Effects of ranolazine on norepinephrine-induced cell death through inhibition of β-adrenoceptor signal pathway in cardiomyocytes

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Directed by Professor Yangsoo Jang

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This certifies that the
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Hye-Jung, Kim

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ABSTRACT

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Ranolazine has been represented a new agent of antianginal therapy with mechanisms of action that blocked intracellular sodium and calcium overload accompanying myocardial ischemia. However, effect of ranolazine on β-adrenoceptor signal transduction system is poorly understood. This study was designed to confirm whether mechanism has related to β-

adrenoceptor antagonist activity of ranolazine on norepinephrine (NE)-induced cardiomyocytes. After cardiomyocytes were preincubated with propranolol (2 μM), ranolazine (3 μM) and mixture of propranolol and ranolazine, cardiomyoxytes were treated with NE (10 μM) for 24 h. The phosphorylation of ERK was decreased about 52±2.5% by ranolazine treatment more than NE only treated cells. The intracellular Ca^{2+} and Na^{+} level were decreased about 40±3% and 17±0.5%, respectively. Ranolazine decreased expression level of the Ca^{2+}/calmodulin dependent protein kinaseII (CaMKII, 57±4%), Na^{+}-Ca^{2+} exchanger (NCX, 21±0.5%) and ryanodine receptor2 (RyR2, 47±1.5%) compared with NE only treated cardiomyocytes. Ranolazine also increased expression level of L-type Ca^{2+} channel (LTCC, 48±3.5%), phospholamban (PLB, 45±2%) and sarcoplasmic reticulum Ca^{2+} ATPase 2a (SERCA2a, 36±0.5%) in NE only stimulated cardiomyocytes, respectively. The number of annexin V/PI positive cell was decreased about 39±1.5% by ranolazine compared with NE only treated cardiomyotes. Ranolazine also inhibited apoptosis through the regulation of pro-apoptotic factor Bax, anti-apoptotic factor Bcl-2 and cytochrome C release. These results demonstrate that ranolazine had an effect on norepinephrine-induced cell death through inhibition of β-adrenoceptor signal pathway in cardiomyocytes.

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

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I. INTRODUCTION

Heart failure (HF) is a condition in which the heart can not pump enough blood to the other organs. This result from narrowed arteries that supply blood to the heart muscle such as coronary artery disease, heart attack, myocardial infarction (MI). HF is associated with unbalances of cellular Ca^{2+} and Na^{+} homeostasis[1]. Ranolazine has been shown to demonstrating

blockade of the late sodium current in ischemic cardiomyocytes. Therefore, ranolazine is thought indirectly to reduce Ca^{2+} overload via the Na^{+} channels, to preserve ionic homeostasis[2]. Acute intravenous administration of ranolazine has also improved left ventricular systolic function in dogs with heart failure[3]. However, sodium current inhibitor mechanism of ranolazine was not clear[4].

The actions of norepinephrine (NE) are carried out via the binding to adrenergic receptors[5]. NE, β-adrenoceptor agonist, is involved in many cardiovascular diseases such as congestive heart failure. Also, NE had a co-mitogenic effect in isolated cardiac fibroblasts, and that it activated mitogen activated protein kinase (MAPK)[6]. Mitogen-activated protein kinases (MAPKs), a large family of serine-threonine kinases, have important functions as mediators of signal transduction and are activated by extracellular stimulus[7,8,9]. Three subgroups of MAPKs have clearly been identified: the extracellular signal-regulated kinase (ERK1/2), the p38 kinase, and the c-jun N-terminal kinases (JNKs). ERK1/2 respond to mitogenic stimulus, whereas p38 kinase and JNKs respond predominantly to cellular stresses or inflammatory cytokines[8,9]. Recently, heterotrimeric G proteins have also been shown to activate various members of the MAPKs family[7,8]. One of the major mechanisms for regulating contractility of the heart is via

β-adrenoceptor stimulation.

The ß-adrenoceptor is the first element in the signal transduction chain mediating adrenergic stimulation of the heart. All known subtypes of ß-adrenoceptor ($ß_1$, $ß_2$, a`nd $ß_3$) are glycoproteins with extracellular amino terminus, seven hydrophobic transmembrane domains, and intracellular carboxy terminus. All three subtypes when activated can cause increases in intracellular levels of cAMP, although many recent studies demonstrate that this is not the sole signaling pathway [10,11]. There are selective agonists and antagonists for all three receptor subtypes[12]. ß-adrenoceptor antagonists are used therapeutically for the treatment of cardiovascular diseases, such as cardiac failure, angina and hypertension, where the beneficial effects result mainly from actions on the heart[13]. Regardless of the underlying mechanism, systemic β-adrenoceptor antagonist can have a number of significant effects on cardiac function[14].

As a second messenger, Ca^{2+} regulates acute physiological function, including contraction of cardiac, skeletal, and smooth muscle and release of hormones and neurotransmitters. Ca^{2+} homeostasis can lead not only to loss of normal physiological control mechanisms but also to pathological changes in cell growth[15]. Cardiac dilatation and pump dysfunction without intrinsic

myocardial systolic failure follows chronic β-adrenoreceptor activation[15]. Increases in Ca^{2+} can transduce signals through various classes of Ca^{2+}-regulated enzymes, one of which is the Ca^{2+}/calmodulin dependent protein kinase (CaMK) family[15]. Ca^{2+}/calmodulin dependent protein kinase II (CaMKII) is a widely expressed protein kinase that modulates various functions ranging from learning and memory of the nervous system, muscle contraction, cell secretion to gene expression[16]. In the heart, CaMKIIδ is the predominant CaMKII isoform and its splice variant δc resides in the cytosol. In addition to its well documented role in the regulation of cardiac excitation contraction (E-C) coupling[17,18,19]. CaMKIIδc has been implicated in apoptotic signaling[20,21,22,23] and in mediating the transition to heart failure[24]. CaMKII modulates an array of key proteins involved in cardiac E-C coupling and Ca^{2+} handling, such as the sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase (SERCA) and its regulator, for example phospholamban (PLB), ryanodine receptor (RyR) Ca^{2+} release channels[24].

β-adrenoceptor stimulus increases apoptosis[25] and reactive oxygen species (ROS)[26] 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 have been known to play an important role in the pathogenesis of several cardiovascular diseases. ROS

are well-known to regulate the transcription of particular genes[27]. However ROS may specifically induce apoptosis[28]. Recent study demonstrated chronic adrenergic stress as being responsible for adverse effects, such as cardiac failure, mainly due to increased cardiomyocyte apoptosis[29]. Apoptosis has been demonstrated to occur in the myocardium in a variety of pathological situations[30]. The number of apoptotic myocytes is increased in myocardium obtained from patients with end-stage heart failure and myocardial infarction[31] and in myocardium from experimental models of myocardial hypertrophy and failure, including the rat[32], the spontaneously hypertensive rat[33], rats with myocardial infarction[34]. Many molecules including proteins in the Bcl-2 and caspase families participate in the apoptotic response to numerous death inducing signals[35].

Therefore, it was examined into confirm whether ranolazine exerts antagonist activity at β-adrenoceptor in norepinephrine (NE)-induced cardiomyocytes. The β-adrenoreceptor antagonist propranolol is able to blunt cardiomyocyte hypertrophic response in hearts. Propranolol has other pharmacological effects such as a Na^+ channel blocking action and an antioxidant effect[36]. In this study, it was expected that ranolazine will is similar response in NE-induced cardiomyocytes such as propranolol. Because NE stimulation increased ERK activation, CaMKII, Ca^{2+} overload, Na^+ overload, Ca^{2+}

channel (LTCC, NCX, SERCA2a, PLB, RyR2) gene expression in cardiomyocytes, ranolazine was examined the effects on inhibition of MAPK/ERK activation, CaMKII, Ca^{2+} overload, Na^{+} overload and Ca^{2+} channel (LTCC, NCX, SERCA2a, PLB, RyR2) gene expression level in NE-induced cardiomyocytes with or without ranolazine. It was examined the effect mechanisms of NE-induced ROS and apoptosis in cardiomyocytes and found that NE-induced cardiomyocyte apoptosis was accompanied by down-regulation of Bcl-2 protein synthesis and activation of β-adrenergic and cytochrome C release.

II. MATERIALS AND METHODS

1. Isolation and culture of rat cardiomyocytes

Neonatal rat cardiomyocytes were prepared by an enzymatic method. Briefly, hearts of one- to two-day-old Sprague-Dawley (SD) rat pups were dissected, minced, enzymatically dispersed with 10 mL of collagenase II (0.5 mg/mL, 262 U/mg, Gibco BRL, Paisley, UK), and centrifuged differentially to yield 5×10^5 cells/mL. After incubation for 4-6 h, the cells were rinsed twice with α-MEM containing 10% fetal bovine serum (FBS) (Gibco BRL, Paisley, UK), and 0.1M BrdU (Sigma Chemical Co., St. Louis, MO, USA) was added to inhibit fibrous growth. Cells were then cultured in a CO_2 incubator at 37°C for 1 day.

2. NE stimulation and treatment with propranolol and ranolazine

Cardiomyocytes were incubated in 5% CO_2 at 37°C for 1day. And then the cells were exchanged with SF α-MEM (Gibco BRL, Paisley, UK) without FBS in CO_2 incubator (Thermo Forma Model 311, Marietta, USA) for 24 h. After cardiomyocytes were preincubated with propranolol (2 μM),

ranolazine (3 μM), mixture of propranolol and ranolazine, it was treatment with NE (10 μM) for 24 h.

3. Measurement of intracellular sodium by flow cytometry

Intracellular sodium was measured by corona green (CoroNa™ Green, AM, Molecular Probes, CA, USA). Cell ($5x10^6$) were cultured for one day in 100mm plate coated with 1.5% gelatin. For intracellular sodium measurement, Corona green stock was individually added to 1 mL of cells at a final concentration of 5 μM for 1 h prior to the time of examination. Incubation was continued at 37°C, 5% CO_2 atmosphere. Before flow cytometric examination, propidium iodide (PI) (sigma chemical St., Louis, MO, USA) was added to final concentration (10 μg/mL). The $1x10^4$ cells were analyzed by sequential excitation of the cells containing corona green and PI at 492-516 nm and 488nm, respectively, using a FACSCalibur system (Becton Dickinson, San Jose, CA, USA) and CellQuest™ software.

4. Measurement of intracellular reactive oxygen species generation

Neonatal rat cardiomyocytes were labeled with 2'7'-dichlorodihydro - fluorescein diacetate (H2DCFDA; Molecular Probe, CA, USA). The probe H2DCFDA (10uM) enters the cell, and the acetate group on H2DCFDA is cleaved by cellular esterases, trapping the nonfluorescent 2'7'-dichlorodihydro-fluorescin (DCFH) inside. Subsequent oxidation by reactive oxygen species yields the fluorescent product DCF. The dye, when exposed to an excitation wavelength of 480nm, emits light at 535nm only when it has been oxidized. Labeled cells were examined using a luminescence spectrophotometer for the oxidized dye.

5. Confocal microscopy and fluorescence measurements

The measurement of the cytosolic free Ca^{2+} concentration was estimated by the confocal microscopy analysis. Neonatal rat cardiomyocytes were plated on a four-well slide chamber coated with 1.5 % gelatin for one day in α-MEM containing 10% FBS (Gibco BRL, Paisley, UK) and 0.1 μM BrdU (Sigma Chemical St., Louis, MO, USA). After incubation, the cells were washed with modified Tyrode's solution with 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 then loaded with 10 μM of the acetoxymethyl

ester of fluo-4 (Fluo-4 AM, Molecular Probes, CA, USA) for 20 min, in the dark at 37°C. Fluorescence images were collected using a confocal microscope (Leica, Solms, Germany) by excitation with a 488 nm line of argon laser, and emitted light was collected through a 510-560 nm band-pass filter. Relative data of intracellular Ca^{2+} was determined by measuring the intensity of the fluorescence.

6. Annexin V/PI staining

Apoptosis was measured by ApoScan™ Annexin V-FITC apoptosis detection Kit (Biobud). The cells were pelleted and analyzed in a FACSCalibur system (Becton Dickinson, San Jose, CA, USA). Excitation wave was set at 488 nm. The emitted green fluorescence of Annexin V (FL1) and red fluorescence of PI (FL2) were collected using 525 and 575 nm band pass filters, respectively. A total of at least $1x10^4$ cells were analyzed per sample. Light scatter was measured on a linear scale of 1024 channels and fluorescence intensity on a logarithmic scale. The amount of early apoptosis and late apoptosis/necrosis was determined as the percentage of Annexin V^+/PI^- and Annexin V^+/PI^+ cells, respectively.

7. Immunoblot anlaysis

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

were detected by enhanced chemiluminescence (ECL) reagent (Amersham Pharmacia Biotech, Piscatawasm NJ, USA). The band intensities were quantified using Photo-Image System (Molecular Dynamics, Uppsala, Sweden).

8. RT-PCR analysis

A. Isolation of total RNA

Total RNA was extracted by using 0.5mL TRIzol reagent (Sigma, St. Louis, MO, USA). Total cellular RNA was extracted by the chloroform, isopropanol, DEPC-treated 75% ethanol procedure. The RNA was suspended in 30uL of Nuclease free water. The quantity and quality of the isolated RNA was determined by OD260/OD280 with DU 640 spectrophotometer (Eppendorf, Hamburg, Germany).

B. RT-PCR assay

Complementary DNA (cDNA) was generated with Reverse Transcription System (Promega, Madison, WI) according to the manufacturer's instructions. 1μg of total RNA was reverse-transcribed in a 20μL reaction mix containing 5 mmol/L $MgCl_2$, 10 mmol/L Tris-HCl (pH. 9.0 at 25 °C), 50

mmol/L KCl, 0.1% Triton X-100, 1 mmol/L dNTP, 20U of RNase inhibitor, 0.5 µg oligo-(dT) 15 primer, 10U of reverse transcriptase for 15 min at 42°C and the reaction was terminated by heating at 99°C for 5 min. To monitor cDNA synthesis, an aliquot of the RT reaction mixture was subjected to PCR for GAPDH. The GAPDH primer sequences were as follows table 1. The PCR condition was a cycle of denaturing at 94°C for 3 min followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 49°C for 30 sec, extension at 72°C for 2 min before a final extension at 72°C for 10 min. The first round of PCR amplification was performed using 1 µL of the cDNA from the reverse transcription. The PCR mix contained 10 pmol/µL of each primer, together with 200 mM Tri-HCl (Ph.8.8), 100 mM KCl, 1.5 mmol/L $MgSO_4$, 1% Triton X-100, 0.1 mM dNTP and 1.25 U of Taq polymerase in a total volume of 25 µL. PCR reactions were carried out in a thermal cycler using the following conditions: 95°C for 3 min, 95°C for 1 min and then followed by the individual conditions for each gene as listed in Table 1. All RT-PCR products were separated by electrophoresis in a 1.2% agarose gel and visualized after staining with ethidium bromide.

Table. 1 PCR primers used in this study

Gene	primer	bp	cycler program
GAPDH	ctcccaacgtgtctgttgtg	450	49°C ,60s/35cyc
	tgagcttgacaaagtggtcg		
CaMKII	tcagatgttttgccacaaagaggtgcctcct	531	60°C ,60s/35cyc.
	ccggatggggtaaaggagtcaactgagagct		
L-type Ca2+ channel	tgtcacggttgggtagtgaa	346	49°C ,60s/35cyc.
	ttgaggtggaagggactttg		
Phospholamban	gctgagctcccagacttcac	339	48°C ,60s/35cyc.
	gcgacagcttgtcacagaag		
NCX-1	tgtctgcgattgcttgtctc	364	48°C ,60s/35cyc.
	tcactcatctccaccagacg		
SERCA2a	tccatctgcctgtccat	351	42°C ,60s/35cyc.
	gcggttactccagtattg		
RyR2	ccaacatgccagaccctact	196	48°C ,60s/35cyc.
	tttctccatcctctccctca		

9. Statistical analysis

Data are expressed as mean±SEM. Student's t-test was used to compare two groups, and examination of more than two groups was done by one-way ANOVA, using the Bonferroni test. A *p*-value <0.05 was considered significant.

III. RESULTS

1. Ranolazine inhibited phosphorylation of ERK in NE-induced cardiomyocytes.

NE activates MAPK signaling pathways via phosphorylation cascades. NE stimulation increased MAPK/ERK activation in cardiomyocytes. Therefore, it was examined the effect of ranolazine with NE stimulation. Although the phosphorylation of ERK was increased about 63±1% by NE for 24 h, phosphorylation of ERK was decreased about 52±2.5% by ranolazine treatment more than that of NE only (Fig. 1).

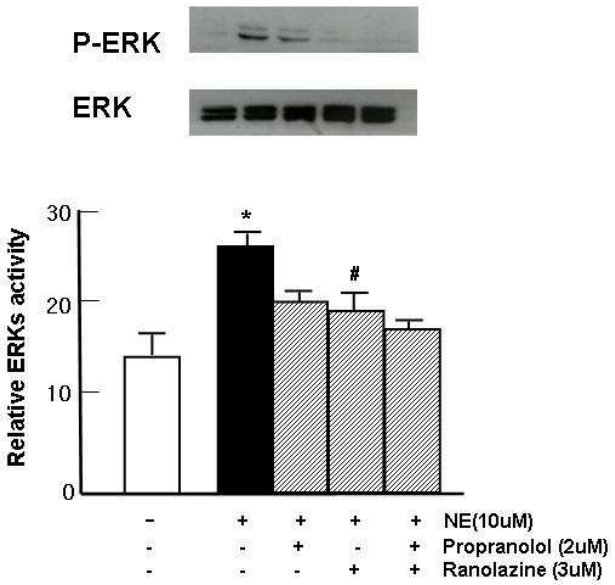

Figure 1. Phosphorylation of ERK in NE induced cardiomyocytes. Relative phosphorylation levels of ERK activity were determined by western blotting. Cardiomyocytes were subjected to NE stimulation with propranonol and ranolazine for 24 h. phosphorylation of ERK was treated during NE stimulation was detected by immunoblotting. Value are mean±SEM. $^{*}p<0.001$ vs. Control. $^{\#}p<0.001$ vs. NE.

2. **Ranolazine decreased CaMKII overexpression in NE-induced cardiomyocytes.**

To investigate possible modulatory effects of sustained β-adrenoceptor stimulation on cardiac CaMKII expression, enzymatically isolated rat ventricular myocytes were cultured in the presence of NE stimulation (norepinephrine 10 μM/mL) for 24 h. During NE stimulation, CaMKII expression is increased. Therefore, it was examined CaMKII expression level in NE- induced cardiomyocytes by RT-PCR. The density of CaMKII was increased about 73±2% in NE-induced cardiomyocytes. After treatment of NE-induced cells with ranolazine, density of CaMKII was dropped about 57±4% compared with NE-induced cardiomyocytes (Fig. 2).

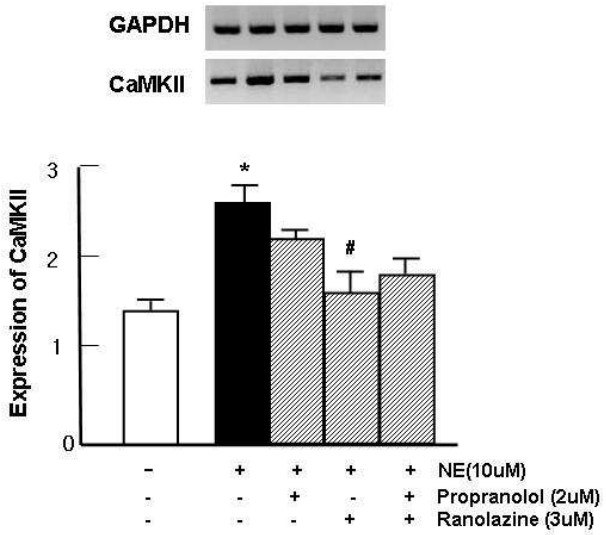

Figure 2. CaMKII expression level in NE-induced cardiomyocytes. Cardiomyocytes treated with ranolazine and propranolol were exposed to NE stimulation for 24 h. Whereas NE treated cells were increased in CaMKII mRNA, ranolazine treated cells were completely decreased in CaMKII mRNA. Value are mean±SEM. $^{*}p<0.001$ vs. Control. $^{\#}p<0.001$ vs. NE.

3. Ranolazine protected intracellular Na^+ overload in NE-induced cardiomyocyte.

It was examined cardiomyocytes at the single cell level for changes in intracellular sodium using the fluorescent corona green (Na^+) dye and flow cytometry. Gating on only the normally sized or positive control, NE treatment in the presence of corona green (Na^+) resulted in cell counts 21±2% increase of M1 region more than control and treating cells of propranolol, ranolazine and mixture of ranolazine and propranolol were 18±3%, 17±0.5%, and 17±2.5% reduced in cell counts of M1 region more than NE-induced cardiomyocytes, respectively (Fig. 3).

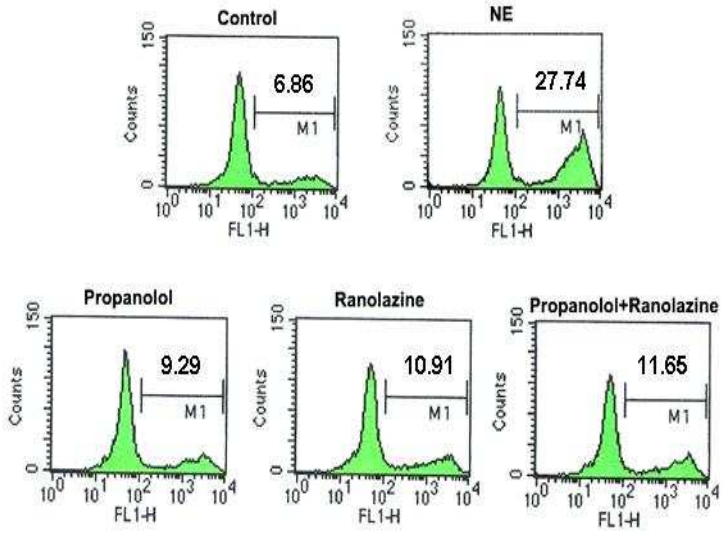

Figure 3. Effects of ranolazine on intracellular Na^+ overload in NE-induced cardiomyocytes. NE treatment in the presence and absence of ranolazine was initially examined for changes in intracellular sodium by flow cytometry using corona green (Na^+). The cells were initially analyzed by gating on only 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. Value are mean±SEM.

4. Ranolazine prevented intracellular Ca^{2+} level in NE stimulation. Intracellular Ca^{2+} level increase by activation of several Ca^{2+} channels in NE-induced cardiomyocytes. To know whether ranolazine treated cells during NE stimulation declined intracellular Ca^{2+} overload, the intracellular Ca^{2+} level was examined with using fluo-4 AM. Intracellular Ca^{2+} level was estimated followed by NE treatment for 24 h and ranolazine was also treated. As shown in Fig. 4, the intracellular Ca^{2+} level during NE stimulus was elevated about 50±3% at 24 h but was dropped about 43±0.5%, 40±3% and 50±1.5% by treatment of NE-induced cells with 2 μM propranolol, 3 μM ranolazine, and mixture of propranolol and ranolazine, respectively.

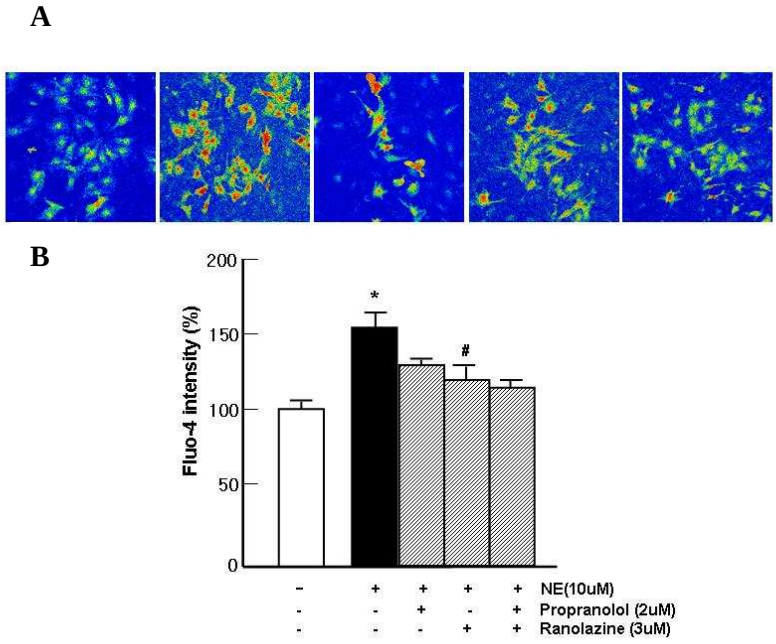

Figure 4. Effects of ranolazine on intracellular Ca^{2+} concentration. Confocal fluorescent images of cardiomyocytes were obtained by loading with fluo-4 AM. The NE-induced cells treated with ranolazine and propranolol were incubated for 24 h. (A) Fluorescence image was obtained by fluo-4 AM and (B) fluorescence intensity was quantified in different cells (n=5) in each condition and analyzed. Value are mean±SEM. $^{*}p<0.001$ vs. Control. $^{\#}p<0.001$ vs. NE.

5. Ranolazine suppressed in Ca^{2+} channel expression by NE-induced cardiomyocyte.

Fig. 5 performs representative example of Ca^{2+} channel in NE-induced cardiomyocytes treated with or without ranolazine. Ca^{2+} entry through 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 LTCC. The density of LTCC was diminished about 63±2% in NE stimulation compared with control cells. After NE-induced cells were treated ranolazine, density of LTCC was elevated about 48±3.5% more than NE stimulation cells (Fig. 5).

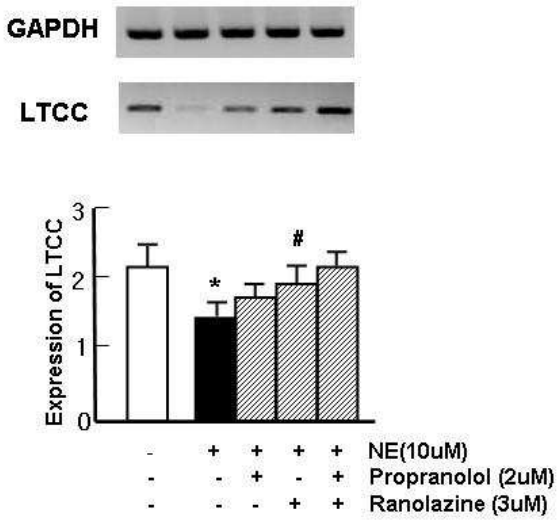

Figure 5. L-type Ca^{2+} channel expression level in NE-induced cardiomyocytes. Cardiomyocytes treated with ranolazine and propranolol were exposed to NE stimulation for 24 h. The mRNA expression of genes was established by separating amplification products in agarose gel electrophoresis and visualized them by ethidium bromide staining. Each expression was quantified by scanning densitometer. Value are mean±SEM. $^{*}p<0.001$ vs. Control. $^{\#}p<0.001$ vs. NE.

6. Ranolazine inhibited intracellular Ca^{2+} overload in NE-induced cardiomyocytes.

Previously, it was confirmed that ranolazine regulates intracellular Ca^{2+} overload via the inhibition of Na^{+} overload. It was examined Ca^{2+} channels related to intracellular Ca^{2+} regulation, which is important in cell function, changes in expression levels of one of the representative Ca^{2+} channels, the NCX1, and its Ca^{2+} handling, such as SERCA2a and its regulators, PLB, RyR2 were tested in cardiomyocytes undergoing NE. RyR2 and NCX gene expression in NE-induced cardiomyocytes with treated ranolazine reduced about 47±1.5%, 21±0.5% compared with NE-induced cells (Fig. 6A). The expression level of SERCA2a and PLB became higher about 36±0.5%, 45±2% compared with NE-induced cells (Fig. 6B). These result represented that ranolazine prevented changes in SERCA2a, PLB, NCX and RyR2 expression levels.

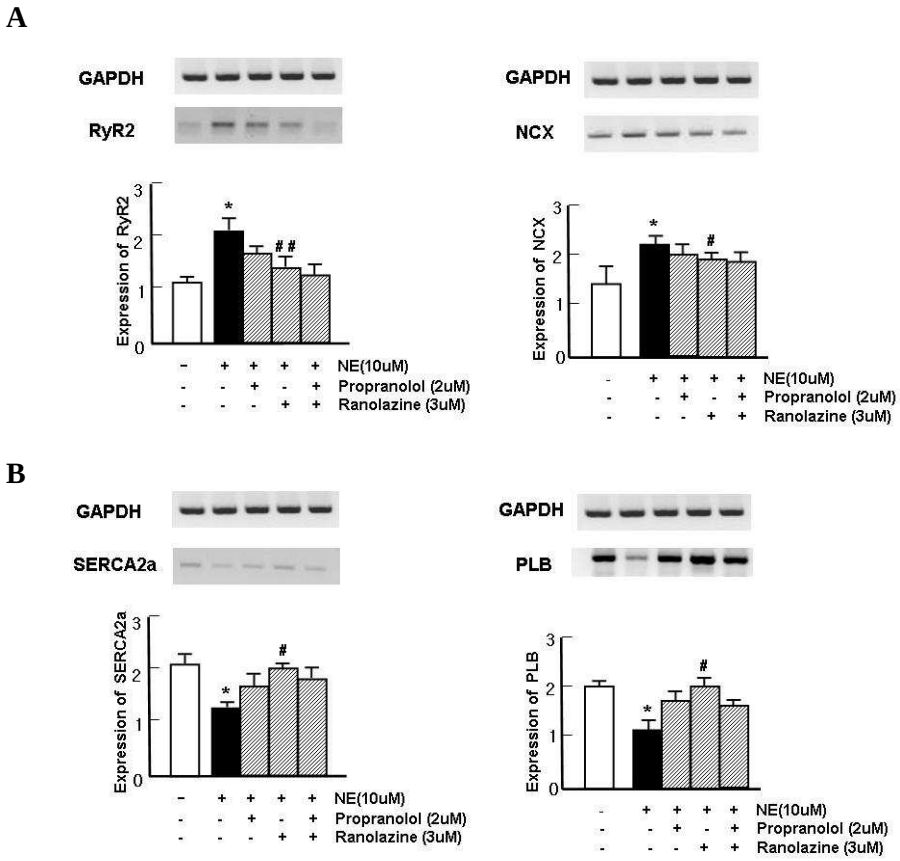

Figure 6. Effects of ranolazine in Ca^{2+} channel of NE-induced cardiomyocytes. Expression levels of the RyR2, NCX1, SERCA2a and PLB were estimated in cardiomyocytes subjected to NE with or without ranolazine, propranolol and analyzed. Value are mean±SEM. $^{*}p<0.001$ vs. Control. $^{\#\#}p<0.05$ vs. NE. $^{\#}p<0.001$ vs. NE.

7. Ranolazine decreased reactive oxygen species production in NE – induced cardiomyocyte.

ROS, including hydrogen peroxides (H_2O_2), hydroxyl radials, are normally generated in the mitochondria and have been identified as important mediators that regulate signal transduction[37,38]. Intracellular ROS by NE-induced cardiomyocytes was comparatively assessed by means of DCF fluorescence. Significant enhancement in fluorescence intensity was detected in the NE treated cell for 24 h as compared to both control cells (Fig. 7A). ROS production was increased by 71±0.5% with NE (10 μM) treatment for 24 h in cardiomyocytes (Fig. 7B). Although ROS production of ranolazine treated cells decreased by 52±1.5%, ROS production of propranolol treated cells was decreased more than ROS production of ranolazine treated cells.

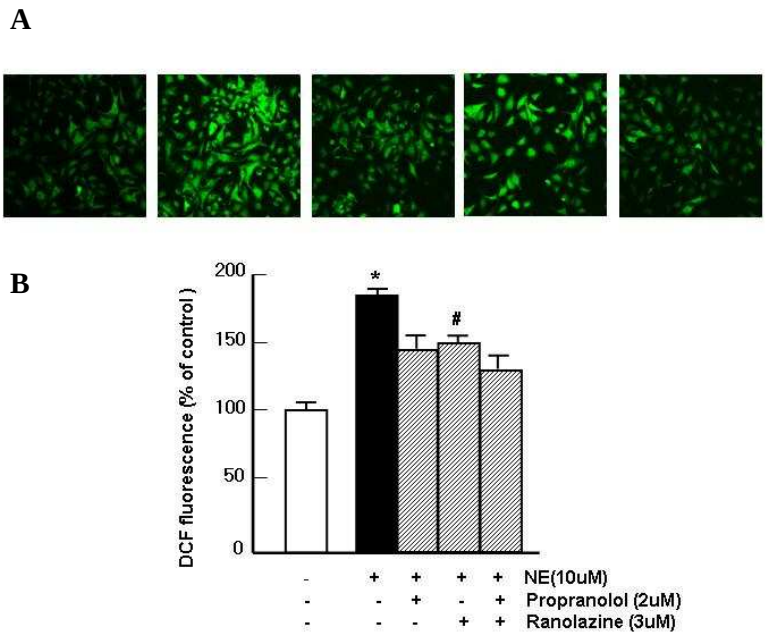

Figure 7. Reactive Oxygen species (ROS) production. After preincubated with propranolol (2 μM), ranolazine (3 μM), mixture of propranolol and ranolazine in cardiomyocytes, it was treated with 10 μM NE for 24 h. (A) Fluorescence image and (B) fluorescence intensity was obtained by using DCF fluorescence. Value are mean±SEM. $^{*}p<0.001$ vs. Control. $^{\#}p<0.001$ vs. NE.

8. Ranolazine prevented NE- induced apoptosis of cardiomyocyte.

Apoptosis is characterized by a variety of morphological features such as loss of membrane asymmetry and attachment, condensation of the cytoplasm and nucleus, and internucleosomal cleavage of DNA. One of the earliest indications of apoptosis is the translocation of the membrane phospholipid phosphatidylserine (PS) from the inner to the outer leaflet of the plasma membrane. Once exposed to the extracellular environment, binding sites on PS become available for Annexin V, Ca^{2+} dependent phospholipid binding protein, with a high affinity for PS. The translocation of PS precedes other apoptotic processes such as loss of plasma membrane integrity, DNA fragmentation, and chromatin condensation. Estimate of apoptosis, the number of annexin V positive cardiomyocytes, was investigated after exposure to NE with and without propranolol and ranolazine (Fig. 8). The number of annexin V positive cardiomyocytes was decreased about 39±1.5% by ranolazine compared with NE treated cells. In contrast, propranolol did not show such an effect.

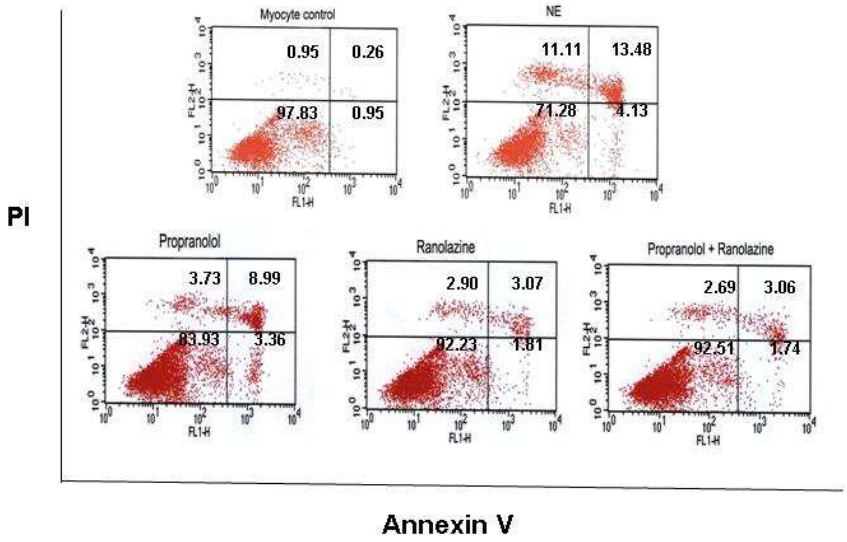

Figure 8. Annexin V/PI staining for apoptosis analysis Cardiomyocytes were exposed to NE for 24 h with propranolol, ranolazine and mixture of ranolazine and propranolol. Effect of ranolazine and propranolol in NE-induced apoptosis was detected by the annexin V-PI staining assay using flow cytometry.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

9. Ranolazine inhibited apoptosis through the regulation of pro-apoptotic factor Bax, anti-apoptotic factor Bcl-2 in NE-incuced cardiomyocyte.

Representative immunoblot of cytochrome C and Bcl-2/Bax separated by SDS-polyacrylamide gel electrophoresis, transferred onto a nitrocellulose membrane. β-actin is used as an internal control. NE-induced cytochrome C release from mitochondria to cytosolic fraction. Although cytochrome C was released into the cytosol from the mitochondrial intermembrane space during NE stimulation, ranolazine treated cells 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 protein expression occurred in NE-induced cardiomyocyte, but ranolazine treated cells did exhibit evident changes in the expression of both Bax and Bcl-2 proteins (Fig. 9B-C).

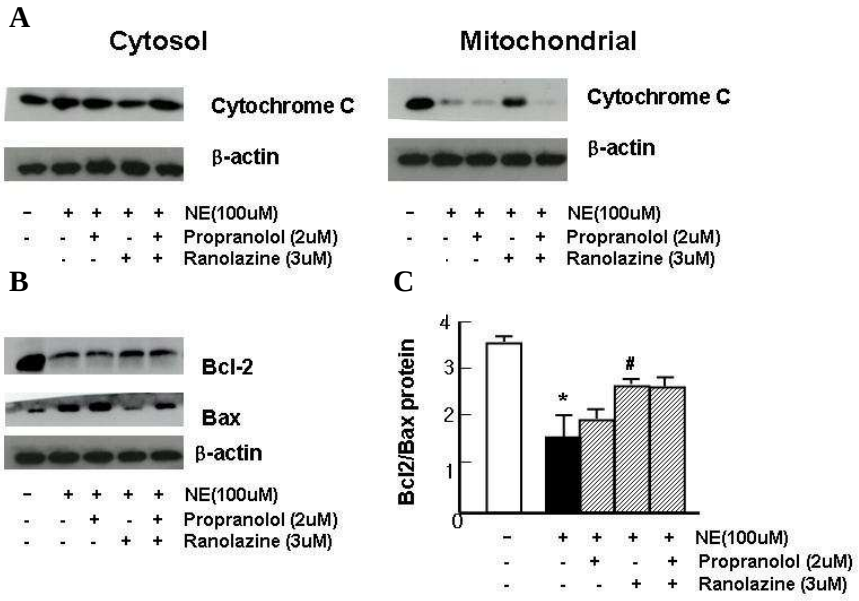

Figure 9. Bcl-2/Bax ratio and cytochrome C release. Cardiomyocytes were exposed to NE for 24 h with propranolol, ranolazine and mixture of ranolazine and propranolol. (A) Mitochondria/ Cytosolic cytochrome C and the (B) Bcl-2/Bax protein ratio were detected by western blotting and (C) densitometric analysis.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

IV. DISCUSSION

These results showed that ranolazine decreased cell death through the β-adrenoceptor signaling pathway in NE-induced cardiomyocytes. Also it was showed that ranoalzine led to inhibition of Ca^{2+}, Na^{+} overload and regulation of intracellular Ca^{2+} regulatory proteins in NE stimulation. Ultimately, ranolazine actions inhibited apoptosis of cardiomyocytes.

NE activated MAPK signaling pathway. To determine whether NE-induced cardiomyocytes treated with ranolazine involved in MAPK signaling pathway. It was performed ERK protein phosphorylation confirm by western blotting. Although the phosphorylation of ERK was elevated by NE for 24 h, phosphorylation of ERK was dropped by ranolazine treatment more than that of NE only (Fig. 1).

Mann and his co-workers advanced this thesis by showing that NE exerts a direct toxic effect on cardiomyocytes in vitro[39]. β-adrenoceptor apoptotic effects are associated with increases in intracellular Ca^{2+} and Ca^{2+} calmodulin-dependent protein kinase II (CaMKII) activity[40]. Especially, CaMKII is the major cardiac isoform. Also CaMKII expression is increased by β-adrenoceptor stimulation[41]. To confirm whether CaMKII expression level increased by β-adrenoceptor stimulation, it was examined CaMKII

gene expression in NE-induced cardiomyocyte. And then, CaMKII gene expression level confirmed to treated ranolazine in NE-induced cardiomyocytes. Whereas NE treatment was enhanced in CaMKII mRNA, ranolazine treatment cells were completely reduced in CaMKII mRNA (Fig. 2).

Stefan W and his co-workers mentioned CaMKII regulates Na^+ channel function in cardiomyocyte, most likely by association with and phosphorylation of Na^+ channels. Moreover, phosphorylation of Na^+ channel enhances intracellular Na^+ overload[42]. and For test of Na^+ overload, it was examined cardiomyocytes at the single cell level for changes in intracellular Na^+ using the fluorescent corona green (Na^+) dye and flow cytometry. Gating on only the normally sized or positive control, NE treatment in the presence of corona green (Na^+) resulted in cell counts increase of M1 region compared with normal control. And, treating cell of propranolol, ranolazine and co-treatment of ranolazine and propranolol was reduced in cell counts of M1 region compared with NE-induced cardiomyocytes (Fig. 3). The stimulation of β -adrenoceptors by NE causes a marked increase in both intracellular Na^+ concentration and intracellular Ca^{2+} concentration in cardiomyocytes.

Ca^{2+} is arguably the most important second messenger in cardiac muscle. Changes in the intracellular Ca^{2+} concentration have both acute and chronic effects on cardiac function[40]. 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[42]. It was observed that intracellular Ca^{2+} overload increased with NE-induced cardiomyocyte and decreased with ranolazine, propranolol and co-treatment of ranolazine and propranolol (Fig. 4). Ca^{2+} ions play an important role in normal cardiac function, and several Ca^{2+} regulating proteins are connected with Ca^{2+} homeostasis in cardiomyocytes. β-adrenoceptor stimulation by the sympathetic NE play a pivotal role in modulation of cardiac function in response to stress. Previous studies have shown that ATP-induced increase in $[Ca^{2+}]i$ is potentiated by NE. This increase of the ATP response by NE treatment has been demonstrated to be due to Ca^{2+} entry through the sarcolemmal (SL) LTCC. In this regard, it is pointed out that NE has been shown to phosphorylate the LTCC and enhance the inward Ca^{2+} influx Major Ca^{2+} cycling proteins involved in LTCC, RyRs, SERCA2a, NCX and PLB. The LTCC provides the primary Ca^{2+} influx mechanism and thus plays an important role in Ca^{2+} regulation[43]. There is substantial disparity in the levels of expression of LTCC in NE-induced cardiomycytes. The expression

level of LTCC was increased in NE stimulation compared with control cells. After treatment of NE-induced cells with ranolazine, expression level of LTCC increased (Fig. 5). The 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[15]. Decreased expression of the SERCA2 gene[44] and PLB gene[45] may contribute to the diminished contractile function occurring in animal models and in human beings with severe congestive heart failure. NCX and RyR2 expression level is increased by β-adrenoceptor stimulation[46]. This result showed that NCX and RyR2 expression levels were elevated by NE-induced cardiomyocytes and ranolazine blocked these changes in the levels of expression (Fig. 6A). The expression level of SERCA2a and PLB were reduced by NE-induced cardiomyocytes and enhanced by treated ranolazine cells (Fig. 6B).

There are many reports documenting that NE induce apoptosis in vitro and in vivo[42]. NE can induce ROS and apoptosis of cardiomyocytes by activation of the β-adrenergic pathway[43]. Regardless of underlying mechanisms, the enhanced β-adrenoceptor stimulation may importantly contribute to the increased incidence of chronic diseases, such as hypertension and related diseases, congestive heart failure, sudden cardiac death, the insulin

resistance syndrome and obesity[47]. A significant increase in fluorescence intensity was detected in the NE treated cell for 24 h as compared to both control groups. It was clearly resulted in increased oxidativestress as measured in NE-induced cardiomyocytes. It was found that oxidativestress decrease in treating ranolazine cells. But, ROS production of propranolol treated cells is decreased more than ROS production of ranolazine treated cells (Fig. 7).

Apoptosis is triggered when something is amiss with the cell: DNA damage, detachment from neighbors, growth factor deprivation, infection, or a host of other signs. In the present study, ranolazine was examined the effect mechanisms of NE-induced apoptosis in cardiomyotes. It was found that NE-induced apoptosis was accompanied by down-regulation of Bcl-2 protein synthesis and activation. To examine that NE-induced apoptosis was detected by annexin V/PI staining (Fig. 8). Very little apoptosis was detected in control cultured cardiomyocytes. Cells underwent significant apoptosis when exposed to NE, but pretreatment with ranolazine and co-treatment of ranolazine and propranolol diminished the apoptosis rate significantly. In contrast, propranolol did not effect. Cytochrome C plays a central role in apoptosis, signaling the cell to begin the process of cell death. Cytochrome C release from the mitochondria also has been observed in NE-induced

apoptosis (Fig. 9A). The anti-apoptotic protein Bcl-2 plays an important role in controlling cell death. It was observed that NE-treated cardiomyocytes decreased the Bcl-2 protein level, whereas Bax protein level was increased. However, treating ranolazine cells was protected to NE stimulation (Fig. 9B). These results are in agreement with previous reports showing that Bcl-2 acts upstream of the caspase cascade. Interestingly, if the rise in intracellular Na^+ is prevented using ranolazine, a complete inhibition of apoptosis is observed. Thus, the rise in intracellular Na^+ appears to have an effect in the initial signaling of the apoptotic program.

Ⅴ. CONCLUSION

This study suggests that ranolazine was involved in β-adrenoceptor activation and was prevented from NE-induced apoptosis. In NE treated cardiomyocytes, phosphorylation of ERK was inhibited in ranolazine treated cells compared with NE only treated cells. Ranolazine also reduced intracellular Ca^{2+} and Na^{+} level by regulating CaMKII in NE stimulation and affected Ca^{2+} channel related gene expression. Although ranolazine was a little influence in NE induced ROS production, ranolazine had a protective effect on apoptosis in NE stimulus. In NE treated cardiomyocytes, ranolazine diminished apoptotic protein expression level in comparison with NE only treated cells. Ranolazine was related in β-adrenoceptor signal pathway in the prevention of NE-mediated cardiomyocyte apoptosis during heart failure.

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ABSTRACT (in Korean)

신생백서 심근세포에서 베타 아드레날린 수용체 신호기전 저해를 통한 노르에피네프린 자극 세포사멸의 라놀라진 효과

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김 혜 정

라놀라진은 칼슘과부하가 유도되어 생기는 지속적인 나트륨 유동을 특이적으로 저해하는 것으로 알려져 항 협심증 치료제로 대두되고 있지만, 라놀라진의 지속적인 나트륨 유동 저해 메커니즘이 명백하게 밝혀지진 않았다. 베타 아드레날린 수용체가 심근세포에서 심장기능 조절에 중요한 역할을 하는 것을 바탕으로 노르에피네프린을 처리하여 베타 아드레날린 수용체를 자극한 심근세포에서 라놀라진이 대표적인 베타 아드레날린 수용체

길항제인 프로프라노롤과 유사한 반응이 나타날 것으로 기대했다. 따라서, 본 연구에서는 베타 아드레날린 수용체를 자극하는 노르에피네프린을 통해 지속적 나트륨 유동, 칼슘 과부하, 세포사멸이 유도된 심근세포에서 라놀라진의 베타 아드레날린 수용체 길항제 활성을 조사하기 위해 2 μM 프로프라노롤, 3 μM 라놀라진 그리고 프로프라노롤과 라놀라진을 함께 처리한 심근세포에 24시간 동안 10 μM 노르에피네프린을 처리하였다. 라놀라진은 노르에피네프린에 의해 유도되어 증가된 세포 내 칼슘과 나트륨 농도를 각각 40±3%, 17±0.5% 감소 시켰고, 노르에피네프린 자극세포에서 라놀라진이 베타 아드레날린 수용체 신호기전에서 나트륨 채널을 조절하는 것으로 알려진 CaMKII (Ca^{2+}/calmodulin dependent protein kinase II) 발현을 57±2% 감소시켰으며, 칼슘 관련인자인 L-Type Ca^{2+} channel, phspholamban, SERCA2a의 발현을 각각 48±3.5%, 45±2%, 36±0.5% 증가하였으며, Na^{+}-Ca^{2+} exchanger, ryanodine receptor2의 발현을 각각 21±0.5%, 47±1.5% 감소하여 노르에피네프린에 의한 라놀라진 효과가 확인되었다. 마지막으로, annexin V/PI staining을 통하여 apoptosis를 분석한 결과 라놀라진 처리한 군이 노르에피네프린만 처리한 군에 비해 39±1.5% 감소하였으며, 전사멸인자인 Bax, 항사멸인자인 Bcl-2, 그리고 cytochrome C의 단백질발현이 2배 이상 저해된 것을

확인함으로 인해 라놀라진의 세포사멸 억제효과를 증명하였다. 이상의 결과로 라놀라진이 노르에피네프린에 의해 유도된 베타 아드레날린 수용체 신호기전에 영향을 줌으로 써 심장기능 이상을 완화시키는 것이 증명되었다.

핵심 되는 말: 심근세포, 라놀라진, 베타 아르레날린 수용체, 노르에피네프린, 세포사멸