Enhanced calreticulin expression is critical for calcium-dependent apoptosis in postnatal heart

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ABSTRACTS

Enhanced calreticulin expression is critical for calcium-dependent apoptosis in postnatal heart

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Background : Calreticulin (CRT) is one of the major Ca^{2+} binding chaperone proteins of endoplasmic reticulum (ER) and an unusual luminal ER protein. As a modulator of Ca^{2+} homeostasis, CRT has an important role in early stages of cardiac development. In contrast, postnatally high level of expression of CRT leads to impaired development of the cardiac conductive system and may be responsible for the pathology of the complete heart block. In this study, the molecular mechanisms that affect Ca^{2+}-dependent signal cascades were investigated on CRT-overexpressing neonatal rat cardiomyocytes. Especially, it was addressed that CRT may play a critical role in the activation of Ca^{2+}-dependent apoptosis.

Methods and Results : CRT-overexpressing cardiomyocytes showed 2-fold lower proliferation in 3-day culture by MTT assay. Suppression of CRT by siRNA for CRT enhanced proliferation compared with control. In the overexpressing cells, the intracellular calcium concentration was significantly increased by a fluorescence spectrophotometry using the Ca^{2+} binding dye, Fluo-4 AM. The level of SERCA2a mRNA were suppressed and the

phosphorylation of Akt and ERKs significantly decreased compared with control. CRT-overexpressing cells significantly decreased the activity of anti-apoptotic factor, Bcl-2 and increased the activities of pro-apoptotic factor, Bax, p53 and caspase-8, leading to dramatic increase of caspase-3 activity.

Conclusion : In the developed neonatal rat cardiomyocytes, CRT overexpression lost the functions for intracellular calcium regulations and relaxation-contraction, leading to calcium-dependent apoptosis in matured cardiomyocytes.

Key words: calreticulin, cardiomyocytes, apoptosis, calcium overload

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I. INTRODUCTION

Ca^{2+} is a universal signaling molecule that affects various physiological processes such as secretion, contraction-relaxation, cell motility, cytoplasmic and mitochondrial metabolism, synthesis, modification, and folding of proteins, gene expression, cell cycle progression, and apoptosis. Especially, the endoplasmic reticulum (ER) plays a central role in maintaining intracellular Ca^{2+} homeostasis[1,2]. Calreticulin (CRT) is one of the major Ca^{2+} binding chaperone proteins of ER and an unusual luminal ER protein[1-4]. Recently, CRT was identified as a new cardiac embryonic gene[5]. The protein is highly expressed in embryonic heart and it is essential during cardiac development[5]. CRT also plays an important role upstream of calcineurin-dependent transcriptional processes during cardiac development[6]. As a modulator of Ca^{2+} homeostasis, CRT has an important role in early stages of cardiac development.

In contrast, postnatally high level of expression of CRT leads to impaired development of the cardiac conductive system and may be responsible for the pathology of the complete heart block. In the study with transgenic mice overexpressing CRT in the heart, these animals develop bradycardia associated with sinus node dysfunction, complete heart block, and death due to intractable heart failure[5,7-9]. Electrocardiograms demonstrated that the P-R interval of transgenic mice is significantly prolonged with subsequent development of complete AV nodal conduction block. These findings indicate that CRT may play a role in the pathology and development of the conductive system[7]. Furthermore, CRT overexpressor hearts have very low levels of connexin 43, a major component of gap junctions responsible for cell-cell communication[7]. Recent studies on H9c2 cells indicate that over-expression of CRT in cardiomyocytes affects the Akt signaling pathway and promotes apoptosis[8]. The most significant findings are that the phenotype of CRT overexpresser mouse is very similar to that seen in children with complete heart block. The cause and molecular mechanism involved in the complete heart block is not known at present. Activation of cardiac muscle is mediated by the specialized electrical system of heart. It consists of the sinoatrial and the atrioventricular nodes, the activation of which depends on an inward Ca^{2+}current, and of the bundle branches and Purkinje network, the activation of which depends on an inward Na^{+} current.

Even though the phenotype of CRT overexpresser mouse is very similar to that seen in children with complete heart block, the molecular mechanism of CRT involved in the complete heart block is not known at present. In this study, the molecular mechanisms that affect Ca^{2+}-dependent signal cascades were investigated on CRT-overexpressing cardiomyocytes. Especially, it was addressed that CRT may play a critical role in the activation of Ca^{2+}-dependent apoptosis.

II. MATERIALS AND METHODS

1. Isolation of neonatal rat cardiomyocytes

Neonatal rat cardiomyocytes were isolated and purified by the modifications of previously described methods. Hearts of 1-2 day-old Sprague Dawley rat pups were dissected, and the ventricles were washed with Dulbecco's phosphate-buffered saline solution (pH 7.4, Gibco BRL) lacking Ca^{2+} and Mg^{2+}. Using micro-dissecting scissors, hearts were minced until the pieces were approximately 1 mm^3 and treated with 10 ml of collagenase I (0.8 mg/ml, 262 units/mg, Gibco BRL) for 15 minutes at 37°C. The supernatant is then removed and the tissue is treated with fresh collagenase I solution for an additional 15 minutes. The cells in the supernatant were transferred to a tube containing cell culture medium (a-MEM containing 10% fetal bovine serum, Gibco BRL). The tubes were centrifuged at 1200 rpm for 4 minutes at room temperature, and the cell pellet was resuspended in 5 ml of cell culture medium. The above procedures were repeated 7-9 times until there little tissue was left. Cell suspensions were collected and incubated in 100 mm tissue culture dishes for 1-3 hours to reduce fibroblast contamination. The non-adherent cells were collected and seeded to achieve a final concentration of $5x10^5$ cells /ml. After incubation for 4-6 hours, the cells were rinsed twice with cell culture medium and 0.1 M BrdU added. Cells were then cultured with 10% (v/v) FBS in a CO2 incubator at 37°C.

2. Transfection

Transfections of CRT cloned into the eukaryotic expression vector pEGFP-N3 (Invitrogen, Groningen, The Netherlands) were performed using LIPOFECTAMIN PLUS™ reagent (Gibco-BRL). Breifly, neonatal rat cardiomyocytes cultured in a 60 mm culture plate ($5X10^5$ cells/ plate) were

washed twice with serum-free MEM. LIPOFECTAMIN PLUS™ reagent was diluted with serum-free MEM and combined with 2g of DNA for the each plate. The DNA and LIPOFECTAMIN PLUS™ reagent was added into the each plate containing fresh medium on cells. After 12 hour incubation in a CO_2 incubator at 37°C, the medium are exchanged with 10% FBS-MEM.

3. siRNA transfection

To inhibition of specific gene expression was used small RNA molecules. This was consisted with 21 nucleotides based on the rat CRT sequence. The forward and reverse strands (5'-ggaugaugaauucacacautt-3') and (5'-augug ugaauucaucaucctt-3') were commercially synthesized. Ambion (Austin, Tex.), and other reagents were also obtained from Ambion. For transfection, cells were trypsinized and suspended in normal growth medium to 106 cells/ml. And then siPORT™, NeoFX™ and siRNA were each diluted for 10 min. After 10 min incubation, combined the diluted siPORT™, NeoFX™ and the diluted RNA at a final concentration of 10 nmol/L. Newly formed transfection complex was dispensed into the empty wells of a culture plate and overlaid cell suspensions on complexes. Finally, cells were incubated for 8-72 hours at 37°C 5% CO_2 incubator.

4. Confocal microscopy and fluorescence measurements

The measurement of cytosolic free Ca^{2+} concentration was performed by the confocal microscopy analysis. Neonatal rat cardiomyocytes were plated on glass coverslips coated with laminin (5 mg/cm^2) for 1 day in cell culture medium (a-MEM containing 10% fetal bovine serum, Gibco BRL) and 0.1 M BrdU. After incubation, the cells were washed with PBS. Cells were then loaded with 5 mM of the acetoxymethyl ester of Fura-2 (Fura-2 AM, Molecular Probes, Eugene, OR) for 20 min, in

the dark and at room temperature, by incubation in modified Tyrode's solution. Fluorescence images were obtained using an argon laser confocal microscope (Leica, Solms, Germany). Fluorochrome was excited by 488 nm line of argon laser and emitted light was collected through a 510-560 nm bandpass filter. Relative changes of free intracellular Ca^{2+} were determined by measuring fluorescent intensity.

5. Immunoblot analysis

Proteins were separated by SDS-PAGE using 10-12% polyacrylamide gel and then electrotransferred to methanol-treated polyvinylidene difluoride membranes. The blotted membranes were washed twice with water and blocked by incubation with 5% nonfat dried milk in PBS buffer (8.0 g NaCl, 0.2 g KCl, 1.5 g NaH_2PO_4, 0.2 g K_2HPO_4 per liter). After the 1 hour incubation at room temperature, the membranes were probed overnight at 4°C with polyclonal antibodies against CRT, p-PKC, p-ERK and p-Akt followed by goat anti-rabbit IgG-peroxidase and goat anti-mouse IgG-peroxidase. The blots were detected using enhanced chemiluminescence kits (ECL, Amersham Pharmacia Biotech.).

6. RT-PCR analysis

The expression levels of various proteins were analyzed by the reverse transcription polymerase chain reaction (RT-PCR) technique. For the RNA preparation, confluent rat neonatal cardiomyocytes were cultured for 48 hours in serum-free MEM after transfection with CRT. Total RNA was prepared by Ultraspect-II^{TM} RNA system (Biotecx Laboratories, Inc., USA) and single-stranded cDNA was then synthesized from isolated total RNA by Avian Myeloblastosis virus (AMV) reverse transcriptase. A 20 μl reverse transcription reaction mixture containing 1 mg of total RNA, 1x reverse transcription buffer

(10 mM Tris-HCl, pH 9.0, 50 mM KCl, 0.1% Triton X-100), 1 mM deoxynucleoside triphosphates (dNTPs), 0.5 unit of RNase inhibitor, 0.5 mg of oligo(dT), and 15 units of AMV reverse transcriptase was incubated at 42°C for 15 min, heated to 99°C for 5 min, and then incubated at 0-5°C for 5 min. PCRs were performed for 35 cycles with 3' and 5' primers based on the sequences of various genes. The actin primers (5'-catcacatactcacaacgctcaac-3' and 5'-catagcacgatggtcgattgtcgt-3') were used as the internal standard. The signal intensity of the amplification product was normalized to its respective actin signal intensity.

7. TUNEL (Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling) assay

For the detection of apoptosis, the TUNEL method was performed using the ApopTag® Peroxidase *In Situ* Apoptosis Detection Kit (Chemicon, USA), according to the protocols of the manufacturer. Briefly, for cardiac myocytes, cells were fixed in 1% paraformaldehyde in PBS for 10 minutes and washed 2 times with PBS, permeabilized with ethanol and acetic acid. The cells were next incubated with equilibration buffer, digoxigenin-conjugated nucleotide mix and TdT at 37°C for 60 min. The activity of TdT was ended by addition of sodium citrate buffer. After 10 min incubation, the cells were incubated with anti-digoxigenin peroxidase conjugate for 30 min at room temperature and washed 2 times with PBS. And then peroxidase substrate was applied enough to completely cover the cells. In order to find the optimal staining time, the color development was observed by looking under the light microscope. After washing of dH_2O and dehydration of xylene, the cells were mounted under a glass coverslip in a mounting medium. Finally TUNEL-positive or -negative cells were counted in a light microscope.

8. Caspase-3 assay

Relative caspase-3 activity was determined using ApopTarget™ Capase-3 Colorimetric Protease Assay, according to manufacturer's instructions (Biosource). This assay is based on the generation of free DEVD-*p*NA chromophore when the provided substrate is cleaved by caspase-3. Upon cleavage of the substrate by caspase-3, free *p*NA light absorbance can be quantified using a microplate reader at 405 nm. Briefly, the cultured neonatal cardiomyocytes ($3X10^6$) after different treatments were treated with lysis buffer including 1M DTT, and cell extracts were centrifuged to eliminate cellular debris. Aliquots (5μl) of the cell extracts were incubated at 37°C for 2 hours in the presence of the chromophore substrate. Free DEVD- *p*NA was determined colometrically. The comparison of absorbance of *p*NA from apoptotic sample with the control allows determination of the fold increase in capase-3 activity.

III. RESULTS

1. The effects of CRT overexpression on the proliferation of neonatal rat cardiomyocytes

Rat neonatal cardiomyocytes were transfected with the expression vector for rat CRT cDNA to investigate the functional roles of CRT during cardiomyocytes proliferation. After the CRT gene was identified from a rat heart cDNA by PCR, the PCR product was inserted into an eukaryotic expression vector, pEGFP-N3, for overexpression. Then it was transfected into neonatal cardiomyocytes for 24 hours by using LIPOFECTAMIN PLUS^{TM} system. The expression and transfection efficiency of CRT gene was confirmed by a confocal microscopy and an immunoblot analysis with anti-CRT antibody. Figure 1 showed that the purified cardiomyocytes had fibroblast-like morphology with photometrical examination and the intracellular localization and efficiency of CRT was examined using immunofluorescence. The CRT overexpression level increased about 3-fold higher than that of control.

To test the cytotoxicity of overexpressed CRT on cardiomyocytes, control and CRT gene-transfected cells were cultured, respectively and cell proliferation was measured by MTT assay. Proliferation rate of cells overexpressing CRT was significantly decreased through 5 days. For the further effects of CRT on the proliferation, siRNA for CRT was treated as negative control. Because expression of CRT was down-regulated, siRNA treatment group has little change compared to the control (Figure 2).

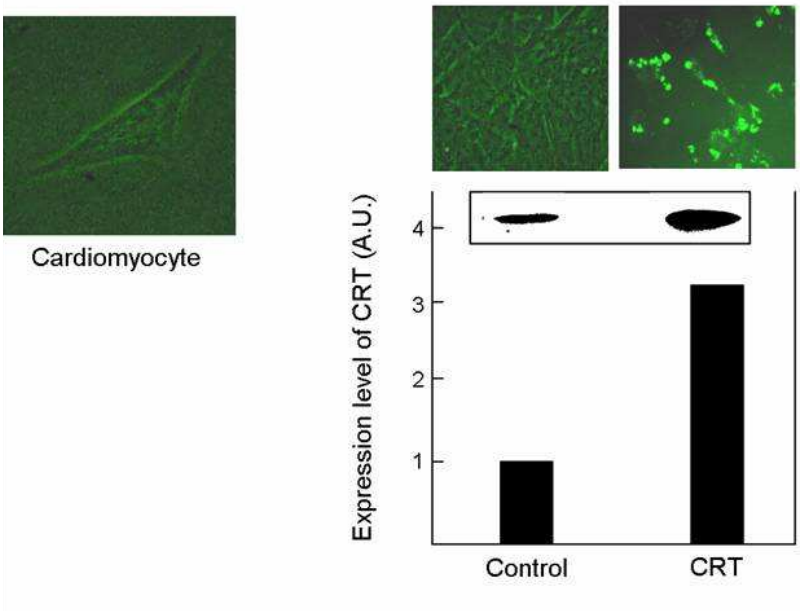

Figure 1. Isolation of neonatal cardiomyocytes and calreticulin expression. Left. Neonatal cardiomyocytes morphology of normal condition. Right. CRT was transiently transfected with pEGFP-N3. Localization of pEGFP-N3 was monitored using a Leica confocal microscope and CRT detected by immunoblot analysis using anti-CRT antibody.

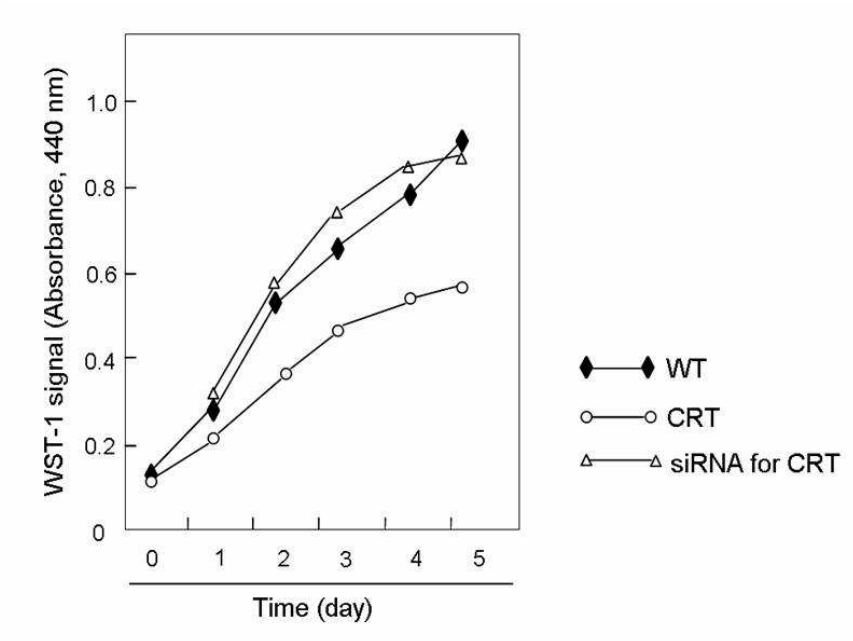

Figure 2. Overexpression of CRT depresses proliferation of neonatal rat cardiomyocytes. Neonatal cardiomyocytes were transfected CRT with or without siRNA for CRT through 5 days. Cell viability was assessed by the MTT assay. WT, wild- type.

2. Effects of CRT overexpression on the intracellular calcium overload of neonatal cardiomyocytes

To examine intracellular Ca^{2+} overload effects of cells overexpressing CRT, the fluorescence intensity of cells was measured after loading Fura-2 AM in control and gene-transfected cells, respectively. As shown in Figure 3, cells overexpressing CRT showed significantly higher intracellular calcium levels compared with the control. Both cells transfected with both CRT and siRNA for CRT and cells transfected with siRNA for CRT alone had similar levels with the control in intracellular calcium level, suggesting that cells overexpressing CRT disrupted the intracellular calcium homeostasis.

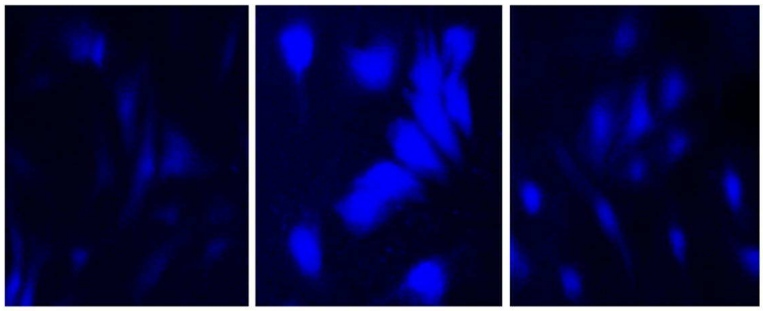

Figure 3. Overexpression of CRT increases intracellular calcium in neonatal cardiomyocytes. 2M Fura-2 loaded on cardiomyocytes and then cultured in a 20 min in the humidified chamber. Relative intracellular calcium intensity was depended on manufacturer's manuals. WT, wild- type.

3. Effects of CRT overexpression on the mRNA level of SERCA in neonatal rat cardiomyocytes.

To reveal whether intracellular calcium elevation induced overexpression of CRT affected sarcoplasmic/endoplasmic rericulum Ca^{2+} ATPase 2a (SERCA2a) expression level, the mRNA level of SERCA2a were examined and compared between control cells and cells transfected with the CRT gene. While SERCA2a expression was inhibited by CRT overexpression, other treatments had less influence on the basal level (Figure 4). These results indicated that CRT disrupted intracellular Ca^{2+} homeostasis by down-regulation SERCA2a.

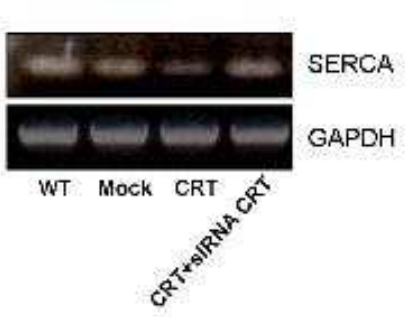

Figure 4. Overexpression of CRT depressed SERCA2a gene expression. The mRNA levels of SERCA2a was determined using reverse transcription- polymerase chain reaction. WT, wild-type; Mock, pEGFP-N3 vector only expression.

4. Effects of CRT overexpression on the apoptosis of neonatal cardiomyocytes

The effect CRT on apoptosis was examined using the TUNEL method (Figure 5). Cells overexpressing CRT increased up to 10 folds in the number of TUNEL-positive cells, whereas in its absence or CRT overexpression under siRNA for CRT treatment, increase was not observed. These results suggested that CRT overexpression enhanced the number of apoptotic cells by overload of the intracellular calcium.

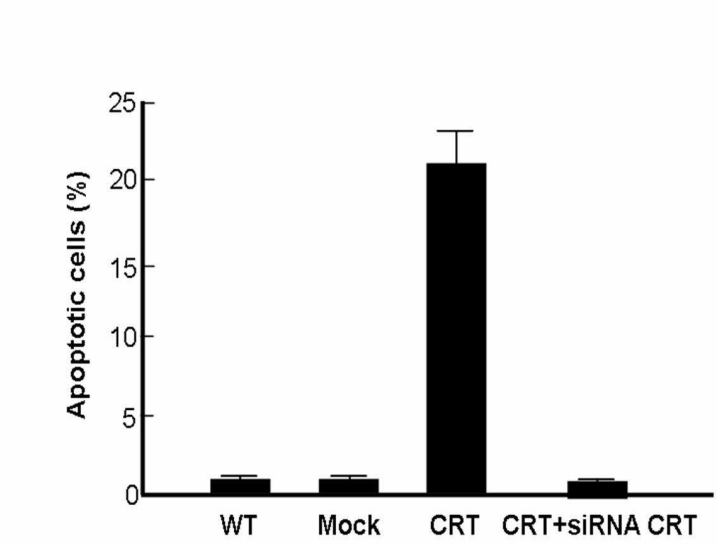

Figure 5. TUNEL assay for control and CRT gene expression on the apoptosis. The neonatal rat cardiomyocytes were transfected with 0.4g of CRT per well and then cultured with -MEM containing 5% FBS for 2 days. Staining of DNA strand break was detected by cytochemically. WT, wild-type; Mock, pEGFP-N3 vector only expression.

5. Effects of CRT overexpression on the activities of Akt and ERKs

After overexpressing CRT into cells, protein kinases related with proliferation were detected by immunoblot analysis. The serine-threonine protein kinase, Akt has been well known as an important mediator of cell survival against apoptotic stimuli. Also several studies suggest that activation of the extracellular signal regulated kinase (ERK1/2) pathway is a resistance marker to apoptosis. Figure 6 showed that phosphorylation levels of ERK and Akt were decreased only in the CRT overexpressing cells. It suggested that proliferation inhibited by overexpressing CRT was related with the activities of ERK and Akt.

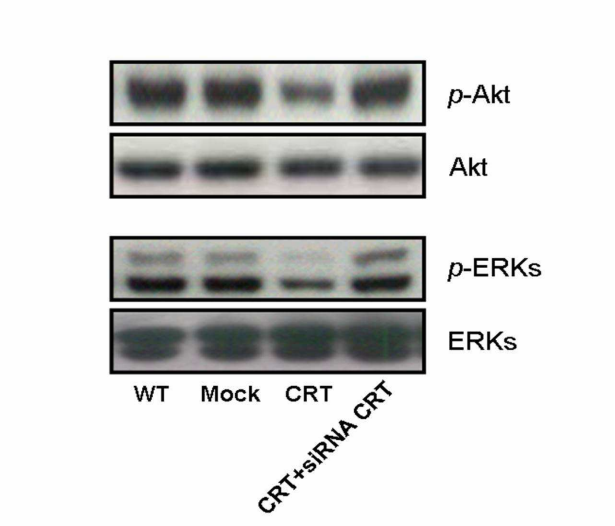

Figure 6. Effects of the activities of Akt and ERKs. Neonatal rat cardiomyocytes were transfected CRT with or without siRNA for 48 hours. Samples were harvested, lysed and immunoblotted with anti-phospho-Akt and anti-phospho-ERK antibody. WT, wild-type; Mock, pEGFP-N3 vector only expression.

6. Effects of CRT overexpression on apoptosis regulatory protein expression

To investigate the effect of CRT on anti-proliferation of cardiomyocytes, both anti-apoptotic factor, bcl-2, and pro-apoptotic factors bax, p53 and caspase-8 from mitochondria were examined in cells overexpressing CRT. As shown in Figure 7, bcl-2 was activated and bax, p53 and caspase-8 were suppressed by CRT overexpression. The apoptotic signals were completely inversed by treatment of siRNA for CRT indicating that overexpressed CRT increased apoptotic signals.

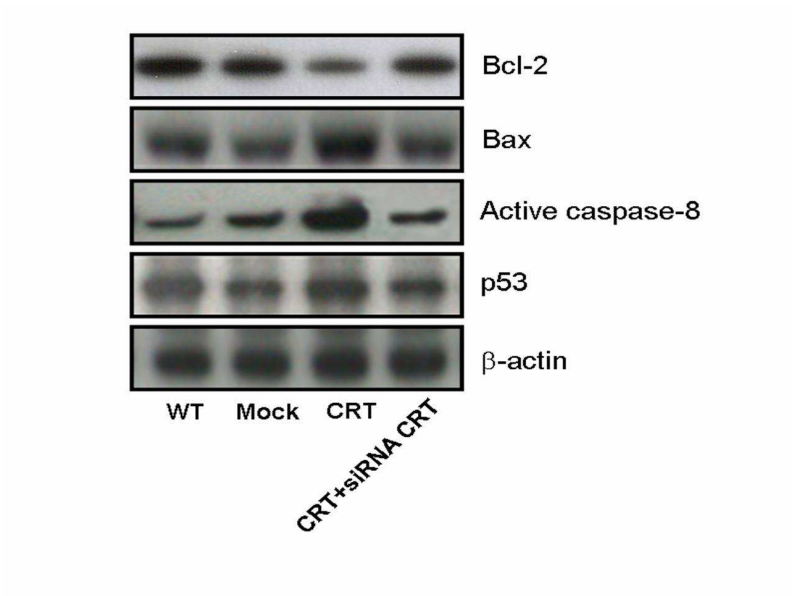

Figure 7. Effects of CRT on apoptosis regulatory protein expression. Estimation of pro- and anti-apoptotic members of the Bcl2 family and pro-caspase was done by immunoblot analysis. WT, wild-type; Mock, pEGFP-N3 vector only expression.

7. Effects of CRT overexpression on the activity of caspase-3

Because caspase-3 activity is the last signal in diverse apoptotic pathways, caspase-3 activity was also measured in cells overexpressing CRT by measuring spectrophoto-metrically (at 405 nm) amount of *p*NA cleaved from the substrate DEVD-*p*NA. Figure 8 showed that caspase-3 activity increased about 5 folds compared with control and cells transfected with CRT cultured with siRNA for CRT. These results indicated that CRT overexpression plays a pivotal role in the apoptotic signal transductions by increasing the intracellular calcium level.

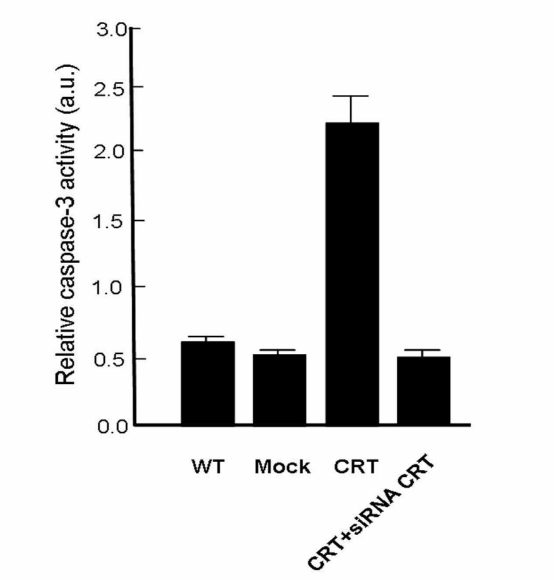

Figure 8. Expression of CRT on the activity of caspase-3. Caspase-3 activity was analyzed for 2 days. The caspase-3 activity was determined by measuring spectrophotometrically at 405 nm amount of *p*NA cleaved from the substrate DEVD-*p*NA. WT, wild-type; Mock, pEGFP-N3 vector only expression.

IV. DISCUSSION

CRT is a highly conserved protein that its analogies from diverse species in functionally important domains. This suggests that it has had important biological functions. Although CRT was firstly identified as a major calcium binding protein chaperone in the endoplasmic reticulum[10], it also acts as a regulator of integrin and nuclear hormone receptor. Recently several reviews have mentioned that CRT may be a new cardiac embryonic gene because the protein is highly expressed in embryonic hearts but not developed heart. In addition to the experiments with CRT overexpression with transgenic mouse showed the possibility that CRT is related with congenital heart block in humans[7-9], but exact physiological function of CRT remains obscure.

In this study, it was shown that CRT overexpression in the developed cells neonatal cardiomyocytes leads to cardiac apoptosis relate on Ca^{2+} dependent pathway. Apoptosis was influenced on several signaling pathways that contain SAPK, MAPK, and protein kinase B/Akt pathways[11]. In control cells and cells with CRT gene overexpression, the latter has significantly suppressed in proliferation through 5 days. In the case of cells with CRT overexpression, the total cellular calcium content examined on the basis of measuring equilibrium calcium was 350% of that in control cells. These results indicate that CRT in developed heart may cause calcium-dependent apoptotic signaling. Recently Nakamura et al.[12]and Printon et al.[13] supported these observations that cells deficient in CRT are resistance to apoptosis compared with cells expressing CRT. SERCA is an integral membrane protein that catalyzes the ATP-dependent transport of Ca^{2+} from the cytosol to the lumen of ER or SR[14]. SERCA in the heart plays a critical role in the beating function of the heart and promotes muscle relaxation

and contraction by lowering and providing the cytosolic calcium concentration through active calcium transport. Decreases in SERCA pump expression and activity have been observed in a variety of pathological conditions of the heart[15]. SERCA is composed of three homologous proteins[12] and SERCA2 has two splicing variants, SERCA2a and SERCA2b. The former are specifically expressed in cardiac muscle and the latter are non-muscle tissues. In this study, it was confirmed that SERCA2a expression level was decreased nearly 3 folds compared with wild type and mock control by RT-PCR analysis. To further confirm apoptotic effect induced CRT overexpression, TUNEL assay, caspase-3 assay, and immunoblot assay on pro-and anti apoptotic factors were carried out. In TUNEL and caspase-3 assays, it was obtained a same aspect that cells with CRT overexpression significantly increase the apoptosis but not in siRNA treatment group for CRT. According to results on immnunoblot assay for pro- and anti-apoptotic factors, the expression of Bax and p53 were increased upon CRT overexpression and activation of caspase-8 showed the same tendency. But bcl-2 and activation of important factor Akt and ERK were decreased in identical condition.

V. CONCLUSION

It was demonstrated that overexpressed CRT modulated activity on SERCA2a and then cells with CRT overexpression undergo a change on intracellular calcium level. In the developed neonatal rat cardiomyocytes, CRT overexpression lost the functions about intracellular calcium regulations and relaxation-contraction. Thus it was concluded that CRT overexpression causes calcium-dependent apoptosis as a key protein in matured cardiomyocytes.

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ABSTRACTS (IN KOREAN)

출생 후 심장에서 과발현된 calreticulin이 칼슘의존성 세포사멸에 미치는 영향

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배경: Calreticulin(CRT)은 소포체에 존재하며 Ca^{2+}과 결합하는 샤프롱 단백질의 한 종류이며 특이하게 관내 소포체 단백질로도 존재한다. Ca^{2+} 항상성을 유지시키는 조절자로서, CRT는 심장 발달의 초기 단계에 중요한 역할을 하지만, 출생 후에도 높은 수준으로 발현될 경우 심장 전도계의 발달 부진을 일으켜 심장 완전 차단의 병리학적인 원인이 될 수 있다. 본 연구에서는 CRT를 과발현시킨 신생 백서의 심근세포에서 Ca^{2+} 항상성에 영향을 미칠 신호 전달 체계에 관한 분자 수준의 기작을 조사하고, CRT가 Ca^{2+}의존성 세포사멸의 활성에 어떠한 영향을 미치는가에 대하여 조사하였다.

방법 및 결과: CRT를 과발현시킨 심근세포를 3일간 배양시켜 MTT 분석을 한 결과, 대조군에 비해 2배 낮은 증식이 확인되었고, CRT 유전자 특이적 siRNA에 의한 CRT 억제는 CRT 과발현 심근세포에 비해 증식을 증가시켰다. CRT가 과발현된 세포에서 세포내 Ca^{2+}농도는 Ca^{2+}과 결합하는 Fura-2 AM 염료의 사용으로 형광분광광도법에 의해, 상당히 증가되어졌음을 밝혔다. SERCA2a mRNA의 발현 정도는 감소하였으며, Akt와 ERK의 인산화도 CRT를 과발현시킨 심근세포에서 두드러지게 감소되었다. 또한, 항세포사멸 인자인 Bcl-2의 활성이 감소되었고, 세포사멸 촉진 인자인 Bax, p53, caspase-8의 활성은

증가되어졌으며, caspase-3의 활성은 급격한 증가를 나타내었다.
결론: 신생 백서 심근세포에서, CRT 과발현은 세포 내 Ca^{2+}조절과 이완-수축에 관한 기능을 감소시키며, Ca^{2+} 의존적 세포사멸을 일으킨다.

핵심되는 말 : calreticulin, 심근세포, 세포사멸, 칼슘 과부하