Tissue Engineering and Regenerative Medicine, Vol. 6, No. 12, pp 1148-1158 (2009)

| Original Article |

Effects of Ranolazine on Norepinephrine-Induced Cell Death by Inhibition of the β-adrenoceptor Signal Pathway in Cardiomyocytes

Kyung-Eun Kim[1,†], Heesang Song[2,†], Hye-Jung Kim[3,†], Min-Ji Cha[3], Byeong-Wook Song[3], Eunju Choi[3], Onju Ham[3], ChangYeon Lee[3], SeongYong Choi[3], Se-Yeon Lee[3], Yangsoo Jang[3,4], Tae Woong Kim[5] and Ki-Chul Hwang[3,*]

[1]Department of Pediatrics, National Medical Center, Korea, [2]Research Institute of Science of Aging, Yonsei University, Seoul, Republic of Korea, [3]Cardiovascular Res Institute, Brain Korea 21 Project for Medical Sci, Yonsei Univ College of Medicine, Seoul 120-752, Korea, [4]Div of Cardiology, Dept. of Internal Medicine, College of Medicine, Yonsei Univ, 250 Seongsanno, Seodaemun-gu, Seoul, 120-752 Korea, [5]Department of Biochemistry and Research Institute of Life Sciences, Kangwon National University, Chuncheon 200-701, Republic of Korea

(Received: July 24th, 2009; Accepted: August 5th, 2009)

Abstract: Ranolazine blocks the intracellular sodium and calcium overload accompanying myocardial ischemia and is used in antianginal therapy. The effects of ranolazine on the β-adrenoceptor signal transduction system are poorly understood. The present study was designed to confirm whether the mechanism was associated with a β-adrenoceptor antagonist activity of ranolazine on norepinephrine (NE)-induced cardiomyocytes. After cardiomyocytes were preincubated with propranolol and ranolazine, cells were treated with NE for 24 hours. The phosphorylation of ERK was decreased by ranolazine treatment, in comparison with NE-only treated cells. Intracellular Ca^{2+} and Na^{+} levels decreased by 40±3% and 17±0.5%, respectively, compared to control. Ranolazine decreased expression of Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) by 57±4%, the Na^{+}-Ca^{2+} exchanger (NCX) by 21±0.5%, and the ryanodine receptor 2 (RyR2) by 47±1.5%, compared with NE-only treated control cells. Ranolazine also increased expression of the L-type Ca^{2+} channel (LTCC) by 48±3.5%, phospholamban (PLB) levels by 45±2%, and the sarcoplasmic reticulum Ca^{2+} ATPase 2a (SERCA2a) by 36±0.5%, compared to levels in NE-only stimulated cells. The number of annexin V/PI-positive cells fell by 39±1.5% after ranolazine treatment, compared with levels in NE-only treated control cells. Ranolazine also inhibited apoptosis by regulating the levels of the pro-apoptotic factor Bax, the anti-apoptotic factor Bcl-2, and cytochrome C release. These results demonstrate that ranolazine had an effect on NE-induced cell death through inhibition of the β-adrenoceptor signal pathway in cardiomyocytes.

Key words: *cardiomyocyte, ranolazine, β-adrenoceptor, norepinephrine, apoptosis*

1. Introduction

Heart failure (HF) is associated with imbalances in cellular Ca^{2+} and Na^{+} homeostasis.[1] Ranolazine has been shown to block the late sodium current in ischemic cardiomyocytes. Therefore, ranolazine is thought to indirectly reduce Ca^{2+} overload *via* an action on Na^{+} channels, preserving ionic homeostasis.[2] Acute intravenous administration of ranolazine improved left ventricular systolic function in animal models of heart failure.[3] However, the sodium current inhibitor mechanism of ranolazine has not been defined.[4] The actions of norepinephrine (NE) involve binding to adrenergic receptors.[5,6] NE, a β-adrenoceptor agonist, plays a role in many cardiovascular diseases including congestive heart failure. Also, NE has a co-mitogenic effect in isolated cardiac fibroblasts, and activates mitogen-activated protein kinases (MAPKs).[7] The MAPKs, a large family of serine-threonine kinases, play important roles as mediators of signal transduction and are activated by extracellular stimuli.[8,9] Three subgroups of MAPKs have been clearly identified; these are the extracellular signal-regulated kinases (ERK1/2), the p38 kinase, and the c-jun N-terminal kinases (JNKs). ERK1/2 responds to mitogenic stimuli, whereas p38 kinase and JNKs respond predominantly to cellular stresses or inflammatory cytokines.[9,10] Recently, heterotrimeric G proteins have also been shown to activate various members of the MAPK family.[8,9] One of the major mechanisms for regulating contractility of the heart involves β-adrenoceptor stimulation.

*Tel: +82-2-2228-8523; Fax: +82-2-365-1878
e-mail: kchwang@yuhs.ac (Ki-Chul Hwang)
†These authors contributed equally to this work

The β-adrenoceptor is the first element in the signal transduction chain mediating adrenergic stimulation of the heart. All known subtypes of the β-adrenoceptor (β_1, β_2, and β_3) are glycoproteins with extracellular amino-termini, seven hydrophobic transmembrane domains, and intracellular carboxy-termini. All three subtypes, when activated, can cause increases in intracellular levels of cAMP, although many recent studies have demonstrated that this is not the sole signaling pathway.[11,12] Selective agonists and antagonists exist for all three receptor subtypes.[13] The β-adrenoceptor antagonists are used therapeutically for treatment of cardiovascular diseases such as cardiac failure, angina, and hypertension, and the beneficial effects result mainly from action on the heart.[14] Regardless of the underlying mechanism, systemic β-adrenoceptor antagonists can have a number of significant effects on cardiac function.[15]

As a second messenger, Ca^{2+} regulates acute physiological functions, including contraction of cardiac, skeletal, and smooth muscles, and release of hormones and neurotransmitters. Variations in Ca^{2+} homeostasis can lead not only to loss of normal physiological control mechanisms but also to pathological changes in cell growth. Cardiac dilatation and pump dysfunction without intrinsic myocardial systolic failure follows chronic β-adrenoreceptor activation.[16] Increases in Ca^{2+} levels can transduce signals through various classes of Ca^{2+}-regulated enzymes, one of which is the Ca^{2+}/calmodulin-dependent protein kinase (CaMK) family.[16] Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) is a widely expressed protein kinase that modulates various functions including learning and memory in the nervous system, muscle contraction, cell secretion, and gene expression.[17] In the heart, CaMKIId is the predominant CaMKII isoform and a splice variant thereof, dc, resides in the cytosol. In addition to a well-documented role in regulation of cardiac excitation-contraction (E-C) coupling,[18-20] CaMKIIdc has been implicated in apoptotic signaling[21-24] and in mediating the transition to heart failure.[25] CaMKII regulates an array of key proteins involved in cardiac E-C coupling and Ca^{2+} processing, such as the sarcoplasmic/endoplasmic reticulum Ca^{2+}ATPase (SERCA) and regulators thereof, for example phospholamban (PLB) and the ryanodine receptor (RyR) Ca^{2+} release channels.[20,21,25]

A β-adrenoceptor stimulus increases apoptosis[26] and the levels of reactive oxygen species (ROS)[27] in cardiomyocytes. ROS, generated by a variety of extracellular and intracellular mechanisms, have gained attention as novel signal mediators that regulate signal transduction events and are known to play important roles in the pathogenesis of several cardiovascular diseases. ROS are recognized to regulate the transcription of particular genes.[28] However, ROS may specifically induce apoptosis.[29] Recent work has demonstrated that chronic adrenergic stress is associated with adverse effects, such as cardiac failure, mainly because of increased cardiomyocyte apoptosis.[30] Apoptosis has been demonstrated to occur in the myocardium in a variety of pathological situations.[31] The number of apoptotic myocytes is increased in myocardium obtained from patients with end-stage heart failure and myocardial infarction,[32] and in myocardium from experimental models of myocardial hypertrophy and failure, including the rat,[33] the spontaneously hypertensive rat,[34] and rats with myocardial infarction.[35] Many molecules, including proteins in the Bcl-2 and caspase families, participate in the apoptotic response to numerous death-inducing signals.[36]

We therefore sought to confirm whether ranolazine exerted an antagonist activity at the β-adrenoceptor level in norepinephrine (NE)-induced cardiomyocytes. The β-adrenoreceptor antagonist propranolol can blunt the cardiomyocyte hypertrophic response in the heart. Propranolol has other pharmacological effects, such as a Na^+ channel-blocking action and an antioxidant effect.[37] In the present study, it was expected that ranolazine would have an effect similar to that of propranolol in NE-induced cardiomyocytes. Because NE stimulation increased ERK activation, CaMKII level, $Ca^{2+}$and Na^+ overload, and Ca^{2+} channel (LTCC, NCX, SERCA2a, PLB, and RyR2) gene expression in cardiomyocytes, we tested whether ranolazine inhibited MAPK/ERK activation, CaMKII expression, Ca^{2+} and Na^+ overload, and Ca^{2+} channel gene expression in NE-induced cardiomyocytes. The mechanisms of NE-induced ROS expression, and apoptosis, were examined in cardiomyocytes and it was found that NE-induced apoptosis was accompanied by downregulation of Bcl-2 protein synthesis and activation of β-adrenergic and cytochrome C release mechanisms.

2. Materials and Methods

2.1 Isolation and Culture of Rat Cardiomyocytes

Neonatal rat cardiomyocytes were prepared by an enzymatic method. Briefly, hearts of 1-2-day-old Sprague-Dawley (SD) rat pups were dissected, minced, enzymatically dispersed in 10 mL of collagenase II solution (0.5 mg/mL, 262 U/mg, Gibco BRL, Paisley, UK), and differentially centrifuged to yield 5×10^5 cells/mL. After incubation for 2 h, cells were rinsed twice in a-MEM (Gibco) containing 10% (v/v) fetal bovine serum (FBS) (Gibco), and 0.1 M BrdU (Sigma Chemical Co., St. Louis, MO) was added to inhibit fibrous cell growth. Cells were next cultured in a CO_2 incubator at 37°C for 1 day.

2.2 NE Stimulation and Treatment with Propranolol and Ranolazine

Cardiomyocytes were incubated in 5% (v/v) CO_2 at 37°C for 1 day. The cells were next placed into a-MEM without FBS in a CO_2 incubator (Thermo Forma Model 311, Marietta, USA) for 24 h. Cells were then pre-incubated with propranolol (2 μM), or ranolazine (3 μM), or a mixture of propranolol and ranolazine, and exposed to NE (10 μM) for 24 h.

2.3 Measurement of Intracellular Sodium Level by Flow Cytometry

Intracellular sodium was measured by the Corona green method (CoroNa™ Green AM, Molecular Probes, Eugene, OR, USA). Cells (5×10^6) were cultured for 1 day on a plate 100 mm in diameter coated with 1.5% (w/v) gelatin. For intracellular sodium measurement, Corona green stock solution was added to 1 mL of cells to yield a final concentration of 5 μM stain, at 1 h prior to assay. Incubation was continued at 37°C under a 5% (v/v) CO_2 atmosphere. Before flow cytometric examination, propidium iodide (PI) (Sigma) was added to a final concentration of 10 μg/mL. Cells (1×10^4) were analyzed by sequential excitation at 492-516 nm and 488 nm, respectively, using a FACSCalibur system (Becton Dickinson, San Jose, CA), and Cell Quest™ software for evaluation purposes.

2.4 Measurement of Intracellular Reactive Oxygen Species Generation

Neonatal rat cardiomyocytes were labeled with 2'7'-dichlorodihydro-fluorescein diacetate (H_2DCFDA; Molecular Probes). The probe (10 μM) enters the cell, and the acetate group on H_2DCFDA is cleaved by cellular esterases, thus trapping nonfluorescent 2'7'-dichlorodihydro-fluorescein (DCFH) internally. Subsequent oxidation by ROS yields the fluorescent product DCF. The dye, when exposed to an excitation wavelength of 480 nm, emits light at 535 nm only after oxidization. Labeled cells were examined by luminescence spectrophotometry.

2.5 Confocal Microscopy and Fluorescence Measurements

Cytosolic free Ca^{2+} concentration was estimated by confocal microscopy. Neonatal rat cardiomyocytes were plated in a four-well slide chamber coated with 1.5% (w/v) gelatin for 1 day in a-MEM containing 10% (v/v) FBS and 0.1 μM BrdU. After incubation, 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, 1.0 g/L glucose, 0.05 g/L NaH_2PO_4, and 1.0 g/L $NaHCO_3$. Cells were next loaded with 10 μM of the acetoxymethylester of fluo-4 (Fluo-4 AM, Molecular Probes) for 20 min, in the dark at 37°C. Images were collected using a confocal microscope (Leica, Solms, Germany) by excitation with the 488 nm line of an argon laser, and emitted light was collected through a 510-560 nm bandpass filter. Relative levels of intracellular Ca^{2+} were determined by measuring fluorescence intensity .

2.6 Annexin V/PI Staining

Apoptosis was measured using an ApoScan™ Annexin V-FITC apoptosis detection kit (Biobud, Seoul, Korea). Cells were pelleted and analyzed in the FACSCalibur system. The excitation frequency was 488 nm. The green fluorescence emitted by Annexin V (FL1) and the red fluorescence of PI (FL2) were measured using 525 nm and 575 nm bandpass filters, respectively. A total of at least 1×10^4 cells was analyzed in each sample. Light scattering was measured on a linear scale using 1,024 channels and the fluorescence intensity was assessed on a logarithmic scale. The levels of early apoptosis and late apoptosis/necrosis were measured as percentages of Annexin V^+/PI^- and Annexin V^+/PI^+ cells, respectively.

2.7 Immunoblot Analysis

Drug-treated cells were washed once in PBS and lysed in a lysis buffer (Cell Signaling, Beverly, MA) containing 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% (v/v) Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 mg/mL leupeptin, and 1 mM PMSF. The protein concentration in each fraction was determined using a BCA protein assay kit (Pierce Biotechnology, Rockford, IL). Proteins were separated in 10-15% (w/v) SDS-polyacrylamide gels and electrotransferred to methanol-treated polyvinylidene difluoride membranes (Millipore Co, Bedford, MA). After blocking the membrane with Tris-buffered saline-Tween 20 (TBS-T containing 0.1% [v/v] Tween 20) and 10% (w/v) skim milk for 1 h at room temperature, each membrane was washed twice with TBS-T and incubated with antibodies against ERK1 (Santa Cruz Biotechnology, Santa Cruz, CA), phospho-ERK1/2 (Santa Cruz Biotechnology), anti-cytochrome C (Santa Cruz Biotechnology), Bcl2 (Santa Cruz Biotechnology), and Bax (Stressgen Biotechnologies, Victoria, BC, Canada) for 1 h at room temperature or overnight at 4°C. Each membrane was washed three times with TBS-T for 5 min on each occasion, and next incubated for 1 h at room temperature with appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies. After extensive washing, bands were detected using

an enhanced chemiluminescence (ECL) reagent (Amersham Pharmacia Biotech, Piscataway, NJ). Band intensities were quantified using a Photo-Image System (Molecular Dynamics, Uppsala, Sweden).

2.8 RT-PCR Analysis

2.8.1 Isolation of Total RNA

Total RNA was extracted using 500 μL/60 mm-diameter plate of TRIzol (Sigma). Chloroform (100 μL) was added to the extract and each sample was vortexed for about 10 sec. Next, centrifugation at 12,000 g, at 4°C for 15 min, caused three layers to appear, and the transparent upper layer was collected in a new tube. Each sample next received 250 μL 2-propanol, and vortexing for about 30 sec was repeated. Centrifugation at 12,000 g, at 4°C for 10 min, followed. The supernatant was discarded and the pellet washed in 75% (v/v) ethanol admixed with diethylpyrocarbonate (DEPC; Sigma), and dissolved in water. Centrifugation at about 7,500 g, at 4°C for 5 min, followed. The supernatant was again discarded and the pellet dried at room temperature for about 7 min. Finally, each pellet was dissolved in 30 μL nuclease-free water (NFW). The quality and quantity of RNA were estimated by calculation of OD_{260}/OD_{280} ratios using a DU 640 spectrophotometer (Eppendorf, Hamburg, Germany).

2.8.2 cDNA Synthesis

Complementary DNA (cDNA) was synthesized using the RT&GO™ kit. One microgram of RNA was added to 1 μL of the anchored primer dT 25V, 2 μL dithiothreitol (DTT), and NFW, to yield a final volume of 9 μL. To prevent development of secondary structure, the mixture was incubated for 5 min at 70°C and 8 μL RT-&GO™ mastermix was next added. Each sample was incubated at 42°C for 1 h. Next, reverse transcriptase was added and incubation continued at 70°C for 15 min. As described above after isolation of total RNA, sample quality and quantity were estimated by calculation of OD_{260}/OD_{280} ratios.

2.8.3 PCR Analysis

One microgram of cDNA, and 10 pmol of each primer (forward and backward; Table 1), 0.1 mM of a dNTP mixture, 1.25 U Taq polymerase, and 10 X reaction buffer were mixed with NFW to give a final volume of 25 μL. PCR conditions were as follows. A cycle of denaturation at 94°C for 3 min was followed by 35 elongation cycles each featuring denaturation at 94°C for 30 sec, annealing at 48-60°C for 30 sec, and elongation at 72°C for 30 sec. The reaction was next held at 72°C for 10 min. Primers used are shown in Table 1. PCR products were separated by electrophoresis in 1.2% (w/v) agarose gels (Biorad, Seoul, Korea) and Gel-Doc (Biorad) was used to visualize bands after staining with ethidium bromide (Sigma).

2.9 Statistical Analysis

Data from three individual experiments are expressed as means±SEs. Statistical analysis was performed using one-way ANOVA, employing the Bonferroni test for between-group comparisons. A P value <0.05 was considered significant.

3. Results

3.1 Ranolazine Inhibited Phosphorylation of ERK in NE-Induced Cardiomyocytes

Table 1. PCR primers used in this study

Gene	primer	bp	cycler program
GAPDH	CTCCCAACGTGTCTGTTGTG TGAGCTTGACAAAGTGGTCG	450	49°C, 60s/35cyc.
CaMKII	TCAGATGTTTTGCCACAAAGAGGTGCCTCCT CCGGATGGGGTAAAGGAGTCAACTGAGAGCT	531	60°C, 60s/35cyc.
L-type Ca2+ channel	TGTCACGGTTGGGTAGTGAA TTGAGGTGGAAGGGACTTTG	346	49°C,60s/35cyc.
Phospholamban	GCTGAGCTCCCAGACTTCAC GCGACAGCTTGTCACAGAAG	339	48°C,60s/35cyc.
NCX-1	TGTCTGCGATTGCTTGTCTC TCACTCATCTCCACCAGACG	364	48°C, 60s/35cyc.
SERCA2a	TCCATCTGCCTGTCCAT GCGGTTACTCCAGTATTG	351	42°C, 60s/35cyc.
RyR2	CCAACATGCCAGACCCTACT TTTCTCCATCCTCTCCCTCA	196	48°C, 60s/35cyc.

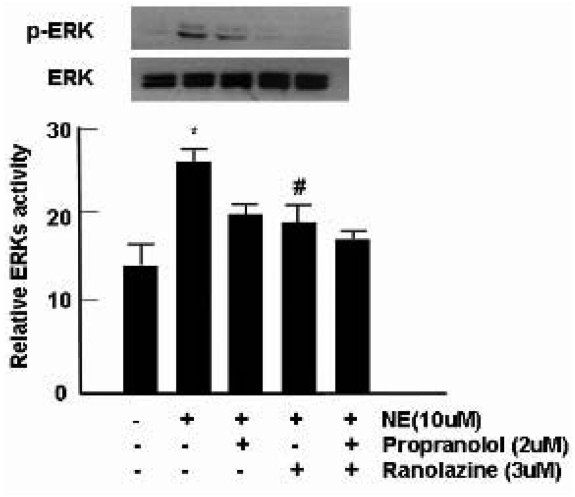

Figure 1. Phosphorylation of ERK in NE-induced cardiomyocytes. Relative phosphorylation levels of ERK activity were determined by western blotting. Cardiomyocytes were subjected to NE stimulation with propranonol and ranolazine for 24 hr. phosphorylation of ERK was treated during NE stimulation was detected by immunoblotting. Values are mean±SEM.

NE stimulation increased MAPK/ERK activation in cardiomyocytes. Therefore, we explored the effect of ranolazine after NE stimulation. Although phosphorylation of ERK increased by 63±1% after treatment with NE for 24 h, ERK phosphorylation fell by 52±2.5% after ranolazine treatment (Fig. 1), compared with the level seen in NE-induced cardiomyocytes.

3.2 Ranolazine Decreased CaMKII Overexpression in NE-Induced Cardiomyocytes

During NE stimulation, CaMKII expression was increased. Therefore, we examined the CaMKII expression level in NE-induced cardiomyocytes using RT-PCR. The level of CaMKII increased by 73±2% in NE-induced cardiomyocytes. After treatment of NE-induced cells with ranolazine, the level of CaMKII fell by 57±4% compared with that in NE-induced cardiomyocytes (Fig. 2).

3.3 Ranolazine Protected NE-Induced Cardiomyocytes from Intracellular Na^+ Overload

We examined cardiomyocytes at the single-cell level for changes in intracellular sodium concentration using the fluorescent Corona green (Na^+) dye test and flow cytometry. With gating for only normal-sized or positive control cells, NE treatment in the presence of Corona green (Na^+) resulted in a rise of 21±2% in cells in the M1 stage, compared with control cells and those treated with propranolol, ranolazine, or a mixture of ranolazine and propranolol, where reductions of

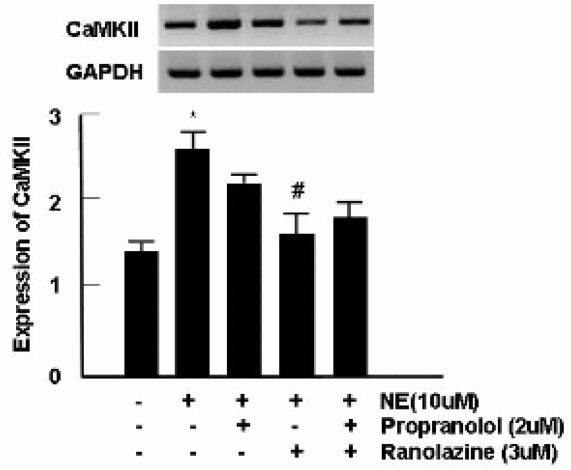

Figure 2. CaMKII expression in NE-induced cardiomyocytes. Cardiomyocytes treated with ranolazine and propranolol were exposed to NE stimulation for 24 hr. Whereas NE treated cells were increased in CaMKII mRNA, ranolazine treated cells were completely decreased in CaMKII mRNA. Values are mean±SEM.

18±3%, 17±0.5%, and 17±2.5%, respectively, in M1 cells were noted, compared to the level in NE-induced cardiomyocytes (Fig. 3).

3.4 Ranolazine Prevented Intracellular Ca^{2+} Accumulation after NE Stimulation

Intracellular Ca^{2+} level was increased by activation of several Ca^{2+} channels in NE-induced cardiomyocytes. As shown in Fig. 4, the intracellular Ca^{2+} level during NE stimulus was elevated by 50±3% at 24 h but fell by 43±0.5%, 40±3%, and 50±1.5% after treatment of cells with 2 μM propranolol, 3 μM ranolazine, and a mixture of propranolol and ranolazine, respectively.

3.5 Ranolazine Suppressed Ca^{2+} Channel Expression in NE-Induced Cardiomyocytes

Ca^{2+} entry through the L-type Ca^{2+} channel (LTCC) into cardiomyocytes is known to be the initiating event of the E-C coupling process. LTCC are critically involved in excitation/secretion coupling whereby membrane depolarization activates the LTCC. The density of LTCC was diminished by 63±2% after NE stimulation compared with that of control cells. After NE-induced cells were treated with ranolazine, the density of LTCC was elevated by 48±3.5% in comparison with NE-stimulated cells (Fig. 5).

3.6 Ranolazine Inhibited Intracellular Ca^{2+} Overload in NE-Induced Cardiomyocytes

We examined the expression of Ca^{2+} channels related to

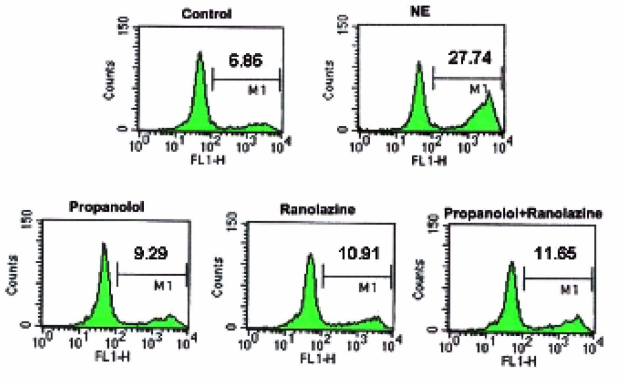

Figure 3. Effects of ranolazine on intracellular Na^+ overload in NE-induced cardiomyocytes. NE treatment in the presence and absence of ranolazine was initially examined for changes in intracellular sodium by flow cytometry using corona green (Na^+). The cells were initially analyzed by gating on only the normal cell or NE treated cells on a side scatter histogram plot. Subsequently, this population of cells was then analyzed on a corona green (Na^+) fluorescence histogram. An increase in corona green (Na^+) fluorescence indicates an increase in intracellular sodium. Only the NE treated cells increased in intracellular sodium, indicating Na^+ overload. Values are mean±SEM.

intracellular Ca^{2+} regulation, which is important in cell function, and sought changes in expression levels of one of the representative Ca^{2+} channels, NCX1, and Ca^{2+} control within cells, involving SERCA2a and regulators thereof, including PLB and RyR2. Tests were conducted on cardiomyocytes exposed to NE. RyR2 and NCX gene expression levels in NE-induced cardiomyocytes treated with ranolazine were reduced by 47±1.5% and 21±0.5%, respectively, compared to levels in NE-treated cells (Fig. 6A). The expression levels of SERCA2a and PLB were higher, by 36±0.5% and 45±2%, respectively, than levels in NE-induced cells (Fig. 6B). These results showed that ranolazine prevented changes in SERCA2a, PLB, NCX, and RyR2 expression levels.

3.7 Ranolazine Decreased Reactive Oxygen Species Production in NE-Induced Cardiomyocytes

ROS, including hydrogen peroxide (H_2O_2) and hydroxyl radicals, are normally generated in mitochondria and are important mediators of signal transduction.[37,38] Significant enhancement in fluorescence intensity was detected in NE-treated cells after 24 h of culture, compared to levels in control cells (Fig. 7A). ROS production was increased by 71±0.5% after NE (10 μM) treatment of cardiomyocytes for 24 h (Fig. 7B). Although ROS production in ranolazine- treated cells decreased by 52±1.5% with respect to the level in NE-exposed cells, ROS production in propanolol-treated cells fell more than did ROS synthesis in ranolazine-treated cells.

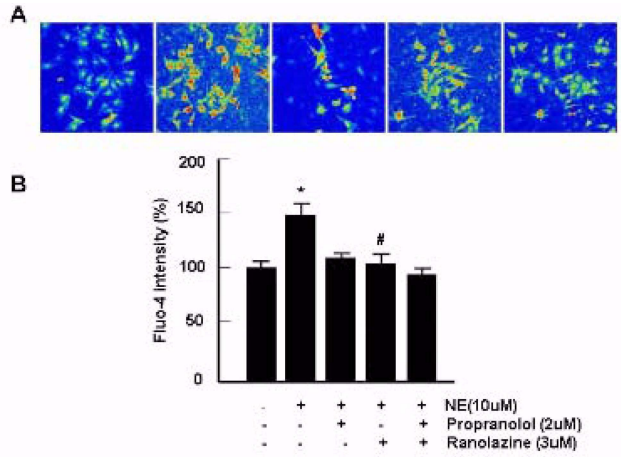

Figure 4. Effects of ranolazine on intracellular Ca^{2+} concentration. Confocal fluorescent images of cardiomyocytes were obtained by loading with fluo-4 AM. The NE-induced cells treated with ranolazine and propranolol were incubated for 24 hr. (A) Fluorescence image was obtained by fluo-4 AM and (B) fluorescence intensity was quantified in different cells (n=5) in each condition and analyzed. Values are mean±SEM.

3.8 Ranolazine Prevented NE-Induced Apoptosis of Cardiomyocytes

The level of apoptosis, as measured by the proportion of Annexin V-positive cardiomyocytes, was investigated after exposure of cells to NE with or without propranolol and ranolazine (Fig. 8). The number of Annexin V-positive cardiomyocytes fell by 39±1.5% after ranolazine treatment, compared with the level seen in NE-treated cells. In contrast,

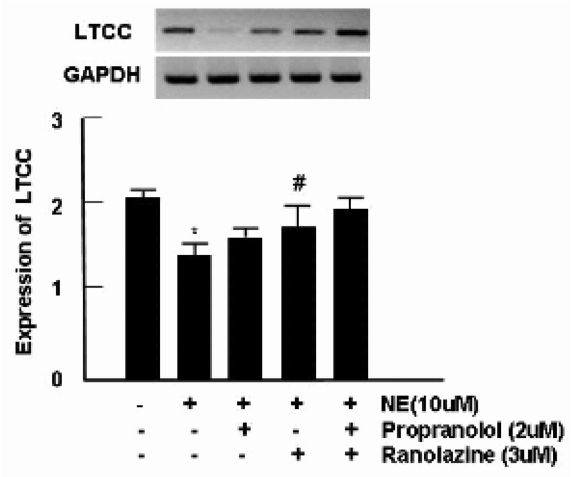

Figure 5. L-type Ca^{2+} channel expression level in NE-induced cardiomyocytes. Cardiomyocytes treated with ranolazine and propranolol wasexposed to NE stimulation for 24 hr. The mRNA expression of genes was established by separating amplification products in agarose gel electrophoresis and visualized them by ethidium bromide staining. Each expression was quantified by scanning densitometer. Values are mean±SEM.

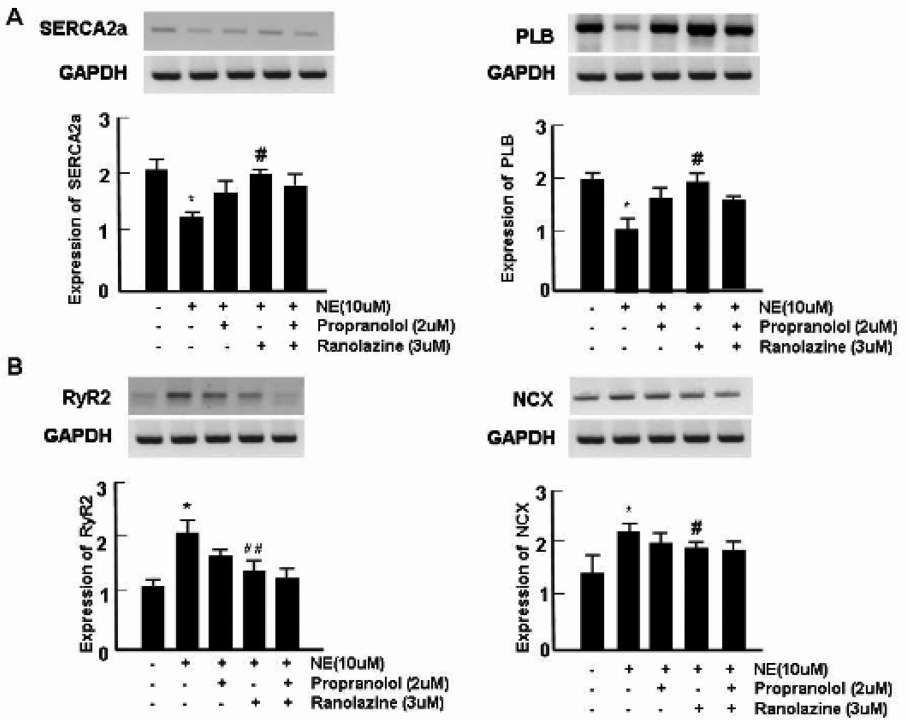

Figure 6. Effects of ranolazine on Ca^{2+} channel levels in NE-induced cardiomyocytes. Expression levels of the RyR2, NCX1, SERCA2a and PLB were estimated in cardiomyocytes subjected to NE with or without ranolazine, propranolol and analyzed. Values are mean±SEM.

propranolol showed no such effect.

3.9 Ranolazine Inhibited Apoptosis by Regulation of levels of the Pro-Apoptotic Factor Bax and the Anti-Apoptotic Factor Bcl-2, in NE-Induced Cardiomyocytes

NE induced cytochrome C release from mitochondria to the cytosolic fraction. Although cytochrome C was released into the cytosol from the mitochondrial intermembrane space during NE stimulation, treatment of cells with ranolazine during NE stimulus blocked cytochrome C release into the cytosol (Fig. 9A). A notable attenuation of Bcl-2 protein expression and a remarkable enhancement of Bax expression occurred in NE-induced cardiomyocytes, but ranolazine-treated cells did not exhibit evident changes in the expression of either Bax or Bcl-2 (Fig. 9B-C).

4. Discussion

In the present study, we showed that ranolazine decreased cell death mediated by the β-adrenoceptor signaling pathway in NE-induced cardiomyocytes. Also, it was demonstrated that ranolazine inhibited Ca^{2+} and Na^{+} cell overload, and influenced the levels of intracellular Ca^{2+}-regulatory proteins after NE stimulation. Ultimately, ranolazine inhibited apoptosis of cardiomyocytes.

NE activates the MAPK signaling pathway. To determine whether ranolazine treatment of NE-induced cells affected MAPK signaling pathway activity, we explored ERK protein phosphorylation using Western blotting. Although phosphorylation of ERK was elevated by NE exposure for 24

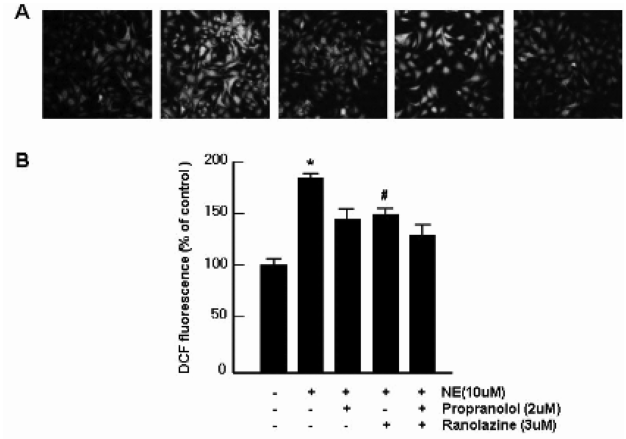

Figure 7. Reactive oxygen species (ROS) production. After preincubated with propranolol (2 μM), ranolazine (3 μM), mixture of propranolol and ranolazine in cardiomyocytes, it was treatedwith 10 μM NE for 24 hr. (A) Fluorescence image and (B) fluorescence intensity was obtained by using DCF fluorescence. Value are mean±SEM.

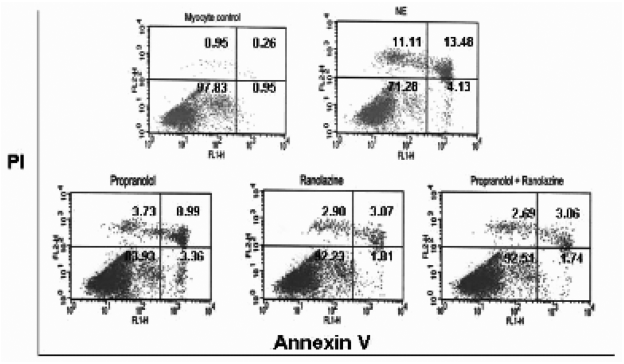

Figure 8. Annexin V/PI staining in apoptosis analysis. Cardiomyocytes were exposed to NE for 24 hr with propranolol, ranolazine and mixture of ranolazine and propranolol. Effect of ranolazine and propranolol in NE-induced apoptosis was detected by the annexin V-PI staining assay using flow cytometry. Values are mean±SEM.

h, phosphorylation fell after ranolazine treatment, to a level lower than that shown after exposure to NE (Fig. 1).

Mann and colleagues have suggested that NE exerts a direct toxic effect on cardiomyocytes *in vitro*.[40] β-adrenoceptor apoptotic effects were associated with increases in intracellular Ca^{2+} and Ca^{2+} calmodulin-dependent protein kinase II (CaMKII) activity.[41,42] CaMKII is the major cardiac isoform. Also, CaMKII expression was increased by β-adrenoceptor stimulation.[43] To confirm that CaMKII expression was enhanced by β-adrenoceptor stimulation, we examined CaMKII gene expression in NE-induced cardiomyocytes. The gene expression level was confirmed to be affected by ranolazine treatment of NE-induced cardiomyocytes. Whereas NE treatment enhanced CaMKII mRNA levels, ranolazine treatment completely inhibited mRNA transcription from the gene encoding CaMKII (Fig. 2).

Stefan and co-workers showed that CaMKII regulated Na^+ channel function in cardiomyocytes, most likely by association with and phosphorylation of Na^+ channels. Moreover, phosphorylation of Na^+ channels enhanced intracellular Na^+ overload.[44] To test for Na^+ overload, we examined cardiomyocytes at the single-cell level for changes in intracellular Na^+ concentration using the fluorescent Corona green (Na^+) dye test coupled with flow cytometry. With gating for only normal-sized or positive control cells, NE treatment in the presence of Corona green (Na^+) resulted in an increase in the proportion of cells in the M1 stage, compared with normal control cells. Also, treatment of cells with propranolol, ranolazine, or both ranolazine and propranolol, reduced the cell proportion in M1 compared with that of NE-induced cardiomyocytes (Fig. 3). The stimulation of β-adrenoceptor expression by NE caused a marked increase in both intracellular Na^+ and Ca^{2+} concentrations in cardiomyocytes.

Ca^{2+} is arguably the most important second messenger in cardiac muscle. Changes in intracellular Ca^{2+}concentration have both acute and chronic effects on cardiac function.[42] β-adrenergic modulation of voltage-sensitive Ca^{2+} channels in myocardial cells initiates a slow inward current, carried mainly by Ca^{2+}, that profoundly influences cardiac function.[44] It has been observed that intracellular Ca^{2+} overload increased in NE-induced cardiomyocytes and fell after treatment with ranolazine, propranolol, or both materials in combination (Fig. 4). Ca^{2+} ions play an important role in normal cardiac function,

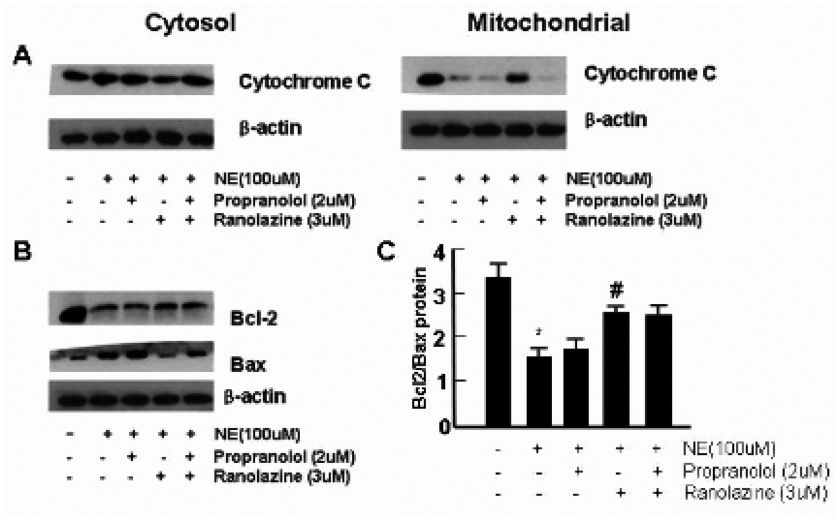

Figure 9. Bcl-2/Bax ratio and cytochrome C release. Cardiomyocytes were exposed to NE for 24 hr with propranolol, ranolazine and mixture of ranolazine and propranolol. (A) Mitochondria/ Cytosolic cytochrome C and the (B) Bcl-2/Bax protein ratio were detected by western blotting and (C) densitometric analysis. Values are mean±SEM. $^{*}p<0.001$ vs. Control. $^{\#}p<0.05$ vs. NE.

and several Ca^{2+}-regulating proteins are associated with Ca^{2+} homeostasis in cardiomyocytes. β-adrenoceptor stimulation by sympathetic NE plays a pivotal role in modulation of cardiac function in response to stress. Previous studies have shown that the ATP-induced increase in $[Ca^{2+}]$ is potentiated by NE. This increase in the ATP response mediated by NE has been demonstrated to be attributable to Ca^{2+} entry through the sarcolemmal (SL) LTCC. In this regard, it has been emphasized that NE has been shown to phosphorylate the LTCC and to enhance the inward Ca^{2+} influx. Major Ca^{2+}-cycling proteins involved in LTCC action are RyRs, SERCA2a, NCX, and PLB. The LTCC provides the primary Ca^{2+} influx mechanism and thus plays an important role in Ca^{2+} regulation.[45] Substantial disparities in the levels of expression of LTCC in NE-induced cardiomyocytes have been noted. The expression level of LTCC was increased after NE stimulation, compared with control cells. After treatment of NE-induced cells with ranolazine, the level of LTCC expression increased further (Fig. 5). SERCA plays an important role in regulating cytoplasmic Ca^{2+} levels in cardiomyocytes and is the most important contributor to the lowering of Ca^{2+} levels during cardiac relaxation.[16] Decreased expression of the SERCA2a[46] and PLB genes[47] may contribute to the diminished contractile function occurring in animal models of heart disease and in humans with severe congestive heart failure. NCX and RyR2 expression levels were increased by β-adrenoceptor stimulation.[48] We showed that NCX and RyR2 expression levels were elevated in NE-induced cardiomyocytes and that ranolazine blocked these changes (Fig. 6A). The expression levels of SERCA2a and PLB were reduced in NE-induced cardiomyocytes but were enhanced by treatment with ranolazine (Fig. 6B).

Many studies have shown that NE induces apoptosis *in vitro* and *in vivo*.[44] NE can induce ROS generation and apoptosis of cardiomyocytes by activation of the β-adrenergic pathway.[45] Regardless of the underlying mechanisms, an enhanced ß-adrenoceptor stimulation may significantly contribute to chronic diseases, such as hypertension and related conditions, congestive heart failure, sudden cardiac death, insulin-resistance syndrome, and obesity.[49] A significant increase in fluorescence intensity was detected in cells treated with NE for 24 h, as compared to both control groups. This was clearly caused by increased oxidative stress in NE-induced cardiomyocytes. Oxidative stress diminished after treatment with ranolazine. However, ROS production in propranolol-treated cells decreased more than did ROS production in ranolazine-treated cells (Fig. 7).

Apoptosis is triggered when something is amiss in the cell. DNA damage, cell detachment from neighbors, growth factor deprivation, infection, and a host of other triggers, have been described. In the present study, ranolazine was used to examine the mechanisms of NE-induced apoptosis in cardiomyocytes. It was found that NE-induced apoptosis was accompanied by downregulation of Bcl-2 protein synthesis and activation of Bax. NE-induced apoptosis was detected by Annexin V/PI staining (Fig. 8). Very little apoptosis was detected in control cultured cardiomyocytes, but cells underwent significant apoptosis when exposed to NE. Pretreatment with ranolazine, or co-treatment with ranolazine and propranolol, significantly reduced the apoptosis rate. In contrast, propranolol alone had no such effect. Cytochrome C plays a central role in apoptosis, signaling the cell to commence the process of cell death. Cytochrome C release from mitochondria has also been observed during NE-induced apoptosis (Fig. 9A). The anti-apoptotic protein Bcl-2 plays an important role in controlling cell death. It has been observed that NE-treated cardiomyocytes showed lower Bcl-2 protein levels, whereas the Bax protein level was increased. However, ranolazine-treated cells were protected against NE stimulation (Fig. 9B). These results are in agreement with previous reports showing that Bcl-2 acts upstream of the caspase cascade. Notably, if the rise in intracellular Na^{+} is prevented using ranolazine, a complete inhibition of apoptosis is observed. Thus, a rise in intracellular Na^{+} appears to act in initial signaling of the apoptotic program.

5. Conclusion

This study suggests that ranolazine is involved in β-adrenoceptor activation and prevents NE-induced apoptosis. In NE-treated cardiomyocytes, phosphorylation of ERK was inhibited in ranolazine-treated cells compared with cells treated with NE alone. Ranolazine also reduced intracellular Ca^{2+} and Na^{+} levels by regulating CaMKII expression after NE stimulation and affected Ca^{2+} channel-related gene expression. Although ranolazine had only a minor influence on NE-induced ROS production, the drug had a protective effect on apoptosis occurring after NE stimulus. In NE-treated cardiomyocytes, ranolazine diminished apoptotic protein expression levels in comparison with NE-only-treated cells. Ranolazine may thus act on the β-adrenoceptor signaling pathway to prevent NE-mediated cardiomyocyte apoptosis during heart failure.

Acknowledgments: This research was supported by a grant from the Korea Health 21 R&D Project, Ministry of Health & Welfare, Republic of Korea and the Technology Development Program (2008-2011) for Agriculture and Forestry, Ministry for

Agriculture, Forestry and Fisheries, Republic of Korea.

References

1. S Sossalla, S Wagner, EC Rasenack, *et al*., Ranolazine improves diastolic dysfunction in isolated myocardium from failing human hearts-role of late sodium current and intracellular ion accumulation, *J Mol Cell Cardio*, **45**, 32 (2008).
2. K Abozguia, K Clarke, L Lee, *et al*., Modification of myocardial substrate use as a therapy for heart failure, *Nat Clin Pract Cardiovasc Med*, **3**, 490 (2006).
3. HN Sabbah, MP Chandler, T Mishima, *et al*., Ranolazine, a partial fatty acid oxidation (pFOX) inhibitor, improves left ventricular function in dogs with chronic heart failure, *J Card Fail*, **8**, 416 (2002).
4. LL Eckhardt, TC Teelin, CT January, Is ranolazine an antiarrhythmic drug?, *Am J physiol heart circ physiol*, **294**, H1989 (2008).
5. BD Waterhouse, HC Moises, HH Yeh, *et al*., Norepinephrine enhancement of inhibitory synaptic mechanisms in cerebellum and cerebral cortex: mediation by b-adrenergic receptors, *J Pharmacol Exp Ther*, **221**, 495 (1982).
6. KH Byun, W Chang, S Lim, *et al*., Gh mediates a1-adrenoreceptor-induced transactivation of integrin signaling and hypertrophy in cardiac myocytes, *Tissue Eng Regen Med*, **5**, 622 (2008).
7. M Leicht, W Briest, HG Zimmer, Regulation of norepinephrine-induced proliferation in cardiac fibroblasts by interleukin-6 and p42/p44 mitogen activated protein kinase, *Mol Cell Biochem*, **243**, 65 (2003).
8. T Force, R Hajjar, FD Monte, *et al*., Signaling pathways mediating the response to hypertrophic stress in the heart, *Gene Expr*, **7**, 337 (1999).
9. T Force, JV Bonventre, Growth factors and mitogen-activated protein kinases, *Hypertension*, **31**, 152 (1998).
10. PH Sugden, A Clerk, "Stress-responsive" mitogen-activated protein kinases (c-Jun N- terminal kinases and p38 mitogen-activated protein kinases) in the myocardium, *Circ Res*, **83**, 345 (1998).
11. RJ Lefkowitz, KL Pierce, LM Luttrell, Dancing with different partners: protein kinase A phosphorylation of seven membranespanning receptors regulates their G protein-coupling specificity, *Mol Pharmacol*, **62**, 971 (2002).
12. S Galandrin, M Bouvier, Distinct signaling profiles of b1 and b2-adrenergic receptor ligands toward adenylyl cyclase and mitogen-activated protein kinase reveals the pluridimensionality of efficacy, *Mol Pharmacol*, **70**, 1575 (2006).
13. SPH Alexander, A Mathie, JA Peters, Guide to receptors and channels (GRAC), *Br J Pharmacol*, **150**, S12 (2007).
14. DA Schwinn, MG Caron, RJ Lefkowitz, The beta-adrenergic receptor as a model for molecular structure-function relationships in G-protein-coupled receptors, *The Heart and Cardiovascular System*, **52**, 1657 (1992).
15. OE Osadchii, GR Norton, R McKechnie, *et al.,* Cardiac dilatation and pump dysfunction without intrinsic myocardial systolic failure following chronic beta-adrenoreceptor activation, *Am J Physiol Heart Circ Physiol*, **292**, H1898 (2007).
16. T Zhang, S Miyamoto, JH Brown, Cardiomyocyte calcium and calcium/calmodulin-dependent protein kinase II: friends or foes?, *Recent Prog Horm Res*, **59**, 141 (2004).
17. MO Hengart, The biochemistry of apoptosis, *Nature*, **407**, 770 (2000).
18. AP Braun, H Schulman, The multifunctional calcium/ calmodulin dependent protein kinase: from form to function, *Annu Rev Physiol*, **57**, 417 (1995).
19. L Li, H Satoh, KS Ginsburg, *et al*., The effect of Ca2+ Almodulindependent protein kinase II on cardiac excitation–contraction coupling in ferret ventricular myocytes, *J Physiol*, **501**, 17 (1997).
20. LS Maier, T Zhang, L Chen, *et al*., Transgenic CaMKII dC overexpression uniquely alters cardiac myocyte Ca2+ handling: reduced SR Ca2+ load and activated SR Ca2+ release, *Circ Res*, **92**, 904 (2003).
21. T Guo, T Zhang, R Mestril, *et al*., Ca2+/calmodulin-dependent protein kinase II phosphorylation of ryanodine receptor does affect calcium sparks in mouse ventricular myocytes, *Circ Res*, **99**, 398 (2006).
22. T Zhang, JH Brown, Role of Ca2+/calmodulin-dependent protein kinase II in cardiac hypertrophy and heart failure, *Cardiovasc Res*, **63**, 476 (2004).
23. Y Yang, WZ Zhu, ML Joiner, *et al*., Calmodulin kinase II inhibition protects against myocardial cell apoptosis in vivo, *Am J Physiol*, **291**, H3065 (2006).
24. WZ Zhu, SQ Wang, K Chakir, *et al*., Linkage of β1-adrenergic stimulation to apoptotic heart cell death through protein kinase A-independent activation of Ca2+/calmodulin kinase II, *J Clin Invest*, **111**, 617 (2003).
25. X Chen, X Zhang, H Kubo, *et al*., Ca2+ influx-induced sarcoplasmic reticulum Ca2+ overload causes mitochondrial-dependent, *Circ Res*, **97**, 1009 (2005).
26. T Zhang, LS Maier, ND Dalton, *et al*., The dC isoform of CaMKII is activated in cardiac hypertrophy and induces dilated cardiomyopathy and heart failure, *Circ Res*, **92**, 912 (2003).
27. C Communal, WS Colucci, K Singh, p38 Mitogen-activated protein kinase pathway protects adult rat ventricular myocytes against β-Adrenergic receptor-stimulated apoptosis, *J Biol Chem*, **275**, 19395 (2000).
28. E Schraml, P Quan, I Stelzer, *et al*., Norepinephrine treatment and aging lead to systemic and intracellular oxidative stress in rats, *Exp Gerontol*, **42**, 1072 (2007).
29. S Bonello, C Zahringer, R Belaibe, *et al*., Reactive oxygen species activate the HIF-1alpha promoter via a functional NFkappa B site, *Arterioscler Thromb Vasc Biol*, **27**, 755 (2007).
30. K England, TG Cotter, Direct oxidative modifications of signalling proteins in mammalian cells and their effects on apoptosis, *Redox Rep*, **10**, 237 (2005).
31. F Qin, N Rounds, W Mao, *et al*., Antioxidant vitamins prevent cardiomyocyte apoptosis produced by norepinephrine infusion in ferrets, *Cardiovasc Res*, **51**, 736 (2001).
32. P Anversa, J Kajstura, G Olivetti, Myocyte death in heart failure, *Curr Opin Cardiol*, **11**, 245 (1996).
33. G Olivetti, F Quaini, R Sala, *et al*., Acute myocardial infarction in humans is associated with the activation of programmed

myocyte cell death in the surviving portion of the heart, *J Mol Cell Cardiol*, **28**, 2005 (1996).
34. E Teiger, TV Dam, L Richard, *et al*., Apoptosis in pressure overload-induced heart hypertrophy in the rat, *J Clin Invest*, **97**, 2891 (1996).
35. Z Li, OHL Bing, X Long, *et al*., Increased cardiomyocyte apoptosis during transition to heart failure in the spontaneously hypertensive rat, *Am J Physiol*, **272**, H2313 (1997).
36. W Cheng, J Kajstura, JA Nitahara, *et al*., Programmed myocyte cell death affects the viable myocardium after infarction in rats, *Exp Cell Res*, **226**, 316 (1996).
37. I Takeshi, T Kouichi, K Sayaka, *et al*., Protective effect of propranolol on mitochondrial function in the ischaemic heart, *Br J Pharmacol*, **136**, 472 (2002).
38. MO Hengart, The biochemistry of apoptosis, *Nature*, **407**, 770 (2000).
39. T Finkel, Reactive oxygen species and signal transduction, *IUBMB Life*, **52**, 3 (2001).
40. HU Simon, A Haj-Yehia, F Levi-Schaffer, Role of reactive oxygen species (ROS) in apoptosis induction, *Apoptosis*, **5**, 415 (2000).
41. DL Mann, RL Kent, B Parsons, *et al*., Adrenergic effects on the biology of the adult mammalian cardiocyte, *Circulation*, **85**, 790 (1992).
42. Y Byun, W Chang, S Lim *et al*., Gh partially mediates a1-adrenergic receptor-stimulated cardiac hypertrophy independent of Gq regulation, *Tissue Eng Regen Med*, **5**, 630 (2008).
43. WZ Zhu, SQ Wang, K Chakir, *et al*., Linkage of b1-adrenergic stimulation to apoptotic heart cell death through protein kinase a-independent activation of Ca2þ/Calmodulin kinase II, *J Clin Invest*, **111**, 617 (2003).
44. JW Bassani, RA Bassani, DM Bers, Relaxation in rabbit and rat cardiac cells: species-dependent differences in cellular mechanisms, *J Physiol,* **476**, 279 (1994).
45. CC Fan, H Koenig, The role of polyamines in beta-adrenergic timulation of calcium influx and membrane transport in rat heart, *J Mol Cell Cardiol*, **20**, 789 (1998).
46. YC Fu, CS Chi, SC Yin, *et al*., Norepinephrine induces apoptosis in neonatal rat endothelial cells via down-regulation of Bcl-2 and activation of beta-adrenergic and caspase-2 pathways, *Cardiovasc Res*, **1**, 61143 (2004).
47. M Meyer, W Schillinger, B Pieske, *et al*., Alterations of sarcoplasmic reticulum proteins in failing human dilated cardiomyopathy, *Circulation*, **92**, 778 (2005).
48. Z Li, OHL Bing, X Long, *et al*., Increased cardiomyocyte apoptosis during transition to heart failure in the spontaneously hypertensive rat, *Am J Physiol*, **272**, H2313 (1997).
49. C Communal, K Singh, DR Pimentel, *et al*., Norepinephrine stimulates apoptosis in adult rat ventricular myocytes by activation of the beta-adrenergic pathway, *Circulation*, **98**, 1329 (1998).