The death mechanism of cardiomyocytes by hypoxic stress and the role of heat shock proteins

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Directed by Professor Yangsoo Jang

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LIST OF ABBREVIATIONS

HSP : heat shock protein

MAPK : mitogen-activated protein kinase

MKP-1 : mitogen-activated protein kinase phosphatase-1

ERK : extracellular signal-regulated kinase

JNK : c-jun N-terminal kinase

HPX : hypoxia

NMX : normoxia

SD : serum deprivation

HSP-IH : Heat shock protein 70 inhibitor-1

Abstract

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Ischemia/hypoxia is a major factor of cardiovascular diseases, which are generated from apoptotic cells. Many studies have suggested that the mitogen-activated protein kinases (MAPKs) as well as heat shock proteins (HSPs) may be important regulators of apoptosis in response to hypoxia stress in cardiomyocytes. However, the precise mechanisms involved in stress recognition and progression to apoptosis in response to hypoxia or serum

deprivation remains largely uncertain. Here we investigated the cell death mechanisms of cardiomyocytes and the involvement of HSP70, MKP-1 and ERK under hypoxia. Neonatal rat cardiomyocytes exposed to simulated hypoxia were used as an *in vitro* model to delineate the roles of HSP70, MAPKs, as well as MKP-1 in apoptosis. Exposure of the cardiomyocytes to hypoxia with serum deprivation reduced cell viability, as measured by the MTT assay, and stimulated apoptosis as evidenced by caspase-3 activation and DNA fragmentation. Under hypoxia condition, serum deprivation is required to complete the apoptotic pathway in cardiomyocytes. Hypoxia insult resulted in an inactivation of ERK, while MKP-1 expression was increased at early time. Induction of HSP70 also increased shortly after the onset of hypoxia. Under hypoxia condition, interestingly, inhibition of HSP70 significantly increased the MKP-1 expression and caspase-3 activity, on the other hand, ERK phosphorylation was reduced more than only hypoxic group. Cardiomyocytes pretreated with vanadate, a MKP-1 inhibitor, showed a significant increase in ERK phosphorylation and attenuation of the caspase-3 activity under hypoxia. To confirm the roles of HSP70 under hypoxia, recombinant HSP70 protein fused with a protein transduction domain TAT was transduced into cardiomyocytes. After HSP70 transduction into cardiomyocytes, MKP-1 expression was significantly reduced under hypoxic

condition whereas ERK phosphorylation was recovered. Our results suggest that HSP70 has a protective role while MKP-1 expression is proapoptotic in cardiomyocytes under hypoxia and that these proteins are correlated precisely with ERK activity.

Key words : Cardiomyocytes, HSP70, MKP-1, ERK, Hypoxia, and Apoptosis.

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I.INTRODUCTION

The function of apoptosis in regulating cardiovascular diseases has recently been extensively investigated as a possible mechanism explaining the pathophysiological significance [1,2]. It is known that cellular apoptosis is associated with cardiovascular diseases such as myocardial infarction [3] and reperfusion [4], heart failure [5], diabetes [6], cardiac hypertrophy [7] and left ventricular remodeling [8]. Recent studies have focused on the signaling events that influence this cardiac cell death [1,9,10]. Recent reports have also supported

that cellular apoptosis derives to death of cardiomyocytes in response to a range of stresses such as hypoxia [11], free radical stress [12], adrenergic over stimulation [13] and viral infection [14].

Heat shock proteins (HSPs) are induced in response to stresses caused by agents, such as high temperature, hypoxia/ischemia, oxidative stress and pressure overload [15]. HSPs are highly conserved molecules that fulfill a range of functions, including cytoprotection, intracellular assembly, folding and translocation of oligomeric proteins [16]. These proteins are induced in most of mammalian cells and categorized into several families that are named on the basis of their approximate molecular weight (e.g. HSP90, HSP70, HSP60 and HSP27). Transcription of HSP gene is regulated by heat shock factors (HSFs), which form homotrimers under various stress conditions; these then move to the nucleus where they bind to a specific heat shock element (HSE) within the promoter region of the HSP gene [17,18]. The stress response is only transient, because a prolonged and inappropriate presence of protein-binding molecules would adversely influence protein homeostasis and a variety of intracellular functions. Especially, it is well established that HSPs are induced in response to hypoxia and may act as important regulators against cardiac cell apoptosis [2,19]. Two HSPs, the strongly stress-induced HSP70 and HSP27 are the best-studied members of the HSP family in human cells. Upregulation of these

proteins can be cytoprotective and can increase the survival of cells exposed to hypoxia known as an inducer of apoptosis [20].

Pathophysiological hypoxia stress causes cells to lose viability. Hypoxia is also an important factor of apoptotic cell death [21] and gene expressions in cardiomyocytes. Hypoxic stress also activates a variety of signaling pathways such as the cAMP-protein kinase A (PKA) [22], p42/44 mitogen-activated protein kinase (MAPK) [23], stress-activated protein kinase (SAPK; p38 kinase) [24] and phosphatidylinositol 3-kinase/Akt [25], leading to different cellular events in several cell types. In cardiac myocytes, MAPK pathways have been known as important downstream transducers of hypoxia/ischemia stimuli. Three major members of the MAPK family, ERK1/2, JNK1/2 and p38 MAPK, are involved in the regulation of a large variety of cellular processes such as cell growth, death and survival. ERK1/2 activation is observed during ischemia/hypoxia in heart and plays an important role in prevention of cardiac myocytes apoptosis, both in cultured cardiomyocytes and in the intact heart [26,27]. The p38 MAPK pathway belongs to the most investigated, but also the most controversial signaling pathway in cardiac responses to hypoxic insult. It has been reported that activation of p38 during ischemia/hypoxia is shown to be involved in proapoptotic signals [26]. In contrast, others showed that p38 activation during a short preconditioning treatment protects cardiomyocytes

from ischemic cell death [28], suggesting a protective role of p38-MAPK activation during ischemia. In some studies, it was found that p38 MAPK mediated cytoprotection through phosphorylation of the small heat shock protein αβ-crystallin [29], and through activation of NF-kB and subsequent release of anti-apoptotic cytokines [30]. Whether p38 acts as a cytoprotective or proapoptotic agent is likely to depend on both the intensity and the duration of p38 MAPK activation. Some studies also documented the possible role of another MAP kinase pathway, JNK, in cardiac apoptosis. JNK activation correlates with many cell lines including cardiac myocytes apoptosis in response to mechanical stress [31], cytokines [32], and oxidative stress [27,33]; however, the precise role of JNK in apoptosis and pathophysiology of ischemia/hypoxia stress remains unresolved.

MAPKs are regulated by MAPK phosphatase-1 (MKP-1) that has dual catalytic activity toward phosphotyrosine- and phosphothreonine-containing proteins, and is known to inactivate ERKs and JNKs [34,35]. The MKPs play a major role in regulating the activity of MAPKs. MKP-1 is transcribed as an immediate early response gene following various stimuli [36-38], but its role in the cardiomyocytes is poorly understood. Recently, a few studies have addressed the role of MKP-1 in cardiac growth and hypertrophic response [39]. Transgenic mice expressing physiological levels of MKP-1 in the heart

showed no activation of p38, JNK1/2, or ERK1/2; diminished developmental myocardial growth; and attenuated hypertrophy in response to aortic banding and catecholamine infusion [39]. Nowadays, it has been studied that MKP-1 regulates MAPKs by HSP70 under heat stress [34]. HSP70 suppressed ERK activation by both protecting dual-specificity phosphatases, which was independent of the chaperone activity [34]. HSP70 can also inhibit JNK activation induced by a number of stresses, including heat shock [40], ethanol [41], and ultraviolet (UV) irradiation [42]. In addition, it has been suggested that HSP70 modulates JNK activity via inhibition of its dephosphorylation [34,43]

We investigated here the cell death mechanisms of cardiomyocytes and the involvement of HSP70, MKP-1 and ERK under hypoxia. We make *in vitro* apoptotic condition with controlling serum deprivation in hypoxia condition. In this condition, hypoxia stress transiently induced HSP70 in cardiomyocytes, and concurrently induced MKP-1 expression. This transient induction of HSP70 and MKP-1 influenced the activation of MAPKs. The MAPKs were inactivated at early time of hypoxic condition in cardiomyocytes. Especially, pretreatment of a MKP-1 inhibitor, vanadate, significantly recovered the ERK1/2 phosphorylation under the hypoxic condition. Interestingly, when cardiomyocytes was treated with HSP70 inhibitor, MKP-1 expression level was significantly increased whereas ERK1/2 phosphorylation was reduced.

Our results indicate that expression of MKP-1 by hypoxic stress to dephosphorylation of ERK1/2 is the initial event that commits the cardiomyocytes to the apoptotic pathway and it is regulated by HSP70. Hypoxia exposing of cardiomyocytes triggers numerous signaling events, which contribute to either cell death or survival. This study suggests that the HSP70 signal pathway plays an important role in the regulation of MKP-1 expression and ERK activation in hypoxia-induced cardiomyocytes death.

II. MATERIALS AND METHODS

1. Materials

Polyclonal heat shock protein (HSP70, 60, 27 and HSC70) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Also, anti-phospho-p38, phospho-JNK, phospho-ERK, p38, JNK and ERK were obtained from Santa Cruz Biotechnology. Monoclonal anti-β-actin antibody was from Sigma (St. Louis, MO, 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. HSP inhibitor I was purchased from Calbiochem (San Diego, CA, USA). MKP-1 inhibitor (sodium orthovanadate) was obtained from Sigma (St. Louis, MO, USA). Caspase-family colormertric substrate set plus for caspse-3 activity test was from BioVision, Inc (Mountain View, CA, USA).

2. Animals

Neonatal Sprague-Dawley rat 1~2days old were used for this study. All animal procedures were carried out according to a protocol approved by the Yonsei University Animal Care Committee.

3. Cell culture

Ventricular myocytes from the hearts of neonatal Spraue-Dawley rats (1~2 days old) were cultured according to the previously described methods with minor modifications [44-46]. The isolated heart tissues were washed with phosphate-buffered saline (PBS) in order to deplete red blood cell 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. The fresh 0.1% collagenase type II solution was added and the incubation procedure was repeated until the tissue was totally digested. The resulting supernatant was collected and equal volume of minimum Essential Medium Alpha Medium (α-MEM, Gibco BRL) containing 10% fetal bovine serum (FBS) and 100U/ml penicillin/streptomycin (Gibco BRL) was added. Cell pellets were obtained by centrifugation. The cells resuspended in fresh medium containing 10% FBS were preplated in culture dish and incubated for at least 1hr at 37°C in 5% CO_2 incubator. The fibroblasts adhered to the dish surface, and the cardiomyocytes remained unattached. Unattached cardiomyocytes were replated on primary culture dish (Falcon, Bedford, MA, USA) and incubated in α-MEM supplemented with 10% FBS. Cells were maintained in a 5% CO_2 incubator for 24hr, at which spontaneously contracting confluent cells were noted. In order to reduce fibroblast contamination, we used α-MEM with

0.1mM 5-bromo-2′-deoxyuridine (Brd-U) (Sigma, St. Louis, MO, USA)

4. Cell treatment and experimental hypoxia

After overnight incubation, fresh serum-free or 10% serum contained medium were added. MAPK inhibitors were then added, and cells were returned to the incubator or placed in the hypoxia chamber. The airtight humidified chamber (Anaerobic Environment, ThermoForma, Marietta, OH, USA) which was maintained at 37^{o}C and continuously gassed with a mixture of 10% CO_2, 5% H_2 and 85% N_2 was used. For concurrent normoxic conditions, cells were placed in a Forma Scientific incubator gassed with 95% air, 5% CO_2 at 37^{o}C.

5. Troponin I staining

Cardiac troponin I was identified on the cardiomyocytes. For assessment, the cardiomyocytes were cultured and incubated for 24hr and α-MEM was discarded and rinsed by PBS. After then, cells were fixed by 50% acetone/ 50% methanol for 2min and rinsed by PBS. Second fixed was for 10min by H_2O_2 and washed by PBS. Cardiomyocytes were incubated for 24hr with a 1:100 dilution of anti-cardiac troponin I antibody (Santa Cruz Biotechnology) at room temperature, washed by PBS with 1% triton X-100 for 5min and

followed by a 1:200 dilution of biotin-conjugated anti-mouse IgG antibody (DAKO, Carpinteria, CA, USA) for 30min and 1:200 dilution of horse-radish peroxidase conjugated streptavidine (DAKO, Glostrup, Denmark). Cells were washed by PBS for 5min and treated Large Volume DAB substrate system (Lab Vision Corporation, Fremont, CA, USA). These cardiomyocytes were carried out counter staining with Mayer's Hematoxyline and mounted.

6. Propidium iodide staining

Primary cultured rat cardiomyocytes were plated in 4-well chamber slide and incubated for 24hr in 5% CO_2 incubator. The cells were washed and cultured with serum-free α-MEM for 24hr, and accessed propidium iodide (PI) staining manner. The mediums were discarded, and cells were fixed with 1% paraformaldehyde for 5min. Second fixation was carried out by 70% ethanol for 1min. Fixed cardiomyocytes were washed with PBS and added 200ul of PBS. After then, PI (100ug/ml PBS) was treated on the cells. After 3min, the cells were observed by fluorescent microscope.

7. DNA fragmentation

Cultured and hypoxia treated cardiomyocytes were gently homogenized in 0.5ml of DNA homogenization buffer, and transferred the homogenate to a

1.5ml tube. After adding 31.25ul 10% SDS, incubate at 65^{o}C for 30min. 87.3ul of 8M potassium acetate (pH 8.0) were added. Mixture was incubated on ice for 1hr and obtained supernatant by centrifugation at 14,000rpm for 10min. The supernatant was added an equal volume of phenol : chloroform : isoamylalcohol (25:24:1), and transferred upper phase to new tube, and also added equal volume chloroform : isoamylalcohol (24:1). Transferred upper phase was stored overnight at -80^{o}C with 2.5vol of cold 100% ethanol. Next day, sample was centrifuged and obtained as white pellet. The 1 X TE buffer and 2ul RNase (1ug/ml) were added, and the mixture was incubated at 37^{o}C for 1hr. The phenol extraction step was repeated and upper phase was collected. Sample was treated with 0.1vol of 3M sodium acetate (pH 5.2) and 2.5vol of cold 100% ethanol, and incubated at -70^{o}C for at least 1hr. After obtaining the pellet by centrifugation, sample was dried, resuspended with dH_2O, and stored at -20^{o}C. Finally, it was fractionated on a 1.5% agarose gel and stained with ethidium bromide.

8. MTT assay

Cell viability was determined by the MTT assay. The 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) (5mg/ml) was dissolved in PBS. Cells were plated in 4-well dish for 24hr. They were then

transferred to hypoxic chamber. After 6hr, 24hr or 48hr, culture medium was aspirated and 0.5ml of MTT solution was added. The plates were then replaced in the incubator. After 3hr, the MTT solution was aspirated and MTT/formazan was extracted with 0.5ml dimethylsulfoxide (DMSO) to dissolve. Optical densities at 570nm were measured using extraction buffer as a blank. Because MTT is metabolized to formazan by living, viable cells, a reduction of measured OD 570 compared to untreated healthy control cells indicates a loss of cell growth and viability.

9. Caspase-3 activity assay.

The activity of caspase-3 in the cardiomyocytes was determined spectrophotometrically with an Apoalert™ CPP32/caspase-3 assay kit (BD Science, Palo Alto, CA, USA) by measuring the release of the chromophore, pnitroanilide (*p*NA), following hydrolysis of DEVD-*p*NA. Primary cultured cardiomyocytes were incubated in α-MEM with or without FBS. Samples were transferred to hypoxic condition and incubated for 24hr. After then, cells were harvested, resuspended in chilled cell lysis buffer and incubated on ice for 10min. Cells were centrifuged and supernatants were transferred to a fresh tube. Protein concentration was determined by the Bradford assay (Bio-Rad, Hercules, CA, USA). Equal amounts of protein in the lysates were incubated

with substrate. It was proper to diluted 100~300ug protein in 50ul cell lysis buffer for each assay. The 50ul of 2 X Reaction Buffer containing 10mM dithiothreitol (DTT) was added and each samples were incubated with 5ul of DEVD-*p*NA (4mM) at 37^{o}C for 1hr. Caspase-3 activity was read in a microtiter plate reader at 405nm, and a standard curve was generated using as standard CPP32 chromogenic substrate.

10. Immunoblot analysis

Primary cultured rat cardiomyocytes were treated for various experimental conditions with hypoxia. At the end of the various treatments, harvested cells were solubilized in a Cell Lysis Buffer (Cell signaling, Beverly, MA, USA) with 1mM PMSF for 15min at 4^{o}C and the protein lysate concentrations were measured by Bradford protein assay kit (Bio-Rad). The same amounts of proteins from whole cell lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto methanol-treated PVDF membranes (Millipore Co, Bedford, MA, USA). After blocking the membrane with Tris-buffered saline-tween 20 (TBS-T, 0.1% tween 20) containing 5% skim milk (Bio-Rad) for 60min at room temperature, they were washed twice with TBS-T and incubated with primary antibodies for 1hr at room temperature or for overnight at 4^{o}C. The

membranes were washed three times with TBS-T for 10min, and then incubated for 45min at room temperature with horseradish peroxidase (HRP)-conjugated secondary antibodies. After extensive washing, the bands were detected by enhanced chemiluminescence (ECL) reagent (Santa Cruz Biotechnology). The band intensities were quantified using Phospho-Image System (Molecular Dynamics, Uppsala, Sweden)

11. Gene construction and protein purification of HSP70-TAT

TAT-beta-galactosidase (TAT-β-gal) and the 6 X His-TAT cloning vector (pTAT-HA, where HA is hemagglutinin) were kindly provided by Dr. Steven Dowdy (Cancer Center, UCSD, USA). The pTAT-HA vector contains an ampicillin resistance marker for selection after transformation, a T7 polymerase promoter, an N-terminal 6-histidine leader before the TAT domain, and an HA tag. HSP70-TAT fusions were generated by insertion of the HSP70 open reading frame (ORF) DNA into the pTAT-HA plasmid. The HSP70 cDNA was removed from the pcDNA3.1-HSP70 plasmid by double digestion with *Xho*I and *Eco*RI. The HSP70 DNA was then ligated into the pTAT-HA vector by double digestion of the multiple cloning sites within pTAT-HA with *Xho*I and *Eco*RI. Plasmids were transformed into *E. coli* DH5α. Individual clones were isolated and analyzed for the correct HSP70 by DNA sequencing.

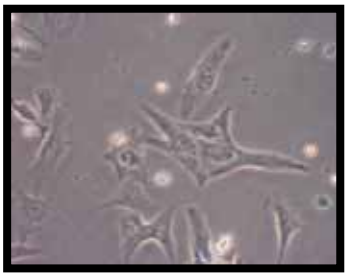
The recombinant plasmid pTAT-HSP70 was introduced into *E. coli* strain BL21 (DE3). *E. coli* harboring pTAT-HSP70 was inoculated into LB media (1% tryptone, 0.5% yeast extract, and 0.5% NaCl) containing 50ug/ml of ampicillin to a density of A600 of 1.0 at 37^{o}C. Recombinant proteins were induced with 0.5mM of isopropyl-b-D-thiogalactopyranoside (IPTG) at 30^{o}C for 6hr. The cells were centrifuged at 6,000rpm for 20min, and then cell pellets were resuspended in buffer A (50mM NaH_2PO_4, 300mM NaCl, 6M urea, pH8.0). Cells were disrupted by ultrasonication and the lysates were centrifuged at 15,000rpm for 30min. The supernatant was applied to a Ni-NTA agarose resin column (Qiagen, Hilden, Germany) pre-equilibrated in buffer A at a flow rate of 1ml/min. The flow-through was discarded and proteins were washed with additional column volumes of buffer A 10 times. The TAT-HSP70 was eluted from the column with buffer B (50mM NaH_2PO_4, 300mM NaCl, 300mM imidazole, pH 8.0). The protein fractions were pooled and desalted into 1 ml PBS on PD-10 columns. The protein fractions were identified by 15% SDS-PAGE analysis.

III. RESULTS

1. Confirmation of primary cultured rat cardiomyocytes

The mammalian heart is composed of many cell types. Approximately 75% of the cells are non-myocardial cells, such as fibroblasts and red blood cells. Myocardial cells, also known as cardiomyocytes, make up the remaining 25% of the cells in the organ. Despite their smaller numbers, cardiomyocyte make up the largest volume of the heart [47]. Common problem with cultured cardiomyocytes is contamination with fibroblasts. Because of these reasons, we first checked morphological characteristics of cardiomyocytes by microscopy (Fig. 1-A). Rhythmically contracting areas appeared at 1day after plating. Using immunocytochemistry, the presence of cardiac-specific protein Troponin I and its spatial organization were also examined in contracting cells. Fig. 1-B shows positive immunostaining of dispersed cardiomyocytes with anti-cardiac Troponin I monoclonal antibody. It was shown that cardiomyocytes have a star-shaped profile. Therefore, we just used more than 90% pure cardiomyocytes in the following experiments.

A



B

Figure 1. The morphological character of neonatal rat cardiomyocytes. Neonatal rat cardiomyocytes were incubated in a CO_2 incubator at 37^{o}C for 24hr. (A) Cardiomyocytes were observed by phase-contrast inverted microscope. Rhythmically contracting areas appeared at 1day after plating. (B) Cardiomyocytes were stained with anti-cardiac troponin I monoclonal antibody. It was shown that cardiomyocytes have a star-shaped profile.

2. Cell death by hypoxia insult with serum deprivation in cardiomyocytes

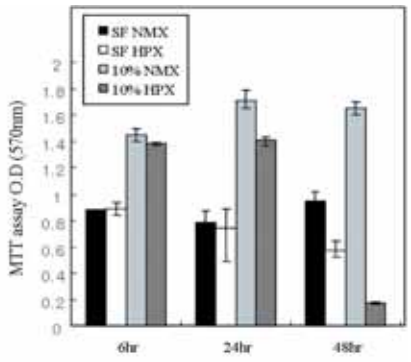
Apoptosis and necrosis are two distinct forms of cell death. Nevertheless, there is increasing evidence that most cardiac diseases are related with apoptosis. In this reason, we decided to make apoptotic condition *in vitro*, in order to identify hypoxia-induced cardiomyocytes death. Many of studies have shown that hypoxia insult generates apoptotic cell death. However, we found that only hypoxia (*in vitro*) could not induce apoptotic death of cardiomyocytes in preliminary data [48]. Therefore, we made appropriate apoptotic condition by controlling serum concentration with hypoxia because the cardiomyocytes are very sensitive to serum concentration containing culture medium. The cardiomyocytes were treated in two experimental conditions, hypoxia with serum-deprived (SD) media or hypoxia with 10% serum-containing media, and then cell viability was determined by MTT assay (Fig. 2-A). It was shown that serum-deprived cardiomyocyte underwent cell death but the differences between hypoxia and normoxia were not observed within 24hr. In the presence of serum, hypoxia for up to 24hr did not induce significant cell death. The remarkable differences of cell viability between hypoxic group and normoxic group were shown after 48hr. The death rate of cardiomyocytes was increased in the combination group of hypoxia

and 10% serum more than serum-deprived group with hypoxia. These results suggest that serum in culture medium is an important factor to determine cell death pattern in cardiomyocytes. To further examine the contribution of serum to cardiomyocytes apoptosis under hypoxic condition, cardiomyocytes were subjected to hypoxia with different serum concentration (0, 0.5 or 10%) in culture medium for 24hr. The combination of hypoxia and serum deprivation significantly increased the level of caspase-3 activity (Fig.2-B). Under hypoxic condition, caspase-3 activities of cardiomyocytes in serum deprivation or 0.5% serum condition were increased about 4-folds and 8-folds, respectively. On the other hand, in the presence of 10% serum with hypoxia, caspase-3 activity level was increased by about 1.7-folds compared to control normoxia (Fig.2-B). These results indicate that caspase-3 activation by hypoxia depends on serum concentration in neonatal cardiomyocytes, and these cells are much more sensitive to serum deprivation than to hypoxia.

In order to determine the apoptotic effect of hypoxia with serum deprivation in cardiomyocytes, we examined the propidium iodide (PI) staining and DNA fragmentation. The PI-stained nuclei were shown in cardiomyocytes under normal condition (Fig. 3-A). Treatment with serum deprivation under hypoxia induced morphological changes (i.e. fragmentation of nucleus) consistent with apoptosis, whereas no apoptotic signal was shown in cardiomyocytes under

normoxia condition. Cultured cardiomyocytes exposed to hypoxia with serum deprivation for 24 h exhibited the typical apoptotic DNA fragmentation (Fig. 3-B). The 100bp DNA fragments were used as marker.

A



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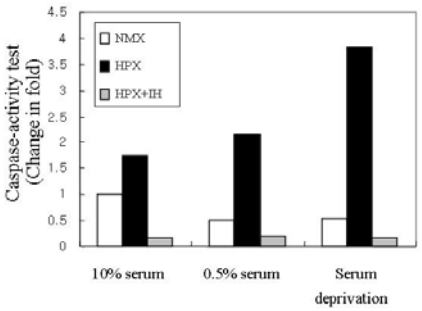

Figure 2. Viability and caspase-3 activity of neonatal rat cardiomyocytes incubated under hypoxia with different serum concentration. (A) Primary cardiomyocytes were incubated with or without 10% serum in a hypoxic chamber for the indicated time. Cell viability was assessed by the MTT assay. Data are average of three independent experiments. (B) Primary cardiomyocytes were incubated with different serum concentration in hypoxic chamber for 24hr. Caspase-3 activity was measured. Results are representative of three independent experiments. NMX, normoxia; HPX, hypoxia; SD, serum deprivation; 10% serum, 10% serum containing; IH, treatment of caspase-3 inhibitor.

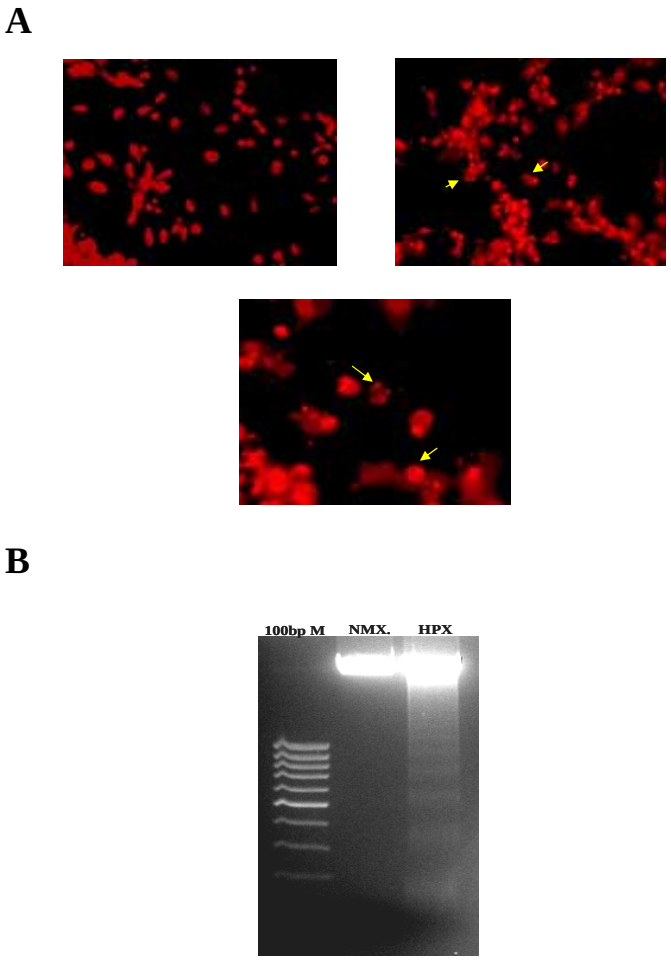

Figure 3. Hypoxia-induced apoptosis in cardiomyocytes. Cultured cardiomyocytes were incubated with serum-free α-MEM under hypoxic condition for 24hr. (A) Cardiomyocytes were stained with propidium iodide (PI). The arrows indicate PI-stained nuclei. (B) DNA fragmentation patterns under hypoxia. The 100bp DNA ladders were used as marker. NMX, normoxia; HPX, hypoxia.

3. Expression of HSPs under hypoxic condition

The accumulation of HSPs in cardiac cells enhances myocardial resistance to hypoxia/ischemia injury [49,50] and HSP70 is synthesized in the myocardium [52] following transiently induced ischemia. To investigate whether expression of HSPs increases in cardiomyocytes under hypoxic stress, cultured cardiomyocytes were exposed to hypoxia for varying periods of time. Expression of HSP70 gradually increased after the onset of serum deprivation under hypoxic conditions for 6hr (Fig. 4). The levels of HSP60 and HSC70 (constitutive form of HSP70) remained unaffected.

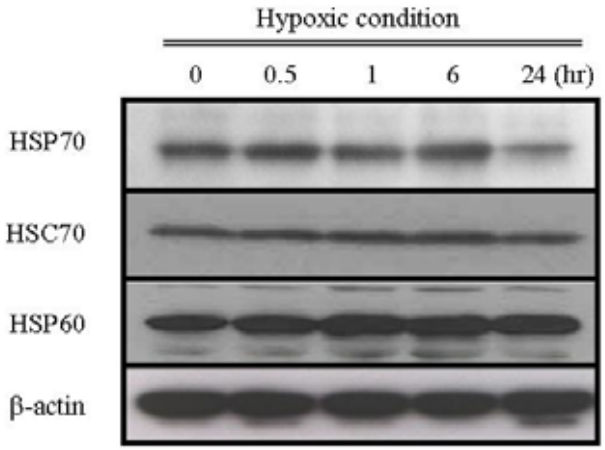

Figure 4. The expression of heat shock proteins in cardiomyocytes under hypoxic condition. Primary cardiomyocytes were incubated in hypoxia chamber. At the indicated times, samples were harvested, lysed and immunoblotted with anti-HSP70, HSP60, and HSC70.

4. MAPKs and MKP-1 expression in hypoxia

To further investigate whether the death mechanism of cardiomyocytes by hypoxia was involved in MAPKs signaling, we primarily examined the effect of hypoxia on the activation of three MAPKs and the expression of one phosphatase MKP-1. Cardiomyocytes were treated with serum deprivation under hypoxia for various times, and then MAPKs were determined by immunoblot with phospho-specific antibodies. As shown in Fig. 5-A, the phosphorylations of ERK and JNK were significantly reduced at early time. Phosphorylation of p38 MAPK was slightly reduced. It has been known that the expression level of MKP-1 was also examined under same condition as it is correlated with regulation of MAPKs activation [34,35]. Interestingly, the expression of MKP-1 was induced at early time in cardiomyocytes in hypoxia (Fig.5-B). These results suggest that MAPKs may play an important role in cardiomyocytes death under hypoxia and their activations are regulated by MKP-1.

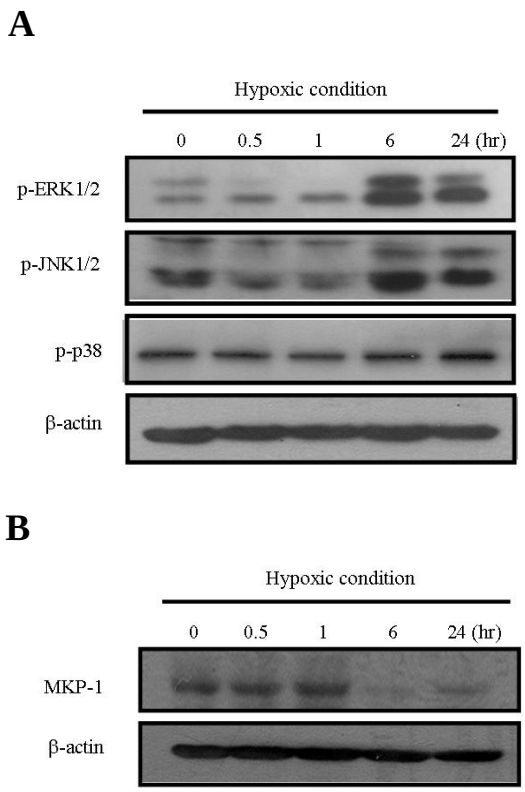

Figure 5. Phosphorylation of MAPKs and expression of MKP-1 under hypoxic condition. Primary cardiomyocytes were incubated in hypoxia chamber. At the indicated times, samples were harvested, and phosphorylation of MAPKs (A) and expression of MKP-1 (B) were determined by immunoblotting.

5. MAPKs and MKP-1 is regulated by HSP70

Since HSP70 induction is simultaneous event with MAPKs inactivation and MKP-1 expression in early time of hypoxic insult, as shown in Fig. 4 and 5, we hypothesized that the crosstalk between HSP70 induction and MAPKs, MKP-1 under hypoxia would be important for regulation of cell death in cardiomyocytes. So the effect of HSP70 inhibitor was studied. Primary cultured cardiomyocytes were pretreated with HSP Inhibitor I (HSP70 inhibitor, final 1uM), and then incubated under hypoxia for 1hr. Interestingly, the phosphorylation patterns of MAPKs were changed (Fig. 6). The JNK phosphorylation was recovered by HSP70 inhibitor treatment whereas phosphorylations of p38 and ERK were decreased under inhibitor-treated condition compared with only hypoxic condition.

To delineate the effect of HSP70 on the MKP-1 expression under hypoxia, cardiomyocytes were pretreated with HSP70 inhibitor and harvested after hypoxia for 1hr, and then immunoblot was examined. MKP-1 expression was significantly increased against the HSP70 inhibitor treatment, indicating regulation of HSP70 on the MKP-1 expression (Fig. 7). We next examined whether MKP-1 expression affects the HSP70 induction. Cardiomyocytes were treated with 100uM vanadate (MKP-1 inhibitor) and subjected to hypoxia. As shown in Fig. 8, no significant changes of HSP70 protein level

were observed with vanadate. These data suggest that hypoxia-induced MKP-1 expression is regulated by HSP70 in cardiomyocytes, at least in part.

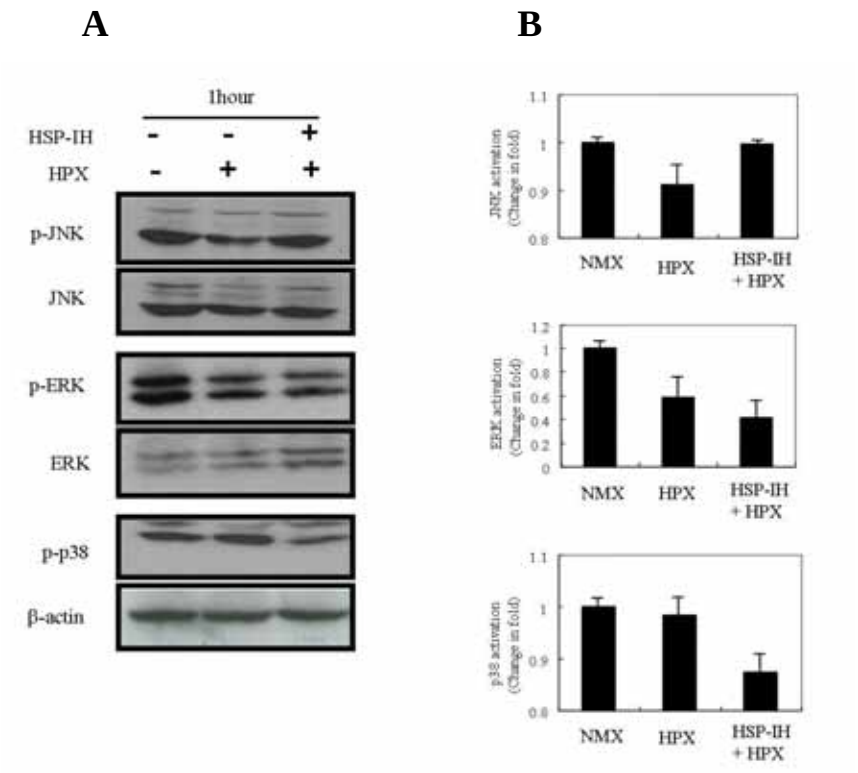

Figure 6. Effect of HSP70 inhibitor on MAPKs phosphorylation in cardiomyocytes under hypoxia. Cardiomyocytes were pretreated with 1uM HSP70 inhibitor for 1hr, and subjected to hypoxia for 1hr. Samples were harvested, and phosphorylation of MAPKs was determined by immunoblotting (A). Each signal was quantified by scanning densitometry (B).

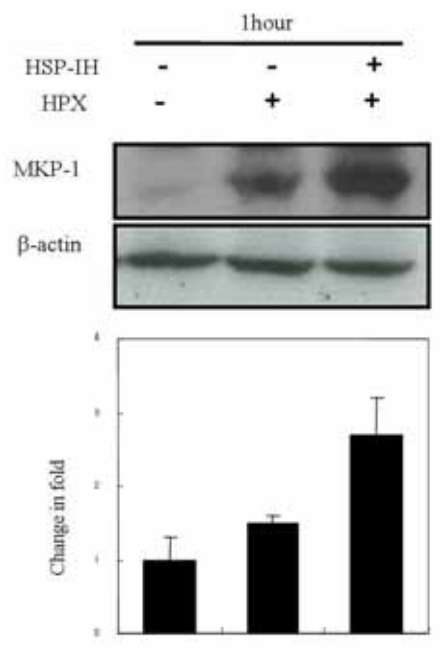

Figure 7. Effect of HSP70 inhibitor on the expression of MKP-1 under hypoxia. Cardiomyocytes were pretreated with 1uM HSP70 inhibitor for 1hr, and subjected to hypoxia for 1hr. Samples were harvested, and MKP-1 level was determined by immunoblotting.

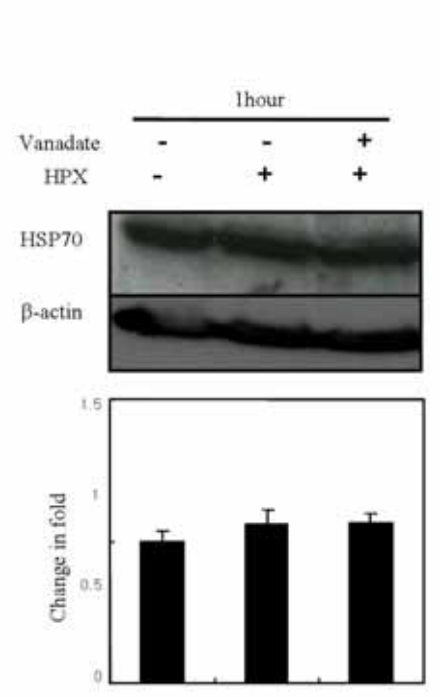

Figure 8. Effect of MKP-1 inhibitor on the HSP70 induction under hypoxia. Cardiomyocytes were pretreated with 100uM vanadate (MKP-1 inhibitor) for 1hr, and subjected to hypoxia for 1hr. Samples were harvested, and HSP70 level was determined by immunoblotting.

6. HSP70 transduction into cells and activation of ERK

To confirm the role of HSP70 on the activation of ERK and MKP-1, we used the recombinant HSP70 protein fused with a protein transduction domain (PTD), TAT (Fig. 9). Protein transduction is a technology by which proteins can be directly transferred into cells and TAT is one of the most widely studied PTD [52]. Cardiomyocytes were pretreated with TAT-HSP70 fusion protein for 1hr and incubated in hypoxia by a time-course. The fusion protein was transduced into cardiomyocytes (Fig. 10-A). Because TAT-HSP70 is fusion protein with TAT, transduced fusion protein compared with endogenous HSP70 was shown as upper shift band in immunoblot. After TAT-HSP70 transduction into cardiomyocytes, MKP-1 expression was significantly reduced under hypoxic condition (Fig. 10-B). In addition, ERK activation was recovered. These suggest that HSP70 upregulates the activation of ERK pathway via the downregulation of MKP-1 expression, indicating a potential roles of these proteins in cardiomyocytes apoptosis under hypoxia.

A

B

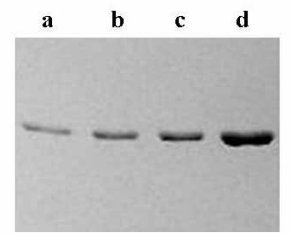

Figure 9. Construction and purification of TAT-HSP70. (A) pTAT-HSP70 expression vector (B) Purification of TAT-HSP70 fusion protein over Ni-NTA resin. The crude lysate from a 1L culture was applied to the resin in the presence of 10mM imidazole, washed, and eluted with (a)100, (b)150, (c)200 and (d)300mM imidazole. Ten microliters of each fraction were resolved by SDS–PAGE and stained with Coomassie blue.

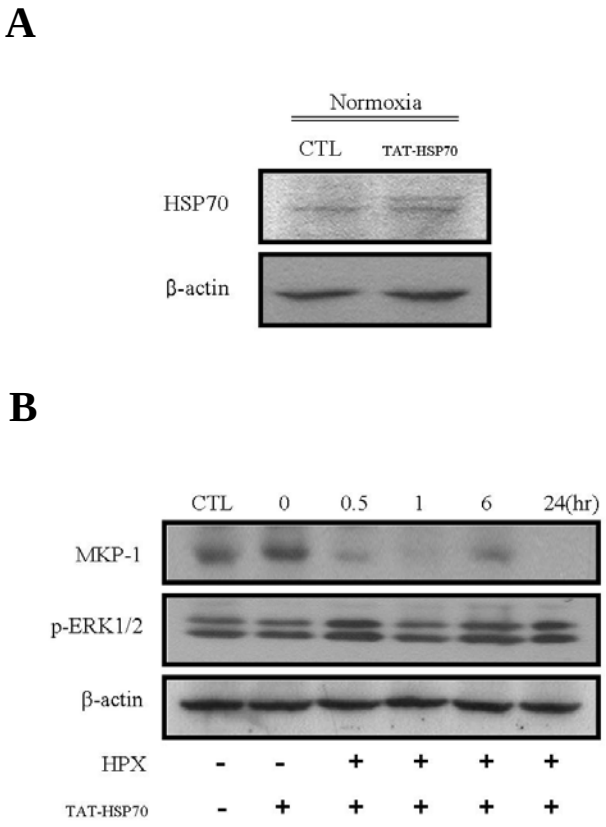

Figure 10. TAT-HSP70 transduction into cardiomyoctyes and its effect on the ERK activation and MKP-1 expression under hypoxia. (A) After transduction of 1uM TAT-HSP70 into cardiomyocytes, immunoblot analysis was performed using anti-HSP70 antibody. (B) Cardiomyocytes were treated with 1uM TAT-HSP70 and subjected to hypoxia. At the indicated time, samples were harvested, and the levels of phospho-ERK and MKP-1 were determined by immunoblotting. CTL, control; HPX, hypoxia; TAT-HSP70. HSP70 fusion protein.

7. MKP-1 inactivates ERK

Recently, it has been reported that MAPKs may be regulated by MKP-1 and other dual-specificity phosphatases [34,35,38]. To examine the potential role of MKP-1 on the activation of MAPKs under hypoxia condition, we measured the phosphorylation of three MAPKs in cardiomyocytes treated with MKP-1 inhibitor. The phospho-ERK levels showed a dramatic increase at 1hr after treatment with MKP-1 inhibitor under hypoxia, suggesting MKP-1 regulates the ERK activation in hypoxia (Fig. 11). However, neither p38 MAPK nor JNK were significantly affected by MKP-inhibitor. These results, therefore, suggest that the MKP-1 phosphatase is induced in cardiomyocytes under hypoxia, and that expression of this phosphatase specifically regulates ERK activation.

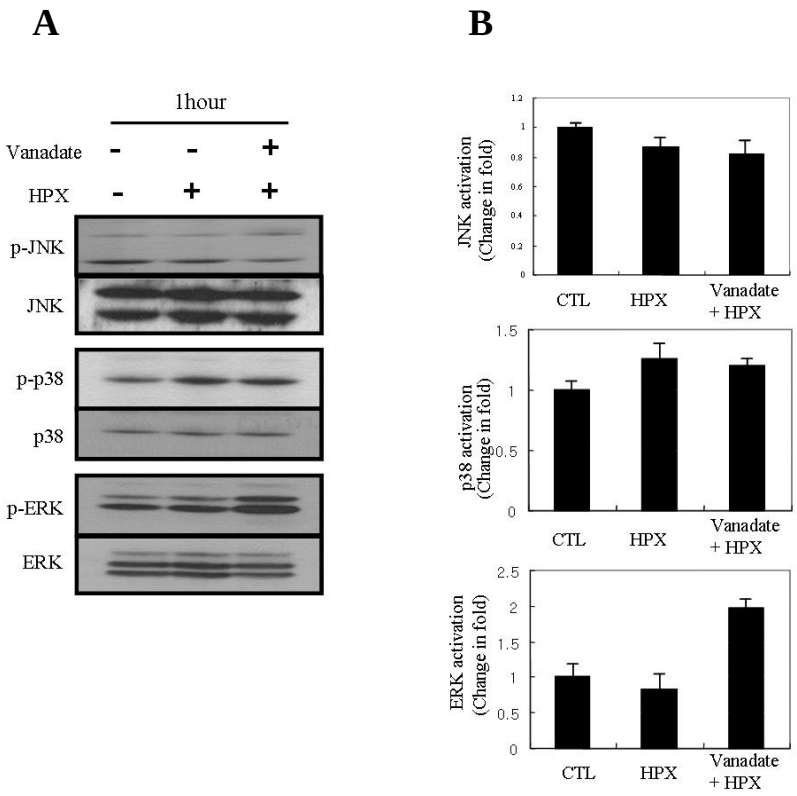

Figure 11. Effect of MKP-1 inhibitor on the activation of MAPKs under hypoxic condition. Cardiomyocytes were pretreated with 100uM vanadate for 1hr, and subjected to hypoxia for 1hr. Samples were harvested, and phosphorylation of MAPKs was determined by immunoblotting (A). Each signal was quantified by scanning densitometry (B).

8. MKP-1 inhibition increases caspase-3 activity

To further confirm whether MKP-1 was really involved in apoptotic events, we examined the effect of MKP-1 inhibitor on the caspase-3 activity in cardiomyocytes under hypoxia. Surprisingly, MKP-1 inhibitor could significantly decrease the caspase-3 activity under hypoxia whereas HSP70 inhibitor increased it (Fig.12). These data suggest that MKP-1 can activate caspase-3 pathway following cell death in cardiomyocytes.

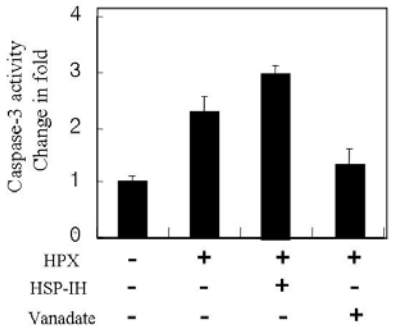

Figure 12. Effect of HSP70 and MKP-1 on the caspase-3 activity in cardiomyocytes under hypoxia. Primary cardiomyocytes were incubated with 100uM vanadate or 1uM HSP70 inhibitor for 1hr and subjected to hypoxia for 1hr. Caspase-3 activity was measured using Caspase-3 fluorescent assay kit.

IV. DISCUSSION

The present study showed that HSP70 protects cardiomyocytes from hypoxic insult by causing a sustained activation of ERK via inhibiting the expression of MKP-1 expression under hypoxic condition. Generally upregulation of HSP70 can be cytoprotective and can increase the survival of cells exposed to a wide range of lethal stimuli by decreasing apoptosis via the caspase cascade [53]. In mammalian cells, HSP70 interacts with several regulatory proteins such as transcription factors, and kinases, some of which are involved in apoptotic events [54]. Indeed, HSP70 binds to Apaf-1, thereby preventing the recruitment of procaspase-9 to the apoptosome [55,56]. In our data, it also appears to regulate ERK and MKP-1. Many studies have addressed the role of MAPK pathway against hypoxia in different cell types. The MAPK signaling cascade has been implicated as a regulator of cardiomyocyte apoptosis in vitro as well as in heart. However, considerable disagreement persists as to the functional effects attributed to these signaling, given that both pro- and anti-apoptotic regulatory roles have been reported [26-28].

MKP-1 is also induced by various stresses and mitogenic. MKP-1 was originally identified based on its specificity toward the ERK, but MKP-1 is a general MAPK phosphatase that can also dephosphorylate JNK [35]. Indeed, the

ability of MKP-1 to inactivate JNK has been linked in some cells to inhibition of apoptosis caused by stimuli [57,58]. Because MKP-1 is a specific inhibitor of MAP kinases, it may be involved in defense mechanisms against stress-induced apoptosis in cells. However, our data showed that expression of MKP-1 is involved in the cellular apoptosis against hypoxia stress in cardiomyocytes. Furthermore, this study demonstrated that induction of HSP70 in response to hypoxia may play an antiapoptotic role via inhibiting MKP-1 expression, as far as we believe, for the first time. These data support the hypothesis that HSPs are involved in not only mitochondria-mediated caspase pathways but also MAPKs pathway in hypoxia-induced cell death events.

It is unclear whether MAPKs and MKPs directly affect mitochondria-mediated caspase pathways. However, recently it has been reported that JNK and MKP-1 is associated with regulation of mitochondrial pathways of apoptosis [59]. In this study, hypoxic insult significantly induced MKP-1 expression and ERK inactivation at early time in cardiomyocytes. In this time, caspase-3 activity was also increased. When cardiomyocytes were treated with MKP-1 inhibitor under hypoxia, ERK phosphorylation was recovered and caspase-3 activity was decreased. These results indicate that ERK and MKP-1 cascade can contribute the apoptotic process through caspase-3

pathway. Actually, it has been well known that MAPKs-related apoptosis links with NF-κB [30,60]. NF-κB is a pleiotropic transcription factor implicated in the regulation of diverse biological phenomena, including apoptosis, cell survival, cell growth, and the cellular responses to ischemia/hypoxia [61,62]. Many of signaling cascades activate NF-κB by activating the IκB kinase (IKK) complex a major component of the canonical pathway. These signaling interactions occur largely via signaling crosstalk involving the mitogen-activated protein kinase/extracellular signal regulated kinase kinases (MEKKs) that are components of MAPK signaling pathways [63]. Accordingly, we could assume that the roles of ERK on the neutralizing apoptosis or cell survival under lethal stress are correlated with crosstalk of NF-κB.

Although we have found that HSP70 regulates MKP-1 expression, their relationship is not clear. The level of MKP-1 expression was defined using HSP70 inhibitor and HSP70 protein transduction. But its activity was not shown. One possibility is that HSP70 interacts with some molecule which regulates transcription of MKP-1. MKP-1 is early response gene, and its transcription involves the synergistic action of multiple cis-acting elements in the proximal promoter. So HSP70 might directly bind to MKP-1 transcription factors or cis-acting elements. Another possibility is MKP-1 is regulated by direct interaction with HSP70. These mechanisms require further studies.

We now show MKP-1 expression is involved in hypoxia-induced cardiac cell death and this is correlated precisely with ERK1/2 activity. And MKP-1 expression is regulated by HSP70. Thus, this study suggests a molecular mechanism on the beneficial role of HSP70 in hypoxia-induced apoptotic cardiomyocytes.

V. CONCLUSION

We have demonstrated that HSP70 exert modulatory effects at distinct points along the apoptosis of cardiomyocytes under hypoxia. Immunoblot analyses showed that hypoxia with serum deprivation induced expression of MKP-1 and HSP70. MKP-1 inhibition is associated with a significant decrease of caspase-3 activity and attenuation of apoptosis, while HSP70 inhibition caused an increase in caspase-3 activation and apoptosis during hypoxia. The inhibition of HSP70 also significantly enhanced the expression of MKP-1 by hypoxia. Consistently, transduction with HSP70 into cardiomyocytes under hypoxia suppressed MKP expression and recovered ERK phosphorylation. These results indicate that MKP-1 is proapoptotic during hypoxia stress, whereas HSP70 limits hypoxia-induced apoptosis through the inhibition of MKP-1 expression. Furthermore, induction of MKP-1 in hypoxic condition is regulated by the HSP70 and this correlated precisely with ERK activity.

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ABSTRACT (KOREAN)

저 산소 자극에 의한 심근세포의 사멸 기작과 열 충격 단백질의 역할 규명

<지도교수 장 양 수>

연세대학교 대학원 의과학과

신 보 희

허혈 또는 저 산소 자극은 심혈관계 질환의 중요한 요인으로 알려져 있으며, 이러한 요인들은 세포 사멸을 유발하는 신호 기작을 유도한다. 심근 세포에서 저 산소 자극에 의한 세포 사멸에 대한 조절 기전이 열 충격 단백질 또는 MAP kinas들을 통해 이루어 진다는 연구 결과가 많이 보고되어 있으나 세포 내에서의 이들 단백질 상호간의 기전은 아직까지 명확하게 알려져 있지 않다. 본 연구에서는 저 산소 자극에 의한 심근 세포의 사멸을 열 충격 단백질 70 (HSP70)과 MKP-1 그리고 ERK의 신호 기작 및 이들 단백질간의 상호작용과 관련하여 조사하였다. 신생 백서로부터 심근 세포를 분리하였으며, MTT와 caspase-3 activity assay를 통해 무혈청 배지

에서 배양된 심근 세포에서 세포 사멸이 활발히 일어나는 것을 확인할 수 있었다. 심근 세포에서 저 산소 자극에 의해 짧은 시간 내에 MKP-1의 발현이 증가 되며 반대로 ERK는 불활성 되는 것을 확인하였다. 또한 같은 시간대에서 열 충격 단백질 70의 발현이 증가되었다. 열 충격 단백질의 발현을 저해시켰을 때, MKP-1의 발현과 caspase-3의 활성이 증가되었으며, 반대로 ERK가 불활성 되었다. 또한 저 산소 자극 조건에서 심근 세포에 MKP-1 저해제인 vanadate를 처리한 결과 ERK의 활성이 현저히 회복되었고, 반대로 caspase-3의 활성은 감소하였다. 이러한 결과들을 바탕으로 열 충격 단백질 70의 역할을 다시금 확인하고자 저 산소 자극 조건에서 열 충격 단백질 70과 단백질 전달 도메인인 TAT을 조합하여 재조합 단백질을 만들어, 심근 세포 내로 전달한 후 MKP-1의 발현 양을 측정한 결과 그 양이 현저하게 감소하였고 반면에 ERK의 활성은 증가하였다. 본 연구 결과를 통해, 저 산소 자극으로 인해 유도되는 심근 세포의 사멸이 유발될 때 MKP-1이 ERK의 활성을 저해하고 이때 열 충격 단백질 70이 MKP-1의 발현을 억제하여 심근 세포를 보호하는 역할을 수행함을 알 수 있었다.

핵심되는 말 : 심근 세포, 열 충격 단백질 70, MKP-1, ERK, 저 산소 자극, 세포 사멸