Diabetes mellitus mitigates cardioprotective effects of remifentanil preconditioning in ischemia-reperfused rat heart in association with anti-apoptotic pathways of survival

Jang-Eun Cho

Department of Medicine

The Graduate School, Yonsei University

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Directed by Professor Young-Lan Kwak

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This certifies that the Doctoral dissertation of Jang-Eun Cho is approved.

Thesis Supervisor : Young-Lan Kwak

Hyun-Chul Lee

Byung-Chul Chang

Yon-Hee Shim

Helen Ki Shin

The Graduate School
Yonsei University
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<ABSTRACT>

Diabetes mellitus mitigates cardioprotective effects of remifentanil preconditioning in ischemia-reperfused rat heart in association with anti-apoptotic pathways of survival

Cho Jang-Eun

Department of Medicine
The Graduate School, Yonsei University

(Directed by Professor Kwak Young-Lan)

Diabetes mellitus has been known to mitigate ischemic or pharmacologic preconditioning in ischemia-reperfusion (I/R) injuries. Remifentanil is a widely used opioid in cardiac anesthesia that possesses a cardioprotective effect against I/R. We evaluated whether diabetes affected remifentanil preconditioning induced cardioprotection in I/R rat hearts in view of anti-apoptotic pathways of survival and Ca^{2+} homeostasis. Streptozotocin-induced, diabetic rats and age-matched wild-type Sprague-Dawley rats were subjected to a left anterior descending coronary artery occlusion for 30 min followed by 1 h of reperfusion. Each diabetic and wild-type rat was randomly assigned to the sham, I/R only, or remifentanil preconditioning group. Myocardial infarct size, activities of ERK1/2, Bcl2, Bax and cytochrome c, and gene expression influencing Ca^{2+} homeostasis were assessed. Remifentanil preconditioning significantly reduced myocardial infarct size compared to I/R only in wild-type rats but not in diabetic rats. Remifentnil

preconditioning increased expression of ERK1/2 and anti-apoptotic protein Bcl-2 and decreased expression of pro-apoptotic proteins, Bax and cytochrome c, compared to I/R only in wild-type rats. In diabetic rat hearts, however, remifentanil preconditioning failed to recover the phosphorylation state of ERK1/2 and to repress apoptotic signaling. In addition, diabetes minimized remifentanil induced modulation of abnormal changes in sarcoplasmic reticulum (SR) genes and proteins in I/R rat hearts. In conclusion, diabetes mitigated remifentanil induced cardioprotection against I/R, which might be associated with reduced recovery of the activities of proteins involved in anti-apoptotic pathways including ERK1/2 and the abnormal expression of SR genes as a result of I/R in rat hearts.

Key words : diabetes mellitus, remifentanil, preconditioning, heart.

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I. INTRODUCTION

Ischemic- and pharmacologic preconditioning involving multiple salvage signal transduction pathways and anti-apoptotic pathways of survival have been proposed as important therapeutic targets for increasing tolerance to ischemia-reperfusion (I/R) injury.[1-5]

Diabetes mellitus (DM) is a commonly encountered co-morbid disease in cardiac surgical patients, that is associated with the pathogenesis as well as adverse prognosis in ischemic heart disease.[6-10] Indeed, DM has been known to attenuate certain anesthetic induced cardioprotective effects mediated by the mitochondrial adenosine triphosphate-sensitive potassium (K_{ATP}) channels and mitochondrial permeability transition pore.[11-12]

Remifentanil is an ultra-short-acting opioid that is gaining popularity in the practice of modern anesthesia, and has been shown to confer cardioprotection against I/R injury,[4, 13-14] sharing common mechanisms with other anesthetic

agents and via activation of protein kinase C (PKC) and opioid receptors.[15-18] Therefore, the possibility exists that DM could also affect the cardioprotective effects of remifentanil against I/R injury and so far, evidence is limited regarding the mechanisms of the effect of DM on opioid-induced cardioprotection.

The aim of the present study was to determine whether DM affected remifentanil-induced cardioprotective actions against I/R injury in terms of protein activity involved in anti-apoptotic pathways of survival including extracellular signal-regulated kinases (ERK) 1/2 in the rat heart. Since proteins involved in apoptosis significantly affect Ca^{++} homeostasis, we concomitantly evaluated the effects of remifentanil on protein and gene expression in the sarcoplasmic reticulum (SR).

II. MATERIALS AND METHODS

The animal experimental procedures used in this study were approved by the Committee for the Care and Use of Laboratory Animals at the Yonsei University College of Medicine and performed in accordance with the Committee's guidelines and regulations for animal care.

2.1 Animal preparation

Male Sprague-Dawley rats (250-300 g), including streptozotocin-induced DM and age-matched non-diabetic control rats, were used in this study. All animals were anesthetized with a single i.p. injection of pentobarbital (60 mg/kg). Anesthesia was maintained by repeated doses of pentobarbital (25 mg kg^{-1}) every 60-90 min. After tracheal intubation, rats were artificially ventilated (Harvard Apparatus 683, Holliston, MA) with 100% oxygen. A heparinized catheter was inserted into the right femoral artery for continuous monitoring of mean arterial pressure, and a lead-II electrocardiogram monitored heart rate *via* subcutaneous stainless steel electrodes. These were connected to a monitoring system (ML845 PowerLab with ML132; AD Instruments, Colorado Springs, CO). The left femoral vein was cannulated to infuse either remifentanil or saline. Rectal temperature was maintained at 38°C during the entire experiment using a heating pad connected to a rectal probe.

2.2 Induction of diabetes

DM was induced by an intraperitoneal injection of streptozotocin (60 mg/kg) dissolved in 0.1 M citrate buffer (pH 4.5).[19] Rats matched for age at the time of streptozotocin-administration were used as non-DM control animals. All animals were given unlimited food and water and were not supplemented with insulin or anti-hyperglycemic agents. Blood glucose was confirmed by glucometer (Accu-Check, Roche, Germany). Rats in the DM groups (5%) were

excluded from the study if blood glucose was less than 300 mg/dL after 3 weeks.[20]

2.3 Induction of ischemia and reperfusion

After performing a left thoracotomy and pericardiotomy, the hearts were exposed and a suture was passed around the left anterior descending (LAD) coronary artery by inserting a small curved prolene 6.0 needle into the margin of the pulmonary cone, exiting through the middle of a line linking the cone to the atrium. The suture ends were threaded through a small vinyl tube to prepare a snare. After surgical preparation, the rat was allowed to stabilize for 20 min. In all groups, the LAD coronary artery was occluded for 30 min by tightening the snare. Myocardial ischemia was confirmed by the appearance of a regional cyanosis on the epicardium distal to the snare and akinesia or bulging in this area. After 30 min of ischemia, the snare was released and reperfusion was allowed for a period of 1h. The thread passing around the LAD coronary artery was left in place. Hemodynamic parameters were measured at the following time points: 1) 10 min before ischemia (baseline), 2) at the end of ischemia (or no ischemia, T-I), and 3) at the end of 1h reperfusion (T-R).

2.4 Study groups

This study consisted of six series of experiments. Rats were randomly assigned to receive one of the six treatments. The groups were as follows: I/R only: rats that were not treated with remifentanil preconditioning in the non-diabetic (Con-I/R) and diabetic conditions (DM-I/R); Remifentanil-preconditioning: rats were subjected to infusion of remifentanil 20 min before 30 min of occlusion followed by 10 min washout in the non-diabetic (Con-Remi) and diabetic conditions (DM-Remi). Remifentanil was infused intravenously at a rate of 6 $\mu g \cdot kg^{-1}$ min^{-1}. ‘Sham: rats were treated similarly,

except that the coronary suture was not tied, and remifentanil was not administered in the non-diabetic (Con-Sham) and diabetic conditions (DM-Sham).

2.5 Infarct size measurement

At the end of the reperfusion period, the hearts were excised and immersed in 2% triphenyltetrazolium chloride (TTC) (Sigma Chemicals, ST. Louis, MO) stains for 20 min at 37°C. The infarct myocardium, which does not take up TTC stain when dehydrogenase enzymes are drained off, remained pale in color. The hearts were sliced and photographed, and infarct size was determined by dividing the total necrotic area of the left ventricle (LV) by the total LV area.[21] The boundaries of unstained areas were traced in a blinded fashion and quantified with NIH images, version 1.61.

2.6 Immunoblot analysis

At the end of the reperfusion period, tissue preparation was performed as previously described.[22] Tissue specimens were pulverized and dissolved in lysis buffer (Cell Signaling, Danvers, MA). The solution was vigorously homogenized with Pyrex Potter-Elvehjem Tissue Grinders (BLD science, Garner, NC) and then centrifuged at 12,000 x g for 10 min at 4°C, and supernatant was transferred to a new tube and stored at -70°C. Protein concentrations were determined using the Bradford protein assay kit (BioRad, San Diego, CA). Proteins were separated in a 12% SDS-polyacrylamide gel and transferred to PVDF membrane (Millipore Co, Billerica, MA). After blocking the membrane with Tris-buffered saline-Tween 20 (TBS-T, 0.1% Tween 20) containing 5% non-fat dried milk for 1 h at room temperature, membranes were washed twice with TBS-T and incubated with primary antibodies for 1 h at room temperature or for overnight at 4°C. The following primary antibodies

were used: rabbit anti- ERK1/2 (42 and 44 kDa), mouse anti-phospho ERK, mouse anti- B cell leukemia/lymphoma (Bcl)-2, mouse anti-cytochrome C, goat anti-rat SR Ca^{2+}-ATPase (SERCA2), mouse phospholamban (PLB), rabbit calsequestrin (CSQ), anti-ryanodine receptor (RyR), phosphorylated PLB at serine-16 (PLB-Ser), phosphorylated PLB at threonine-17 (PLB-Thr) (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti- Bcl-2-associated X protein (Bax) (Assay Designs, Ann Arbor, MI) and mouse anti-β actin antibodies (Sigma Chemicals, ST. Louis, MO). The membranes were washed three times with TBS-T for 10 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 (Santa Cruz Biotechnology, Santa Cruz, CA). β-actin gene was used as the standards for equality of the protein samples. The band intensities were quantified using NIH image, version 1.61. Each experiment was performed at least three times.

2.7 RT-PCR analysis

Tissue samples were vigorously homogenized with Pyrex Potter-Elvehjem Tissue Grinders (BLD science, Garner, NC) in TRI Reagent (Sigma Chemicals, ST. Louis, MO). Total RNA was prepared by the UltraspectTM-II RNA system (Biotecx Laboratories, Inc., Houston, TX) and single-stranded cDNA was then 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 9.0, 50 mM KCl, and 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 was incubated at 42°C for 15 min, heated to 99°C for 5 min, and then incubated at 0-5°C for 5 min. All primer pairs for DNA

sequencing of proteins related to calcium homeostasis were shown in Table 1. The PCR conditions were 94°C for 3 min, 94°C for 1 min, 41-49°C for 1 min and 72°C for 2 min, for a total of 35 cycles, with a final extension for 10 min at 72°C. GAPDH gene (primers 5'-accacagtccatgccatcac-3' and 5'-tccaccaccctgttgctgta-3', 450 bp) was used as the internal standard. The signal intensity of the amplification product was analyzed using the UVlband software (UVltec, Cambridge, UK).

To confirm functional changes of SR proteins related to alteration of mRNA expression, protein levels of SERCA2a, PLB, RyR, and CSQ were reexamined after RT-PCR.

Table 1. The nucleotide sequence of all primer pairs for proteins related to Ca^{2+} homeostasis

Genes	Primer sequence	Size (bp)
L-type Ca^{2+}-channel	Sense: 5′- TGTCACGGTTGGGTAGTGAA-3′	346
	Antisense: 5’-TTGAGGTGGAAGGGACTTTG-3’	364
NCX	Sense: 5′-TGTCTGCGATTGCTTGTCTC-3′	196
	Antisense: 5′-TCACTCATCTCCACCAGACG-3′	
SERCA2a	Sense: 5′-TCCATCTGCCTGTCCAT-3′	339
	Antisense: 5′-GCGGTTACTCCAGTATTG-3′	
PLB	Sense: 5′-GCTGAGCTCCCAGACTTCAC-3′	351
	Antisense: 5′-GCGACAGCTTGTCACAGAAG-3′	352
RyR2	Sense: 5′-CCAACATGCCAGACCCTACT-3′	
	Antisense: 5′-TTTCTCCATCCTCTCCCTCA-3′	
Calsequestrin	Sense: 5′-TCAAAGACCCACCCTACGTC-3′	
	Antisense: 5′-CCAGTCTTCCAGCTCCTCAG-3′	

2.8 Statistical analysis

Data are presented as mean ± S.E.M. of more than three separate experiments performed in triplicate. Where results of blots and RT-PCR are shown, a representative experiment is depicted. Data analysis was performed with Prism v3.0 – statistical software (GraphPad Software, San Diego, CA). Comparisons between the two groups were analyzed using student’s t test for

body and heart weight, and blood glucose levels. Comparisons between multiple groups were performed with two-way ANOVA with Bonferroni post-hoc test for multiple comparisons. Infarct sizes were analyzed between the groups using ANOVA with Student-Newman-Keula post-hoc test for multiple comparisons. Statistical significance was defined as $P < 0.05$.

III. RESULTS

3.1 Animal data

All streptozotocin-treated rats were diabetic with mean plasma glucose levels of 430 ± 10 mg/dL (P<0.05 *vs*. Control). Heart weight and body weight were lower in the DM group than in the Control group (P<0.05 *vs*. Control) (Table 2).

Table 2. Characteristics of control and diabetic rats

	Control	**Diabetes**
Body weight (g)	355 ± 12	210 ± 24[a]
Heart weight (mg)	840 ± 16	520 ± 31[a]
Heart weight/body weight (mg/g)	2.4 ± 0.1	2.5 ± 0.1
Blood glucose level (mg/dL)	115 ± 10	430 ± 10[a]

Comparison between 2 groups were analyzed using Student's t test. Values are presented as mean ± S.E.M.

[a] P < 0.05 vs control group, n=20.

3.2 Hemodynamic parameters and infarct size measurement

The heart rate and mean arterial pressure are summarized in Table 3. The mean arterial pressure significantly decreased after I/R compared to baseline values in all groups, except in the Con-I/R group. At the end of reperfusion, the mean arterial pressure was significantly lower than baseline value in the DM-Remi group. Other mean arterial pressure and heart rate values revealed no statistically significant differences in either inter- or intragroup comparisons.

Table 3. Hemodynamic parameters

	Baseline		Ischemia (30 min)		Reperfusion (1 h)	
	HR	**MAP**	**HR**	**MAP**	**HR**	**MAP**
Con-I/R	382 ± 28	139 ± 14	391 ± 47	101 ± 17	384 ± 21	119 ± 15
Con-Remi	376 ± 47	134 ± 17	367 ± 57	92 ± 15[a]	371 ± 55	104 ± 16
DM-I/R	312 ± 41	149 ± 10	302 ± 48	89 ± 19[a]	325 ± 44	116 ± 14
DM-Remi	309 ± 32	145 ± 16	311 ± 31	81 ± 22[a]	301 ± 45	97 ± 18[a]

Comparison between groups were analyzed using two-way ANOVA with Bonferroni post-hoc test. Data are means ± S.E.M. HR, heart rate (bmp); MAP, mean arterial pressure (mmHg). Con, control; DM, diabetes; Remi, remifentanil administrated during the preconditioning period; I/R, ischemia/reperfusion.

[a] $P < 0.05$ vs baseline, n=10

Myocardial infarct sizes of the four groups are shown in Figure 1. Mean infarct size of the LV in the Con-I/R group (45.2 ± 4.0%) and DM-I/R group (47.0 ± 4.0%) was similar. In the Con-Remi group, infarct size of the LV was significantly reduced to 18.7 ± 1.3% ($P<0.01$) compared to the Con-I/R group, while it was not reduced in the DM-Remi group (31.8 ± 5.7%), which was significantly greater compared to that of the Con-Remi group ($P<0.05$).

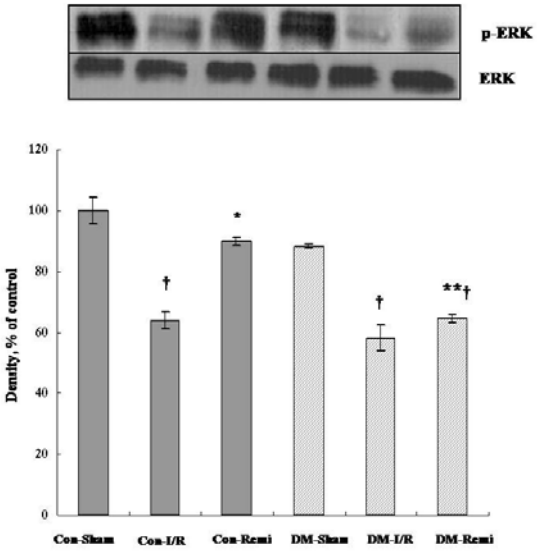


Fig. 1. Infarct size was analyzed with planar morphometry in 2,3,5-triphenyltetrazolium (TTC) stained sections and expressed as a ratio of the left ventricular area. *Closed bars*, data from the control rat hearts; *striped bars*, data from the diabetic rat hearts. Values are presented as mean ± S.E.M. **P* < 0.01, ***P* <0.05 compared to the Con-I/R and DM-I/R group, respectively; †*P* <0.05 Con-Remi vs. DM-Remi group.

3.3 Western blotting analysis on phosphorylation of ERK1/2 and apoptosis proteins

Phosphorylations of ERK1/2 were similar between the Con-Sham and DM-Sham groups. I/R significantly decreased phosphorylation of ERK1/2 in both the Con-I/R and DM-I/R groups. In the Con-Remi group, phosphorylations of ERK1/2 were significantly higher than in the Con-I/R group and were similar to those in the Con-Sham group. In the DM-Remi group, ERK1/2 phosphorylations increased compared to those in the DM-I/R group but were significantly lower than in the DM-Sham group (Fig 2).

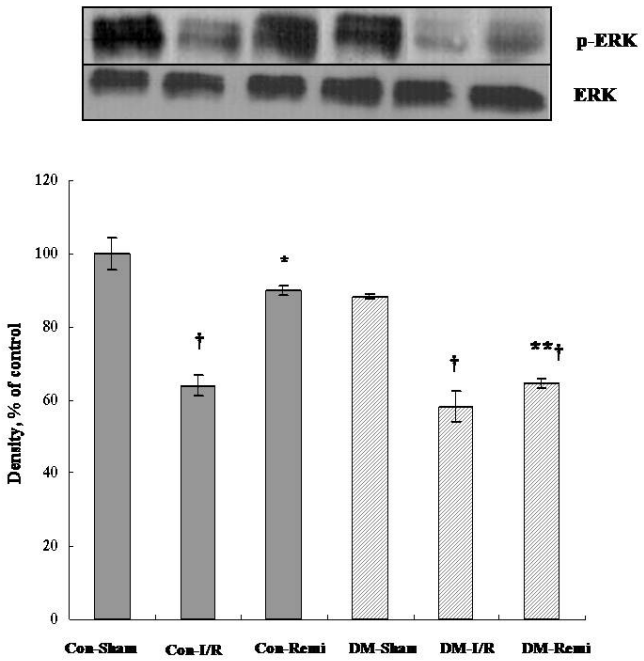


Fig. 2. Western blot analysis of ERK 1/2 and p-ERK1/2. Each signal was quantified by scanning densitometry. *Closed bars*, data from the control rat hearts; *striped bars*, data from the diabetic rat hearts. Values are presented as mean ± S.E.M. **P* <0.01, ***P* <0.05 compared to Con-I/R and DM-I/R groups, respectively; †*P* < 0.01 compared to Con-Sham and DM-Sham group respectively.

Levels of baseline Bax and cytochrome c were significantly higher in the DM-Sham group than in the Con-Sham group, although the level of Bcl-2 was similar between the groups. I/R increased expression of the pro-apoptotic proteins, Bax and cytochrome c, and decreased expression of the anti-apoptotic

protein, Bcl-2, in both the Con-I/R and DM-I/R groups. In the Con-Remi group, expression of Bax and cytochrome c decreased and expression of Bcl-2 increased significantly compared to the Con-I/R group and the levels were comparable to the Con-Sham group. In the DM-Remi group, Bcl-2 level increased compared to the DM-I/R group, but levels of Bax and cytochrome c did not change (Fig 3).

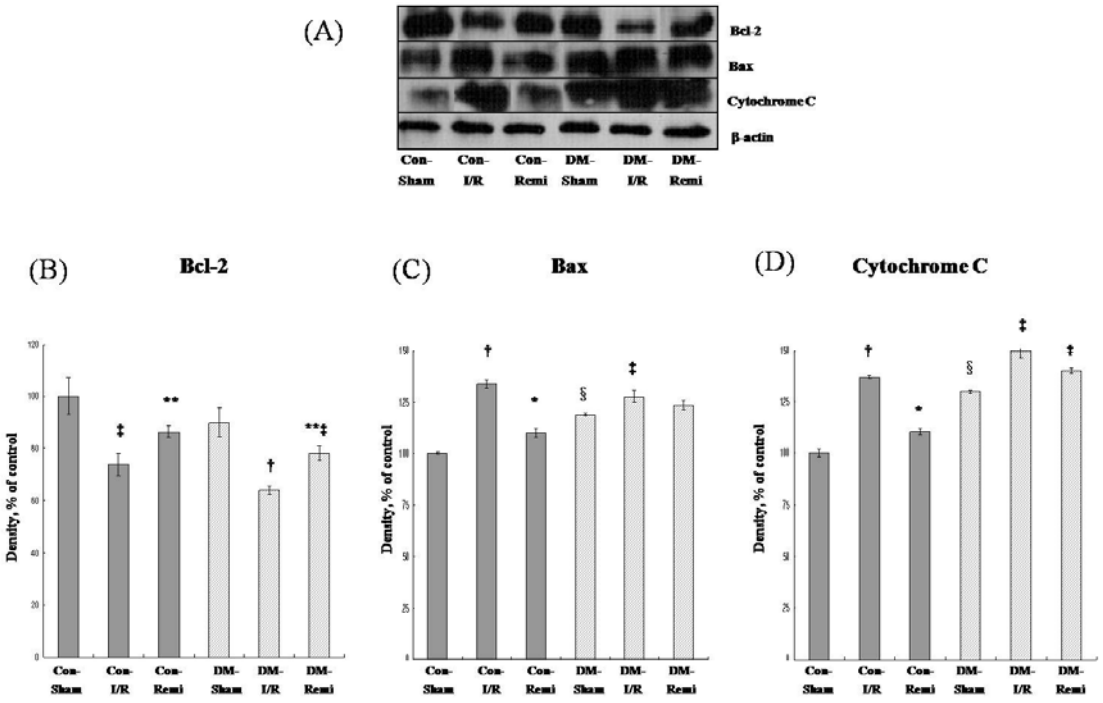
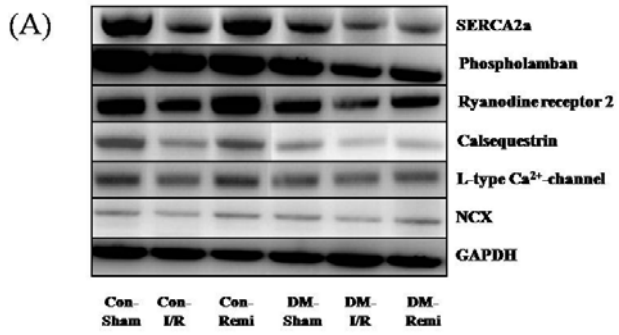


Fig. 3. Western blot analysis of apoptotic proteins. (A): Representative quantitative immunoblots. B-D: Histograms of Bcl-2 (B), Bax (C), and cytochrome c (D). Each signal was quantified by scanning densitometry. *Closed bars*, data from the control rat hearts; *striped bars*, data from the diabetic rat hearts. Values are presented as mean ± S.E.M. **P* <0.01, ***P* <0.05 compared to Con-I/R and DM-I/R groups, respectively; †*P* < 0.01, ‡*P* <0.05

compared to Con-Sham and DM-Sham groups, respectively; §P <0.05 Con-Sham vs. DM-Sham group.

3.4 Expression of genes and proteins influencing the calcium homeostasis

Gene expressions for SR proteins, except RyR 2, were lower in the DM-Sham group than in the Con-Sham group. In the Con-I/R and DM-I/R group, gene expressions for SR proteins, SERCA2a, PLB, RyR 2, and CSQ decreased compared to the Con-Sham and DM-Sham group, respectively. mRNA transcription levels for SR proteins were up-regulated in the Con-Remi group compared to the Con-I/R group, which were similar to those in the Con-Sham group. In the DM-Remi group, mRNA transcription levels for SR proteins were not up-regulated compared to the DM-I/R group, except for SERCA2a, which was significantly up-regulated. L-type Ca^{2+} channels and Na^{+}-Ca^{2+} exchanger (NCX) were unaltered by I/R and remifentanil preconditioning in both the Control and DM groups (Fig 4).



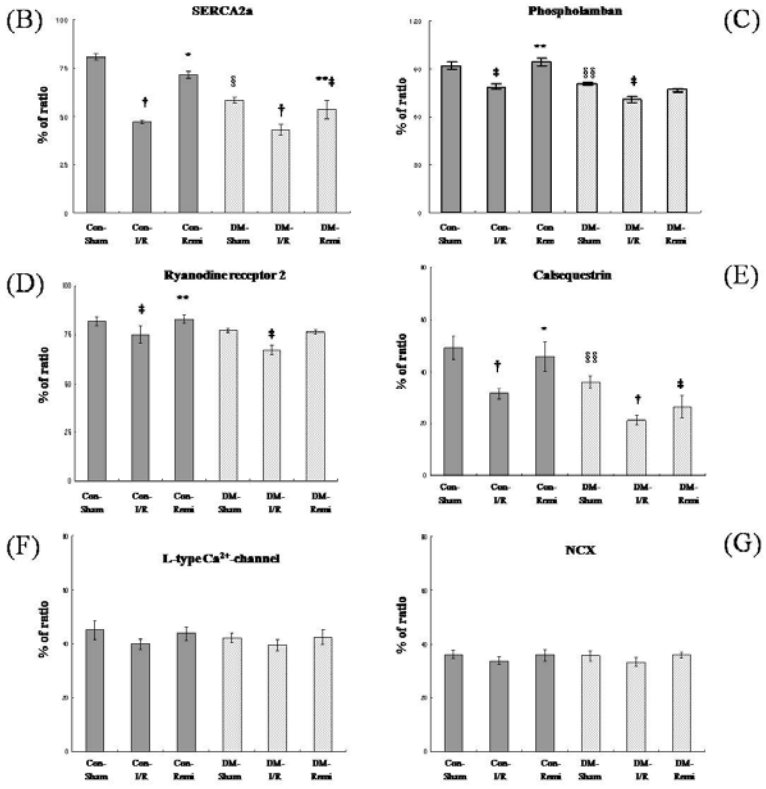


Fig. 4. Analysis of the gene expressions related to Ca^{2+} homeostasis. (A): Representative gel image photographs of mRNA levels. B-G: Histograms of SERCA2a (B), phospholamban (C), ryanodine receptor 2 (D), calsequestrin (E), L-type Ca^{2+}-channel (F), and NCX (G). GAPDH was used as an internal standard. *Closed bars*, data from the control rat hearts; *striped bars*, data from the diabetic rat hearts. Values are presented as mean ± S.E.M. **P* <0.01, ***P* <0.05 compared to the Con-I/R and DM-I/R groups, respectively; †*P* < 0.01, ‡*P* <0.05 compared to the Con-Sham and DM-Sham groups, respectively; §*P* <0.01, §§*P* <0.05 Con-Sham vs. DM-Sham group.

To confirm the functional changes of SR proteins related to alteration of mRNA expression, we examined SERCA2, PLB, RyR, and CSQ protein levels. Most SR proteins were lower in the DM-Sham group than in the Con-Sham group, and significantly decreased, except PLB, after I/R in both the Con-I/R and DM-I/R groups. Those protein levels increased in the Con-Remi group compared to the Con-I/R group, while the effect of remifentanil preconditioning was minimized in the DM-Remi group, except on SERCA2 (Fig 5). Although PLB protein contents were unaltered, they were lower in the DM-Sham group than in the Con-Sham group, and reduced by I/R in both the DM-I/R and Con-I/R groups when phosphorylated PLB proteins were normalized to total PLB. Remifentanil-treatment increased PLB proteins in the Con-Remi group, although it was lower than in the Con-Sham group. The effect of remifentanil preconditioning on PLB proteins was mitigated in the DM-Remi group (Fig. 6).

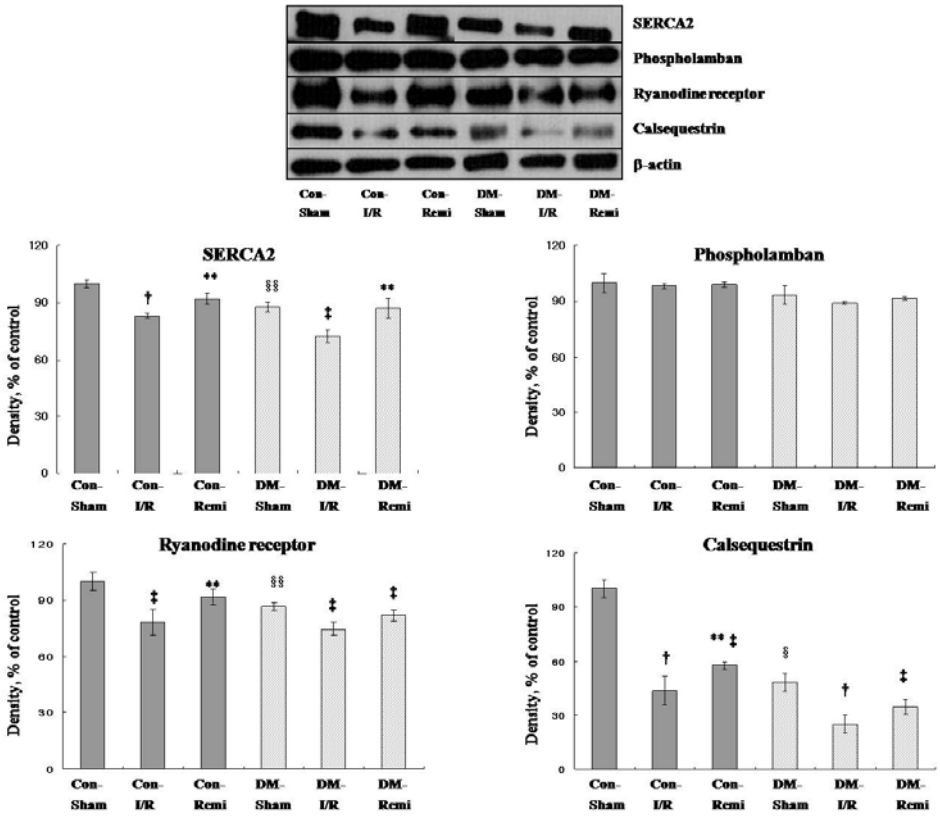


Fig. 5. Western blot analysis of SR proteins. Each signal was quantified by scanning densitometry. β-actin was used as an internal standard. *Closed bars*, data from the control rat hearts; *striped bars*, data from the diabetic rat hearts. Values are presented as mean ± S.E.M. ***P* <0.05 compared to the Con-I/R and DM-I/R groups, respectively; †*P* < 0.01, ‡*P* <0.05 compared to Con-Sham and DM-Sham groups, respectively; §*P* <0.01, §§*P* <0.05 Con-Sham vs. DM-Sham group.

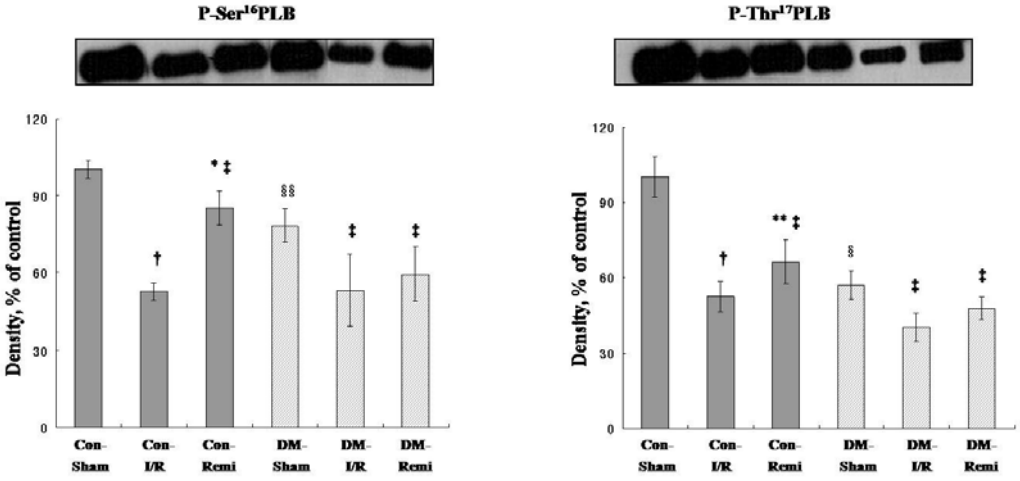


Fig. 6. Western blot analysis of PLB-Ser and PLB-Thr. Each signal was quantified by scanning densitometry. Total PLB was used as an internal standard. *Closed bars*, data from the control rat hearts; *striped bars*, data from the diabetic rat hearts. Values are presented as mean ± S.E.M. **P* <0.01, ***P* <0.05 compared to Con-I/R group, respectively; †*P* < 0.01, ‡*P* <0.05 compared to the Con-Sham and DM-Sham groups, respectively; §*P* <0.01, §§*P* <0.05 Con-Sham vs. DM-Sham group.

IV. DISCUSSION

The current study demonstrates that streptozotocin-induced type I DM significantly reduced the cardioprotective effect of remifentanil preconditioning and resulted in significantly larger myocardial infarction, which was associated with reduced recovery of ERK1/2 and anti-apoptotic protein activities and alleviated suppression of pro-apoptotic protein activities in DM the rat heart compared to the wild-type rat heart. In addition, in the DM rat heart, remifentanil preconditioning failed to recover suppressed gene expressions of SR proteins by I/R, unlike the wild-type rat hearts. The mean arterial pressure was significantly lower than baseline in DM-Remi group at the end of reperfusion. However it was not considered to affect the results, because it was within normal range, and not significantly different from other groups at the end of reperfusion.

With regard to the effect of anesthetic agents on the extent of cardioprotection in diabetic animals, previous reports observed that DM abolished morphine[23] and isoflurane[24]-induced cardioprotection, which were in agreement with our results. DM also inhibited the protective effects of early and late ischemic preconditioning.[25-26] As early and late ischemic preconditioning and anesthetic agent-induced preconditioning pose a few common pathways, the lack of cardioprotective effect in various conditions in DM might be accompanied by impaired activation of similar signaling pathways. In several recent studies, DM impaired activation of mitochondrial K_{ATP} channels[27] and abolished delayed cardioprotection by the κ-opioid receptor agonist via impaired heat-shock protein 70 production[28]. Also, inhibition of the mitochondrial permeability transition pore, a final action site of pro-survival kinase signaling pathways, could restore sevoflurane-induced cardioprotection blocked by hyperglycemia.[29] These results may imply that DM has an impact on survival kinases and

apoptosis related pathways in preconditioning induced myocardial protection.

The activation of pro-survival kinases, such as AKt and the two isoforms of ERK1 and ERK2, have been demonstrated to confer powerful cardioprotection against I/R injury and ERK1/2 are established players in the anti-apoptotic defense network.[30] In the current study, DM significantly attenuated remifentanil induced recovery of activities of ERK1/2, which were reduced by I/R. Morphine-induced activation of ERK1/2 was also reported to be lessened by DM.[23] In addition, activation of ERK1/2 inhibited the conformational change in the pro-apoptotic protein, Bax and cytochrome c- induced caspase activation, thereby preventing apoptosis.[31] The anti-apoptotic protein Bcl-2 is known to attenuate cellular injury by inhibiting cytochrome c translocation, preventing injurious Ca^{2+} release from the endoplasmic reticulum[32] and inhibiting Bax translocation from the cytoplasm to the mitochondria.[33] In this study, levels of the pro-apoptotic proteins Bax and cytochrome c were greater in the diabetic heart, although the level of anti-apoptotic protein Bcl-2 was similar between non-diabetic and diabetic rats. After I/R, expression of Bcl-2 decreased and expression of Bax and cytochrome c increased in both the Con-I/R and DM-I/R groups and remifentanil preconditioning normalized I/R induced changes comparably to those of the Con-Sham group in wild-type rats, while only Bcl-2 levels recovered, which was still lower than the DM-Sham group in diabetic rats. All of these findings indicated that DM reduced the cardioprotective effect of remifentanil preconditioning by affecting the anti-apoptotic pathways of survival in I/R rat hearts.

Ca^{2+} is an important messenger in intracellular signal transduction and the balance between Bcl-2 and Bax has been demonstrated to affect mitochondrial Ca^{2+} homeostasis, which is important in determining whether cells survive or undergo apoptosis. SR plays a central role in regulating intracellular Ca^{2+} concentration and contains SR Ca^{2+}-cycling proteins such as RyR, SERCA2a,

PLB, and CSQ. Intracellular Ca^{2+} homeostasis is altered in the diabetic heart, as the result of several abnormalities including decreased SERCA2a activity in SR[34-35] and NCX[36] which lead to Ca^{2+} overload and it may be involved in the pathogenesis of diabetic cardiomyopathy.[37-42] In the current study, the expressions of SR genes were significantly decreased after I/R as previous studies-found,[43-44] and most expressions of SR genes were lower in DM rats. Moreover, remifentanil preconditioning induced restoration of levels of all SR gene expressions was not to be observed in DM rats. Altered expressions of SR genes might be associated with altered SR function seen in diabetic hearts, which possibly leads to the reduced protective effects of remifentanil preconditioning in the current study. This is in accordance with previous reports that found contractile dysfunction in the diabetic rat heart was related to alteration of SR protein expressions.[37, 42] In support of our results, several reports suggested that depressed SERCA activity is common to type I diabetes in animal models,[40] and SERCA activity is decreased with a concomitant change in mRNA for the protein.[37, 45] The mechanisms regulating expression of these genes are unknown and further studies are needed to investigate the transcription mechanism that regulates the expressions of SR genes by DM in remifentanil preconditioning. DM related changes in myocardial intracellular Ca^{2+} homeostasis is still somewhat controversial. In a recent report, impaired SERCA activity was found to contribute to cardiomyocyte dysfunction, but NCX expression was not changed in insulin resistant animals.[41] In another study, PLB increased and NCX expression decreased in rat models of diabetic cardiomyopathy induced during 12 week period,[37] but total PLB protein and NCX mRNA expression were not altered in diabetic rat models induced during 3 weeks in the current study. These discrepancies may be caused by differences during the period in which diabetes was induced. This might have caused cardiomyocyte dysfunction at an early stage rather than cardiomyopathy in our

models.[41] Although the PLB protein contents were unaltered, all phosphorylated PLB proteins normalized to total PLB were attenuated by DM and I/R in this study, which means that the PLB proteins in DM and I/R hearts were predominantly in the unphosphorylated form. Interestingly, we observed that remifentanil restored levels of SERCA2a gene and proteins, even in diabetic rats. Therefore, further evidence is needed to demonstrate whether SERCA function is especially associated with cardioprotective mechanisms of remifentanil in other animal models.

The limitation of this study is as follows. Although the differences in remifentanil induced myocardial protective effect could be clearly demonstrated, the accuracy of the infarct size measurement could have been affected by the relatively short duration of reperfusion period. Also, we did not measure the infarct size by determining the necrotic area at risk which is a more accurate method.

V. CONCLUSION

In the diabetic rat heart, remifentanil preconditioning-induced myocardial protective effects were less effective than in wild type rat heart due to reduced recovery of ERK1/2 activity and anti-apoptotic pathways of survival, as well as attenuated recovery of suppressed gene expressions SR proteins by I/R.

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< ABSTRACT(IN KOREAN)>

Remifentanil의 전조건화를 통한 심근 보호에 제 1형 당뇨가 미치는 영향

<지도교수 곽 영 란>

연세대학교 대학원 의학과

조 장 은

서론 : 당뇨는 심근의 허혈-재관류 손상에 대한, 약물성 전조건화의 심근 보호 효과를 감소시키는 것으로 알려져있다. Remifentanil은 심장 수술의 마취에 널리 쓰이는 아편양제제로 심근의 허혈-재관류 손상에 대한 심근 보호 효과가 입증되어있다. 본 연구는 허혈-재관류를 시행한 쥐의 심장에서 remifentanil 전조건화의 심근 보호 효과에 당뇨가 미치는 영향을 세포 사멸와 칼슘 항상성의 관점에서 밝히고자 하였다.

대상 및 방법 : 정상쥐와 streptozotocin으로 1형 당뇨를 유도한 당뇨쥐를 각각 허혈-재관류만 시행한 군 (Con-I/R, DM-I/R), Remifentanil 전조건화를 시행한 군 (Con-Remi, DM-Remi), 허혈-재관류 및 Remifentanil 전조건화를 시행하지 않는 군(Con-Sham, DM-Sham)으로 나누었다. 심근 허혈은 좌전 하행지를 30분 동안 결찰하여 시행하였고, 1시간 동안 재관류 하였다. Remifentanil 전조건화는 좌전 하행지 결찰 30분 전부터, Remifentanil을 6μg/kg/min의 속도로 20분간 투여하였다.심근 경색 부위는 전체 좌심실 영역에 대한 비율(%)을 염색약 (triphenyltetrazolium chloride)을 처리하여 측정하

였다. 세포 사멸와 관련된 단백질 (ERK 1/2, Bcl2, Bax, cytochrome c)의 발현은 Western blot, 칼슘 항상성 관련 단백질의 유전자 발현은 RT-PCR을 이용하여 측정하였다.

결과 : 심근경색부위는 당뇨쥐에서 remifentanil의 심근보호효과가 감소하여, 정상쥐에 비해 심근경색부위가 유의하게 증가되었다. 당뇨는 허혈-재관류에 의해 감소하는 ERK1/2의 활동을 remifentanil이 회복시키는 효과를 감소시켰다. 전구세포사멸 단백질 활동의 억제는 DM-Remi군에서 Con-Remi군에 비하여 감소하였다. 허혈-재관류에 의한 SR protein의 유전자 발현 억제의 회복은 DM-Remi군에서 Con-Remi군에 비하여 감소되었다.

결론 : 당뇨는 허혈-재관류에 대한 remifentanil 전조건화의 심근 보호 효과를 감소시키며, 이는 세포 사멸 관련 단백질의 활동과, 허혈-재관류에 따른 근질세망 단백질 유전자의 비정상 발현에 의한 칼슘 항상성 변화의 회복이 감소하는 것과 관계가 있다.

핵심되는 말 : 당뇨, remifentanil, 전조건화, 심장.