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Regulation of heat shock-induced p53 signaling in OC2 human oral cancer cells

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中文摘要

背景 已知熱處理應用於腫瘤治療已數個世紀；然而，其作用機制仍莫衷一是。本實驗之目的乃在探討熱處理誘發之 p53 訊息傳導。

材料與方法 人類口腔癌細胞株 OC2 或其未經熱處理細胞之蛋白質溶液經不同的熱處理(37、39、41 及 43℃，三小時)。利用免疫轉漬技術檢測調控細胞週期的主要調控分子之表現。以免疫沉澱法分析蛋白質間之相互關係。HSP70 之組態則以 Non-denaturing 電泳檢測。實驗結果經掃描後以 NIH 影像分析系統(NIH, Bethesda, MD, USA)分析。所有數據皆經 ANOVA 分析。

結果 熱處理誘發 p53 積存、p53 於 serine 15 位置磷酸化且增加其下游標的基因 p21、Bax/Bcl-2 之表現。p53 之表現於熱處理後 12 小時達最高峰且至少延續達 24 小時。熱緊迫顯著增加 p53 與 MDM2 間之負回饋調控機制且增加 ERKs 的致活作用。PD98059，MEK (MAPK 或 ERK kinase)之特異抑制劑，顯著的強化了熱緊迫所誘發之 p53 磷酸化作用及 HSP70 的表現。HSP70 係以單體存在且於熱處理後形成寡體。同時，HSP70 複合物之表現例如 p53、p21 與 Bax 皆於熱處理後顯著增加。

結論 本研究顯示熱處理誘發之細胞週期停滯及細胞凋亡係藉由 p53 之訊息傳導。此外，熱處理誘發之 p53 訊息傳導與 HSP70 表現與 ERKs 之活性無關。

關鍵詞 過熱症、p53 腫瘤抑制蛋白質、HSP70、PD98059、細胞凋亡

Abstract

Background It is well known that heat treatment used in tumor therapy existed for centuries, however, the heat-induced signaling pathway remained less well characterization. The aim of this study is to investigate the regulation of heat shock-induced p53 signaling in OC2 cells, a human oral cancer cell line.

Materials and methods OC2 cells or cell lysates were incubated at different temperatures (37, 39, 41 and 43℃) for 3 hours. The Western blot was employed to quantify differences in master regulative molecule of cell cycle. Immunoprecipitation was used to detect the relationship among proteins. Non-denaturing electrophoresis was applied to detect the conformation of HSP70. Patterns of changes in expression were scanned and analyzed using the NIH image 1.56 software. All the data were analyzed by ANOVA.

Results Heat shock induced the accumulation of p53, phosphorylation of p53 at serine 15 and increased the expression of its downstream target genes, p21 and Bax/Bcl-2. The p53 signaling reached the peak at 12 hours and prolonged for at least 24 hours after heat treatment. Heat shock significantly induced the negative control mechanism between p53 and MDM2 60 kDa fragment, and trigged the activation of ERKs. PD98059, a specific inhibitor of MEK (MAPK or ERK kinase), markedly enhanced the heat shock-induced p53 phosphorylation and HSP70 expression. HSP70 existed in monomeric form and oligomerized after heat shock. In the meanwhile, the expression of HSP70 complexes such as p53, p21 and Bax were significantly induced by heat shock.

Conclusion This study demonstrates that heat-induced cell cycle arrest and apoptosis are mediated by activation of p53 signaling. Furthermore, the p53 signaling and HSP70 expression induced by heat shock are independently of ERKs activation.

Keywords hyperthermia, HSP70, tumor suppressor protein p53, PD98059, apoptosis

Introduction

Heat shock induced the tumor suppressor gene p53 accumulation and contributed to cell cycle arrest or apoptosis[1-2]. The tumor suppressor protein p53 has several biological effects involving DNA replication and repair, cell cycle arrest and apoptosis after DNA damage. The p53 protein in normally expressed at low levels in a complex with mouse double minute 2 (MDM2) as a latent inactive form[3]. When cells exposed to an appropriate stress, p53 will be phosphorylated at its N-terminal region[4], lost its association with MDM2 and results in increasing p53 stability and accumulation. Subsequent activation of p53-target genes, particularly the cyclin-dependent-kinase inhibitor $p21^{WAF1/CIP1}$ and Bax, results in arrest of cell cycle in the G1 phase and activation of apoptosis[5-6].

The MAP (mitogen-activated protein) kinases are serine/threonine protein kinases, which play pivotal roles in a variety of cell functions in many cell types[7]. MAP kinases are central transducers of extracellular signals from hormones, growth factors, cytokines, and environmental stresses. Three major mammalian MAP kinase subfamilies have been described: including extracellular signal-regulated kinase 1/2 (ERK1/2), c-Jun-N-terminal kinase (JNK) and p38 kinase. MAP kinase isoforms ERK1 and ERK2 mediate key events throughout the cell and phosphorylate transcription factors[8-9], cytoskeletal proteins, and other protein kinases and enzymes[7, 10]. Recently, ERKs were found to have a direct role in phosphorylation of p53 at serine 15 in response to cisplatin[11] and UV radation[12]. Inactivation of ERKs decreased the phosphorylation of p53 at serine 15[11-12].

The heat shock proteins (HSPs) are one of the most popular subjects about hyperthermia in these years. HSPs are a heterogeneous group of proteins, function as molecular chaperones to preventing protein aggregation and refolding soluble misfolded protein molecules, induced in cells exposed to sublethal heat shock or stress[13-14]. They can be classified according to their molecular mass into five major families, the small HSP (molecular mass < 40 kDa), HSP60, HSP70, HSP90 and HSP100. Each family is comprised of several members and exhibits a distinct constitutive and inducible expression pattern. The HSP70 family is constitutive expressed (HSP73) in all cells and can be induced (HSP72) by stress[15]. In nonstressed cells, HSP72 was primary nuclear, localized in heterochromatic region and in nucleoli. HSP73 was distributed throughout the cell, with most cytoplasmic label associated with mitochondria. After stress, HSP72 concentrated in nuclei and nucleoli and HSP73 localized to nuclei, nucleoli and cytoplasm, with increased label over mitochondria[16]. Using specific oligonuclotides to block the expression of HSP70 triggered cancer cell apoptosis[17-19]. It seems not surprising that HSP70 is supposed to represent 'general survival protein' and makes cells more resistant to apoptosis. However, the function of HSPs in tumor cells remains controversial. Previous studies showed that HSPs expression had been correlated with a number of cell types to resist[19-20] or to induce[21-22] apoptosis.

The aim of this study is to determine the regulation of heat shock-induced p53 signaling. We found that heat shock induced p53 prtoein accumulation and phosphorylation at serine residue 15; in turn, upregulation its downstream gene, p21 and Bax/Bcl-2. Inactivation of ERKs by specific

inhibitor markedly enhanced heat shock-induced phosphorylation serine 15 of p53 protein and expression of HSP70. It indicated that heat-induced p53 phosphorylation and HSP70 expression are independently of ERKs activation. The HSP70 complexes including p53, p21 and Bax were increased after heat shock. Furthermore, heat-induced HSP70 complex formed not only in cultured cells but also in extracted cell lysate. The monomeric HSP70 aggregated to form oligomer after heat shock.

Materials and methods

Cell culture

Human oral cancer cell line OC2[23] incubated in RPMI1640 medium (Gibco BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum, streptomycin (10,000 U/ml) and penicillin (10,000 U/ml), and buffered with sodium bicarbonate (2.0 gm/liter) as manufacturer recommended. Cells were washed with PSB and renewed fresh medium before heat treatment (37, 39, 41 and 43℃ for 3 hours).

Western blotting

The expression of HSP70, p53 (Sigma Chemical Co., St. Louis, MO), p53-pSer15, p53-pSer392 (Calbiochem Co. Ltd, San Diego, CA) and its regulators or downstream genes including phosphor-ERK1/2 (Cell Signaling Technology, Beverly, MA), MDM2, p21, Bax, Bcl-2 (Santa Cruz Biotechnology Inc., Santa Cruz, CA) were analyzed by Western blotting. In immunoblotting the HSP70 antibody purchased from Sigma Chemical Co. localizes both the constitutive (HSP73) and the inducible (HSP72) forms of HSP70. Briefly, cells were washed twice with cold PBS before extracting with 2.5% Triton X-100 (Sigma). Samples were heated at 95℃ for 5 min in Laemmli buffer then chilled on ice. Subsequently after electrophoresis (50 μg /lane), the proteins were electro-blotted to ECL nitrocellular membrane (Amersham Pharmacia Biotech, Buckinghamshire, England). The proteins of interest were detected with an ECL western blotting detection reagent (Amersham). Briefly, nonspecific binding on the nitrocellular membranes were blocked with 5 % non-fat dry milk in 20 mM Tris and 150 mM NaCl prior to incubating with primary antibodies against specific antigens. After incubation with the conjugated second antibody, the blotted nitrocellular membranes were exposed to x-ray films and images of blotted patterns were analyzed with NIH image software (free download from National Institutes of Health, U.S.A.). Blots were routinely re-probed with anti-actin to ensure equivalence of loading. If necessary, membranes were strip by incubation at 50℃ for 30 min in a solution of 62.5 mM Tris-HCl, pH6.7, 2% SDS and 100 mM 2-mercaptoethanol.

Nondenaturing Electrophoresis

Mobility in a nonodenaturing gel depends on the size as well as shape and intrinsic charge of the protein. Separation of proteins by nondenaturing electrophoresis described as gel electrophoresis in Western blotting, except use reagents that do not contain SDS and do not boil sample.

Immunoprecipitation

Cells were washed twice with cold PBS prior to being extracted with lysis buffer (1.5 mM

$MgCl_2$, 1% Triton X-100, 50 mM Hepes, pH 7.6, 1 mM EGTA, 10% glycerol, 150 mM NaCl, 1 mM NaVO3, 10 mM NaF, 10 mM beta-glycerol-phosphate, 1 μg/ml leupeptin, 1 μg/ml pepstatin and 2.5 μg/ml aprotinin). Lysates were passed through 25G needle 3 times. Proteins (300 μg) were incubated with specific antibodies including p53 (Sigma), p21, Bax, MDM2 (Santa Cruz Biotechnology), phosphor-ERK (Cell Signaling Technology), HSP70 (Calbiochem) at 4℃ for 6 hours while mixing end over end on rotator. After incubation, samples were combined 10% protein A-Sepharose bead (Sigma) slurry in PBS containing 0.1% BSA, 10% glycerol and 0.02% sodium azide, and incubated at 4℃ for 1 hour. Beads washed 3 times with cold lysis buffer prior to be analyzed immunoprecipitates by electrophoresis.

Statistical analysis

All data were analyzed by ANOVA (analysis of variance) and expressed as mean ± standard deviation. A P-value of less than 0.05 is considered statistically significant.

Results

p53 phosphorylation and signaling in response to heat shock

Previous study reported that heat-induced G1 arrest was not found in cells, which are deficient in p53 function, indicating that p53 function is essential for G1 arrest after heat shock[24]. To investigate the effect of heat shock on p53 signaling, we performed two independent heat treatments. First, OC2 cells incubated at different temperatures (37, 39, 41 and 43℃) for 3 hours. Lysate were collected for detecting the expression of p53 signaling. As expected, heat shock significantly induced p53 protein accumulation and phosphorylated of p53 at serine 15 ($p53^{Ser15}$); in turn, upregulated the expression of its downstream genes $p21^{WAF1/CIP1}$and Bax/Bcl2 ($p<0.05$) (Fig 1A). Heat shock significantly induced the expression of p21 at 39 and 43℃, and the ratio of Bax/Bcl2 significantly increased to about six-folds after heat treatment at 43℃ for 3 hours ($p<0.05$). The ratio of Bax/Bcl-2 at 43℃ was resulted from significant upregulation of Bax (3.82±0.54 folds) and downregulation of Bcl-2 (0.65±0.11 folds) ($p<0.05$). Second, cells were cultured in incubator at 37℃ for an additional period (0, 3, 6, 12 and 24 hours) after heat treatment (43℃, 3 hours). Data show that the expression of p53 and $p53^{Ser15}$ reached the peak at 12 hours and prolonged for 24 hours (Fig. 1B). Results suggest that heat shock induced cell cycle arrest and apoptosis mediated by p53 signaling.

Upregulation of p53-MDM2 complex formation by heat shock

Phosphorylation of tumor suppressor protein p53 at serine 15 occurs after DNA damage and this leads to reduce interaction of p53 with its negative regulator, the oncoprotein MDM2. We found that the expression of 60 kDa MDM2 fragment significantly induced by heat shock ($p<0.05$) (Fig. 2A). Human tumor cell lines often expression high levels of a 60 kd MDM2 isoform in the absence of apoptosis [25]. This helps to explain why the OC2 cells used in this study expressed high levels of 60 kd MDM2 fragment.

Whether 60 kDa MDM2 fragment still possess its function to regulate p53? Previous study indicated that MDM2 cleavaged by caspase-3-like proteases may result in losing the ability to promote p53 degradation[26]. Others suggested that the functions of MDM2 are not effected by the

degradation[27-28]. To study this subject, immunoprecipitation was used in this study. Results showed that accumulation of MDM2 60 kDa fragment-p53 complex significantly triggered to about 2 folds at 41 and 43℃ ($p<0.05$) (Fig. 2B). We suggest that the role of 60 kDa MDM2 fragment are not effected by the degradation, at least in heat shock-induced p53 signaling.

Inactivated ERKs enhances heat shock-induced p53 phosphorylation at serine 15

Heat shock activated ERKs and PD98059, a specific inhibitor of MEK (MAPK or ERK kinase), inhibited heat shock-induced activation of ERKs[29]. Previous studies indicated that ERKs have a direct role in phosphorylation of p53 at serine 15 in response to cisplatin[11] and UV radation[12]. Inactivation of ERKs decreased the phosphorylation of p53 at serine 15[12-13]. On the contrary, MEK1 inhibition with PD98059 inhibited ERK activation but was without effect on p53 stabilization in response to DNA damage[30]. We therefore investigated the induction of phosphorylated p53 in OC2 cells during hyperthermia and its correlation with ERKs activation. We found that heat treatment (37, 39, 41 and 43℃ for 3 hours) significantly increased the expression of ERKs and phosphorylated ERKs as previous studies[29] ($p<0.05$) (Fig. 3A). To further determine whether phosphorylation of p53 at serine 15 requires ERKs, PD98059 was used in this study. Cells were pretreated with PD98059 (10 μM) for 1 hour at 37℃ before heat treatment (37 and 43℃ for 3 hours). PD98059 inhibited the expression of phosphorylated ERK completely. Data showed that there are dual and opposing roles of PD98059 in regulating the phosphorylation of p53 in response to heat shock. Inactivation of ERKs significantly upregulated the expression of p53 and decreased serine 15 phosphorylation of p53 at 37℃ ($p<0.05$). Conversely, PD98059 significantly enhanced the heat-induced phosphorylation of p53 at serine 15, but there is no difference of p53 expression at 43℃ ($p<0.05$) (Fig. 3B). Taken together, the intrinsic kinase activities of ERKs required for serine 15 phosphorylation of p53 at 37℃ as previous studies[11-12], whereas inactivated of ERKs markedly enhanced heat shock-induced p53 phosphorylation at serine 15 in response to heat shock.

Elevated HSP70 levels were preceded by transcription, which was associated with heat shock transcription factor 1 (HSF1) phosphorylation and activation. HSF1 activity can be inhibited through the phophorylation of HSF1 serine residues by ERKs[31]. Inhibition of ERKs by its specific inhibitor did not influence HSF1 activation[32]. Our result showed that cells pretreated with PD98059 significantly increased the level of heat-induced HSP70, but there is no significantly influence at 37℃ (Fig. 3B). Herein, we suggest that heat-induced p53 signaling and HSP70 expression are independently of ERKs activation.

Heat shock triggered HSP70 complexes formation

The expression of HSP70 significantly increased at 43℃ ($p<0.05$) (Fig. 4A). HSP70 expression reached the peak at 3 hours and sustained for 24 hours ($p<0.05$) (Fig. 4B). It is well known that HSPs can bind to other proteins to assist proteins to maintain their physiological functions. Hsp70 has been detected in complexes with proteins including SV40 large T antigen, adenovirus E1A protein, cellular c-myc and tumor suppressor protein p53[33-35]. To study the relationship between HSP70 and p53 signaling proteins, immunoprecipitation was performed in this experiment. Result showed that heat shock significantly induced HSP70 complex formation

including p53 (Fig. 4C), p21 and Bax (Fig. 4D) ($p<0.05$). In an independent experiment, cells which without heat treatment were washed twice with cold PBS prior to being extracted. The lysate (300μg) was incubated at 37, 39, 41 and 43℃ for 3 hours, respectively. Immunoprecipitation was applied to detect HSP70-p53 complex formation. Data showed that heat shock significantly increased the HSP70-p53 complex formation at 41 and 43℃ ($p<0.05$) (Fig. 4E). We found that heat shock trigged HSP70 complex formation not only in cultured cell but also in extracted lysate.

Heat shock induce HSP70 oligomerization

Angelidis and colleagues reported that HSP70 formed high molecular mass aggregates in temperature-dependent manner after heat treatment[36]. The molecular chaperone HSP70 is in a mixture of monomer-self associated oligomer equilibrium in the presence of ADP, while in ATP it is monomeric[36-39]. The aggregated forms of HSP70 can not be active as chaperones[36]. To determine the conformation of HSP70 after heat shock in OC2 cells, non-reducing electrophoresis was performed and probed with a HSP70-specific antibody in this study. Cells were incubated at 37, 39, 41 and 43℃ for 3 hours prior to be extracted. Result showed disagreement with previous studies[36-39] that HSP70 existed in non-aggregated form (37 and 39℃) and oligomerized after heat shock at 41 and 43℃ (Fig.5).

Discussion

Phosphorylation of the p53 tumor suppressor protein plays an important role in regulation its activity. When cells exposed to DNA damage agents, several different serine residues in p53 were phosphorylated. Phosphorylation of serine 392 may be important for p53 oligomerization[40-41]. While, serine 15 and 37 become phosphorylated after DNA damage and this phosphorylation reduces MDM2 biding to p53[42-43]. Wang and Chen indicated that heat shock induced p53 accumulation and phosphorylated p53 at serine 15 and serine 20, however, there is no difference at serine392[44]. The expression of p53 phosphorylated p53 at serine 15 ($pSer^{15}$) and serine 392 ($pSer^{392}$) were detected. However, no signal can be detected at serine 392 in this experiment.

It is well known that changed the structure of protein caused the hydrophobic region explored, which buried deep side the molecule in normal, and leaded molecular aggregation and lost its function. The tumor suppressor protein p53 is temperature sensitivity for conformation flexible, small changes in temperature may directly affect p53 function[45-46]. When temperature above 40℃ makes it very easy to change its conformation and exposes its hydrophobic region[47]. This helps to elucidate why HSP70-p53 complex significantly increased after heat shock in this study.

Using HSP70 antisense oligomer to abrogate the expression of HSP70 in cells deficient p53 function resulted in cell death independent of p53 tumor suppressor protein[48-49]. It indicates that HSP70-induced cell death did not mediate by p53 protein. What is the role of HSP70 played in heat-induced p53 signaling? Mutant p53 protein formed stable complexes with heat shock-induced family member HSP70 and complexes dissociation when incubated with ATP[37-40]. A shift of a temperature-sensitive p53 mutant to wild-type form requires ATP hydrolysis and

involves HSP70[50]. This suggests that the role of HSP70 is regulating p53 protein conformation.

Herein, base on the above combined results, we constructed a model that explains the effect of heat shock on p53-related pathway (Fig. 6). Heat shock induced the mutation of p53 protein conformation and increased the expression of HSP70. In turn, trigged HSP70 chaperone associated with mutated p53 protein (p53mut). The HSP70-p53mut complex dissociated to release HSP70 and wild-type p53 (p53wt) while in the presence of ATP. Wild-type p53 stimulated the transcription of the MDM2 gene, which created a negative feedback loop for p53 deactivation. In the meanwhile, wild-type p53 was phosphorylated at serine 15 by heat shock to upregulate its downstream genes, p21 and Bax. Heat shock induced the activation of ERKs, however heat shock-induced p53 signaling is independent of ERKs activation.

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Fig. 1. p53 phosphorylation and signaling in response to heat shock. OC2 cells were washed twice with PBS and changed medium prior to treatment with various temperatures (37, 39, 41 and 43℃) for 3 hours in incubator. (A) Heat shock triggered p53 accumulation and p53 phosphorylation at serine 15, in turn upregulation its downstream genes p21 and Bax/Bcl-2. (B) The dynamic changing of p53 after heat shock. The expression and phosphorylation of p53 reached the peak at 12 hours after heat treatment, and lasting for 24 hours. Triplicate experiments were performed and only the representative blot is shown.

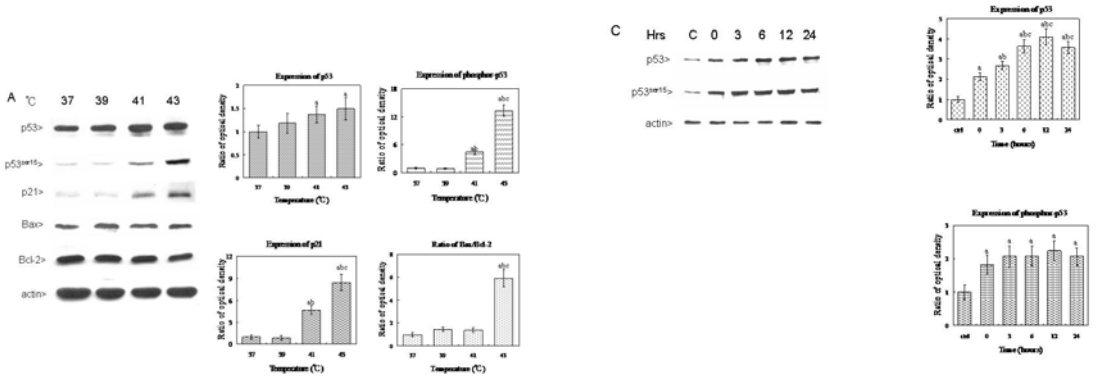


Fig. 2. Upregulation of p53-MDM2 complex formation by heat shock. (A) The expression of 60 kDa MDM2 fragment significantly induced by heat shock. (B) Immunopricitation was used to determine whether 60 kDa MDM2 fragment still possess its function to regulate p53. p53-MDM2 complex accumulation significantly induced at 41 and 43℃. Triplicate experiments were performed and only the representative blot is shown.

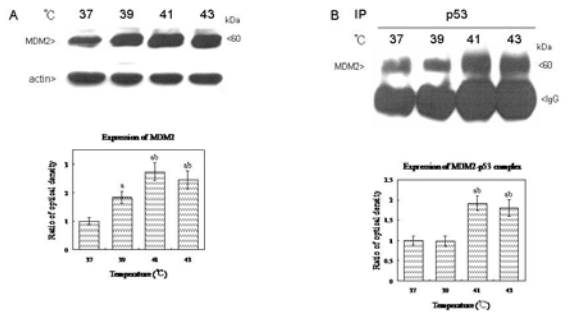


Fig. 3. Inactivated ERKs enhances heat shock-induced p53 phosphorylation at serine 15. (A) Heat treatment significantly increased the expression and phosphorylation of ERKs. (B) Cells were cultured with 10 μM PD98059, a specific inhibitor of MEK (MAPK or ERK kinase), for 1 hour prior to heat treatment (37 and 43℃ for 3 hours). The heat shock-induced phosphorylation of p53 at serine 15 ($p53^{Ser15)}$ and expression of HSP70 were markedly enhanced when ERKs activation was suppressed. Triplicate experiments were performed and only the representative blot is shown.

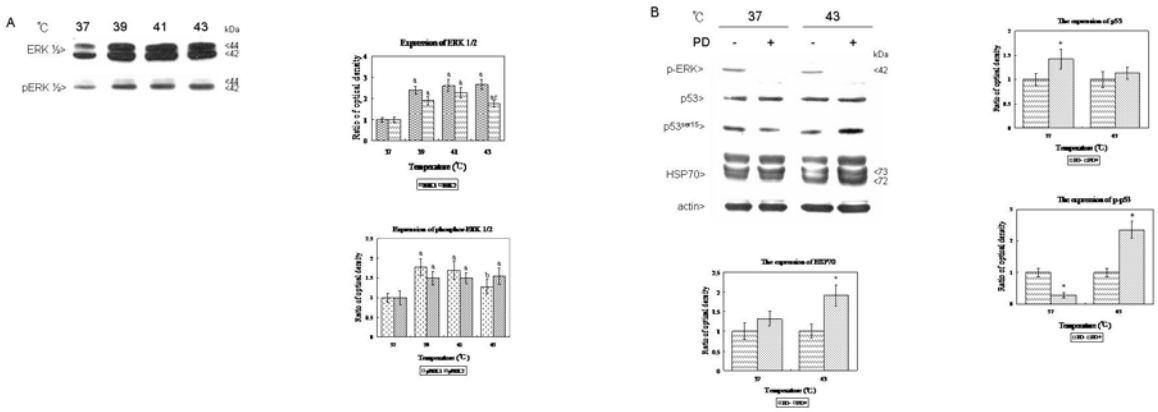


Fig. 4. Heat shock triggered HSP70 complexes formation. (A and B) Expression of HSP70 induced by heat shock and reached the peak at 3 hours after heat treatment and sustained for 24 hours. (C and D) Immunopricipitation (IP) was used to study the relationship between HSP70, p53 and p53 downstream proteins. The capable of heat shock-induced HSP70 binding to proteins include p53, p21 and Bax. (E) Cell lysate collected from untreated OC2 cells were incubated at 37 to 43℃ for 3 hours. The formation of HSP70-p53 complex was significantly induced by heat shock at 41 and 43℃. Triplicate experiments were performed and only the representative blot is shown.

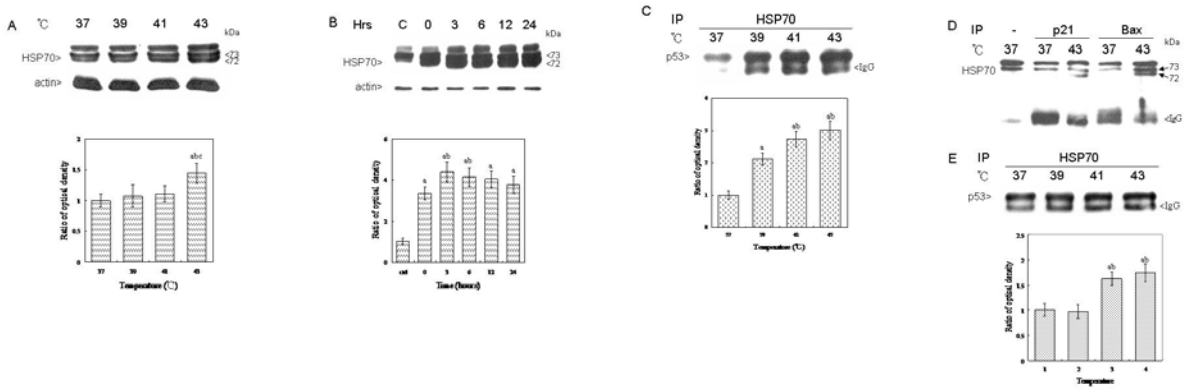


Fig. 5. Heat shock-induced HSP70 oligomerization. Cells were heat treated at indicated temperature for 3 hours prior to be extracted. HSP70 conformation was detected by using non-reducing electrophoresis. Western blotting was performed using HSP70-specific monoclonal antibody purchased from Calbiochem Co. Ltd. HSP70 oligomerized after heat shock at 41 and 43℃. Triplicate experiments were performed and only the representative blot is shown.

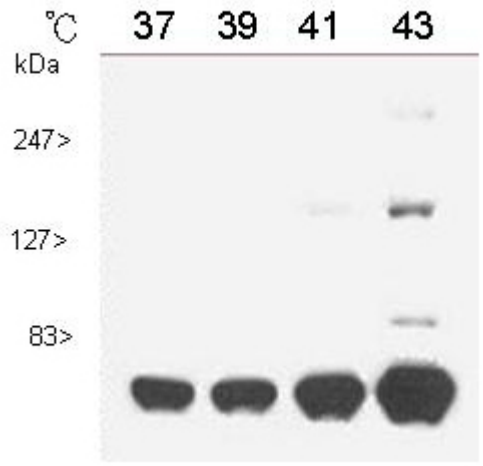
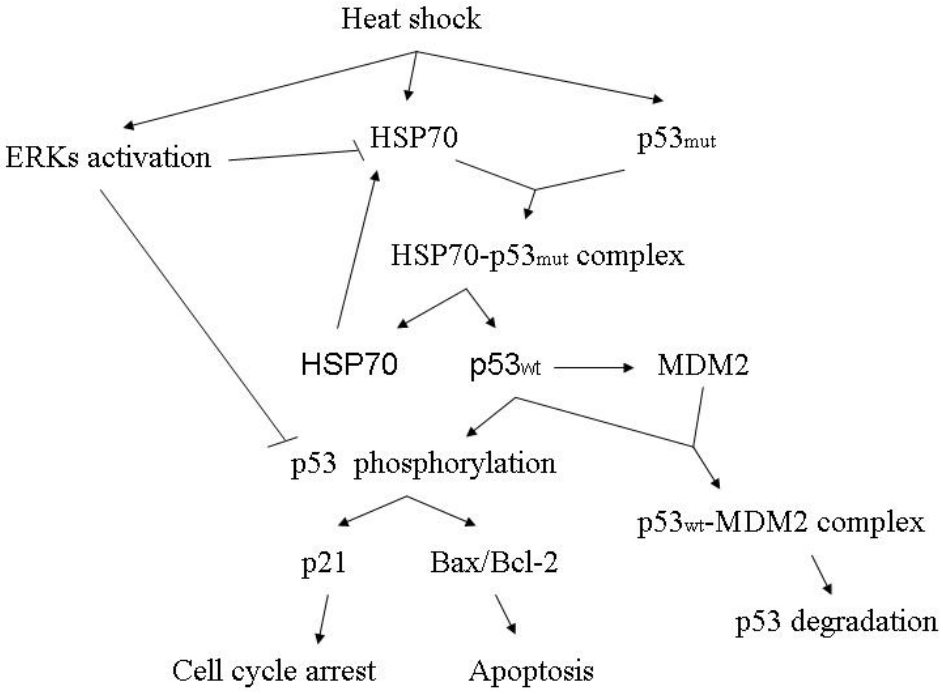


Fig. 6. A scheme depicting the signaling in regulating cell-cycle arrest and apoptosis of heat shock.


Heat shock
ERKs activation
HSP70
p53mut
HSP70-p53mut complex
HSP70
p53wt
MDM2
p53 phosphorylation
p53wt-MDM2 complex
p21
Bax/Bcl-2
p53 degradation
Cell cycle arrest
Apoptosis