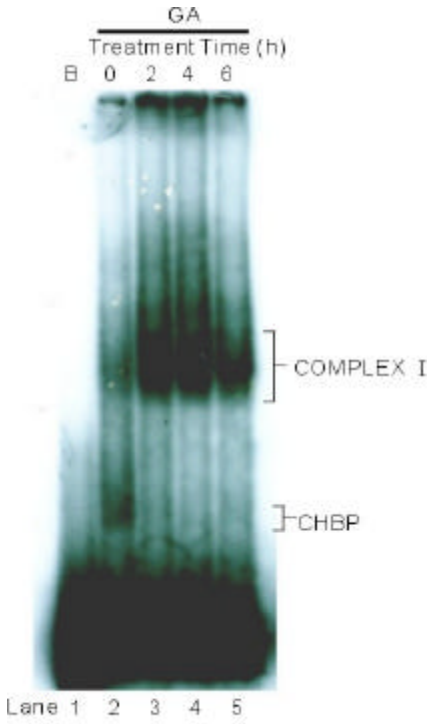
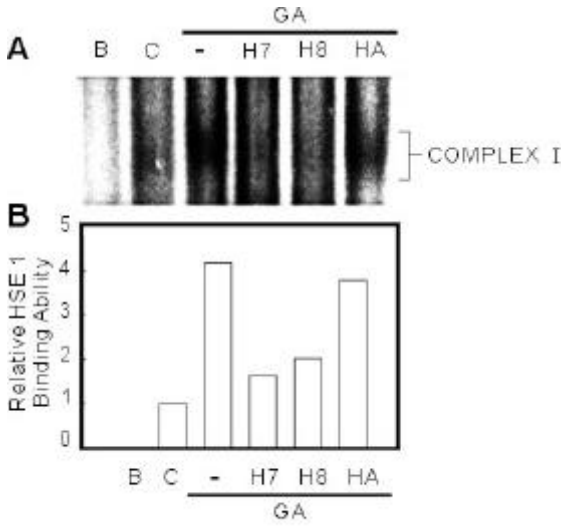


Fig. 8


GA
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Relative HSE 1 Binding Ability
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H8
HA
GA

Fig. 9