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Involvement of Calcium in the Differential Induction of Heat Shock Protein 70 by Heat Shock Protein 90 Inhibitors, Geldanamycin and Radicicol, in Human Non-Small Cell Lung Cancer H460 Cells

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Abstract Both geldanamycin (GA) and radicicol (RA) are HSP90 binding agents that possess antitumour activities. Although the in vitro data indicated that the inhibitory constant of RA is much bigger than that of GA, the in vivo data on drug efficacy might reveal different results. We have recently shown that treatment with GA induces a heat-shock response and that calcium mobilization may be involved in the process. By using induction of HSP70 as the endpoint assay, we found changes in upstream signaling mediators, including HSF1 and calcium mobilization, as well as possible involvement of protein kinase in human non-small cell lung cancer H460 cells treated with GA and RA. Our results demonstrated that calcium mobilization, a calcium dependent and H7-sensitive protein kinase, along with HSF1 activation by phosphorylation, are all involved in the HSP70 induction process triggered by the drugs. However, only GA, but not RA, can provoke a rapid calcium mobilization and thereby result in an instant induction of HSP70. Furthermore, the rapid calcium influx, followed by instant HSP induction, could be achieved in GA- or RA-treated cells placed in a medium containing excessive calcium while the response was completely abolished in cells depleted of calcium. Taken together, our findings suggest that differential calcium signaling may account for the differential induction of HSP and the action of GA and RA. J. Cell. Biochem. 97: 156–165, 2006.

Key words: geldanamycin; heat-shock factor-1; heat shock protein; radicicol; intracellular calcium; extracellular calcium

The 90 kDa heat-shock proteins (HSP90s) are molecular chaperones that play critical roles in post-translational controls of the stability and function of many key regulators of cell growth, differentiation, and apoptosis [Pratt, 1998; Pratt and Toft, 2003]. Together with the fact that HSP90 is essential in maintaining transformation, this chaperone has emerged as an important target in cancer treatment and its inhibitors are being vigorously sought after [Goetz et al., 2003; Chiosis et al., 2004]. Recently, the anticancer activities of several HSP90 binding agents (collectively termed as the HBAs) had been validated in preclinical and clinical tests [Workman, 2004a,b].

The most thoroughly studied HBAs are two structurally distinct natural products, geldanamycin (GA) and radicicol (RA). GA, a benzoquinone ansamycin, and RA, a macrocyclic lactone, are antibiotics isolated from *Streptomyces hygroscopicus* and *Monosporium bonorden*, respectively, decades ago. The antitumour activities of both compounds were attributable to direct inhibition of HSP90 [Kwon et al., 1995; Schulte et al., 1996, 1998]. Thanks to extensive crystallographic and biochemical analyses, it is

Abbreviations used: GA, geldanamycin; HBAs, HSP90 binding agents; HSF1, heat-shock factor-1; HSP70, heat shock protein 70; HSP90, heat shock protein 90; HSC70, HSP70 cognate; NSCLC, non-small cell lung cancer cells; PKC, protein kinase C; RA, radicicol; $[Ca^{2+}]_i$, cytosolic (intracellular) calcium concentration; $[Ca^{2+}]_o$, extracellular calcium concentration; H7, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine.

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now clear that GA and RA bind to the ATP/ADP-binding domain of HSP90, locking the chaperone in its ADP-bound conformation and compromising its function [Stebbins et al., 1997; Roe et al., 1999]. GA binds to full-length dimeric HSP90 with a $K_d = 1.2$ μM [Roe et al., 1999], and 17-allylamino,17-demethoxygeldanamycin (17AAG), a derivative of GA that has been validated in clinical trials, has a much greater affinity for the HSP90 chaperone complex found in malignant cells with an IC_{50} as low as 5 nM [Kamal et al., 2003]. In contrast, RA shows a significantly higher affinity for HSP90 with a K_d of 19 nM [Roe et al., 1999], but it lacks antitumour activities in vivo. The in vivo effective doses documented for the antiproliferative actions of GA and RA may be different. Furthermore, the binding of HBAs to HSP90 would result in the degradation of a wide range of cancer-associated proteins that are well-characterized client proteins of the molecular chaperone and elicit a heat-shock response [Blagosklonny, 2002].

The heat-shock response is a universal defense system present in all cells; it is characterized by an increased expression of heat shock proteins (HSPs), including the most highly conserved and extensively studied HSP90 and HSP70, which function as molecular chaperones in regulating cellular homeostasis and promoting cell survival. The induced expression of HSPs in response to various stimuli is regulated by heat shock transcription factor-1 (HSF1), which exists as an inert monomer held inactive when complexes with HSP90, and disruption of the HSP90-HSF1 chaperone complex facilitates the activation of HSF1 [Bharadwaj et al., 1999]. Upon activation, HSF1 undergoes a multistep process that involves the conversion of HSF1 from a monomer to a trimeric nuclear protein complex, which binds to heat-shock elements of the heat shock genes, thus triggering their transactivation [Pirkkala et al., 2001]. Several lines of evidence suggest that phosphorylation play a role in the activation of HSF1 [Holmberg et al., 2002], and hyperphosphorylation of HSF1 correlates with its transcriptional activation [Morimoto, 1998]. Recent mechanistic work in vivo has demonstrated that activation of HSF1 is necessary for the induction of the heat-shock response by HBAs [Bagatell et al., 2000]. Although GA and RA exhibit similar antiproliferative activities by modulating HSP90, the signaling evoked by these drugs is still unclear.

Calcium is an ubiquitous intracellular signal responsible for controlling a diverse range of cellular processes, such as gene transcription, muscle contraction, and cell proliferation. It is known that intracellular calcium is essential for the multi-step activation of HSF1 [Price and Calderwood, 1991; Choi et al., 1994]. In addition, the observation that heat shock elevated $[Ca^{2+}]_i$ in a time-and temperature-dependent manner, and removal of external calcium could obliterate this rise in $[Ca^{2+}]_i$, preventing phosphorylation and translocation of HSF1 and reduce the expression of HSP70, indicates that the increase in $[Ca^{2+}]_i$ was due to calcium influx in heat shocked cells [Kiang et al., 2000]. Recently, we found that chelating $[Ca^{2+}]_i$ with BAPTA-AM would result in significant suppression of GA-induced expression of HSP70. Additionally, depletion of $[Ca^{2+}]_o$ by EGTA would also reduce HSP70 induction [Shu et al., 2005]. These findings strongly suggest that calcium signaling might also be involved in the pathway of heat-shock response triggered by GA. This study aims to determine the difference between the heat-shock response elicited by GA and RA in H460 cells and to investigate the contribution made by $[Ca^{2+}]_i$ mobilization in these processes. Our results indicate that $[Ca^{2+}]_i$ mobilization plays a crucial role and that differential calcium signaling provoked by GA and RA may account for the different heat shock responses.

MATERIALS AND METHODS

Materials

Geldanamycin (GA) and Radicicol (RA) were purchased from Sigma (St. Louis, MO) and dissolved in dimethylsulfoxide at a stock concentration of 10 mM and stored in the dark at −20°C. All cultureware was purchased from Falcon (Franklin Lakes, NJ) and Corning (Corning, NY), and culture media from Gibco (Grand Island, NY). 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H7) was purchased from Sigma (St. Louis, MO). [^{35}S]methionine (specific activity >800 Ci/mmol) from Amersham (Buckinghamshire, England). Mouse monoclonal antibodies against human HSP70 or actin were separately purchased from Stressgene (Victoria, BC, Canada) and Sigma. Rabbit polyclonal antibody against HSF1 was purchased from ABR (Golden, CO). Horseradish peroxidase-

conjugated goat anti-mouse and anti-rabbit IgG antibodies were purchased from Sigma and Amersham, respectively. Chemicals for electrophoresis were from Bio-Rad (Richmond, CA). Chemicals related to intracellular calcium concentration ($[Ca^{2+}]_i$) detection assay such as Indo-1-acetoxymethylester (Indo-1-AM) were from Molecular Probes (Eugene, OR).

Cell Culture and Drug Treatments

Human non-small cell lung cancer (NSCLC) H460 cells were purchased from the American Type Culture Collection (Manassas, VA) and propagated in RPMI-1640 MEM supplemented with 10% fetal bovine serum, 100 U/ml penicillin G, 10 μg/ml streptomycin and 10 mM HEPES. Exponentially growing cells at 80–90% confluence were used, maintained at 37°C under a 5% CO_2 atmosphere. To study the short-term effects of GA and RA treatment, cells were treated with 0.5 μM GA or RA for 0, 0.5, 1, 3, 5, 7, 60, 120, 180, 240, and 300 min, then allowed to recover for 6 h. In experiments using protein kinase inhibitor, the cells were pre-incubated with 60 μM H7, a serine/threonine protein kinase inhibitor, for 1 h, followed by treatment with the drugs in the presence of the inhibitor and allowed to recover for 6 h before subsequent biochemical assays.

Metabolic Labeling and Gel Electrophoresis

De novo protein synthesis was revealed by [^{35}S]methionine labeling at a concentration of 20 μCi/ml. At the end of various treatments, the cells were labeled with [^{35}S]methionine for 1 h before they were harvested. The cells were washed twice with PBS and lysed with 2× SDS electrophoresis sample buffer.

Protein concentrations of cell lysates were determined using bovine serum albumin as standard. The cell lysates were then resolved by 7.5 or 10% SDS–PAGE. After electrophoresis, the gels were fixed, dried, and exposed to X-ray films. Protein bands of interest were quantified by densitometric scanning (Molecular Dynamics, Sunnyvale, CA).

Western Blot Analysis

After treatment, cells were washed twice with PBS, lysed in 2× SDS electrophoresis sample buffer and the lysates (100–200 μg) were resolved by SDS–PAGE as described above. The proteins were electroblotted onto nitrocellulose membranes by semidry transfer (Bio-Rad). After blocking with TTBS (0.5% Tween 20, 20 mM Tris-HCl, pH 7.4, and 0.5 M NaCl) containing 5% nonfat dry milk for 2 h at room temperature or overnight at 4°C, the membranes were immunblotted with primary antibodies. The primary antibodies used were: anti-HSP70, 1:1,000 dilutions; anti-actin, 1:1,000 dilutions; anti-HSF1, 1:10,000 dilutions. After overnight incubation with primary antibodies at 4°C, the membranes were washed three times with TTBS and probed with horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG (1:2,000 dilutions) for 2 h at room temperature. The proteins were then visualized with ECL Western blotting detection reagents (Perkin Elmer, Boston, MA) and the signal intensity was quantified by densitometry.

Intracellular Calcium Measurements

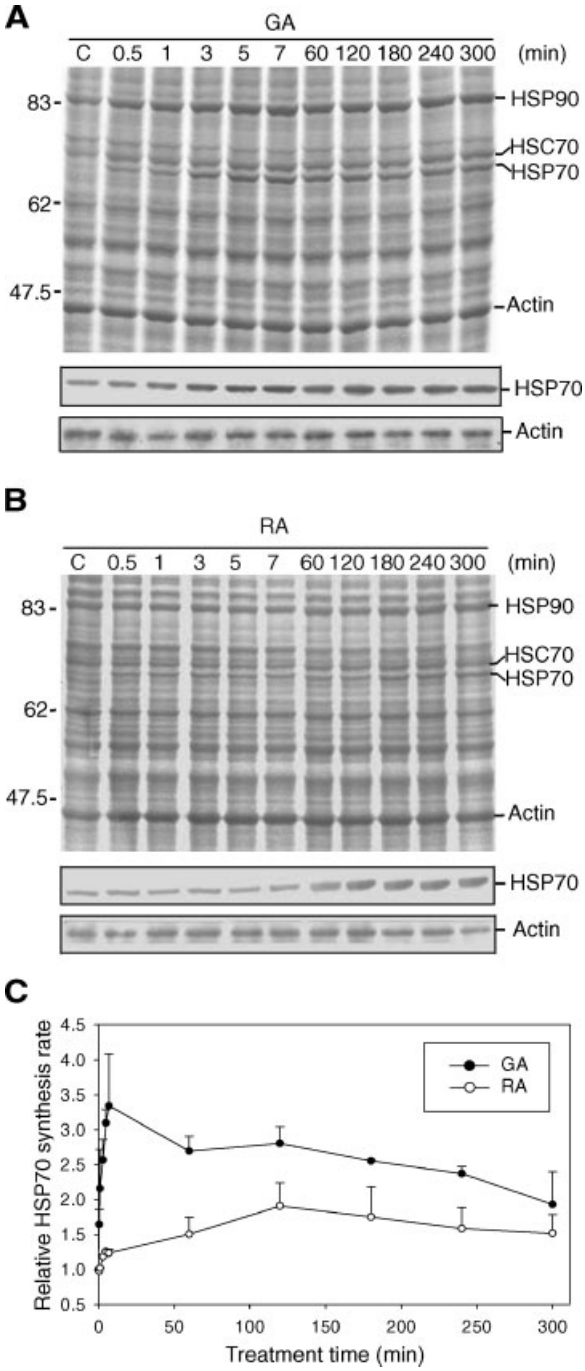
$[Ca^{2+}]_i$ was determined by fluorescence ratio imaging of indo-1/AM, according to the methods of Nuccitelli et al. [1993]. Confluent monolayers of cells were cultured on coverslip in a 35 mm dish for 24 h. Before fluorescence measurement, the cells were washed with HEPES-buffered saline (140 mM NaCl, 2 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM HEPES, pH 7.2), and then loaded with calcium binding dye, 4 mM indo-1-AM dissolved in normal saline buffer containing 0.1% pluronic acid and 1% FBS, to mimic normal culture medium $[Ca^{2+}]$ conditions for 45 min at room temperature in the dark. After loading the dye, excess fluorescence signals were removed by washing the cells twice with PBS before measuring $[Ca^{2+}]_i$, by single-cell dual-wavelength microfluorymetry (PhoCal Pro, Life Science Resources, UK) with an excitation wavelength of 340 nm. The fluorescence signals at emission wavelengths of 405 nm (Indo-1 in calcium–binding form) and 490 nm (Indo-1 in free form) were measured simultaneously by two photomultipliers and integrated in 100 ms intervals. The $[Ca^{2+}]_i$ was estimated by the equilibrium equation $[Ca^{2+}]_i = K_d[(R - R_{min})/(R_{max} - R)](S_{f2}/S_{b2})$ [Grynkiewicz et al., 1985], where R is the experimental value of fluorescence, K_d is the dissociation constant, 0.25 μM. R_{min} and R_{max} are minimal fluorescence (all the indicators are in the Ca^{2+}-free form) and maximal fluorescence (indicators are saturated with calcium), respectively. The factor S_{f2}/S_{b2} is the ratio of the

fluorescence intensity measured when all the indicators are free (S_{f2}) or Ca^{2+}-bound (S_{b2}). Both measurements were taken at 490 nm.

RESULTS

Time-Dependent Effects of GA and RA on HSP70 Expression and HSF1 Phosphorylation in NSCLC H460 Cells

Human NSCLC H460 cells were treated with 0.5 μM GA or RA and allowed to recover for 6 h.



The de novo synthesis of proteins and protein levels of HSP70 in the treated cells were monitored by metabolically labeling with [^{35}S]methionine and Western blotting, respectively (Fig. 1). As shown in the autoradiographs, the expression of HSP70 was significantly increased. On the other hand, the induction of HSP90s and HSC70, the highly constitutively expressed HSPs, was not as obvious. Induction or suppression of other proteins was not noticeable. The identity and increased expression of HSP70 were verified by the corresponding Western blots (Fig. 1A,B). Quantitative analysis of the autoradiographs indicated that the induction of HSP70 was much faster in GA-treated cells. The rate of HSP70 synthesis reached maximum (approximately 3.3-fold) after only 5 min of treatment and leveled off thereafter. The enhanced synthesis rate sustained for 5 h (300 min) and it was still doubled compared to untreated cells. In contrast, the onset of HSP70 induction begins slowly in RA-treated cells, reaching maximum (approximately twofold) after 2 h (120 min) of drug treatment before leveling off (Fig. 1C).

Since the induction of HSP70 most likely resulted from activation of HSF1 through phosphorylation, the effects of GA and RA on this process were also monitored by immunoblotting techniques. Latent and phosphorylated HSF1 exhibited protein bands with apparent molecular weights of 80 kDa (lower band) and 90 kDa (up-shifted band), respectively (Fig. 2). It was found that phosphorylation of HSF1 became detectable within 1 min upon treatment in GA-treated cells but the response was again much slower in cells treated with RA (Fig. 2). Considered in perspective, our data showed that treatment with GA would lead to instant

Fig. 1. Effects of GA and RA on de novo protein synthesis and expression of HSP70 in NSCLC H460 cells. Cells were respectively treated with 0.5 μM GA (**A**) or RA (**B**) for the indicated time durations. After treatment, cells were allowed to recover for 6 h in the absence of drugs. For de novo protein synthesis, cells were labeled with [^{35}S]methionine for 1 h before harvested. Equal amounts of labeled and unlabeled cell lysates were resolved by 10% SDS–PAGE and then respectively processed for autoradiography (**upper panels**, A and B) and Western blot analysis (**lower panels**, A and B) using anti-HSP70 as the primary antibody. Equal loading was confirmed by the amount of actin. **C**: Bands of interest in autoradiographs shown in A and B were quantified by densitometric scanning, and the relative rate of synthesis was presented as a sum of the pixel values of each band, using actin as internal control. Data represent the mean ± SD of three independent experiments.

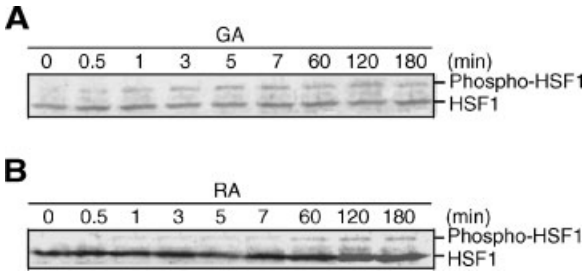


Fig. 2. Effect of GA and RA on HSF1 phosphorylation in NSCLC H460 cells. Cells were respectively treated with 0.5 μM GA (**A**) or RA (**B**) for the indicated durations and allowed to recover for 6 h before being harvested. The cells were then lysed and equal amounts of cell lysates (~160 μg) were resolved by 7.5% SDS–PAGE. The gels were processed for Western blot analysis using primary antibodies against HSF1. The up-shifted bands represent the hyperphosphorylation form of HSF1.

phosphorylation-activation of HSF1, which in turn resulted in rapid induction of HSP70, and that response in RA-treated cells were substantially delayed. Thus, the mechanisms underlying the induction of HSP70 by GA and RA would be different, albeit the fact that both drugs are proven inhibitors of HSP90.

Inhibitory Effects of PKC Inhibitor H7 on GA- or RA-Induced HSP70 Expression and HSF1 Phosphorylation

It has been suggested that a number of protein kinases are responsible for the phosphorylation-activation of HSF1 and we have reported that the GA-induced expression of HSP70 in H460 cells would be attenuated by the presence of H7, a serine/threonine protein kinase inhibitor [Shu et al., 2005]. For short-term treatments, cells were treated for 5 min as both HSP70 induction and HSF1 phosphorylation would become evident in GA-treated cells (but not in RA-treated cells). For long-term treatments, cells were treated by GA for 5 h (300 min) or RA for 2 h (120 min) since these treatment durations would result in approximately twofold induction of HSP70. Cells were pre-incubated with 60 μM of H7 for 1 h before they were subjected to drug treatments. Induction and expressed levels of HSP70 were respectively monitored by metabolic labeling and Western blotting as described in the previous section. The data indicated that, in cells pre-treated with H7, the de novo synthesis of HSP70 was completely abolished and the corresponding accumulation of HSP70 was completely suppressed (Fig. 3A). Simultaneously, HSF1 phosphorylation in the samples

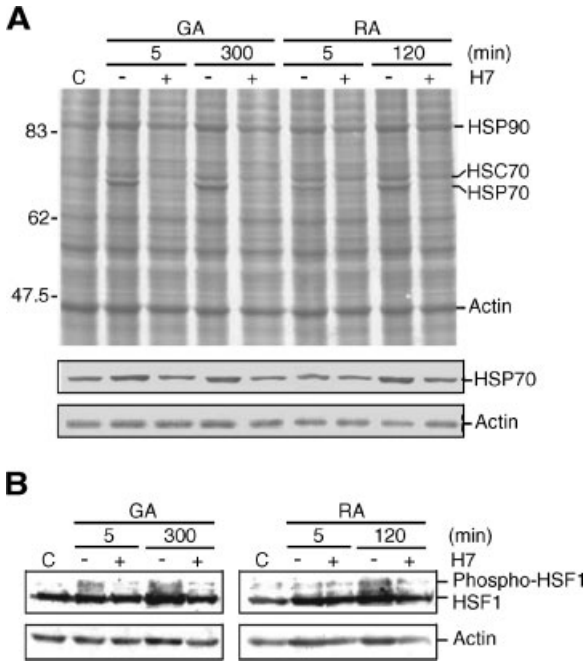


Fig. 3. Effects of protein kinase inhibitor H7 on GA- and RA-induced HSP70 synthesis and HSF1 hyperphosphorylation in NSCLC H460 cells. Cells were pre-incubated with 60 μM H7 for 1 h followed by treatment with 0.5 μM GA (for 5 and 300 min) or 0.5 μM RA (for 5 and 120 min). The cells were subsequently allowed to recover for 6 h. H7 was always present in the medium except during [^{35}S]methionine labeling. **A**: Labeled and unlabeled samples were then respectively processed for autoradiography and Western blot analysis as previously described. Autoradiograph with de novo synthesis of HSC70, HSP70, HSP90 and actin marked (**upper panel**), and Western blot analysis of HSP70, HSP70 band was indicated, equal loading was confirmed by the amount of actin (**lower panel**). **B**: Hyperphosphorylation of HSF1 was examined by Western blot analysis. Equal amounts of cell lysate (~160 μg) were resolved by 7.5% SDS–PAGE, and the membranes were probed using anti-HSF1 as the primary antibody. Up-shifted bands represent the hyperphosphorylation form of HSF1 while actin is used as a loading control.

was monitored by the appearance of up-shifted protein bands revealed by immunoblotting. As shown in Figure 3B, phosphorylation of HSF1 induced by treatments with GA and RA was largely neutralized by pretreatment with H7 (Fig. 3B). These results suggested that induction of HSP70 by GA and RA was commonly mediated by the phosphorylation-activation of HSF1.

Changes in $[Ca^{2+}]_i$ in H460 Cells Treated with GA and RA

Led by our previous finding that GA treatment would elicit a calcium spike signal in H460 cells [Shu et al., 2005], the following experiments were designed to elucidate that how

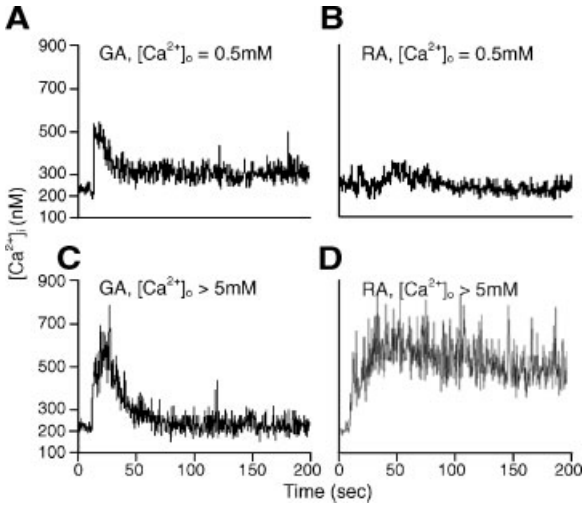


Fig. 4. Effects of $[Ca^{2+}]_o$ on $[Ca^{2+}]_i$ mobilization in GA- and RA-treated HSCLC H460 cells. Prior to drug treatment, cell culture medium were replaced with normal medium ($[Ca^{2+}]_o = 0.5$ mM) (**A** and **C**) and Ca^{2+}-enriched medium (normal medium with 5 mM $CaCl_2$, $[Ca^{2+}]_o > 5.0$ mM) (**B** and **D**). The cells were then treated with the drugs (at time 0) while $[Ca^{2+}]_i$ was being monitored by single-cell dual-wavelength microfluorometry as described in Materials and Methods. The experiments were performed at least three times and representative graphs are shown.

calcium signaling is involved in the induction processes. Two different concentrations of extracellular calcium ($[Ca^{2+}]_o$) were used and the changes in $[Ca^{2+}]_i$ were monitored by microspectrophotometry. In normal culture medium ($[Ca^{2+}]_o = 0.5$ mM), GA treatment elicited a sharp increase in $[Ca^{2+}]_i$ in 10 s and the signal spike subsided to basal level within 30 s (Fig. 4A); on the other hand, RA treatment did not affect calcium homeostasis at all (Fig. 4B). In calcium-enriched medium ($[Ca^{2+}]_o > 5.0$ mM), the calcium signal spike in GA-treated cells was slightly stronger in magnitude (Fig. 4C), whereas that in the RA-treated cells was completely different (Fig. 4D). Upon treatment with RA, $[Ca^{2+}]_i$ was promptly elevated and maintained at a high level for more than 3 min (Fig. 4D). In calcium-free medium, $[Ca^{2+}]_i$ mobilization was not observed in GA- or RA-treated cells (data not shown).

Effects of $[Ca^{2+}]_o$ on HSP70 Induction in H460 Cells Treated with GA and RA

Based on the fact that conventional PKC isozymes require calcium influx for activity [Nishizuka, 1995] and that $[Ca^{2+}]_i$ mobilization is essential for HSP induction in GA-treated cells [Shu et al., 2005], we further examined whether the differential HSP70 induction by GA and RA could be attributed to the differences in calcium signaling. As shown in Figure 5, in normal culture medium, significant induction of HSP70 could be detected in cells subjected to short and long-term treatments with GA, and long but not short-term treatment with RA (Fig. 5, left panel). In contrast, significant induction of HSP70 became ubiquitous in all samples in calcium-enriched medium (Fig. 5, middle panel). In calcium-free medium, however, de novo synthesis of HSP70 was entirely abolished (Fig. 5, right panel). These results strongly indicated that calcium influx is crucial for the expression of HSP70, regardless of basal or induced, or the forms of induction. The

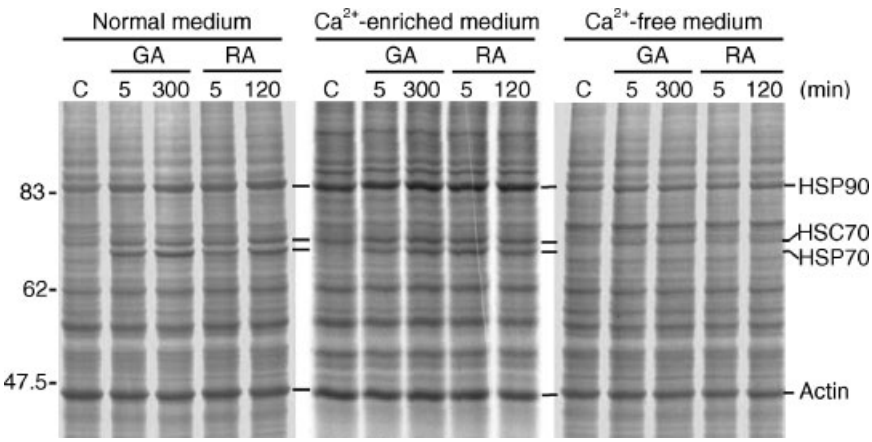


Fig. 5. Effects of $[Ca^{2+}]_o$ on GA- and RA-induced HSP70 synthesis in NSCLC H460 cells. Prior to drug treatment, cell culture medium were replaced with those containing different concentrations of calcium: normal medium ($[Ca^{2+}]_o = 0.5$ mM, **left panel**), Ca^{2+}-enriched medium ($[Ca^{2+}]_o > 5$ mM, **middle panel**) and Ca^{2+}-free medium ($[Ca^{2+}]_o = 0$, **right panel**). The cells in different media were then respectively treated with 0.5 μM GA (for 5 and 300 min) or 0.5 μM RA (for 5 and 120 min), allowed to recover for 6 h and labeled with $[^{35}S]$methionine for 1 h, then processed for autoradiography as previously described.

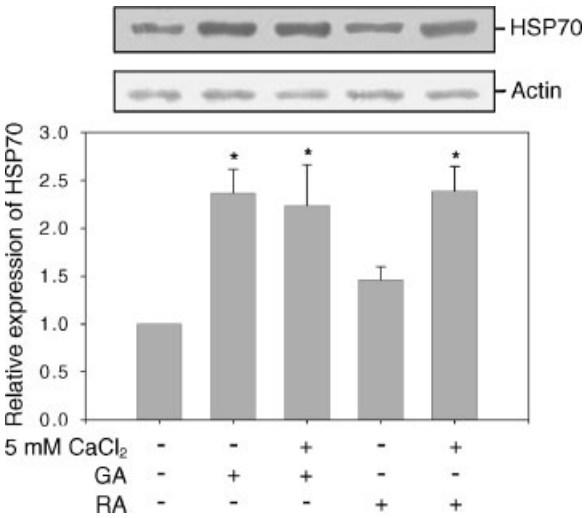


Fig. 6. Effects of excess $[Ca^{2+}]_o$ on induction of HSP70 in GA- and RA-treated HSCLC H460 cells. The cells were treated with 0.5 μM GA or RA for 5 min in normal medium or Ca^{2+}-enriched medium, and allowed recovering for 6 h in normal medium in the absence of the drugs. After recovery, the cells were harvested, lysed, and the cells lysates were resolved by SDS–PAGE. The gels were processed for Western blot analysis by using anti-HSP70 as the primary antibodies (**upper panel**). The visualized protein bands were quantified by densitometric scanning, and normalized with actin (**lower panel**). The data shown are representative of three reproducible experiments. Data represent the mean ± SD of three independent experiments.

protein levels of HSP70 in cells exposed to calcium-enriched medium and treated for 5 min with GA and RA were also determined. Under such conditions, short-term treatment with RA was also able to elicit enhanced induction of HSP70, and the protein level was as high as that of treatment with GA (Fig. 6). All in all, these results support the idea that there are common mediators between GA and RA pathways and that different calcium influxes might be able to account for the differential effects of GA and RA on HSP70 induction.

DISCUSSION

In this report, we studied the mechanistic effects of GA and RA on HSP70 induction in NSCLC H460 cells. The HBAs, GA, and RA are specific inhibitors of HSP90 that are recently being exploited as molecular targets for cancer treatment [Bagatell and Whitesell, 2004; Workman, 2004a,b]. Despite the fact that both drugs are effective inducers of HSPs [Zou et al., 1998; Bagatell et al., 2000; Griffin et al., 2004] and that their derivatives (e.g., 17AAG and KF25706) are being developed for therapeutic uses [Workman, 2004b], the differential inductions of HSPs by the GA and RA have not been investigated. In our study, we found that the kinetics of HSP70 induction is drastically different in GA and RA treated cells. Given time for gene transactivation and protein translation (i.e., the recovery time), we found that increased expression of HSP70 could be achieved within 5 min of drug exposure in GA-treated cells. On the other hand, a much more prolonged treatment time (i.e., 1 h) was required for the detection of HSP70 induction in cells treated with RA. We have also shown that the preceding hyperphosphorylation/activation of HSF1 occurs in a similar time-dependent manner, supporting the proposition that induction of HSPs is governed by the activation of this transcription factor [Holmberg et al., 2002].

Activation of HSF1 in mammalian cells is a complex process, largely regulated by protein phosphorylation/dephosphorylation. In normal cells, HSF1 monomers are bound to the HSP90 chaperone complex and are constitutively phosphorylated. Upon sensing stress, HSF1 undergoes a transition to form homotrimers, translocates to the nucleus, and transactivates HSE-containing (heat shock) genes [Morano and Thiele, 1999]. Both nuclear translocation and transcriptional activity acquisition required the phosphorylation step [Holmberg et al., 2002; Kim et al., 2005]. Depending on the treatments, a number of protein kinases were suggested to act as signaling mediators in this process [Ding et al., 1996; Hung et al., 1998; Holmberg et al., 2001]. The involvement of PKC in HSP70 induction and the modulation of the heat shock response by PKC isozymes have been documented [Holmberg et al., 1998]. Recently, we have shown that depletion of intracellular calcium ($[Ca^{2+}]_i$) with BAPTA-AM, a chelator of $[Ca^{2+}]_i$, significantly decreased GA-induced HSP70 expression. Additionally, the induction of HSP70 could be diminished by protein kinase inhibitor H7 and PKC-specific inhibitors (Ro-31-8425 and Go-6976), suggesting that both calcium and PKC are involved in GA-induced synthesis of HSP70 in NSCLC H460 cells [Shu et al., 2005]. Herein, we showed that induction of HSP70 by GA and RA can be completely abolished by the presence of protein kinase inhibitor, H7 and depletion of external calcium. The results indicated that the induction

processes, although different in kinetics, may share common mediators/modulators.

Cytosolic (or intracellular) calcium, is a focal point of many signal transduction pathways and changes in $[Ca^{2+}]_i$ regulate many cellular functions, including proliferation, differentiation, and death [Berridge et al., 2003]. Calcium homeostasis is a balance between extracellular calcium (i.e., $[Ca^{2+}]_o$) and intracellular storage sites such as the ER (i.e., $[Ca^{2+}]_{ER}$) and mitochondria (i.e., $[Ca^{2+}]_{mit}$), which is tightly regulated by the concerted actions of cohorts of cross-membrane calcium channels/pumps [Strehler and Treiman, 2004]. In general, cells treated with agents that disturb calcium homeostasis would result in the induction of glucose-regulated proteins but not the HSPs [Lee, 2001]. It is now clear that calcium and/or calcium-dependent protein kinases play very important roles in the process of HSP induction. In addition, it has been reported that $[Ca^{2+}]_i$ can be directly involved in HSP70 induction [Ding et al., 1996] and that increased synthesis of HSP70, elicited by a variety of inducers, is attenuated in cells placed in media devoid of calcium [Chen et al., 1996; Kiang et al., 2000]. Furthermore, chelating $[Ca^{2+}]_i$ by BAPTA-AM or $[Ca^{2+}]_o$ by EGTA, resulted in significant reduction of GA-induced expression of HSP70 [Shu et al., 2005], suggesting a positive/stimulatory role of $[Ca^{2+}]_i$ in the process. However, calcium ionophore A23187, which drastically elevates $[Ca^{2+}]_i$ in the treated cell, could inhibit the induction of HSP70 by heat shock [Elia et al., 1996] as well as activation of HSF-1 [Soncin et al., 2000]. On the contrary, another ionophore ionomycin causes an increase in $[Ca^{2+}]_i$ and activates HSF1, resulting in the induction of HSP70 [Sreedhar and Srinivas, 2002]. Thus, the effect of $[Ca^{2+}]_i$ disturbance on HSP induction is still controversial, and the different effects of A23187 and ionomycin may be caused by different experimental approaches and the applied dosages. In this study, we found that removal of $[Ca^{2+}]_o$ leads to total abolishment GA- and RA-induced expression of HSP70, indicating that calcium influx is essential for the induction processes triggered by GA and RA in NSCLC 460 cells. We further showed that GA induced a prompt calcium influx, which fittingly correlated with rapid HSF1 phosphorylation and HSP70 induction. In contrast, induction of HSP70 by RA, which was unable to induce the $[Ca^{2+}]_i$ spike signal, was much slower. However, finding that depletion of $[Ca^{2+}]_o$ also interrupted RA-induced synthesis of HSP70 suggested that calcium influx is also required for the process and RA may eventually cause mobilization of $[Ca^{2+}]_i$ by a mechanism that remains to be elucidated. In cells placed in ectopic calcium (i.e., excess $[Ca^{2+}]_o$), a rapid calcium influx was detected in RA-treated samples and the response was accompanied by rapid induction of HSP70. The results further support the notion that calcium mobilization is a co-stimulator of HSP70 induction in cells treated with HBAs.

In summary, we have shown that the HBAs were able to activate HSF1 and bring about the induction of HSP70. However, kinetics of the processes triggered by GA and RA are different (i.e., GA is faster and more effective in its induction of the heat shock response than RA), which may be attributed to discrepancies in their respective abilities to elicit calcium mobilization. Additionally, our present study provides the first piece of corroborating evidence indicating that calcium mobilization and related protein kinase activities are general mediators of signaling for GA and RA. Our findings implied that there may be additional targets to which the HBAs bind and evoke calcium mobilization, which in turn induce subsequent responses. Further studies will be required to address this question and the mechanisms underlying these observations. Understanding HBA drug actions at a molecular level would undoubtedly aid drug development for the management of malignant diseases and our findings may help shed some new light on this process.

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