Mol. Cells, Vol. 25, No. 3, pp. 390-396

Molecules and Cells

Enhanced Calreticulin Expression Promotes Calcium-dependent Apoptosis in Postnatal Cardiomyocytes

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(Received August 21, 2007; Accepted January 22, 2008)

Calreticulin (CRT) is one of the major Ca^{2+} binding chaperone proteins of the endoplasmic reticulum (ER) and an unusual luminal ER protein. Postnatally elevated expression of CRT leads to impaired development of the cardiac conductive system and may be responsible for the pathology of complete heart block. In this study, the molecular mechanisms that affect Ca^{2+}-dependent signal cascades were investigated using CRT-overexpressing cardiomyocytes. In particular, we asked whether calreticulin plays a critical role in the activation of Ca^{2+}-dependent apoptosis. In the cells overexpressing CRT, the intracellular calcium concentration was significantly increased and the activity of PKC and level of SECAR2a mRNA were reduced. Phosphorylation of Akt and ERKs decreased compared to control. In addition the activity of the anti-apoptotic factor, Bcl-2, was decreased and the activities of pro-apoptotic factor, Bax, p53 and caspase 8 were increased, leading to a dramatic augmentation of caspase 3 activity. Our results suggest that enhanced CRT expression in mature cardiomyocytes disrupts intracellular calcium regulation, leading to calcium-dependent apoptosis.

Keywords: Apoptosis; Calcium Overload; Calreticulin; Cardiomyocytes.

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Introduction

Ca^{2+} is a universal signaling molecule that affects diverse cellular functions such as secretion, contraction-relaxation, cell motility, cytoplasmic and mitochondrial metabolism, the synthesis, modification, and folding of proteins, gene expression, cell cycle progression, and apoptosis. The endoplasmic reticulum (ER) plays a central role in maintaining intracellular Ca^{2+} homeostasis (Berridge, 2002; Michalak et al., 2002a). Calreticulin (CRT) is one of the major Ca^{2+} binding chaperone proteins of the ER and an unusual luminal ER protein (Ma and Pan, 2003; Papp et al., 2003). Recently, CRT was identified as a new embryonic cardiac gene highly expressed in embryonic heart (Mesaeli et al., 1999). CRT also plays an important role upstream of calcineurin-dependent transcriptional processes during cardiac development (Milan et al., 1994). As a modulator of Ca^{2+} homeostasis, CRT has an important role in the early stages of cardiac development. Postnatally high levels of expression of CRT lead to impaired development of the cardiac conductive system and may be responsible for complete heart block. In a study of transgenic mice overexpressing CRT in the heart, animals developed bradycardia associated with sinus node dysfunction, complete cardiac block, and death due to intractable heart failure (Kageyama et al., 2002; Mesaeli et al., 1999; Nakamura et al., 2001; Salameh et al., 2004). Electrocardiograms demonstrated that the P-R interval of the transgenic mice was prolonged in CRT overexpressors, with subsequent development of complete AV nodal conduction block. These finding indicate that CRT plays a role in the

Abbreviation: CRT, calreticulin.

development and pathology of the conductive system (Salameh et al., 2004). Furthermore, CRT overexpressing hearts have very low levels of connexin 43, a major component of the gap junctions responsible for cell-cell communication. Recent studies on H9c2 cells indicate that over-expression of CRT in cardiomyocytes affects the AKt signaling pathway and promotes apoptosis (Kageyama et al., 2002). The most significant findings are that the phenotype of calreticulin overexpressing mice is very similar to that seen in children with complete heart block. The molecular mechanism involved in complete heart block is not known at present. Activation of cardiac muscle is mediated by the specialized electrical system of the heart. This 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, whose activation depends on an inward Na^{+} current.

Although the phenotype of calreticulin overexpressing mice is very similar to that seen in children with complete heart block, the molecular basis of calreticulin involvement in the complete heart block is not known. In this study, the effects on Ca^{2+}-dependent signal cascades were investigated in calreticulin overexpressing cardiomyocytes. In particular, we addressed the possibility that calreticulin plays a critical role in the activation of apoptotic pathways.

Materials and Methods

Isolation of neonatal rat cardiomyocytes We isolate and purify neonatal rat cardiomyocytes by previously described methods (Hwang et al., 2004). Briefly, hearts of 1–2 day-old Sprague Dawley rat pups are dissected, and the ventricles washed with Dulbecco's phosphate-buffered saline solution (pH 7.4, Gibco BRL) lacking Ca^{2+} and Mg^{2+}. Using micro-dissecting scissors, hearts are minced to pieces of approximately 1 mm^3 and treated with 10 ml of collagenase I (0.8 mg/ml, 262 units/mg, Gibco BRL) for 15 min at 37°C. The supernatant is then removed and the tissue is treated with fresh collagenase I solution for an additional 15 min. The cells in the supernatant are transferred to a tube containing cell culture medium (a-MEM containing 10% fetal bovine serum, Gibco BRL). The tubes are centrifuged at 1200 rpm for 4 min at room temperature, and the cell pellets are resuspended in 5 ml of cell culture medium. The above procedures are repeated 7–9 times until little tissue is left. The cell suspensions are collected and incubated in 100 mm tissue culture dishes for 1–3 h to reduce fibroblast contamination. The non-adherent cells are collected and seeded to achieve a final concentration of 5×10^5 cells/ml. After incubation for 4–6 h, the cells are rinsed twice with cell culture medium and 0.1 μM BrdU is added. The cells are then cultured with 10% (v/v) FBS in a CO_2 incubator at 37°C.

Transfection Transfections of CRT cloned into the eukaryotic expression vector pEGFP-N3 (Invitrogen) were performed using LIPOFECTAMINE PLUS™ reagent (Gibco-BRL). Briefly, neonatal rat cardiomyocytes cultured in a 60 mm culture plate (5×10^5 cells/plate) were washed twice with serum-free MEM. LIPOFECTAMINE PLUS™ reagent was diluted with serum-free MEM and combined with 2 μg of DNA for each plate. The DNA and LIPOFECTAMINE PLUS™ reagent were added to each plate containing fresh medium and cells. After 12 h incubation in a CO_2 incubator at 37°C, the medium was changed to 10% FBS-MEM.

Flow cytometric analysis of the cell cycle For cell cycle analysis and detection of apoptosis, cardiomyocytes (5×10^6 cells/plate) were harvested and washed twice in cold PBS. Cell pellets were fixed with 70% ethanol at 4°C for at least 12 h and washed twice by cold PBS. Then the fixed cells were suspended in PBS containing RNase A (1 mg/ml, Sigma-Aldrich) and kept at 37°C for 30 min. After being centrifuged at 1,000 × *g* for 5 min, samples were re-suspended in PBS containing propidium iodide (50 μg/ml, Sigma-Aldrich) and stained in the dark for 30 min. Data were obtained using a FACSCalibur (Becton Dickinson) and analyzed with ModFit LT 3.0 software (Variety Software House).

Confocal microscopy and fluorescence measurements Measurements of cytosolic free Ca^{2+} concentration were performed by confocal microscopy. Neonatal rat cardiomyocytes were plated on glass coverslips coated with laminin (5 mg/cm^2) for 1 d in cell culture medium (a-MEM containing 10% fetal bovine serum, Gibco BRL) and 0.1 μM BrdU. After incubation, the cells were washed with modified Tyrode's solution containing: 0.265 g/l $CaCl_2$, 0.214 g/l $MgCl_2$, 0.2 g/l KCl, 8.0 g/l NaCl, 1g/l glucose, 0.05 g/l NaH_2PO_4, and 1.0 g/l $NaHCO_3$. They were then loaded with 5 mM of the acetoxymethyl ester of Fluo-4 (Fluo-4 AM, Molecular Probes) for 20 min, in the dark and at room temperature in modified Tyrode's solution. Fluorescence images were obtained using an argon laser confocal microscope (Leica). The fluorochrome was excited by the 488 nm line of the 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 fluorescence intensity.

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 1 hour incubation at room temperature, the membranes were probed overnight at 4°C with polyclonal antibodies against CRT, PKC, p-PKC, ERK, p-ERK, Akt, p-Akt, Bcl-2, Bax, caspase-8, p53 and β-actin 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.).

RT-PCR analysis The expression levels of various genes were analyzed by the reverse transcription polymerase chain reaction (RT-PCR) technique. Confluent rat neonatal cardiomyocytes were cultured for 48 h in serum-free MEM after transfection with CRT. Total RNA was prepared with an Ultraspect™-II RNA system (Biotecx Laboratories, Inc.) and single-stranded cDNA was synthesized from the isolated total RNA with Avian Myeloblastosis Virus (AMV) reverse transcriptase. The 20 μl samples of the reverse transcription reaction mixtures containing 1 μg of total RNA, 1× 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 μg of oligo(dT)15, and 15 units of AMV reverse transcriptase were 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 the various genes. GAPDH primers (5′-accacagtccatgccatcac-3′ and 5′-tccaccaccctgttgctgta-3′) were used as an internal standard. The signal intensities of the amplification products were normalized to the corresponding GAPDH signal intensities.

Caspase 3 assay Relative caspase 3 activity was determined using the ApopTarget™ Capase-3 Colorimetric Protease Assay, according to the manufacturer's instructions (Biosource). This assay is based on the generation of the free DEVD-*p*NA chromophore when the substrate provided 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, cultured neonatal cardiomyocytes (3×10^6) were harvested in lysis buffer (1 M DTT), and cell extracts were centrifuged to eliminate cellular debris. Aliquots (50 μl) of the cell extracts were incubated at 37°C for 2 h in the presence of the chromophore substrate. Free DEVD-*p*NA was determined colorimetrically. Fold increases in caspase-3 activity were derived from a comparison of the absorbance of the *p*NA from apoptotic samples with the uninduced control (Cieslak and Lazou, 2007).

Statistical analysis Data are expressed as means ± SE. Statistical analyses were performed by one-way ANOVA, using the Bonferroni test for comparison of several groups. A value of $p < 0.05$ was considered significant.

Results

Effects of CRT on the viability of neonatal cardiomyocytes To investigate the functions of CRT in cardiomyocytes proliferation, the CRT gene was isolated from a rat heart cDNA library by PCR, and the PCR product was inserted into the eukaryotic expression vector, pEGFP-N3 and transfected into neonatal cardiomyocytes for 24 h. To confirm its expression and the transfection efficiency, we used confocal microscopy, and immunoblot analysis with anti-CRT antibody. The intracellular location of CRT was observed and CRT expression was found to be about 2.5-fold higher than that of the control (Fig. 1A). To assess the cytotoxicity of the overexpressed CRT in cardiomyocytes, control and gene-transfected cells were cultured, and cell proliferation was measured by MTT assay. The proliferation of cells overexpressing CRT was significantly decreased over 5 days. To further examine the effects of CRT on proliferation, we used siRNA for CRT. Although expression of CRT was down-regulated, siRNA treatment group had little effect on growth (Fig. 1B). We performed cell cycle analysis to know whether the decreased growth caused by overexpressing CRT was due to cell death related to apoptosis, and noted an increase of apoptosis (19.8%). siRNA for CRT did not affect the cell cycle (Fig. 1C). We also examined protein kinases related to proliferation and survival by immunoblot analysis. The serine-threonine protein kinase Akt is an important mediator of cell survival in the face of apoptotic stimuli, and several studies suggest that activation of the extracellular signal regulated kinase (ERK1/2) pathway confers resistance to apoptosis. The signaling pathways involving ERK and Akt are also important for regulating proliferation in various types of cell including fibroblasts, cardiomyocytes, vascular smooth muscle cells and cancer cells (Elia et al., 2007; Nishimura et al., 2007; Olson et al., 2008); thus inhibition of the ERK and Akt pathways causes apoptosis, arrest of the cell cycle and suppression of cell proliferation (Bonaccorsi et al., 2004; Kim et al., 2007; Moon et al., 2007). Figure 1D shows that ERK and Akt phosphorylation levels were decreased in the CRT overexpressing cells, but unchanged when siRNA for CRT was also present.

Effects of CRT on the intracellular calcium overload of neonatal cardiomyocytes To examine the intracellular Ca^{2+} levels of cells overexpressing CRT, we measured fluorescence intensity after loading them with fluo-4 AM. The cells overexpressing CRT had significantly higher intracellular calcium level than control but it was unchanged when siRNA for CRT was also present (Fig. 2A). To see whether overexpression of CRT affected PKC, p-PKC and SERCA2a expression, we examined the phosphorylation of PKC and expression of PKC and SERCA2a. PKC activity and expression of PKC and SERCA2a were inhibited by CRT overexpression, while siRNA had less effect on basal levels of p-PKC, PKC and SERCA2a (Fig. 2B). These results indicate that CRT influences PKC and SERCA2a activities and alters intracellular Ca^{2+} homeostasis.

Effects of CRT on apoptosis of neonatal cardiomyocytes The effect of CRT on apoptosis was examined by the TUNEL method (Fig. 3A). The number of TUNEL-positive cells increased up to 10 fold in the CRT overexpressing cells, whereas additional treatment with siRNA for CRT decreased the enhanced number of apoptotic cells. We also showed that treatment with the broad spectrum

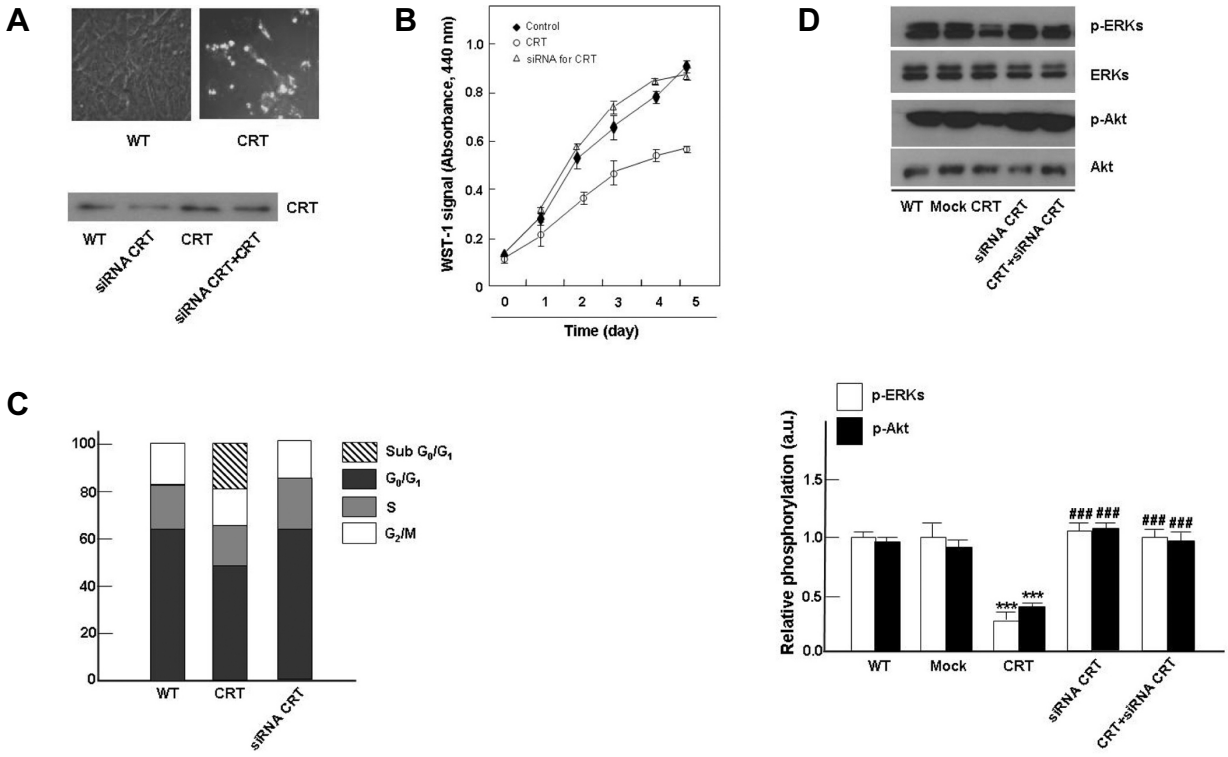

Fig. 1. Effects of CRT on proliferation of neonatal cardiomyocytes. **A.** Confocal microscopy of GFP in pEGFP-CRT-transfected cardiomyocytes, and detection of endogenous and exogenous CRT by immunoblot. **B.** Overexpression of CRT depresses proliferation of neonatal cardiomyocytes. Neonatal cardiomyocytes were transfected with CRT with or without siRNA for CRT for 5 d. Cell viability was assessed by the MTT assay. **C.** Cells were treated for 2 d under the same conditions as for the MTT assays and the cell cycle was analyzed by flow cytometry. **D.** CRT was transfected in the presence or absence of siRNA for 48 h. Samples were harvested, lysed and immunoblotted with anti-phospho-Akt and anti-phospho-ERK antibody. WT, wild-type; Mock, pEGFP-N3 vector only expression. *** $p < 0.001$ vs WT, ### $p < 0.001$ vs. CRT. Results shown are the means ± SE of three independent experiments.

caspase inhibitor Z-VAD-FMK and the caspase 8-specific inhibitor Z-IETD-FMK reduced the number of apoptotic cells to roughly 4% and 7%, respectively. These results suggest that CRT overexpression enhances apoptosis by increasing intracellular calcium levels. CRT overexpression also stimulated release of the pro-apoptotic factors, bax, p53 and proteinases from mitochondria and lowered release of the anti-apoptotic factor bcl-2 (Fig. 3B). Caspase-3 activity was also induced by CRT overexpression (Fig. 3C). These findings suggest that CRT overexpression stimulates apoptosis by increasing intracellular calcium levels.

Discussion

Calreticulin is a major ER protein that plays important roles in cardiac development and pathology (Michalak et al., 2002b). Although CRT was firstly identified as a major calcium binding protein chaperone in the endoplasmic reticulum (Michalak et al., 1999), several reviews have because the protein is highly expressed in embryonic suggested that it may be an early cardiac gene product hearts but not in mature hearts. In addition experiments with CRT-overexpressing transgenic mice suggested the possibility that CRT is related to congenital heart block in humans (Kageyama et al., 2002; Nakamura et al., 2001; Salameh et al., 2004), but the exact physiological function of CRT remains obscure. In this study, we demonstrated that enhanced CRT expression in postnatal cardiomyocytes leads to Ca^{2+}-dependent apoptosis.

Studies with calreticulin-deficient cells suggest that this protein participates in apoptosis. Cells deficient in CRT are relatively resistant to apoptosis (Nakamura et al., 2000; Pinton et al., 2001) and mice with a targeted disruption of the calreticulin gene die in utero with decreased ventricular cell mass due to increased apoptosis of cardiac myocytes (Rauch et al., 2000). In the CRT transgenic heart, CRT-dependent cardiac block involves damage to both L-type Ca^{2+} channels and gap junction connexin-40 and connexin-43, due to defective regulation of Ca^{2+} homeostasis (Nakamura et al., 2001). Overexpression of CRT suppresses Akt signaling and causes differentiation-induced apoptosis in H9c2 cells (Kageyama et al., 2002). The present study showed that cells overexpressing CRT

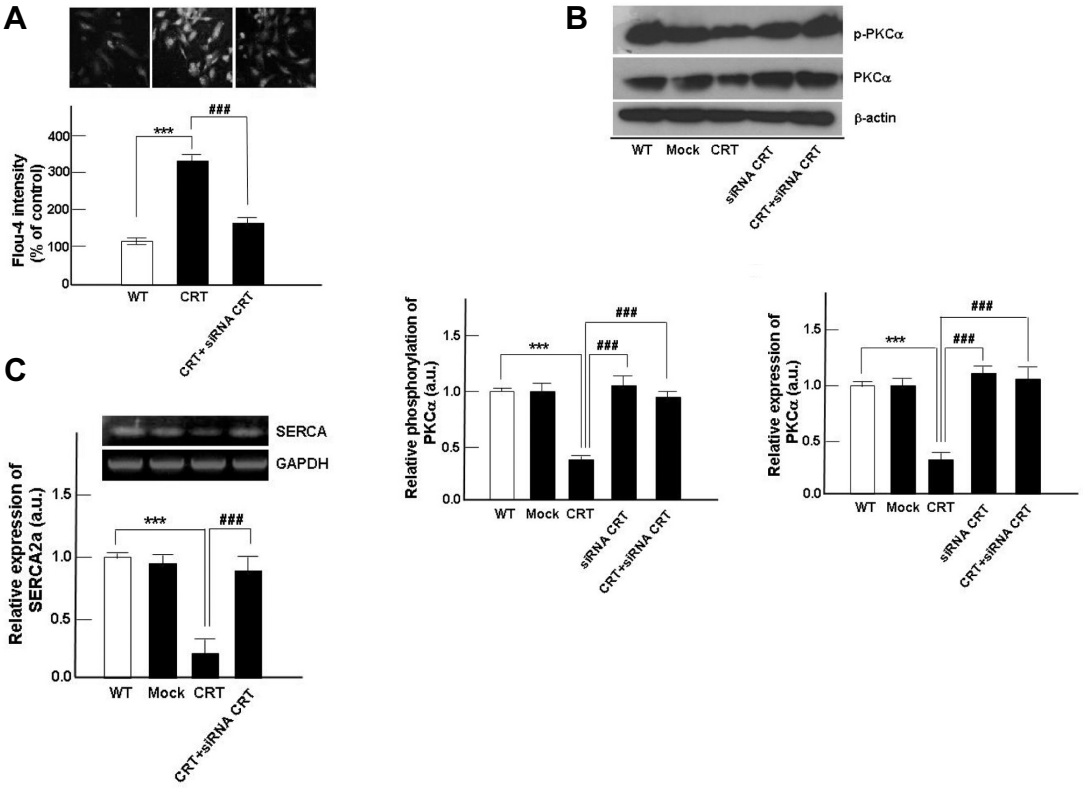

Fig. 2. Effects of CRT on Ca^{2+} homeostasis of neonatal cardiomyocytes. **A.** Overexpression of CRT increased intracellular Ca^{2+} in neonatal cardiomyocytes. The 2 μM Fluo-4 was loaded into the cardiomyocytes and they were cultured for 20 min in a humidified chamber. Relative intracellular Ca^{2+} intensity was determined from the manufacturer's manual. *** $p < 0.001$ vs. WT, $^{###}$ $p < 0.001$ vs. CRT. Results shown are the means ± SE of ten independent experiments. **B.** Overexpression of CRT depresses the activity of PKC and the mRNA level of SERCA2a. PKCα phosphorylation and expression were detected by immunoblot analysis using anti-PKC antibody. Phosphorylation and expression of PKC were calculated relative to β-actin. *** $p < 0.001$ vs. WT, $^{###}$ $p < 0.001$ vs. CRT. **C.** SERCA2a gene expression was determined by RT-PCR. WT, wild-type; Mock, pEGFP-N3 vector only expression. *** $p < 0.001$ vs. WT, $^{###}$ $p < 0.001$ vs. CRT. Results shown are the means ± SE of three independent experiments.

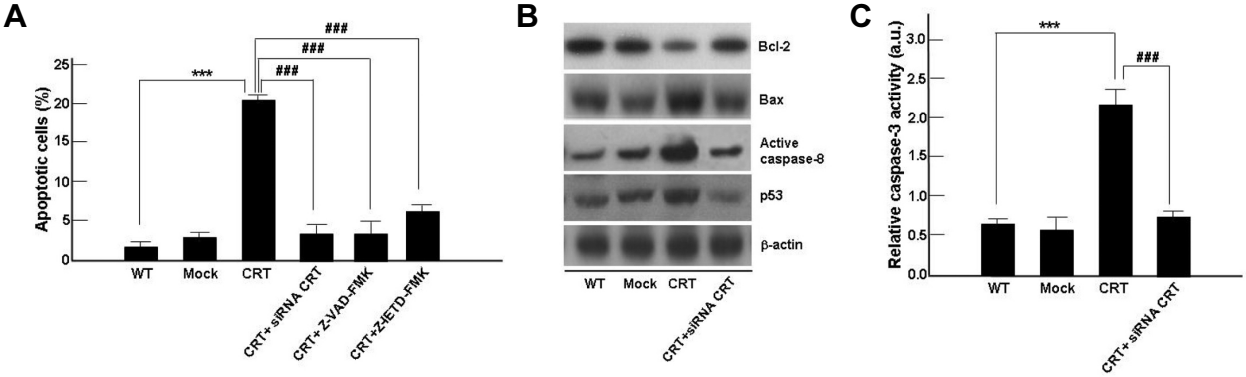

Fig. 3. Effects of CRT on apoptosis of neonatal cardiomyocytes. **A.** TUNEL assays of CRT-overexpressing cells. The neonatal cardiomyocytes were transfected with 0.4 μg of CRT per well in the presence or absence of Z-VAD-FMK (100 μM) and Z-IETD-FMK (50 μM) or siRNA, and then cultured with α-MEM containing 10% FBS for 2 d. DNA strand breaks were detected cytochemically. WT, wild-type; Mock, pEGFP-N3 vector only expression. *** $p < 0.001$ vs. WT, $^{###}$ $p < 0.001$ vs. CRT. **B.** Effects of CRT on expression of apoptosis regulatory proteins. Pro- and anti-apoptotic members of the Bcl2 family and pro-caspases were assayed by immunoblot analysis. **C.** Effect of overxpression of CRT on the activity of caspase-3. Caspase-3 activity was determined by measuring spectrophotometrically at 405 nm the amount of *p*NA cleaved from the substrate DEVD-*p*NA. WT, wild-type; Mock, pEGFP-N3 vector only expression. *** $p < 0.001$ vs. WT, $^{###}$ $p < 0.001$ vs. CRT. Results shown are the means ± SE of three independent experiments.

have altered Ca^{2+} homeostasis and undergo increased apoptosis (Figs. 2 and 3), confirming that enhanced CRT is related to cell death in postnatal cardiomyocytes (Groenendyk et al., 2004).

We also tested whether CRT affects PKC activation. CRT overexpression inhibited not only the expression but also the phosphorylation of PKCα, a PKC isoform activated by Ca^{2+}. Although the link between PKC expression and activity still remains unresolved in skeletal muscle, some reports have pointed to decreased expression of PKC in disused muscle (Pierno et al., 2007), while suppression of PKCα by antisense treatment led to inactivation of PKCα and triggered caspase-dependent apoptosis (Hsieh et al., 2003). It is known that CRT is phosphorylated in response to α-adrenergic stimulation of PKC *in vivo* and that it is an excellent substrate for all PKC isoforms *in vitro* (Wuytack et al., 2002). The interaction between CRT and the sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) is controversial. SERCA is composed of three homologous proteins (Periasamy and Huke, 2001) and SERCA2 has two splicing variants, SERCA2a and SERCA2b. The former are specifically expressed in cardiac muscle and the latter in non-muscle tissues. We confirmed that the SERCA2a expression level of cells overexpressing CRT decreased nearly 3 fold. We also confirmed the apoptotic effect induced by CRT overexpression, by TUNEL assays, caspase 3 assays, and immunoblot assays of pro- and anti-apoptotic factors (Fig. 3).

In conclusion, we have demonstrated that overexpression of CRT modulates the activity of PKC and SERCA2a and that intracellular calcium levels change in the overexpressing cells. The neonatal cardiomyocytes thus are defective in intracellular calcium regulation and relaxation-contraction. Our findings show that CRT overexpression causes apoptosis of mature cardiomyocytes.

Acknowledgments This work was supported by a faculty research grant of the Yonsei University College of Medicine for 2006, and the 2007 Hannam University Research Fund.

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