

Review

Chaperone-inducing compounds

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INTRODUCTION

Virtually all organisms respond to up-shifts in temperature (heat shock) by synthesizing a set of proteins called heat shock proteins (HSPs). These HSPs are induced not only by heat shock but also by various other environmental stresses (Schlesinger et al 1982). Usually, HSPs are also expressed constitutively at normal growth temperatures and have basic and indispensable functions in the life cycle of proteins as molecular chaperones (Hartl 1996; Hartl and Hayer-Hartl 2002; Morimoto 2002), as well as play a role in protecting cells from environmental deleterious stresses (Parsell and Lindquist 1993). Molecular chaperones are able to inhibit the aggregation of partially denatured proteins and refold them as demonstrated in *in vitro* and *in vivo* studies (Minami et al 1996; Michels et al 1997). Therefore, molecular chaperones are considered to be endogenous cytoprotective factors, lifeguards, or guardians of proteome (Morimoto and Santro 1998; Jaattela 1999; Ohtsuka and Hata 2000; Ohtsuka and Suzuki 2000; Muchowski 2002).

Many lines of study indicate that molecular chaperones have beneficial functions at both the cellular and tissue level for the organism. For example, induction of molecular chaperones in animals by whole body hyperthermia or by gene transfer can protect the brain and heart from tissue injury induced by ischemia (Liu et al. 1992; Plumier et al 1997; Brown and Sharp 1999; Carroll and Yellon 1999). A moderate overexpression of molecular chaperones resulted in an extended life span in nematoda and the fruit fly (Smith 1958, Lithgow et al. 1995, Tatar et al.

1997; Yokoyama et al 2002; Hsu et al 2003; Walker and Lithgow 2003; Morley and Morimoto 2004). Also, molecular chaperones can suppress the aggregate formation of mutant proteins that cause neurodegenerative diseases, such as spinocerebellar ataxia 1 (SCA1) (Cummings et al 1998), spinal and bulbar muscular atrophy (SBMA) (Kobayashi et al 2000), familial amyotrophic lateral sclerosis (FALS) (Takeuchi et al 2002), and Huntington's disease (Jana et al 2000), as demonstrated by model experimental systems. Although the substrates of the molecular chaperones are usually normal or wild-type proteins, molecular chaperones can also deal with mutant proteins in some cases; that is, they can help even mutant proteins fold correctly and maintain normal function by inhibiting non-productive folding pathways (Jeoung et al 1991; Tang et al 1997).

To date, several chemical compounds are known to have positive effects on the induction of HSPs, such as geranylgeranylacetone (GGA) (Hirakawa et al 1996), bimeclocholol (BRLP-42) (Vigh et al 1997), herbimycin-A (Morris et al 1996), rebamipide (Hahm et al 1997), and carbenoxolone (Nagayama et al 2001). GGA, an acrylic isoprenoid, is clinically used as an antiulcer drug and can induce HSPs via HSF1 activation. Pretreatment of animals with GGA markedly suppresses ischemia-reperfusion injury of the liver, small intestine, and heart (Rokutan 2000). Induction of HSPs by GGA, however, is somewhat cell-type specific, occurring especially in gastrointestinal mucosal cells. Bimeclocholol, a hydroxylamine derivative, is a co-inducer of

HSPs. Bimoclomol itself has no HSP inducing activity, but when cells are heat shocked in the presence of bimoclomol, HSPs are induced at higher levels than with heat shock alone (Vigh et al 1997). Bimoclomol, however, is reported to show protective activities against various forms of stresses at the levels of cell, tissue, or organism (Nanasi and Jednakovits 2001; Lubbers et al 2002). Herbimycin-A, rebamipide, and carbenoxolone have not been extensively examined with respect to the induction of HSPs. It should be very useful and beneficial to find other nontoxic chaperone inducers for the prevention and treatment of various pathological states, such as stress ulcers and ischemia-induced injuries, and of diseases associated with protein misfolding and protein aggregation.

INDUCTION OF HSPs BY HERBAL MEDICINES

We obtained five herbal medicines (TJ-9, TJ-41, TJ-48, TJ-60, and TJ-3020) from Tsumura Co. Ltd., and prepared six mixtures by mixing equal amounts of two herbal medicines (941=TJ-9+TJ-41, 4841=TJ-48+TJ-41, 948=TJ-9+TJ-48, 4160=TJ-41+TJ-60, 960=TJ-9+TJ-60, and 4860=TJ-48+TJ-60) (Table 1). Each herbal medicine and

Table 1 Herbal medicines (Kampo medicines) and their mixtures used in this study^{a)}

Product number of Tsumura Co. Ltd.	Name of herbal medicine
TJ-9	Sho-saiko-to
TJ-41	Hochu-ekki-to
TJ-48	Juzen-taiho-to
TJ-60	Keishi-ka-shakuyaku-to
TJ-3020	Koujin
Mixtures ^{b)}	
941	TJ-9+TJ-41
4841	TJ-48+TJ-41
948	TJ-9+TJ-48
4160	TJ-41+TJ-60
960	TJ-9+TJ-60
4860	TJ-48+TJ-60

a) These herbal medicines were kindly supplied by Tsumura Co. Ltd., Japan. They are extract granules for ethical use. Mixtures were prepared by mixing equal amounts of each herbal medicine.

the six mixtures were tested for the induction of HSPs. In the first stage of screening, we asked whether these herbal medicines could enhance the induction of HSPs in the presence or absence of mild heat shock. Expression levels of HSPs in HeLa cells treated with herbal medicines for 24 hr with or without mild heat shock (42°C for 2 hr) were monitored by western blotting with anti-Hsp70 antibody. As shown in Fig. 1, four mixtures of herbal medicines (941, 948, 960, and 4841) had enhancing effects on the induction of Hsp70 by mild heat shock; the expression level of Hsp70 was higher than that of heat shock alone [50-400 µg of herbal medicine+heat(+) in Fig. 1(a) to 1(d)]. On the other hand, three mixtures (948, 960, and 4841) each had a positive effect on the induction of Hsp70 by itself; the expression level of Hsp70 was higher than that of the non-treated control [50-400 µg of herbal medicine+heat(-) in Fig. 1(b), 1(c), and 1(d)]. There was no obvious dose-dependency in the induction of Hsp70 under our present experimental conditions at this stage. TJ-48 alone or TJ-60 alone had some positive effect on the induction of Hsp70 (data not shown).

Since the results shown in Fig. 1 appear to be attributable to the specific constituents in these herbal medicines, we chose ten representative

Table 2 Representative constituents in herbal medicines^{a)}

Constituents	Mixtures			
	941	948	960	4841
▲ ^{b)} saikosaponin	○	○	○	○
▲ glycyrrhizin ◇ ^{c)}	○	○	○	○
▲ liquiritin	○	○	○	○
▲ ginsenoside	○	○	○	○
baicalein	○	○	○	○
zingerol	○	○	○	○
▲ paeoniflorin ◇	-	○	○	○
atractylodin	○	○	-	○
atragalosidie	○	○	-	○
▲ wogonin	○	○	○	-

a) ○ : The constituent is included in the mixtures.

- : The constituent is not included in the mixtures.

b) ▲ : The constituent that was tested in this study.

c) ◇ : The constituent that has a positive effect on the induction of HSPs demonstrated in this study.

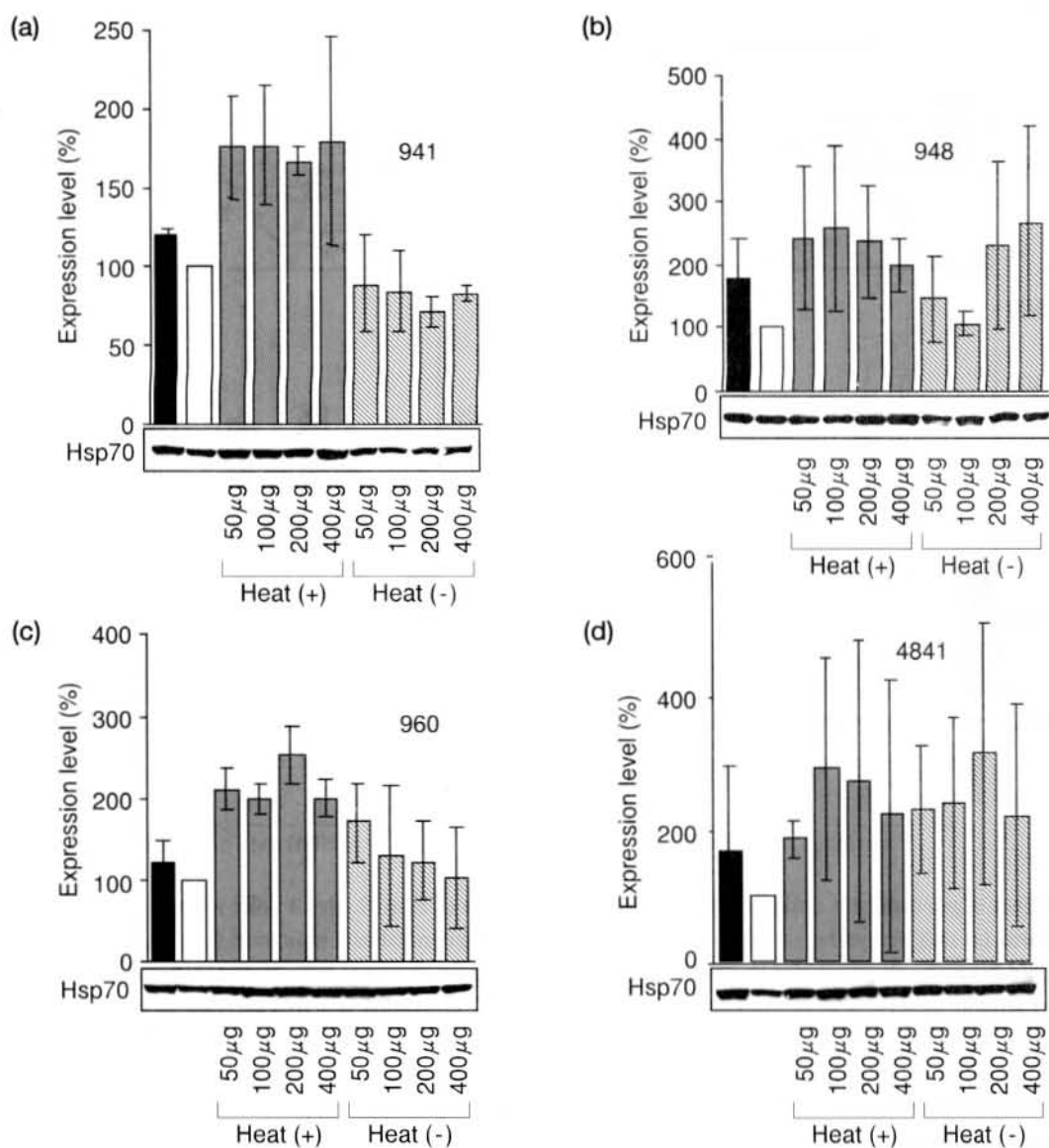


Figure 1 Induction of Hsp70 by mixtures of herbal medicines. HeLa cells were treated with 941 (a), 948 (b), 960 (c), or 4841 (d) mixtures at 37°C for 24 hr at the concentrations indicated ($\mu\text{g/ml}$), with or without mild heat shock. Heat shock (42°C for 2 hr) was given for the final 2 hr of herbal medicine treatment, and cells were cultured for further 3 hr at 37°C. Hsp70 was detected by western blotting and the relative intensity of each band was measured by densitometry. Each bar indicates the mean $\pm$ SD of the relative amount of Hsp70 from at least three independent experiments. The relative amount of Hsp70 in non-treated control cells was taken as 100% (Control, open bar). Typical western blot data are shown under each graph. All four mixtures of herbal medicines had enhancing effects on the induction of Hsp70 [heat(+)]; the relative amount of Hsp70 is much more than that of heat shock alone (Heat, closed bar). Three mixtures (b, 948; c, 960; d, 4841) could induce Hsp70 without heat shock [heat(-)]; the relative amount of Hsp70 is much more than that of the non-treated control (Control, open bar).

constituents, as listed in Table 2, having previously defined chemical structures. The difference between 941 and the other three mixtures (948, 960, and 4841) is the presence or absence of paeoniflorin; it is not included in mixture 941. Therefore, we expected that paeoniflorin would be able to induce HSPs by itself. We examined the effect of six constituents including paeoni-

florin on Hsp70 induction (indicated by closed triangles in Table 2). Among these six constituents, paeoniflorin and glycyrrhizin had positive effects on the induction of Hsp70 (Fig. 2). Both paeoniflorin and glycyrrhizin had an enhancing effect on the Hsp70 induction by mild heat shock; the expression level of Hsp70 was higher than that of heat shock alone [10–80 μg of

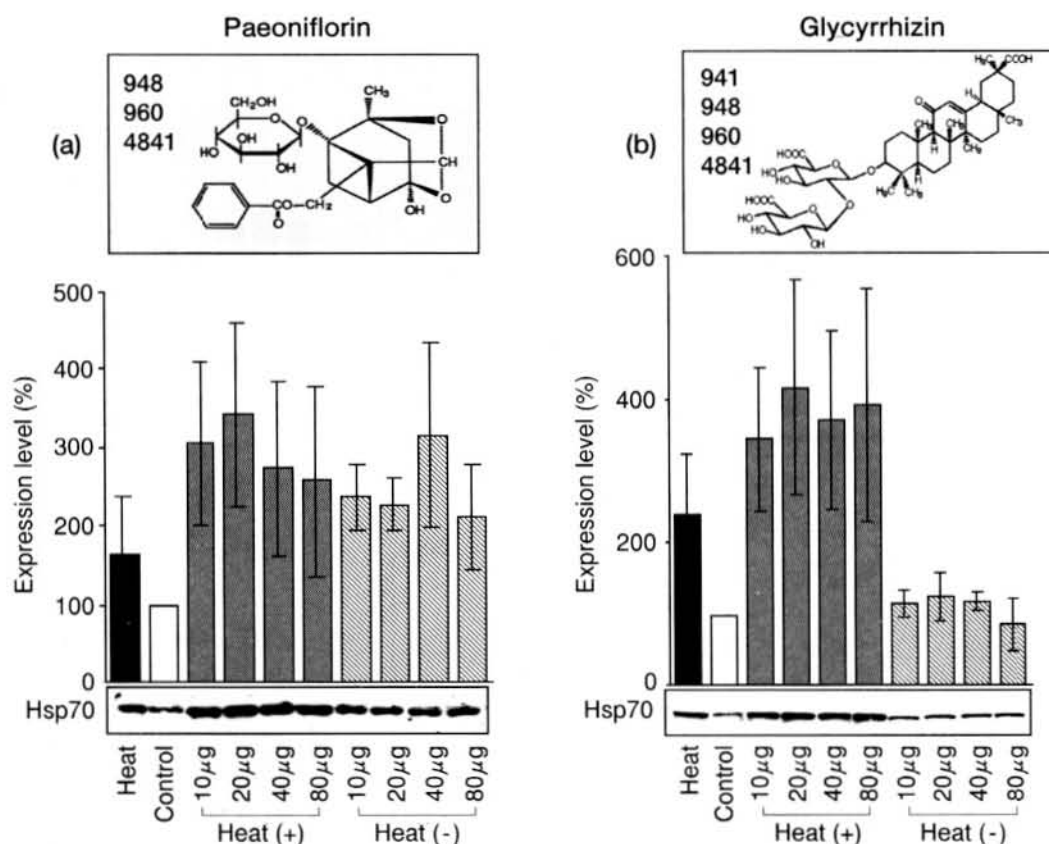


Figure 2 Effect of paeoniflorin and glycyrrhizin on the induction of Hsp70. HeLa cells were treated with paeoniflorin (a) and glycyrrhizin (b) at 37°C for 24 hr at the concentrations indicated (μ g/ml), with or without mild heat shock. Heat shock (42°C for 2 hr) was given in the final 2 hr of paeoniflorin or glycyrrhizin treatment, and cells were cultured for further 3 hr at 37°C. Hsp70 was detected by western blotting, and the relative intensity of each band was measured by densitometry. Each bar indicates the mean $\pm$ SD of the relative amount of Hsp70 from at least three independent experiments. The relative amount of Hsp70 in non-treated control cells was taken as 100% (Control, open bar). Typical western blot data are shown under each graph. The structures of paeoniflorin and glycyrrhizin are shown over each graph. Paeoniflorin is included in 948, 960, and 4841 mixtures but not in the 941 mixture. Glycyrrhizin is included in all four mixtures. Both paeoniflorin and glycyrrhizin had enhancing effects on the induction of Hsp70 [heat(+)]; the relative amount of Hsp70 is much more than that of heat shock alone (Heat, closed bar). Paeoniflorin but not glycyrrhizin could induce Hsp70 without heat shock [heat(-)]; the relative amount of Hsp70 is much more than that of the non-treated control (Control, open bar).

paeoniflorin+heat(+) in Fig. 2(a), and 10–80 μ g of glycyrrhizin+heat(+) in Fig. 2(b)]. According to our expectation, paeoniflorin alone could induce Hsp70 without the necessity of added heat shock [10–80 μ g of paeoniflorin+heat(-) in Fig. 2(a)], but glycyrrhizin alone could not induce Hsp70 [10–80 μ g of glycyrrhizin+heat(-) in Fig. 2(b)]. In this case, again, we could not get obvious dose-dependent induction of Hsp70 in the range of 10–80 μ g/ml of paeoniflorin. Paeoniflorin was able to induce Hsp40 and Hsp27 as well as Hsp70 (lanes 4–7 in Fig. 3(a) and 3(b)). Similar results were also obtained in

NRK cells with respect to the induction of HSPs by these herbal medicines (data not shown).

The concentration of paeoniflorin required for the induction of HSPs was relatively high (more than 10 μ g/ml=20.8 μ M) under our experimental conditions (10% fetal bovine serum). When cells were incubated in serum-free medium, however, 1–5 μ g/ml of paeoniflorin could induce HSPs (data not shown). Therefore, it seems that paeoniflorin might bind to or be inactivated by serum protein(s).

HSF1 ACTIVATION BY PAEONIFLORIN

Induction of HSPs by heat shock is usually regulated by the trans-acting heat shock transcription factor 1 (HSF1) and cis-acting heat shock element (HSE) present at the promoter region of each heat shock gene (Wu et al 1994). Activation of HSF1 is a multi-step process, including relocalization into the nucleus, oligomerization, acquisition of a DNA-binding competent state, and phosphorylation, until it finally accumulates in the characteristic structures to form HSF1 granules in the nucleus (Sarge et al 1993; Cotto et al 1997; Morimoto 1998). Although the phosphorylation of HSF1 is not always necessary for its activation, in some cases, its phosphorylation can be used as a good

indicator of cells being under stressful conditions. Phosphorylation of HSF1 is usually detected as an upward band-shift by western blotting (Sarge et al 1993). As shown in Fig. 3, treatment of HeLa cells or IMR-32 cells with paeoniflorin (80 μ g/ml) for 4-8 hr resulted in the clear-cut up-shift of the HSF1 band (lanes 3 and 4 in Fig. 3(a)-3(d)). On the other hand, glycyrrhizin could not induce the up-shift of the HSF1 (lanes 5 and 6 in Fig. 3(c) and 3(d)). These results clearly indicate that paeoniflorin but not glycyrrhizin is able of promoting the phosphorylation of HSF1. Following the treatment of cells with paeoniflorin for 16-24 hr, the phosphorylated form of HSF1 disappeared (lanes 6 and 7 in Fig. 3(a) and 3(b)), indicating dephosphorylation of HSF1.

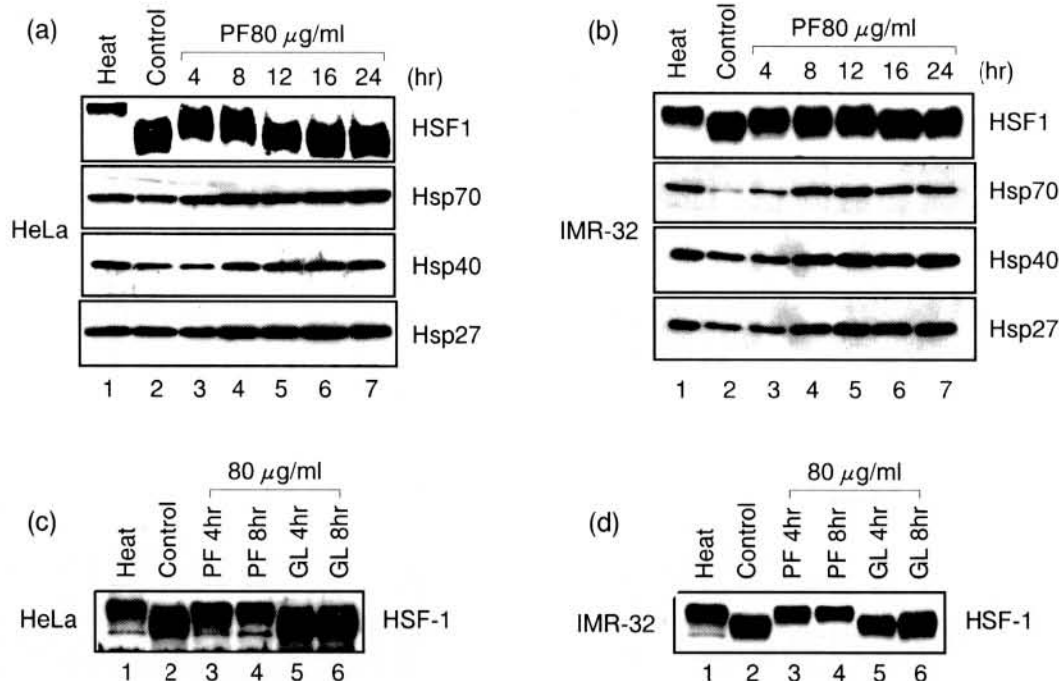


Figure 3 Phosphorylation of heat shock factor 1 (HSF1) and induction of HSPs in HeLa cells (a and c) and IMR-32 cells (b and d) by paeoniflorin. (a), (b): lane 1, cells were heated at 42°C for 2 hr; lane 2, non-treated control cells. Cells were treated with 80 μ g/ml of paeoniflorin (PF) for 4 hr (lane 3), 8 hr (lane 4), 12 hr (lane 5), 16 hr (lane 6), and 24 hr (lane 7). After treatments, the cell lysate was analyzed by western blotting with anti-HSF1 antibody (top of panels a and b), or with anti-Hsp70, anti-Hsp40, or anti-Hsp27 antibodies. (c), (d): lane 1, cells were heated at 42°C for 2 hr; lane 2, non-treated control cells; cells were treated with 80 μ g/ml of paeoniflorin (PF) for 4 hr (lane 3) and 8 hr (lane 4), or with 80 μ g/ml of glycyrrhizin (GL) for 4 hr (lane 5) and 8 hr (lane 6). After treatments, cell lysate was analyzed by western blotting with anti-HSF1 antibody. Treatment of cells with heat shock (lane 1 in panels (a)-(d)) or paeoniflorin (lanes 3 and 4 in panels (a)-(d)) resulted in clear mobility shift of the HSF1 signal as compared with that of non-treated control cells (lane 2 in panels (a)-(d)) for both HeLa and IMR-32 cells. The mobility shift of HSF1 implies phosphorylation of HSF1. Treatment of cells with paeoniflorin for 16 and 24 hr resulted in disappearance of the phosphorylated form of HSF1 (lanes 6 and 7 in panels (a) and (b)), indicating attenuation of HSF1 activity. Glycyrrhizin treatment had no effect on the mobility shift of HSF1 (lanes 5 and 6 in panels (c) and (d)). Hsp70, Hsp40 and Hsp27 were induced by paeoniflorin treatment (lanes 4-7 in panels (a) and (b)).

The acquisition of DNA binding ability of HSF1 by paeoniflorin treatment was demonstrated by a gel mobility shift assay using labeled HSE oligonucleotide. Specific DNA-protein complexes were detected in cells treated with paeoniflorin for 4 and 8 hr (Fig. 4(a), lanes 3 and 4) as well as in heat-shocked cells (42°C for 2 hr, Fig. 4(a), lane 2). The bands of DNA-protein complexes were supershifted in the presence of anti-HSF1 antibody (Fig. 4(a), lanes 8-10), indicating that the protein bound to HSE was HSF1. The specific DNA-protein complex disappeared when cells were treated with paeoniflorin for 12-24 hr (Fig. 4(a), lanes 5-7). In contrast, glycyrrhizin could not induce DNA binding activity of HSF1 (Fig. 4(b), lanes 3-7).

Intracellular localization of HSF1 was examined by indirect immunofluorescence staining. HSF1

was localized both in the cytoplasm and the nucleus at normal growth temperature (Fig. 5(b)). Upon heat shock (42°C for 2 hr), HSF1 was relocated into the nucleus to form typical HSF1 granules (Fig. 5(d)). The HSF1 granules were clearly different from the phase dense nucleolus (compare Fig. 5(c) with 5(d)). When cells were treated with paeoniflorin (80 μ g/ml) for 4-8 hr, evident HSF1 granules were formed (Fig. 5(e) and 5(f)). Interestingly, the HSF1 granules disappeared during continuous treatment of cells with paeoniflorin for 16 and 24 hr (Fig. 5(g) and 5(h)). The time course of DNA binding activity of HSF1 (Fig. 4) and the formation and disappearance of HSF1 granules (Fig. 5) well corresponds to that of phosphorylation and dephosphorylation of HSF1 (Fig. 3). This might imply the activation of HSF1 and attenuation of

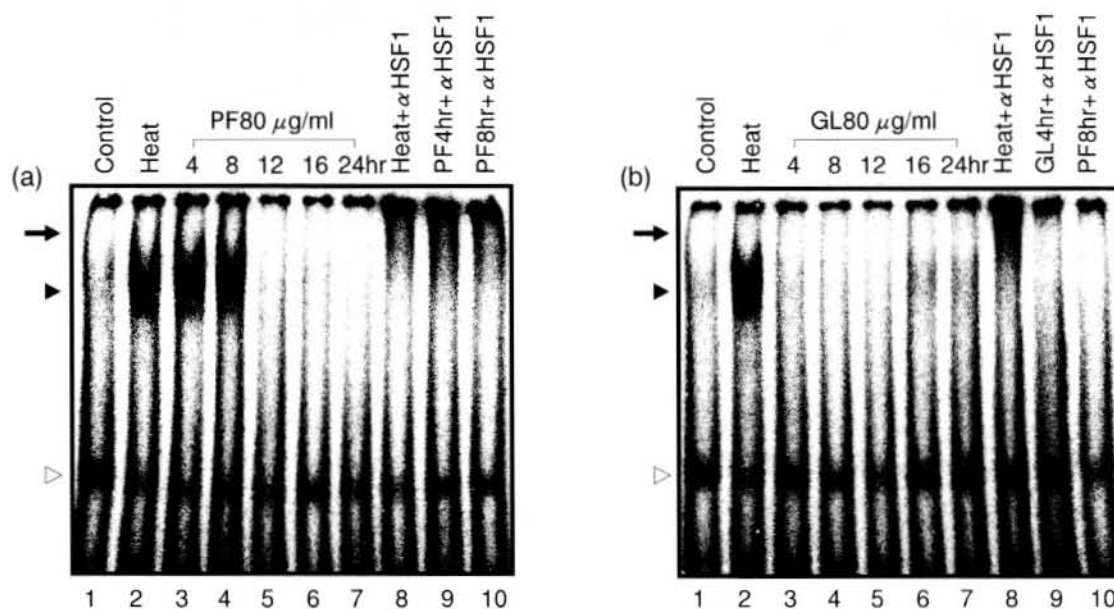


Figure 4 Gel mobility shift assay. (a) Effect of paeoniflorin (PF). Lane 1, extract from control cells; lane 2, extract from heat shocked (42°C for 2 hr) cells; lanes 3-7, extracts from cells treated with 80 μ g/ml of paeoniflorin for 4, 8, 12, 16, and 24 hrs, respectively; lane 8, extract from heat shocked (42°C for 2 hr) cells and then incubated with anti-HSF1 antibody; lane 9, extract from cells treated with 80 μ g/ml of paeoniflorin for 4 hr and then incubated with anti-HSF1 antibody; lane 10, extract from cells treated with 80 μ g/ml of paeoniflorin for 8 hr and then incubated with anti-HSF1 antibody. (b) Effect of glycyrrhizin (GL). Lane 1, extract from control cells; lane 2, extract from heat shocked (42°C for 2 hr) cells; lanes 3-7, extracts from cells treated with 80 μ g/ml glycyrrhizin for 4, 8, 12, 16, and 24 hrs, respectively; lane 8, extract from heat shocked (42°C for 2 hr) cells and then incubated with anti-HSF1 antibody; lane 9, extract from cells treated with 80 μ g/ml of glycyrrhizin for 4 hr and then incubated with anti-HSF1 antibody; lane 10, extract from cells treated with 80 μ g/ml of glycyrrhizin for 8 hr and then incubated with anti-HSF1 antibody. Open arrowheads indicate nonspecific bands. Filled arrowheads indicate HSE-protein complexes. Arrows indicate supershifted complexes with anti-HSF1 antibody.

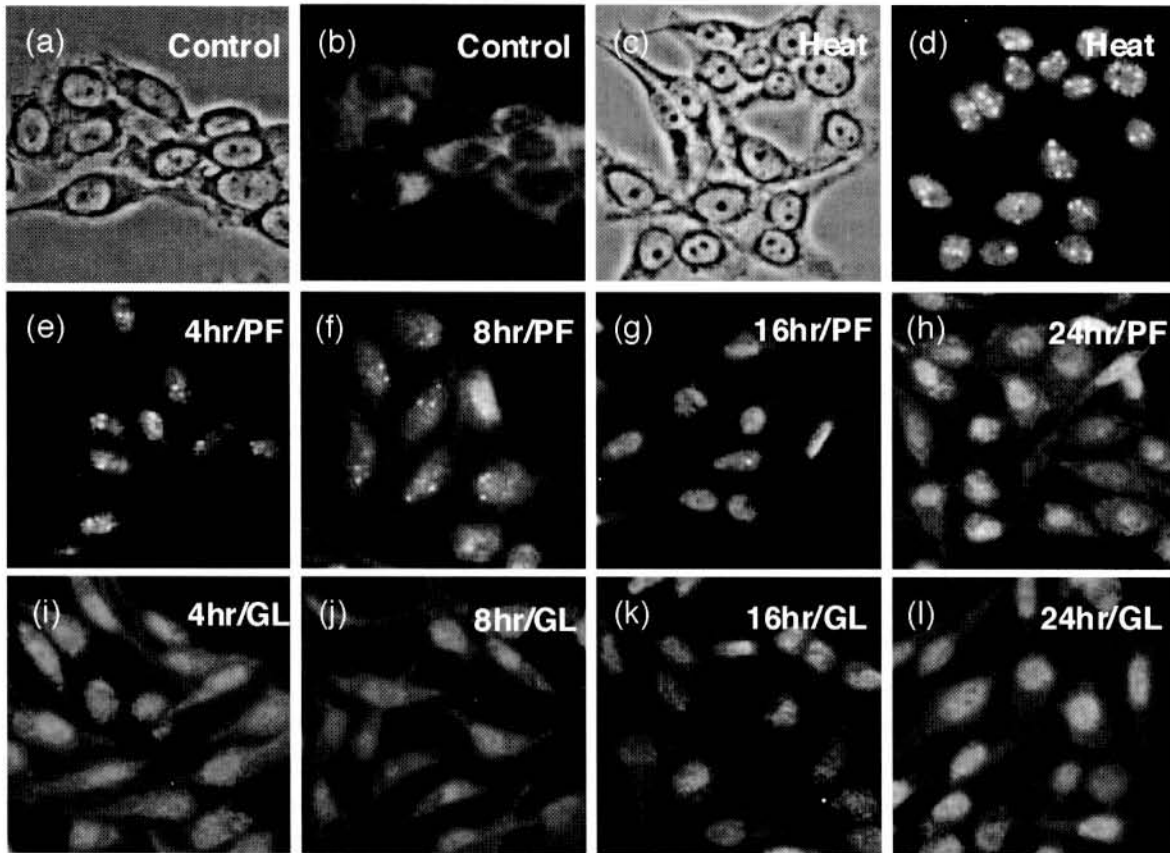


Figure 5 Intracellular localization of HSF1 in HeLa cells. (a), (b): non-treated control cells; (c), (d): cells heated at 42°C for 2 hr; (e), (f), (g), (h): cells treated with paeoniflorin (PF) for 4, 8, 16, and 24 hr, respectively; (i), (j), (k), (l): cells treated with glycyrrhizin (GL) for 4, 8, 16, and 24 hr, respectively. HSF1 is localized both in the cytoplasm and the nucleus in non-treated control cells (b). Treatment of cells with heat shock (d) or with paeoniflorin for 4 and 8 hr (e and f) induced relocalization of HSF1 into the nucleus to form typical HSF1 granules. Continuous treatment of cells with paeoniflorin for 16 and 24 hr resulted in the disappearance of HSF1 granules, indicating attenuation of HSF1 activity. Treatment of cells with glycyrrhizin resulted in the gradual relocalization of HSF1 into the nucleus (i, j, k, and l). The phase-contrast micrographs are shown in (a) and (c); the HSF1 specific fluorescence micrographs of each corresponding field are shown in (b) and (d).

stress response. A similar phenomenon has been reported when cells were continuously heated at 42°C for more than 6 hr (Sarge et al 1996). Thus, the activation of HSF1 and its attenuation during continuous paeoniflorin treatment indicate that cells are being exposed to a mild level of stress. On the other hand, when cells were treated with glycyrrhizin (80 µg/ml), HSF1 appeared to be gradually relocalized into the nucleus, but HSF1 granules were not formed (Fig. 5(i), 5(j), 5(k), and 5(l)). As shown in Fig. 2(b), glycyrrhizin had an enhancing effect on the induction of HSPs by heat shock. This might be explained, in part, by the gradual relocalization of HSF1 by glycyrrhizin treatment. Thus, glycyrrhizin may be called a co-inducer of HSPs. Since HSF1

is not phosphorylated and its DNA binding activity is not induced by the treatment with glycyrrhizin (Figs. 3 and 4), HSF1 might be relocalized into the nucleus irrespective of its phosphorylation and DNA binding competency. In other words, HSF1 might be first relocalized into the nucleus after which the subsequent HSF1 activation steps (oligomerization, acquisition of DNA binding ability, and phosphorylation) might occur in the nucleus under the stress conditions.

From these results, it is strongly suggested that induction of HSPs by the treatment with paeoniflorin is mediated by the activation of HSF1.

INDUCTION OF THERMOTOLERANCE BY PAEONIFLORIN AND PAEONIFLORIN-CONTAINING HERBAL MEDICINE

When living cells are exposed to non-lethal heat shock, they acquire a transient resistance to an otherwise lethal heat challenge as determined by the increase in clonogenic cell survival (Gerner and Schneider 1975). This phenomenon is termed acquired thermotolerance. As shown in Fig. 6(a), thermotolerance was clearly induced by the treatment with paeoniflorin (80 μ g/ml for 24 hr), and the 960 mixture (400 μ g/ml for 24 hr) which contains paeoniflorin. Surviving fractions of these cells were almost the same as those of cells treated with mild heat shock (42°C for 2 hr). In contrast, glycyrrhizin or the 941 mixture (not containing paeoniflorin) could not induce thermotolerance at the same concentrations (Fig. 6(b)). Thus, the development of thermotolerance is well correlated with the induction of HSPs.

PAEONIFLORIN AND GLYCYRRHIZIN ARE NOT TOXIC

For the purpose of clinical use of chaperone-

inducing compounds, they should not be toxic or deleterious. When HeLa cells were cultured in the presence of a mixture of herbal medicines (960 and 941) at a concentration of 400 μ g/ml, and in the presence of paeoniflorin or glycyrrhizin at a concentration of 80 μ g/ml, no appreciable growth inhibition was observed (Fig. 7(a)). Also, the cell cycle phase distributions of HeLa cells treated with these herbal medicines or constituents (24 hr at 37°C) were not significantly different from those of non-treated control cells (Fig. 7(b)). Of course, these herbal medicines and constituents had no apparent effect on the morphology of HeLa cells under the same conditions as described in Fig. 7(b) (data not shown).

DISCUSSION

In the present study, we demonstrated that treatment of cells with paeoniflorin alone resulted in the phosphorylation and acquisition of DNA binding ability of HSF1, relocalization of HSF1 to form characteristic HSF1 granules in the nucleus, and finally the induction of HSPs. These results strongly suggest that the induction of HSPs by paeoniflorin is mediated by the activation of

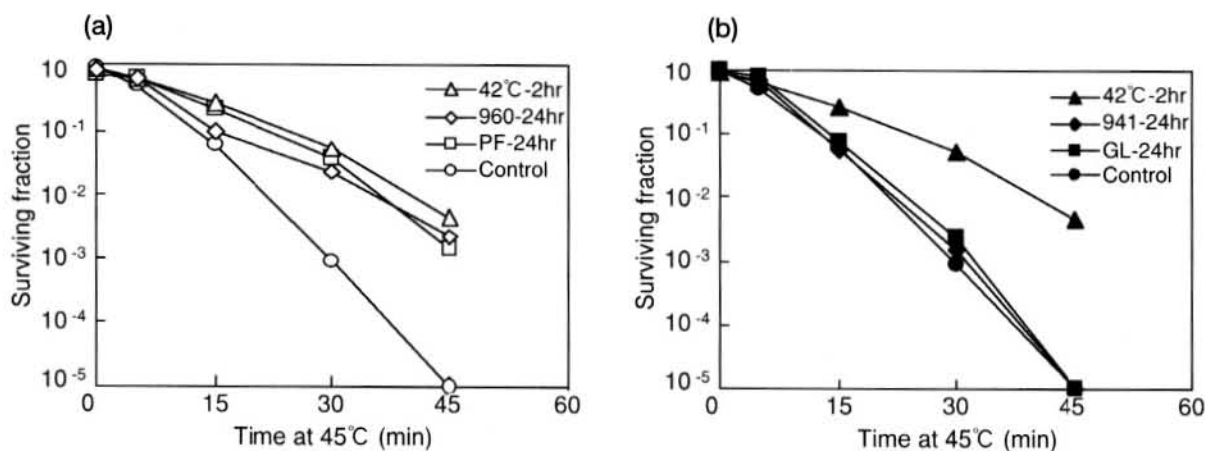


Figure 6 (a) Development of thermotolerance in HeLa cells treated with paeoniflorin and the 960 mixture (containing paeoniflorin). Cells were treated with heat shock (42°C for 2 hr, $\triangle$), 960 mixture (400 μ g/ml for 24 hr, $\diamond$), or paeoniflorin (PF, 80 μ g/ml for 24 hr, $\square$), then heated at 45°C for the indicated period. After 45°C heating, cells were subjected to the clonogenic survival assay. (b) Effect of glycyrrhizin and the 941 mixture (not containing paeoniflorin) on the development of thermotolerance in HeLa cells. Cells were treated with heat shock (42°C for 2 hr, $\blacktriangle$), 941 mixture (400 μ g/ml for 24 hr, $\blacklozenge$), or glycyrrhizin (GL, 80 μ g/ml for 24 hr, $\blacksquare$), then heated at 45°C for the indicated period. After 45°C heating, cells were subjected to the clonogenic survival assay. Survival curves of non-treated control cells are indicated by open and closed circles in panel (a) and panel (b), respectively. All data points represent the means of three independent experiments.

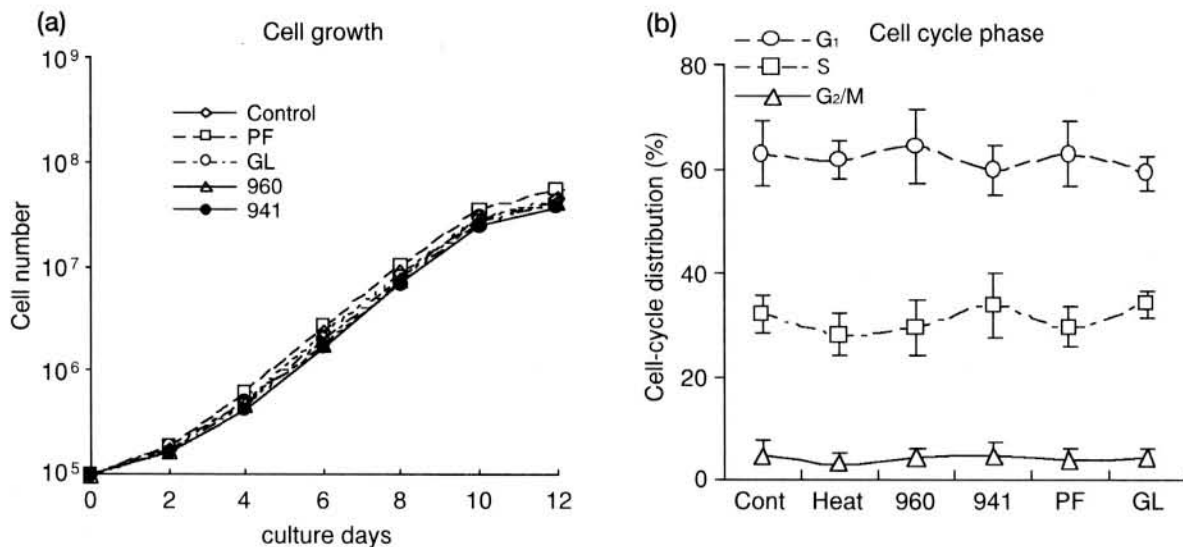


Figure 7 Effect of herbal medicines and constituents on cell growth and cell cycle. (a) HeLa cells (1×10^6) were inoculated in triplicate on day 0 and incubated at 37°C in the presence of paeoniflorin (PG, $80 \mu\text{g/ml}$, $\square$), glycyrrhizin (GL, $80 \mu\text{g/ml}$, $\circ$), 960 mixture ($400 \mu\text{g/ml}$, $\triangle$), or 941 mixture ($400 \mu\text{g/ml}$, $\bullet$) for up to 12 days. Every two days, the number of cells was counted with a hemocytometer. Each data point represents the mean of three independent experiments. No appreciable growth inhibition was observed in cells treated with herbal medicines and constituents. (b) HeLa cells were treated with heat shock (42°C for 2 hr), 960 mixture ($400 \mu\text{g/ml}$ for 24 hr), 941 mixture ($400 \mu\text{g/ml}$ for 24 hr), paeoniflorin (PF, $80 \mu\text{g/ml}$ for 24 hr), or glycyrrhizin (GL, $80 \mu\text{g/ml}$ for 24 hr). Then cell cycle phase distribution was analyzed by flow-cytometry. $\circ$, G₁ phase; $\square$, S phase; $\triangle$, G₂/M phase. Each data point represents the mean of three independent experiments. There are no apparent differences between non-treated control cells and cells treated with herbal medicines and constituents.

HSF1. Although glycyrrhizin alone could not induce HSPs, it promoted relocalization of HSF1 into the nucleus. This might explain why glycyrrhizin could enhance the induction of HSPs in combination with mild heat shock. Also, development of thermotolerance is well correlated with the expression of HSPs. These results clearly indicate that paeoniflorin is a chaperone inducer and glycyrrhizin a chaperone co-inducer. Precise molecular mechanisms of the positive effect of these compounds on the induction of HSPs, however, remain to be elucidated.

It has been reported that several chemical compounds can induce or enhance the expression of HSPs. Geranylgeranylacetone (GGA) is shown to induce HSPs in a primary culture of gastric mucosal cells under the condition of low serum concentration (Hirakawa 1996). GGA, however, could not induce HSPs in cultured rat hepatocytes (Ikeyama et al 2001). Therefore the positive effect of GGA on the induction of HSPs might be cell-type specific. When orally adminis-

tered, however, GGA enhanced the induction of HSPs in the rat liver in combination with heat shock and protected the liver from the injury caused by ischemia-reperfusion (Yamagami et al 2000). Bimoclomol (BRLP-42), a hydroxylamine derivative, acts as a co-inducer of HSPs, enhancing the amount of HSPs following heat shock compared with heat shock alone (Vigh et al 1997). Bimoclomol is shown to bind directly to HSF1 and induce a prolonged binding of HSF1 to HSE (Hargitai et al 2003). It has been reported from results in experimental animal models that bimoclomol has potential therapeutic use in the treatment of diabetic peripheral neuropathy (Biro et al 1997), cardiac dysfunction (Lubbers et al 2002), and cerebrovascular disorders (Erdo and Erdo 1998). Since paeoniflorin and glycyrrhizin had no appreciable toxicity at the concentrations used in this study, they might be used for the prevention and treatment of various pathological states, such as stress ulcers and ischemia-induced injuries, and of diseases associated with protein

misfolding and protein aggregation.

Peony plants, such as *Paeonia suffruticosa*, *P. lactiflora*, *P. veitchii*, and *P. obovata*, have been utilized in traditional Chinese medicines or herbal medicines in China and Japan. Paeoniflorin, isolated from *P. lactiflora*, is one of the major constituents of peony plants. Peony extracts and their constituents have been shown to have various biological and bio-modulating activities including improvement of memory (Ohta et al 1993), antioxidant activity (Okubo et al 2000), anti-epileptic activity (Tsuda et al 1997), anti-mutagenic properties (Sakai et al 1990), and anti-hyperglycemia (Hsu et al 1997). Glycyrrhizin, tripartenoid saponin composed of one molecule of glycyrrhetic acid and two of glucuronic acid, is a main constituent of the hydrophilic fraction of licorice (*Glycyrrhiza glabra*) extracts, which have also been used as herbal medicines. Glycyrrhizin is known to have a wide range of pharmacological actions, such as anti-viral (Pompei et al 1979), anti-carcinogenic (Nishino et al 1984), anti-allergic (Takeda et al 1991; Park et al 2004), and anti-inflammatory activities (Inoue et al 1986). Although the molecular mechanisms of these pharmacological functions of paeoniflorin and glycyrrhizin are not yet fully understood, these activities might be ascribed in part to their positive effect on the induction of molecular chaperones. In the future, a wide variety of pharmacological activities of paeoniflorin and glycyrrhizin ought to be studied in relation to their properties of chaperone inducer and co-inducer. Also, paeoniflorin and paeoniflorin-containing herbal medicines might be used clinically as chaperone inducers, and glycyrrhizin and glycyrrhizin-containing herbal medicines as chaperone co-inducers. We are now studying whether paeoniflorin and glycyrrhizin can induce or enhance the expression of HSPs in the whole organism.

In this study, we tested a limited number of herbal medicines and constituents. It may be of value to search for other chaperone inducers and co-inducers in herbal medicines.

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