Tissue Engineering and Regenerative Medicine, Vol. 6, No. 13, pp 1191-1197 (2009)

| Original Article |

Novel Lactam Type Pyridine Derivatives Improves Myocardium Dysfunction Derived from Ischemic Injury

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(Received: July. 27st, 2009; Accepted: September. 24th, 2009)

Abstract : The extended acute myocardial ischemia (AMI) results in cardiac myocytes death. In the present study, we show that lactam pyridine derivative, SK-D80375, have the effects on cell survival in hypoxic cardiomyocytes and might be used as an anti-ischemic drug. The lactam pyridine derivatives are inhibitors of the late sodium current, which decreases sodium-dependent intracellular calcium overload in ischemia/reperfusion-injured hearts. We found that pretreatment with SK-D80375 significantly decreased the level of intracellular Ca^{2+} and the expression level of the Na^{+}-Ca^{2+} exchanger by 39±2.5% and 19±0.5%, respectively in hypoxic cardiomyocytes compared to untreated controls. In addition, the expression level of sarcoplasmic reticulum Ca^{2+} ATPase 2a was significantly increased by 37±1.5% in SK-D80375-treated hypoxic cardiomyocytes compared to untreated controls. The induction of Hsp70 was observed in SK-D80375-treated hypoxic cardiomyocytes with dose-dependent manner and the highest level of Hsp70 was induced at the concentration of 2.5 μM SK-D80375. The echocardiographic analysis showed that heart function was significantly improved in SK-D80375-injected ischemic hearts. These results demonstrate that lactam pyridine derivative, SK-D80375, have beneficial effects on hypoxia-induced cell death, therefore, might be used as a novel anti-ischemia drug.

Key words: *cardiomyocyte, lactam pyridine derivative, ischemia, apoptosis*

1. 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 Ca^{2+} pump including NCX, PMCA4 and CREM 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 $[Ca^{2+}]_i$ and $[Ca^{2+}]_m$ occur and attenuation of $[Ca^{2+}]_m$, but not $[Ca^{2+}]_i$, 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

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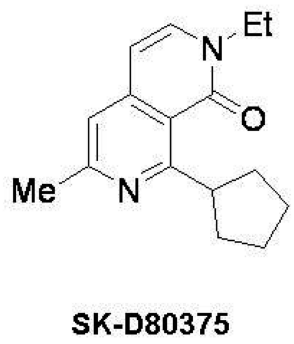

Figure 1. Chemical structure of a lactam pyridine derivative SK-D80375.

shown to reverse the sustained rise in systolic and diastolic calcium caused.[15-17] Moreover, the special mechanism is to inhibit late I_{na} thus preventing sodium overload of the cell. In fact, ranolazine, currently used lactam pyridine drug for treatment of anti-ischemia, prevents reverse mode sodium-calcium exchange (NCX) and diastolic accumulation of calcium possibly resulting in improved diastolic tone and improved coronary blood flow. And, it has been shown to decrease post-ischemic contracture in rabbit isolated perfuse hearts subjected to ischemia and reperfusion.[18-21]

We hypothesized that novel lactam pyridine derivatives (Fig 1) induced the HSP70 reducing the Ca^{2+} overload and finally may cause the myocardial cell survival as an effective cardioprotective agent against severe hypoxia. We confirmed that the effect of new anti-ischemia drug is lactam pyridine derivatives on the cell survival effect.

2. Materials and Methods

2.1 Isolation and Culture of Rat Cardiomyocytes

Cardiomyocytes were prepared from Sprague-Dawley neonatal rat hearts. To deplete red blood cells, isolated heart tissues were washed with Dulbecco's phosphate-buffered saline solution (pH 7.4, Invitrogen). Hearts were minced with micro-dissecting scissors to 0.5 mm^3 and treated with 4 ml of collagenase II (1.4 mg/ml 270 units/mg, Invitrogen) for 5 minutes in a 37°C humidified chamber. The supernatant was removed and washed with 10% FBS DMEM. Cell pellets were obtained by centrifugation, and resuspended in an equal volume of fresh medium containing 10% FBS. Remaining tissue was treated with fresh collagenase II solution for an additional 5 minutes. The incubation procedure was repeated until tissue was totally digested. The resulting supernatant was centrifuged at 2000 rpm for 2 minutes at room temperature. The cell pellet was resuspended in 5 ml of cell culture medium and plated for 2 hours at 37°C in a 5% CO_2 incubator. Non-adherent cells were identified as cardiomyocytes. Unattached cardiomyocytes were replated on 100 mm culture dish (5×10^5 cell/ml) and incubated in a-MEM supplemented with 10% FBS. Cells were then cultured in a CO_2 incubator at 37°C To reduce fibroblast contamination, α-MEM with 0.1 mM 5-bromo-2'-deoxyuridine (Brd-U) (Sigma) was used.[22-23]

2.2 Measurement of Cytosolic Free Ca^{2+}

The measurement of cytosolic free Ca^{2+}concentration was estimated by confocal microscopy analysis. Neonatal rat cardiomyocytes were plated on a four well slide chamber coated with 1.5% gelatin for 1 day in α-MEM containing 10% fetal bovine serum (Gibco BRL) and 0.1 μM BrdU (Sigma). After incubation, 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 g/L glucose, 0.05 g/L NaH_2PO_4, and 1.0 g/L $NaHCO_3$. Cells were then loaded with 10 μM acetoxymethyl ester fluo-4 (Fluo-4 AM, Molecular Probe) for 20 minutes, at 37°C, in the dark. Fluorescence images were collected using confocal microscope excited by 488 nm argon laser and emitted light was collected through a 510-560 nm band-pass filter. The relative intracellular Ca^{2+} concentration was determined by measuring fluorescent intensity.

2.3 MTT Assay

NRVCMs were plated in triplicate wells of 96 well plates at a density of 5×10^4 cells per well. 90% confluent NRVCMs were fed with 10% FBS DMEM. The NRVCMs were placed in the hypoxia chamber and washed twice by degassed serum-free DMEM. After degassed fresh serum-free DMEM were exchanged, cells incubated under hypoxia. Time dependently incubation was performed, the incubation period, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (Sigma, USA) was added to each well to a final concentration of 0.5 mg/mL and was incubated at 37°C for 2 hours 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.

2.4 RT-PCR Analysis

Total RNA was prepared using the Ultraspect™-II RNA system (Biotecx Laboratories Inc.) and single-stranded cDNA was synthesized from isolated total RNA by Avian myeloblastosis virus (AMV) reverse transcriptase. A 20 μl reverse transcription reaction mixture containing 1 μg of total RNA, 1X reverse transcription buffer (10 mM Tris–HCl, pH

Table 1. PCR primers used in this study.

Primer	sequence
NCX	TGTCTGCGATTGCTTGTCTC TCACTCATCCACCAGACG
PMCA4	AGCAGTTGCGTCAGTCAGAA GCTTTGTAGAGGGCTGTTGG
CREM	CCGTATGACCATGGAAACAG CAGGTCCAAGTCAAACACAG
SERCA	TCCATCTGCCTGTCCAT GCGGTTACTCCAGTATTG
GAPDH	CTCCCAACGTGTCTGTTGTG TGAGCTTGACAAAGTGGTCG

9.0, 50 mM KCl, 0.1% Triton X-100), 1 mM deoxynucleoside triphosphates (dNTPs), 0.5 unit of RNase inhibitor, 0.5 μg of oligo(dT)15, and 15 units of AMV reverse transcriptase were incubated at 42°C for 15 minutes, heated at 99°C for 5 minutes, and then incubated at 4°C for 5 minutes. PCRs were performed for 30 cycles with 3' and 5' primers shown in Table 1. GADPH was used as an internal standard. The amplification product signal intensity was normalized to its respective GADPH signal intensity.

2.5 Immunoblot Analysis

Cells were washed once in PBS and lysed in buffer (Cell Signaling Technology) containing 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM Na_2-EDTA, 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 phenylmethylsulfonyl fluoride. Protein concentrations were determined using the Bradford Protein Assay Kit (Bio-Rad). Proteins were separated in a 12% sodium dodecyl sulfate-polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (Millipore). After membrane blocking with Tris-buffered saline-Tween 20 (TBS-T, 0.1% Tween 20) containing 5% nonfat dried milk for 1 hour at room temperature, the membrane was washed twice with TBS-T and incubated with primary antibody for 1 hour at room temperature or overnight at 4°C. The membrane was washed three times with TBS-T for 10 minutes and incubated for 1 hour atroom temperature with horseradish peroxidase-conjugated secondary antibodies. After extensive washing, bands were detected by enhanced chemiluminescence reagent (Santa Cruz Biotechnology). Band intensities were quantified using the Photo-Image System (Molecular Dynamics).

2.6 Myocardial Infarction Induction and SK-D80375 Injection

All animal experimental procedures were approved by the Committee for Care and Use of Laboratory Animals, Yonsei University College of Medicine, and performed in accordance with Guidelines and Regulations for Animal Care. Myocardial infarction was produced in male Sprague-Dawley rats (200±30 g) by left anterior descending coronary artery surgical occlusion, according to previously described procedures. Briefly, after anesthesia with ketamine (10 mg/kg) and xylazine (5 mg/kg), hearts were exteriorized by opening the chest at the third and fourth ribs. After 1 hour of occlusion, ischemic heart was reperfused. SK-D80375 was injected by intra-peritoneally at everyday.

2.7 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.

2.8 Statistical Analysis

Data are expressed as means±SE. Statistical analysis of two

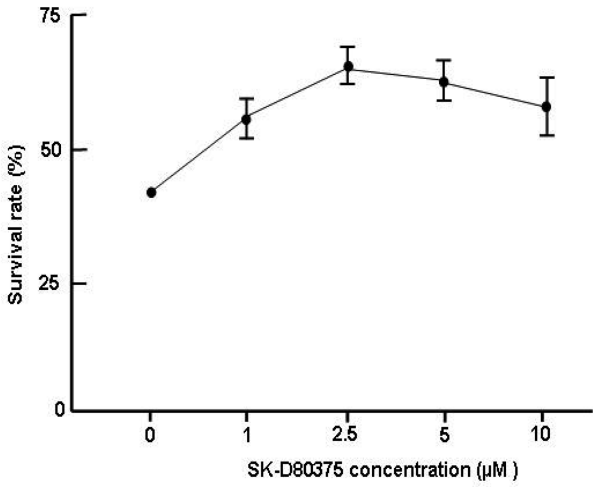

Figure 2. Measurement of cardiomyocyte viability during hypoxia. Cardiomyocytes were incubated in the absence or presence of SK-D80375 under hypoxia for 24 hr. 1-10 μM SK-D80375 were simultaneously treated with hypoxia (less than 1% O_2). Cell survival rate was detected by MTT assay. Each bar came from six wells of 96-well plate and represented the mean±SE.

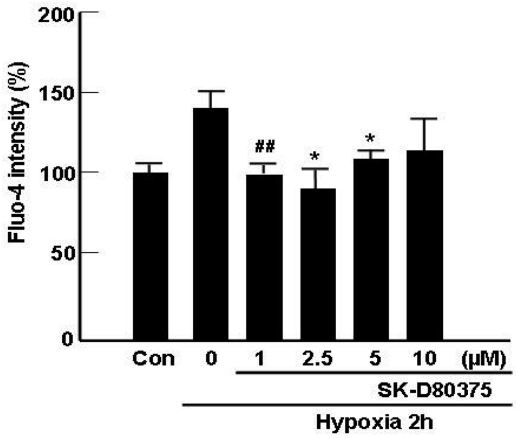

Figure 3. Effects of SK-D80375 on intracellular Ca^{2+} concentration. Confocal fluorescent images of neonatal rat cardiomyocytes were obtained by loading with fluo-4 AM. The cells with SK-D80375 were incubated in an anaerobic chamber for 24 hr. The mean±SE of eight independent experiments is reported (##p<0.001 vs hypoxia and *p<0.05 vs hypoxia).

groups were estimated by Student′s t-test. Across group analysis was performed with one-way ANOVA, using the Bonferroni test. A p <0.05 was considered significant.

3. Results

3.1 Effect of SK-D80375 on Cell Survival in Hypoxic Cardiomyocytes

To determine the effects of SK-D80375 on survival of cardiomyocytes under hypoxic conditions, we evaluated the survival rate of hypoxic cardiomyocytes. The structure of SK-D80375 is shown in Fig 1. As shown in Fig 2, cell survival was significantly increased in SK-D80375-treated hypoxic cardiomyocytes compared to untreated controls. In particular, 2.5-5 μM concentration of SK-D80375 have shown the highest effect on cell survival.

3.2 Effect of SK-D80375 on Cytosolic Ca^{2+} Overload in Hypoxia

Altered Ca^{2+} homeosasis play important roles in hypoxia-induced cardiomyocytes injury. To know whether SK-D80375 treatment decreased intracellular Ca^{2+} overload in hypoxic cardiomyocytes, the intracellular Ca^{2+} level was examined using the fluo-4. As shown in Fig 3, the intracellular Ca^{2+} during hypoxia was increased about 1.5-fold at 2 hours after hypoxia, which was significantly decreased by treatment with 2.5 μM SK-D80375 (39±2.5%).

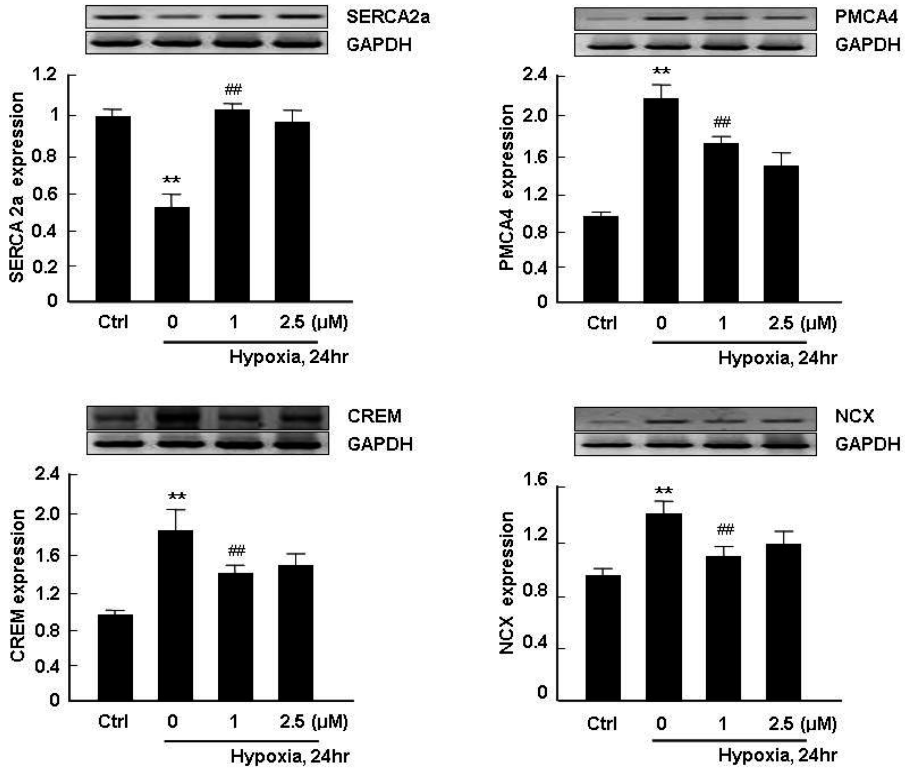

Figure 4. Effects and SK-D80375 on Ca^{2+} regulating proteins. Expression levels of the SERCA2a, PMCA4, CREM and NCX were estimated in cardiomyocytes subjected to Hypoxia with or without SK-D80375 and analyzed (**p<0.001 vs control and ##p<0.001 vs hypoxia).

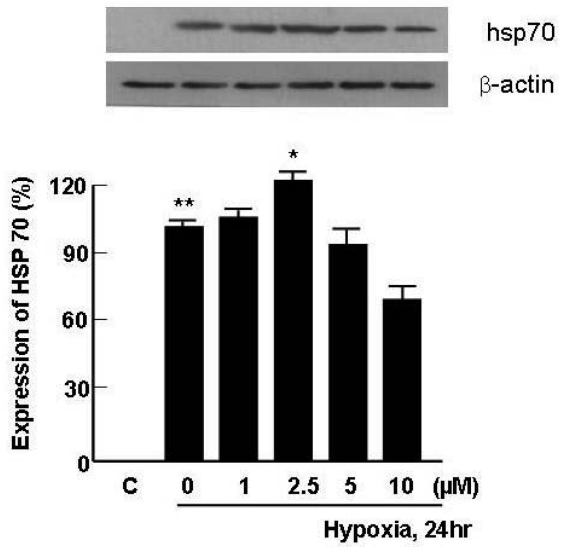

Figure 5. Effects of SK-D80375 on expression of hsp70. Cardiomyocytes were subjected to hypoxia with or without SK-D80375 for 24 hr. HSP70 was treated during hypoxia and was detected by immunoblotting (**p<0.001 vs control and ##p<0.001 vs hypoxia).

3.3 SK-D80375 Regulates the Ca^{2+} Channel Expression

Since various calcium handling proteins are associated with the intracellular Ca^{2+} levels in cardiomyocytes, we investigated the alteration of those proteins in hypoxic cardiomyocytes by RT-PCR. The expression level of SERCA2a was decreased in hypoxic cardiomyocytes, which was significantly rescued by SK-D80375 treatment. In addition, the expression levels of other calcium regulating proteins such as, PMCA4, CREM, and NCX were significantly increased in cardiomyocytes by hypoxic stress. These alterations were also rescued by SK-D80375 (Fig 4). These result demonstrated that SK-D80375 protects apoptosis of cardiomyocytes through the maintaining Ca^{2+} homeostasis.

3.4 Effect of SK-D80375 on the Expression of Heat Shock Protein 70 ($H_{SP}70$)

Since $H_{SP}70$ mediated cardiac protection from ischemic injury and restored Ca^{2+} homeostasis,[21] we examined the effect of SK-D80375 on $H_{SP}70$ induction in hypoxic cardiomyocytes. Although the expression of $H_{SP}70$ was increased by 24 hours hypoxia as shown in Fig 5, $H_{SP}70$ was increased by SK-D80375 treatment with more than that of hypoxia only. The induction of $H_{SP}70$ was observed at 1-10 μM concentrations of SK-D80375 and the highest induction was caused by 2.5 μM treatment.

3.5 Effect of SK-D80375 on the Heart Function

Heart function was estimated by echocardiographic analysis of the rat heart one week after ischemia and the administration of therapeutics, SK-D80375. There were three groups (n=5/group): control, MI, MI+SK-D80375. 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 SK-D80375 (3 mg/kg) in the MI+SK-D80375 group relative to the MI group (Fig 6).

4. Discussion

In the previous study, we showed that maternal administration of 2 hours of hypoxic condition caused apoptotic cell death in rat heart *in vivo* in a dose-dependent manner.[24] 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, we have shown that ischemic condition induces calcium

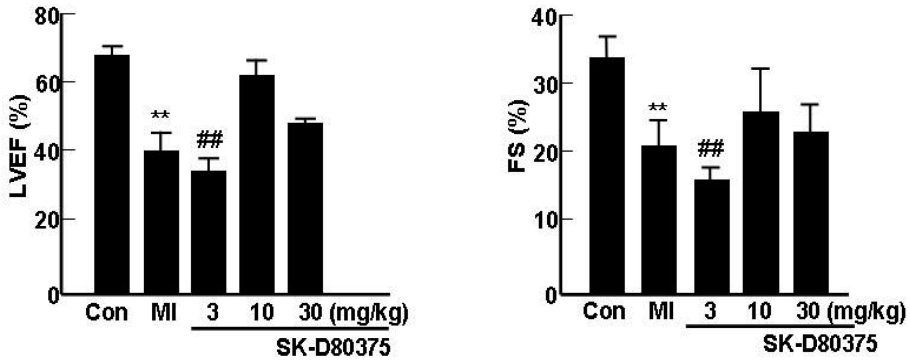

Figure 6. Two-dimensional-echocardiography of left ventricle. Each value is the mean±SE of the five independent experiments (**p<0.001 vs control and ##p<0.001 vs MI).

Table 2. Echocardiography.

Variables	Control (n=5)	MI (n=5)	MI-375 (n=5)
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	100±0.00
IVSs, mm	2.00±0.33	1.33±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‡	6.100±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).

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.[25] It is a racemic mixture composed with aromatic and pyridine. A lactam ring is part of the structure of several antibiotic families.[24] 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.

We observed that the effects of lactam ring and pyridine combined drugs on cell survival are higher than ranolazine. And, we demonstrated that new lactam pyridine derivatives, SK-D80375 had an effect as a new anti-ischemia drugs. As shown in Fig 2, the hypoxic cell death was reduced in cardiomyocytes treated with SK-D80375 with dose-dependent manner. During hypoxia, intracellular Ca^{2+} caused by activation of several calcium channels and pumps is increased in various cells including cardiomyocytes.[6] To address the role of SK-D80375 about Ca^{2+} homeostasis in hypoxic conditions, intracellular Ca^{2+} level was estimated in hypoxic cardiomyocytes. As shown in Fig 3, the intracellular Ca^{2+} was increased about 1.5-fold in hypoxia only, which was significantly decreased by treatment with SK-D80375. We also examined the expression levels of calcium-related proteins in cardiomyocytes (Fig 4). We observed that the expression levels of Ca^{2+} regulating proteins include NCX, PMCA4 and CREM are altered in hypoxic cardiomyocytes , but those altered levels were restored to normal levels in SK-D80375 treated cardiomyocytes. In fact, the Ca^{2+} regulating proteins include NCX, PMCA4 and CREM plays a major role in Ca^{2+} homeostasis and contributes to abnormal intracellular Ca^{2+} handling in a failing heart.[8] Entry of Ca^{2+} triggers further release the Ca^{2+} via ryanodine receptor (RyR) in the SR, leading to a sudden increase in $[Ca^{2+}]_i$.[10] The NCX activity is also increased after preconditioning as indicated by a shorter decay time of the $[Ca^{2+}]$i, and a greater activity determined in the Ca^{2+} flux study.

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.[26] 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.[27] 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.[28-29] 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.[28] Upon stress such as heat, which induces cell death, the inducible form Hsp70 is activated and protects the cell from apoptosis.[29] Although the expression of Hsp70 was increased in cardiomyocytes by hypoxia as shown in Fig 5, Hsp70 was more induced in cardiomyocytes treated with 2.5 μM SK-D80375 than that of hypoxia only.

Heart function was estimated by echocardiographic analysis in one week after ischemia and the administration of therapeutics, SK-D80375. There were three groups (n=5/group): control, MI and MI+SK-D80375. 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 SK-D80375 (Fig 6, Table 2)

The SK-D80375 on hypoxic condition brought about the reduction of calcium overload in cardiomyocytes. It is postulated that therapy with SK-D80375 could prove useful in attenuating cardiac remodeling and in reducing the development of calcium overload in myocardial infarction.

5. Conclusion

This study suggests that SK-D80375 could be useful in attenuating cardiac remodeling and in reducing the

development of calcium overload in myocardial infarction. Echocardiographic studies have shown restored cardiac contractility in diabetic model after treatment with SK-D80375. SK-D80375 were expected as a new anti-ischemia drug for the prevention of ischemia -mediated cardiomyocyte apoptosis.

Acknowledgements: This research was supported by a grant of the Korea Health 21 R&D Project, Ministry of Health & Welfare, Republic of Korea A085136).

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