

**Role of heat shock protein 90  
in angiotensin II-induced  
myocardial hypertrophy**

**Kyung Hye Lee**

**Department of Medical Science**

**The Graduate School, Yonsei University**

**Role of heat shock protein 90  
in angiotensin II-induced  
myocardial hypertrophy**

**Directed by Professor Yangsoo Jang**

**Doctoral Dissertation**

**submitted to the Department of Medical Science,  
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partial fulfillment of the requirements for the  
degree of Doctor of Philosophy**

**Kyung Hye Lee**

**December 2006**

**This certifies that the Doctoral Dissertation  
of Kyung Hye Lee is approved.**

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Thesis Supervisor : Yangsoo Jang

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Thesis Committee Member

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Thesis Committee Member

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Thesis Committee Member

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Thesis Committee Member

**The Graduate School**

**Yonsei University**

**December 2006**

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**December 2006**  
**Kyung Hye Lee**

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**Abstract**

**Role of heat shock protein 90  
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*Department of Medical Science  
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Angiotensin II (AngII), vasoactive octapeptide, has a modulatory role on hypertrophic effect in cardiac cells. Hypertrophy is well characterized by an increased cell size and protein synthesis without cell division. Numerous studies have evidenced that AngII induces nuclear factor- $\kappa$ B (NF- $\kappa$ B) activation in cardiac cells and that activation of NF- $\kappa$ B is required for hypertrophic process. Interestingly, recent reports proposed that Hsp90 function was required for NF- $\kappa$ B activation. In this study, we investigated the role of Hsp90 on AngII-induced cardiac hypertrophy and molecular mechanism NF- $\kappa$ B pathway. Hsp90 inhibitors inhibited AngII-induced cellular hypertrophy and NF- $\kappa$ B luciferase activity in cardiac cells. Hsp90 inhibitor also inhibited the phosphorylation of I $\kappa$ B $\alpha$  and level of IKK $\alpha$ / $\beta$  induced by

AngII. Interestingly, accumulation of I $\kappa$ B $\alpha$  cleavage fragment was found in Hsp90 inhibitor-treated group. This I $\kappa$ B $\alpha$  cleavage, when pretreated with z-IETD-fmk, an inhibitor of caspase-8, was recovered. I $\kappa$ B $\alpha$  is directly cleaved by caspase-8 *in vitro*. Cleaved I $\kappa$ B $\alpha$  fragment does not contain the amino terminus bearing two lysine residues which can be ubiquitinated by proteasome complex. So, even if I $\kappa$ B $\alpha$  is phosphorylated by any kinases, it can not go through proteosomal degradation, resulting in the maintenance of I $\kappa$ B $\alpha$  interaction with NF- $\kappa$ B. Hsp90 inhibitor induces cardiac cell apoptosis with caspase-8 activation and increase of FasL expression. However, the level of c-Flip<sub>s</sub> was decreased by Hsp90 inhibitor. When caspase-8 inhibitor was added into Hsp90 inhibitor-treated cells, NF- $\kappa$ B activation was recovered. These results suggest that Hsp90 sustains NF- $\kappa$ B activity by blocking the caspase-8 activation, thereby representing a potential molecular switch where apoptosis is inhibited without affecting hypertrophy in cardiac cells.

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Key Words : hypertrophy, hsp90, caspase-8, I $\kappa$ B $\alpha$  cleavage

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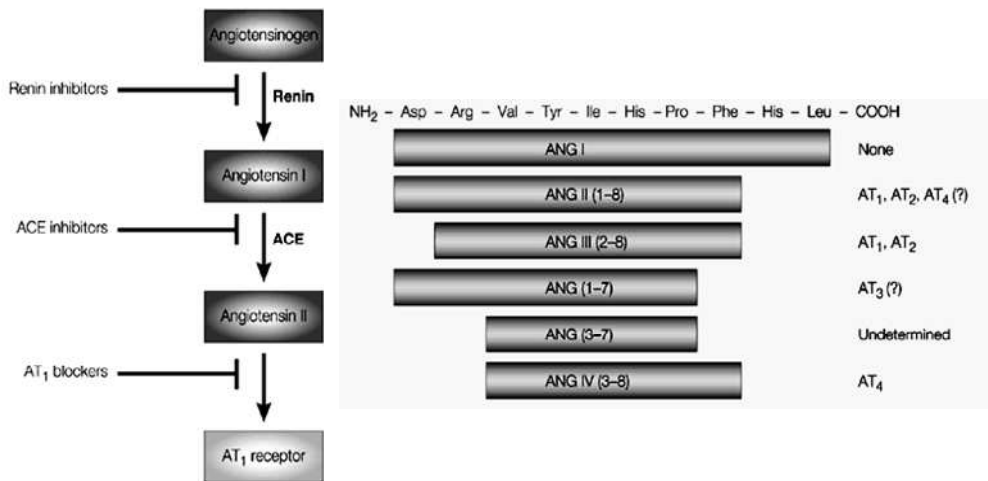
**(Directed by Professor Yangsoo Jang)**

## **I. INTRODUCTION**

Cardiac hypertrophy is an increase in the size of cardiac myocytes to generate increased muscle mass, usually driven by increased workload for the heart. Although it is an adaptive response to physical exercise, excessive hypertrophy can result in arrhythmia, dilated cardiomyopathy, fibrotic disease, heart failure and sudden death.<sup>1</sup> Hypertrophy is well characterized by an increased cell size and protein synthesis without cell division. Cardiac hypertrophy is induced by various growth factors,

vasoactive peptides, cytokines and several neurohormones such as epinephrine, norepinephrine, aldosterone and catecholamines.<sup>2</sup>

Angiotensin II (AngII), vasoactive octapeptide, is the effector peptide of the renin-angiotensin system (RAS) and is involved in electrolyte homeostasis regulating cardiac growth. The system was well represented by Zaman MA *et al* and the diagram was quoted in Fig. 1. Renin released from the juxtaglomerular cells of the kidney cleaves the liver-derived angiotensinogen to produce the inactive decapeptide angiotensin I (AngI), which is then converted to the active octapeptide AngII by angiotensin-converting enzyme (ACE). The RAS can be considered as a system in which circulating AngII is delivered to target tissues or cells and can be mediated several biological actions.<sup>3</sup>



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**Fig. 1. Renin-angiotensin system and angiotensin peptides.**

Action of AngII are mediated by angiotensin type 1 (AT1) and angiotensin type 2 (AT2) receptors, which are G-protein coupled seven transmembrane glycoproteins. AT1 receptor is present in many tissues and organs, including the heart, blood vessels, kidney, and adipocytes, whereas the AT2 receptor is expressed mainly in the fetus and has low levels of expression after birth. AT1 receptor is responsible for most of the pathophysiological actions of AngII.<sup>4</sup> Among other pathological factors, AngII is an important humoral factor responsible for cardiac hypertrophy, and is associated with the induction of immediate-early genes such as *c-fos*, *c-jun* and *Egr-1* which is followed by activation of late genes such as  $\beta$ -myosin heavy chain,  $\alpha$ -skeletal and  $\alpha$ -smooth muscle actin and atrial natriuretic factor (ANF).<sup>2</sup> Ang II-induced cellular hypertrophy is mainly mediated via stimulation of AT1 receptor. So, pharmacological agents that antagonize the RAS at the level of ACE or the AT1 receptor have come into prominence in the treatment of hypertension, cardiac hypertrophy, heart failure and cardiac remodeling. Practically, administration of either an ACE inhibitor or an AT1 receptor antagonist not only significantly induces the regression, but also prevents the development of cardiac hypertrophy in experimental animal models. Previous studies have shown that AngII also induces myocyte apoptosis.

Numerous studies have evidenced that AngII induces nuclear factor- $\kappa$ B (NF- $\kappa$ B) activation in cultured cardiac cells<sup>5,6</sup>, and reported that activation of NF- $\kappa$ B is required for hypertrophic growth of primary rat neonatal ventricular

cardiomyocytes.<sup>7,8</sup> NF- $\kappa$ B is a ubiquitously-expressed cytoplasmic transcription factor whose primary mode of regulation is through nuclear translocation. Most abundant forms of NF- $\kappa$ B transcription factor is heterodimer of p65 and p50 in five mammalian family members : RelA/p65, cRel, RelB, NF- $\kappa$ B1 (p50/p100), NF- $\kappa$ B2 (p52/p100).<sup>9</sup> In un-stimulated cells, NF- $\kappa$ B is sequestered in the cytoplasm by interaction with inhibitor of  $\kappa$ B (I $\kappa$ B) which masks nuclear localization signal in Rel homology domain (RHD) of NF- $\kappa$ B . Phosphorylation on ser-32 and ser-36 of I $\kappa$ B $\alpha$  leads to its ubiquitination and degradation, permitting NF- $\kappa$ B to translocate to the nucleus and stimulates transcription of specific target genes. One of the best known pathway on I $\kappa$ B $\alpha$  phosphorylation is activation of I $\kappa$ B kinase (IKK) complex, which is composed of catalytic IKK $\alpha$  and IKK $\beta$  and regulatory IKK $\gamma$ /NEMO, and these possess essential kinase activity to phosphorylate I $\kappa$ B $\alpha$ . IKK complex is regulated by NF- $\kappa$ B-inducing kinase (NIK) or other kinases. NF- $\kappa$ B also regulates expression of many genes that are involved in anti-apoptotic pathway, cell cycle progression and cell survival. Activation of NF- $\kappa$ B can promote cell survival by inducing the expression of genes encoding anti-apoptotic proteins such as Bcl-2 family. It was also reported that the cellular FADD-like IL-1 $\beta$ -converting enzyme (FLICE)-inhibitory protein (c-FLIP) which can interfere with procaspase-8 activation was induced by NF- $\kappa$ B transcriptional activation.<sup>10,11</sup> Caspase-8 is a cysteine protease that initiates activation of other caspases. Procaspase-8, an inactive precursor, is present in cytosol as monomer, and proximity of inactive caspase-8 enhances auto-



processing itself resulting heterotetrameric active caspase-8. This proximity was provided by death receptor ligation offering death domain recruitment including death effector domain of caspase-8. As an important modulator of caspase-8, c-FLIP appears to be an important regulator determining the life and death in various types of cells.<sup>12</sup>

Heat shock proteins (Hsps) has been previously shown to regulate the NF- $\kappa$ B cascade. Hsps have been implicated in protective cardiac responses to ischemia, hypoxia and other kinds of stressful or injurious stimuli. Several groups have reported that Hsps regulates NF- $\kappa$ B activation. Recent reports proposed that Hsp90 function was required for IKK activation and its homeostasis. After TNF- $\alpha$  stimulates the cells, Hsp90 recruits IKK complex to the TNF- $\alpha$  receptor and cytokines are expressed by NF- $\kappa$ B activation. Interestingly, it is found that Hsp70 regulates development of cardiac hypertrophy.<sup>13</sup> When cells are subjected to heat shock or other stresses, heat shock proteins helps to maintain protein stability and to re-nature of target for degradation unfolded proteins. The cardiotrophin-1 (CT-1), an hypertrophic stimuli, treatment causes an increase in the Hsp70 and Hsp90 in cardiac cells.<sup>14</sup> It was also reported that Hsp70 was induced in acute hypertension through increased blood pressure,<sup>15</sup> and Hsps were up-regulated by hypertrophic stimuli, AngII, in vascular smooth muscle cells.<sup>16</sup> Especially, recent studies demonstrated that heat shock treatment suppresses AngII-induced NF- $\kappa$ B activation,<sup>17</sup> and revealed liposomal delivered Hsp70 blocks NF- $\kappa$ B activation in

renal tubular cells.<sup>18</sup> However, disruption of Hsp90 function resulted in blockage of the NF- $\kappa$ B activation.<sup>19</sup> Other studies also suggest that Hsp90 regulates the stability and function of receptor-interaction proteins (RIP) and IKK $\beta$ , the key components of the NF- $\kappa$ B activation pathway. Despite these evidences in our understanding of the important roles of Hsps in NF- $\kappa$ B cascade, no information is currently available regarding the crosstalk between Hsp90 and NF- $\kappa$ B cascade in the modulation of cardiac hypertrophy and cell death induced by AngII. In this study, we investigated the role of Hsp90 on AngII-induced cardiac hypertrophy, as well as the associated signaling mechanisms. In particular, this work demonstrated how Hsp90 regulates NF- $\kappa$ B signal and caspase cascade in regulating the cardiac hypertrophy or death induced by AngII.

## II. MATERIALS AND METHODS

### 1. Materials

Angiotensin II was purchased from Sigma (St. Louis, Mo, USA). Geldanamycin, 17-AAG (17-(Allylamino)-17-dimethoxygeldanamycin), MG-132 (Z-Leu-leu-leu-aldehyde) and cycloheximide were obtained from A.G. Scientific (San Diego, CA, USA). Heat shock protein inhibitor I (3,4-Methylenedioxy-benzylidene- $\gamma$ -butyrolactam) was purchased from Calbiochem (San Diego, CA, USA). Recombinant rat tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) was purchased from Biosource (Camarillo, CA, USA). As calpain inhibitors, PD150606 (3-(4-Iodo phenyl)-2-mercapto-(Z)-2-propenoic Acid), calpeptin (Benzoyloxycarbonyl dipeptidyl aldehyde), calpastatin were also purchased from Calbiochem (San Diego, CA, USA). Caspase-3 inhibitor (z-DEVD-fluoromethylketone, z-DEVD-fmk) was purchased from A.G. Scientific. Caspase-8 inhibitor (z-IETD-fmk) was purchased from R&D systems (Minneapolis, MN, USA). Other caspase inhibitors (z-VAD-fmk, z-VDVAD-fmk, z-LEHD-fmk) were purchased from Tocris (Ellisville, MO, USA). Polyclonal heat shock protein antibodies (anti-Hsp70, anti-Hsc70 and anti-Hsp90) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). NF- $\kappa$ B p65, I $\kappa$ B- $\alpha$ , IKK- $\alpha/\beta$  and IKK- $\gamma$  antibodies were also obtained from Santa Cruz Biotechnology. Phospho-I $\kappa$ B- $\alpha$  (ser32), phospho-IKK- $\alpha$  (ser180)/IKK $\beta$  (Ser181) and FLIP antibodies were purchased from Cell Signaling (Beverly, MA, USA).

Horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence (ECL) western blotting detection system were obtained from Santa Cruz Biotechnology and Amersham Biosciences (Uppsala, Sweden), respectively. L-[4,5-<sup>3</sup>H]Leucine and  $\gamma$ -<sup>32</sup>P-labeled dATP were also obtained from Amersham Biosciences.

## **2. Cell culture**

Rat neonatal ventricular cardiac myocytes were prepared as described previously with minor modifications.<sup>21</sup> All animal procedures were carried out according to a protocol approved by the Yonsei University Animal Care Committee. Heart from neonatal (1-2 days old) Sprague-Dawley rats were excised, and washed with phosphate-buffered saline (PBS) in order to deplete red blood cells and minced. The minced myocardial tissues were incubated with 0.1% collagenase type II (Gibco BRL, Grand Island, NY, USA) with PBS at 37°C repeatedly. Ten 10 min digestions were carried out, the cells were collected by centrifugation and resuspended in plating media, minimum Essential Medium Alpha Medium ( $\alpha$ -MEM, Gibco BRL) containing 10% fetal bovine serum (FBS) and 100 U/ml penicillin/streptomycin (Gibco BRL). Cells were pre-plated for 1 h at 37°C in 5% CO<sub>2</sub> incubator to allow removal of fibroblasts. Unattached cardiac myocytes were plated in gelatin coated dishes and incubated for 24 h in plating media and then spontaneously contracting confluent cells could be seen. In order to reduce fibroblast contamination, 0.1 mM 5-

bromo-2'-deoxyuridine (Brd-U) (Sigma, St. Louis, Mo, USA) was added in the media. Rat heart-derived embryonic myoblast H9c2 cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS and 100 U/ml penicillin/streptomycin (Gibco BRL).

### **3. [<sup>3</sup>H]Leucine incorporation**

[<sup>3</sup>H]Leucine incorporation was measured as described previously.<sup>20</sup> Cells were grown in 24-well plates and made quiescent in serum-free medium for 24 h. Cells were then treated with AngII for 24 h after pretreatment with or without inhibitors for 1 h. The stimulated cells were pulsed with 1 µCi/ml [<sup>3</sup>H]leucine during the last 4 h before harvest. After being washed with ice-cold PBS, cells were treated with 5% trichloroacetic acid for 30 min and then washed twice with ice-cold PBS. Finally, cells were solubilized in 500 µl of 1 N NaOH for 30 min. After neutralization with 0.5 N HCl and following add of aquazol (PerkinElmer, Wellesley, MA), an aliquot was taken to determine the incorporated radioactivity by liquid scintillation counting. Radioactivity was measured in a liquid scintillation counter, Beckman LS 3801 (Beckman, Fullerton, CA, USA) or 1450 microbeta Trilux (Wallac, Turku, Finland).

### **4. Immunocytochemistry assay**

Cells were plated in the 4-well slide chamber (Sonic Seal Slide, Nalge Nunc,

Rochester, NY, USA) and grown with serum free  $\alpha$ -MEM for 24 h. After 48 h treatment of 100 nM of AngII or 1% of FBS, cells were fixed with 4% paraformaldehyde. And then after the washing with PBS containing 0.2% NP-40, cells were blocked with 10% goat serum-containing PBS and incubated with rabbit polyclonal ANF antibody for overnight. After three times washing with PBS, cells were incubated with FITC-conjugated goat anti-rabbit antibody as the secondary antibody for 1 h. Photographs of cells were taken under fluorescence by immunofluorescence microscopy (Olympus, Melville, NY, USA).

## **5. NF- $\kappa$ B luciferase activity Assay**

Cells were plated at  $6 \times 10^4$  in 24-well plate 24 h prior to transfection. Cells were transfected with 100 ng of pNF- $\kappa$ B/LUC luciferase vector, and pcDNA3.1/lacZ was co-transfected to cells as an internal control of transfection efficiency detection. All transfections were performed using Lipofectamine plus<sup>TM</sup> reagent (Invitrogen, Carlsbad, CA, USA). The media were changed 3 h after transfection, and the cells were grown overnight in fresh complete media. After recovery, cells were starved and exposed to AngII or several inhibitors. Luciferase activity was determined using Luciferase Reporter Assay Kit (BD Biosciences, Franklin Lakes, NJ, USA). The luciferase activity was measured using luminometer Victor 3 (Perkin Elmer, Wellesley, MA, USA). The activity of the NF- $\kappa$ B reporter luciferase was standardized to that of  $\beta$ -galactosidase activity. It was determined using ONPG for

its substrate by measuring absorbance at 420 nm.

## **6. Nuclear protein extraction**

Nuclear and cytosolic extracts were prepared as described previously<sup>21</sup>. Briefly, AngII-treated cells were washed, and then scraped into 1 ml of ice-cold PBS, and pellet at 3,000 rpm at 4°C for 5 min. The pellets were suspended in 10 mM Tris (pH 8.0), with 1.5 mM MgCl<sub>2</sub>, 1 mM DTT, 0.1% NP-40 and incubated on ice for 15 min. Nuclei were separated from cytosol by centrifugation at 12,000 rpm at 4°C for 15 min. The cytosolic supernatants were removed, and the precipitated pellets were suspended in 10 mM Tris (pH 8.0) containing 50 mM KCl, 100 mM NaCl and incubated on ice for 30 min. After agitation for 30 min at 4°C, the lysate was centrifuged at 12,000 rpm for 5 min at 4°C, and the supernatant was collected. The nuclear protein concentration was determined using Bradford protein assay kit (Bio-Rad).

## **7. SDS-PAGE and immunoblot analysis**

Cells were treated with AngII in the presence or absence of appropriate inhibitors for the indicated time periods at 37°C. The medium was aspirated, and the cells were rinsed with cold PBS and immediately scraped into 1.5 ml tubes at -80°C. Harvested cells were solubilized in a lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM Na<sub>2</sub>EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate,

1 mM  $\beta$ -glycerophosphate, 1 mM  $\text{Na}_3\text{VO}_4$ , 1 mM phenylmethylsulfonyl fluoride, 1  $\mu\text{g}/\text{ml}$  aprotinin, and 1  $\mu\text{g}/\text{ml}$  leupeptin) and centrifuged at 12,000 rpm for 20 min at 4°C. The protein concentrations of the supernatants were determined using a Bradford protein assay kit (Bio-Rad). Cell lysates containing equal amounts of protein were subjected to SDS–polyacrylamide gel electrophoresis, and the proteins were transferred to polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA, USA). After blocking in 10 mM Tris–HCl buffer (pH 8.0) containing 150 mM sodium chloride, 0.1% Tween 20, and 5% (w/v) nonfat dry milk, the membrane was treated with appropriate primary antibodies, followed by incubation with appropriate horseradish peroxidase-conjugated secondary antibodies. The bands were detected using an enhanced chemiluminescence reagent kit (Santa Cruz Biotechnology, and Amersham Biosciences) and quantified using densitometry. All of Western blot analyses were repeated at least three times in independent experiments.

## **8. Immunoprecipitation**

Cells were washed once in PBS and lysed in a lysis buffer (Cell signaling) containing 20 mM Tris (pH7.5), 150 mM NaCl, 1 mM  $\text{Na}_2\text{EDTA}$ , 1 mM EGTA, 1% triton, 2.5 mM sodium pyrophosphate, 1 mM  $\beta$ -glycerophosphate, 1 mM  $\text{Na}_3\text{VO}_4$ , 1 mg/ml leupeptin and 1 mM PMSF. Protein concentrations were determined using Bradford protein assay kit (Bio Rad). Cell extract (200  $\mu\text{g}$ ) was pre-cleared with



protein A/G agarose plus (Santa Cruz Biotechnology) and incubated with 2  $\mu\text{g}$  of antibody on a rotating platform plate at 4°C for 2 h. Immunoprecipitation was facilitated by the addition of 15  $\mu\text{l}$  of protein A/G agarose at 4°C for 1 h on a rotating platform plate. After serial washes, it was subjected to SDS-PAGE and detected using immunoblot analysis.

### **9. In vitro cleavage assay**

Caspase reaction was performed with 1  $\mu\text{g}$  of GST-I $\kappa$ B $\alpha$  and 1  $\mu\text{g}$  of caspase-8 or 1 unit of caspase-1, 2, 3, 4, 5, 6, 7, 9, or 10 in 20 mM HEPES (pH 7.4), 100 mM NaCl, 0.5% NP-40, 10 mM DTT, 1 mM PMSF for 1 h at 37°C. Samples were subjected to 10% SDS-PAGE and analyzed by immunoblot assay with anti-I $\kappa$ B $\alpha$  antibody.

### **10. IKK kinase assay**

Immunoprecipitation was performed using 2  $\mu\text{g}$  of rabbit anti-IKK $\alpha/\beta$  antibodies and protein A/G agarose plus. Precipitate sample were incubated with GST-I $\kappa$ B $\alpha$  (1  $\mu\text{g}$ ) and purified active caspase-8 (0.5  $\mu\text{g}$ ) in kinase buffer containing 20 mM HEPES (pH 7.4), 10 mM MgCl<sub>2</sub>, 300  $\mu\text{M}$  sodium *ortho*-vanadate, 10 mM  $\beta$ -glycero phosphatate, 1 mM NAF, 1 mM DTT, 1 mM PMSF, 1 mM benzamidine, 10  $\mu\text{g}/\text{ml}$  aprotinin, 1  $\mu\text{g}/\text{ml}$  leupeptin. Total volume of reaction mixture was 50  $\mu\text{l}$  as follows: 1  $\mu\text{g}$  GST-I $\kappa$ B $\alpha$ , 1  $\mu\text{l}$   $\gamma$ -[<sup>32</sup>P]-ATP (>3000 Ci/mmol, 250 Ci/25 $\mu\text{l}$ ), Enzyme reaction was performed at 30°C for 40 min and then reaction mixtures were loaded on 12.5%

SDS-PAGE. After electrophoresis, the gel was vacuum-dried and performed autoradiography by exposure to X-ray film at  $-70^{\circ}\text{C}$  for 1 d.

### **11. Cell viability assay**

Cells were plated in 48-well plates at a density of  $1 \times 10^4$  per well. After starving with serum-free DMEM for 24 h, cells were exposed to the appropriate treatment. Cell viability was determined by the MTT assay. The 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) solution (Sigma) was added to each well to a final concentration of 0.5 mg/ml and was incubated at  $37^{\circ}\text{C}$  for 1 h. The formazan crystal was dissolved by adding dimethylsulfoxide (DMSO) and absorbance was measured at the 570 nm with a spectrophotometer.

### **12. JC-1 staining assay**

Cells were plated in 4-well chamber slide at a density of  $1 \times 10^4$  per well. After starving with serum-free DMEM, cells were treated with z-IETD-fmk (100  $\mu\text{M}$ ) for 30 min, and geldanamycin (1  $\mu\text{M}$ ) was treated for 12 h. Samples were incubated with 5  $\mu\text{g/ml}$  of 5,5',6,6'-tetrachloro-1,1',3,3'-teterathylbenzimidazolocarboyanine iodide (JC-1) (Molecular Probe, Eugene, OR, USA) for 10 min and washed with PBS. JC-1 is a cationic, lipophilic dye for the detection of changes in mitochondrial inner membrane electrochemical potential in living cells. Electrochemical potential gradient causes concentrated dye in mitochondria emit red fluorescence, while break

down of the gradient makes dye dispersed in the cytosol, leading to green fluorescence. Mitochondrial JC-1 aggregates were visualized with LSM 510 Meta (Carl Zeiss, Thornwood, NY, USA) confocal microscope for the excitation at 488 nm and the emission at 530 nm and 590 nm.

### **13. Measurement of caspases activity**

The activity of caspases in the cells were determined with ApoAlert™ Caspase-3 and ApoAlert™ Caspase-8 Fluorescent assay kits (BD Biosciences, Palo Alto, CA, USA) by fluorescent detection (at 505 nm) of 7-amino-4-trifluoromethyl coumarin (AFC) after cleavage from the peptide substrate DEVD-AFC and IETD-AFC, respectively. Cells were incubated in DMEM without FBS and treated with geldanamycin for 15 h. After then, cells were harvested, resuspended in chilled cell lysis buffer and incubated on ice for 10 min. Cells were centrifuged and supernatants were transferred to a fresh tube. Protein concentration was determined by the Bradford assay (Bio-Rad). The 50 µl of 2× reaction buffer containing 10 mM DTT was added and each sample was incubated with 5 µl of DEVD-AFC (1 mM) or IETD-AFC (1 mM) at 37°C for 1 h. The AFC-substrate conjugate usually emits blue light, however, cleavage of the substrate by the appropriate caspase liberates AFC, which fluorescence yellow-green ( $\lambda$  max=505 nm). Caspase-3 and caspase-8 activity were read in a Luminescence Spectrometer LS50B (Perkin Elmer Life and Analytical Sciences, Boston, MA, USA) with a 400 nm excitation filter and 505 nm

emission filter.

#### **14. RT-PCR Analysis**

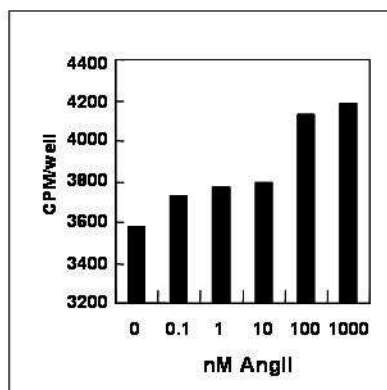
RT-PCR was used to measure the expression of FasL. Cells were plated on 100 mm tissue culture dishes, and starved with serum deprived DMEM for 24 h. After geldanamycin treatment for 15 h, cells were harvested and total RNA was purified by 1 ml of TRIZOL® Reagent (Invitrogen, Carlsbad, CA, USA). 1 µg of total RNA was reverse transcribed with 200 units of Molony Murine Leukemia (M-MLV) Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) for 50 min using oligo(dT)<sub>12-18</sub> in a 20 µl reaction. The first-strand cDNA (2 µl) was amplified by *ExTaq* (TaKaRa, Shiga, Japan) with 10 pmol of primer sets and 0.25 mM dNTP. PCR condition was as follows: 30 cycles of denaturation at 94°C for 30 s, annealing at 54°C for 30 s, and extension at 72°C for 30 s. The primer sequences were as follows: 5'-AAGGCGGCCTTGTGATCA-5' (forward), 5'-TGCAGGCATTAAGGACCA-5' (reverse).

### **III. RESULTS**

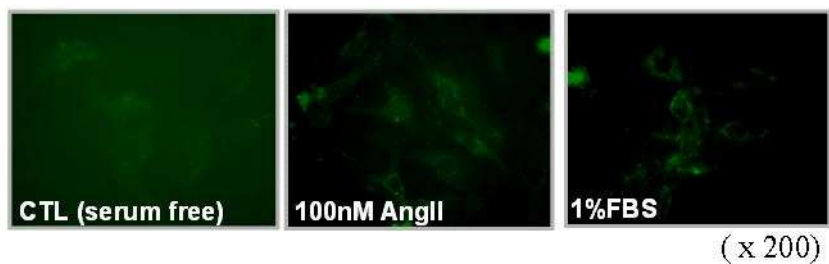
#### **1. AngII induces hypertrophy and p65 NF- $\kappa$ B nuclear translocation in cardiac cells**

AngII stimulation induces long-term changes in cardiovascular tissues including cell growth and hypertrophy. Cellular hypertrophy is an adaptive process for synthesis of proteins without DNA replication, leading to protein accumulation. To confirm the hypertrophic effect of AngII in cardiac cells, [ $^3$ H] leucine incorporation assay was performed in rat neonatal cardiac myocytes. At the cellular level, the hypertrophic response is characterized by an increase in protein synthesis which can be analyzed by measurement of [ $^3$ H] leucine incorporation into cells. Cardiac myocytes cultured in serum-free condition were treated with AngII (0.1 ~ 1000 nM) for 48 h. AngII increased [ $^3$ H] leucine incorporation in a concentration-dependent manner in cardiac myocytes (Fig. 2A). The specific marker of hypertrophy, atrial natriuretic factor (ANF) was also visualized by immuno cytochemical staining assay using specific anti-ANF antibody. ANF was detected around nucleus in AngII-treated group compared with untreated cells (Fig. 2B). We showed the same result in rat heart-derived myoblast H9c2 cells, and no significant difference was observed between primary cardiac cells and H9c2 cells (data not shown). These findings clearly demonstrate that in agreement with the previous reports, AngII treatment of cardiac cells resulted in a significant increase in cellular hypertrophy.

A.



B.



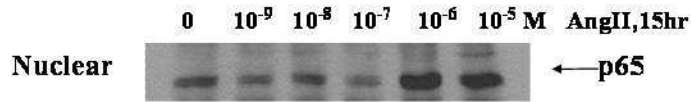
**Fig. 2. AngII induces hypertrophy in cardiac cells.** A. Cells were treated with AngII and assessed for hypertrophy by [<sup>3</sup>H]leucine incorporation assay in three independent experiments. Incorporated radioactivity was determined by liquid scintillation counting. B. Cells were incubated with 100 nM AngII or 1% FBS for 48 h. Cells were stained for ANF protein using polyclonal anti-ANF antibody and FITC-conjugated secondary antibody and then visualized by fluorescence microscopy.

## 2. AngII induces activation of NF- $\kappa$ B in cardiac cells

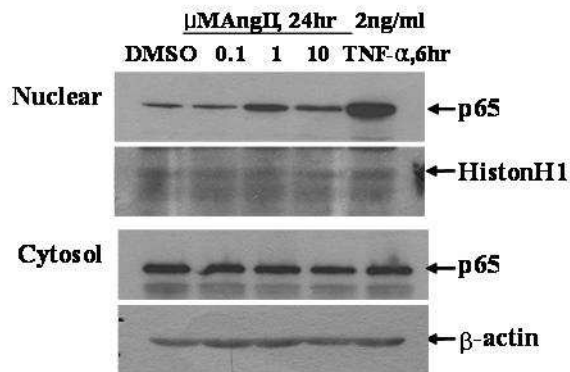
It has been well reported that NF- $\kappa$ B is activated during hypertrophy in cardiac cells. To validate the NF- $\kappa$ B activation on AngII-induced hypertrophy, NF- $\kappa$ B p65 subunit of nuclear fraction were visualized by immunoblot analysis. AngII stimulated the nuclear translocation of p65 NF- $\kappa$ B in neonatal cardiac myocytes (Fig. 3A). As shown in Fig. 3B, p65 NF- $\kappa$ B subunit was translocated to nucleus at 1  $\mu$ M AngII in H9c2 cells in the same manner with primary cardiac myocytes. TNF- $\alpha$ , is a well known cytokine that activates NF- $\kappa$ B, also induced p65 nuclear translocation.

To confirm the transcriptional activity of AngII-induced nuclear translocation of p65, NF- $\kappa$ B luciferase activity assay was examined. Because of low efficiency of transfection in rat neonatal cardiac myocytes, H9c2 was used to perform luciferase activity assay. After cells were transiently transfected with a luciferase reporter construct (pNF- $\kappa$ B/LUC luciferase vector) possessing consensus pNF- $\kappa$ B binding sites, AngII was treated. NF- $\kappa$ B luciferase activity was enhanced by the addition of AngII in a concentration-dependent manner (Fig. 4A). To confirm whether the translocation of p65 is due to AngII-induced phosphorylation of I $\kappa$ B $\alpha$ , immunoblot assay was performed using phospho-specific I $\kappa$ B $\alpha$  antibody. I $\kappa$ B $\alpha$  was decreased in AngII- or TNF- $\alpha$ -treated group and its reduction is a result of proteosomal degradation (Fig. 4B). It was shown that pretreatment of MG132, a proteosomal inhibitor, blocked I $\kappa$ B $\alpha$  degradation. Theses are evidences that AngII activates NF- $\kappa$ B through phosphorylation and degradation of I $\kappa$ B $\alpha$ .

A.



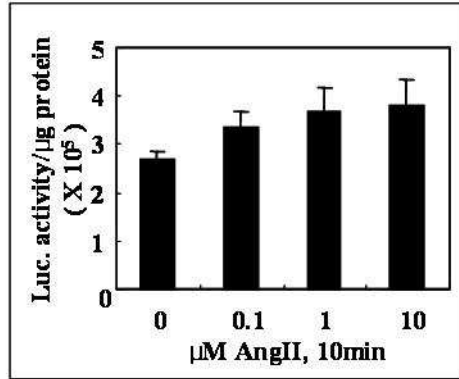
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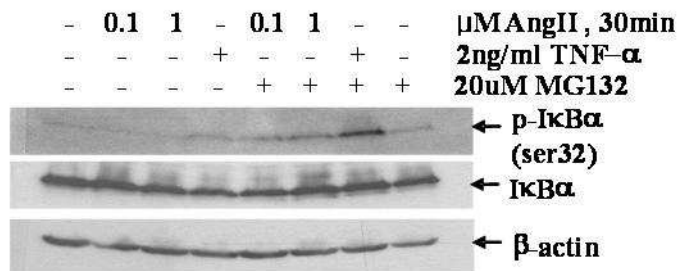
**Fig. 3. AngII induces nuclear translocation of p65 NF- $\kappa$ B in cardiac cells.** Neonatal rat cardiac myocytes (A) or H9c2 cells (B) were cultured in presence or absence of AngII for the indicated times. Cells harvested and extracted the nuclear protein. The p65 nuclear translocation was visualized by immunoblot assay using anti-p65 NF- $\kappa$ B antibody. Equal amounts of nuclear and cytosolic protein were confirmed using Histon H1 and  $\beta$ -actin antibody, respectively.



A.



B.



**Fig. 4. AngII activates NF-κB in cardiac cells.** A. Cells transfected with 100 ng of pNF-κB/LUC luciferase vector were treated with AngII as indicated concentration for 24 h. Luciferase activity was determined using Luciferase Reporter Assay Kit and measured using luminometer. B. Cells were incubated with 1 μM AngII for 30 min or 2 ng/ml TNF-α for 10 min, either in the presence or absence of 20 μM MG132. Immunoblot assay was performed with anti-phospho-IκBα antibody.

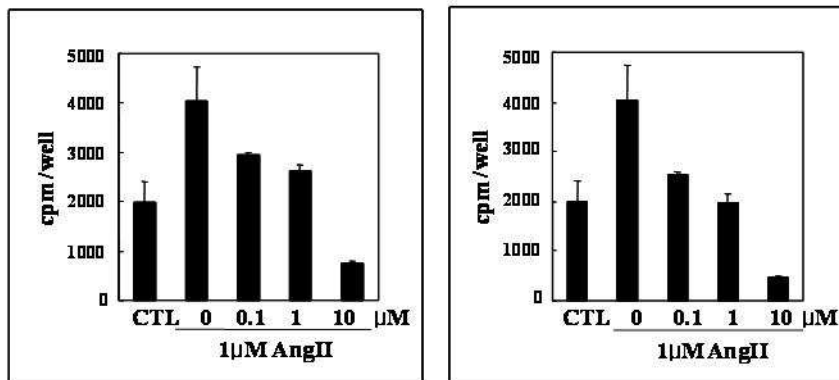
### **3. Hsp90 inhibitor suppresses AngII-induced hypertrophy and NF- $\kappa$ B activation in cardiac cells**

Heat shock proteins (Hsps) are a group of chaperone proteins that helps to maintain protein stability and to re-nature or target for degradation of unfolded proteins when cells are subjected to heat shock or other stresses. Since cardiac hypertrophy is an adaptive response of the heart for increasing workload, it is supposed that Hsps are induced and have a role as chaperone during hypertrophic stress. To investigate the role of Hsp90 in AngII-induced hypertrophy in cardiac cells, specific inhibitors of Hsp90 (geldanamycin;GA or 17-AAG) were treated prior to AngII treatment. These compounds are specific inhibitors of Hsp90 by blocking the binding of ATP to Hsp90. GA reduced AngII-induced [ $^3$ H]leucine incorporation in a concentration-dependent manner (Fig. 5A). The pretreatment of 17-AAG also significantly inhibited AngII-induced [ $^3$ H]leucine incorporation in similar pattern with that of GA (Fig. 5B). These findings clearly demonstrate that blockage of Hsp90 function inhibits AngII-induced cardiac hypertrophy. Recent reports illustrated that Hsp90 modulated NF- $\kappa$ B activity in TNF $\alpha$ -induced activation of NF- $\kappa$ B. To understand the role of Hsp90 on activity of NF- $\kappa$ B in AngII-induced hypertrophy, NF- $\kappa$ B luciferase assay was performed using GA. GA has an effect on lowering the activity of NF- $\kappa$ B in AngII-induced activation in cardiac cells (Fig. 6A). NF- $\kappa$ B activity is controlled by translocation of NF- $\kappa$ B through I $\kappa$ B $\alpha$  degradation or modification of p65 such as phosphorylation after nuclear localization. To assess

whether Hsp90 regulates the nuclear localization of NF- $\kappa$ B, cells were fractionated into nuclear and cytosolic extract after treatment of 17-AAG in cardiac cells. As shown in Fig. 6B, 17-AAG inhibited the AngII-induced p65 nuclear translocation. However, the inhibitor of Hsp70 did not affect the p65 nuclear translocation. These data represent that blockage of Hsp90 activity inhibits the activation of NF- $\kappa$ B, and Hsp90 might be an important regulator of NF- $\kappa$ B pathway in AngII-induced hypertrophy in cardiac cells.

### A. Geldanamycin

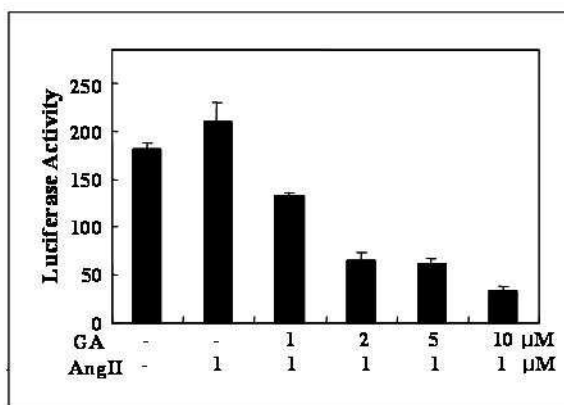
### B. 17AAG



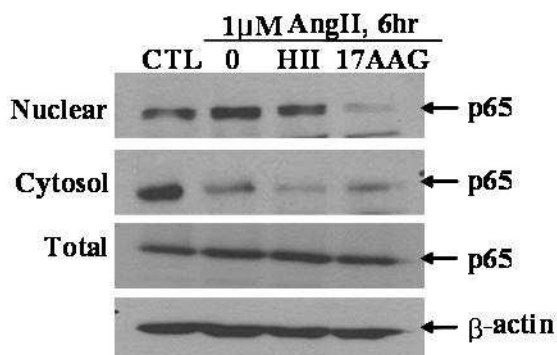
**Fig. 5. Blockage of Hsp90 function inhibits AngII-induced cardiac hypertrophy.**

Cells were treated with indicated concentration of geldanamycin (A) or 17-AAG (B) prior to incubation with 1 μM AngII for 24 h. The [<sup>3</sup>H]leucine incorporation assay was performed and radioactivity measured with liquid scintillation counter

A



B



**Fig. 6. Blockage of Hsp90 function inhibits AngII-induced NF-κB activation in cardiac cells.** A. After transfection with pNF-κB Luc vector for 24 h, cells were starved then exposed to various concentrations of GA for 1 h prior to AngII treatment. B. Cells were treated with 1 μM 17-AAG or 100 μM Hsp70 inhibitor (HII) for 1 h prior to addition of 1 μM AngII. Cells were harvested and fractionated into nuclear and cytosolic protein. The NF-κB p65 subunit level was determined by

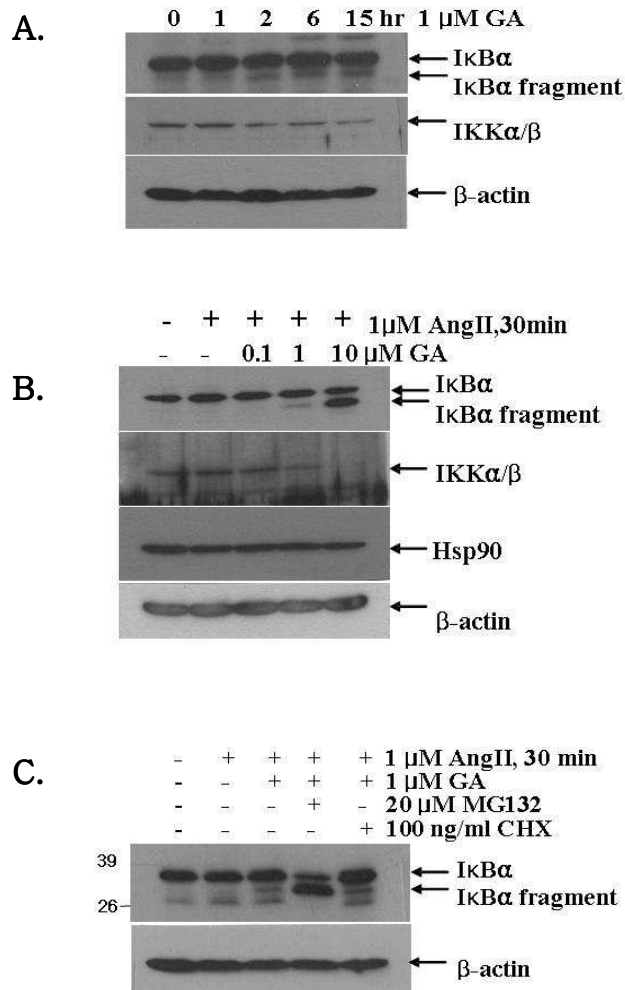
immunoblot assay using anti-p65 specific antibody.

#### **4. Hsp90 inhibitor cleaves I $\kappa$ B $\alpha$ through caspase-8 activation in cardiac cells**

##### **1) I $\kappa$ B $\alpha$ cleavage by Hsp90 inhibitor**

Hsp90 inhibitors suppressed AngII-induced hypertrophy and nuclear translocation of NF- $\kappa$ B activation in cardiac cells (Fig. 6). These results suggest that Hsp90 inhibitor can modulate some signal proteins in the NF- $\kappa$ B activation pathway. In order to determine if Hsp90 inhibitor affects the p65 NF- $\kappa$ B subunit, the protein level of p65 was measured by immunoblot analysis. The p65 level exhibited little or no change after treatment with GA (data not shown). However, immunoblot analysis of the whole extract revealed that the smaller band of I $\kappa$ B $\alpha$  began to appear in GA-treated H9c2 cells (Fig. 7A, B). Suppression of Hsp90 activity also strongly affected the time-course of the cleavage of I $\kappa$ B $\alpha$  (Fig. 7A) The smaller band of I $\kappa$ B $\alpha$  became to appear by 1  $\mu$ M GA treatment, and 10  $\mu$ M GA induced an increase of a significant amount of the smaller band of I $\kappa$ B $\alpha$  (Fig. 7B). GA acts as a specific inhibitor of Hsp90 by blocking the binding of ATP to Hsp90. In this experiment, Hsp90 showed no differences in the GA-treated cells compared to the control cells (Fig. 7A, B). To determine if GA also affects IKK $\alpha$ / $\beta$  stability as known, the protein level of IKK $\alpha$ / $\beta$  was also examined with specific antibody to IKK $\alpha$ / $\beta$ . IKK $\alpha$ / $\beta$  was significantly decreased in a time-dependent and concentration-dependent manner (Fig. 7A, B). To observe whether the smaller band of I $\kappa$ B $\alpha$  is the product of proteosomal degradation or *de novo* protein synthesis, a proteasome inhibitor, MG132 (20  $\mu$ M) and a protein synthesis inhibitor, cycloheximide (CHX, 100 ng/ml) were added with prior to

treatment with GA. These inhibitors did not exhibit a decrease of the smaller band of I $\kappa$ B $\alpha$ , but MG132 generated a large amount of the smaller band of I $\kappa$ B $\alpha$ , interestingly (Fig. 7C). These results suggest that the smaller band might be a cleavage product of I $\kappa$ B $\alpha$  due to treatment with GA.



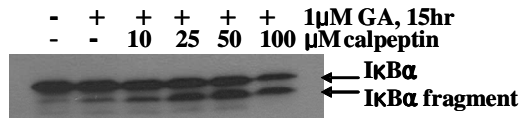
**Fig. 7. Hsp90 inhibitor induces I $\kappa$ B $\alpha$  cleavage in cardiac cells.** H9c2 cells were exposed to GA for indicated time (A) or with various concentrations (B). Cells were harvested and subjected to immunoblot analysis. H9c2 cells were incubated with 1  $\mu$ M GA for 15 h in the presence or absence of 20  $\mu$ M MG132 or 100 ng/ml of cycloheximide (CHX) (C). I $\kappa$ B $\alpha$  was determined by immunoblot analysis using anti-I $\kappa$ B $\alpha$  antibody.



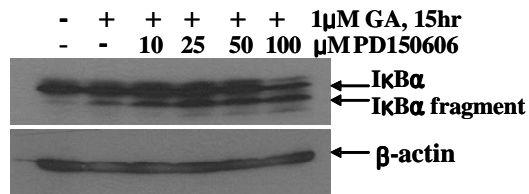
## 2) Effects of calpain inhibitors on the I $\kappa$ B $\alpha$ cleavage

In previous study by Schaecher *et al*, a calcium-activated neutral proteinase, calpain, exhibited cleavage of I $\kappa$ B $\alpha$  in human peripheral blood mononuclear cells (PBMC) under activation with anti-CD3/CD28 (HIT3A/CD28.2), and 150  $\mu$ M calpeptin, a calpain inhibitor, blocked cleavage of I $\kappa$ B $\alpha$ .<sup>22</sup> To investigate whether calpain could mediate cleavage of I $\kappa$ B $\alpha$ , various calpain inhibitors were added 1 h prior to treatment with GA. Calpain inhibitors (calpeptin and PD150606) were treated as the indicated concentrations and cleavage of I $\kappa$ B $\alpha$  was analyzed by the immunoblot assay using I $\kappa$ B $\alpha$  antibody. Contrary to previous reports, cleavage of I $\kappa$ B $\alpha$  was not decreased by calpain inhibitors (Fig. 8A, B). When another calpain inhibitor (1  $\mu$ M calpastatin) was treated, same result was shown (Fig. 8C). These results suggest that GA-induced I $\kappa$ B $\alpha$  cleavage is not mediated by calpain in H9c2 cells.

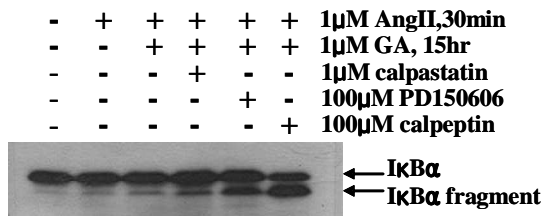
A.



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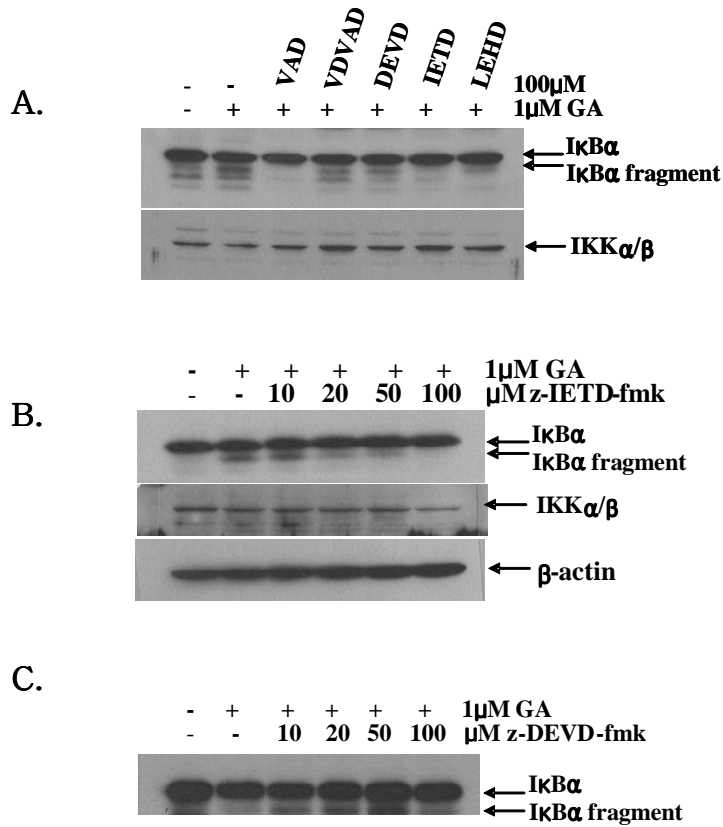
**Fig. 8. Effects of calpain inhibitors on the I $\kappa$ B $\alpha$  cleavage.** Cells were treated with calpeptin (A) or PD150606 (B) with various concentrations prior to the incubation of 1  $\mu$ M GA for 15 h. Cells were pretreated with 1  $\mu$ M calpastatin or 100  $\mu$ M PD150606 or 100  $\mu$ M calpeptin for 30 min and further incubation was carried with 1  $\mu$ M GA for 15 h prior to the addition of 1  $\mu$ M of AngII for 30 min (C). Samples were harvested and I $\kappa$ B $\alpha$  level was determined by immunoblot analysis.

### 3) Caspase-8 induces I $\kappa$ B $\alpha$ cleavage in GA-treated cardiac cells

Barkett *et al.* have already revealed that caspase-3 specifically cleaved I $\kappa$ B $\alpha$  at a conserved Asp-Ser sequence *in vitro*.<sup>23</sup> In order to determine whether caspases are responsible for the cleavage of I $\kappa$ B $\alpha$ , various membrane permeable caspase inhibitors were incubated with GA-treated H9c2 cells. z-VAD-fmk, z-VDVAD-fmk, z-DEVD-fmk, z-IETD-fmk and z-LEHD-fmk were used for inhibition of a broad spectrum of caspase, caspase-2, caspase-3, caspase-8 and caspase-9, respectively. GA-induced cleavage of I $\kappa$ B $\alpha$  was significantly inhibited by exposure of 100  $\mu$ M of a pan-caspase inhibitor (z-VAD-fmk) (Fig. 9A). Whereas pre-treatment of a caspase-8 inhibitor (z-IETD-fmk) reduced I $\kappa$ B $\alpha$  cleavage, cells treated with z-VDVAD-fmk, z-DEVD-fmk, and z-LEHD-fmk did not exhibit inhibition of I $\kappa$ B $\alpha$  cleavage (Fig. 9A). It was confirmed that I $\kappa$ B $\alpha$  cleavage was decreased by the addition of z-IETD-fmk, in a concentration-dependent fashion (Fig. 9B). And, on the contrary, caspase-3 inhibitor slightly increased GA-mediated cleavage of I $\kappa$ B $\alpha$  in H9c2 cells (Fig. 9C).

Caspases has been known to recognize and cleave specific substrates that contain a DXXD-G/S/A consensus peptide motif and cleave after the second aspartic acid of DXXD. Because a putative sequence for recognition by caspases is present in the amino-terminus as DRHD<sup>31</sup>-S<sup>32</sup>, the cleavage product of I $\kappa$ B $\alpha$  should be 3-5 kD less compared to intact I $\kappa$ B $\alpha$  in immunoblot analysis by using the antibody against the carboxyl-terminus of I $\kappa$ B $\alpha$ . To further investigate the effect of caspase inhibitors on the GA-induced decrease of IKK $\alpha/\beta$ , immunoblot analysis with anti-IKK $\alpha/\beta$  antibody was performed. A GA-mediated decrease of IKK $\alpha/\beta$  was not recovered by

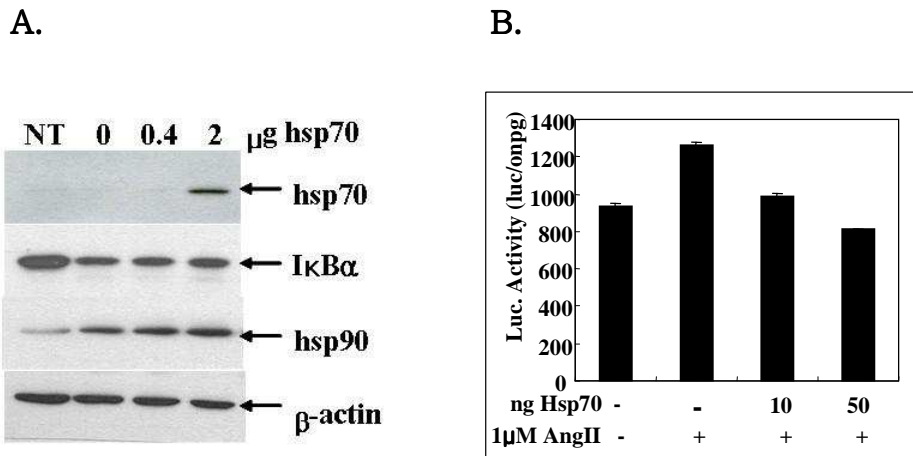
caspase inhibitors, suggesting that caspase activity is not required for a decrease of IKK $\alpha$ / $\beta$  levels (Fig. 9A, B). Taken together, I $\kappa$ B $\alpha$  was a target of caspase-8 during the GA-induced inhibition of hypertrophy.



**Fig. 9. IκBα cleavage by caspase-8 in GA-treated cardiac cells.** A. Cells were incubated with 100 μM z-VAD-fmk, z-VDVAD-fmk, z-DEVD-fmk, z-IETD-fmk and z-LEHD-fmk for 30 min prior to 1 μM GA for 15 h. Cells were pretreated with indicated doses of z-IETD-fmk (B) or z-DEVD-fmk (C) for 30 min, and then exposed with 1 μM GA for 15 h. Cells were harvested and immunoblot analysis was performed using anti-IκBα or IKKα/β antibodies.

## 5. Hsp70 is not involved in I $\kappa$ B $\alpha$ cleavage

Hsp90 inhibitors are well-known inducers of Hsp70. Hsp70 induction was appeared at approximately the same time as that of I $\kappa$ B $\alpha$  cleavage (data not shown). Because of this result, the following experiment was carried out to reveal whether Hsp70 causes I $\kappa$ B $\alpha$  cleavage or not. The pcDNA3.1/Hsp70 was delivered by lipofectamine plus into H9c2 cells, and the level of I $\kappa$ B $\alpha$  was detected by an immunoblot analysis (Fig. 10A). Hsp70 was over-expressed by 2  $\mu$ g of pcDNA3.1/Hsp70 successfully. Enhanced Hsp70 expression had an effect on increasing the level of I $\kappa$ B $\alpha$  and Hsp90. Similar to this result, the effect of Hsp70 on I $\kappa$ B $\alpha$  accumulation has been well illustrated. However, I $\kappa$ B $\alpha$  cleavage was not determined at the Hsp70-overexpressing cells. Thus, Hsp70 is not involved in the cleavage of I $\kappa$ B $\alpha$  during the GA-induced increase of Hsp70 in H9c2 cells. As expected, Hsp70 induction reduced NF- $\kappa$ B activity in accordance with I $\kappa$ B $\alpha$  accumulation (Fig. 10B).



**Fig. 10. Hsp70 is not involved in IκBα cleavage.** A. H9c2 cells were transiently transfected with indicated amount of pcDNA3.1/Hsp70. After 48 h, the cells were harvested and the protein levels of Hsp70, Hsp90 and IκBα were evaluated with immunoblot analysis. B. H9c2 cells were transfected with 100 ng of p-NF-κB/LUC luciferase vector and pcDNA3.1/lacZ, and simultaneously co-transfected with pcDNA3.1/Hsp70. After 24 h, cells were starved for 24 h and treated with 1 μM of AngII for 30 min. The NF-κB luciferase activity was performed with Luciferase Reporter Assay Kit.

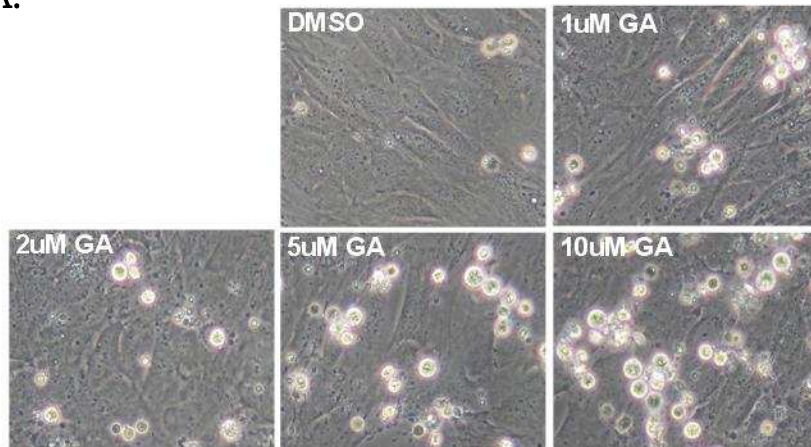
## 6. Hsp90 inhibitor induces apoptosis in cardiac cells

We previously showed that GA induced caspase-8-mediated I $\kappa$ B $\alpha$  cleavage. Caspases have been known to be activated during apoptosis, a form of regulated programmed cell death. Cell death was observed in rat cardiac myoblast cell line H9c2 cells with a phase contrast microscope (Fig. 11A). After exposure to GA, the cells developed a rounded shape and detached from the bottom of the culture dish. To investigate the effect of GA on cell death, the cells were treated with various doses of GA, and cell viability was determined using the MTT assay (Fig. 11B). The viability was decreased in a concentration-dependent manner, and about 73% of the cells were viable in cell treated with 1  $\mu$ M GA compared with the DMSO-treated cells. When the cells were treated with 1  $\mu$ M GA for 15 h and then stained using an ApopTag®Plus Peroxidase *In Situ* Apoptosis Detection Kit, the cells were stained with TdT enzyme bounded HRP-conjugated at the breaks of DNA (Fig. 12A). JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-teterathylbenzimidazole carbocyanine iodide) is up-taken into the mitochondria, and can be used as an indicator of mitochondrial potential, as a distinction between normal and apoptotic cells. Photographs demonstrated that treatment with GA results in green fluorescence shift from red as compared with the DMSO-treated cells (Fig. 12B). GA did not give the mitochondrial potential disruption under the treat of 100  $\mu$ M z-IETD-fmk in H9c2 cells. This showed that GA induces mitochondrial damage, a part of which was mediated by caspase-8, indicating that Hsp90 inhibition could activate mitochondrial

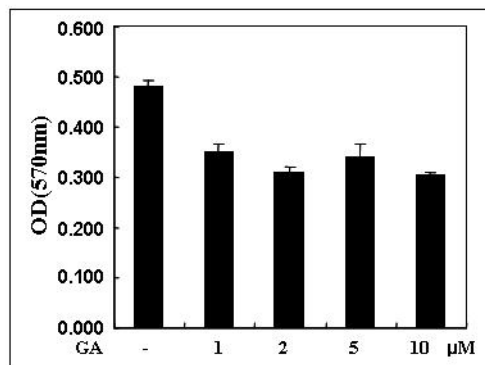


depolarization-dependent apoptosis through caspase-8.

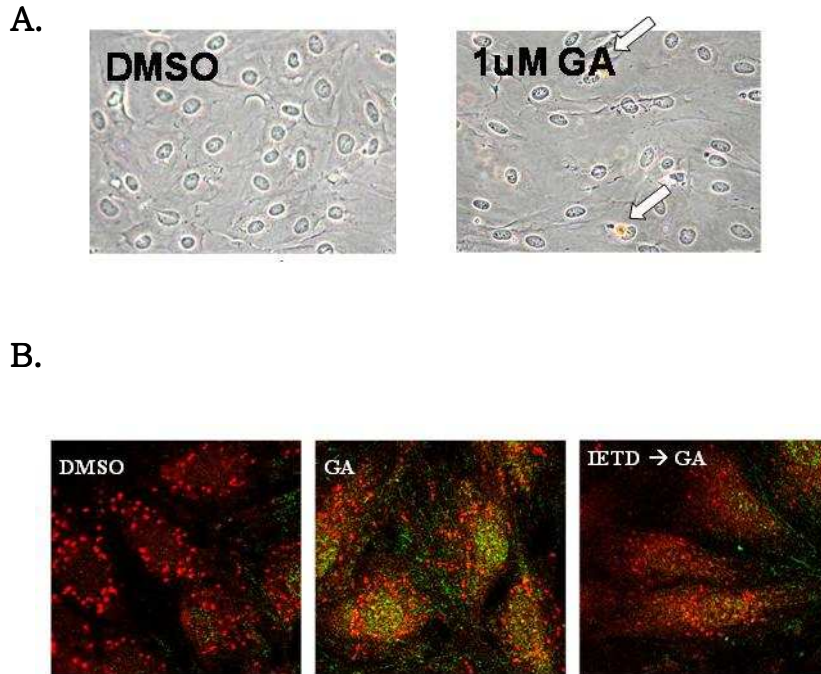
A.



B.



**Fig. 11. GA induces cell death in cardiac cells.** A. Morphological changes of cells were monitored with phase contrast microscope and photographed after treatment with GA. B. H9c2 cells were incubated with various concentrations of GA. After 24 h, viable cells were quantified by an MTT assay.



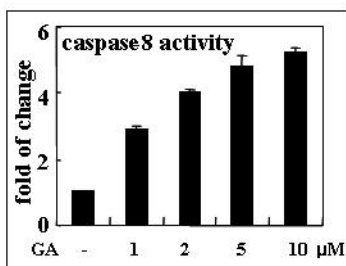
**Fig. 12. GA induces apoptosis in cardiac cells.** A. H9c2 cells were treated with 1  $\mu\text{M}$  GA for 15 h and incubated with TdT enzyme bounded HRP-conjugated antibody. B. H9c2 cells were incubated with 1  $\mu\text{M}$  GA in the presence or absence of 100  $\mu\text{M}$  z-IETD-fmk. The cells were treated with 5  $\mu\text{g}/\text{ml}$  of JC-1 for 10 min. The disruption of mitochondrial membrane potentials was measured using fluorescence microscopy on the excitation at 488 nm and the emission at 530 nm and 590 nm.

In order to know whether GA activated caspases, caspase activity assay and immunoblot analysis were performed. Caspase-8 activity increased in a dose-dependent manner and began to increase at 1  $\mu$ M GA (Fig. 13A). A fragment of active caspase-3 was detected after treatment with 5 and 10  $\mu$ M GA (Fig. 13B). The protein level of 55 kD pro-caspase-8 decreased at 10  $\mu$ M GA (Fig. 13B). These results show that GA induces caspase-3- and caspase-8-dependent apoptosis in H9c2 cells. Recent reports have suggested that caspase activation was not the only mechanism of Hsp90 inhibition-induced apoptosis in Hodgkin's lymphoma cell lines. Cytochrome C, which normally resides exclusively in the intermembrane space of mitochondria, is released into the cytosol during apoptosis. Release of cytochrome C from the mitochondria inactivates the electron transfer chain and triggers activation of caspase-3 through Apaf-1. GA induced the cytosolic release of cytochrome C in a concentration-dependent manner in H9c2 cells (Fig. 13B).

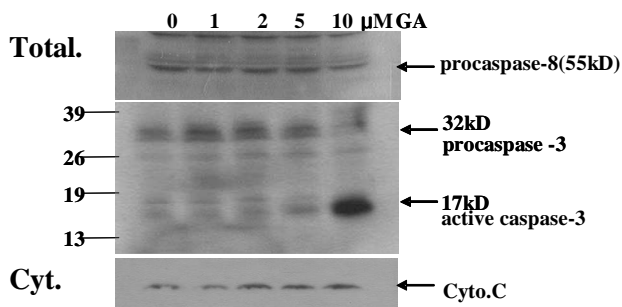
To verify whether caspase activation is necessary in GA-induced apoptosis, an MTT assay was carried out after pre-treating with caspase inhibitors in GA-exposed H9c2 cells. GA-inhibited cell viability was recovered by caspase inhibitors, z-VAD-fmk, z-VDVAD-fmk, z-DEVD-fmk, z-IETD-fmk and z-LEHD-fmk (Fig. 14A), and various doses of z-IETD-fmk, an inhibitor of caspase-8 (Fig. 14B). However, caspase is not a necessary mediator to induce apoptosis, because of its partial recovery. This implies that GA might mediate caspase-independent apoptosis in part. Mitochondrial proteins, EndoG and AIF were increased at the 1  $\mu$ M GA-treated

nuclear fraction (data not shown). EndoG and AIF are released from the mitochondria into the cytosol, and translocated to the nucleus to induce apoptosis unrelated to caspases. These results demonstrate that GA induced not only caspase-dependent apoptosis but also caspase-independent apoptosis in H9c2 cells.

A.

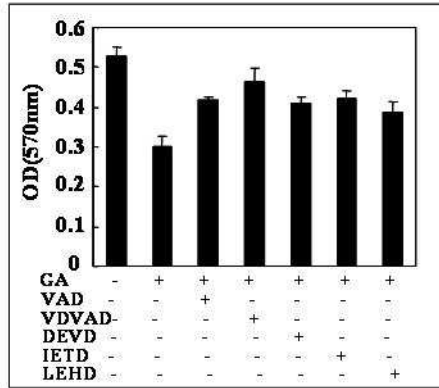


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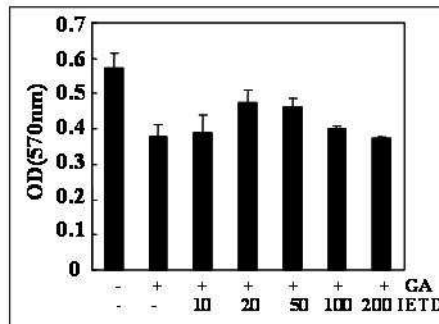


**Fig. 13. Caspase-8 activation by GA in cardiac cells.** A. H9c2 cells were treated with GA for 15 h. Total cell lysate was incubated with 5 μl of IETD-AFC (1 mM) for 1 h. Caspase-8 activity was quantified by fluorescence spectrometer at 400 nm excitation filter and 505 nm emission filter. B. H9c2 cells were treated with GA for 15 h. Total cell lysate was subjected to SDS-PAGE and analyzed using immunoblot assay. The level of cytosolic cytochrome C was also analyzed by immunoblot assay.

A.



B.



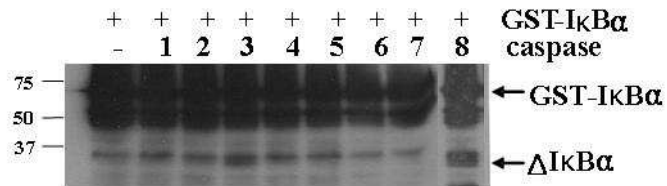
**Fig. 14. Caspase-independent cell death by GA in cardiac cells.** A. H9c2 cells were incubated with 100  $\mu$ M of z-VAD-fmk, z-VDVAD-fmk, z-DEVD-fmk, z-IETD-fmk, and z-LEHD-fmk for 1 h prior to treatment with 10  $\mu$ M GA. After 24 h, cell viability was determined by an MTT assay.

## 7. Caspase-8 directly cleaves I $\kappa$ B $\alpha$ protein

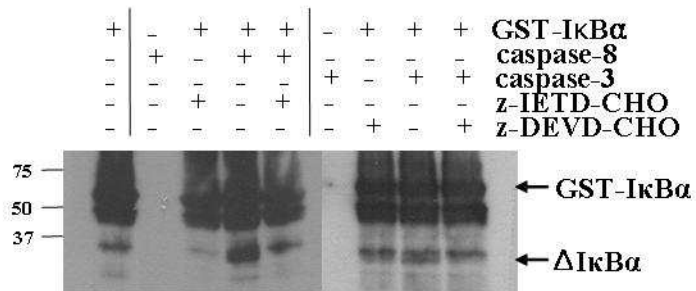
In order to confirm that caspase-8 is directly responsible for I $\kappa$ B $\alpha$  cleavage, purified GST-I $\kappa$ B $\alpha$  and active caspases were incubated in test tube, then visualized by an immunoblot assay using an I $\kappa$ B $\alpha$  antibody. Because GST is tagged at the NH<sub>2</sub>-terminus of I $\kappa$ B $\alpha$ , the cleavage product has a mass of the same thing compared to the cleavage fragment of I $\kappa$ B $\alpha$  without GST in an immunoblot assay using a carboxyl-terminus-specific I $\kappa$ B $\alpha$  antibody. I $\kappa$ B $\alpha$  was cleaved by caspase-3 and caspase-8 directly, and the cleavage was inhibited by z-DEVD-fmk and z-IETD-fmk, respectively (Fig. 15). The other caspases had no effect on the cleavage of I $\kappa$ B $\alpha$ . Because z-DEVD-fmk showed no change in the GA-induced I $\kappa$ B $\alpha$  cleavage in the cells (Fig. 9C) and GA activated caspase-3 and caspase-8 in H9c2 cells (Fig. 13), GA activated both caspase-3 and caspase-8, but GA-mediated caspase-3 activation could not cleave I $\kappa$ B $\alpha$ , which suggests that GA-mediated caspase-3 is not sufficient to cleave I $\kappa$ B $\alpha$  *in vivo*. These data show that the amino-terminus of I $\kappa$ B $\alpha$  was cleaved by caspase-3 and caspase-8 *in vitro*, but the GA-induced active caspase-8 only had the ability to cleave I $\kappa$ B $\alpha$ .



A.



B.



**Fig. 15. Caspase-8 directly cleaves IκBα protein.** A. Purified GST- IκBα (100 ng) was treated with purified active caspases for 1 h. Samples were subjected to SDS-PAGE and cleaved IκBα was analyzed by immunoblot assay. B. Purified GST- IκBα (100 ng) was incubated in the absence or presence of 20 μM z-IETD-CHO or z-DEVD-CHO for 30 min. After the mixture was treated with purified active caspase-8 or caspase-3 for 1 h, cleaved IκBα was analyzed by immunoblot analysis.

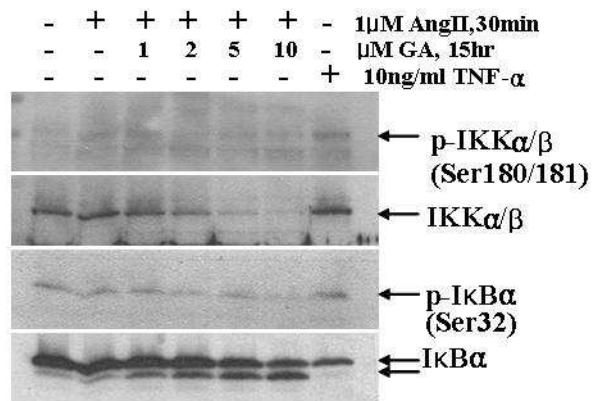
## 8. Hsp90 inhibitor inhibits I $\kappa$ B $\alpha$ phosphorylation and degradation in cardiac cells

### 1) Hsp90 inhibitor inhibits AngII-induced IKK $\alpha/\beta$ activation

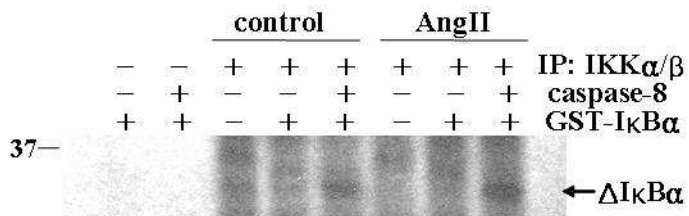
The expression level of IKK $\alpha/\beta$  was decreased and I $\kappa$ B $\alpha$  cleavage was increased after GA exposure (Fig. 7). To investigate whether AngII-induced IKK activity changes along with IKK $\alpha/\beta$ , an immunoblot assay was examined using specific IKK $\alpha/\beta$  and phospho-IKK $\alpha/\beta$  (Ser181/182) antibodies. Compared to phosphorylated IKK $\alpha/\beta$ , the protein level of IKK $\alpha/\beta$  was dramatically decreased (Fig. 16A upper two panels). To elucidate whether the GA-induced IKK dephosphorylation has an effect on I $\kappa$ B $\alpha$  phosphorylation, a phospho-I $\kappa$ B $\alpha$  antibody (Ser32) was used for an immunoblot assay. I $\kappa$ B $\alpha$  was phosphorylated by treatment of 10 ng/ml of TNF- $\alpha$ , and the expression level was reduced without cleavage, which indicated that I $\kappa$ B $\alpha$  might be reduced by proteosomal degradation through ubiquitination. However, GA cleaved I $\kappa$ B $\alpha$  and reduced its phosphorylation, suggesting that inactive IKK $\alpha/\beta$  could not phosphorylate I $\kappa$ B $\alpha$  (Fig. 16 lower two panels). The fact that the phospho-I $\kappa$ B $\alpha$  level did not completely decrease although most of the IKK $\alpha/\beta$  level was decreased indicates that other kinases are present to phosphorylate I $\kappa$ B $\alpha$ .

Interestingly, AngII failed to phosphorylate cleaved I $\kappa$ B $\alpha$ , indicating that the N-terminal deleted fragment of I $\kappa$ B $\alpha$  could not be phosphorylated, at least on Ser32. To confirm whether the I $\kappa$ B $\alpha$  fragment has a possibility of being phosphorylated by AngII, an IKK activity assay was performed (Fig. 17). First, an *in vitro* cleavage

assay was performed with 1  $\mu\text{g}$  of purified GST-I $\kappa$ B $\alpha$  and 1  $\mu\text{g}$  of purified active caspase-8. The cells treated with 1  $\mu\text{M}$  AngII for 30 min were immunoprecipitated with a specific antibody for IKK $\alpha/\beta$ , and the precipitates were incubated with a mixture of the *in vitro* cleavage reaction. Immunoprecipitates with the anti-IKK $\alpha/\beta$  antibody phosphorylated not only GST-I $\kappa$ B $\alpha$  but also cleaved I $\kappa$ B $\alpha$ , and its phosphorylation was enhanced by the addition of AngII (Fig. 17), which demonstrated that the caspase-8-mediated I $\kappa$ B $\alpha$  fragment can be phosphorylated by active IKK $\alpha/\beta$ . Although the fragment of I $\kappa$ B $\alpha$  possessed the phosphorylation residues, ser32 and ser36, because caspase-8 cleaved at the front of ser32 of I $\kappa$ B $\alpha$ , only ser36 was assumed to be a target of phosphorylation. Taken together, these results suggest that inhibition of IKK activity could be responsible for the blocking of I $\kappa$ B $\alpha$  phosphorylation by AngII, and I $\kappa$ B $\alpha$  proteolysis by caspase-8 repressed phosphorylation of cleaved I $\kappa$ B $\alpha$  on ser32.



**Fig. 16. GA inhibits AngII-induced IKK $\alpha$ / $\beta$  activation.** H9c2 cells were treated with GA and AngII. The cells were harvested and IKK $\alpha$ / $\beta$  was determined by immunoblot analysis.



**Fig. 17. Phosphorylation of I $\kappa$ B $\alpha$  fragment by IKK $\alpha$ / $\beta$ .** H9c2 cells were treated with 1  $\mu$ M AngII. The cells were harvested and immunoprecipitated with against IKK $\alpha$ / $\beta$  antibody. Mixture of GST-I $\kappa$ B $\alpha$  (1 $\mu$ g) incubated with active caspase-8 (1 $\mu$ g) was incubated with the immunoprecipitates and kinase assay was performed as described in materials and methods.

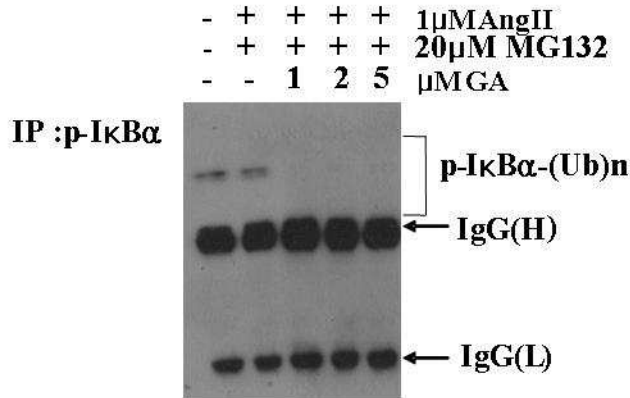
## **2) Hsp90 inhibitor inhibits AngII-induced ubiquitination of I $\kappa$ B $\alpha$ and maintains its interaction with p65 NF- $\kappa$ B**

Since I $\kappa$ B $\alpha$  has lysine residues in the amino-terminus modulated by ubiquitin ligase at Lys21 and Lys22, it might be deleted when the proteolytic cleavage of I $\kappa$ B $\alpha$  is processed by caspase-8. To determine whether ubiquitination of I $\kappa$ B $\alpha$  is disrupted by treatment with GA, an immunoblot assay with ubiquitin-specific antibody was performed, and followed by immunoprecipitation with a phospho-I $\kappa$ B $\alpha$  antibody. AngII induced poly-ubiquitination of phosphorylated I $\kappa$ B $\alpha$ . Even though I $\kappa$ B $\alpha$  was phosphorylated and ubiquitinated by AngII, it was not degraded in proteosome because of the addition of 20  $\mu$ M MG-132 prior to treatment with AngII. The ubiquitination of I $\kappa$ B $\alpha$  was disrupted by treatment with GA (Fig. 18A). This result indicates that cleavage of N-terminus of I $\kappa$ B $\alpha$  by caspase-8 in GA-treated cells, allowing the release of ubiquitination residue from intact I $\kappa$ B $\alpha$ , decreased ubiquitination chance of phospho-I $\kappa$ B $\alpha$ .

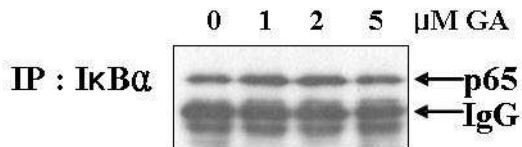
The 26S proteosomal degradation of I $\kappa$ B $\alpha$  is followed by ubiquitination of I $\kappa$ B $\alpha$ . As a result of these processes, I $\kappa$ B $\alpha$  can not interact with NF- $\kappa$ B. Interaction of the I $\kappa$ B $\alpha$  protein with the p65 NF- $\kappa$ B subunit usually results in retention of the NF- $\kappa$ B in the cytoplasm and inhibition of the transcriptional activity of NF- $\kappa$ B.<sup>9</sup> In order to confirm that GA enhanced the interaction between I $\kappa$ B $\alpha$  and NF- $\kappa$ B, an immunoprecipitation assay was examined. The interaction between I $\kappa$ B $\alpha$  and the p65 NF- $\kappa$ B subunit was slightly increased in the presence of 1  $\mu$ M and 2  $\mu$ M GA

(Fig. 18B). Therefore, GA-mediated diminishment of poly-ubiquitination of phospho-I $\kappa$ B $\alpha$  resulted in increased interaction between I $\kappa$ B $\alpha$  and NF- $\kappa$ B. These results show that GA has an effect on the inactivation of NF- $\kappa$ B through the reduction of ubiquitination and maintenance of interaction of I $\kappa$ B $\alpha$  with the p65 NF- $\kappa$ B subunit.

A.



B.



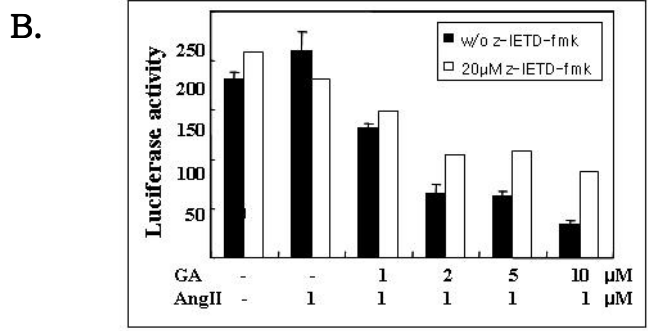
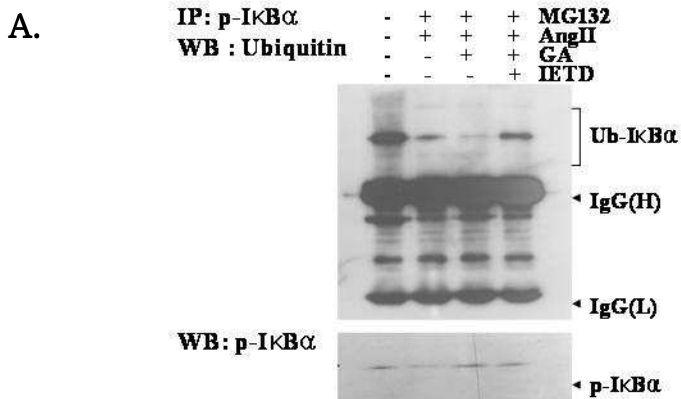
**Fig. 18. GA inhibits AngII-induced ubiquitination of I $\kappa$ B $\alpha$  and maintains its interaction with p65 NF- $\kappa$ B.** A. After treatment with GA for 15 h, H9c2 cells were exposed to 20  $\mu$ M MG132 for 30 min prior to incubation of 1  $\mu$ M AngII for 30 min. Cells were lysed and immunoprecipitated with antibody against phospho-I $\kappa$ B $\alpha$ , and followed by immunoblot analysis with anti-ubiquitin antibody. B. Cell lysate was immunoprecipitated with I $\kappa$ B $\alpha$  antibody and immunoblot analysis was performed using anti-p65 antibody.



## **9. Recovery of NF- $\kappa$ B activation by inhibition of caspase-8 activity**

As shown in Fig. 18, GA-induced caspase-8 activation exerts cleavage of I $\kappa$ B $\alpha$  and subsequent inhibition of ubiquitination. To confirm that these effects were mediated by caspase-8, a caspase-8 inhibitor, z-IETD-fmk was pretreated for 30 min, and the level of ubiquitination of phospho-I $\kappa$ B $\alpha$  was checked (Fig. 19A, upper panel). When caspase-8 was inactivated by z-IETD-fmk, ubiquitinated phospho-I $\kappa$ B $\alpha$  was increased compared with the GA treatment group in the absence of z-IETD-fmk. To confirm that this effect was not a result of an increase of I $\kappa$ B $\alpha$  phosphorylation, the same lysate was visualized by immunoblotting using a phospho-I $\kappa$ B $\alpha$  antibody (Fig. 19A, lower panel). As expected, pretreatment of z-IETD-fmk did not induce phosphorylation of I $\kappa$ B $\alpha$ , indicating that ubiquitination of I $\kappa$ B $\alpha$  was increased by inhibition of I $\kappa$ B $\alpha$  cleavage without phosphorylation of I $\kappa$ B $\alpha$ .

To determine whether recovery of I $\kappa$ B $\alpha$  ubiquitination has an effect on the NF- $\kappa$ B activity, NF- $\kappa$ B luciferase activity was measured (Fig. 19B). GA-mediated NF- $\kappa$ B inactivation was partially recovered by the addition of a caspase-8 inhibitor. Because recovered ubiquitination of phospho-I $\kappa$ B $\alpha$  occurred in z-IETD-fmk-pretreated cells compared with GA-treated H9c2 cells, and correlated with increased NF- $\kappa$ B transcriptional activity (Fig. 19), GA-mediated NF- $\kappa$ B inactivation was due to the blocking of ubiquitination through caspase-8 mediated I $\kappa$ B $\alpha$  cleavage.



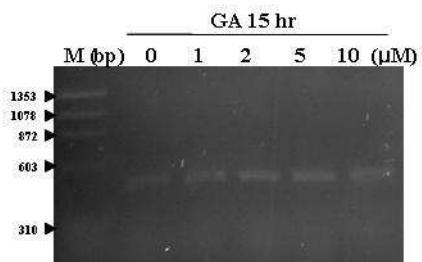
**Fig. 19. Recovery of NF-κB activation by inhibition of caspase-8 activity.** A. H9c2 cells were pre-incubated with 20 μM z-IETD-fmk for 30 min prior to addition of 1 μM GA for 15 h and cells were exposed to MG132. After 30 min treatment with 1 μM AngII, cells were collected and immunoprecipitation analysis was carried out using phospho-IκBα antibody, and followed by immunoblot assay with anti-ubiquitin antibody. B. H9c2 cells were transfected with p-NF-κB/LUC luciferase vector. After 24 h, H9c2 cells were treated with z-IETD-fmk and GA and incubated with 1 μM of AngII for 30 min. NF-κB activity was determined by Luciferase Reporter Assay Kit.

## **10. Hsp90 inhibitor increases FasL expression and decreases c-FLIP<sub>S</sub> expression**

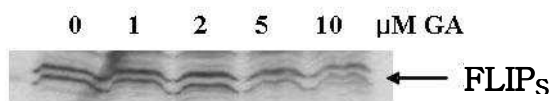
Caspase-8 activation has been well-known to be activated by death receptors such as CD95 (Fas/Apo1). Engagement of the Fas ligand (FasL) results in the recruitment of the Fas-associated death domain and the initiator caspase-8 via a homophilic DED interaction. To determine whether GA-mediated activation of caspase-8 is led by Fas receptor ligation, the FasL transcription level was examined by RT-PCR analysis (Fig. 20A). H9c2 cells were incubated with GA in the indicated doses, and the cells were harvested after 15 h. The synthesized first strand cDNA using total RNA (1 ug) was used for the template for PCR analysis. Thirty cycles of PCR were performed with a primer for FasL. FasL was expressed in only the DMSO- treated group, caused by the starvation with serum-free DMEM for 24 h before exposure to GA. Serum deprivation has been reported to be a cause of Fas-mediated caspase activation. GA enhanced the expression of FasL, in a concentration-dependent manner.

Since it has been observed that c-FLIP<sub>S</sub> is a dominant-negative inhibitor of caspase-8, an immunoblot with c-FLIP<sub>S</sub>, a regulator of Fas-mediated caspase activation, was examined. Otherwise the c-FLIP<sub>L</sub> level did not decrease. GA reduced the expression level of c-FLIP<sub>S</sub> in a concentration-dependent manner (Fig. 20B). These results show that that GA-induced Fas activation through FasL induction and reduction of c-FLIP<sub>S</sub> activates caspases-8 in cardiac cells.

A.



B.



**Fig. 20. Effect of GA on the expression of FasL and c-FLIP<sub>s</sub>.** H9c2 cells were treated with GA for 15 h. A. Transcriptional level of FasL was analyzed by RT-PCR. PCR product was electrophoresed in 1.5% agarose gel electrophoresis. B. The level of FLIPs was determined by immunoblot assay using anti-c-FLIP<sub>s</sub> antibody.

## IV. DISCUSSION

Angiotensin II (Ang II) induces cardiac myocyte apoptosis and hypertrophy, which contribute to heart failure, possibly through enhanced oxidative stress. GPCR stimuli, such as  $G\alpha_q$  overexpression, induce hypertrophy with normal cardiac function in cardiac myocyte at a moderate level in mice, while strong expression of  $G\alpha_q$  leads to apoptosis and cardiac failure.<sup>24</sup> AngII also activates  $G\alpha_q$  signaling which induces peroxynitrite synthesis by NADPH oxidase activity is responsible for DNA damage, which causes an apoptotic response in cardiac cells.<sup>25-27</sup> However, the mechanisms underlying the activation of both pathways and their interactions remain unclear. Recently, several investigations have implicated NF- $\kappa$ B activation as a casual event in the cardiac hypertrophic response, and NF- $\kappa$ B signaling is also critically involved in the apoptotic response of a cell to stress or inflammation, regulating a number of pro- and anti-apoptotic gene products.

NF- $\kappa$ B activation is induced by various kinases, including IKK $\alpha/\beta$ , which phosphorylates I $\kappa$ B $\alpha$  at ser 32 and 36. It has been reported that Hsp90 stabilizes NF- $\kappa$ B activity. Geldanamycin (GA), an inhibitor by binding within the ATP-associated pocket of Hsp90, lowers the expression of several kinases, such as RIP, IKK $\alpha$ , IKK $\beta$  and RAF-1.<sup>28</sup> Interactions between these client proteins with Hsp90 are abolished by GA, which leads to their instabilization and degradation. Yeo *et al* demonstrated that inhibition of Hsp90 inactivated NF- $\kappa$ B in *Helicobacter pylori*-induced activation.<sup>29</sup>

IKK biosynthesis and subsequent NF- $\kappa$ B activation required Hsp90 activity was also examined.<sup>30</sup> These results imply that Hsp90 has a role in sustaining the activity of NF- $\kappa$ B. In this study, GA reduced the expression of IKK $\alpha/\beta$ , which was phosphorylated and activated by AngII. AngII-induced IKK $\alpha/\beta$  phosphorylation and activation was inhibited by GA-induced IKK $\alpha/\beta$  destabilization mediating, NF- $\kappa$ B inactivation. Interestingly, GA also induced cleavage of I $\kappa$ B $\alpha$  at the N-terminal regulatory domain. The I $\kappa$ B $\alpha$  protein is the substrate of calpain and caspases, as cysteine proteases, which play important roles in the regulation of pathological cell death.

It was reported that calpain is an important Ca<sup>2+</sup>-dependent cysteine protease, and has an active role in apoptosis. During hypertrophy, ischemic reperfusion, or heart failure, an increase of intracellular calcium concentration activates calpain, which exerts myocardial damage such as apoptosis or necrosis.<sup>31</sup> Another report suggested that m-calpain was responsible for cleaving caspase-12 and Bcl-xL, therefore inducing apoptosis in an ischemic mimic condition.<sup>32</sup> Moreover, Schaecher *et al.* demonstrated that calpain exhibited cleavage of I $\kappa$ B $\alpha$  in human peripheral blood mononuclear cells (PBMC) under activation with anti-CD3/CD28 (HIT3A/CD28.2), and 150  $\mu$ M calpeptin, a calpain inhibitor, blocked cleavage of I $\kappa$ B $\alpha$ .<sup>22</sup> However, pretreatment of calpain inhibitors increased cell death along with cleavage of I $\kappa$ B $\alpha$  in this study. Calpain inhibitors have been reported to be inducers in caspase-dependent apoptosis, and calpain inhibitor I activates p53-dependent apoptosis,<sup>33, 34</sup> induced by

p53 accumulation. Because a high dose of calpain inhibitor has been reported to inhibit proteosomal degradation, enhanced GA-mediated cleavage of I $\kappa$ B $\alpha$  by calpain inhibitors was considered by blocking the ubiquitin-proteosomal pathway. It is supposed that accumulation of I $\kappa$ B $\alpha$  might accelerate the cleavage, but the exact mechanism remains to be elucidated.

Our results indicated that cleavage of I $\kappa$ B $\alpha$  was mediated by GA-induced activation of caspase-8. Because I $\kappa$ B $\alpha$  has a sequence for the target of caspase at D<sup>28</sup>RHD<sup>31</sup>S, caspase-8 has a chance to cleave the substrate releasing N-terminal 31 amino acids, which include ubiquitin recognizing two lysine residues. Since the C-terminal fragment of I $\kappa$ B $\alpha$  still possesses the ability to interact with the p65 NF- $\kappa$ B subunit, cleaved-I $\kappa$ B $\alpha$  can inactivate NF- $\kappa$ B. However, the I $\kappa$ B $\alpha$  fragment has no chance to recycle as proteosomal degradation and resynthesis; the I $\kappa$ B $\alpha$  fragment remains associated with NF- $\kappa$ B as a constitutive inhibitor of NF- $\kappa$ B. Thus, NF- $\kappa$ B suffers from irreversible inactivation by GA-induced caspase-8 activation.

While caspase-8 has been implicated as a regulator of death receptor-mediated apoptosis, it was reported that caspase-8 was activated by treatment with mistletoe lectin I, an active component in plant extracts used in adjuvant cancer therapy, in CD95 (Apo-1,Fas)-resistant Jurkat cells.<sup>35</sup> This means that caspase-8 is involved in death receptor-independent apoptosis. Contrary to the above suggest, down regulation of Akt signaling stimulated Fas ligand (Apo1/CD95) transcription in vascular smooth muscle cells (VSMCs).<sup>36</sup> In VSMCs, FasL is not present in normal

state, but Akt signaling blockage by wortmannin or adeno viral delivered as a dominant negative mutant of Akt induces FasL expression and apoptosis. Akt phosphorylates and inactivates not only forkhead transcriptional factor (FKHRL1), which transactivates FasL, but also caspase-9, which activates JNK, an important regulator of Fas-mediated apoptosis. Uriarte *et al* also reported that inhibition of Akt induced FasL and caspase-8 activation and reduced c-FLIP<sub>S</sub> in Jurkat T cells. Reduced c-FLIP<sub>S</sub> allowed the recruitment of caspase-8 to death-inducing signaling complex (DISC) and activated caspase-8.<sup>37</sup> Georgakis *et al* showed that 17AAG, a derivative of GA, exerted Akt downregulation in Hodgkin's lymphoma cells.<sup>38</sup> 17AAG also induces apoptosis and cell cycle arrest in Hodgkin's lymphoma cell lines. Akt, an important serine/threonine protein kinase in anti-apoptosis, has an active complex with Hsp90 preventing dephosphorylation of Akt.<sup>39</sup> Disruption of Hsp90 by 17AAG led to reduce c-FLIP<sub>S</sub> rather than c-FLIP<sub>L</sub> but had no effect on c-IAP, XIAP and Bcl-2 expression.<sup>38</sup> c-FLIP<sub>S</sub> is well characterized as having an inhibitory function on FADD-bound p43/41 caspase-8 intermediated, mature active caspase-8 (heterodimer of p21/p10) inhibited by c-FLIP<sub>L</sub>.<sup>40</sup> It was also examined whether a decrease of FLIP sensitized Fas receptor-mediated apoptosis in Jurkat cells.<sup>41</sup> In these results, FasL expression was increased in GA-treated H9c2 cells, and the decrease of Akt was also induced by GA. When Akt was repressed by GA or Akt inhibitor III, phosphorylated JNK decreased in H9c2 cells (Data not shown). However, whether Akt inhibition and subsequent phosphorylation of JNK were



sufficient to activate caspase-8 and cleave I $\kappa$ B $\alpha$  remain to be elucidated. In our additional results, GA inactivated NF- $\kappa$ B and down-regulated c-FLIP $_S$  in H9c2 cells, indicating that GA-mediated inactive NF- $\kappa$ B suppressed c-FLIP expression. Consequently, GA-mediated increased FasL and reduced c-FLIP $_S$  led Fas-mediated caspase-8 activation, which was responsible for cleaving I $\kappa$ B $\alpha$ .

Otherwise, Hsp90 suppressed TNF- $\alpha$ -mediated Bid cleavage, which is well described as being responsible for active caspase-8 in many cell types. By direct interaction with Hsp90, the complex of Hsp90 and caspase-8 had no effect on Bid cleavage,<sup>42</sup> meaning that Hsp90 might have effects on inhibition of caspase-8 activation in addition to blocking cleavage of the substrate by activated caspase-8. Other reports emphasize that I $\kappa$ B $\alpha$  cleavage is important in apoptosis.<sup>43</sup> Thus, the protective effect of Hsp90 on I $\kappa$ B $\alpha$  cleavage through blocking activation of caspase-8 is important to inhibit cardiac cell death.

Heat shock response transactivates HSF-1, which induces activation of heat shock proteins. A heat shock protein such as Hsp90 is constitutively highly expressed, but Hsp70 or Hsp27 levels are induced rapidly. The heat shock response inhibits NF- $\kappa$ B activation through reducing phospho-IKK and enhancing phosphatase activity. It is assumed that the dual action is responsible for the decrease of I $\kappa$ B $\alpha$  phosphorylation.<sup>44</sup> Ran *et al* observed that interaction between Hsp70 and IKK $\gamma$  occurred, and inhibited NF- $\kappa$ B activation.<sup>45</sup> Overexpressed Hsp72 blocks NF- $\kappa$ B activation and the subsequent expression of TNF- $\alpha$ .<sup>46</sup> Therefore, contrary to

Hsp90's role on NF- $\kappa$ B, Hsp70 has a negative effect on NF- $\kappa$ B.

To know whether cleavage of I $\kappa$ B $\alpha$  is responsible for Hsp70 induction, Hsp70 overexpression was conducted transiently (Fig. 10A). Hsp70 overexpression had an effect on the accumulation of I $\kappa$ B $\alpha$  leading to inactivation of NF- $\kappa$ B but not by cleavage. HIII (heat shock protein inhibitor I; 3,4-methylenedioxy-benzylidene- $\gamma$ -butyrolactam), is a benzylidene lactam compound that inhibits synthesis of heat shock proteins, including Hsc70, Hsp70 and Hsp105.<sup>47,48</sup> As HIII was administered before MG132 or GA was treated, contradictory results were produced. When Hsp90 and Hsp70 were inhibited at the same time, I $\kappa$ B $\alpha$  cleavage was enhanced by inhibition of Hsp70. Conversely, HspII had an inhibitory effect on MG-132-induced I $\kappa$ B $\alpha$  cleavage, in addition to MG-132-mediated-cell death. It is supposed that Hsp70 is not responsible for I $\kappa$ B $\alpha$  cleavage although Hsp70 induction coincides with the cleavage. In this research, we described a protective role of Hsp90 on the NF- $\kappa$ B signal transduction pathway and NF- $\kappa$ B-mediated cardiac hypertrophy. Specifically, we have shown that Hsp90 affected the inhibition of caspase-8, and the I $\kappa$ B $\alpha$  protein can serve as a direct substrate for GA-induced active caspase-8. In this study, a new mechanism was proposed that Hsp90 modulates the hypertrophic signal, protecting the NF- $\kappa$ B signaling from I $\kappa$ B $\alpha$  cleavage by caspase-8.

## V. CONCLUSION

This study demonstrated that Hsp90 exert modulatory effects at distinct points along the hypertrophy by angiotensin II (AngII) in cardiac myocytes. Blockage of Hsp90 function inhibited AngII-induced cardiac hypertrophy and NF- $\kappa$ B activation. Hsp90 inhibitor also induced cleavage of I $\kappa$ B $\alpha$  at the N-terminal regulatory domain through caspase-8 activation in cardiac cells. Caspase-8 activation was carried out through FasL induction and reduction of c-FLIP<sub>s</sub>. Caspase-8 directly cleaved the I $\kappa$ B $\alpha$  protein which has a sequence (-DRHDS-) for the target of caspase. Since N-terminal small fragment of I $\kappa$ B $\alpha$  cleaved by caspase-8 includes ubiquitin-recognizing two lysine residues, C-terminal fragment of I $\kappa$ B $\alpha$  has no chance to recycle as ubiquitination and proteosomal degradation. C-terminal fragment of I $\kappa$ B $\alpha$  also remains associated with NF- $\kappa$ B as a constitutive inhibitor of NF- $\kappa$ B. In summary, this study shows that caspase-8 is involved in I $\kappa$ B $\alpha$  degradation and NF- $\kappa$ B inhibition, and Hsp90 regulates the hypertrophic signal through modulating caspase-8 activation and I $\kappa$ B $\alpha$  cleavage.

## REFERENCES

1. Marian AJ, Roberts R. Recent advances in the molecular genetics of hypertrophic cardiomyopathy. *Circulation* 1995;92:1336-1347.
2. Schaub MC, Hefti M., Harder B.A., Eppenberger H.M. Various hypertrophic stimuli induce distinct phenotypes in cardiomyocytes. *J Mol Med* 1997;75:901-920.
3. Zaman MA, Oparil S, Calhoun DA. Drug targeting the rennin-angiotensin-aldosterone system. *Nat Rev Drug Discov* 2002;1:621-636.
4. Abhiram P, Arshed AF. Renin-Angiotensin System and Angiotensin Receptor Blockers in the Metabolic Syndrome. *Circulation* 2004;110: 1507-1512.
5. Brasier AR, Jamaluddin M, Han Y, Patterson C, Runge MS. AngiotensinII induces gene transcription through cell-type-dependent effects on the nuclear factor- $\kappa$ B (NF- $\kappa$ B) transcription factor. *Mol Cell Biochem* 2000;212:155-169.
6. Rouet-Benzineb P, Gontero B, Dreyfus P, Lafuma C. AngiotensinII induces nuclear factor- $\kappa$ B activation in cultured neonatal rat cardiomyocytes through protein kinase C pathway. *J Mol Cell Cardiol* 2000;32:1767-1778.
7. Li Y, Ha T, Gao X, Kelly J, Williams DL, Browder IW, et al. NF- $\kappa$ B activation is required for the development of cardiac hypertrophy in vivo. *Am J Physiol Heart Circ Physiol* 2004;287:H1712-H1720.
8. Purcell NH, Tang G, Yu C, Mercurio F, Didonato JA, Lin A. Activation of NF-

- $\kappa$ B is required for hypertrophic growth of primary rat neonatal ventricular cardiomyocytes. *Proc Natl Acad Sci USA* 2001;98:6668-6673.
9. Gosh S, Karin M. Missing pieces in the NF- $\kappa$ B puzzle. *Cell* 2002;109:S81-S96.
  10. Kataoka T. The caspase-8 modulator c-FLIP. *Crit Rev Immunol* 2005;25:31-58.
  11. Mora AL, Corn RA, Stanic AK, Goenka S, Aronica M, Stanley S, et al. Antiapoptotic function of NF- $\kappa$ B in T lymphocytes is influenced by their differentiation status : role of Fas, c-FLIP, and Bcl-xL. *Cell Death Differ* 2004;10: 1032-1044.
  12. Tschopp J, Irmeler M, Thome M. Inhibition of Fas death signals by FLIPs. *Curr Opin Immunol* 1998;10:552-558.
  13. Nishizawa J, Nakai A, Komeda M, Ban T, Nagata K. Increased preload directly induces the activation of heat shock transcription factor1 in the left ventricular overloaded heart. *Cardiovasc Res* 2002;55:341-348.
  14. Latchman DS. Cardiotriphon-1 induces heat shock protein accumulation in cultured cardiac cells and protects them from stressful stimuli. *J Mol Cell Cardiol* 1998;30: 849-855.
  15. Xu Q, Li DG, Holbrook NJ, Udelsman R. Acute hypertension induced heat-shock protein70 gene expression in rat aorta. *Circulation* 1995;92:1223-1229.
  16. Patton WF, Erdjument-Bromage H, Marks AR, Tempst P, Tauman MB. Components of the protein synthesis and folding machinery are induced in

- vascular smooth muscle cells by hypertrophic and hyperplastic agents: identification by comparative protein phenotyping and microsequencing. *J Biol Chem* 1995;270:21404-21410.
17. Chen Y, Arrigo AP, Currie RW. Heat shock treatment suppresses angiotensinII-induced activation of NF- $\kappa$ B pathway and heart inflammation: a role for IKK depletion by heat shock. *Am J Physiol Heart Circ Physiol* 2004;287:H1104-1114.
  18. Meldrum KK, Burnett AL, Meng X, Misseri R, Shaw M, Gearhart JP, et al. Liposomal delivery of heat shock protein 72 into renal tubular cells blocks nuclear factor- $\kappa$ B activation, tumor necrosis factor- $\alpha$  production, and subsequent ischemia-induced apoptosis. *Circ Res* 2003;92:293-299.
  19. Lewis J, Devin A, Miller A, Lin Y, Rodriguez Y, Neckers L, et al. Disruption of Hsp90 function results in degradation of the death domain kinase, receptor-interacting protein(RIP), and blockage of tumor necrosis factor-induced nuclear factor- $\kappa$ B activation. *J Biol Chem* 2000;275:10519-10526.
  20. Zheng Y, Song HJ, Kim CH, Kim HS, Kim EG, Sachinidis A, Ahn HY. Inhibitory effect of epigallocatechin 3-O-gallate on vascular smooth muscle cell hypertrophy induced by angiotensin II. *J Cardiovasc Pharmacol* 2004;43:200-208.
  21. Kang KH, Lee KH, Kim MY, Choi KH. Caspase-3-mediated cleavage of the NF-kappa B subunit p65 at the NH2 terminus potentiates naphthoquinone analog-induced apoptosis. *J Biol Chem*. 2001;276:24638-44.

22. Schaecher K, Goust JM, Banik LN. The effect of calpain inhibition on I $\kappa$ B $\alpha$  degradation after activation of PBMCs: Identification of the calpain cleavage sites. *Neurochemical Research* 2004;20:1443-1451.
23. Barkett M, Xue D, Horvitz HR, Gilmore TD. Phosphorylation of I $\kappa$ B- $\alpha$  inhibits its cleavage by caspase CPP32 *in vitro*. *J Biol Chem* 1997;272:29419-29422
24. Berghe TV, Kalai M, Loo GV, Declercq W, Vandenabeele P. Disruption of HSP90 function reverts tumor necrosis factor-induced necrosis to apoptosis. *J Biol Chem* 2003;278:5622-5629.
25. Yeo M, Park HK, Lee KM, Lee KJ, Kim JH, Cho SW, et al. Blockage of HSP90 modulates Helicobacter pylori-induced IL-8 production through the inactivation of transcriptional factors of AP-1 and NF- $\kappa$ B. *Biochem Biophys Res Commun* 2004;320:816-824.
26. Broemer M, Krappmann D, Scheidereit C. Requirement of Hsp90 activity for I $\kappa$ B kinase (IKK) biosynthesis and for constitutive and inducible IKK and NF- $\kappa$ B activation. *Oncogene* 2003;23:5378-5386.
27. Perrin C, Vergely C, Rochette L. Calpains and cardiac diseases. *Ann Cardiol Angeiol* 2004;653:259-266.
28. Nakagawa T, Yuan J. Cross-talk between two cysteine protease families: activation of caspase-12 by calpain in apoptosis. *J Cell Biol* 2006;150:887-894.
29. Zhu DM, Uckun FM. Calpain inhibitor II induces caspase-dependent apoptosis in human acute lymphoblastic leukemia and non-Hodgkin's lymphoma cells as

- well as some solid tumor cells. *Clin Cancer Res* 2000;6:2456-2463.
30. Atencio IA, Ramachandra M, Shabram P, Demer GW. Calpain inhibitor 1 activates p53-dependent apoptosis in tumor cell lines. *Cell Growth Differ* 2000;11:247-253.
  31. Bantel H, Engels IH, Voelter W, Schulze-Osthoff K, Wesselborg S. Mistletoe lectin activates caspase-8/FLICE independently of death receptor signaling and enhances anticancer drug-induced apoptosis. *Cancer Res* 1999;59:2083-2090.
  32. Suhara T, Kim HS, Kirshenbaum LA, Walsh K. Suppression of Akt signaling induces Fas ligand expression: involvement of caspase and jun kinase activation in Akt-mediated Fas ligand regulation. *Mol Cell Biol* 2002;22:680-691.
  33. Uriarte SM, Joshi-Barve S, Song Z, Gobejishvilli L, Jala VR, Haribabu B et al. Akt inhibition upregulates FasL, downregulates c-Flips and induces caspase-8-dependent cell death in Jurkat T lymphocytes. *Cell Death Differ* 2005;12:233-242.
  34. Georgakis GV, Li Y, Rassidakis GZ, Martinez-Valdez H, Medeiros LJ, Younes A., Inhibition of Heat shock protein 90 function by 17-Allylamino-17-Demethoxy-Geldanamycin in Hodgkin's Lymphoma cells down-regulated Akt kinase, dephosphorylates extracellular signal-regulated kinase, and induces cell cycle arrest and cell death. *Clin Cancer Res* 2006;12:584-590.
  35. Sato S, Fujita N, Tsuruo T. Modulation of Akt kinase activity by binding to Hsp90. *Proc Natl Acad Sci U S A* 2000;97:10832-10837.



36. Wajant H. Targeting the FLICE inhibitory protein (FLIP) in cancer therapy. *Mol Interv* 2003;3:124-127.
37. Tran SE, Meinander A, Holmstrom TH, Rivero-Muller A, Heiskanen KM, Linnau EK, et al. Heat stress downregulates FLIP and sensitizes cells to Fas receptor-mediated apoptosis. *Cell Death Differ* 2003;10:1137-1147.
38. Zhao C, Wang E. Heat shock protein 90 suppresses tumor necrosis factor alpha induced apoptosis by preventing the cleavage of Bid in NIH3T3 fibroblasts. *Cell Signal* 2004;16:313-321.
39. Kim KW, Kim BJ, Chung CW, Jo DG, Kim IK, Song YH, Kwon YK, Woo HN. Caspase cleavage product lacking amino-terminus of IkappaBalpha sensitizes resistant cells to TNF-alpha and TRAIL-induced apoptosis. *J Cell Biochem* 2002;85:334-45.
40. Malhotra V, Wong HR. Interaction between the heat shock response and the nuclear factor- $\kappa$ B signaling pathway. *Crit Care Med* 2002;30:S89-S95.
41. Ran R, Lu A, Zhang L, Tang Y, Zhu H, Xu H, et al. Hsp70 promotes TNF-mediated apoptosis by binding IKK $\gamma$  and impairing NF- $\kappa$ B survival signaling. *Genes & Dev* 2004;18:1466-1481.
42. Meldrum KK, Burnett AL, Meng X, Misseri R, Shaw M BK, Gearhart HP et al. Liposomal delivery of heat shock protein 72 into renal tubular cells blocks nuclear factor- $\kappa$ B activation, tumor necrosis factor- $\alpha$  production, and subsequent ischemia-induced apoptosis. *Cir Res* 2003;92:293-299.

43. Koishi M, Yokota S, Mae T, Nishimura Y, Kanamori S, Horii N, et al. The effects of KNK437, a novel inhibitor of Heat shock protein synthesis, on the acquisition of thermotolerance in a murine transplantable tumor in vivo. *Clin Cancer Res* 2001;7:215-219.
44. Yokota S, Kitahara M, Nagata K. Benzylidene lactam compound, KNK437, a novel inhibitor of acquisition of thermotolerance and heat shock protein induction in human colon carcinoma cells. *Cancer Res* 2000;60:2942-2948.
45. Adams JW, Sakata Y, Davis MG, et al. Enhanced Galphaq saignaling: a common pathway mediates cardiac hyopertrophy and apoptotic heart failure. *Proc Natl Acad Sci U S A* 1998;95:10140-10145.
46. Grishko V, Pastukh V, Solodushko V, Gillespie M, Azuma J, Schaffer S. Apoptotic cascade initiated by angiotensinII in neonatal cardiomyocytes: role of DNA damage. *Am J Physiol Heart Circ Physiol* 2003;285:H2364-H2372.
47. Qun F, Patel R, Yan C, Liu W. NADPHY oxidase is involved in angiotensin II-induced apoptosis in H9c2 cardiac muscle cells: Effects of apocynin. *Free Radic Biol Med* 2006;40:236-246.
48. Akishita M, Nagai K, Xi H, Yu W, Sudoh N, Watanabe T et al. Renin-angiotensin system modulates oxidative stress-induced endothelial cell apoptosis in rats. *Hypertension* 2005;45:1188-1193.

## ABSTRACT (KOREAN)

# 안지오텐신 II 자극에 의한 심근세포의 비대기전

< 지도교수 장 양 수 >

연세대학교 대학원 의과학과

이 경 혜

심근비대는 심근압력 과부하에 의해 발생하여 세포분열 없이 세포의 크기만 증가하는 현상으로서 비대 후 부정맥이나 심근경색 등의 과정을 겪게 된다. 8개의 아미노산으로 구성된 안지오텐신 II는 NF- $\kappa$ B의 활성을 증가시킴으로써 심근비대를 초래하며 동시에 세포사멸 (apoptosis)을 유도하는 것으로 알려져 있다. 열 충격 단백질들 가운데 하나인 Hsp90은 세포사멸에 대한 보호 기전과 동시에 심근비대 과정에서 NF- $\kappa$ B 활성을 유지하는데 기여한다고 알려져 있다. 따라서 본 연구에서는 안지오텐신 II에 의한 심근비대 과정에서 NF- $\kappa$ B 신호전달 과정에 대한 Hsp90의 영향과 이의 분자기전을 규명하고자 하였다. Hsp90의 특이적인 저해제인 geldanamycin과 17-AAG는 안지오텐신 II에 의해 유도되는 심근비대를 억제하였으며, NF- $\kappa$ B의 활성도 억제함을 확인하였다. 심근세포에 Hsp90

저해제를 처리하였을 때 NF- $\kappa$ B의 상위단계인 IKK $\alpha/\beta$ 의 발현 수준과 NF- $\kappa$ B의 억제자인 I $\kappa$ B $\alpha$ 의 인산화가 감소되는 것을 확인하였다. 특히 흥미롭게도 Hsp90 저해제에 의한 I $\kappa$ B $\alpha$  단백질의 절단현상이 새롭게 관찰되었고, 이런 현상은 caspase-8의 억제제인 z-IETD-fmk에 의하여 감소하였으며, caspase-8가 I $\kappa$ B $\alpha$  단백질의 절단에 직접 관여함을 확인하였다. Caspase-8에 의한 I $\kappa$ B $\alpha$  단백질의 절단은 I $\kappa$ B $\alpha$ 의 아미노 말단에서 유발되고 이는 유비퀴틴화 잔기의 탈락을 유발하여 I $\kappa$ B $\alpha$ 가 인산화되더라도 유비퀴틴화 되지 못해 I $\kappa$ B $\alpha$ 가 지속적으로 세포 내에 축적됨으로써 NF- $\kappa$ B 활성을 억제하는 것으로 사료된다. Hsp90 저해제에 의해 I $\kappa$ B $\alpha$ 의 절단과 동시에 심근세포의 apoptosis가 유발되는데, caspase-8의 활성이 관찰되었다. 이때 FasL의 발현이 증가되고 FLIP<sub>s</sub>의 발현이 감소함을 확인하였는데, 이를 통해 caspase-8의 활성이 유도되어 I $\kappa$ B $\alpha$ 의 절단을 증가시킨 것으로 생각된다. 이와 같은 결과들을 통하여 안지오텐신 II에 의한 심근세포의 사멸과 비대의 두 가지 경로에서 Hsp90은 caspase-8의 활성을 억제함으로써 NF- $\kappa$ B의 활성을 유지하여 apoptosis를 억제하면서 심근비대를 초래하는 것으로 사료된다.

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핵심되는 말 : 심근비대, 열 충격 단백질90, 캐스페이즈-8, I $\kappa$ B $\alpha$  절단