Cardioprotective Effect of Survivin in Doxorubicin-Induced Myocardial Injury Model

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Graduate Program in Science for Aging

Molecular Biology

Cardioprotective Effect of Survivin in Doxorubicin-Induced Myocardial Injury Model

Directed by Professor Seok-Min Kang

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Apoptosis has been known to be an important mechanism of doxorubicin-induced cardiotoxicity. Survivin, belongs to the inhibitor of apoptosis protein (IAP) family is associated with apoptosis and alteration of cardiomyocyte

molecular pathways. Recent studies have shown that reduction of survivin expression is associated with less favorable cardiac remodeling in animal models. However, the mechanism by which survivin mediates the cardioprotective effect against doxorubicin-associated injury has not been fully determined. Therefore, we investigated the anti-apoptotic effect and molecular cellular mechanisms of survivin using a protein delivery system in doxorubicin-induced cardiomyocyte injury model.

We demonstrated that treatment of doxorubicin resulted in a significant decrease of survivin expression in the H9c2 cardiomyocyte cell line and Sprague Dawley rat hearts. In addition, Sp1 play a role in the upregulation of survivin expression, doxorubicin treatment increases a degradation of Sp1 protein via proteasome-mediated proteolysis. Transcription of survivin decreases by activation of p53. The effect of doxorubicin is consistent with the observed increase p53 protein level and the phosphorylation level at Ser^{15} in the cardiomyocyte. Following doxorubicin treatment of the cardiomyocyte, the survivin promoter has reduced levels of bound Sp1, but an increased level of bound p53. Moreover, the upstream signaling Sp1 protein, such as Akt, mTOR and p70s6k, were also decreased by doxorubicin treatment.

We constructed a recombinant survivin fused to the protein transduction domain (PTD) derived from HIV-TAT protein. And, Purified recombinant TAT-

survivin protein were efficiently delivered to H9c2 cardiomyocyte cell line and Sprague Dawley rat hearts via intraperitoneal injection, and its transduction showed an anti-apoptotic effect, demonstrated by reduced caspase-3 activity, and apoptotic index concomitantly with increased cell viability against doxorubicin-associated injury. Moreover, TAT-Survivin protein-pretreated hearts demonstrated decreased less cardiomyocyte vacuolation and myofibrillar disarray, compared with doxorubicin-associated injury hearts.

We evaluated the effect of TAT-survivin protein on cardiac functional recovery using Langendorff system. After ischemia-reperfusion injury, cardioprotective effect of TAT-survivin such as functional recovery measured by LVDP (left ventricular developed pressure) and RPP (rate pressure product) was observed.

Furthermore, the survivin pretreatment using PTD-mediated delivery has a potential cardioprotective effect against doxorubicin-induced cardiomyocyte apoptosis through the mechanism involving a decrease in the phosphorylation of p38 MAP kinase, mitochondrial Smac release, and increased expression of Bcl2 and activated nuclear translocation of CREB.

Present study suggest that firstly, survivin may be an important protein in the process of doxorubicin-induced cardiomyocyte injury, and secondly, TAT-survivin protein has a potentially protective effect against doxorubicin-induced

cardiomyocyte apoptosis. Taken together, these results may provide the insight for future development of drugs targeted on myocardial protection under cardiovascular diseases.

Key Words: Apoptosis; Survivin; TAT-HIV; Doxorubicin; Cardiomyocyte

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Ⅰ. INTRODUCTION

Doxorubicin is a quinine-containing anthracycline anticancer drug, is the most commonly used chemotherapeutic agent. Doxorubicin is frequently included in chemotherapy regimens for the treatment of stomach, lung, breast, ovarian, thyroid cancer, solid tumors, soft tissue sarcoma multiple myeloma and Hodgkin's lymphoma. Although it has strong anticancer effect, doxorubicin is

also known to cause the cardiotoxicity that leads to hypotension, arrhythmia, depression of left ventricular function and heart failure [1-3]. A variety of studies have been tried to find out the mechanisms about doxorubicin-induced cardiotoxicity, and apoptosis, including generation of reactive oxygen species, leading to DNA damage or lipid peroxidation have been inferred as the most important mechanism to explain doxorubicin-induced cardiotoxicity [4,5]. Therefore, understanding of doxorubicin-induced apoptosis signaling and investigation of the possible candidate molecules, which have an anti-apoptotic effect against doxorubicin-associated injury should be crucial.

Survivin is the smallest member of the inhibitor of apoptosis gene family. Survivin gene contain one BIR1 (baculovirus IAP repeat) domain and an extended C-terminal–helix. The 16.5 kDa cytoplasmic protein is believed to play a role in cell cycle regulation and its encoding region was localized in chromosome 17q25 [6]. Expression of the survivin gene is largely regulated at the transcription level [7]. The promoter region of survivin gene contains many transcription factors binding sites. These transcription factors include NF-κB, GATA-1, Stat3, E2F, c-myc, KLF5, DEC1, Sp1, Sp3, HIF-1α and tumor suppressor p53 and Rb [7-14]. It has been reported that down-regulation of survivin transcription by the DNA-damaging agent doxorubicin is mediated by p53 induction [15]. It is also known that p53 suppresses survivin gene expression both directly and indirectly [7-9,16,17]. Conversely, accumulated

evidences have demonstrated that Sp1 and Sp3 transcription factors transactivate the survivin promoter [18].

The survivin is selectively expressed in cells during the G2/M phase of the cell cycle in a cell cycle-dependent and its mRNA and protein are degraded at G1 by ubiquitin-dependent proteasome [13,19]. And, survivin is overexpressed during development and in most human cancer tissues [20]. It has been reported that survivin was able to suppress caspase-3 even though *in vitro* binding experiment was failed and survivin is known to interact with Smac, which is the inhibitor of other IAPs [21]. Since the main role of survivin is considered as the suppression of the caspase and apoptosis, survivin may be one of appropriate target for the doxorubicin-induced cardiomyocyte apoptosis signal cascade. Survivin has been found to be cardioprotection [22-24]. In the spontaneously hypertensive rat, reduction of survivin expression is associated with increased apoptosis and cardiac remodeling [25], and heart failure developed in survivin cardiac specific knock-out mice [26]. In addition, reduction of survivin expression in the failing human heart is associated with pre-mature cardiac death, due to a decreased number of cardiomyocyte and progressive heart failure [27].

The contribution of the phophatidylinositide-3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) signaling pathway have been demonstrated not only in various cancer cells [28,29], but also in normal cells including

cardiomyocyte [30]. In the later cases, survivin plays a role in the insulin-treated cardioprotection effect in the ischemia/reperfused heart through the PI3K/Akt/mTOR signaling pathway. The microtubule localization of survivin and the enhancement of microtubule structure stability with the overexpression of survivin are important evidences to manifest that survivin might be one of targets for doxorubicin injury because those cytoskeletal proteins are essential to heart contraction [31]. Moreover, it was reported that the inhibition of the survivin resulted in similar biochemical interaction with the treatment of doxorubicin [32]. Furthermore, it has been demonstrated that the survivin gene therapy prevents myocytes apoptosis and attenuates left ventricular systolic dysfunction in the doxorubicin-induced heart model [33]. Although its mechanism is not clearly understood, survivin is considered to be key factor regulating cell survival and suppression of apoptosis.

HIV-TAT protein is one of the smallest proteins with protein transduction domain (PTD), which contain 11 amino acids (YGRKKRRQRRR) from 47 to 57aa of HIV–TAT protein essential for its transduction function. HIV–TAT-fusion protein containing N-terminal PTD has been reported to transduce in vitro into almost all cell types tested, including all blood cells, hepatocellular carcinoma cells and leukemic T cells [34]. In the present study, a recombinant survivin fused to the PTD derived from HIV-TAT protein were delivered into the H9c2 cardiomyocyte cell line or Sprague Dawley rat hearts, because the

transfection efficacy to cardiomyocyte with the non-viral carrier is extremely poor. And then, we evaluated anti-apoptotic effect of PTD-mediated transduction of the recombinant survivin against doxorubicin-associated injury with the apoptosis-related signals.

Ⅱ. Material and Method

1. A chemical and antibodies

Doxorubicin was purchased from Tocris. Anti-survivin, anti-caspase-3 (cleaved form), anti-phospho-Akt (Ser^{473}), anti-Akt, anti-phospho-mTOR (Ser^{2481}) anti-phospho-CREB (Ser^{133}), anti-CREB, anti-phospho-p38 (Thr^{180}/Tyr^{182}), anti-p38, anti-phospho-p53 (Ser^{15}) and anti-mTOR antibodies were obtained from Cell Signaling. Anti-Sp1, anti-phospho-p70S6K (Thr^{421}/Ser^{424}), anti-p70S6K, anti-Smac, anti-Lamin B, anti-β-actin and anti-GAPDH antibodies were purchased from Santa Cruz Biotechnology. Anti-p53, anti-VDAC1, anti-Bcl-2, anti-Bax antibodies were obtained from Abcam. Anti-his-tag antibody was obtained from Thermo. Anti-cytochrome c antibody was included in ApoAlert® Cell Fractionation Kit (Clontech).

2. Construction and protein purification of TAT-survivin

The survivin fragment was inserted into the BamHI and XhoI sites of the pHis/TAT vector [35] for TAT-survivin fusion protein expression. *Escherichia coli* BL21 pLysS (Novagen) was transformed to pHis/TAT-survivin plasmid, and then grown for 24 hours at 37℃ in LB broth supplied with 100 μg/ml kanamycin and 34 μg/ml chloramphenicol and while shaking at 200 rpm. Protein expression was induced by the addition of 1 mM β-D-1-

thiogalactopyranoside (IPTG) for 80 minutes while shaking at 37℃. The pHis/TAT-survivin fusion protein were then isolated using a urea-denaturing protein purification protocol. The bacterial pellet was harvested and resuspended in buffer (8 M urea, 100 mM NaCl, and 20 mM Hepes, pH 8.0). The clarified lysate was loaded onto a Ni-IDA affinity column (Macherey-Nagel). TAT-survivin protein was eluted with imidazole in buffer. The proteins were loaded onto a PD-10 desalting column in order to exchange the buffer to phosphate buffer saline (PBS).

3. Animal care

Sprague-Dawley rats (ORIENT-Charles River Technology), weighing 200-250 g, were used for all experiments. Animals were housed in plastic cages with soft bedding under a 12/12 hours reversed light and dark cycle, and freely accessed to food and water. 8 weeks old rats were randomly divided into three groups and they were pretreated with 2 mg/kg of TAT-survivin for 1 hour and then 15 mg/kg of doxorubicin by intraperitoneal injection. After, rats were treated again with 2mg/kg of TAT-survivin at 1, 3, 5 and 7 days. Anesthesia was induced with 5% isoflurane in a gas mixture of 70% nitrous oxide and 30% oxygen, and was maintained using 2% in the same gas mixture during the operation. Rectal temperature was maintained at 37.0±0.51℃ with a homeothermic blanket throughout the procedure (Harvard Apparatus). During

these procedures, every effort was made to minimize usage and their sufferings.

4. Langendorff –perfused rat heart

Heart were removed from anesthetized Sprague Dawley rats and mounted on a Langendorff apparatus, perfused with a modified Krebs-Henseleit buffer containing (mmol/L) 112 NaCl, 5 KCl, 1 KH_2PO_4, 1.2 $MgSO_4$, 25 $NaHCO_3$, 11.5 Glucose, 2 Pyruvate, 1.25 $CaCl_2$ and 0.026 Na2EDTA. The buffer was saturated with 95% O2/5% CO2 (pH 7.4, 37℃) as previously described [36]. A 50% ethanol-filled balloon was inserted into the left ventricular cavity and coupled to a pressure transducer. The balloon was inflated until a left ventricular end diastolic pressure (LVEDP) reached 10 mm Hg. A 4-0 silk suture with its ends through a small plastic tube was placed around the left coronary artery, approximately 2–3 mm from its origin. After 30 minutes stabilization, myocardial ischemia was initiated by clamping the plastic tube onto the surface of the heart. After 30 minutes of ischemia, the snare was released and coronary perfusion was restored for 10 to 40 minutes. Successful coronary occlusion and reperfusion were verified by visual inspection of the color in the apex and typical electrocardiogram changes. Contractile performance was evaluated on the basis of heart rate, left ventricular systolic pressure (LVSP), LVEDP and left ventricular developed pressure (LVDP) with a hemodynamic analyzing system.

5. Cell culture

The rat heart-derived myoblast cell line, H9c2 (2-1) cardiomyocyte was obtained from the American Type Culture Collection (ATCC). H9c2 cardiomyocyte were cultured in Dulbecco's modified Eagle's Medium (DMEM) supplement with 10% fetal bovine serum (FBS) and 1% penicillin streptomycin (Gibco) at 37℃ in humidified atmosphere of 5% CO_2. All experiments were performed using cells between 10 to 25 passage numbers.

6. Transduction of pHis/TAT-survivin fusion protein into cells and hearts

H9c2 cardiomyocyte were incubated for 24 hours in 100 mm culture plate and changed to 0.5% FBS DMEM for 24 hours starvation. After starvation, for the transduction of TAT-survivin, cells were treated with various concentration of pHis/TAT-survivin fusion protein. The cells were washed by using PBS for 2 times while centrifuged.

Sprague Dawley rats were randomly divided and they were treated with 1 to 10 mg/kg of TAT-survivin for 1 hour by intraperitoneal injection. After sacrifice, heart was isolated and transduced TAT-survivin was detected by immune blot using survivin or His-tag antibody and immunohistochemistry using His-tag antibody.

7. Subcellular fractionation

Cell pellets were separated into cytoplasmic, nucleic, membrane and mitochondrial fractions using the Mitochondria Isolation Kit (Qiagen) according to the manufacturer's instructions. After washing, cells were suspended in lysis buffer to disrupt the plasma membrane without solubilizing it and to aid in the isolation of cytosolic proteins. Plasma membranes and compartmentalized organelles, such as nuclei, mitochondria, and the endoplasmic reticulum, remained intact and were pelleted by centrifugation at 1000 x g for 10 minutes. The resulting pellet was resuspended in disruption buffer, repeatedly passed through a narrow-gauge needle (26 or 21 gauge), and re-centrifuged to pellet nuclei, cell debris, and unbroken cells at 1000 x g for 10 minutes. The supernatant which contains mitochondria and the microsomal fraction was recentrifuged to pellet mitochondria at 6000 x g for 10 minutes. After removal of the supernatant, mitochondria and nuclei were dissolved using a lysis buffer and analyzed by SDS-PAGE as outlined below.

8. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA isolated from cells using QIAzol-Reagent (Qiagen) was reverse transcribed using Omniscript Reverse Transcriptase (Qiagen). The cDNAs was amplified using TaKaRa Ex Taq^{TM} polymerase (Takara). Levels of mRNA were

analyzed by RT-PCR using the following primers. PCR products were separated by electrophoresis in a 1% agarose gel containing Gel-red (Biotium).

The primer sequence is

Rat survivin F, 5'-ATG GGT GCT ACG GCG CTG CCC-3'

Rat survivin R, 5'-TCA GCG TAA GGC AGC CAG CTG-3'

Rat Sp1 F, 5'- GGA GAA AAC AGC CCA GGA TGC-3'

Rat Sp1 R, 5'-CTC ATC CGA ACG TGT GAA GC-3'

Rat Bcl-2 F, 5'-GAC GCG AAG TGC TAT TGG T-3'

Rat Bcl-2 R, 5'-TCA GGC TGG AAG GAG AAG AT-3'

Rat GAPDH F, 5'-AAT GCA TCC TGC ACC ACC AAC TGC-3'

Rat GAPDH R, 5'-GGA GGC CAT GTA GGC CAT GAG GTC-3'.

9. Immunoblot analysis

The cells were solubilized in a cell lysis buffer (Cell signaling) and centrifuged at 14,000 rpm for 1 hour at 4℃. The proteins samples were separated by a SDS–polyacrylamide gel and transferred to polyvinylidene difluoride membranes. After blocking in TBS-tween 20 (TBS-T, 0.1% tween 20) containing 10% non-fat dry milk for 1 hour at room temperature, and incubated with primary antibodies for overnight at 4℃ using Rocker machine. The membrane was washed three times with TBS-T for 7 minutes, and incubated for 1 hour at room temperature with appropriate horseradish peroxidase-conjugated secondary antibodies. The antigen-antibody bands were detected using enhanced chemiluminescence reagent kit (Amersham pharmacia biotech) and quantified by densitometry.

10. Cell viability, caspase-3 activity and TUNEL assays

Cell viability was measured by the classical 2-(4,5-dimethyltriazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (Amresco). The formazan crystals were dissolved by adding dimethylsulfoxide and absorbance was measured at the 570 nm with a spectrophotometer. The activity of caspase-3 in the cells was determined spectrophotometrically with an Apoalert^{TM}CPP32/caspase-3 assay kit (BD Biosciences, USA) by measuring

the release of the chromophore, p-nitroanilide (pNA), following hydrolysis of DEVD-pNA. Terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling (TUNEL) analysis was performed with a commercially available kit according to the manufacturer's instructions (Intergen).

11. Immunofluorescence microscopy

The cells were incubated on Lab-Tek chamber slides (Nalgene Nunc, USA), and were fixed with 3% paraformaldehyde for 10 minutes at room temperature and washed with PBS. The cells were permeabilized in 0.5% Triton X-100 buffer (0.5% Triton X-100, 20 mM Hepes-KOH, pH 7.9, 50 mM NaCl, 3 mM $MgCl_2$, 300 mM sucrose) in PBS for 10 minutes and washed with PBS. They were blocked with PBS containing 0.3% goat serum and 5% bovine serum albumin for 1 hour at room temperature and then incubated for 1 hour with mouse monoclonal survivin antibody or rabbit monoclonal Sp1 antibody. The cells were washed with PBS once and incubated with FITC-488, goat anti-mouse IgG or Rhodamine Red-X, goat anti-rabbit IgG (dilution 1:500; Invitrogen) as secondary antibody for 1 hour in dark room. After washing, the cells were mounted with ProLongantifade reagent containing DAPI. The immunoreactive signals were visualized by confocal laser scanning microscope LSM700 (Carl Zeiss, Germany).

12. Chromatin immunoprecipitation (ChIP)

ChIP assay was performed according to Hsu *et al.* with minor modifications [37]. Briefly, formaldehyde-treated nuclear lysates were subjected to immunoprecipitation with anti-Sp1 and anti-p53 antibodies. The cross-linked chromatin complex was reversed in the presence of proteinase K and DNA fragments were purified. The DNA fragment (257-bp) of survivin promoter region (between -265 and -9) was amplified by PCR using a pair of primers as depicted in Fig. 5A: Rat survivin promoter F, 5'-AGG ACA CAA CTC CCA GCA AG- 3'; Rat survivin promoter R, 5'-CGC CAC AAT CCC TAA TTC AA- 3'. PCR condition was as follows: at 95℃ for 30 sec; at 56℃ for 30 sec; and at 72℃ for 60 sec. After 36 cycles of PCR, products were analyzed by 2% agarose gel electrophoresis. For input data (5%), 25 μl aliquots of 500 μl samples were taken before immunoprecipitation.

13. Tissue staining and immunohistochemistry

Rat hearts were perfused with saline, removed, and fixed in 4% paraformaldehyde for 24 hours in 4℃. Then, they were embedded in paraffin and prepared in 4 μM cross sections. Rat hearts were stained with conventional hematoxylin and eosin (H&E) and Masson's trichrome for analysis of histology and fibrosis. For immunohistochemical analyses, sections were

deparaffinzed in xylene, rehydrated in graded ethanol solutions and washed with distilled water as described from other study [30]. Section were blocked with 5% goat serum in antibody diluent for 30 minutes and incubated overnight at 4℃ with following antibodies: survivin, cleaved caspase-3 and His-tag (1:150). After rinsing three time in PBS, RTU horseradish peroxidase streptavidin (SA570A, Vector Laboratories) was applied and the slides are incubated for 10 minutes. For color development, 3,3'-diaminobenzidine (DAB) was used.

14. Statistical analysis

Data are expressed as means ± S.E. One-way ANOVA with Bonferroni post hoc correction was used for comparison between the groups. Values of *p* less than 0.05 were considered statistically significant.

Ⅲ. RESULTS

1. Doxorubicin induces apoptosis in the H9c2 cardiomyocyte cell line

We first investigated the effect of doxorubicin on cell viability in the H9c2 cardiomyocyte cell line. Cells were treated with various concentrations of doxorubicin with 0.5% FBS for 24 hours, cell viability gradually decreased to about 49.8 ± 0.4% with up to 1 μM of doxorubicin (Fig. 1A and B). The treatment of 5 μM of doxorubicin did not further decrease cell viability compared to that of 1 μM of doxorubicin. We next studied whether the apoptosis were altered by doxorubicin treatment. The activation of caspase-3 was dramatically increased when the cells were treated with no more than 1 μM of doxorubicin (Fig. 1C). Also, TUNEL assay showed that there were increased apoptosis index in doxorubicin-induced H9c2 cardiomyocyte cell line (Fig. 1D and E). These data suggest that doxorubicin treatment of H9c2 cardiomyocyte resulted significant increase in cell death.

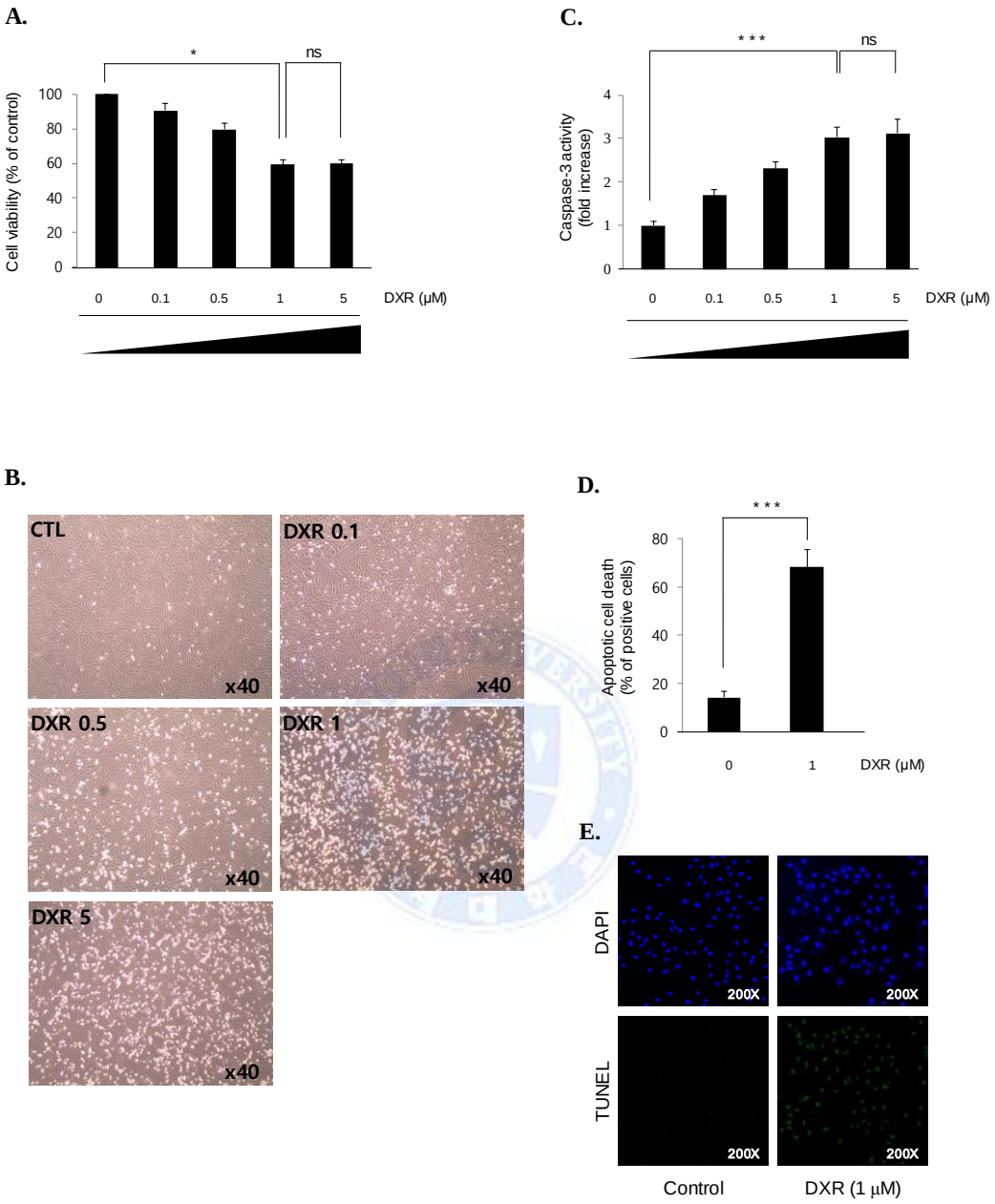

Figure 1 legend (the following page)

Fig.1. Effect of doxorubicin on cell viability and apoptosis in the H9c2 cardiomyocyte cell line. (A, B and C) The H9c2 cardiomyocyte were treated with various concentration of doxorubicin (DXR) for 24 hours. (A) Cell viability was assessed by the MTT assay. (B) Cells were photograped with microscope of Nikon coolpix 4500. (C) Caspase-3 activity was determined using caspase-3 activity assay kit. (D and E) Cells were treated with 1 μM of of doxorubicin for 24 hours. Apoptotic cells were measured by the TUNEL assay. The results present the means of three independent experiments. **P* < 0.05; ****P* < 0.001. Ns: no significant.

2. Doxorubicin decreases survivin expression level in the H9c2 cardiomyocyte cell line

We next investigated the effect of doxorubicin on survivin expression in the H9c2 cardiomyocyte cell line. Cells were treated with various concentrations of doxorubicin for 24 hours. As shown in Fig. 2A, survivin protein levels were decreased by doxorubicin in a concentration-dependent manner. And, the immunofluorescence microscopy image showed that the protein of survivin was down-regulated by doxorubicin (1 μM) treatment (Fig. 2B). Fig. 2C shows the time-dependent decrease of survivin protein level, when treated with 1 μM of doxorubicin for variable time. We further studied changes in mRNA level of survivin by doxorubicin treatment. RT-PCR showed that there were decreased in survivin mRNA levels (Fig. 2D). These data suggest that doxorubicin decreases survivin protein level and mRNA level in H9c2 cardiomyocyte cell line.

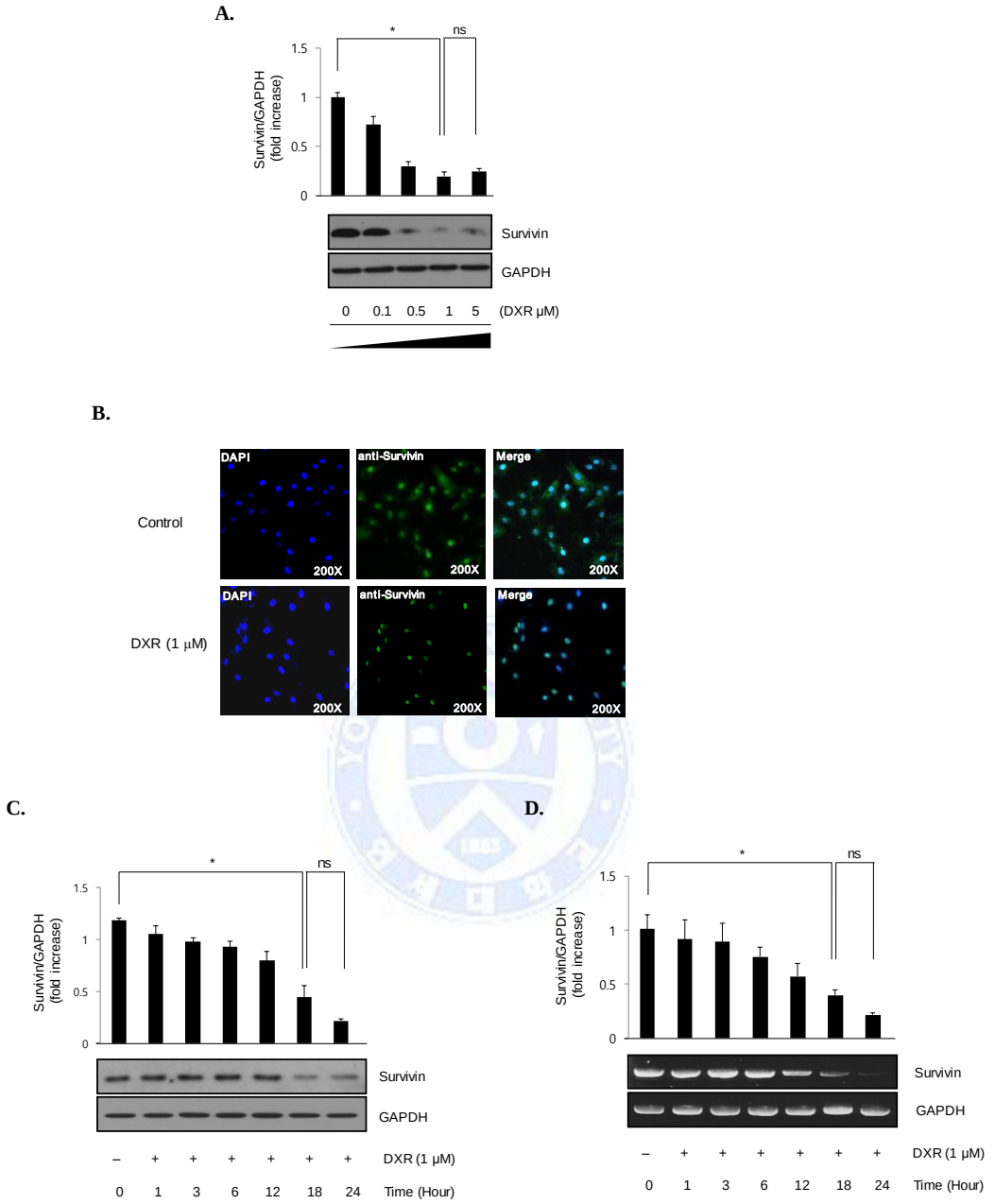

Figure 2 legend (the following page)

Fig. 2. Effect of doxorubicin on survivin protein and mRNA level in the H9c2 cardiomyocyte cell line. (A) The H9c2 cardiomyocyte cell line were treated with various concentration of doxorubicin (DXR) for 24 hours. Equal amounts of protein were separated by SDS-PAGE gel, and immunoblot analysis was performed using anti-survivin antibody. (B) The cells were treated with 1 μM of doxorubicin treatment for 24 hours. Survivin proein was also observed with confocal microscopy using primary anti-survivin antibody and FITC-conjugated secondary antibody (200x). (C and D) The cells were treated with 1 μM of doxorubicin treatment for the indicated time point. (C) Whole cell lysates were separated by SDS-PAGE gel and analyzed by immunoblotting with antibodies against survivin and GAPDH, and (D) total RNA was analyzed by RT-PCR (28 cycles) using primers specific to survivin and GAPDH gene. The results present the means of three independent experiments. $*P < 0.05$. Ns: no significant.

3. Doxorubicin decreases Sp1 protein level in the H9c2 cardiomyocyte cell line

It has been reported that Sp1 plays a role in the up regulation of survivin expression in cancer cells at the transcriptional level [38], we first examined Sp1 level in doxorubicin-treated H9c2 cardiomyocyte cell line. When the cells were treated with various amounts of doxorubicin for 12 hours, Sp1 protein level gradually decreased to about 0.42±0.1 with up to 1 μM of doxorubicin (Fig. 3A). Fig. 3B shows the time-dependent decrease of Sp1 protein level, when treated with 1 μM of doxorubicin for variable time. However, there was no change observed in Sp1 mRNA with the same condition (Fig. 3C). And, the immunofluorescence microscopy image showed that the protein of Sp1 was down-regulated by doxorubicin treatment (Fig. 3D). These results indicate that Sp1 protein is regulating survivin expression level.

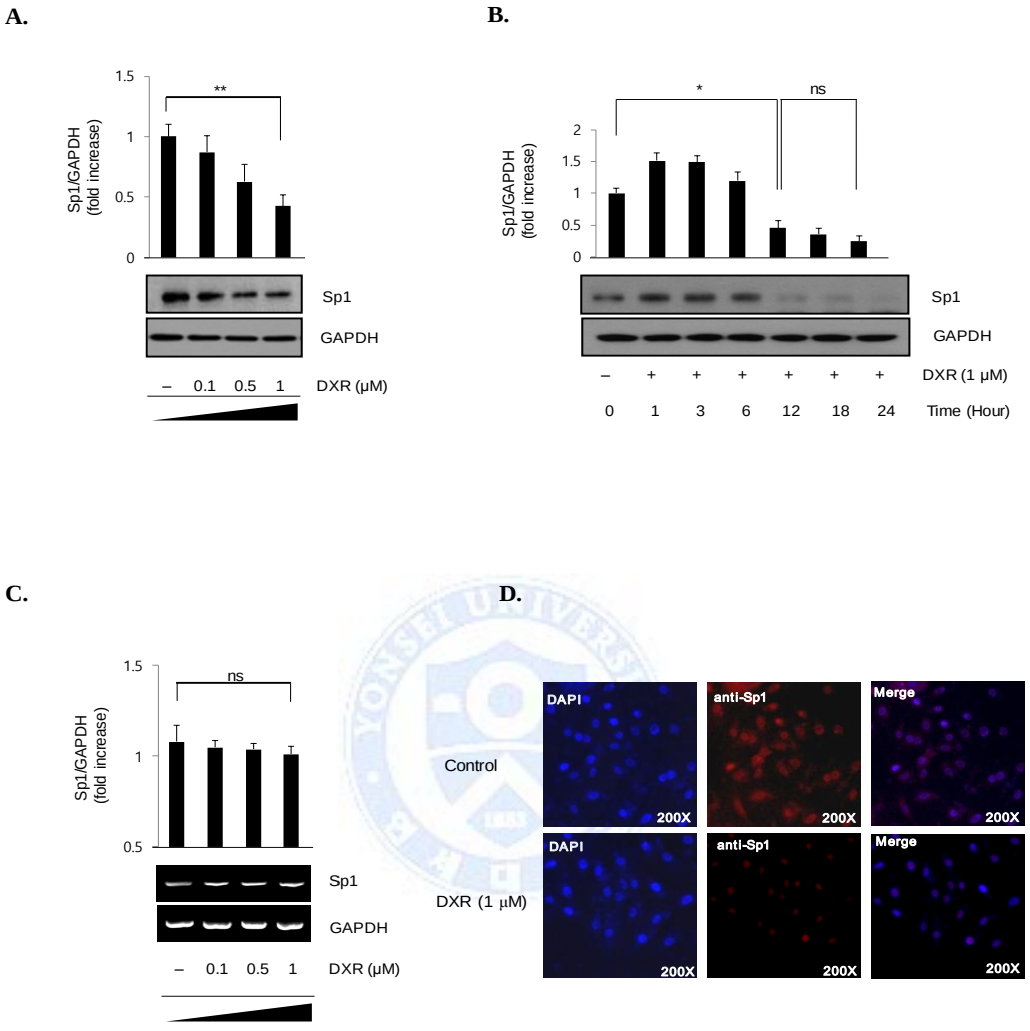

Figure 3 legend (the following page)

Fig. 3. Effect of doxorubicin on Sp1 protein and mRNA level in the H9c2 cardiomyocyte cell line. (A) The H9c2 cardiomyocyte cell line were treated with various concentration of doxorubicin (DXR) for 12 hours. Equal amounts of protein were separated by SDS-PAGE gel, and immunoblot analysis was performed using anti-Sp1 antibody. (B) The cells were treated with 1 μM of doxorubicin treatment for the indicated time point. Whole cell lysates were separated by SDS-PAGE gel and analyzed by immunoblot analysis with antibodies against anti-Sp1 and anti-GAPDH antibody. (C) Cells were treated with various concentration of doxorubicin for 12 hours. Total RNA was analyzed by RT-PCR (28 cycles) using primers specific to Sp1 and GAPDH gene. (D) The cells were treated with 1 μM of doxorubicin treatment for 12 hours. Sp1 protein was also observed with confocal microscopy using primary anti-Sp1 antibody and Rhodamine-conjugated secondary antibody (200x). The results present the means of three independent experiments. $*P < 0.05$, $**P < 0.01$. Ns: no significant.

4. Doxorubicin increases p53 protein level and its phosphorylation in the H9c2 cardiomyocyte cell line

Previously study showed that repression of the anti-apoptosis survivin transcription by activation of p53 [15]. Further, it has showed that doxorubicin increase in p53 protein level and in the phosphorylation level at Ser^{15} in various human cancer cell lines [16]. Therefore, we investigated the effect of doxorubicin on p53 activation and protein level in the H9c2 cardiomyocyte cell line. This is correlates with doxorubicin-stimulated increase in p53 phosphorylation (Ser^{15}) level at 1 hour, followed by an increase in p53 protein level at 12 hours (Fig. 4A, B and C).

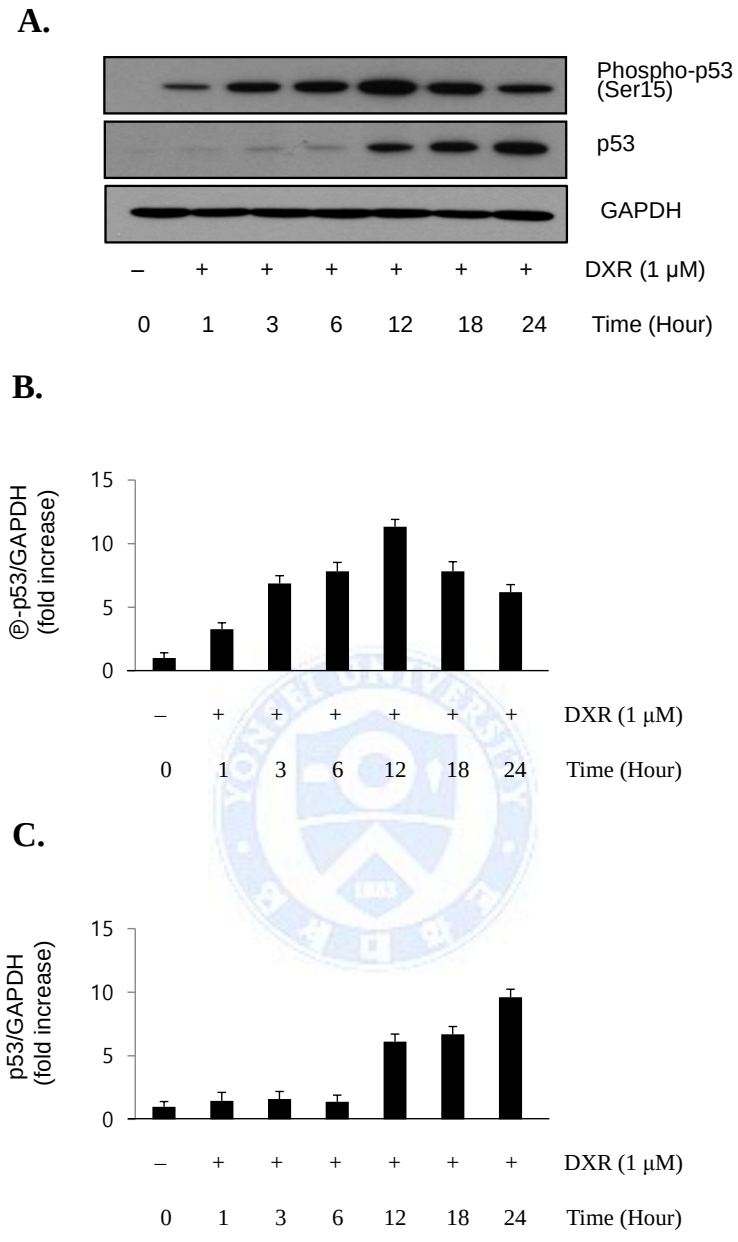

Figure 4 legend (the following page)

Fig. 4. Effect of doxorubicin on p53 activation and protein level in the H9c2 cardiomyocyte cell line. (A, B and C) The H9c2 cardiomyocyte cell line were treated with 1 μM of doxorubicin (DXR) treatment for the indicated time point. Whole cell lysates were separated by SDS-PAGE gel and analyzed by immunoblot analysis with antibodies against anti-p53, anti-phospho-p53 (Ser^{15}) and anti-GAPDH antibody. The results present the means of three independent experiments.

5. Promoter sequence analysis of survivin gene

The survivin gene promoter contains several Sp1 and p53 binding sites with some variations between species as shown in Fig. 5, indicating the evolutionarily conserved participation of Sp1 and p53 in the survivin gene regulation.

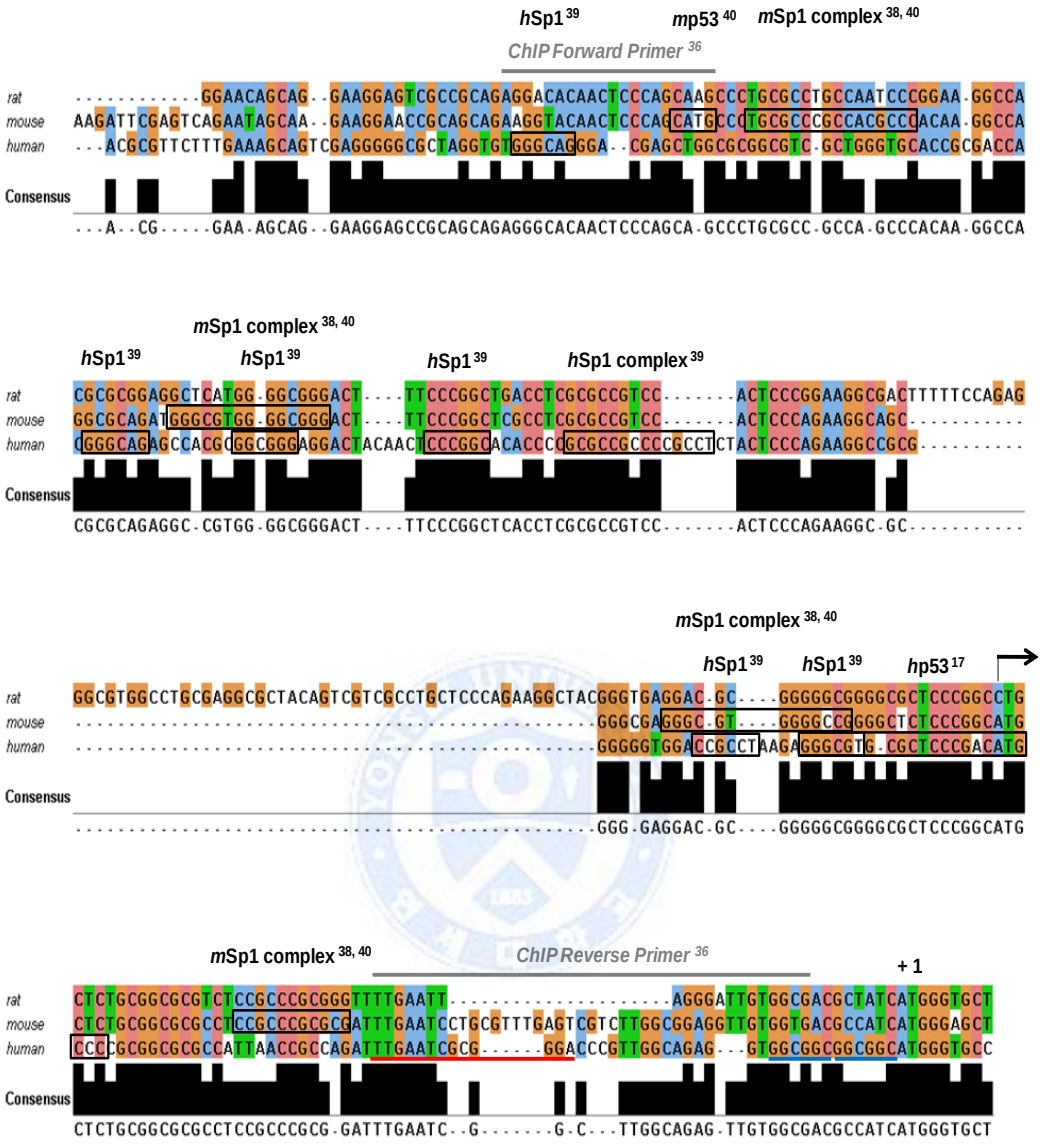

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Fig. 5. ClustalW alignment of the proximal promoter regions of the human, mouse and rat survivin gene. Multiple sequence alignment (ClustalW2) of the proximal promoter regions of the human, mouse and rat survivin genes. Black arrow indicates the transcriptional start site and +1 points out the translation start codon. Canonical p53, Sp1, Sp1-like sites of the human and mouse survivin genes are boxed [15,39-41]. Two gray bars indicate the primers for ChIP analysis [37]; a red bar corresponds to human CHR sequence; two blue bars highlight human CDE regions [42].

6. Doxorubicin suppresses survivin expression by the transcriptional activation of p53

In order to determine whether Sp1 and/or p53 are recruited to the endogenous survivin promoter region in response to doxorubicin, we first examined Sp1 or p53 levels in doxorubicin-induced H9c2 cardiomyocyte cell line. When the cells were treated with 1 μM of doxorubicin for 8 hours, p53 activation and protein level gradually increased. However, there was no change observed Sp1 protein level with same condition (Fig. 6A). We next performed ChIP experiments using in H9c2 cardiomyocyte cell line. The fixed cell extracts were incubated with anti-Sp1 or anti-p53 antibodies. The DNA-protein complexes captured by the antibodies were PCR-amplified to detect the presence of the survivin promoter. Anti-Sp1 or anti-p53 antibodies detected the survivin promoter if Sp1 or p53 alone or in a complex that is bound to the survivin promoter. Data presented in Fig. 6B demonstrate that in the absence of doxorubicin the survivin promoter was occupied by Sp1, but not by p53. Following doxorubicin treatment of the H9c2 cardiomyocyte, the survivin promoter had reduced levels of bound Sp1, but an increased level of bound p53. This observation is in accordance with the findings of Esteve et al. using human HCT116 cell line [16] and recently Hsu et al. using rat C6 glioma cell line [37].

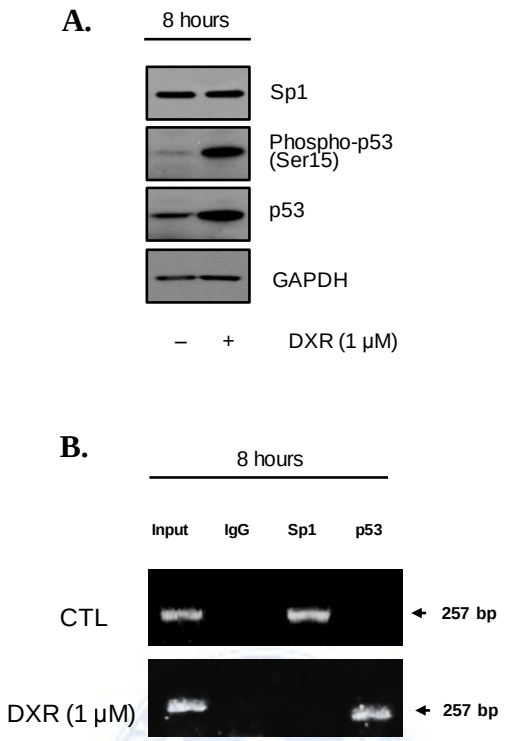

Fig. 6. Effect of doxorubicin on the transcriptional activity of Sp1 and p53 in the cardiomyocyte cell line. (A and C) The H9c2 cardiomyocyte cell line were treated with 1 μM of doxorubicin for 8 hours. (A) Whole cell lysates were separated by SDS-PAGE gel and analyzed by immunoblot analysis with antibodies against anti-Sp1, anti-p53, anti-phospho-p53 (Ser^{15}) and anti-GAPDH antibody. (B) Cross-linked cell lysates were subjected to ChIP analysis with anti-Sp1 or anti-p53 antibody. RT-PCR (36 cycles) was performed with ChIP primer as listed in Materials and Methods. The results present the means of three independent experiments.

7. Doxorubicin induces protein degradation of Sp1 by associated with proteasome-mediated proteolysis

To evaluate whether doxorubicin could down-regulate Sp1 protein level in the H9c2 cardiomyocyte cell line caused by proteasome activation, cells were pretreated with MG132 (50 nM) for 1 hour and then doxorubicin add for 12 hours. The reduction of Sp1 protein due to doxorubicin treatment was significantly prevented by pretreatment of proteasome inhibitor MG132 (Fig. 7A). In the same condition, MG132 pretreatment also inhibited the doxorubicin-mediated down-regulation of survivin in both protein and mRNA levels (Fig. 7 B and 7C, respectively). These data suggest that doxorubicin treatment triggers a degradation of Sp1 protein via proteasome-mediated proteolysis seems to block this process through an unknown mechanism.

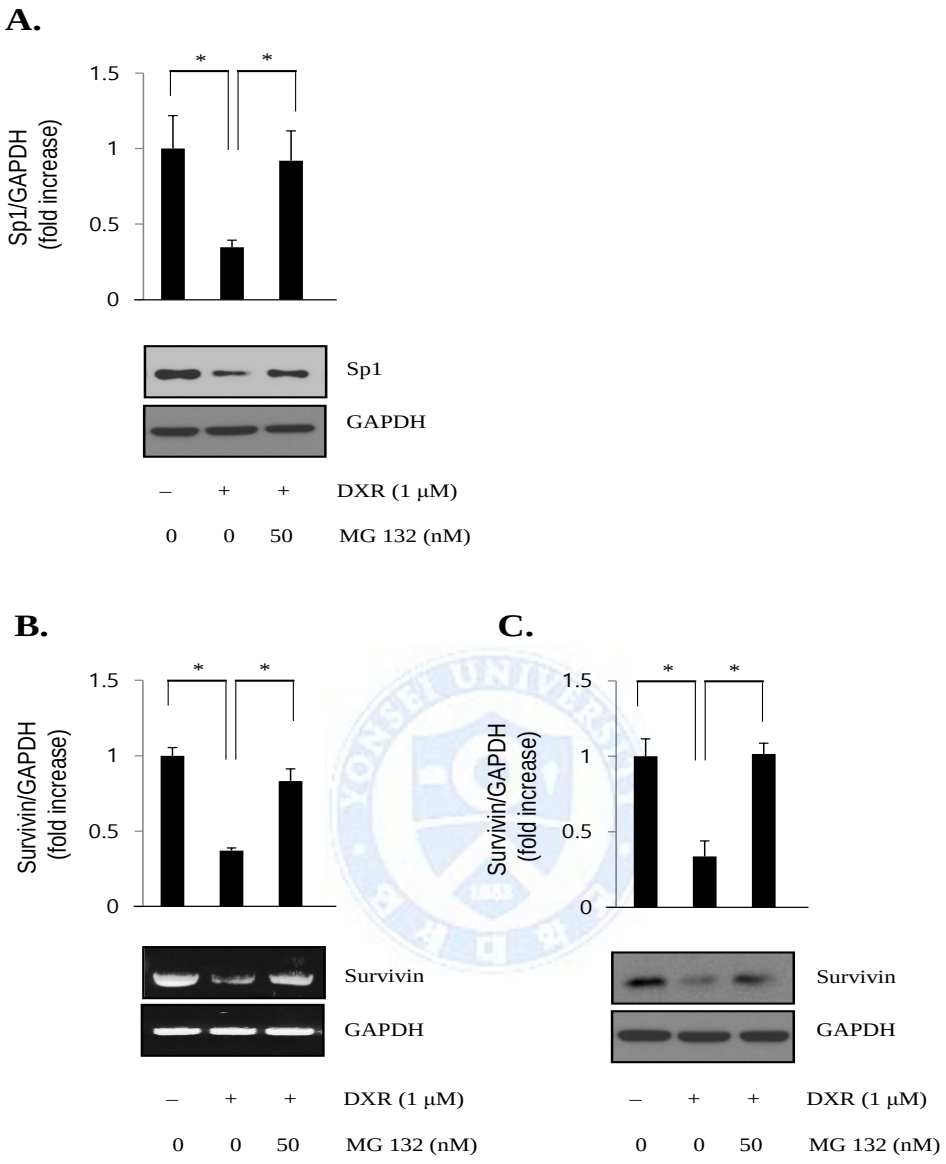

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Fig. 7. Effect of doxorubicin on Sp1 protein levels. (A to C) The H9c2 cardiomyocyte cell line were pretreated with proteasome inhibitor MG132 (50 nM) for 1 hour and treated with 1 μM doxorubicin (DXR) for either 12 hours (A) or 24 hours (B and C). Whole cell lysates were analyzed by immunoblot analysis using anti-Sp1 and anti-survivin antibodies and total RNA was analyzed by RT-PCR (28 cycles) with specific primers to survivin gene. Values are means ±S.D. *$p < 0.05$.

8. Doxorubicin increases protein degradation of Sp1 via inactivation of Akt/mTOR/p70S6K pathway

Accumulating evidence has suggested that insulin-treated cardiovascular protections are closely related to the activation of PI3K/Akt/mTOR pathway [43-47]. And, IGF-1 treatment leads to induced nuclear abundance of Sp1 through PI3K/Akt/mTOR pathway in HCF-7 Cells [48]. We first verified the effect of doxorubicin on the Akt/mTOR/p70S6K pathway in the H9c2 cardiomyocyte cell line. As shows in Fig. 6A, B and C, phosphorylation of Akt, mTOR and p70S6K were decreased by treatment of H9c2 cardiomyocyte with doxorubicin.

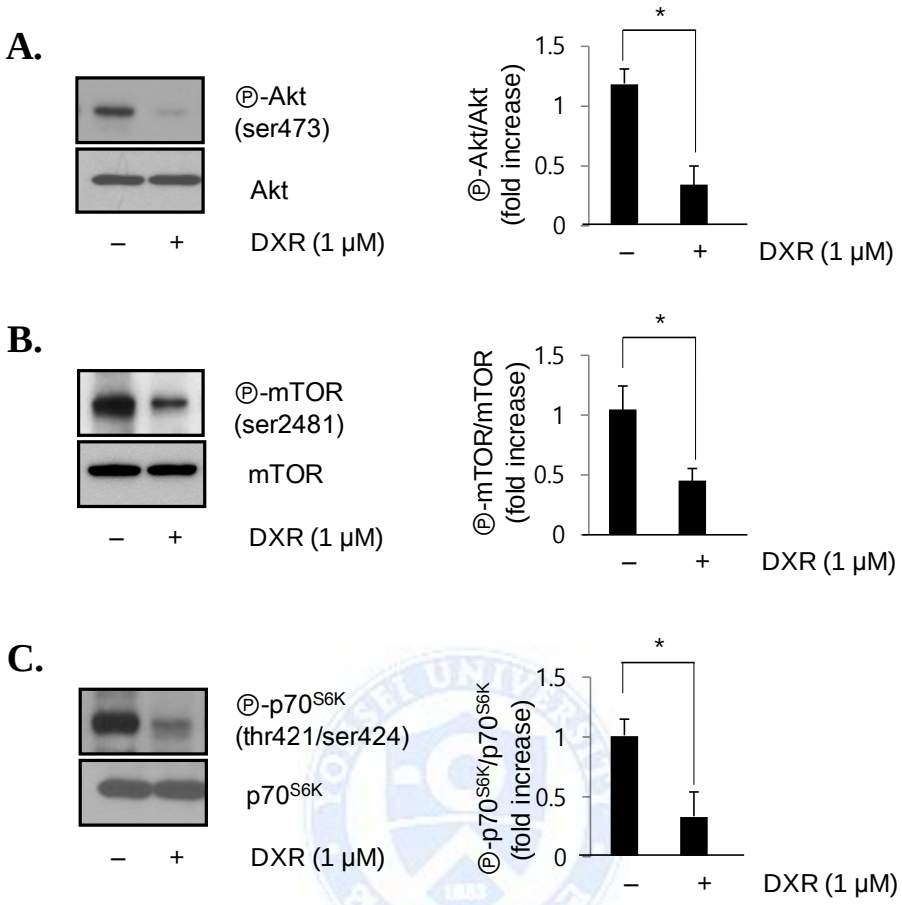
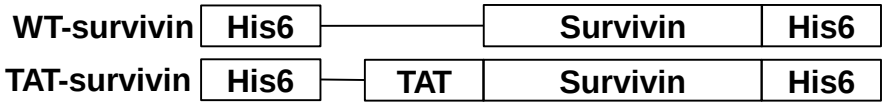


Fig. 8. Effects of doxorubicin on Sp1 upstream pathway in the H9c2 cardiomyocyte cell line. (A, B and C) The H9c2 cardiomyocyte cell line were treated 1 μM doxorubicin (DXR) for 12 hours. Whole cell lysates were analyzed by immunoblot analysis for Akt, mTORC1 and p70S6K activation using antibodies listed in Materials and Methods. Note that these blots represent one of three independent experiments. Values are mean ±S.D. *$p < 0.05$.

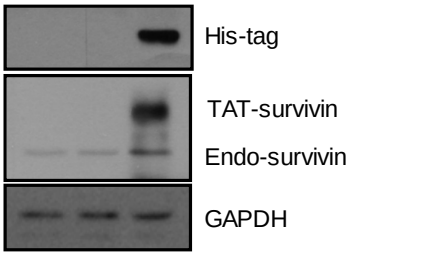
9. Delivery of TAT-survivin fusion protein into the H9c2 cardiomyocyte cell line

We constructed a recombinant survivin fused to the protein transduction domain derived from HIV-TAT protein (Fig. 9A). TAT-survivin protein was transduced into the H9c2 cardiomyocyte cell line efficiently in a concentration-dependent manner (data not shown). TAT-survivin (1 μM) were detected by immunoblot analysis using anti-His-tag or anti-survivin antibody (Fig. 9B). And the confocal microscopy image using primary anti-His-tag antibody and Rhodamine-conjugated secondary antibody also showed efficient transduction of TAT-survivin protein (Fig. 9C). The colors stained with red and blue in the image represent the transduced TAT-survivin proteins and nuclei, respectively.

A.



B.



– – + TAT-survivin (1 μM)

– + – Wt-survivin (1 μM)

C.

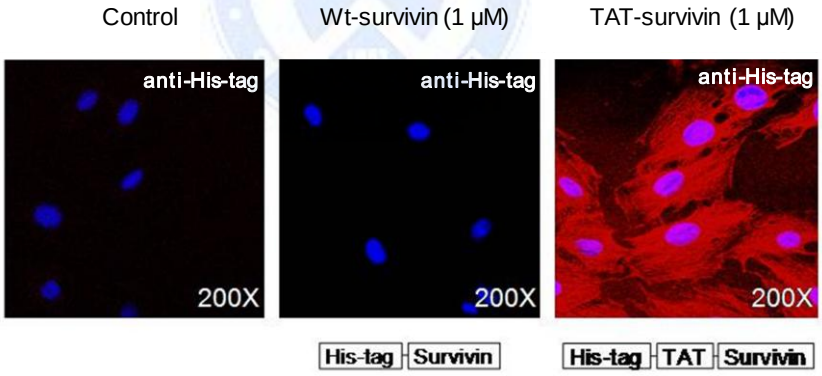


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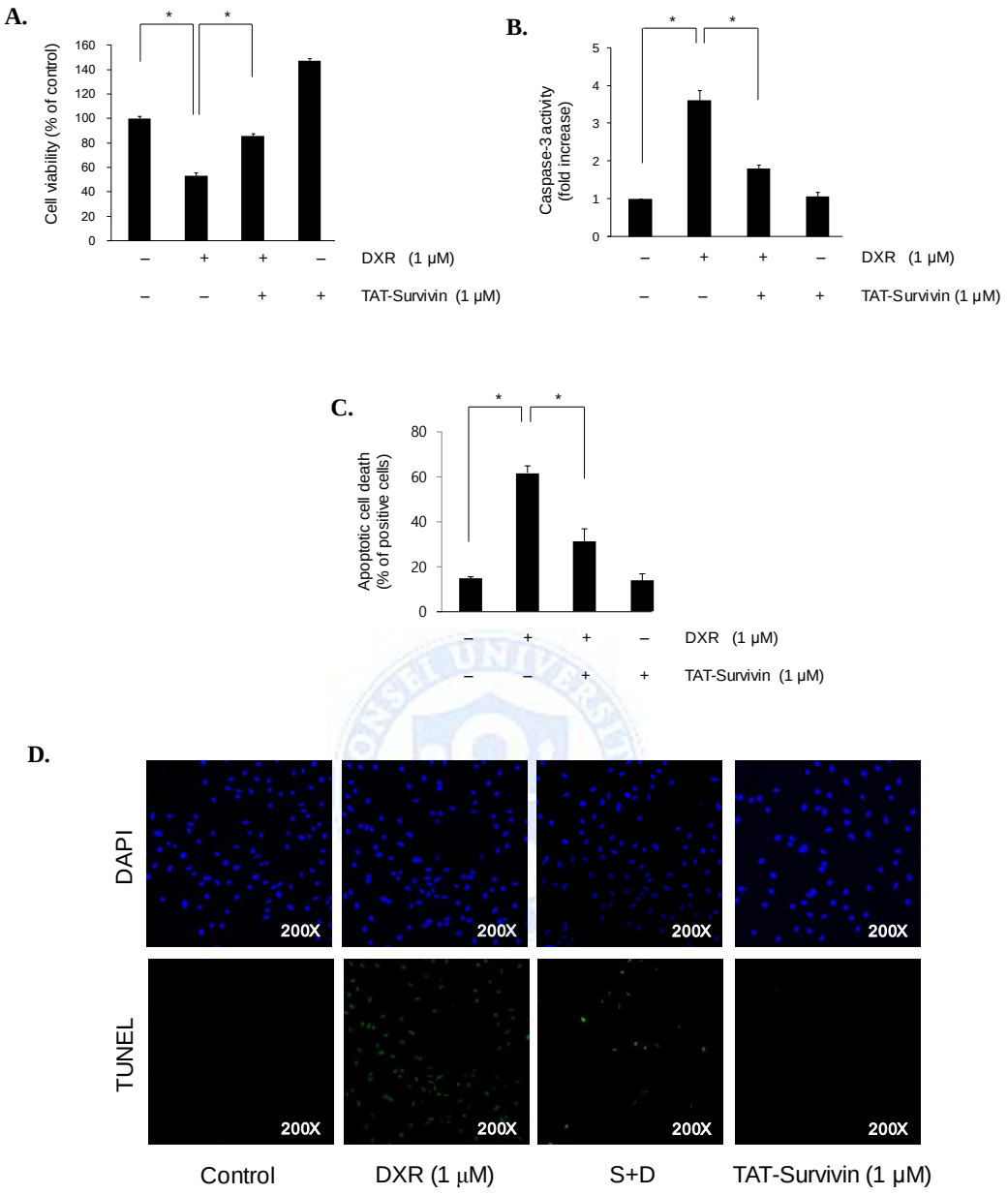
Fig.9. Intracelluar delivery of recombinant TAT-survivin protein in the H9c2 cardiomyocyte cell line. (A) Survivin gene was into the BamHI and XhoI sites of the pHis/TAT vector for TAT-survivin fusion protein expression. (B) The H9c2 cardiomyocyte cell line were treated with TAT-survivin protein for 1 hour. Immunoblot analysis was performed with anti-His-tag or anti-survivin antibody. Normalized densitometric intensities of levels for transduced protein are shown as average fold changes. (B) Protein transduction was also observed with confocal microscopy using primary anti-His-tag antibody and FITC-conjugated secondary antibody.

10. TAT-survivin protects H9c2 cardiomyocyte cell line from doxorubicin-induced apoptosis

We examined whether the intracellular delivery of TAT-survivin in this manner would protect H9c2 cardiomyocyte cell line against doxorubicin-induced injury. As shown in Fig. 10A, significant protective effect of the TAT-survivin protein against doxorubicin-induced cell death was observed in the H9c2 cells. Caspase-3 activity was increased more than 3-fold after doxorubicin treatment. However, TAT-survivin transduction significantly decreased caspase-3 activity (Fig. 10B). Also, TUNEL assay demonstrated the anti-apoptotic effect of TAT-survivin against doxorubicin-induced injury (Fig. 10C and D). Next, we investigated the subcellular localization of proapoptotic mitochondrial protein Smac and cytochrome C, endogenous inhibitors of IAPs, released from mitochondria during apoptosis. TAT-survivin protein inhibited the release of Smac and cytochrome C from the mitochondria to the cytoplasm induced by doxorubicin treatment (Figs. 10E and F).



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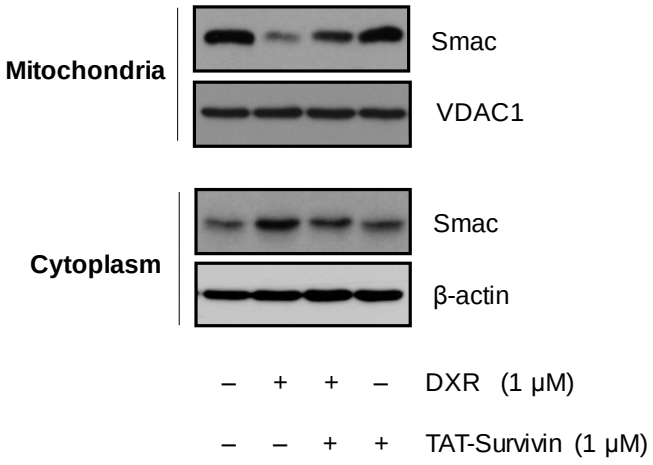
E.



F.

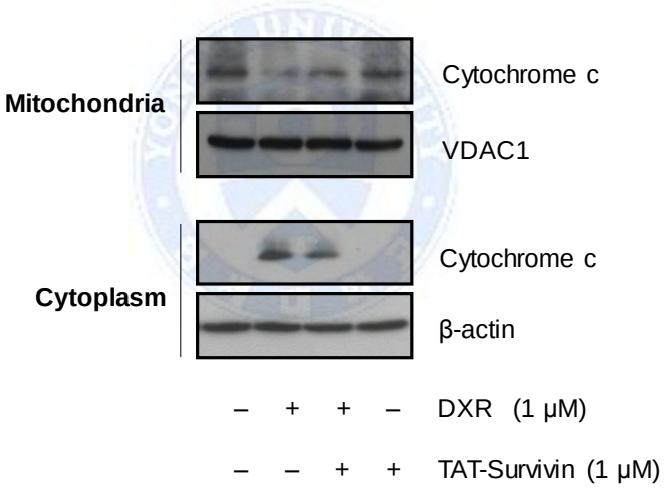


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Fig. 10. Effect of TAT-survivin transduction on cell viability and apoptosis under doxorubicin treatment in the H9c2 cardiomyocyte cell line. (A to F) The H9c2 cardiomyocyte cell line were treated with 1 μM of TAT-survivin for 1 hour and then subjected to doxorubicin (DXR) treatment for 24 hours. (A) Cell viability was assessed by the MTT assay. (B) Caspase-3 activity was determined using caspase-3 activity assay kit. (C and D) Apoptotic cells were measured by the TUNEL assay. (E and F) Mitochondrial and cytoplasm fractions were prepared, and equal amounts of protein were separated by SDS-PAGE gel. The release of Smac or cytochrome c from mitochondria were detected using anti-Smac or anti-cytochrome c antibody. VDAC1 bands show that equal amounts of sample were loaded. Note that blots represent one of three independent experiments.The results present the means of three independent experiments. Values are mean ±S.D. *$P < 0.05$.

11. TAT-survivin transduction leads to recovery of Bcl-2 expression

Apoptotic signals were evaluated to define the effect of TAT-survivin transduction on the doxorubicin-induced apoptosis. To get information about the relationship between doxorubicin-induced apoptosis and apoptosis related signals, we first measured the changes of Bcl-2, Cleaved caspase-3 and Bax by immunoblot analysis. As shown in Fig. 11, Cleaved caspase-3 and Bax protein level were increased following doxorubicin treatment (Fig. 11 A and C), whereas Bcl-2 level was decreased (Fig. 11B). Treatment of TAT-survivin protein led to significant recovery of protein level of Bcl-2 in doxorubicin stimulus (Fig. 11B). Moreover, TAT-survivin transduction significantly decreased cleaved caspase-3 protein level (Fig. 11A). However, there was no change observed in Bax protein with TAT-survivin treatment in same condition (Fig. 11C). RT-PCR showed that the expression of Bcl-2 was down-regulated by doxorubicin treatment, which was significantly recovered by TAT-survivin transduction (Fig. 11D).

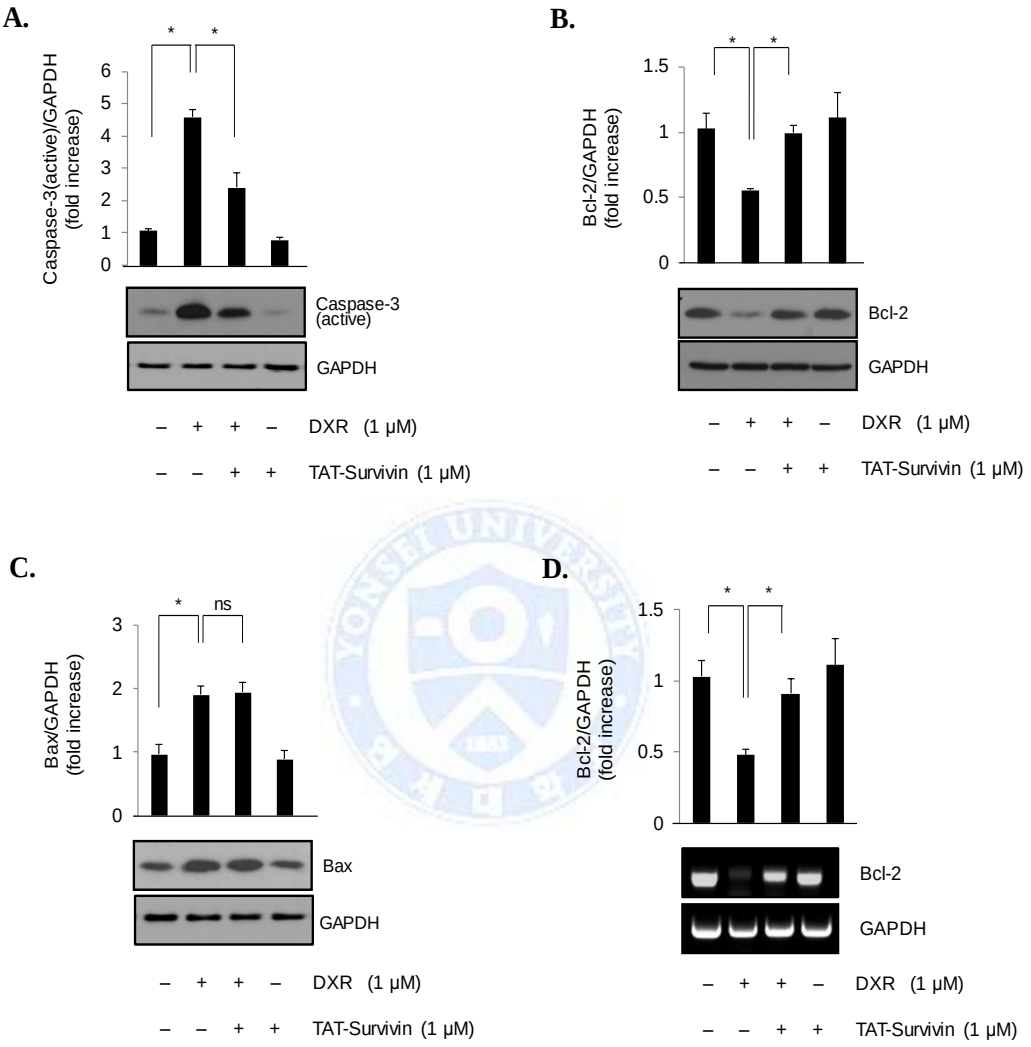

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Fig.11. Effects of TAT-survivin on Bcl-2 expression in the H9c2 cardiomyocyte cell line. (A to D) The cells were treated with 1 μM of TAT-survivin for 1 hour and then subjected to doxorubicin (DXR) treatment for 24 hours. Whole cell lysates were immunoblotted with (A) anti-Cleaved caspase-3, (B) anti-Bax and (C) anti-Bcl-2 antibody. (D) Total RNA was purified from cells and subjected to RT-PCR using primers specific for Bcl-2. Note that blots represent one of three independent experiments. Values are mean ±S.D. *P < 0.05.

12. TAT-survivin restores the transcriptional activity of CREB by p38 inactivation

The activation of cyclic adenosine monophosphate response element-binding protein (CREB), which is known as the transcription factor of Bcl-2 was quantified in both whole cell lysate and nuclear protein. As shown in Fig. 13A, doxorubicin prevented the nuclear translocation of CREB. However, a significant restoration of CREB nuclear translocation was observed in the treatment of TAT-survivin protein (Fig. 12A). The phosphorylated form of p38 MAP kinase, which is known as the upstream signal regulator of doxorubicin-induced apoptosis was decreased with TAT-survivin treatment (Fig. 12B).

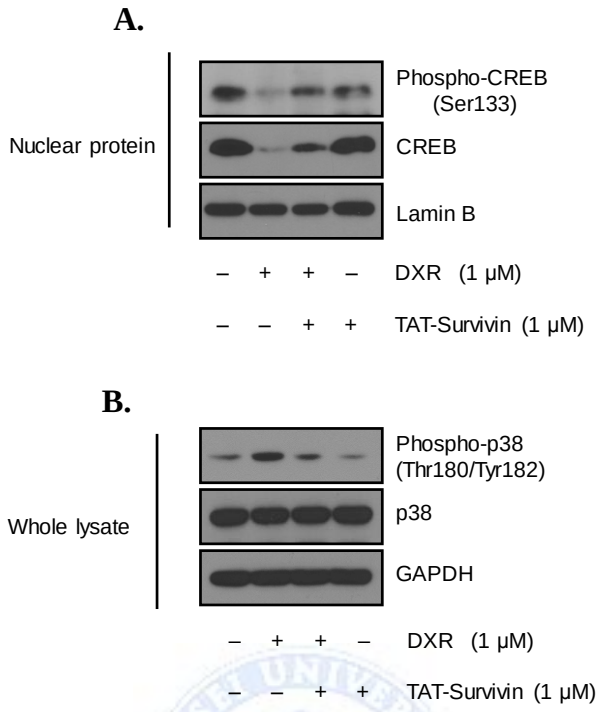

Fig.12. Effect of TAT-survivin on CREB and p38 MAP kinase in doxorubicin-treated H9c2 cardiomyocyte cell line. (A and B) The cells were treated with 1 μM of TAT-survivin for 1 hour and then subjected to doxorubicin (DXR) treatment for 24 hours. (A) Equal amounts of nuclear protein were separated by SDS-PAGE gel, and immunoblot analysis was performed using anti-phospho-CREB (Ser^{133}) or anti-CREB antibody. LaminB bands show that equal amounts of sample were loaded. (B) Whole lysates were immunoblotted with anti-phospho-p38 (Thr^{180}/Tyr^{182}) or anti-p38 antibody. Note that blots represent one of three independent experiments.

13. Doxorubicin induces apoptosis in myocardial rat model.

To investigate the effect of doxorubicin on apoptosis in Sprague Dawley rat model. Sprague Dawley rat were treated at 1, 3, 5 and 7 days with 15 mg/kg of doxorubicin (Fig. 13A). After sacrifice, injured heart was isolated and paraffin embedded for Haemotoxylin and Eosin staining, immunohistochemistry for cleaved caspase-3 and Masson's trichrome staining. Fig. 13 shows the representative images of each group treated with doxorubicin or saline below. The results clearly show the heart affected by multifocal areas of patchy and scattered cardiomyocyte with vacuolation in doxorubicin-induced Sprague Dawley rat model (Fig. 13B). And, immunohistochemistry revealed that cleaved-caspase-3 expression was increased in heart from rats treated with doxorubicin compared to that of the saline-treated group (Fig. 13C). However, vacuolated cardiomyocyte are not observed adjacent to areas of fibrosis (Fig. 13D).

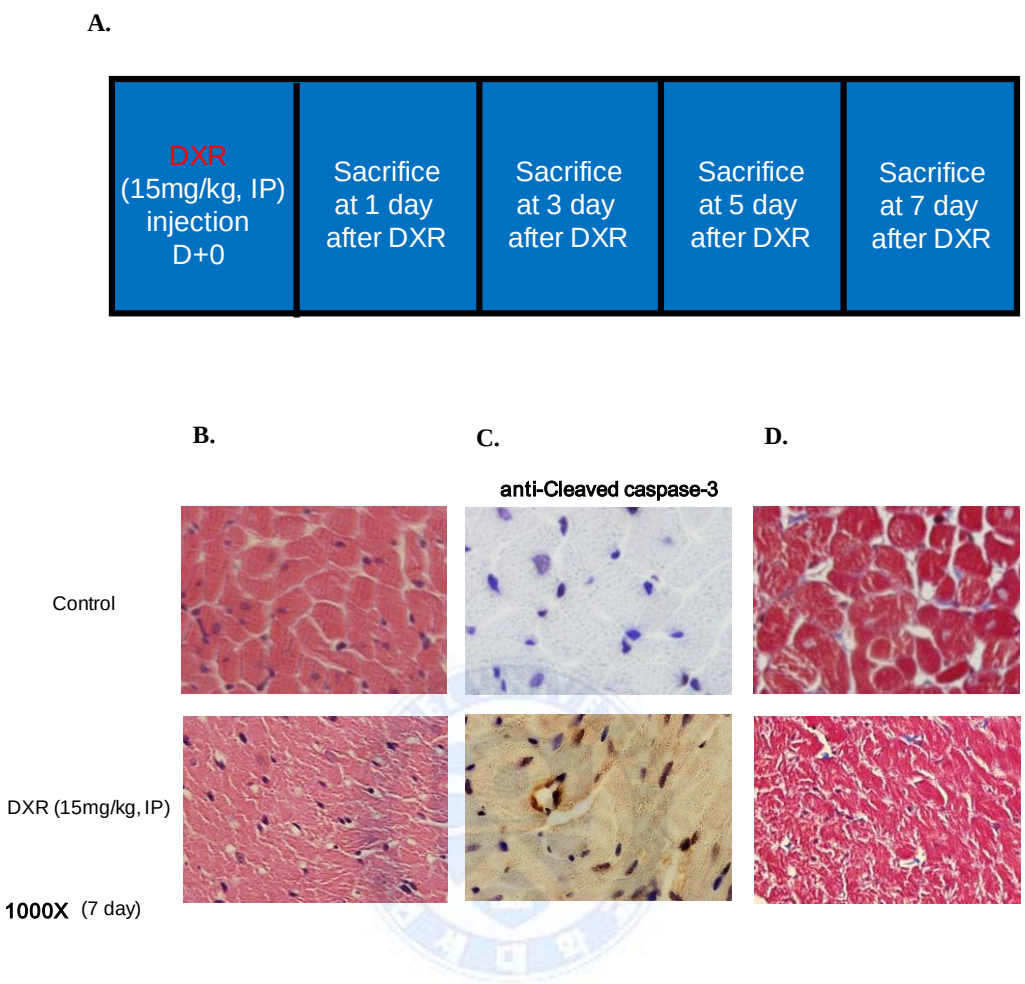

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Fig.13. Effect of doxorubicin on apoptosis in myocardial rat model. (A) Diagram of experimental protocol. Sprague Dawley rats (n=10) were treated with 15 mg/kg of doxorubicin (DXR) by intraperitoneal (IP) injection. Photomicrographs show representative myocardial section 7 day after the injection stained with (B) Haemotoxylin and Eosin, (C) immunohistochemistry for cleaved caspase-3 and (D) Masson's trichrome staining (Control, Saline group vs. DXR, doxorubicin (15 mg/kg) group).

14. Doxorubicin decreases survivin expression levels in myocardial injury rat model

We next investigated the effect of doxorubicin on survivin expression in myocardial rat model. Sprague Dawley rat were treated at 1, 3, 5 and 7 days with 15 mg/kg of doxorubicin by intraperitoneal injection. As shown in Fig. 14A, survivin protein levels were decreased by doxorubicin in a time-dependent manner. And, the immunohistochemistry microscopy image showed that the protein of survivin was down-regulated by doxorubicin treatment (Fig. 14B). We further studied changes in mRNA level of survivin by doxorubicin treatment. RT-PCR showed that there was decreased in survivin mRNA levels (Fig. 14C). These data suggest that doxorubicin decreases survivin protein and mRNA level in myocardial rat model.

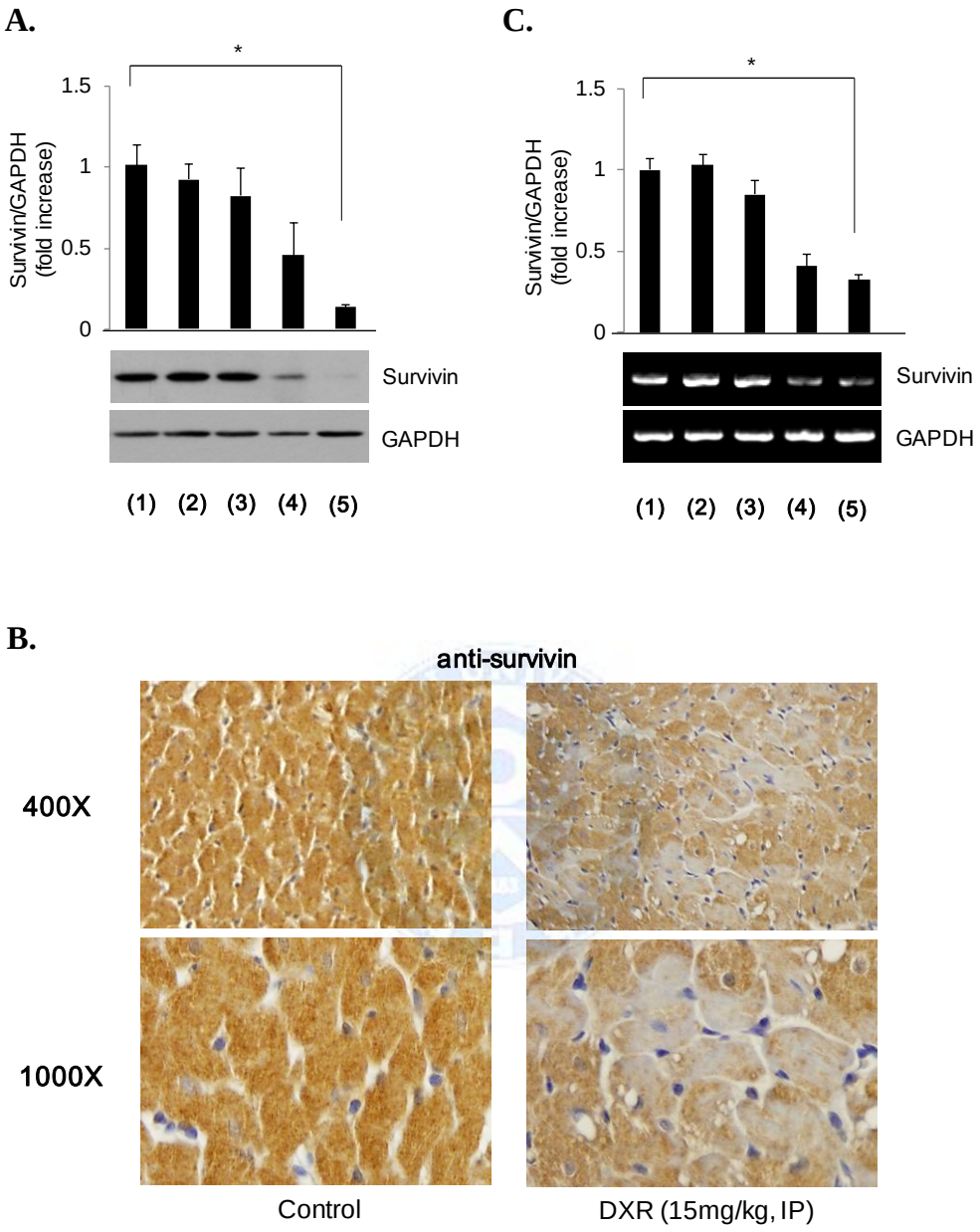

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Fig.14. Change of survivin protein and mRNA in doxorubicin-induced myocardial rat model. (A, B and C) Sprague Dawley rats (n=10) were treated with 15 mg/kg of doxorubicin (DXR) by intraperitoneal (IP) injection. After sacrifice, injured heart was isolated and survivin protein measured by (A) immunoblot analysis or (B) immunohistochemistry analysis and (C) survivin mRNA measured by RT-PCR. Lane 1, saline control; Lane 2, Doxorubicin (15 mg/kg) day 1; Lane 3, Doxorubicin (15 mg/kg) day 3; Lane 4, Doxorubicin (15 mg/kg) day 5; Lane 5, Doxorubicin (15 mg/kg) day 7. Values are mean ±S.D. *$P < 0.05$.

15. Doxorubicin suppresses survivin gene by modulating transcription factors Sp1 and p53 in myocardial injury rat model

We previously show that doxorubicin decreases survivin gene by Sp1 or p53 in the H9c2 cardiomyocyte cell line. We next examined on Sp1 or p53 in doxorubicin-induced myocardial rat model. Sprague Dawley rats were treated with 15 mg/kg of doxorubicin by intraperitoneal injection. As shown in Figs. 15A and B, Sp1 protein levels were decreased by doxorubicin in a time-dependent manner. However, in the results present study showed that doxorubicin-stimulated increase in p53 phosphorylation (Ser^{15}) level at 5 day, followed by an increase in p53 protein level at 7 day (Figs. 15A, C and D).

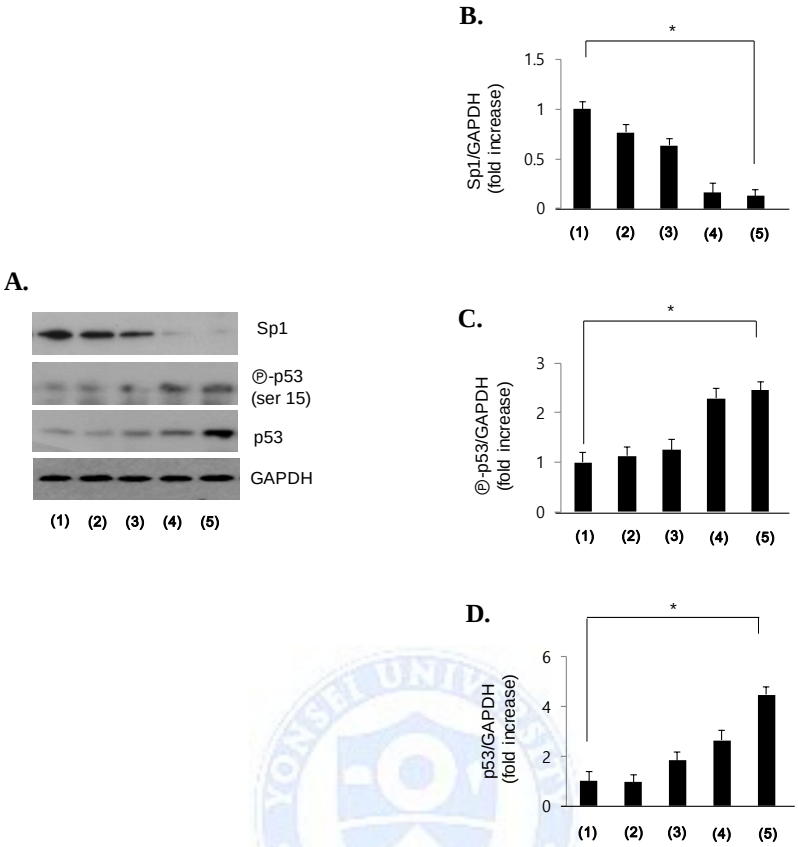

Fig.15 Effect of doxorubicin on Sp1 and p53 in myocardial rat model. (A, B and C) Sprague Dawley rats (n=10) were treated with 15 mg/kg of doxorubicin by intraperitoneal injection. After sacrifice, injured heart was isolated and Sp1 protein, phospho-p53 (Ser^{15}) and p53 protein level measured by immune blot analysis. Lane 1, saline control; Lane 2, Doxorubicin (15 mg/kg) day 1; Lane 3, Doxorubicin (15 mg/kg) day 3; Lane 4, Doxorubicin (15 mg/kg) day 5; Lane 5, Doxorubicin (15 mg/kg) day 7. Values are mean ±S.D. *$P < 0.05$.

16. Doxorubicin inhibits activation of the Akt/mTOR/p70S6k pathway in myocardial injury rat model

Our result present study shown that doxorubicin decreases activation of Akt, mTOR and p70S6k in the H9c2 cardiomyocyte cell line. Therefore, we examined the effect of doxorubicin on the Akt/mTOR/p70S6k pathway in myocardial rat model. Sprague Dawley rats were treated with 15 mg/kg of doxorubicin by intraperitoneal injection. The results in Fig. 16A, B and C shows that activation of Akt, mTOR and p70S6k were decreased by treatment of myocardial rat model with doxorubicin.

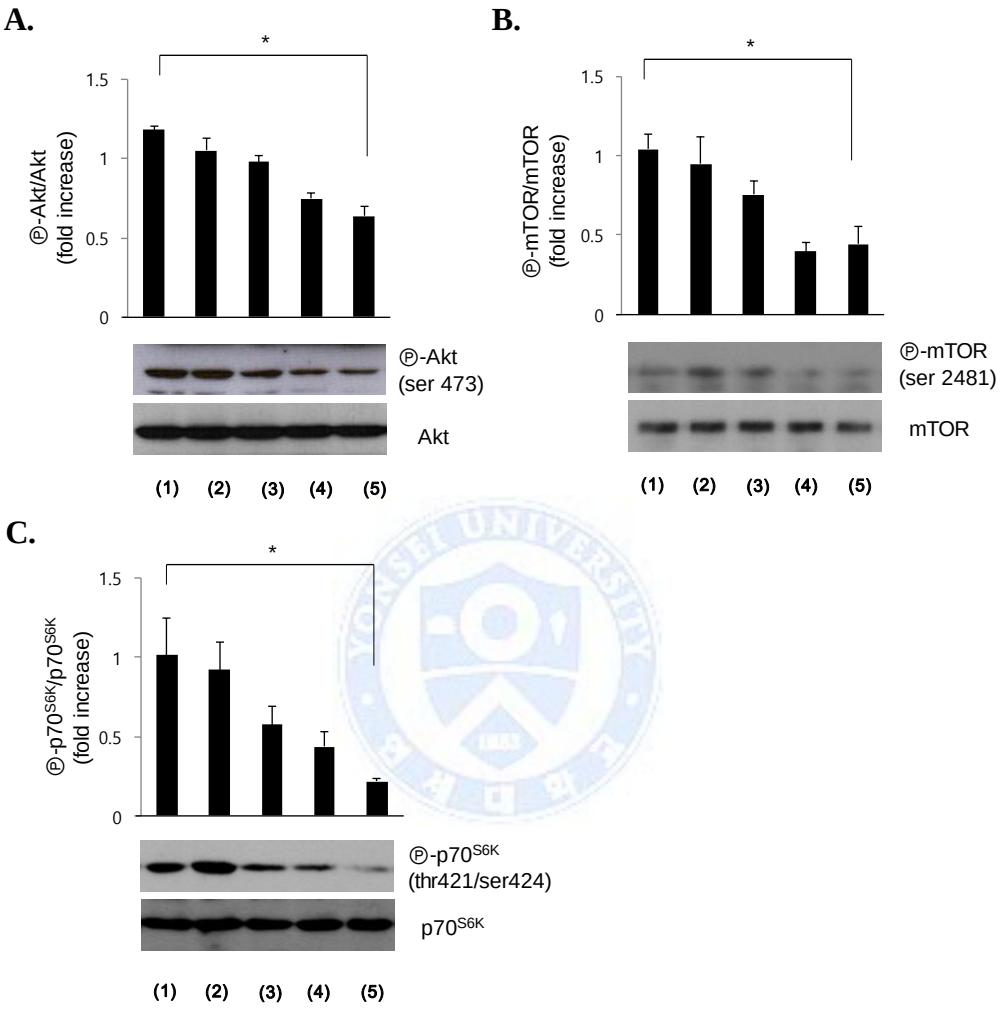

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Fig.16. Effect of doxorubicin on Sp1 upstream in myocardial rat model. (A, B and C) Sprague Dawley rats (n=10) were treated with 15 mg/kg of doxorubicin by intraperitoneal injection. After sacrifice, injured heart was isolated and phospho-Akt (Ser^{473}), Akt protein, phospho-mTOR (Ser^{2481}), mTOR protein, phospho-p70S6k (Thr^{421}/Ser^{424}) and p70S6k protein level measured by immunoblot analysis. Lane 1, saline control; Lane 2, Doxorubicin (15 mg/kg) day 1; Lane 3, Doxorubicin (15 mg/kg) day 3; Lane 4, Doxorubicin (15 mg/kg) day 5; Lane 5, Doxorubicin (15 mg/kg) day 7. Values are mean ±S.D. *$P < 0.05$.

17. Delivery of TAT-survivin fusion protein into the Sprague Dawley rat hearts

Sprague Dawley rats were treated with various concentrations of TAT-survivin by intraperitoneal injection. After sacrifice, heart was isolated and transduced TAT-survivin was detected by immunoblot analysis using anti-survivin or anti-His-tag antibody and immunohistochemistry using anti-His-tag antibody. TAT-survivin protein was transduced into myocardial rat heart efficiently in a concentration-dependent manner (Fig. 17A). And the microscopy image using primary anti-His-tag antibody also showed efficient transduction of TAT-survivin protein (Fig. 17B).

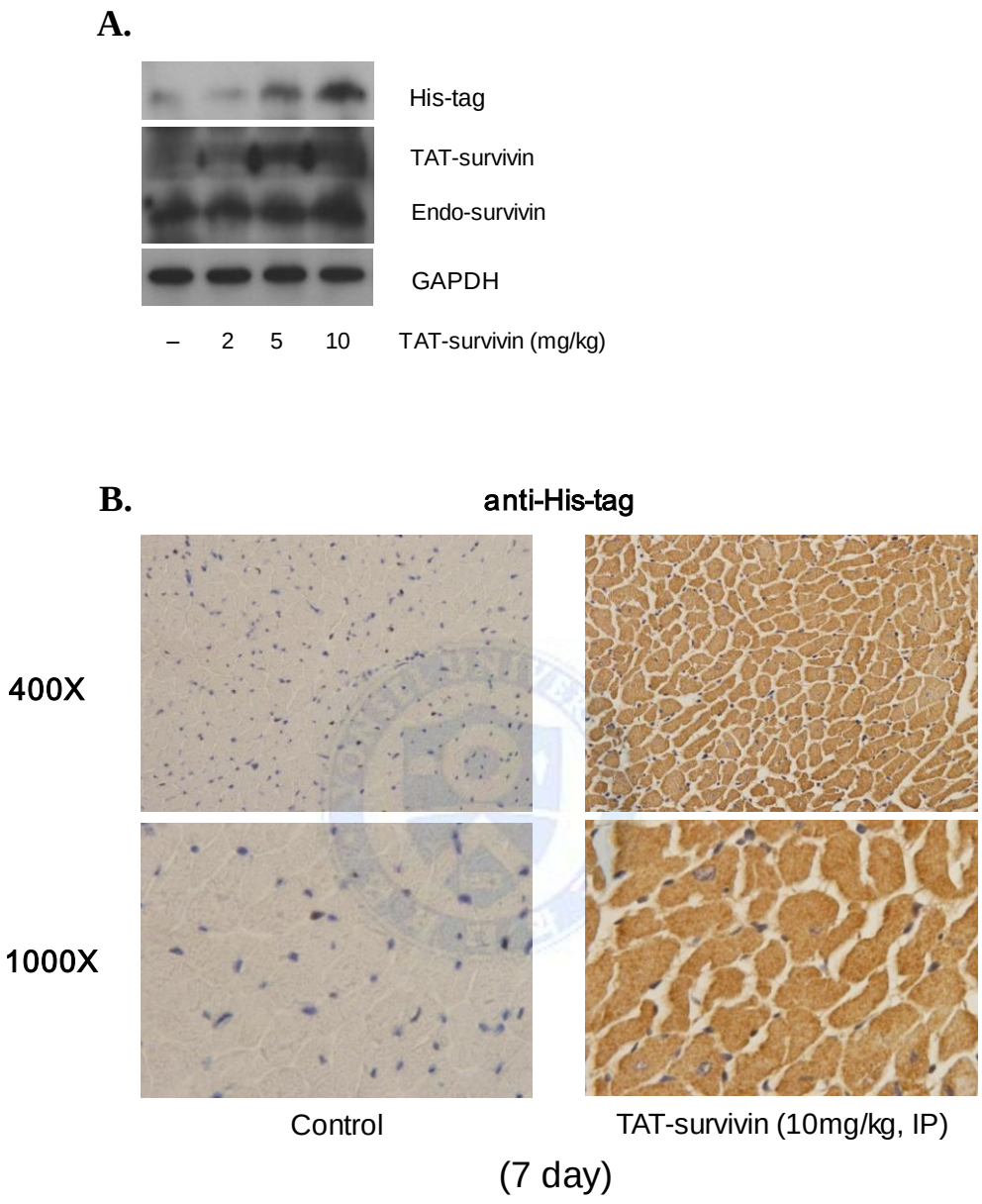

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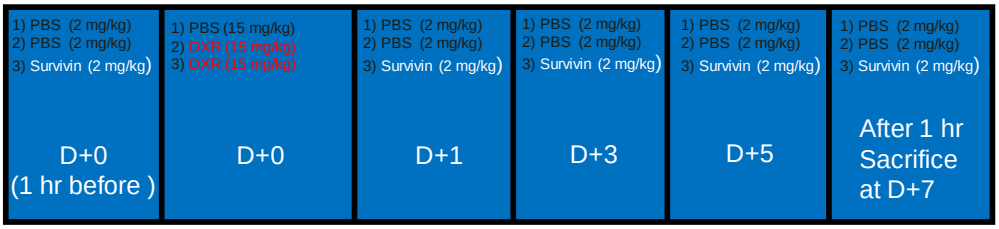
Fig.17. PTD delivery of TAT-survivin into the Sprague Dawley rat hearts. (A and B) Sprague Dawley rats (n=10) were treated with various concentrations of TAT-survivin by intraperitoneal (IP) injection. After 1 hour, heart was isolated and TAT-survivin was detected by (A) immunoblot analysis using anti-survivin or anti-His-tag antibody and (B) immunohistochemistry analysis using anti-His-tag antibody.

18. TAT-survivin protects rat cardiomyocyte from doxorubicin-induced in myocardial injury rat model

We investigated the protective effect of survivin in doxorubicin-induced myocardial rat model. Sprague Dawley rat were pretreated with 2 mg/kg of TAT-survivin for 1 hour and then 15 mg/kg of doxorubicin by intraperitoneal injection. After, rats were treated again with 2 mg/kg of TAT-survivin at 1, 3, 5 and 7 days (Fig. 18A). After sacrifice, hearts were isolated and paraffin embedded for immunohistochemistry analysis for survivin and Masson's trichrome staining. As shown in Fig. 18B, survivin protein levels were decreased by doxorubicin. However, the survivin protein was decreased by doxorubicin treatment, which was significantly recovered by TAT-survivin transduction. Haemotoxylin and Eosin staining image showed that the heart affected by multifocal areas of patchy and scattered cardiomyocyte with vacuolation by doxorubicin treatment, which was also significantly recovered by TAT-survivin protein (Fig.18C).

A.



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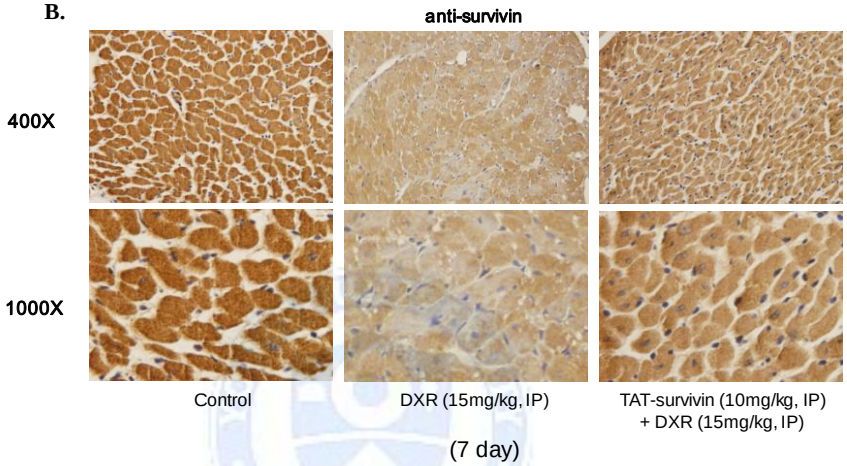

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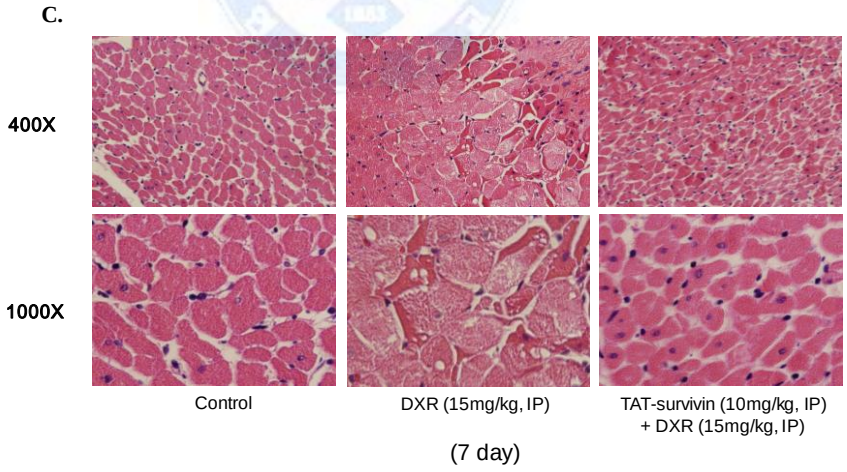

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Fig.18. Effect of TAT-survivin on cardiotoxicity in doxorubicin-induced myocardial rat model. (A) Diagram of experimental protocol. (B and C) Sprague Dawley rat (n=10) were pretreated with 2 mg/kg of TAT-survivin for 1 hour and then 15 mg/kg of doxorubicin (DXR) by intraperitoneal (IP) injection. After, rats were treated again with 2 mg/kg of TAT-survivin at 7 day. (B) Photomicrographs showing representative myocardial section stained with immunohistochemistry for survivin and (C) Haemotoxylin and Eosin staining demonstrated less cardiomyocyte vacuolation and myofibrillar disarray.

19. TAT-survivin protein-pretreated hearts demonstrated decreased caspase-3 activity in doxorubicin-induced myocardial injury rat model.

Next, we investigated the protective effect of survivin on caspase-3 in doxorubicin-induced myocardial rat model. Sprague Dawley rat were pretreated with 2 mg/kg of TAT-survivin for 1 hour and then 15 mg/kg of doxorubicin by intraperitoneal injection. After, rats were treated again with 2 mg/kg of TAT-survivin at 1, 3, 5 and 7 days. After sacrifice, hearts were isolated and paraffin embedded for immunohistochemistry analysis for cleaved-caspase-3. Apoptosis were detected by caspase-3 activity and immunoblot analysis using anti-cleaved caspase-3 antibody. As shown in Figs. 19A and B, cleaved caspase-3 levels were increased by doxorubicin treatment, which was significantly decreased by TAT-survivin transduction. Also, caspase-3 activity assay demonstrated the anti-apoptotic effect of TAT-survivin against doxorubicin-induced injury (Fig. 19C).

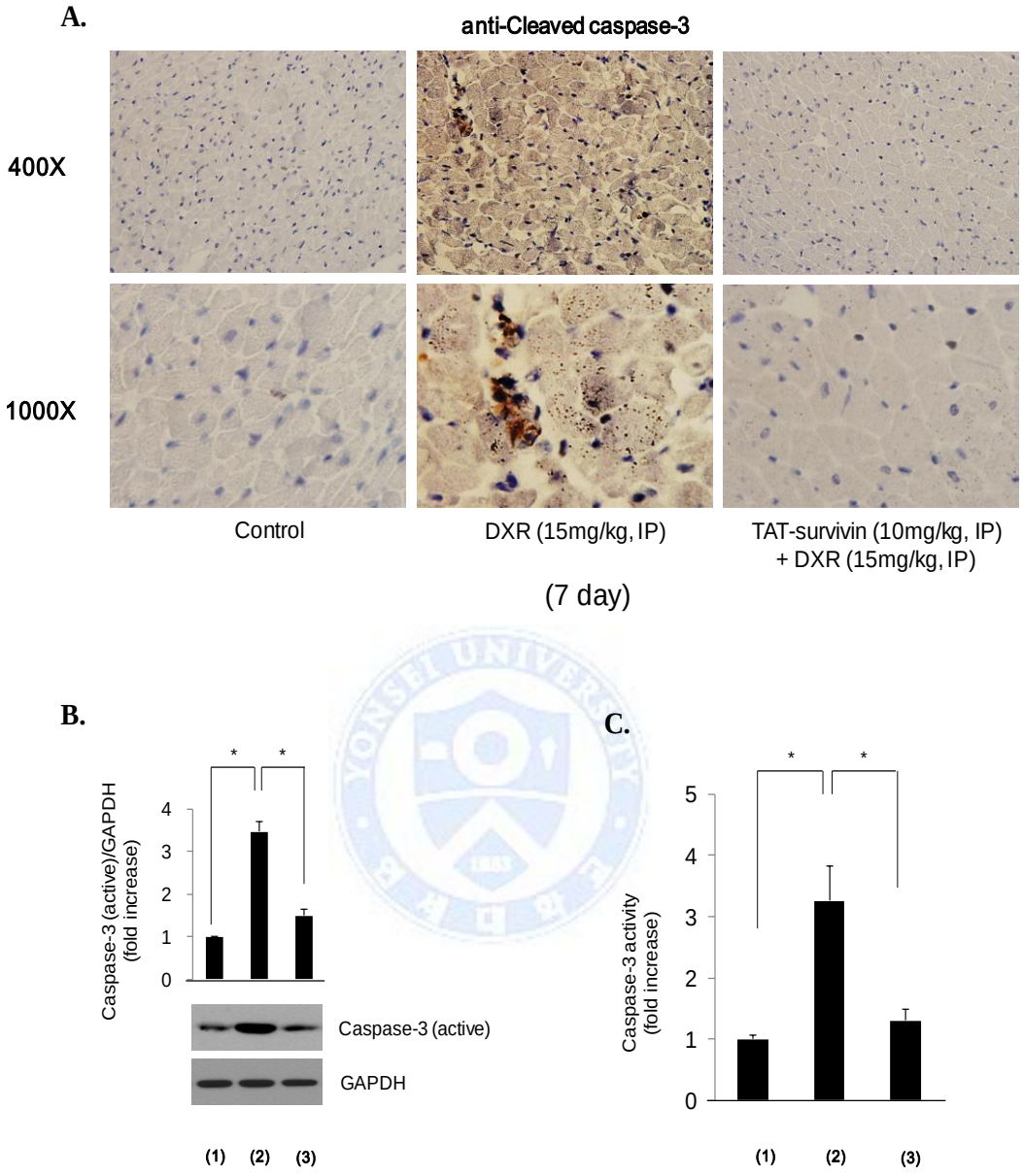

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Fig. 19. Effect of TAT-survivin on doxorubicin-induced decrease of caspase-3 activity in myocardial rat model. (A, B and C) TAT-Survivin was intraperitoneally (IP) injected into Sprague Dawley rat (n=10). After 1 hour, doxorubicin (DXR) was intraperitoneally injected. And then, TAT-Survivin was injected after 1, 3, 5 and 7 days. Heart was isolated and caspase-3 levels were measured by (A) immunohistochemistry analysis, (B) immunoblot analysis for cleaved caspase-3 and (C) caspase-3 activity. Lane 1, saline control; Lane 2, Doxorubicin (15 mg/kg); Lane 3, Doxorubicin (15 mg/kg) and TAT-Survivin (2 mg/kg) 0, 1, 3, 5, 7 days. Values are mean ±S.D. *$P < 0.05$.

20. Left ventricle Functional recovery after TAT-survivin delivery measured by the Langendorff system.

Next, we investigated left ventricle (LV) functional recovery after TAT-survivin pretreatment in the acute doxorubicin and ischemia/reperfusion injury model. To test directly the effects of TAT-survivin on LV functional recovery in doxorubicin-induced myocardial rat model, we used an *ex vivo* Langendorff system. Briefly, isolated Sprague Dawley rat hearts were perfused with KH buffer for 30 minutes, followed by global ischemia for 20 minutes and reperfusion for 40 minutes (Fig. 20A). There was significantly decrease in LVDP between control and doxorubicin-induced hearts after perfusion for 40 minutes, which was significantly decreased by TAT-survivin transduction (Figs. 20B and 20D). However, there was no change observed in heart rate with the same condition (Fig. 20C). To further evaluate the cardiac function after ischemia/reperfusion, we also calculated the rate pressure product (RPP). RPP was significantly improved in the doxorubicin/TAT-survivin pretreated group, compared with doxorubicin/saline group (Fig. 20E).

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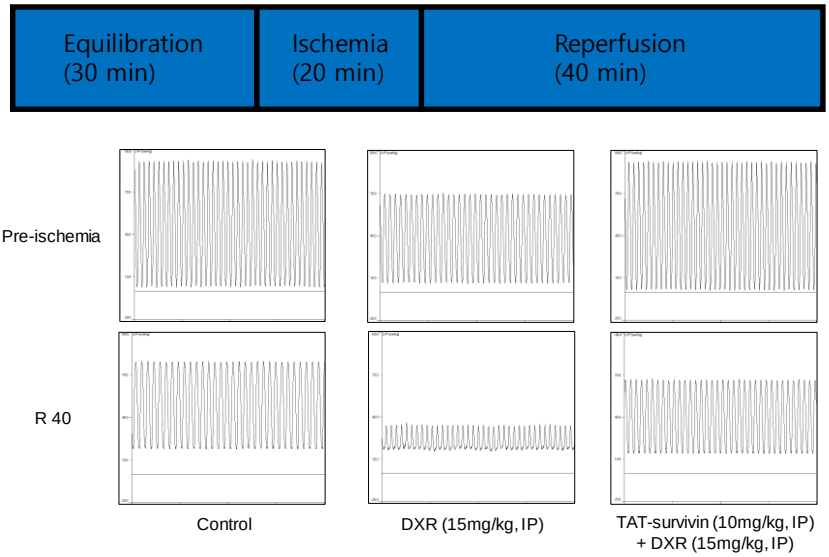
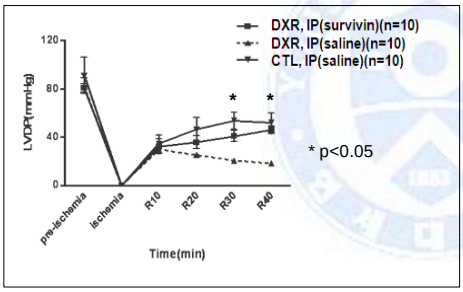
A.



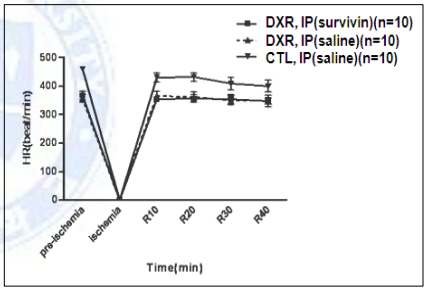
B.

LVDP (left ventricular developed pressure)
: LV systolic pr. – LV diastolic pr.



C.

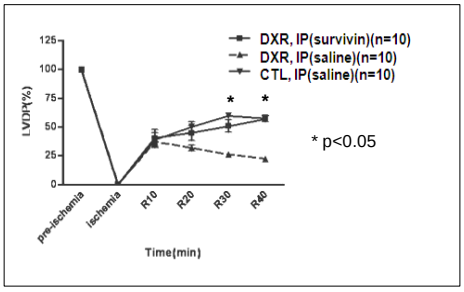
HR (Heart rate)



D.

Functional recovery

LVDP at the end of reperfusion /
LVDP at preischemic period (%)



E.

RPP (Rate Pressure Product) = LVDPxHR

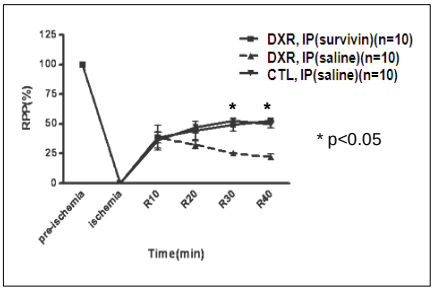


Figure 20 legend (the following page)

Fig. 20. Hemodynamic parameters in each groups measured by Langendorff system. (A) Diagram of I/R experimental protocol. Hemodynamic parameters were assessed after 20 minutes of global ischemia followed by 10, 20, 30, 40 minutes of reperfusion. (B) Measurements of left ventricular developed pressure (LVDP) and (C) Heart rate (HR). (D) LVDP (%; recovery LVDP) was calculated based on equation: LVDP at the end of reperfusion / LVDP at preischemic period and (E) Rate pressure product (RPP) was calculated based on the equation: RPP = LVDP x heart rate. CTL, saline control; DXR, Doxorubicin (15 mg/kg) and saline 0, 1, 3, 5, 7 days; DXR/Survivin, Doxorubicin (15 mg/kg) and TAT-Survivin (2 mg/kg) 0, 1, 3, 5, 7 days. Shown as mean ± S.D $*P < 0.05$. IP: intraperitoneally injection.

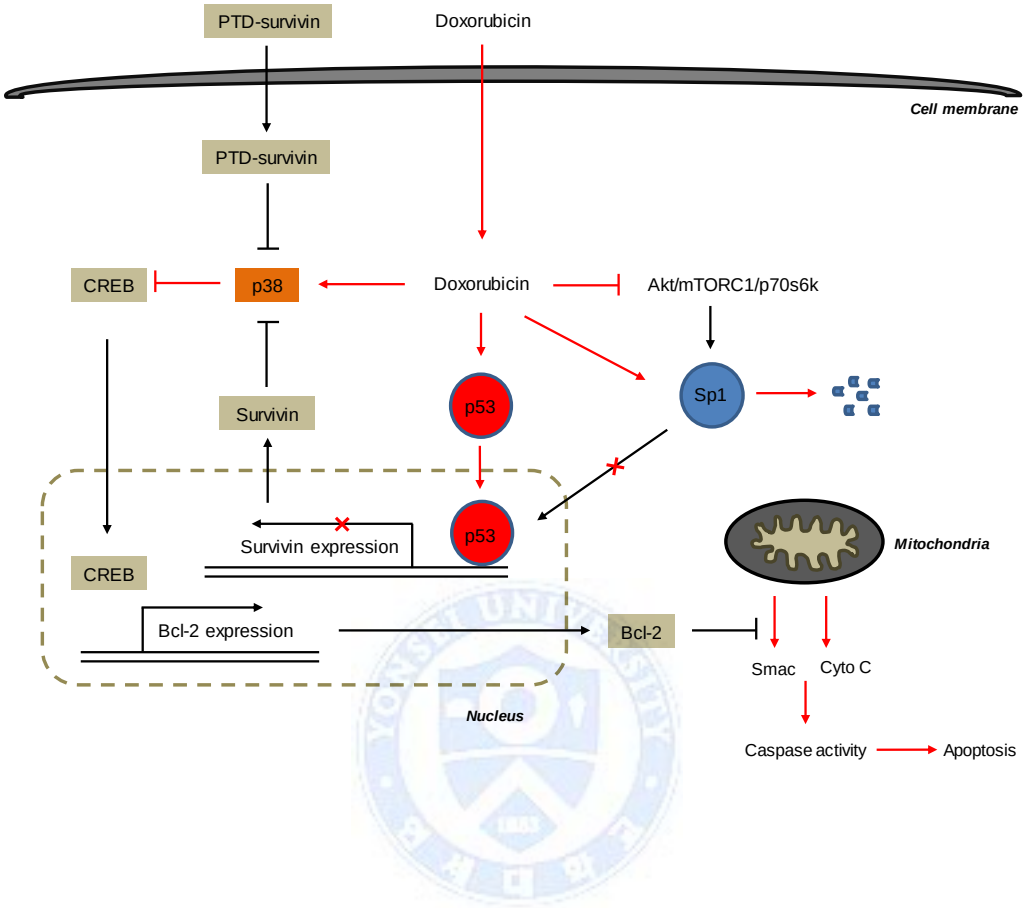


Fig. 21. Schematic representation regarding the protective effect of survivin in doxorubicin-induced cardiomyocyte death. This figure is derived from our experimental findings and other previously reported studies. Pro-apoptotic signaling cascades induced by doxorubicin are shown as red lines. Black lines represent pathways that are pro-survival or carry an anti-apoptotic signal. Protective proteins including survivin are illustrated by the gray boxes.

Ⅳ. DISCUSSION

The major finding of this study are as follows. First, survivin may be an important protein in the process of doxorubicin-treated cardiac dysfunction in H9c2 cell line and a rat model. Second, TAT-survivin protein has a potentially cardioprotective effect against doxorubicin-induced cardiomyocyte apoptosis.

Doxorubicin is the most commonly used anticancer drugs, but its cumulative and dose-dependent cardiotoxicity has been a major concern in clinical practice for decades [1]. This doxorubicin-treated cardiotoxicity leads to cardiomyocyte apoptosis, progressive cardiomyopathy, and congestive heart failure [3]. Although intensive investigations on doxorubicin-treated cardiotoxicity have been performed, the underlying mechanisms have not been completely elucidated. However, a rapidly expanding body of evidence supports the notion that cardiomyocyte death by apoptosis and necrosis is a primary mechanism of doxorubicin-treated cardiotoxicity [3,5]. Our study is the first to demonstrate that doxorubicin inhibits survivin expression in H9c2 cardiomyocyte, a rat-derived noncancerous myoblast cell line and Sprague Dawley rat heart. And, the promoter region of survivin gene has been studied exclusively in various cancer cell lines, mostly originated from mouse or human [15,39-41]. In the present study, we demonstrated that doxorubicin inhibited

survivin expression by Sp1 protein degradation against doxorubicin-mediated toxicity of cardiomyocyte. In the study, similar to our result, it has been demonstrated that doxorubicin induces Sp1 down-regulation in doxorubicin-resistant MCF-7 cell line, whereas Sp1 mRNA is not changed by doxorubicin treatment [49]. Moreover, in the present study we have shown that in the absence of doxorubicin the survivin promoter was occupied by Sp1 protein, but not by p53 protein. Following doxorubicin treatment of the cardiomyocyte, the survivin promoter had reduced levels of bound Sp1, but an increased level of bound p53. In consistent with our ChIP data, in which Sp1 acted as a transcriptional activator, it has been demonstrated that p53 appears on the promoter without the loss of Sp1 to mediate dual function of both transcription activator and repressor after 9 hours of doxorubicin treatment [16]. The increased level of p53 protein can be accomplished by enhancing protein stability instead of inducing transcription or translation. Our results supported the notion that DNA damage, e.g. doxorubicin stimuli, induces phosphorylation of p53 at serine^{15} and leads to a reduced interaction between p53 and its negative regulator, the oncoprotein MDM2, which inhibits p53 accumulation by targeting it for ubiquitination and proteasomal degradation [46,50]. Surprisingly, the doxorubicin was increased phosphorylation of p53 at Ser^{15} in the H9c2 cardiomyocyte cell line and Sprague Dawley rat heart. To better understand the

mechanisms for cardiomyopathy associated by doxorubicin, more studies will be required to identify the specific kinases that are activated by doxorubicin to mediate the Ser^{15} phosphorylation and define the precise correlation between the post-translational modification of p53 and its function as transcription factor [16,51]. It has also been suggested that chromatin modification in the promoter region may play a crucial role in the survivin gene silencing by p53 [9]. As shown in Fig. 7, we designed an experiment using a potent proteasome inhibitor MG132 to inhibit doxorubicin-stimulated degradation of the Sp1 protein. Our findings are supported by a recent publication that showed MG132 pretreatment of doxorubicin-resistant MCF-7 cells inhibited the doxorubicin-induced Sp1 down-regulation [49]. Insulin-like growth factor-I (IGF-I) has been reported to induce the expression of survivin by increasing the translation of mRNA through mTOR-dependent p70S6K activation, rather than by regulating gene transcription or protein stability [28,52]. In a more complicated study by Song et al., IGF-I has been suggested to reverse suppression of survivin gene expression by TGF-β [52], whose survivin gene down-regulation is depend on two cell cycle repressor elements in the survivin promoter region, a cell cycle-dependent element (CDE) and a cell cycle genes homology region (CHR); and two transcription factors Smad2 and Smad3 [42]. In this study, it was successfully confirmed by immunoblot analysis that doxorubicin-treated

decreases survivin expression by Sp1 degradation through Akt/mTOR/p70s6K pathway in the H9c2 cardiomyocyte cell line and Sprague Dawley rat heart.

Survivin known as strong anti-apoptosis factor were successfully delivered into the H9c2 cardiomyocyte cell line and Sprague Dawley rat heart using PTD system, leading to significant protective effect against doxorubicin-treated apoptosis. The transduction of TAT-survivin significantly reduced cell death and caspase activity with less cardiomyocyte vacuolation and myofibrillar disarray, compared with doxorubicin-injury rat heart model. Our results suggest that intracellularly delivered survivin suppresses caspase associated cell death, at least in part, by cytochrome C or procaspase-3 following preventing its correct function, thereby allowing the cells better tolerate potentially lethal doxorubicin injury. In additional experiments, we evaluated the effect of TAT-survivin protein on cardiac functional recovery using Langendorff system. After ischemia-reperfusion injury, cardioprotective effect of TAT-survivin such as functional recovery measured by LVDP (left ventricular developed pressure) and RPP (rate pressure product) was observed. Our finding agrees with a previous observation that survivin gene therapy improves left ventricular systolic dysfunction in doxorubicin-treated cardiomyopathy by reducing apoptosis [33]. The induction of Bcl-2, which is known as an anti-apoptosis factor, also manifested anti-apoptotic effect of TAT-survivin. Both protein and

mRNA level of Bcl-2 were increased by TAT-survivin transduction. We also demonstrated that the activation of CREB, which is the transcription factor of Bcl-2, was also increased by TAT-survivin. The CREB has been established as a cellular transcription factor which binds to a specific DNA site called cyclic adenosine monophosphate response element (CRE) that regulating the transcription of several proteins including the expression of IAPs family [53,54], leading to an upregulation of Bcl-2 expression that is related to anti-apoptosis. Other reports showed that activation of CREB also increased the expression level of the IAP family [55]. The present findings, protein delivery of TAT-survivin, suggest that Bcl-2 may be at least in part involved in the protection mechanism with TAT-survivin against doxorubicin insult, and that recovery of Bcl-2 level may be attributed to the CREB reactivation by TAT-survivin treatment. Furthermore, TAT-survivin transduction recovered its translocation of Smac into mitochondria from cytoplasm. Smac is a mitochondrial protein that promotes apoptosis by inhibiting IAPs. Survivin is considered to inhibit Smac by binding each other in cytosol [21], but the transduction of survivin also suppressed the release of Smac from mitochondria in our study. Although the interaction between Smac and survivin in the mitochondria is not proven, this result can be explained by the activation of Bcl-2. It is reported that Bcl-2 attenuated the release of Smac from mitochondria [56]. We also observed that

doxorubicin-induced cytochrome C release from mitochondria is inhibited by TAT-survivin protein transduction. Besides its interaction with Smac as already reported, survivin may also bind to other upstream proteins of Bcl-2 and regulate the release of mitochondrial proteins including Smac and cytochrome C. Taken together, a mechanism of action of survivin protein for the prevention of Smac and cytochrome C release can be explained by its direct effect on CREB in keeping the level of Bcl-2 high or by regulating the specific protein kinases that activate CREB. According to previous studies the transcriptional activity of CREB requires phosphorylation of serine^{133} which provides positive conditions to bind with CRE [57]. Also, CREB must form a complex such as its binding with transducers of regulated CREB protein 2 (TORC2) to achieve its transcriptional activity. The increase of protein kinase A (PKA) and protein kinase G (PKG)-Iα activity induces the translocation of TORC2 into nucleus and also phosphorylates CREB [58]. We observed that TAT-survivin transduction significantly attenuated the phosphorylation of p38 MAP kinase against doxorubicin-associated injury model. Therefore, we speculated that the TAT-survivin could recover the CREB activation and Bcl-2 expression by suppressing p38, although our data did not provide direct evidence for a role of p38 in this event. MAP kinases are known to regulate gene expression, mitosis, differentiation, cell survival, and apoptosis in response to a wide variety of

stimuli [59]. Generally, extracellular regulated kinases (ERKs) activation is involved in cardiac cell proliferation and survival [60]. In contrast, Jun N-terminal kinases (JNKs) is activated by several stimuli that induce cardiomyocyte death [61]. Recent evidence has implicated dual role of p38 as a regulator of cell growth, thus it can promote either cellular survival or death [62]. Many reports suggested that p38 activation is necessary for cell death in response to various stimuli [63,64].

In the opposite, other observations indicated that p38 plays a role in the cell protection against several stresses [65,66]. Several studies have shown that p38 MAP kinase can act a negative regulator of cardiomyocyte death against many other stimuli [62,67,68]. For instance, the p38 activation is associated with protection against cardiomyocyte apoptosis in myocardial infarction model by coronary artery ligation [69]. Comparably, p38 activation protects cardiomyocyte from thermal stress-induced cell death in the isolated perfused heart model [70]. Moreover, the p38 MAP kinase pathway is substantially involved in the protective effect against doxorubicin-induced cardiac cell death [62].

Therefore, a possible mechanism underlying the cardioprotective effect of TAT-survivin, which we can propose, is that doxorubicin-treated p38 activation may suppress other intracellular factor which activates the nuclear translocation

of CREB and transcriptional activity of Bcl-2. This finding corresponds to the results that p38 MAP kinase is shown to crosstalk in PKA pathway which is an upstream positive regulator of CREB [67]. Our results support that doxorubicin-induced p38 activation can suppress the PKA pathway, leading to the inactivation of CREB and downregulation of Bcl-2 gene expression, and this event may be prevented by TAT-survivin protein transduction. Evidence has been demonstrated that p38 MAP kinase mediates apoptotic cell death through downregulation of Bcl family [71,72].

V. CONCLUSION

Clinically, the doxorubicin-induced cardiotoxicity has been one of the most important sequelae of current chemotherapy against various cancers. So many researchers have tried to find the safe and effective way for reducing the cardiotoxicity of anthracyclin treatment. Our findings suggest the doxorubicin was decreased survivin expression, which were demonstrated by the proteasome-mediated degradation of Sp1 protein via the inactivation of Akt/mTOR/p70S6k pathway. And, direct transduction of survivin protein into cardiomyocyte using PTD system can prevent cardiac cells death from doxorubicin. Moreover, the PTD-mediated pretreatment of TAT-survivin showed less cardiomyocyte vacuolation and myofibrillar disarray, which were demonstrated by doxorubicin. We demonstrated LV functional recovery after TAT-survivin pretreatment in the acute doxorubicin and ischemia/reperfusion injury model. We could assume that the protective role of survivin protein is closely associated with the regulation of CREB activation and Bcl-2 gene expression, although its mechanisms are not fully elucidated. Therefore, survivin pretreatment using PTD-mediated delivery may offer potential cardioprotection against doxorubicin and ischemia-reperfusion injury. The schematic diagram represents a model of the intracellular mechanism of the protective effect of survivin in doxorubicin-treated cardiomyocyte death (Fig.

21). Promising further study will prove our hypothesis and reveal biological role of survivin in the cardiac cells.

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ABSTRACT (KOREAN)

독소루비신으로 유도된 심근 손상모델에서 서바이빈 단백질의 심근 보호효과 규명

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이범섭

세포사멸은 독소루비신에 의해 발생한 심장독성에서 중요한 메커니즘으로 알려져 있다. 세포사멸을 억제하는 단백질 중 하나인 서바이빈은 심근세포의 분자적 경로에 따른 세포사멸의 조절에 관련이 있다. 최근 연구에 따르면 동물 모델에서 서바이빈 단백질 발현 감소는 비정상적 심장 리모델링 발생과 연관 있는 것으로

보고되었다. 그러나 독소루비신에 의한 심근손상에서 서바이빈 단백질의 심근보호효과에 대한 기전 연구는 진행되지 않았다. 따라서 본 연구는 독소루비신에 의한 심근손상 모델에서 서바이빈 단백질의 역할과 서바이빈 단백질을 단백질 전달시스템을 사용하여 세포사멸 억제효과와 그에 따른 분자 생물학적 기전을 조사하였다.

본 연구에서 심근세포와 쥐의 심장에서 독소루비신을 처리한 결과 서바이빈 단백질이 유의하게 감소하는 것을 관찰하였다. 또한, Sp1 단백질은 서바이빈 유전자 발현을 유도하는 역할을 하며, 프로테아좀 활성화가 독소루비신에 의해 증가하여 Sp1 단백질의 분해가 되는 것을 확인하였다. p53 단백질의 활성화는 서바이빈 유전자의 전사를 억제하며, 독소루비신에 의해 p53의 인산화와 단백질이 증가하는 것을 관찰하였다. 독소루비신에 의해 심근세포에서 서바이빈 전사에 관여하는 프로모터에 Sp1 단백질이 붙는 수준은 감소하였지만 p53 단백질은 붙는 수준이 증가하는 것을 확인하였다. 또한, Sp1 단백질을 조절하는 상위단백질은 Akt, mTOR, p70s6k 단백질이며, 이들은 독소루비신에 의해 활성화가 감소하는 것을 관찰하였다.

서바이빈 단백질의 세포 보호 효과를 규명하기 위해 서바이빈 유전자를 HIV 바이러스에서 얻은 단백질 전달시스템인 TAT-

유전자와 결합한 단백질을 제작하여 사용하였다. TAT-서바이빈 단백질은 심근세포와 쥐의 심장에 효과적으로 전달되는 것을 관찰 할 수 있었으며, caspase-3 activity, TUNEL assay 그리고 cell viability 방법을 통해 전달된 TAT-서바이빈 단백질의 세포사멸 억제 효과를 확인하였다. 또한, TAT-서바이빈 단백질은 독소루비신에 의해 유도된 심근세포의 공포 형성과 근원섬유의 손상이 억제되는 것을 관찰하였다.

TAT-서바이빈 단백질의 심장 기능 회복 효과를 알아보기 위해 랑겐도르프 시스템을 이용하였다. 허혈-재관류 손상 후 좌심실내압 (LVDP: left ventricular developed pressure)와 RPP (Rate pressure product)를 관찰한 결과, TAT-서바이빈 단백질 투여가 심장기능 회복에 관여하는 것을 관찰할 수 있었다.

또한, 심근세포를 이용한 독소루비신 세포 손상 모델에서 TAT-서바이빈 단백질이 p38 MAPK 단백질의 활성화를 억제하여 Bcl-2의 전사인자인 CREB 단백질이 핵으로 이동되었고, 세포사멸 과정 중 미토콘드리아에서 분비하는 Smac 단백질이 Bcl-2 단백질 발현에 의해 분비가 억제 되는 것을 확인되었다.

본 연구 결과를 통해, 서바이빈 단백질은 독소루비신의 심근 손상 유발에 있어 중요한 단백질임을 알 수 있었고, 향후 임상에서

심근보호 치료약제의 개발에 중요한 타겟으로 사용 될 수 있을 것이다.

핵심 되는 말: 세포사멸, 서바이빈 단백질, TAT 단백질, 독소루비신, 심근세포