Novel lactam type pyridine derivative improves myocardium dysfunction derived from ischemic injury

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Directed by Professor Jun Hee Sul

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Abstract

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The extended acute myocardial ischemia (AMI) results in cardiac myocytes death. It is well known that Ca^{2+} homeostasis within the cardiac myocyte is controlled by regulatory proteins in sarcolemmal and sarcoplasmic reticulum (SR) membranes. Ranolazine has been shown to reverse the sustained rise in systolic and diastolic calcium caused by a well-known enhancer of late I_{Na}. 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 lactam pyridine derivatives are inhibitors of the late sodium current, which decreases sodium-dependent intracellular calcium overload in ischemia/reperfusion injured hearts. This study was designed to confirm whether the mechanism was associated with a β-adrenoceptor antagonist activity of ranolazine on norepinephrine (NE)-induced cardiomyocytes and to compare lactam pyridine derivative, LP-80375, as a therapeutic agent having a protective function like ranolazine. 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 Ca2+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. Ranolazine also inhibited apoptosis by regulating the levels of the pro-apoptotic factor Bax, the anti-apoptotic factor Bcl-2, and cytochrome C release. It was founded that

pretreatment with LP-80375 and ranolazine significantly decreased the level of intracellular Ca^{2+} by 49±3.5% and 42±5.5%. In addition, the expression level of SERCA2a was significantly increased by 56±2.1% in LP-80375-treated hypoxic cardiomyocytes compared to untreated controls. And the expression level of the cAMP (adenosine 3'5' cyclic monophosphate) response element modulator (CREM; 22±0.8%, 11±1.8%), and NCX (20±1.2%, 5±1.2%) was decreased in LP-80375 and ranolazine-treated hypoxic cardiomyocytes compared with hypoxic control. And the highest level of Hsp70 was induced at the concentration of 2.5 μM LP-80375. The echocardiographic analysis showed that heart function was significantly improved in LP-80375-injected ischemic hearts. These results demonstrate that ranolazine had an effect on NE-induced cell death through inhibition of the β-adrenoceptor signal pathway in cardiomyocytes. The present studies also demonstrate that lactam pyridine derivative, LP-80375, have beneficial effects on hypoxia-induced cell death and might be used as a novel anti-ischemia drug.

Key words: cardiomyocyte, lactam pyridine derivative, LP-80375, ischemia, apoptosis

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I. INTRODUCTION

Acute myocardial infarction (AMI) is a leading cause of death in developed countries. Myocardial ischemia is known to produce changes in cardiac function, metabolism and ultrastructure.[1,2] The effect of hypoxia may be more complex because hypoxia have direct effects on the cardiovascular system and may cause

cardiac ischemia. Hypoxia induces physiological responses such as, cell proliferation and vascular remodeling,[3] which is due to an intracellular Ca^{2+}overload. In fact, ischemia is associated with unbalances of cellular Ca^{2+} and Na^{+} homeostasis.[4,5]

It is well known that Ca^{2+} homeostasis within the cardiac myocyte is controlled by regulatory proteins in sarcolemmal and sarcoplasmic reticulum (SR) membranes.[6,7] The Na^{+}-Ca^{2+}exchanger (NCX) and Ca^{2+} pump including cAMP (adenosine 3'5' cyclic monophosphate) response element modulator (CREM) and sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA) plays a major role in Ca^{2+} homeostasis and contributes to abnormal intracellular Ca^{2+} handling in a failing heart.[8,9] Ca^{2+} enters the cell via the L-type Ca^{2+} channel when the sarcolemmal membrane is depolarized.[10] Ca^{2+} released from the SR is transferred to mitochondria, another reservoir of Ca^{2+}, via a coupling of RyR and closely apposed mitochondrial membrane.[11] It has been shown that upon

myocardial ischemia and reperfusion, overload of both intracellular Ca^{2+} concentration and mitochondrial Ca^{2+} concentration occur and attenuation of mitochondrial Ca^{2+} concentration, but not intracellular Ca^{2+} concentration, overload is responsible for improved recovery in contractile functions.[12]

Recently, many studies showed that calcium channel blockers are the most frequently prescribed drugs for the treatment of cardiovascular disease.[13,14] Calcium channel blockers are potent vasodilators, and their use in cardiovascular treatment remains based on the blockade of Ca^{2+} mobilization. Most specifically, some drugs including ranolazine has been shown to reverse the sustained rise in systolic and diastolic calcium caused.[15-17] Cardiomoycytes of dog and guinea pig, ranolazine was shown to cause a concentration, voltage, and frequency-dependent inhibition of late I_{Na}. Most specifically, ranolazine has been shown to reverse the sustained rise in systolic and diastolic calcium caused by a well-known enhancer of late I_{Na}. Moreover, the special mechanism of

action of ranolazine is to inhibit late I_{Na} thus preventing sodium overload of the cell.[18,19] Ranolazine is thought to indirectly reduce Ca^{2+}overload via an action on Na^{+} channels, preserving ionic homeostasis.[20] Acute intravenous administration of ranolazine improved left ventricular systolic function in animal models of heart failure.[21]

Norepinephrine (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).[22] The MAPKs, a large family of serine-threonine kinases, play important roles as mediators of signal transduction and are activated by extracellular stimuli.[23,24] 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.[24,25] Recently, heterotrimeric G proteins have also been shown to activate various members of the MAPK family.[24,25] One of the major mechanisms for regulating contractility of the heart involves β-adrenoceptor stimulation.

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 sub types of ß-adrenoceptor ($ß_1$, $ß_2$, and $ß_3$) 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.[26,27] There are selective agonists and antagonists for all three receptor subtypes.[28] 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.[29] Regardless of the underlying mechanism, systemic β-adrenoceptor antagonist can have a number of significant effects on cardiac function.[30]

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. Cardiac dilatation and pump dysfunction without intrinsic myocardial systolic failure follows chronic β-adrenoreceptor activation.[31] 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.[32] 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.[33] 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.[34-36] CaMKIIδc has been implicated in apoptotic signaling[36-39] and in mediating the transition to heart failure.[40] 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.[41-43]

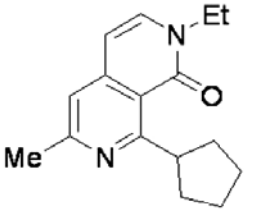
β-adrenoceptor stimulus increases apoptosis[44] and reactive oxygen species (ROS)[33] in cardiomyocytes. ROS generated by a variety of extracellular and intracellular mechanisms, have gained attention as novel signal mediators that regulate signal transduction events and have been known to play an important role in the pathogenesis of several cardiovascular diseases. ROS are well-known

to regulate the transcription of particular genes.[34] However ROS may specifically induce apoptosis.[35] Recent study demonstrated chronic adrenergic stress as being responsible for adverse effects, such as cardiac failure, mainly due to increased cardiomyocyte apoptosis.[36] Apoptosis has been demonstrated to occur in the myocardium in a variety of pathological situations.[37] The number of apoptotic myocytes is increased in myocardium obtained from patients with end-stage heart failure and myocardial infarction[38] and in myocardium from experimental models of myocardial hypertrophy and failure, including the rat,[39] the spontaneously hypertensive rat,[40] rats with myocardial infarction.[45] Many molecules including proteins in the Bcl-2 and caspase families participate in the apoptotic response to numerous deaths inducing signals.[45] 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.[47]

It was confirmed 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.[48] 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 down regulation of Bcl-2

protein synthesis and activation of β-adrenergic and cytochrome C release mechanisms.

It was hypothesized that ranolazine and novel lactam pyridine derivatives (Fig. 1) reduced the Ca^{2+} overload and finally may cause the myocardial cell survival as an effective cardioprotective agent against severe hypoxia. It also confirmed whether lactam pyridine derivatives and ranolazine exerted the effect of new anti-ischemia drug on the cell survival effect.



LP-80375

Figure 1. Chemical structure of a lactam pyridine derivative LP-80375.

II. MATERIALS AND METHODS

1. Isolation and culture of rat cardiomyocytes

Neonatal rat ventricle cardiomyocytes (NRVCM) were isolated from neonatal rat hearts, as previously described.[19] This heart tissue was washed with pH 7.4 Dulbecco's phosphate-buffered saline (D-PBS; Gibco BRL) in order to remove red blood cells. After depletion of the atria, the hearts were sliced up approximately 0.5 mm^2 sized pieces and treated with 4 ml collagenase II (1.4 mg/ml, 270 units/mg Gibco BRL) solution for 5 min. The supernatant was removed, and the pellet was suspended in collagenase II. After cells were incubated in a 37°C humidified atmosphere chamber containing 5% CO_2 for 5 min, gained supernatant was mixed with alpha minimum essential medium (α-MEM; Gibco BRL) then centrifuged at 2000 rpm for 2 minutes. The remaining tissue was treated with fresh collagenase II solution for five min. This incubation procedure was repeated until the tissue was completely digested. The gathering cell pellet was resuspended in α-MEM and attached to a culture dish at 37°C in 5% CO_2 chamber. Two hours later, adherent cells turned into fibroblasts and non-adherent cells became NRVCMs. The NRVCMs were washed twice and replated on 100 mm plates with α-MEM plus 10% FBS at

37°C in 5% CO_2 chamber. To eliminate fibroblast expansion, we used 0.1 mM 5-bromo-2'-deoxyuridine (Brd-U; Sigma-Aldrich)

2. NE stimulation and treatment with propranolol and ranolazine

Cardiomyocytes were incubated in 5% (v/v) CO_2 at 37℃ for 1 day. The cells were next placed into α-MEM without FBS in a CO_2 incubator (Thermo Forma Model 311, Marietta) 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.

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). 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℃ 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.

4. Measurement of intracellular reactive oxygen species generation

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.

5. Measurement of Ca^{2+} level of ischemic NRVCMs

The measurement of cytosolic free Ca^{2+} was performed by confocal microscopy analysis. Neonatal rat cardiomyocytes were plated on glass cover slips coated with laminin (5 mg/cm^2) for 1 day in cell culture medium (α-MEM containing 10% fetal bovine serum, Gibco BRL) and 0.1 μM BrdU. Cardiomyocytes were put in a hypoxic chamber for established ischemic time. After incubation, the cells were washed with modified Tyrode's solution containing 0.54 mM $CaCl_2$, 0.25 mM $MgCl_2$, 4.5 mM KCl, 125 mM NaCl, 5.5mM glucose, 1.2mM NaH_2PO_4 and 24 mM $NaHCO_3$. Cells were then loaded with 5 mM of the acetoxymethyl ester of Fluo-4 (Fluo-4 AM, Molecular Probes, Eugene, OR) for 20 min, in the dark and at room temperature, by incubation in modified Tyrode's solution. Fluorescence images were obtained using an argon laser confocal microscope (Carl Zeiss Inc.). This fluorochrome is excited by the 488 nm line of an argon laser and emitted light is collected through a 510-560 nm band pass filter. Relative changes of free intracellular Ca^{2+} were determined by measuring fluorescent intensity.

6. Measurement of ischemic time (hypoxic injury) of NRVCMs

Cardiomyocytes were plated in triplicate wells of 96 well plates at a density of 5

x 10^4 cells per well. Time dependently incubation was performed, the incubation period, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (Sigma) was added to each well to a final concentration of 0.5 mg/ml and was incubated at 37°C for 2 hr to allow MTT reduction. The formazan crystals were dissolved by adding dimethylsulfoxide (DMSO) and absorbance was measured at the 570 nm with a spectrophotometer. Experiments were performed in triplicate.

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 electro transferred to methanol-treated poly vinylidenedifluoride 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 hr 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 hr 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).

8. RT-PCR Analysis

8.1 Isolation of total RNA

Total RNA was extracted by 500 μl/60 mm plate Tri-reagent (Sigma). After pouring 100 μl chloroform into Tri-reagent and voltexing a sample about 10 sec,

sample was centrifuged at 12000 g, 4°C and 15 min. Three layers were appeared in the tube, transparency upper layer collected in new tubes. And poured 250 μl 2-propanol over the sample, again voltex a sample about 30 sec. Centrifuge was accomplished about 12000 g, 4°C and 10 min. Left the pellet, supernatant was discarded and washed by 75% ethanol (Duksan, Korea)–mixed diethylpyrocarbonate (DEPC; Sigma) water. Centrifugation was also operated about 7500 g, 4°C and 5 min. Dismissed the supernatant, pellet was dried on room temperature about 7 min. Finally, poured the 30 μl nuclease free water (NFW). The quality and quantity of the RNA was detected by OD260/OD280 with DU 640 spectrophotometer (Effendorf, Hambug, Germany)

8.2 cDNA synthesis

Complementary DNA (cDNA) was synthesized with RT-&GO^{TM}. Quantitative 1 μg total RNA was added to 1 μl anchored primer (dT) 25V, 2 μl dithiothreitol (DTT) and NFW, totally 9 μl volume. To prevent secondary structures, incubated the mixture for 5 min at 70°C and added 8 μl of RT-&GO^{TM} mastermix. Sample was incubated the assay at 42°C for 1 hr. At the conclusion, sample was inactivated the reverse transcriptase at 70°C for 15 min. After

isolation of total RNA, sample was detected by optical density (OD) 260/280 with DU 640 spectrophotometer.

8.3 PCR analysis

Quantitative 1 μg cDNA, each 10 pmol primer (forward and backward; Table 1), 0.1 mM dNTP mixture, 1.25 U of Taq polymerase and 10 X reaction buffer were mixed with NFW, lastly total volume of 25 μl. PCR condition was fixing as fellow. A cycle of denaturing at 94°C for 3 min followed by number of 35 cycles with denaturation at 94°C for 30 sec, annealing at 48°C to 60°C for 30 sec, and elongation at 72°C for 30 sec. Then keep up 72°C for 10 min. Primers were appeared Table 1. When PCR assay have finished, PCR product was separated by electrophoresis in a 1.2% agarose gel (Biorad) and Gel-Doc (Biorad) visualized after staining with ethidium bromide (EtBr; Sigma).

Table 1. PCR primer

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.
CREM	CCGTATGACCATGGAAACAG CAGGTCCAAGTCAAACACAG	416	53°C, 60s/35cyc

9. 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 0.54 mM $CaCl_2$, 0.25 mM $MgCl_2$, 4.5 mM KCl, 125 mM NaCl, 5.5 mM glucose, 1.2 mM NaH_2PO_4 and 24 mM $NaHCO_3$. Cells were next loaded with 10 μM of the acetoxy methyl ester of fluo-4 (Fluo-4 AM, Molecular Probes) for 20 min, in the dark at 37℃. 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 band pass filter. Relative levels of intracellular Ca^{2+} were determined by measuring fluorescence intensity.

10. 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 FACS Calibur 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 band pass filters, respectively. Each sample contained at least 1×10^4 cells. 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.

11. Myocardial ischemia protocol and treatment with LP-80375 and ranolazine

The myocardial infarction animal model was created through the method of Lipsic et al.[47] with minor modifications. Under general anesthesia, male Sprague-Dawley rats (230 ± 10 g) of 8 weeks of age were ventilated with positive-pressure (180 mL/min) using a Harvard ventilator (Harvard Apparatus, Millis, MA). The rat heart was exposed through a 2 cm left lateral costal rib incision. The proximal portion of the left coronary artery was ligated with a 6-0 silk suture (ETHICON Inc., Somerville, NJ) placed beneath the left atrium for 1 week. Drugs were injected everyday intra-peritoneally.

12. Echocardiography

The rats were sedated with zoletil (50 mg/kg) and xylazine (5 mg/kg), both of which were given intraperitoneally. Imaging was performed at 15 MHz with a linear transducer interfaced with an ultrasound system (Vivid 7, GE Vingmed Ultrasound, Horten, Norway). Images were acquired in a parasternal short axis view and all data were recorded and, subsequently, analyzed at the end of the study. For each animal, the LV end-systolic dimensions (LVESD) and LV end-diastolic dimensions (LVEDD) were measured from the M-mode tracings, and the LV shortening fraction (FS), ejection fraction (EF) were calculated.

13. Statistical analysis

Data were summarized from 3 individual experiments and expressed as means±S.E. Statistical analyses were performed by one-way ANOVA, using Bonferroni test for comparison of several groups. A value of $P<0.05$ was considered significant.

III. RESULTS

1. Effects of norepinephrine-induced cell death

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

The phosphorylation activity of ERK was dramatically higher in NE-cardiomyocytes than in normal cells. The increasing ERK phosphorylation level induced by NE was significantly inhibited by pretreatment with propranolol and ranolazine, respectively. Although phosphorylation of ERK increased by 63±1% after treatment with NE for 24 hr, ERK phosphorylation fell by 52±2.5% after ranolazine treatment (Fig. 2), compared with the level seen in NE-induced cardiomyocytes.

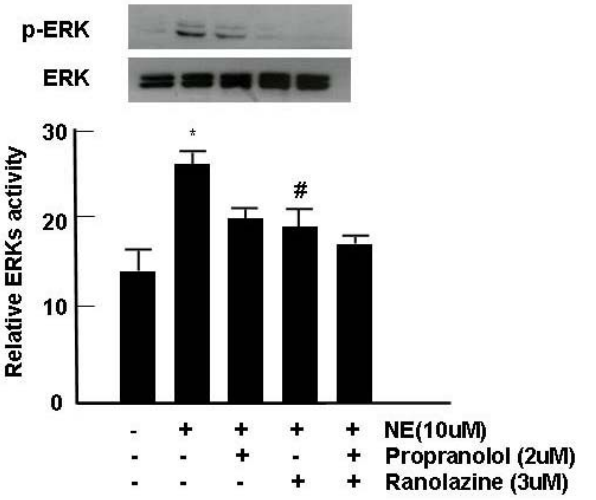


Figure 2. Phosphorylation of ERK in NE induced cardiomyocytes. Relative phosphorylation levels of ERK activity were determined by western blotting. Cardiomyocytes were subjected to NE stimulation with propranonol and ranolazine for 24 hr. phosphorylation of ERK was treated during NE stimulation was detected by immunoblotting. The mean±SE of eight independent experiments is reported ($^{*}p<0.001$ vs. Control and $^{\#}p<0.05$ vs. NE).

1.2. Ranolazine decreased CaMKII over expression in NE-induced cardiomyocytes

CaMKII is activated on the binding of Ca^{2+} and calmodulin complex. In addition to the widely accepted Ca^{2+} /calmodulin- mediated activation, recent studies have demonstrated that CaMKII can be activated in a Ca^{2+} -independent fashion. To test the above hypothesis, we investigated whether activation of 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. 3).

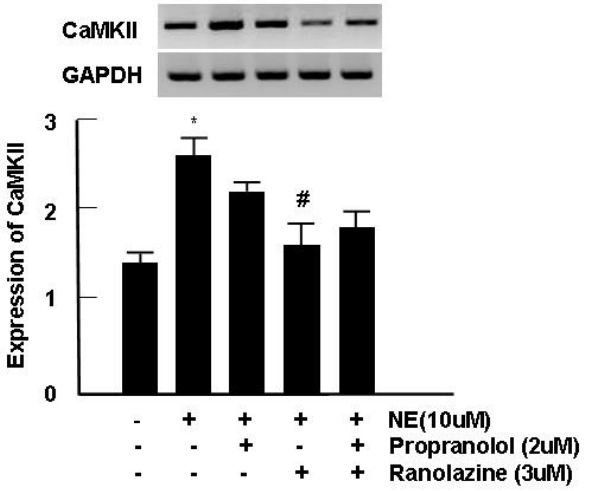


Figure 3. CaMKII expression level in NE-induced cardiomyocytes. Cardiomyocytes treated with ranolazine and propranolol were exposed to NE stimulation for 24 hr. Whereas NE treated cells were increased in CaMKII mRNA, ranolazine treated cells were completely decreased in CaMKII mRNA. The mean±SE of eight independent experiments is reported ($^{*}p<0.001$ vs. Control and $^{\#}p<0.05$ vs. NE).

1.3. Ranolazine protected NE-induced cardiomyocytes from intracellular Na^+ overload

It was 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 18±3%, 17±0.5%, and 17±2.5%, respectively, in M1 cells were noted, compared to the level in NE-induced cardiomyocytes (Fig. 4).

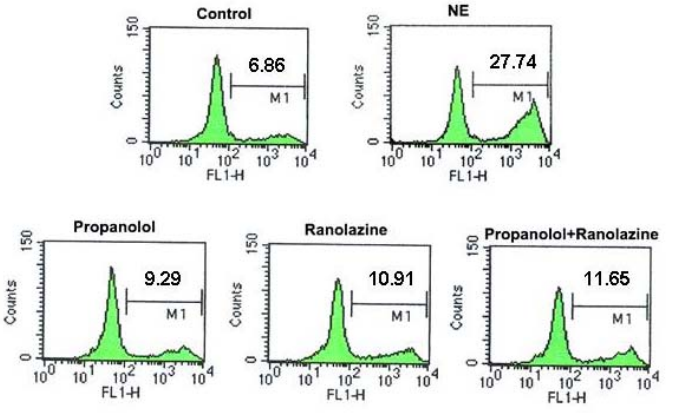


Figure 4. Effects of ranolazine on intracellular Na^+ overload in NE-induced cardiomyocytes. Intracellular sodium was measured by flow cytometry using corona green (Na^+). The cells were initially analyzed by gating on only the normal cell or NE treated cells on a side scatter histogram plot. Subsequently, 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.

1.4. Ranolazine prevented intracellular Ca^{2+} accumulation after NE stimulation

To examine intracellular Ca^{2+} overload in NE-induced cardiomyocytes, we evaluated the fluorescent intensity of cardiomyocytes loaded with fluo-4 AM by using confocal microscopy. As shown in Fig. 5, the intracellular Ca^{2+} level during NE stimulus was elevated by 50±3% at 24 hr 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.

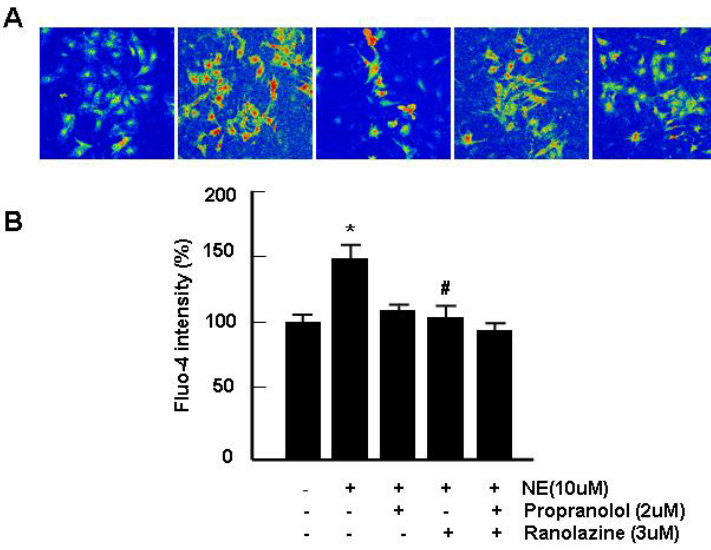


Figure 5. Effects of ranolazine on intracellular Ca^{2+} concentration. Confocal fluorescent images of cardiomyocytes were obtained by loading with fluo-4 AM. The NE-induced cells treated with ranolazine and propranolol were incubated for 24 hr. (A) Fluorescence image was obtained by fluo-4 AM and (B) fluorescence intensity was quantified in different cells (n=5) in each condition and analyzed. The mean±SE of eight independent experiments is reported ($^{*}p<0.001$ vs. Control and $^{\#}p<0.05$ vs. NE).

1.5. Ranolazine suppressed Ca^{2+} channel expression in NE-induced cardiomyocytes.

For assessment of gene expressions related to Ca^{2+} homeostasis, total RNA was isolated and analyzed by semi-quantitative RT-PCR. As illustrated in Fig. 6, compared with normal cardiomyocytes, the levels of gene. 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. 6).

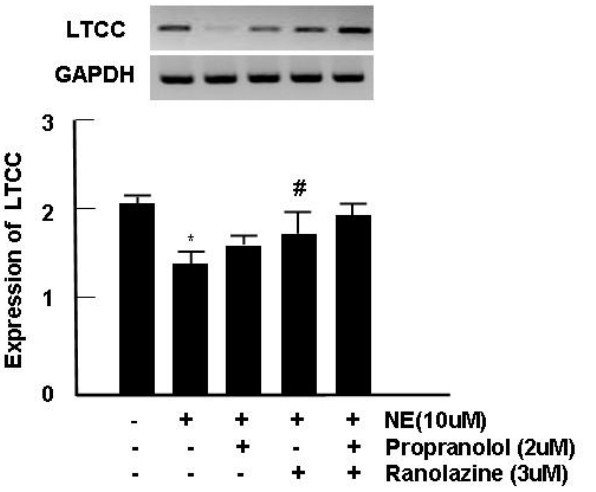


Figure 6. L-type Ca^{2+} channel expression level in NE-induced cardiomyocytes. Cardiomyocytes treated with ranolazine and propranolol was exposed to NE stimulation for 24 hr. The mRNA expression of genes was established by separating amplification products in agarose gel electrophoresis and visualized them by ethidium bromide staining. Each expression was quantified by scanning densitometer. The mean±SE of eight independent experiments is reported ($^{*}p<0.001$ vs. Control and $^{\#}p<0.05$ vs. NE).

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

It was examined the expression of Ca^{2+} channels related to 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. 7A). The expression levels of SERCA2a and PLB were higher, by 36±0.5% and 45±2%, respectively, than levels in NE-induced cells (Fig. 7B). These results showed that ranolazine prevented changes in SERCA2a, PLB, NCX, and RyR2 expression levels.

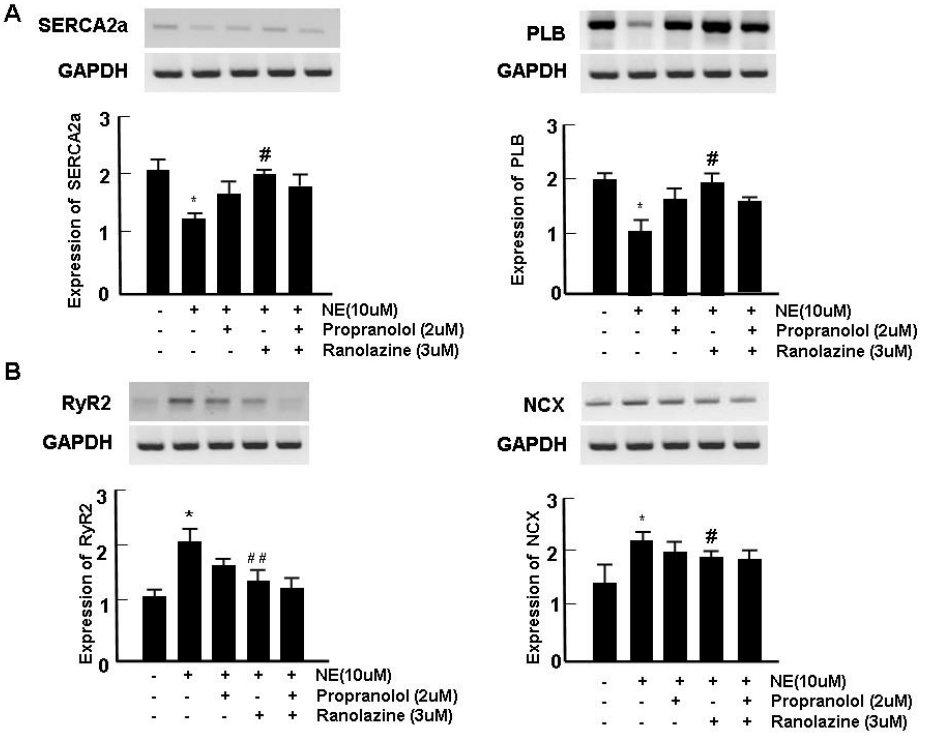


Figure 7. Effects of ranolazine in Ca^{2+} channel of NE-induced cardiomyocytes. Expression levels of the RyR2, NCX1, SERCA2a and PLB were estimated in cardiomyocytes subjected to NE with or without ranolazine, propranolol and analyzed. The mean±SE of eight independent experiments is reported ($^{*}p<0.001$ vs. Control and $^{\#}p<0.05$ vs. NE).

1.7. 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.[35,36] Significant enhancement in fluorescence intensity was detected in NE-treated cells after 24 hr of culture, compared to levels in control cells (Fig. 8A). ROS production was increased by 71±0.5% after NE (10 μM) treatment of cardiomyocytes for 24 hr (Fig. 8B). 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.

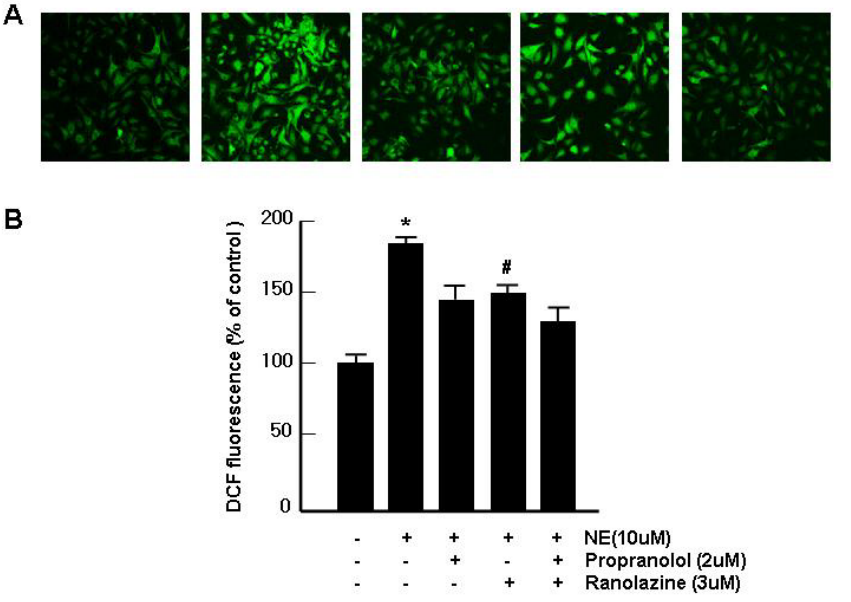


Figure 8. Reactive oxygen species (ROS) production. After preincubated with propranolol (2 μM), ranolazine (3 μM), mixture of propranolol and ranolazine in cardiomyocytes, it was treated with 10 μM NE for 24 hr. (A) Fluorescence image and (B) fluorescence intensity was obtained by using DCF fluorescence. The mean±SE of eight independent experiments is reported ($^{*}p<0.001$ vs. Control and $^{\#}p<0.05$ vs. NE).

1.8. Ranolazine prevented NE-induced apoptosis of cardiomyocytes

Figure 9 showed a representative analysis and regional percentage of annexin V versus PI dot plot of cardiomyocytes, was investigated after exposure of cells to NE with or without propranolol and ranolazine. 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, propranolol showed no such effect. These results demonstrate that delivery of ranolazine effectively enhances viability of cardiomyocytes.

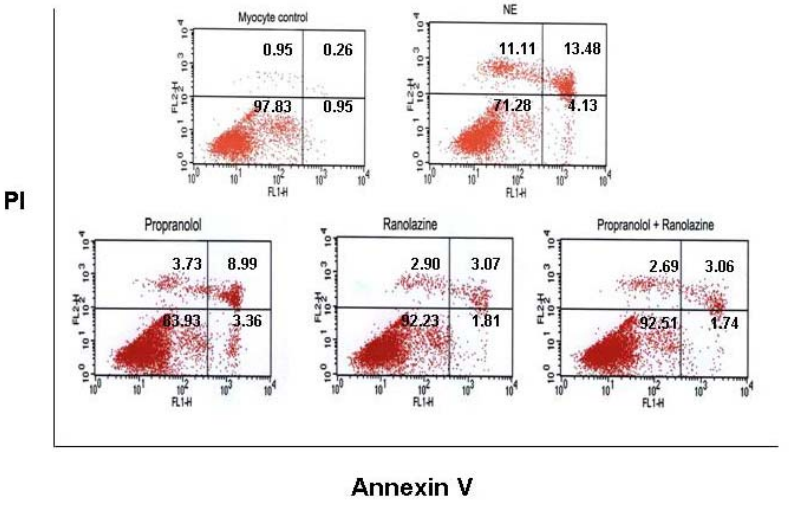


Figure 9. Annexin V/PI staining for apoptosis analysis. Cardiomyocytes were exposed to NE for 24 hr with propranolol, ranolazine and mixture of ranolazine and propranolol. Effect of ranolazine and propranolol in NE-induced apoptosis was detected by the annexin V-PI staining assay using flow cytometry. Values are mean±SEM

1.9. Ranolazine inhibited apoptosis by regulation of levels of the pro-apoptotic factor Bax and the anti-apoptotic factor Bcl-2, in NE-induced cardiomyocytes

As shown in Fig. 10, NE increased expression of pro-apoptotic proteins such as Bcl-2-associated X protein (Bax) and cytochrome c and decreased expression of the anti-apoptotic protein, B cell leukemia/lymphoma-2 (Bcl-2). NE induced cytochrome C release from mitochondria to the cytosolic fraction. Although cytochrome C was released into the cytosol from the mitochondrial inter membrane space during NE stimulation, treatment of cells with ranolazine during NE stimulus blocked cytochrome C release into the cytosol (Fig. 10A). 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. 10B-C).

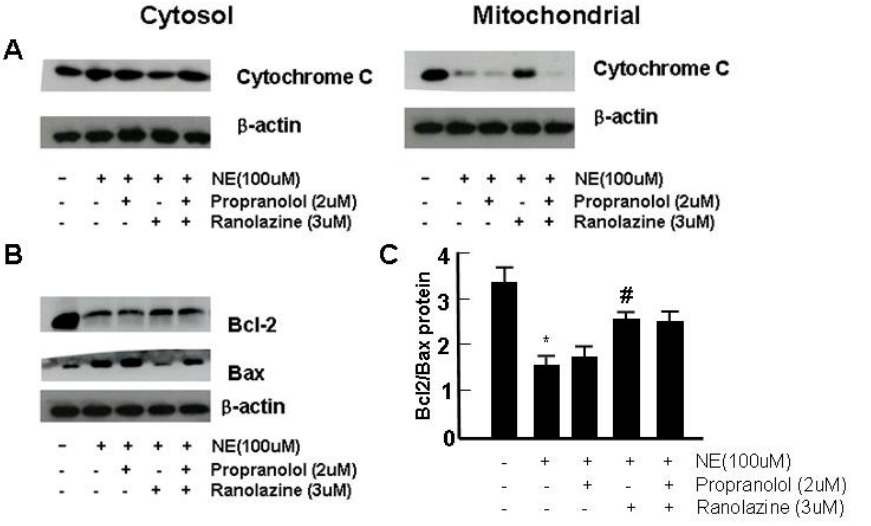


Figure 10. Bcl-2/Bax ratio and cytochrome C release. Cardiomyocytes were exposed to NE for 24 hr with propranolol, ranolazine and mixture of ranolazine and propranolol. (A) Mitochondria/ Cytosolic cytochrome C and the (B) Bcl-2/Bax protein ratio were detected by western blotting and (C) densitometric analysis. The mean±SE of eight independent experiments is reported ($^{*}p<0.001$ vs. Control and $^{\#}p<0.05$ vs. NE).

2. Effects of novel lactam type pyridine derivatives on myocardium dysfunction derived from ischemic injury

2.1. Effects of LP-80375 and ranolazine on cell survival in hypoxic cardiomyocytes

To determine the effects of LP-80375 and ranolazine on survival of cardiomyocytes under hypoxic conditions, we evaluated the survival rate of hypoxic cardiomyocytes. As shown in Fig. 11A, cell survival was significantly increased in ranolazine or LP-80375 -treated hypoxic cardiomyocytes compared to untreated controls. In particular, 2.5 μM concentration of ranolazine has shown the highest effect on cell survival. The cell survival rates at LP-80375 concentrations above 2.5-5 μM were higher than those in the hypoxic group, and the 1 and 10 μM were not significant (Fig. 11B).

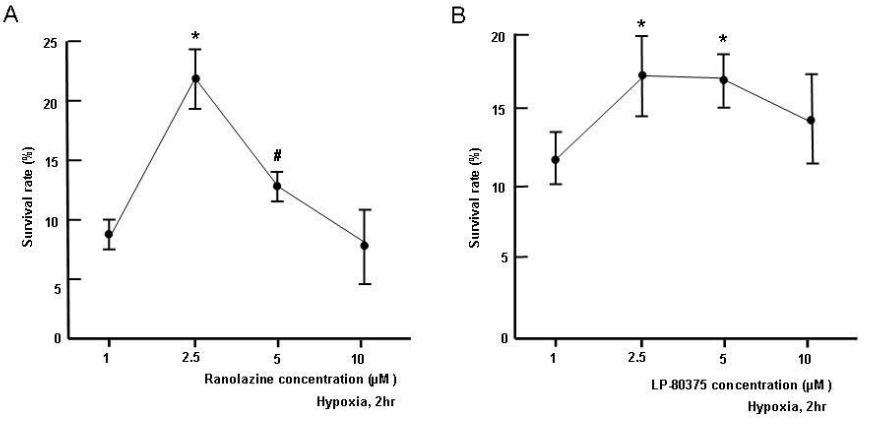


Figure 11. Measurement of cardiomyocyte viability during hypoxia. Cardiomyocytes were incubated in the absence or presence of ranolazine (A) or LP-80375 (B) under hypoxia for 24 hr. 1-10μM LP-80375 and ranolazine were simultaneously treated with hypoxia (less than 1% O_2). Cell survival rate was detected by MTT assay. Each bar came from six wells of 96-well plate and represented the mean±SE. (*p<0.01 vs control and #p<0.05 vs hypoxia)

2.2. Effect of LP-80375 and ranolazine on cytosolic Ca^{2+} overload in hypoxia

To know whether LP-80375 and ranolazine treatment decreased intracellular Ca^{2+} overload in hypoxic cardiomyocytes, the intracellular Ca^{2+} level was examined using the fluo-4. As shown in Fig. 12 A, the intracellular Ca^{2+} during hypoxia was increased about 1.5-fold at 2 hr after hypoxia, which was significantly decreased by treatment with in 2.5 μM ranolazine (49±3.5%). LP-80375 was also significantly decreased hypoxia-induced Ca^{2+} overload as shown in Fig. 12 B.

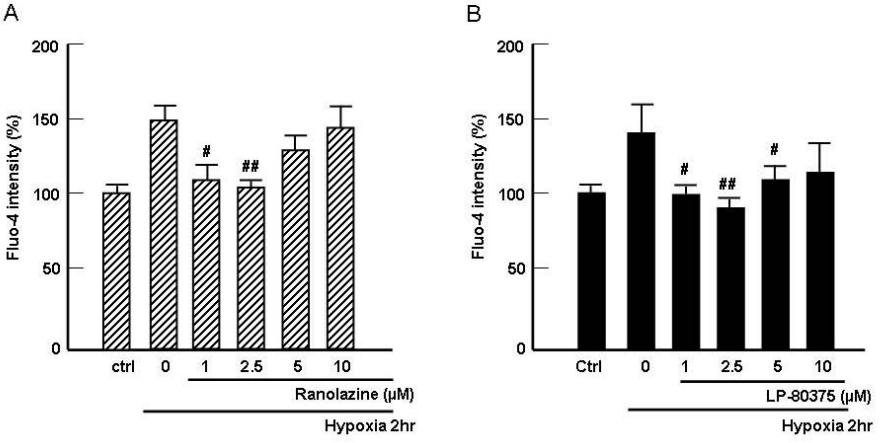


Figure 12. Effects of LP-80375 and ranolazine on intracellular Ca^{2+} concentration. Confocal fluorescent images of neonatal rat cardiomyocytes were obtained by loading with fluo-4 AM. The cells with ranolazine and LP-80375 were incubated in an anaerobic chamber for 2 hr. The mean±SE of eight independent experiments is reported (##p<0.001 vs hypoxia and #p<0.05 vs hypoxia).

2.3. Regulations of LP-80375 and ranolazine on the Ca^{2+} channel expression

It has investigated the alteration of Ca^{2+} handling proteins in hypoxic cardiomyocytes by RT-PCR. The expression level of SERCA2a was decreased in hypoxic cardiomyocytes, which was significantly rescued by LP-80375 treatment (56±2.1%). Compared with LP-80375 and ranolazine, expression level was not increased by ranolazine treatment. In addition, the expression levels of other calcium regulating proteins such as, CREM and NCX were significantly increased in cardiomyocytes by hypoxic stress. LP-80375 and ranolazine decreased expression level of the CREM (22±0.8%, 11±1.8%) and NCX (20±1.2%, 5±1.2%) compared with hypoxic cardiomyocytes.

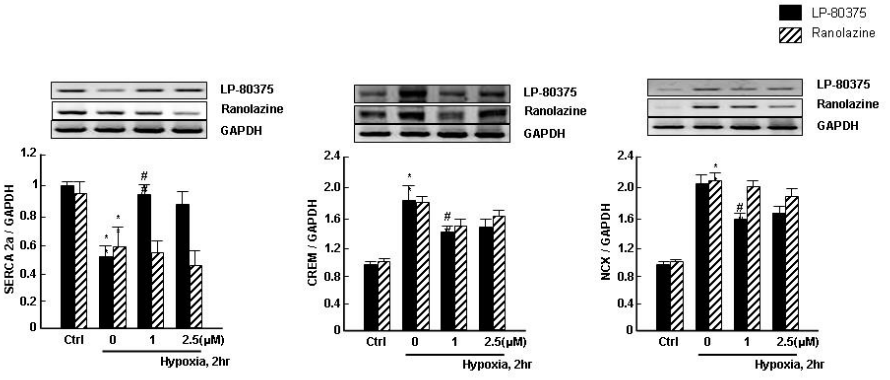


Figure 13. Effects LP-80375 and ranolazine on Ca^{2+} regulating proteins. Expression levels of the SERCA2a, CREM and NCX were estimated in cardiomyocytes subjected to Hypoxia with or without ranolazine, LP-80375 and analyzed(***p*<0.001 vs control and ##*p*<0.001 vs hypoxia).

2.4. Effects of LP-80375 and ranolazine on the expression of heat shock protein 70 (HSP70)

Since Hsp70 mediated cardiac protection from ischemic injury and restored Ca^{2+} homeostasis,[21] we examined the effect of LP-80375 and ranolazine on hsp70 induction in hypoxic cardiomyocytes. Although the expression of Hsp70 was increased by 2 hr hypoxia as shown in Fig. 14, Hsp70 was increased by LP-80375 treatment with more than that of ranolazine. The induction of Hsp70 was observed at 1-10 μM concentrations of two drugs and the highest induction was caused by LP-80375 1 μM, ranolazine 2.5 μM treatment.

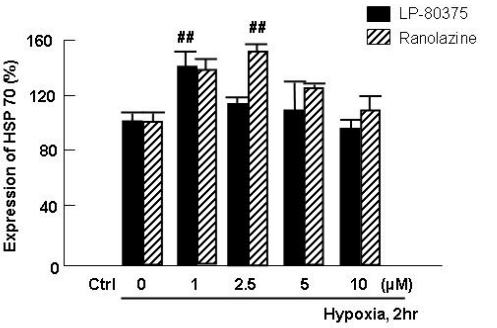


Figure 14. Effects of LP-80375 and ranolazine on expression of hsp70. Cardiomyocytes were subjected to hypoxia with or without LP-80375 and ranolazine for 2 hr. Hsp70 was treated during hypoxia and was detected by immunoblotting (##p<0.001 vs hypoxia).

2.5. Effects of LP-80375 on the heart function

Heart function was estimated by echocardiographic analysis of the rat heart one week after ischemia and the administration of therapeutics, LP-80375 only. There were three groups (n=5/group): control, MI, MI+LP-80375. The results from the echocardiographic examinations were compared between the control group and experimental animal groups. LV function and remodeling indices are summarized in Table 2. LV ejection fraction and LV fractional shortening were improved by treatment with LP-80375 (3 mg/kg) in the MI+LP-80375 group relative to the MI group (Fig. 15).

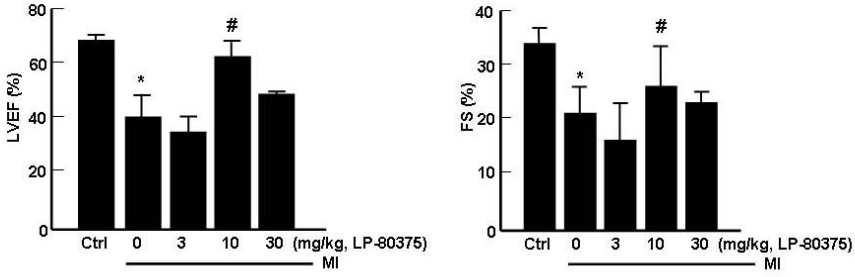


Figure 15. Two-dimensional-echocardiography of left ventricle. Each value is the mean±SE of the five independent experiments (***p*<0.001 vs control and ##*p*<0.001 vs MI).

Table 2. Echocardiography.

Variables	Control	MI	MI+375
HR, bpm	192.67±8.00	189.56±10.77	212±17.00
LVIDd, mm	7.33±0.00	10.33±1.34†	8.50±0.50
LVIDs, mm	4.44±0.23	8.44±1.56†	6.00±1.00
IVSd, mm	1±0.00	0.67±0.33	1.00±0.00
IVSs, mm	2.00±0.33	1.33±0.67	1.50±0.50
LVPWd, mm	1.56±0.11	1.11±0.22	1.50±0.50
LVPWs, mm	2.22±0.11	1.56±0.44	2.50±0.50
FS, %	39.22±1.45	15.45±12.55†	26.50±6.50∬
LVEF, %	75.00±1.67	35.22±24.45†	61.00±6.00∫

Each value is given as mean±SE. LVIDd= left ventricular internal diameter in diastole, LVIDs= left ventricular internal diameter in systole, IVSd= interaventricular septum in diastole, IVSs= intera ventricular septum in systole, LVPWd= left ventricular posterior wall in diastole, LVPWs= left ventricular posterior wall in systole, FS=fractional shortening, LVEF=left ventricular ejection fraction (†*p*<0.01 vs Control, ∫*p*<0.01 vs MI, ∬ *p*<0.05 vs MI).

IV. DISCUSSION

In the present study, it was 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 hr, phosphorylation fell after ranolazine treatment, to a level lower than that shown after exposure to NE (Fig. 2). Mann and colleagues have suggested that NE exerts a direct toxic effect on cardiomyocytes in vitro.[48] β-adrenoceptor apoptotic effects were associated with increases in intracellular Ca^{2+} and Ca^{2+} calmodulin-dependent protein kinase II (CaMKII) activity.[49,32] CaMKII is the

major cardiac isoform. Also, CaMKII expression was increased by β-adrenoceptor stimulation.[50] 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. 3).

Stefan and co-workers showed that CaMKII regulated Na^+ channel function in cardiomyocytes,[51] most likely by association with and phosphorylation of Na^+ channels. Moreover, phosphorylation of Na^+ channels enhanced intracellular Na^+ overload.[52] 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. 4). 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.[32] 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.[52] 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. 5). Ca^{2+} ions play an important role in normal cardiac function, 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.[53] 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. 6). 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.[30] Decreased expression of the SERCA2a[54] and PLB genes[45] 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.[54] We showed that NCX and RyR2 expression levels were elevated in NE-induced cardiomyocytes and that ranolazine blocked these changes (Fig. 7A). The expression levels of SERCA2a

and PLB were reduced in NE-induced cardiomyocytes but were enhanced by treatment with ranolazine (Fig. 7B).

Many studies have shown that NE induces apoptosis in vitro and in vivo.[45] NE can induce ROS generation and apoptosis of cardiomyocytes by activation of the β-adrenergic pathway.[55] Regardless of the underlying mechanisms, an enhanced ßadrenoceptor stimulation may significantly contribute to chronic diseases, such as hypertension and related conditions, congestive heart failure, sudden cardiac death, insulinresistance syndrome, and obesity.[37] A significant increase in fluorescence intensity was detected in cells treated with NE for 24 hr, 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. 8).

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 down regulation of Bcl-2 protein synthesis and activation of Bax. NE-induced apoptosis was detected by Annexin V/PI staining (Fig. 9). 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. 10A). 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. 10B). 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.

It was showed that maternal administration of 2 hr of hypoxic condition caused apoptotic cell death in rat heart in vivo in a dose-dependent manner.[56] However, the effect of hypoxia may be more complex because hypoxia may have direct effects on the cardiovascular system and may cause cardiac ischemia. Although it cannot be excluded that other factors may be involved in maternal hypoxic stress mediated heart apoptosis, ischemic condition induced calcium overload level in cardiomyocytes in this study. Given the finding that hypoxia induced apoptosis in cultured neonatal rat cardiomyocytes; it is likely that there is a connection between myocardial hypoxia and cardiomyocyte apoptosis in vitro in the present study. Ranolazine is a brightly anti-anginal drug for cardiac therapy.[57] It is a racemic mixture composed with aromatic and pyridine. A lactam ring is part of the structure of several antibiotic families.[56] for example, penicillins, cephalosporins, and monobactams. Pyridine is a simple aromatic heterocyclic organic compound with the chemical formula C_5H_5N used

as a precursor to pharmaceuticals.

It was observed that the effects of lactam ring and pyridine combined drugs on cell survival are higher than ranolazine. And, it was demonstrated that new lactam pyridine derivatives, LP-80375 had an effect as a new anti-ischemia drug. As shown in Fig. 11, the hypoxic cell death was reduced in cardiomyocytes treated with LP-80375 and ranolazine with dose-dependent manner. During hypoxia, the increase in intracellular Ca^{2+} concentration observed in various cells including cardiomyocytes and the increment was caused by activation of several calcium channels and pumps.[6] To address the roles of LP-80375 and ranolazine about Ca^{2+} homeostasis in hypoxic conditions, intracellular Ca^{2+} level was estimated in hypoxic cardiomyocytes. Therefore, intracellular dysregulation of Ca^{2+} homeostasis is proposed as the mechanism of cell injury induced by NE treatment. The Ca^{2+} entry across the membrane through L-type Ca^{2+} channels is balanced by an efflux of Ca^{2+} from the cell via NCX.[58] As shown in Fig. 12, the intracellular Ca^{2+} was increased about 1.5-fold in hypoxia only, which was significantly decreased by treatment with similar concentrations of LP-80375 and ranolazine. It was also examined the expression

levels of calcium-related proteins in cardiomyocytes (Fig. 13). It was observed that the expression levels of Ca^{2+} regulating proteins include NCX and CREM are altered in hypoxic cardiomyocytes, but those altered levels were restored to normal levels in LP-80375 treated cardiomyocytes. In fact, the Ca^{2+} regulating proteins include NCX and CREM plays a major role in Ca^{2+} homeostasis and contributes to abnormal intracellular Ca^{2+} handling in a failing heart.[8] Na^{+}/Ca^{2+} exchangers (NCXs) and plasma membrane Ca^{2+} pumps (PMCAs) are crucial for intracellular Ca^{2+} homeostasis and Ca^{2+} signaling. Elevated $[Ca^{2+}]_i$ is a hallmark of hypoxic disease and stroke. all NCXs and PMCAs were significantly regulated at the RNA(NCX) and protein (PMCA) level.[59] Entry of Ca^{2+} triggers further release the Ca^{2+} via ryanodine receptor (RyR) in the SR, leading to a sudden increase in intracellular Ca^{2+} concentration.[10] The NCX activity is also increased after preconditioning as indicated by a shorter decay time of the intracellular Ca^{2+} concentration, and a greater activity determined in the Ca^{2+} flux study. Compared with LP-80375 and ranolazine, expression level was not increased by ranolazine treatment. In addition, the expression levels of other calcium regulating proteins such as CREM and NCX were significantly

increased in cardiomyocytes by hypoxic stress.

Heat shock proteins (HSP) are a family of proteins induced by an increase in temperature as well as other environmental stresses and are well known to play a role in protein folding, translocation, and the assembly of intracellular protein, which may protect against various environmental challenges.[57] Hsp70 (70 kDa family of HSP) is one of the most extensive studied groups. Some studies have shown that over-expression of Hsp70 allows protection against simulated ischemia and metabolic stress.[58] In particular, up-regulation of the inducible Hsp70 ameliorated cardiomyocyte function in the presence of calcium overload. Despite this evidence, the exact mechanism of HSP-mediated myocardial protection remains unknown.[60,61] In normal conditions, Hsp70 family members function as molecular chaperones by assisting in folding and assembly of newly synthesized proteins and by transporting these proteins to various organelles.[62] Upon stress such as heat, which induces cell death, the inducible form Hsp70 is activated and protects the cell from apoptosis.[61] Although the expression of Hsp70 was increased in cardiomyocytes by hypoxia as shown in Fig. 5, Hsp70 was induced in cardiomyocytes treated with 2.5 μM ranolazine and 1 μM

LP-80375.

Heart function was estimated by echocardiographic analysis in one week after ischemia and the administration of therapeutics, LP-80375 only. There were three groups (n=5/group): control, MI and MI+LP-80375. The results from the rat echocardiography were compared between control group and experimental animal groups. Cardiac function was directly assessed in this study, and echocardiographic studies have shown restored cardiac contractility after treatment with LP-80375 (Fig. 6, Table 2).

LP-80375 and ranolazine on hypoxic condition brought about the reduction of calcium overload in cardiomyocytes. It is postulated that therapy with lactam type pyridine derivatives could prove useful in attenuating cardiac remodeling and in reducing the development of calcium overload in myocardial infarction.

V. CONCLUSION

This study suggests that ranolazine is involved in β-adrenoceptor activation and prevents NE-induced apoptosis and lactam type pyridine derivatives could be useful in attenuating cardiac remodeling and in reducing the development of calcium overload in myocardial infarction. Ranolazine also reduced intracellular Ca^{2+} and Na^{+} levels by regulating CaMKII expression after NE stimulation and affected Ca^{2+} channel-related gene expression. Ranolazine may thus act on the β-adrenoceptor signaling pathway to prevent NE-mediated cardiomyocytes apoptosis during heart failure. Echocardiographic studies have shown restored cardiac contractility in diabetic model after treatment with LP-80375. LP-80375 compared with ranolazine was expected as a new anti-ischemia drug for the prevention of ischemia -mediated cardiomyocytes apoptosis.

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Abstract (in Korean)

신규 락탐계 유도체에 의한 허혈성 심근손상 보호

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김 경 은

급성 심근 허혈의 증대(AMI)는 심근 세포의 사멸을 초래한다. 심근세포내의 칼슘항상성은 sarcolemmal와 SR 막에 있는 regulatory proteins 에 의해 통제되는 것으로 알려져 있다. Ranolazine은 칼슘과부하가 유도되어 생기는 지속적인 나트륨 유동을 특이적으로 저해하는 것으로 알려져 항 협심증 치료제로 대두되고 있지만, Ranolazine의 지속적인 나트륨 유동저해 메커니즘이 명백하게 밝혀지진 않았다. 본 연구에서는 베타 아드레날린 수용체를 자극하는 노르에피네프린을 통해 지속적인 나트륨 흐름, 칼슘 과부하, 세포사멸이 유도된 심근세포에서 Ranolazine의 베타 아드레날린

수용체 길항제 활성을 조사하고, Ranolazine과 같은 방어적인 기능이 있는 치료 효과를 가진 lactam pyridine 유도체인 LP-80375을 비교하여 작용기전을 알아보고자 하였다. Ranolazine과 LP-80375는 허혈/재관류된 심장에서 나트륨 의존적인 세포내 칼슘 과부하를 감소시키는 나트륨 흐름을 저해하였다. Ranolazine은 노르에피네프린에 의해 유도되어 증가된 세포 내 Ca^{2+}과 Na^{+} 농도를 각각 40±3%, 16.8±0.5% 감소 시켰으며, 베타 아드레날린 수용체 신호기전에서 Na^{+} 채널을 조절하는 것으로 알려진 CaMKII (56.8±2%) (Ca^{2+}/calmodulin dependent protein kinaseII)와 칼슘 관련인자 (L-Type Ca^{2+} channel (48±3.5%), phspholamban (45±2%), Na^{+}-Ca^{2+} exchanger (21±0.5%), ryanodine receptor2 (47±1.5%), and SERCA2a (36±0.5%) 의 노르에피네프린에 ranolazine 효과가 확인되었다. 마지막으로, annexin V/PI staining을 통하여 apoptosis를 분석해본 결과, Ranolazine을 처리한 군이 39.2±1.5% apoptosis가 감소하였으며, 전사멸인자인 Bax, 항사멸인자인 Bcl-2, 그리고 cytochrome C의 단백질발현 정도를 확인하여 세포사멸 억제효과를 증명하였다. 또, LP-80375가 처리되지 않은 대조군과 비교해봤을 때, 저산소 심근세포에서 각각 49±3.5%와 42±5.5% 만큼 세포내 칼슘 레벨과 Na^{+}-Ca^{2+} exchanger의 발현을 상당히 감소시켰다. 게다가, 근소포체 칼슘 ATPase 2a의 발현은 처리되지 않은 대조군과

비교해 봤을 때 LP-80375 처리된 저산소 심근세포에서 56±2.1%만큼 상당히 증가했다. 또, CREM (22±0.8%, 11±1.8%)과 NCX (20±1.2%, 5±1.2%) 에서 각각 저산소 대조군과 비교하였을 때 감소하였다. Hsp70은 용량 의존적 방법을 한 LP-80375 처리한 저산소 심근세포에서 관찰되었고 Hsp70의 최고치는 2.5μM LP-80375의 농도에서 유도되었다. 심초음파 분석은 LP-80375 주입된 허혈 심장에서 심장 기능이 상당히 증가했음을 보여주었다. 이상의 결과로 Ranolazine이 노르에피네프린 에 의해 유도된 베타 아드레날린 수용체 신호기전에 영향을 줌으로 써 심장기능 이상을 완화시킴을 증명하였으며, lactam pyridine 유도체인 LP-80375가 저산소증이 유도된 세포 사멸을 저해하는 효과를 가진 새로운 항허혈제로의 가능성을 보였다.

핵심되는 말: 심근세포, lactam pyridine 유도체, 허혈, LP-80375, 세포사멸

PUBLICATION LIST

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