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Arctigenin from Fructus Arctii is a novel suppressor of heat shock response in mammalian cells

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Abstract Because heat shock proteins (Hsps) are involved in protecting cells and in the pathophysiology of diseases such as inflammation, cancer, and neurodegenerative disorders, the use of regulators of the expression of Hsps in mammalian cells seems to be useful as a potential therapeutic modality. To identify compounds that modulate the response to heat shock, we analyzed several natural products using a mammalian cell line containing an *hsp* promoter-regulated reporter gene. In this study, we found that an extract from Fructus Arctii markedly suppressed the expression of Hsp induced by heat shock. A component of the extract arctigenin, but not the component arctiin, suppressed the response at the level of the activation of heat shock transcription factor, the induction of mRNA, and the synthesis and accumulation of Hsp. Furthermore, arctigenin inhibited the acquisition of thermotolerance in mammalian cells, including cancer cells. Thus, arctigenin seemed to be a new suppressive regulator of heat shock response in mammalian cells, and may be useful for hyperthermia cancer therapy.

INTRODUCTION

The heat shock response that is characterized by the induction of heat shock proteins (Hsps) is a highly conserved mechanism of gene regulation in response to a wide variety of physiological challenges (Lindquist 1986; Minowada and Welch 1995; Yura and Nakahigashi 1999). Hsps function as molecular chaperones to prevent protein aggregation; they facilitate refolding of dysfunctional proteins, which is critical to the survival of all organisms; and they have been implicated in longevity and aging. Regulation of the cellular level of Hsps has gained attention as a potential therapeutic modality for cancer, ischemia-reperfusion, trauma, transplantation surgery, and diabetes (Favatier et al 1997; Oki and Younes 2004). Consequently, the identification of pharmacologically active small molecules that influence the levels of Hsps has gained some attention. For example, sodium salicylate and indomethacin induce a response to stress and suppress heat- and polyglutamine-induced cytotoxicity in mammalian cells (Ishihara et al 2003, 2004). In contrast to inducers of Hsps, suppressors of Hsp expression are also believed to be useful for cancer therapy using hyperthermia, which is now considered an effective modality in conjugation with radiotherapy or chemotherapy. One of the major difficulties with the use of hyperthermia is, however, the development of thermotolerance, a transient resistance to heat shock induced by prior sublethal heat treatment, in cancer cells (Gerner and Schneider 1975; Liu et al 1996). The inhibition of the acquisition of thermotolerance in cancer cells could be expected to improve the antitumor efficiency of hyperthermia.

We have established a simple screening system for Hsp modulators; mouse cells stably transfected with a plasmid containing the *hsp105* promoter upstream of a luciferase reporter gene (Ishihara et al 2003). To identify compounds that modulate the response of mammalian cells to heat shock, we examined various natural products using a simple screening system. In this study, we found that an extract from Fructus Arctii markedly suppressed the expression of Hsp induced by heat shock. Furthermore, we identified arctigenin (ARC-G) as the active component in the extract, and showed that the compound also inhibited the development of thermotolerance in various cancer cells.

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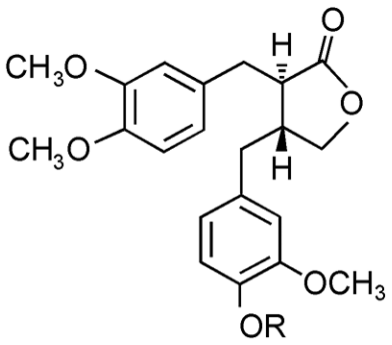

Arctigenin: R=H
Arctiin: R=D-glucose

Fig 1. Structure of arctigenin (ARC-G) and arctiin (ARC).

MATERIALS AND METHODS

Materials

Fructus Arctii (fruits of *Arctium lappa*) obtained from Tochimototenkaido Co Ltd (Osaka, Japan) were extracted with methanol. Arctiin (ARC) and ARC-G (Fig 1) were isolated from the extract and identified with authentic samples by their spectrometric data of infrared spectrometry (IR), mass spectrometry (MS), and nuclear magnetic resonance (Han et al 1994; Takasaki et al 2000). From 1 kg of Fructus Arctii, 0.5 g of ARC-G and 2.5 g of ARC were isolated.

Cell culture conditions and treatments

Mouse fibroblast C3H10T1/2 cells, human cervical carcinoma HeLa cells, and human lung carcinoma A549 cells were obtained from RIKEN Bioresource Center Cell Bank (Tsukuba, Japan). C3H10T1/2 cells stably transfected with a plasmid containing the mouse *hsp105* promoter upstream of a luciferase reporter gene were designated pGL105/C3H cells (Ishihara et al 2003). These cells were cultured in Dulbecco modified Eagle medium (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% fetal calf serum in a humidified atmosphere of 5% CO_2 in air at 37°C.

Extracts and compounds from natural products were dissolved in dimethylsulfoxide (DMSO), and added to the culture medium at the indicated concentrations. Control cells were incubated with DMSO at equivalent concentrations.

Measurement of luciferase activity

pGL105/C3H cells (2×10^5 cells/35 mm dish) were treated with extracts or compounds at 37°C for 24 hours, then further incubated at 37 or 41°C for 3 hours. After the harvesting of cells, luciferase activity was assayed with a luciferase assay reagent (Promega, Madison, WI, USA) using a Turner Designs model TD-20/20 Luminometer (Turner Designs, Sunnyvale, CA, USA).

Neutral red uptake assay

Cells ($7 \times 10^4 \sim 1 \times 10^5$ cells/well) in 24-well plates were incubated for 3 hours in the presence of 50 μg/mL neutral red, then fixed with 1% formaldehyde containing 1% $CaCl_2$ for 1 minute. The dye incorporated into viable cells was extracted with 50% ethanol containing 1% acetic acid and absorbance was measured at 540 nm.

Gel mobility shift assay

Cell extracts (15 μg of protein) were incubated with a ^{32}P-labeled heat shock element (HSE) of the *hsp70* gene (Williams et al 1989) at 25°C for 20 minutes, and then electrophoresed on a native 4% polyacrylamide gel, as described previously (Ishihara et al 2003). The specific heat shock transcription factor(HSF)-HSE complex was detected by adding a 100-fold molar excess of unlabeled HSE.

Reverse transcription–polymerase chain reaction

Cellular RNA was extracted from cells using the messenger RNA (mRNA) GeneElute kit (Sigma, St Louis, MO, USA) according to the manufacturer's instructions. After treatment with RNase-free DNase I at 37°C for 1 hour, a reverse transcription (RT) reaction was performed. Then, a polymerase chain reaction (PCR) was performed with KOD-dash polymerase (Toyobo, Tokyo, Japan) and primers specific for Hsp70 or G3PDH for 25 cycles (95°C, 15 seconds; 55°C, 30 seconds; 68°C, 1 minute). The primers for Hsp70 were 5′-ACTCTGCTGCTTCTCCTTGC-3′ (forward) and 5′-TCACAATGCAATGTCCCTGT-3′ (reverse). The primers for G3PDH were purchased from Toyobo (human, rat, mouse G3PDH RT-PCR primer set). PCR products were analyzed by agarose gel electrophoresis.

Measurement of protein and RNA syntheses of cells

To measure cellular RNA and protein syntheses, cells (2×10^5 cells/35 mm dish) were incubated in uridine- or leucine-deficient Eagle minimal essential medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal calf serum and 1 μCi/mL [4,5-^{3}H] uridine or [5,6-^{3}H] leucine (Amersham Pharmacia Biotech, Baie D'Urfe, Quebec, Canada), respectively, at 37 or 41°C for 3 hours. Cells were then washed, suspended in 0.5 mL of phosphate-buffered saline, and mixed with 0.5 mL of ice-cold 20% (w/v) trichloroacetic acid. The mixture was kept on ice for 30 minutes, then filtered on a glass filter disk (What-

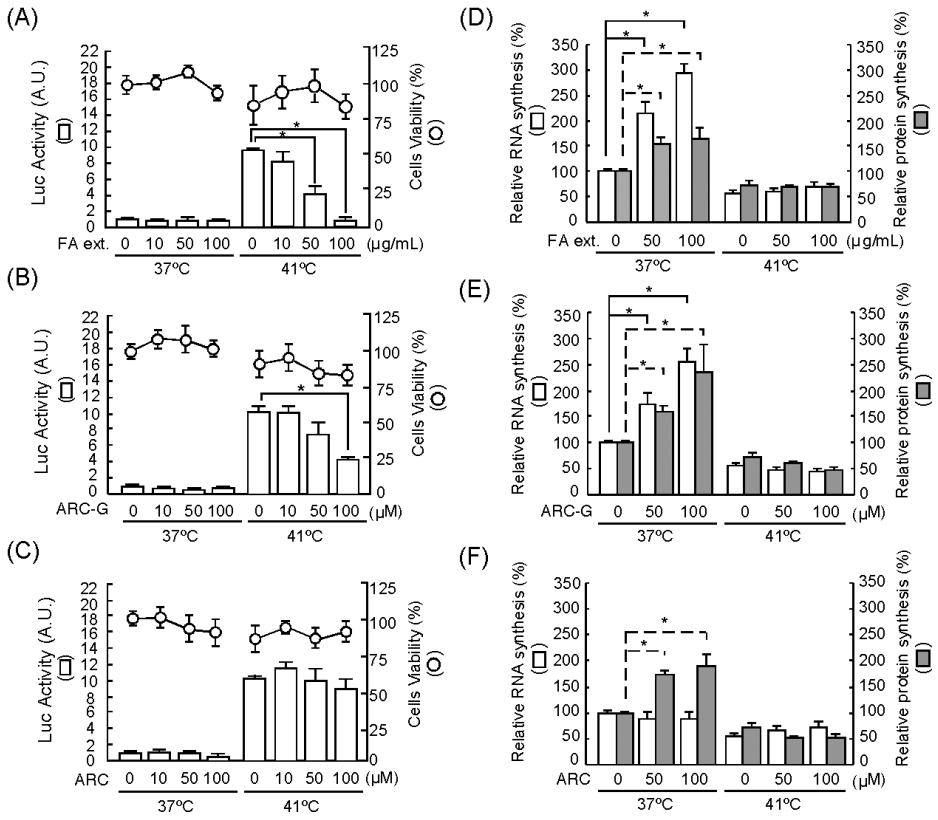

Fig 2. Effects of FA ext., ARC-G, and ARC on the hsp promoter in mammalian cells. pGL105/C3H cells were incubated with or without FA ext. (A), ARC-G (B), or ARC (C) at 37°C for 24 hours, and further incubated at 37° or 41°C for 3 hours. Luciferase activity was measured, and relative activity is shown as a ratio to that of control cells. The viability of cells was assessed with a neutral red uptake assay. Cells were incubated with or without FA ext. (D), ARC-G (E), or ARC (F) at 37°C for 24 hours, and further incubated at 37 or 41°C for 3 hours in the medium containing 1 μCi/mL [4,5-^{3}H] uridine or [5,6-^{3}H] leucine, then radioactivity incorporated into the cells was measured as described in Materials and Methods. Relative RNA and protein syntheses are shown as percentages of those of respective control cells at 37°C. Each value represents the mean ± SEM for three independent experiments. Statistical significance was determined with the Student *t*-test, and asterisks indicate that differences are statistically significant between the two values indicated by lines (*$P < 0.05$).

man GF/A) in a filtration apparatus under vacuum. The filter was washed with ice-cold 10% (w/v) trichloroacetic acid and with ethanol. The radioactivity on a filter was measured using a liquid scintillation counter. Relative RNA and protein syntheses were calculated as radioactivity incorporated into cells (dpm/μg cellular proteins).

Measurement of Hsp70 synthesis

Cells (1.3×10^6 cells/60 mm dish) were incubated in methionine-deficient Eagle minimal essential medium (Invitrogen) supplemented with 10% calf serum and 20 μCi/mL [^{35}S]methionine (Amersham Pharmacia Biotech) at 37 or 45°C for 3 hours, and lysed in 200 μL of extraction buffer (50 mM Tris/HCl pH 8.0, 50 mM NaCl, 5 mM ethylenediamine tetraacetic acid, 1% Nonidet P-40, and 2 mM phenylmethylsulfonyl fluoride) for 20 minutes at 4°C. After centrifugation, the cell extract (200 μg of protein) was mixed with 2.5 μL of anti-Hsp70 monoclonal antibody (Sigma), and kept on ice for 1 hour. Next was added 40 μL of 50% Protein G-Sepharose (Amersham Pharmacia Biotech) preabsorbed with 5% bovine serum albumin, and the mixtures were rotated for 1 hour at 4°C, and centrifuged. The precipitates were washed, 40 μL of 2× sodium dodecyl sulfate (SDS) sample buffer was added, and the mixture was boiled for 5 minutes. The eluted proteins were separated by SDS–polyacrylamide gel electrophoresis (PAGE) and detected by autoradiography.

Western blot analysis

Cells were lysed in 100 μL of 0.1% SDS and boiled for 5 minutes. Cellular proteins (15 μg) were separated by SDS-PAGE, and blotted onto a nitrocellulose membrane. The membrane was washed with Tris-buffered saline (0.1 M Tris-HCl pH 7.5, and 0.9% NaCl) containing 0.1% Tween-20 (TTBS), and incubated with rabbit anti-Hsp105 (Honda et al 1989; Ishihara et al 1999), mouse anti-Hsp70 (Stressgen Biotech, Victoria, British Columbia, Canada, catalog no. SPA-810), or mouse anti-α-tubulin antibody (Sigma) at room temperature for 1 hour. After a wash with TTBS, the membrane was incubated with horseradish peroxidase-conjugated anti-rabbit or anti-mouse immunoglobulin G antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at room temperature for 1 hour. Hsp105α, Hsp70, and α-tubulin were then detected using enhanced chemiluminescence reagent (Santa Cruz Biotechnology). For quantification, films were digitized by scanning into Adobe Photoshop 7 (Adobe Systems, San José, CA, USA), and the intensity of each band (Hsp105α, Hsp70, and α-tubulin) was quantified using the software program NIH Image (http://rsb.info.nih.gov/nih-image/).

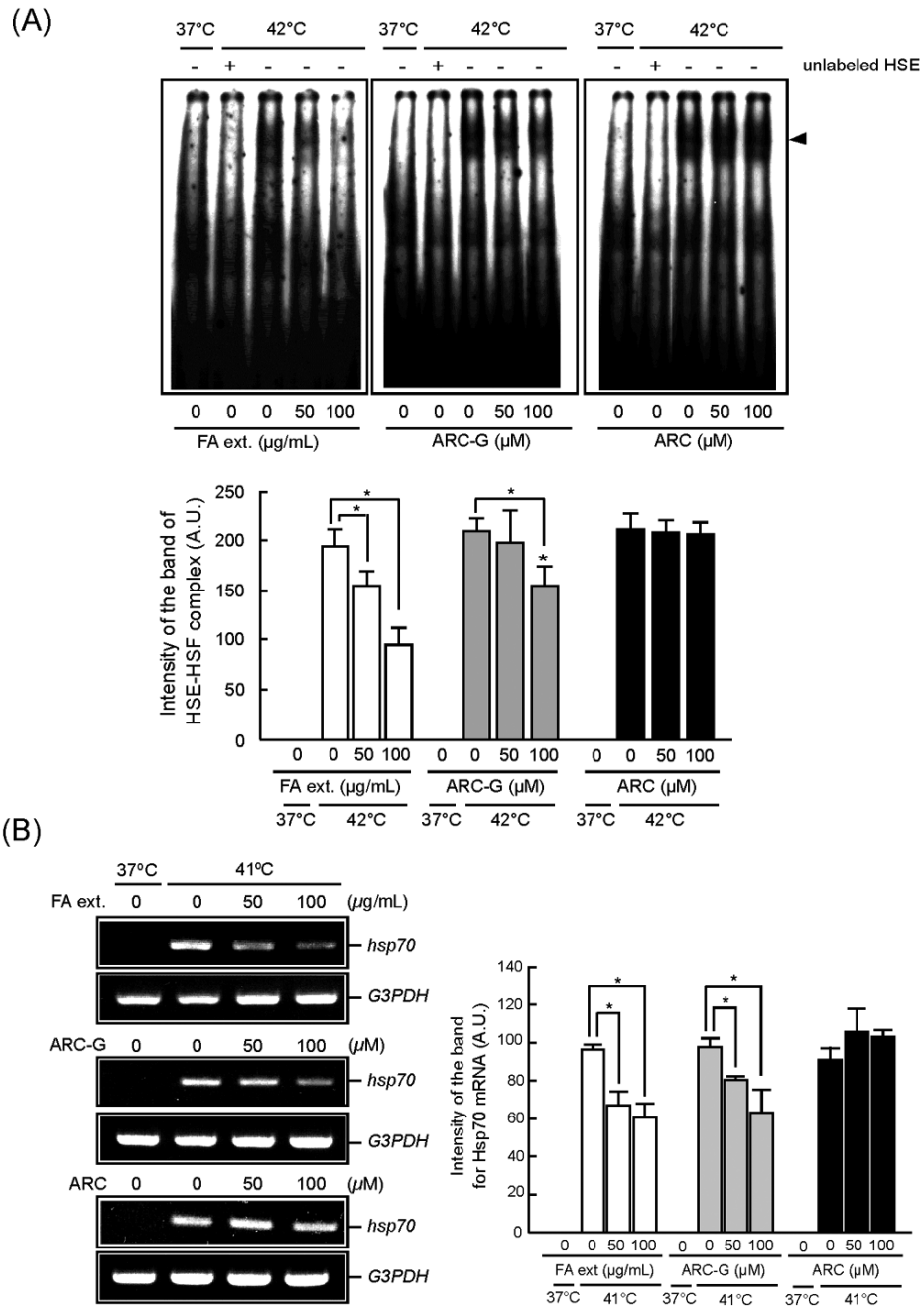

Fig 3. Effects of FA ext., ARC-G, and ARC on the activation of HSF and the expression of Hsp70 mRNA in mammalian cells. (A) C3H10T1/2 cells were incubated with or without FA ext., ARC-G, or ARC at 37°C for 24 hours, and then subjected to heat shock at 42°C for 1 hour. Extracts from these cells were subjected to a gel mobility shift assay using ^{32}P-labeled HSE. A specific HSF-HSE complex was determined by adding a 100-fold excess of unlabeled HSE. The arrowhead indicates the specific HSF-HSE complex, and the intensity of bands of this complex was quantified. (B) Total RNA of cells treated with or without these compounds at 37°C for 24 hours and heat shocked at 41°C for 3 hours was analyzed by RT-PCR. Bands were quantified by densitometry, and the level of hsp70 mRNA is normalized to that of G3PDH. In (A) and (B), each value represents the mean ± SEM for three independent experiments. Statistical significance was determined with the Student *t*-test, and asterisks indicate that differences are statistically significant between the two values indicated by lines (*$P < 0.05$).

Thermotolerance of cells

C3H10T1/2 cells (7×10^4 cells/well) grown on collagen-coated coverslips in 24-well plates were incubated with or without the drug at 37°C for 24 hours, and further incubated at 41 or 42°C for 3 hours to induce thermotolerance or at 37°C for 3 hours as a control. These cells were then exposed to a lethal heat shock at 45°C for 45 minutes or 1 hour, and cell morphology was observed using a phase-contrast microscope.

RESULTS

Extract from Fructus Arctii suppresses heat shock response of mammalian cells

To identify compounds that modulate the heat shock response of mammalian cells, we screened extracts from various natural products using pGL105/C3H cells containing an *hsp105* promoter-regulated luciferase reporter-gene (Ishihara et al 2003). When the cells were incubated with the extracts from Fructus Arctii, Fructus Gardeniae,

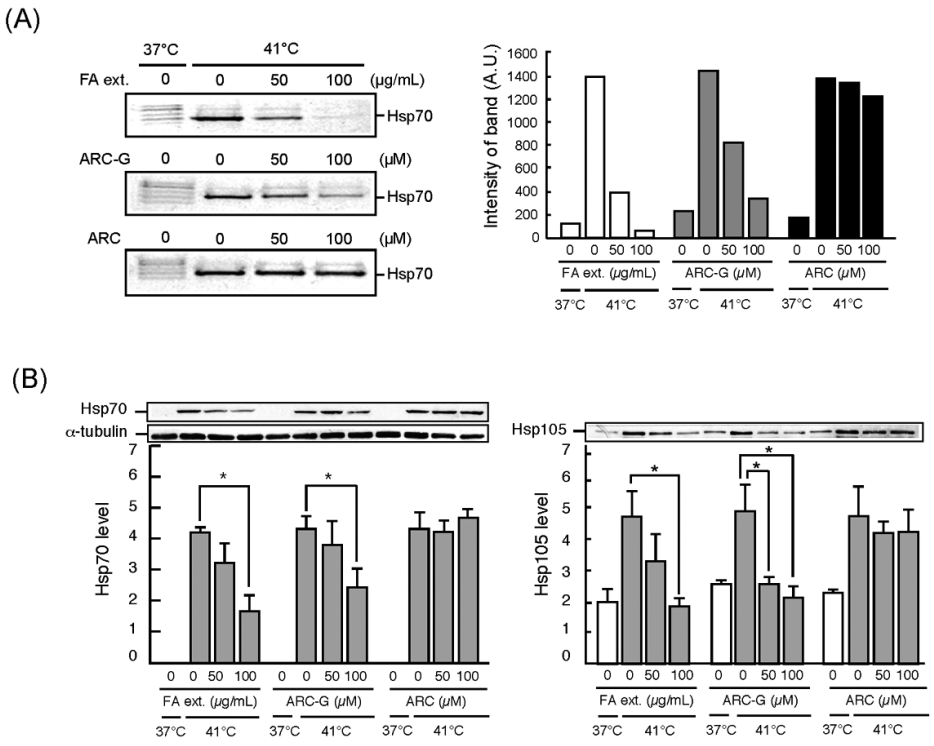

Fig 4. Effects of FA ext., ARC-G, and ARC on the synthesis and accumulation of Hsp70 in mammalian cells. (A) C3H10T1/2 cells were treated with or without FA ext., ARC-G, or ARC at 37°C for 24 hours, and then labeled with [^{35}S]methionine at 37 or 41°C for 3 hours. Cell extracts were immunoprecipitated with anti-Hsp70 antibody, and Hsp70 was separated by SDS-PAGE and detected by autoradiography. Bands were quantified by densitometry, and each value represents the mean for two independent experiments. (B) Cells incubated with or without these compounds at 37°C for 24 hours were further incubated at 37 or 41°C for 3 hours. Cellular proteins were separated by SDS-PAGE, blotted onto nitrocellulose membranes, and immunostained using anti-Hsp105, anti-Hsp70, or anti-α-tubulin antibody. Bands were quantified by densitometry, and the level of Hsp105α or Hsp70 is normalized to that of α-tubulin. Each value represents the mean ± SEM for three independent experiments. Statistical significance was determined with the Student *t*-test, and asterisks indicate that the differences are statistically significant between the two values indicated by lines (*$P < 0.01$).

and Saussurea Involucrate at 37°C for 24 hours, no promoter activity was induced. However, activity that increased the basal level to more than 10-fold after heat shock at 41°C for 3 hours was suppressed by treatment with the Fructus Arctii extract (FA ext.) in a dose-dependent manner (Fig 2A). The extracts from Gardeniae Fructus or Saussurea Involucrate, however, did not suppress or enhance the heat-induced promoter activity (data not shown). Furthermore, although the heat-induced promoter activity was almost completely suppressed by FA ext. at a concentration of 100 μg/mL, no cytotoxic effect of the extract was detected in the neutral red uptake assay (Fig 2A). Because FA ext. contains primarily ARC-G and ARC (Fig 1), we next examined the effects of these compounds on the heat shock promoter using pGL105/C3H cells. ARC-G, but not ARC, at a concentration of 100 μM suppressed the heat-induced promoter activity to approximately 25% of control without having a cytotoxic effect (Fig 2B,C). As the suppression of the heat-induced promoter activity by these compounds may be due to the general suppression of cellular RNA and protein syntheses, we examined effects of these compounds on cellular RNA and protein syntheses (Fig 2D–F). FA ext. (up to 100μg/mL) and ARC-G (up to 100 μM) rather stimulated RNA synthesis at 37°C, but ARC (up to 100 μM) did not affect RNA synthesis. Protein synthesis at 37°C was also stimulated by FA ext., ARC-G, and ARC; however, these compounds did not significantly enhance or inhibit RNA and protein syntheses at 42°C. Furthermore, toxic doses (LD_{50}) of FA ext., ARC-G, and ARG were 240 ± 6 μg/mL, 280 ± 15 μM, and more than 300 μM, respectively (data not shown). Thus, FA ext. as well as its component, ARC-G, seemed to specifically suppress activation of the heat shock promoter induced by heat shock.

Heat shock response is regulated by the activation of HSF, which binds to the conserved HSE in the promoter region of *hsp* genes. Then, we next examined the effect of FA ext. and its components, ARC-G and ARC, on the heat-induced activation of HSF by conducting a gel mobility shift assay (Fig 3A). When C3H10T1/2 cells were heat shocked at 42°C for 1 hour, the activation of HSF was marked. However, treatment with FA ext. at 50 and 100 μg/mL suppressed the activation in a dose-dependent manner. ARC-G, but not ARC, at 100 μM also significantly suppressed the activation of HSF by heat shock. When the expression of Hsp70 mRNA was analyzed by RT-PCR, it was found to be weak in C3H10T1/2 cells at 37°C, but markedly increased by heat shock at 41°C for 3 hours (Fig 3B). Expression of Hsp70 mRNA was also suppressed by FA ext. at 50 and 100 μg/mL and ARC-G at 50 and 100 μM in a dose-dependent manner.

Metabolic labeling experiments further showed that the synthesis of Hsp70 induced by heat shock was markedly suppressed by FA ext. at 50 and 100 μg/mL and ARC-G at 50 and 100 μM, but not ARC (Fig 4A). Western blotting analysis also revealed that FA ext. at 100 μg/mL and

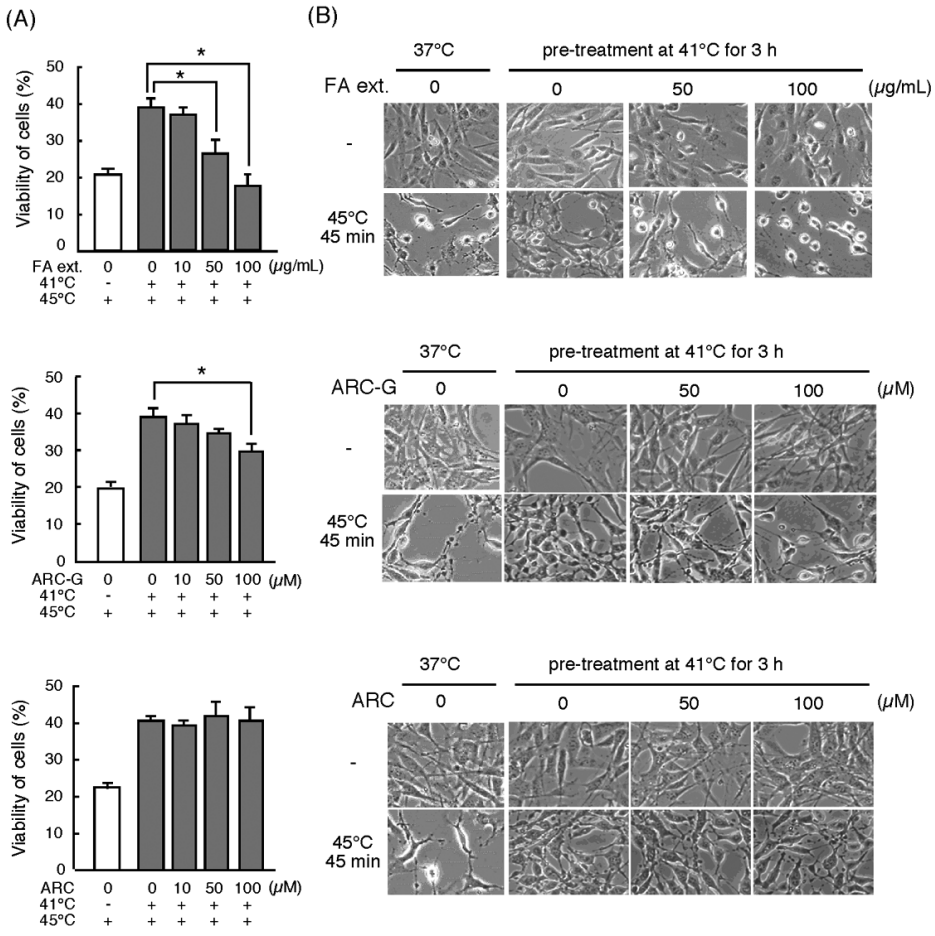

Fig 5. Suppression of thermotolerance of mammalian cells treated with FA ext. and ARC-G. (A) C3H10T1/2 cells incubated with or without FA ext., ARC-G, or ARC at 37°C for 24 hours were further incubated at 41°C for 3 hours, then heat shocked at 45°C for 45 minutes. The viability of cells was measured with a neutral red uptake assay. Each value represents the mean ± SEM for three independent experiments. Statistical significance was determined with the Student *t*-test, and asterisks indicate that differences are statistically significant between the two values indicated by lines ($*P < 0.05$). (B) Cells were incubated with or without these compounds at 37°C for 24 hours, and incubated at 37 or 41°C for 3 hours (upper panels). Then, these cells were further heat shocked at 45°C for 45 minutes (lower panels). Cell morphology was observed using a phase-contrast microscope.

ARC-G at 100 μM significantly suppressed the accumulation of Hsp70 and Hsp105α induced by heat shock (Fig 4B). These findings suggested that FA ext. as well as ARC-G suppressed the response to heat shock at the level of HSF activation, the induction of hsp mRNA, and the synthesis and accumulation of hsp in mammalian cells.

Fructus Arctii extract and arctigenin suppress the thermotolerance of mammalian cells

Thermotolerance is a transient resistance to heat shock induced by pretreatment with a mild heat shock (Gerner and Schneider 1975; Liu et al 1996). Because thermotolerance is suggested to develop concomitantly with the expression of hsp, we next examined whether FA ext. and ARC-G suppressed the thermotolerance of cells. When C3H10T1/2 cells were treated with a lethal heat shock at 45°C for 45 minutes, cell viability determined with the neutral red uptake assay was reduced to approximately 20% of that of control cells (Fig 5A), and a marked decrease in the number of cells attached to dishes was observed by phase-contrast microscopy (Fig 5B). However, when cells were pretreated with a mild heat shock at 41°C for 3 hours to induce thermotolerance and then treated with a lethal heat shock, viability was reduced but remained at approximately 40%, and many cells attached to dishes were observed. However, in cells treated with FA ext. or its constituents at 37°C for 24 hours, the thermotolerance induced by mild heat shock was significantly suppressed by FA ext. at 50 and 100 μg/mL and ARC-G at 100 μM, but not ARC.

Because FA ext. and ARC-G suppressed the heat shock response and development of thermotolerance in mouse C3H10T1/2 fibroblast cells, we further examined whether FA ext. and ARC-G suppress the expression of hsp and the development of thermotolerance in human cancer cells (Fig 6). In human cervical carcinoma HeLa and lung carcinoma A549 cells, FA ext. and ARC-G significantly suppressed the heat-induced accumulation of Hsp70 and the development of thermotolerance.

DISCUSSION

Fructus Arctii is a common herbal medicinal preparation used for treating rheum ailments. It contains primarily ARC-G and ARC, a glycosidic form of ARC-G. These

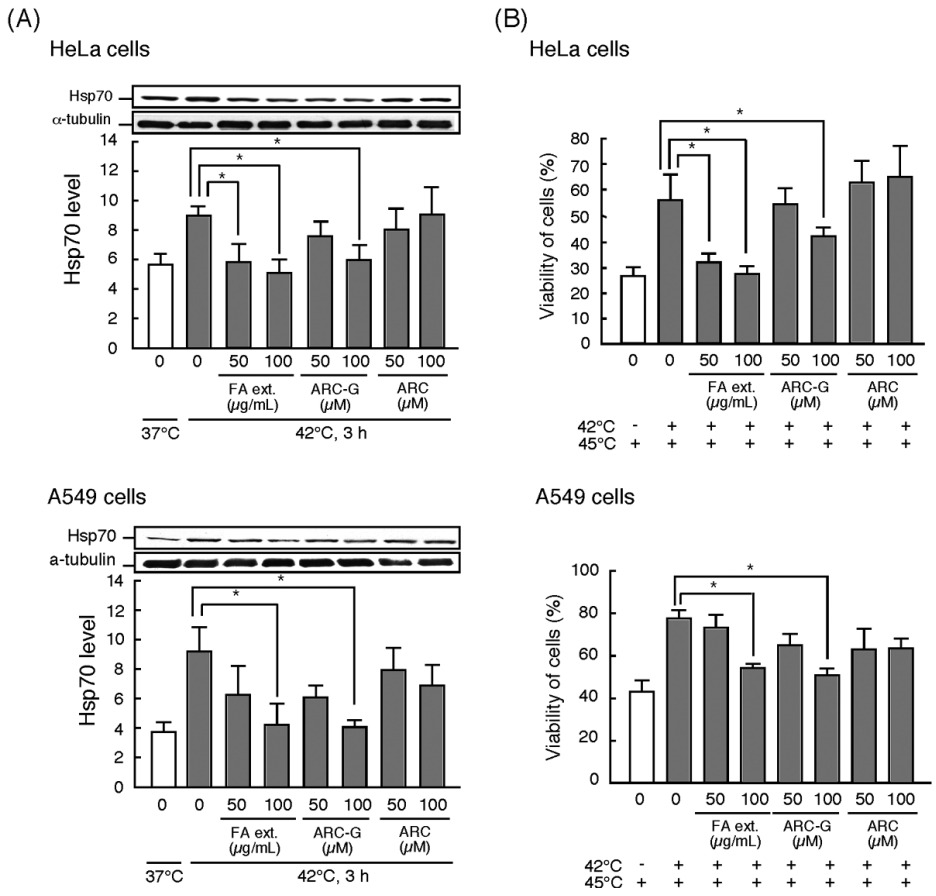

Fig 6. Effects of FA ext., ARC-G, and ARC on thermotolerance of cancer cells. HeLa and A549 cells were incubated with or without FA ext., ARC-G, or ARC at 37°C for 24 hours, and further incubated at 37 or 42°C for 3 hours. (A) Hsp70 in these cells was detected by Western blotting and the density of the bands was quantified. The level of Hsp70 is normalized to that of α-tubulin. (B) After incubation at 37 or at 42°C for 3 hours, cells were further treated with lethal heat shock at 45°C for 1 hour, and the viability of cells was measured with a neutral red uptake assay. In (A) and (B), each value represents the mean ± SEM for three independent experiments. Statistical significance was determined with the Student *t*-test, and asterisks indicate that the differences are statistically significant between the two values indicated by lines (*$P < 0.05$).

compounds have antitumor-promoting and antimutation activities (Takasaki et al 2000). Here we showed that FA ext. and its constituent, ARC-G, suppressed the induction of heat shock response in mammalian cells. The suppression occurred at the levels of HSF activation, mRNA induction, and synthesis and accumulation of hsp. Consistent with reports that the acquisition of thermotolerance correlates with the expression of hsp in a variety of organisms from bacteria to mammals (Li and Werb 1982; Johnston and Kucey 1988), FA ext. and ARC-G also suppressed the thermotolerance of mammalian cells, including human cancer cells. However, although ARC did not suppress the heat shock response and thermotolerance of cells as ARC-G did, this may be due to the permeability of cells to these compounds; the glycosidic form is less able to permeate than the nonglycosidic form. As the suppressive activity of heat shock response by ARC-G was less than that by FA ext., components other than ARC-G may also suppress the heat shock response or simultaneously enhance the inhibitory activity of ARC-G.

Hyperthermia is an effective modality for cancer therapy, in conjugation with radiotherapy or chemotherapy (Levy et al 1998; Kinuya et al 2004). A major difficulty with the use of hyperthermia, however, is the development of thermotolerance in cancer cells, a transient resistance to heat induced by prior sublethal heat treatment. Because it is critical to prevent the induction of thermotolerance when using hyperthermia to treat cancer, a specific inhibitor of Hsp in cancer cells would be useful. Several compounds have been shown to suppress the thermotolerance of mammalian cells. The bioflavonoid quercetin inhibits the expression of hsp, and prevents the acquisition of thermotolerance in human colon cancer cells (Hosokawa et al 1990, 1992; Elia and Santoro 1994). The benzylidene lactam compound KNK437 also inhibits the synthesis of hsp and the acquisition of thermotolerance in human cancer cells (Yokota et al 2000). Stresgenin B isolated from culture broth of *Streptomyces* sp. AS-9 inhibits the heat-induced expression of hsp and thereby suppresses the induction of thermotolerance in several neoplastic cell lines (Akagawa et al 1999). In contrast, paeoniflorin and cerastrol isolated from herbal medicines induce the heat shock response in mammalian cells (Westerheide et al 2004; Yan et al 2004). These compounds have cytoprotective effects against deleterious stressors and may be used for the treatment of various diseases such as ischemia, ulcers, and neurodegenerative disorders.

In addition to these compounds, we herein showed that ARC-G is a new suppressive regulator of heat shock re-

sponse in mammalian cells. Inhibition by FA ext. and ARC-G of the development of thermotolerance in cancer cells may improve the efficacy of clinical fractionated hyperthermia with fewer side effects, because Fructus Arctii is a common herbal medicinal preparation (Leung and Foster 1996).

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