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Transactivation of *hsp70-1/2* in Geldanamycin-Treated Human Non-Small Cell Lung Cancer H460 Cells: Involvement of Intracellular Calcium and Protein Kinase C

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Abstract Geldanamycin is an antitumor drug that binds HSP90 and induces a wide range of heat shock proteins, including HSP70s. In this study we report that the induction of HSP70s is dose-dependent in geldanamycin-treated human non-small cell lung cancer H460 cells. Analysis of the induction of HSP70s specific isoform using LC-ESI-MS/MS analysis and Northern blotting showed that HSP70-1/2 are the major inducible forms under geldanamycin treatment. Transactivation of *hsp70-1/2* was determined by electrophoretic mobility-shift assay using heat shock element (HSE) as a probe. The signaling pathway mediators involved in *hsp70-1/2* transactivation were screened by the kinase inhibitor scanning technique. Pretreatment with serine/threonine protein kinase inhibitors H7 or H8 blocked geldanamycin-induced HSP70-1/2, whereas protein kinase A inhibitor HA1004, protein kinase G inhibitor KT5823, and myosin light chain kinase inhibitor ML-7 had no effect. Furthermore, the protein kinase C (PKC)-specific inhibitor Ro-31-8425 and the Ca^{2+}-dependent PKC inhibitor Gö-6976 diminished geldanamycin-induced HSP70-1/2, suggesting an involvement of the PKC in the process. In addition, geldanamycin treatment causes a transient increase of intracellular Ca^{2+}. Chelating intracellular Ca^{2+} with BAPTA-AM or depletion of intracellular Ca^{2+} store with A23187 or thapsigargin significantly decreased geldanamycin-transactivated HSP70-1/2 expression. Taken together, our results demonstrate that geldanamycin-induced specific HSP70-1/2 isoforms expression in H460 cells through signaling pathway mediated by Ca^{2+} and PKC. J. Cell. Biochem. 94: 1199–1209, 2005.

Key words: geldanamycin; heat-shock protein; intracellular calcium; protein kinase C

Geldanamycin, a benzoquinone ansamycin, is a naturally occurring antiproliferative drug that is active in several tumor cell lines as well as in animal models [Supko et al., 1995; Neckers et al., 1999]. The molecular target of geldanamycin is the HSP90 family chaperones and the binding of geldanamycin to HSP90 leads to the destabilization of the chaperone complex with

Abbreviations used: NSCLC cells, non-small cell lung cancer cells; HSP70, 70 kDa heat shock protein; ESI, electrospray ionization; MS/MS, tandem mass spectrometry; HSF1, heat shock factor 1; EMSA, electrophoretic mobility-shift assay; HSE, heat shock element; PKA, protein kinase A; PKC, protein kinase C; PKG, protein kinase G; MLCK, myosin light-chain kinase; H7, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine; H8, N-(2-[methylamino]ethyl)-5-isoquinoline sulfonamide; HA1004, N-[2-guanidinoethyl]-5-isoquinolinesulfonamide hydrochloride; Ro-31-8425, 2-[8-(Aminomethyl)-6,7,8,9-tetrahydropyrido-[1,2-a]indol-3-yl]-3-(1-methyl-1H-indol-3-yl) maleimid; Gö-6976, 12-(2-Cynnoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5H-indolo(2,3a)pyrrolo(3,4-c)-carbazole; $[Ca^{2+}]_i$, cytosolic Ca^{2+} concentration; $[Ca^{2+}]_o$, extracellular Ca^{2+} concentration; TG, thapsigargin; BAPTA-AM, bis-(o-aminophenoxy) ethane-*N*,*N*,*N*′,*N*′-tetra-acetic acid tetrakis (acetoxymethyl ester).

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its client proteins rendering them available for proteasomal degradation [Whitesell et al., 1997]. HSF1 is one of the client proteins of HSP90, but disruption of the HSP90-HSF1 chaperone complex facilitates the activation of HSF1 [Bharadwaj et al., 1999]. In fact, treatment with geldanamycin, triggers the expression of other chaperone HSPs, including HSP70s, in several cell lines and in rat brain [Sittler et al., 2001; Huang et al., 2002; Lu et al., 2002]. Unfortunately, the induction of specific HSP70 isoform(s) by geldanamycin has not been studied in detail.

Induction of HSPs is the hallmark of the heat shock and is responsible for the protection of cytotoxicity inflicted by adverse environments [Jaattela, 1999; Xanthoudakis and Nicholson, 2000]. The most well studied HSPs are the family with molecular weights around 70 kDa, collectively termed the HSP70s. In humans, the HSP70 family encompasses multiple distinct genes, which encode a group of highly related proteins, including the mitochondria resided GRP75 (HSPA9B locus), the endoplasmic reticulum (ER) resided GRP78 (HSPA5 locus), cytosol/nuclear resided HSC70 (the cognate/constitutive HSP70, HSP72/73; HSPA8 locus), and also cytosol/nuclear resided but highly inducible HSP70 (HSP70/72; HSPA1A, HSPA1B, and HSPA6 loci). Recent data suggested that there are three stress-inducible HSP70-encoding genes in humans. Two of these genes, *hsp70-1* (HSPA1A locus) and *hsp70-2* (HSPA1B locus), are found as a nearly identical tandem pair in the major histocompatibility complex at 6p21.3 region. *hsp70-1/2* codes for an identical protein, the major inducible HSP70 [Milner and Campbell, 1990]. Another inducible *hsp70* gene, *hsp70b′* (HSPA6 locus), is located on chromosome 1 and it is mainly induced by extreme temperature [Leung et al., 1992; Narita et al., 2002]. The HSP70 isoforms, like those of HSP90s, interact with a number of proteins involved in regulating cell proliferation, including several oncoproteins [Jaattela and Wissing, 1992], as well as tumor suppressor proteins: Rb [Nihei et al., 1993] and p53 [Jolly and Morimoto, 2000]. More importantly, isoforms of HSP70s involve different cellular roles in the death/survival toggle switch regulation [Terauchi et al., 2003]. Specific downregulation of endogenous Hsc70 by antisense oligodeoxynucleotide interference, possibly through caspase-3, provoked an increase in apoptosis both in vitro and in the neurulating chick embryo [Rubio et al., 2002]. Selective overexpression of HSP72 (HSP70-1) ameliorated ATP depletion-induced apoptosis by inhibiting caspase activation, possibly through suppressing pro-apoptotic double-stranded RNA dependent protein kinase, in renal epithelial cells [Wang et al., 2002]. Thus, the induction of specific isoform(s) of HSP70 by geldanamycin has important implications for understanding the mechanism of action of this anti-cancer drug.

Different physiological and environmental stressors and the induction of HSPs are integrated through the activation of a single transcription factor, HSF1 [Christians et al., 2002]. In non-stressed mammalian cells, HSF1 exists in an inert non-trimeric form. In response to stress HSF1, assembles into homotrimers, binds to specific heat shock element (HSE) sequence present within inducible *hsp* genes, and becomes hyperphosphorylated [Morimoto, 1998]. The process appears to involve chaperone interactions [Shi et al., 1998; Bharadwaj et al., 1999] as well as phosphorylation/dephosphorylation events [Holmberg et al., 1998; Hung et al., 1998]. Besides phosphorylation of HSF1, many protein kinases, such as DNA-dependent protein kinase, PKA, PKC, as well as members ERK family, are mediators of signaling pathways in cells responding to environmental stimuli [Peterson et al., 1995; Holmberg et al., 1998; Kiang et al., 2000].

Although induction of HSP70 by geldanamycin has been reported in a number of experimental systems, the signaling pathway leading to cellular process remains unclear and may be cell type-specific. This study is to determine the responsiveness of *hsp70-1/2* to geldanamycin-treatment in human non-small cell lung cancer cells and to investigate the role of protein kinases and calcium signaling in the process. We examined the possible involvement of protein kinase(s) in the process with blockers of serine/threonine protein kinase (H-7 and H-8), PKA (H1004), PKG (KT5823), MLCK (ML-7), and PKC (Ro-31-8425 and Gö-6976). To address how Ca^{2+} mobilization contributes to HSP70-1/2 induction, we analyzed the effects with the Ca^{2+} mobilizers including a chelator of extracellular Ca^{2+} (EGTA), a chelator of intracellular Ca^{2+} (BAPTA-AM), a Ca^{2+} ionophore (A23187), and an ER Ca^{2+}-ATPase inhibitor (TG). In this study, we demonstrate that both Ca^{2+} and PKC are involved in the geldanamycin-induced HSP70-1/2 expression.

EXPERIMENTAL PROCEDURES

Materials

Geldanamycin was obtained from Sigma (St. Louis, MO), dissolved in dimethylsulfoxide at a concentration of 1 mM, and stored in the dark at −20°C. [^{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). Mouse monoclonal antibodies against HSP70 were purchased from Stressgen (Victoria, BC, Canada). Monoclonal antibodies against actin and horseradish peroxidase-conjugated goat antibodies against mouse IgG were purchased from Promega (Madison, WI). Synthetic oligonucleotides were ordered from DNAFax (Taipei, Taiwan). Protein kinase inhibitors including H7, H8, HA1004, KT5823, ML-7, Ro-31-8425, Gö-6976, TG, and A23187 were purchased from Calbiochem (La Jolla, CA). BAPTA-AM (cell permeable) and indo-1-acetoxymethylester (Indo-1-AM) were purchased from Molecular Probes (Eugene, OR).

Cells and Drug Treatment

The human non-small cell lung cancer (NSCLC) H460 cells from ATCC were maintained in RPMI-1640 minimum essential medium with 10% fetal bovine serum. The cells, at 80%–90% confluency, were treated with 0.5 μM geldanamycin for various durations as specified in the legends. To study the effects of protein kinase inhibitors or drugs that disturb calcium homeostasis, the cells were respectively preincubated with the compounds for 1 h prior to the geldanamycin treatment. The concentrations used were as follows: 100 μM for H7, 2 μM for ML-7, 100 μM for H8, 100 μM for HA1004, 2 μM for KT5823, 1 μM or 10 μM for Ro-31-8425 and Gö-6976, 15 μM for BAPTA-AM, 1 mM for EGTA, 1 mM for $CaCl_2$, 4 μM for A23187, and 0.1 μM for TG.

Metabolic Labeling, PAGE, and Western Blot Analysis

For the detection of de novo protein synthesis, cells were metabolically labeled with [^{35}S]methionine for 1 h before harvest. The cells were briefly rinsed in PBS and lysed with sample buffer [Laemmli, 1970] or lysis buffer [O'Farrell, 1975] as previously described [Lee et al., 1993]. The cell lysates were resolved by SDS– or two-dimensional PAGE according to the methods described by Laemmli and O'Farrell, respectively [Lee et al., 1993]. The resolved proteins were transferred electrophoretically onto nitrocellulose membranes according to the manufacturer's protocols (OWL Scientific, Woburn, MA). The membranes were blocked with TBS (20 mM Tris/HCl (pH 7.6) and 137 mM NaCl) supplemented with 0.1% (v/v) Tween 20 and 5% (w/v) skim milk powder. The membranes were then incubated with the appropriate primary antibody and the binding of immobilized proteins was detected with horseradish peroxidase-conjugated secondary antibody and enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ). Bands of interest in the autoradiograms and the immunoblots were quantified by densitometric scanning (Molecular Dynamics, Sunnyvale, CA).

Tryptic Digestion of Protein

Protein spots cut from 2D-PAGE was destained with water and processed as standard protocol [Shevchenko et al., 1996]. The gel pieces were dehydrated with acetonitrille, reduced by adding DTT to 10 mM and incubating at room temperature for 1 h, alkylated by adding iodoacetamide to 50 mM and incubating in the dark at room temperature for 1 h. The solution was diluted with Milli-Q water to four times the original volume and 5 ng/μl Sequence Grade Trypsin (Promega) was added. The digestion was incubated at 37°C for 18 h and frozen until MS analysis.

Mass Spectrometry and Data Treatment

Two-dimensional LC-MS/MS was used to identify tryptic peptides from the 2D-PAGE. HPLC system was interfaced directly to a Q-TOF tandem mass spectrometer (MS/MS, Amersham Pharmacia Biotech, Piscataway, NJ) using the nano Z-spray electrospray source. Peptides were eluted by a 55 min gradient of 2%–50% acetonitrile in 1% acetic acid, 1% formic acid. The flow rate was 400 nl/min. Data reduction was performed using MassLynx and ProteinLynx software, and the resulting MS/MS data set was searched against the NCBI database using the Mascot search engine. The MS/MS data was first searched with carbamidomethylation of cysteine as a fixed modification. Tolerances for Mascot searches were 0.2 Da for the MS/MS matching. Proteins were considered identified if at least one matching

peptides had a score above the significance threshold of the search (Mowse probability better than 0.05).

RNA Extraction and Northern Blotting

Total RNA was isolated by using a commercial RNA extraction kit (RNAzol, Gibco BRL, Gaithersburg, MD). The same amount of RNA was fractionated on 1% formaldehyde–agarose gels and transferred to nylon membranes (Hybond-N, Amersham Pharmacia Biotech, Piscataway, NJ) in 10 × SSC (1.5M NaCl/0.15M sodium citrate). The blots were UV cross-linked (Strategen, La Jolla, CA) and hybridized with [α-^{32}P]dCTP-labeled *hsp70* isoform fragments amplified from *hsp70-1/2* primer sets [Wang et al., 1999] and *hsp70b′* primer sets (Stressgen, Cat. no.: STM-507). The blots were then washed with high stringency (0.1 × SSC, 0.1% SDS, at 65°C for 15 min). *Hsp70* mRNA transcripts were detected by autoradiography and quantitative analysis was performed by densitometric scanning using rRNAs to normalize RNA loading amount.

Preparation of Nuclear Extracts and EMSA

Cells were lysed in nuclear extraction buffer (10 mM HEPES/KOH (pH 7.9), 0.5% Triton X-100, 0.5M sucrose, 0.1 mM EDTA, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM DTT, 0.5 mM PMSF) and homogenized by three strokes with a Dounce Homogenizer. After a brief centrifugation, the pellet was lysed in nuclear extraction buffer supplemented with 0.5M NaCl and 5% glycerol at 4°C for 30 min. Nuclear extracts were obtained by centrifugation at 14,000*g* for 20 min and kept at −70°C.

Synthetic oligonucleotides (sense: 5′-GG-C<u>GAA</u>ACCCCTG<u>GAA</u>TA<u>TTC</u>CCGACCTG-3′, and antisense: 5′-CAGGTCGG<u>GAA</u>TA<u>TTC</u>CA-GGGGT<u>TTC</u>GCC-3′, the consensus sequence of HSE is underlined) were used for the detection of protein factors that bind to specific HSE and 5′-end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase. Two micrograms of nuclear extracts were incubated in reaction buffer (10 mM HEPES/KOH (pH 7.9), 12% glycerol, 4 mM Tris-HCl, 1 mM EDTA, 100 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, 300 μg BSA/ml, and 20 μg poly(dI-dC)). Protein–DNA complexes were resolved on 8% non-denaturing polyacrylamide gels. After electrophoresis, the gels were dried and processed for autoradiography.

Measurement of Intracellular Calcium Concentration

The cytoplasmic calcium was determined according to the method of Nuccitelli et al. [1993]. The cells cultured on coverslips in a 35-mm dish were incubated with calcium probe, indo-1-AM, in darkness at 25°C for 45 min prior to experiments in which $[Ca^{2+}]_i$ was monitored by single-cell dual-wavelength microfluorymetry (PhoCal Pro, Life Science Resources, UK). The indo-1 loaded cells were illuminated at an excitation wavelength of 340 nm. The fluorescent intensities at emission wavelength of 405 nm (Ca^{2+}-binding form) and 490 nm (Ca^{2+}-free form) were measured simultaneously by two photomultipliers, and integrated in 100 ms intervals. The concentration of intracellular calcium was estimated from the ratio R of the two emitted fluorescence bands according to equation 1 [Grynkiewicz et al., 1985].

$$\left[Ca^{2+}\right] = K_d[(R - R_{min})/(R_{max} - R)](S_{f2}/S_{b2}) \quad (1)$$

Where K_d: 250 nM, and (S_{f2}/S_{b2}): 2.01 after Grynkiewicz et al., but R_{min} and R_{max}, were determined in situ, according to Kao [1994]. Briefly, the R_{min} is the limiting value of the ratio R when the entire indicator is in the Ca^{2+}-free form and the R_{max} is the limiting value of the ratio R, when the indicator is saturated with calcium.

RESULTS

Induction of HSP70 in Geldanamycin-Treated NSCLC H460 Cells

Cells treated with geldanamycin were metabolically labeled with [^{35}S]methionine and the de novo synthesized proteins were visualized by autoradiography (Fig. 1A,B). The synthesis rate of HSP90, HSC70, and HSP70 was apparently enhanced in the geldanamycin-treated cells while that of other stress proteins, for example, GRP78, remained unaffected (Fig. 1A,B). The induction of the HSPs, in particular the HSP70, is dose-dependent. Enhanced synthesis of HSP70 was detected in cells treated with 0.1 μM and the maximal induction, approximately eight-fold compared to the non-treated cells, was elicited by 0.5 μM geldanamycin in 6 h treatments (Fig. 1A). In cells treated with 0.5 μM geldanamycin, the synthesis rate of HSP70 increased about four-fold at 2 h, reached a maximum after

6 h of exposure but subsided significantly thereafter (Fig. 1B,C). These results showed that induction of HSP70-1/2 is a dose- and time-dependent manner by geldanamycin treatment. It is worthy to note that the overall intensity among different sample lanes remain largely constant, suggesting the drug does not affect the uptake of [^{35}S]methionine nor the general protein synthesis in the treated cells. Moreover, there was only one HSP70 spot being induced in the geldanamycin-treated H460 cells by two-dimensional PAGE analysis and Western analysis using anti-HSP70 antibodies (Fig. 1D). Specific isoforms of HSP70 were further distinguished by LC-ESI-MS/MS analysis (Table I). In eighteen sequenced peptides, there were twelve peptide sequences present in several HSP70 isoforms, but six peptide sequences hit HSP70-1/2 only by Masco searching engine against NCBI database. The data indicate that HSP70-1/2 are the major inducible forms in the geldanamycin-treated H460 cells.

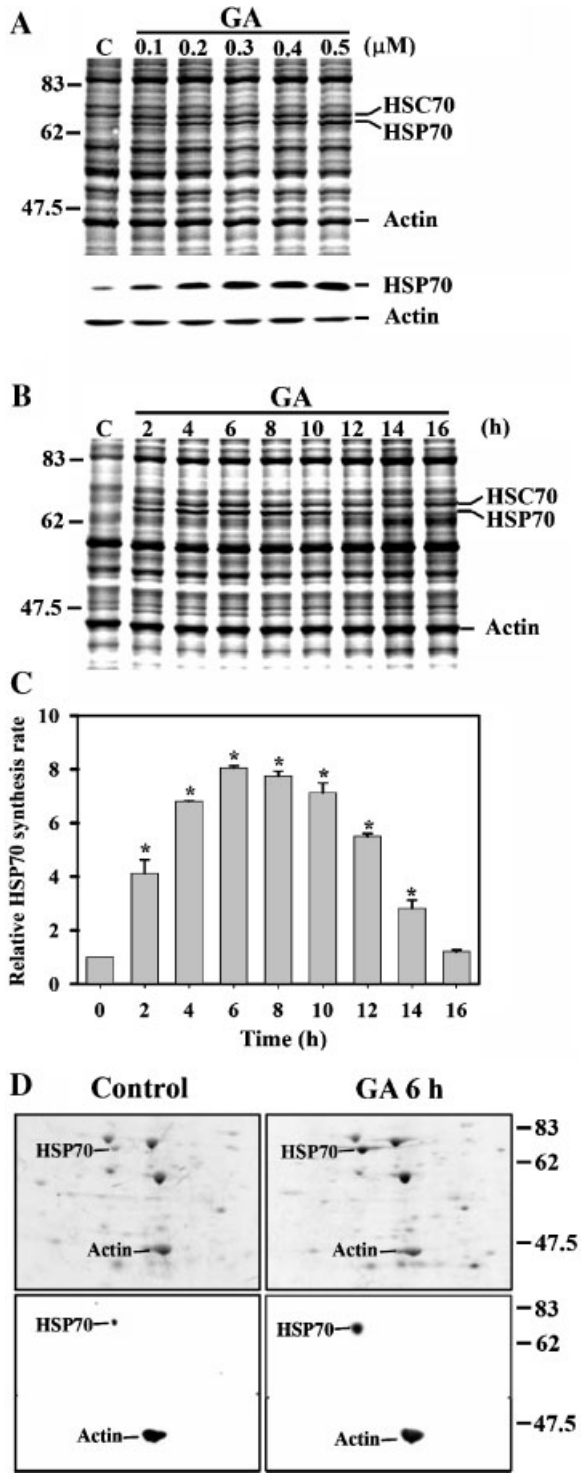

Fig. 1. Induced synthesis of HSP70-1/2 in geldanamycin-treated NSCLC H460 cells. **A**: Cells were treated with various concentrations of geldanamycin for 6 h or **(B)** with 0.5 μM geldanamycin for various durations as indicated. The cells were then metabolically labeled with [^{35}S]methionine in the absence of the drug for 1 h before being harvested. Equal amounts of cell lysates were resolved by SDS–PAGE and the gels were processed for autoradiography and Western blotting. **C**: Autoradiograms in (B) were quantified by densitometric scanning; data were expressed as the relative de novo synthesis rates of HSP70-1/2, using values of actin as internal controls (means ± SD of three separate experiments; *, $P < 0.05$, compared with non-treated cells). **D**: Labeled (**upper panels**) and unlabeled (**lower panels**) cell lysates were resolved by two-dimensional PAGE and processed for autoradiography and Western blotting, respectively. Antibodies against HSP70-1/2 and actin were used as described in the experimental procedures. The experiments were performed at least three times and a representative result is shown.

Transactivation of the *Hsp70-1/2* Gene in Geldanamycin-Treated NSCLC H460 Cells

Induction of HSP70 in geldanamycin-treated cells was studied at the transcriptional level. Two *hsp70* probes, namely *hsp70-1/2* and *hsp70b′*, were used in the Northern analyses. The mRNA level of *hsp70-1/2*, but not *hsp70b′*, significantly increased in geldanamycin-treated H460 cells. The maximum expression of *hsp70-1/2* mRNA was detected after 6 h of treatment, similar to the protein induction profile (Fig. 2A). Our results further suggested that only the *hsp70-1/2* gene was induced and that the HSP70 detected in the previous experiment is indeed the HSP70-1/2 isoform. Transactivation of *hsp70-1/2* in geldanamycin-treated H460 cells was also monitored by EMSA, in which a HSE derived from this gene was used as a probe. We found that protein factors in the nuclear extracts from control cells reacted with

TABLE I. Identification of HSP70-1/2 by LC-ESI-MS/MS Analysis

Spot	Peptide sequence	Score	AA (%)	Identification
HSP70	LLQDFFNGR	11	42	HSP70-1/2
	FELSGIPPAPR	42		
	FGDPVVQSDMK	29		
	VEIIANDQGNR	37		
	LVNHFVEEFK	2		
	NALESYAFNMK	19		
	FEELCSDLFR	44		
	AQIHDLVLVGGSTR	48		
	TTPSYVAFTDTER	47		
	ARFEELCSDLFR	42		
	AFYPEEISSMVLTK	46		
	NOVALNPQNTVFDAK	37		
	ATAGDTHLGGEDFDNR	65		
	IINEPTAAAIAYGLDR	84		
	SINPDEAVAYGAAVQAAILMGDK	97		
	CQEVISWLDANTLAEKDEFEHK	27		
	QTQIFTTYSDNQPGVLIQVYEGER	44		
	EIAEAYLGYPVTNAVITVPAYFNDSQR	47		

The HSP70 spot were cut from 2D-PAGE for in gel digestion and the tryptic peptides were identified by LC-ESI-MS/MS system using the Mascot search engine against NCBI database. Peptide sequences underlined indicate that its hit HSP70-1/2 only and the others hit not only HSP70-1/2, but also other HSP70 family members due to high homology between HSP70 isoforms. AA represents amino acid coverage (%).

the HSE to form two DNA–protein complexes, I and II (Fig. 2B, lane C). The complex II corresponds to the constitutive HSE-binding activity (CHBA) and loss of CHBA is a time dependent manner in stressed cells [Kim et al., 1995]. Similar to previous report, complex II disappeared but the formation of complex I was greatly enhanced by treatment with geldanamycin, suggesting that complex I, possibly HSF–HSE complex, is the transcriptionally active complex that causes the transactivation of *hsp70-1/2* (Fig. 2B). Taken together, these results indicated that the elevation of HSP70-1/2 after geldanamycin treatment are due to de novo synthesis of *hsp70-1/2* through transactivation in NSCLC H460 cells.

Possible Involvement of a Ca^{2+}-Dependent PKC in the Induction of HSP70-1/2 in Geldanamycin-Treated H460 Cells

Possible involvement of protein kinase(s) in the geldanamycin-induced expression of HSP70-1/2 was studied by the inhibitor scanning technique. Cells were pre-incubated with a battery of protein kinase inhibitors prior to the treatment with geldanamycin. Geldanamycin-induced synthesis of HSP70-1/2 was abolished by pre-treatment with serine/threonine protein kinase inhibitors H7 and H8, which inhibit PKC, PKA, PKG, and MLCK; but the process was not affected by PKA inhibitors HA1004, PKG inhibitor KT5823, and MLCK inhibitor ML-7 (Fig. 3A). Suppression of geldanamycin-induced *hsp70-1/2* expression by serine/threonine protein kinase inhibitors H7 and H8 were checked at the protein level by Western blotting (Fig. 3B) as well as at the mRNA level by Northern blotting (Fig. 3C). Furthermore, geldanamycin-induced HSP70-1/2 expression was also attenuated by pre-treatment with broad range PKC inhibitor Ro-31-8425 as well as Ca^{2+}-dependent PKC inhibitor Gö-6976 (Fig. 4). It suggested that Ca^{2+}-dependent PKC signaling pathway is involved in the up-regulation of geldanamycin-induced HSP70-1/2 expression.

Possible Involvement of Ca^{2+} Homeostasis in Geldanamycin-Induced Synthesis of HSP70-1/2 in NSCLC H460 Cells

$[Ca^{2+}]_i$ in H460 cells was continuously monitored by microspectrohotometry. Before geldanamycin exposure, the basal $[Ca^{2+}]_i$ level was about 50 nM. A sharp increase in $[Ca^{2+}]_i$ occurred after the addition of 0.5 μM geldanamycin. The signal spike indicated that $[Ca^{2+}]_i$ rose to 250 nM and returned to basal levels within 10 s in cells exposed to geldanamycin (Fig. 5). This observation suggested that Ca^{2+} homeostasis was affected by geldanamycin. Since Ca^{2+} signaling is involved in a number of early signal transduction pathways in a variety of cellular responses, we decided to re-examine the effects of Ca^{2+} mobilization on geldanamycin-induced HSP70-1/2. As shown in Figure 6A, except affecting $[Ca^{2+}]_o$, (pretreatments with EGTA and $CaCl_2$), all other drugs affect Ca^{2+} homeostasis, including BAPTA-AM,

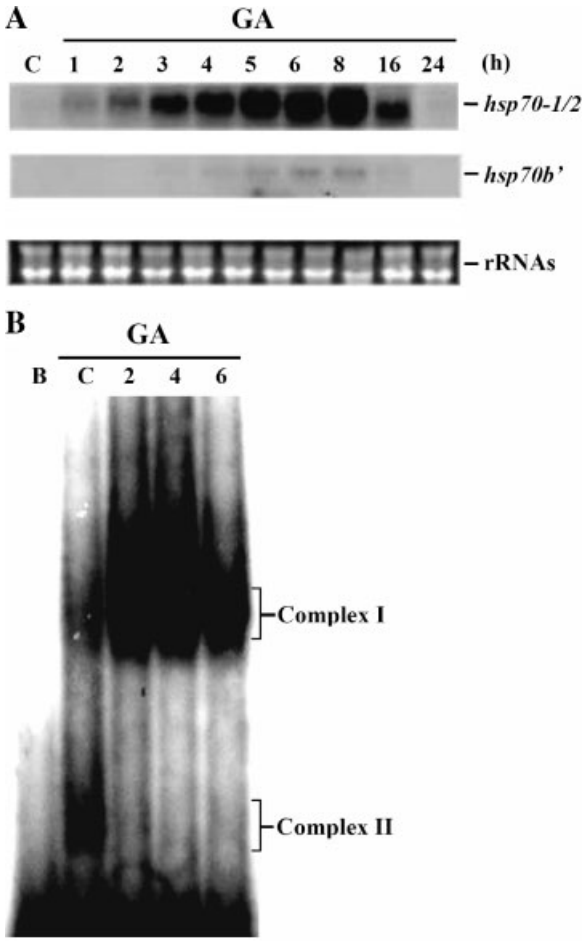

Fig. 2. Transactivation of *hsp70-1/2* in geldanamycin-treated NSCLC H460 cells analyzed by Northern blotting and EMSA. **A**: Cells were treated with 0.5 μM geldanamycin for various durations as indicated. After treatment, total RNA was extracted and analyzed for the expression of *hsp70* mRNA by Northern blotting, using ^{32}P-labeled *hsp70-1/2* and *hsp70b′* probes. **B**: Nuclear extracts were prepared from cells after treatment and the interaction of nuclear factors with the HSE1 derived from human *hsp70-1/2* was examined by EMSA. **Lane B**, blank, contains only labeled HSE1 DNA; **lane C**, control, untreated cell. Complexes I and II were marked as indicated. Similar results were obtained from at least three independent experiments.

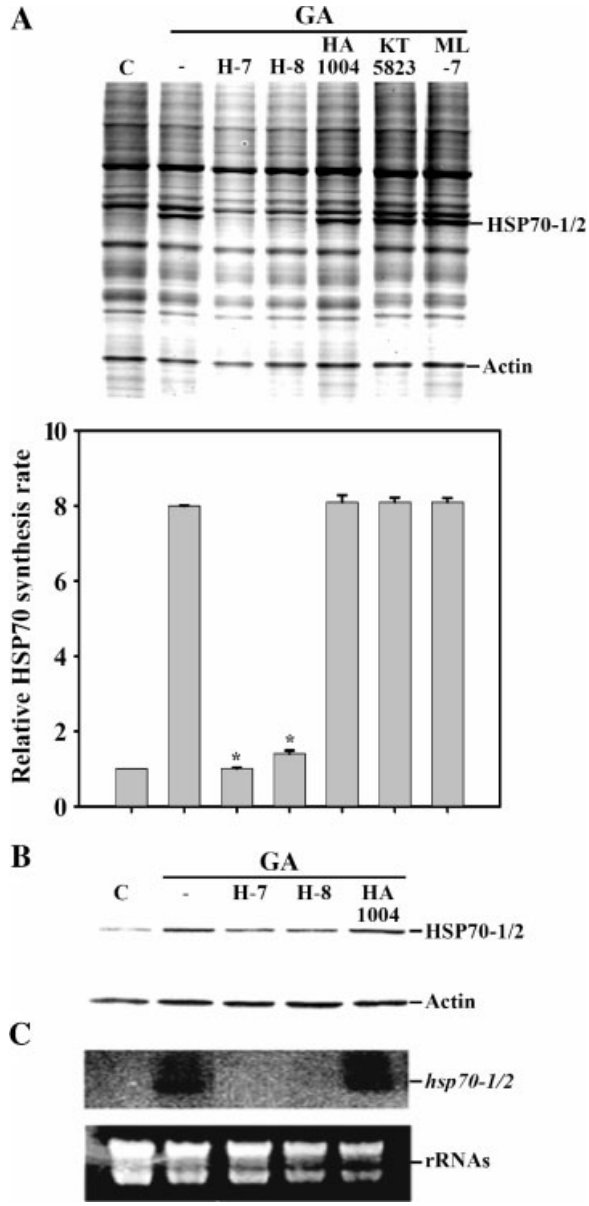

Fig. 3. Effects of protein kinase inhibitors on HSP70-1/2 induction in geldanamycin-treated NSCLC H460 cells. **A**: Cells were respectively pre-incubated with 100 μM H7, 100 μM H8, 100 μM HA1004, 2 μM KT5823, and 2 μM ML-7 for 1 h before they were treated with 0.5 μM geldanamycin for 6 h in the presence of inhibitors. The cells were labeled with [^{35}S]methionine for 1 h before being harvested. Cell lysates were resolved by SDS–PAGE and the resulted gels were processed for autoradiography followed by quantitative analysis as described in the legend of Figure 1 (means ± SD of three separate experiments; *, $P < 0.05$, compared with treatment of geldanamycin only). **B**,**C**: The effects of H7 and H8 on geldanamycin-induced HSP70-1/2 expression were further examined at the protein and mRNA levels by Western and Northern analysis, respectively. The representative results of at least three separate experiments were shown.

A23187, or TG, reduced geldanamycin-transactivated HSP70-1/2 expression. Similar results were obtained when total HSP70-1/2 or *hsp70-1/2* mRNA level were examined (Fig. 6B,C). Ca^{2+} mobilization, therefore, involve in *hsp70-1/2* induction in geldanamycin-treated H460 cells.

DISCUSSION

A number of studies have shown that *hsp70-1/2* and *hsp70b′* are differentially regulated. For instance, *hsp70-1/2* were induced by cadmium [Croute et al., 2000], cisplatin [Jakubowicz-Gil

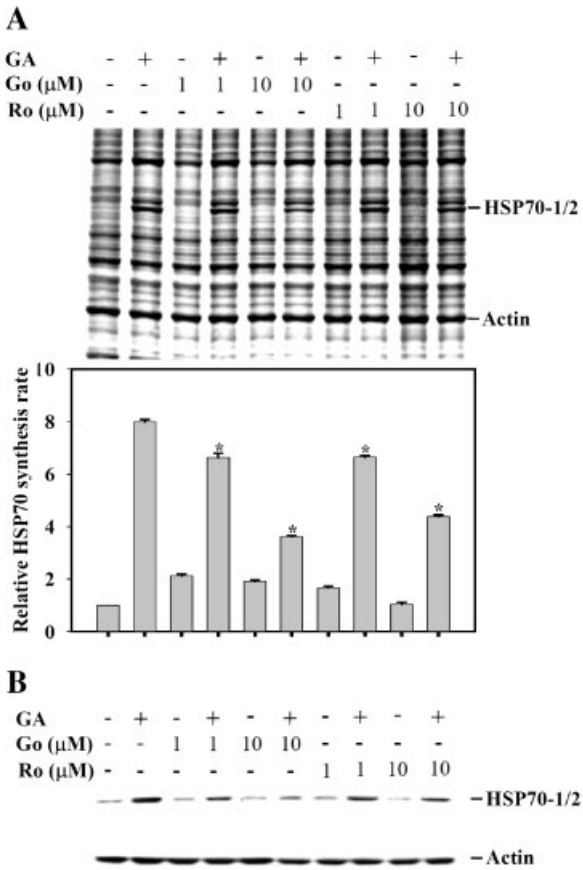

Fig. 4. Effects of PKC inhibitors on HSP70-1/2 induction in geldanamycin-treated NSCLC H460 cells. **A**: Cells were respectively pre-incubated with 1 μM or 10 μM Ro-31-8425 and Gö-6976 for 1 h before they were treated with 0.5 μM geldanamycin for 6 h in the presence of inhibitors. The cells were labeled with [^{35}S]methionine for 1 h before being harvested. Cell lysates were resolved by SDS–PAGE and the resulted gels were processed for autoradiography. **B**: The effects of Ro-31-8425 and Gö-6976 on geldanamycin-induced HSP70-1/2 expression were further examined by Western blot analysis followed by quantitative analysis as described in the legend of Figure 1 (means ± SD of three separate experiments; *, $P < 0.05$, compared with treatment of geldanamycin only). The representative results of at least three separate experiments were shown.

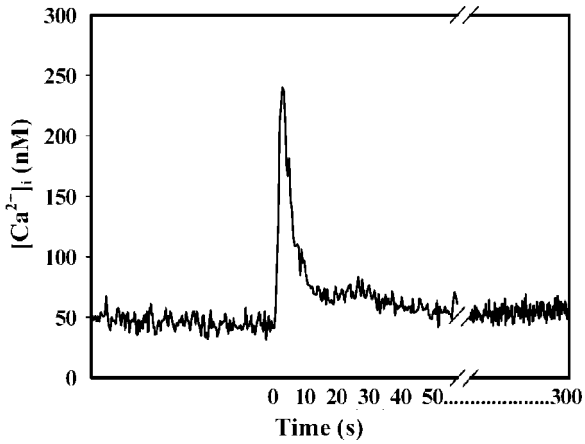

Fig. 5. Calcium mobilization in NSCLC H460 cells treated with geldanamycin. Cells grown on coverslips were loaded with Indo-1-AM for 45 min at 25°C and then stimulated with 0.5 μM geldanamycin at time 0. Changes in $[Ca^{2+}]_i$ were determined by fluorescence microphotospectrometry. The representative results of at least three separate experiments were shown.

et al., 2002], and IL-6 [Febbraio et al., 2002]; while *hsp70b′* was mainly induced by heat stress [Narita et al., 2002]. Therefore, *hsp70-1/2* and *hsp70b′*, like most members of other stress gene families, are differentially regulated depending on the stress conditions and/or cell types. Geldanamycin, a HSP90 inhibitor, a potent inducer of HSP90, is also an inducer of HSP70s [Holmberg et al., 1998; Lu et al., 2002]. The current studies further demonstrated that at least in human NSCLC cells, only the expression of *hsp70-1/2*, but not *hsp70b′* is provoked by the treatment with geldanamycin. *Hsc70*, other gene in the same family, was also slightly affected by geldanamycin in NSCLC H460 cells as shown in the [^{35}S]methionine labeling experiment.

Activation of the HSF1 is responsible for the transactivation of all heat shock genes under stress, and that this factor is hyperphosphorylated when activated [Xia and Voellmy, 1997; Morimoto, 1998]. As mentioned previously, HSF1 may serve as a substrate for a number of protein kinases in different experimental systems and inhibitors and/or activators of specific protein kinases can modulate these stress responses. In this context, PKC is directly or indirectly involved in the enhanced expression of HSP70s in stressed cells. In human peripheral blood monocytes (PBM), treatment with phorbol 12-myristate 13-acetate (PMA), a potent activator of PKC, enhanced synthesis of HSP90 and HSP70s through stabilize *hsps*-specific mRNA, but did not transactivate *hsp* genes by activated HSF1, suggesting PKC is not a transcription activator of HSPs [Jacquier-Sarlin et al., 1995]. On the other hand, PKC inhibitor or transient transfection of a dominant negative PKC, block HSF1 activation under hypoxia treatment in radiation-induced fibrosacroma (RIF) tumor cells [Baek et al., 2001]. In human intestinal epithelial T84 cells, PKC activity was increased by heat stress and pretreatment with PKC inhibitor GF-109203X or Ca^{2+} chelator EGTA or BAPTA-AM would

attenuate heat stress induced-HSP70 expression [Kiang et al., 2002]. Similar to our results, the induced expression of HSP70 could be blocked either by serine/threonine protein kinase inhibitor H-7 or H-8 in the presence of Ca^{2+} or by BAPTA-AM in the absence of Ca^{2+} [Kiang et al., 2002]. Moreover, Gö-6976, which selectively inhibited PKCα and PKCβI or PKCμ-isoforms, reduced geldanamycin-induced HSP70-1/2 expression in our study. PKCβ, γ, ζ, and μ were not detected by Western blot analysis using isozyme-specific anti-PKC antibodies in H460 cells [Lee, 2001], these results suggested that Ca^{2+}-dependent PKC, probably PKCα, may act upstream of the transactivation of *hsp70-1/2* induced by geldanamycin in NSCLC H460 cells. Since there is no report on the causal relationship between geldanamycin inhibition of HSP90 and activation of PKC, these results may pave ground for understanding the coordination of HSP70 and HSP90 chaperones.

A calcium-dependent metabolic process may be involved in the generation of the heat shock signal, and calcium is known to induce DNA-binding activity and phosphorylation of HSF1 [Mosser et al., 1990; Hung et al., 1998]. A23187 is known to cause the release of eicosanoids, which are converted to cyclooxygenase or lipoxygenase products [Smith and McCabe, 1984] and may consequently stimulate adenylate cyclase, giving rise to a mixed Ca^{2+}/cAMP signal [Smith and McCabe, 1984; Elia et al., 1996]. On the other hand, cyclooxygenase and lipoxygenase inhibitors are reported to modulate the heat shock response in human cells [Ito et al., 1996], which suggests the interesting possibility that Ca^{2+} mobilization is directly or indirectly involved in the enhanced expression of HSPs in human cells. We have shown that chelating intracellular Ca^{2+} with BAPTA-AM or depletion of intracellular Ca^{2+} store with A23187 or TG results in significant suppression of geldanamycin-induced expression of *hsp70-1/2*. In addition, depletion of $[Ca^{2+}]_o$ by EGTA also reduced *hsp70-1/2* expression in geldanamycin-treated NSCLC H460 cells. Although the

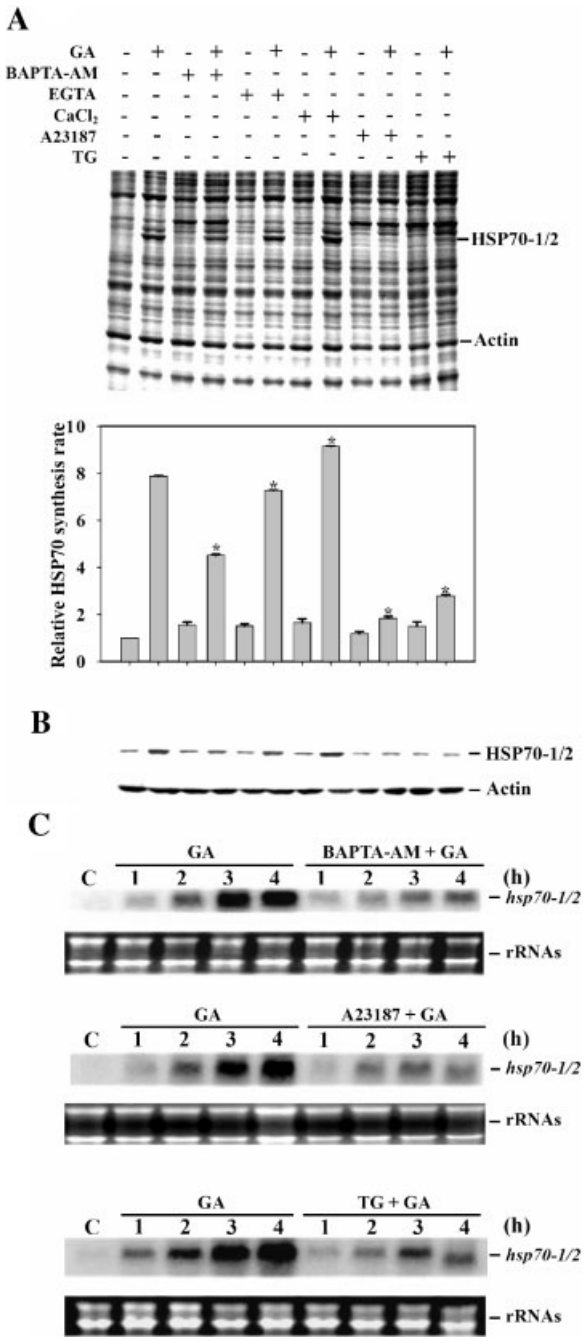

Fig. 6. Effects of $[Ca^{2+}]_i$ destructors on HSP70-1/2 expression in geldanamycin-treated NSCLC H460 cells. **A**,**B**: Cells were respectively pre-incubated with 15 μM BAPTA-AM, 1 mM EGTA, 1 mM $CaCl_2$, 4 μM A23187, or 0.1 μM TG for 15 min prior to the treatment with 0.5 μM geldanamycin for 6 h in the presence of the drugs. The effects of these drugs on the induction of HSP70-1/2 were shown by [^{35}S]methionine-labeled experiments and by Western blot analysis as performed in the previous experiments. The quantitative data were expressed as relative expression level of HSP70 (means ± SD of three separate experiments; *, $P < 0.05$; compared to the cells treated with geldanamycin alone). **C**: Cells were respectively pre-incubated with 15 μM BAPTA-AM, 4 μM A23187, or 0.1 μM TG for 15 min prior to the treatment with 0.5 μM geldanamycin for various durations as indicated. After treatment, total RNA was extracted and processed for Northern blot analysis, using ^{32}P-labeled *hsp70-1/2* as probes. The representative results of at least three separate experiments were shown.

effects of Ca^{2+} mobilization on HSF1 activation remains to be clarified, our results support the notion that a calcium-dependent metabolic process is involved in the transcriptional regulation of the geldanamycin-induced HSP70-1/2 synthesis. Another interesting point is that geldanamycin causes a transient increase of $[Ca^{2+}]_i$ in NSCLC H460 cells. This is the first demonstration that geldanamycin can evoke a transient intracellular Ca^{2+} signal, which plays a crucial role on geldanamycin-induced synthesis of the HSPs.

The regulation of heat shock gene expression has attracted a great deal of interest because of HSPs role in the cell viability and cell proliferation. Understanding of the mechanisms by which mammalian cells detect physiological stress at the molecular level and transduce the stress signal to the transcriptional apparatus is an important tool because of the possible pharmacological manipulation of the stress response.

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