Remifentanil protects myocardium via activation of anti-apoptotic pathways of survival in ischemia-reperfused rat heart

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Summary

Remifentanil is a commonly used opioid in anesthesia with cardioprotective effect in ischemia-reperfused (I/R) heart. We evaluated the influence of remifentanil on myocardial infarct size and expressions of proteins involved in apoptosis in I/R rat heart following various time protocols of remifentanil administration. Artificially ventilated anaesthetized Sprague-Dawley rats were subjected to a 30 min of left anterior descending coronary artery occlusion followed by 2 h of reperfusion. Rats were randomly assigned to one of five groups; Sham, I/R only, remifentanil preconditioning, postconditioning and continuous infusion group. Myocardial infarct size, the phosphorylation of ERK1/2, Bcl2, Bax and cytochrome c and the expression of genes influencing Ca^{2+} homeostasis were assessed. In remifentanil-administrated rat hearts, regardless of the timing and duration of administration, infarct size was consistently reduced compared to I/R only rats. Remifentanil improved expression of ERK 1/2 and anti-apoptotic protein Bcl2, and expression of sarcoplasmic reticulum genes which were significantly reduced in the I/R only rats. Remifentanil reduced expression of pro-apoptotic protein, Bax and cytochrome c. These suggested that remifentanil produced cardioprotective effect by preserving the expression of proteins involved in anti-apoptotic pathways, and the expression of sarcoplasmic reticulum genes in I/R rat heart, regardless of the timing of administration.

Keywords: anti-apoptosis, cardioprotection, ischemia/ reperfusion injury, remifentanil,

Introduction

Despite restoration of coronary blood flow, ischemia-reperfusion (I/R) injury in myocardium triggers stress signaling processes that eventually result in various degree of myocardial damage (Anaya-Prado *et al*. 2002, Fliss and Gattinger 1996). In attempts to lessen the I/R injury, ischemic preconditioning was introduced as an effective treatment modality (Murry *et al*. 1986). Certain anaesthetic agents also possess cardioprotective effects against I/R injury, mimicking ischemic preconditioning, termed pharmacologic preconditioning (Schultz and Gross 2001, Tanaka *et al*. 2004). Remifentanil, is an ultra-short-acting, selective, μ−opioid receptor agonist, gaining increasing popularity in cardiac anaesthesia (Glass 1995, Patel and Spencer 1996). Like several anesthetics, remifentanil was reported to protect myocardium against I/R injury. In the light of remifentanil's preconditioning effect, the activation of opioid receptors (Peart *et al*. 2005, Yu *et al*. 2007, Zhang *et al*. 2004), protein kinase C (PKC) and mitochondrial adenosine triphosphate-sensitive potassium (K_{ATP}) channels were reported to be important mediators of its cardioprotective action (Zhang *et al*. 2005), although those were observed at doses much higher than doses used in clinical practice.

Apoptosis, a programmed cell death, contributes significantly to post-ischemic cardiomyocyte death, (Gottlieb and Engler, 1999) but evidence is lacking regarding the effect of remifentanil on anti-apoptotic pathways of survival in I/R injury. In mammalian cells, among three major mitogen activated protein kinases (MAPKs) signaling pathways, activation of extracellular signal related protein kinases (ERK1/2) exerts beneficial effect on post-ischemic myocardial apoptosis (Yue *et al*. 2000).

Thus, we evaluated the effects of the minimal concentration of remifentanil (Yu *et al*. 2007, Zhang *et al*. 2005) against I/R injury on myocardial infarction in rat in terms of the expression of proteins involved in apoptosis signaling cascades including ERK1/2 following various time protocols of remifentanil administration in rat heart. In addition, since the proteins involved in apoptosis significantly affect Ca^{2+} homeostasis, we concomitantly measured expressions of genes associated with Ca^{2+} homeostasis.

Methods

Animals

Male Sprague-Dawley rats (250-300 g) were anaesthetized with a single i.p. injection of pentobarbital 60 mg kg^{-1}(Yu *et al*. 2007, Zhang *et al*. 2004). Anesthesia was maintained by repeated doses of pentobarbital 25 mg kg^{-1} every 60-90 min. After tracheal intubation, the rats were artificially ventilated (Harvard Apparatus 683, USA) at a rate of 30-35 cycles per minute with oxygen 100% and a tidal volume of approximately 5 ml. The respiratory rate was varied to maintain the end-tidal CO_2 by 30-35 mmHg using a capnograph (Datec, Normocap®, Finland).

The right femoral artery was used for monitoring of mean arterial pressure, and the heart rate was monitored *via* subcutaneous stainless steel electrodes. These were connected to a PowerLab monitoring system (ML845 PowerLab with ML132; AD Instruments, USA). The left femoral vein was cannulated to infuse remifentanil or saline. Rectal temperature was maintained at 38°C during the entire experiment. The animal experimental procedures were approved by the committee for the Care and Use of Laboratory Animals of our hospital and were performed in accordance with the Committee's Guidelines and Regulations for Animal Care.

Experimental protocol

After a left thoracotomy and pericardiotomy, the heart was 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 allowed for a period of 2h. Haemodynamic parameters were measured at following time points; 1) at the end of stabilization before ischemia (baseline), 2) at the end of ischemia (or no ischemia), and 3) after 2 h of reperfusion (Fig 1).

Study groups

This study consisted of five series of experiments. 'I/R': rats did not receive any remifentanil (Ultiva^{TM}, GlaxoSminthKline, Italy) treatment during the entire experiment (n = 7); 'Remifentanil-preconditioning (Pre-R)': rats were subjected to infusion of remifentanil (6 μg $kg^{-1}min^{-1}$) during 20 min before 30 min of occlusion followed by 10 min washout (n = 6); 'Remifentanil-postconditioning (Post-R)': remifentanil was administrated during the first 20 min of reperfusion (n = 7); 'Long-R': remifentanil was administrated from 30 min before ischemia to the first 20 min of reperfusion (n = 9). Control-operated rats (Sham) were administrated similarly, except that the coronary suture was not tied, and remifentanil was not administrated (n = 5).

Infarct size measurement

At the end of the reperfusion period (2h), the heart was excised and immersed in 2% triphenyltetrazolium chloride (TTC) (Sigma, USA) stain for 20 min at 37°C. The infarct myocardium, which does not take up TTC stain when the dehydrogenase enzymes are drained off, remains pale in color. The heart was sliced and photographed, and infarct size was determined by dividing the total necrotic area of the left ventricle (LV) by the total LV area (Kang *et al.* 2006, Hwang *et al.* 2004, Gnecchi *et al.* 2006). The boundary of unstained area was traced in a blinded fashion and quantified with NIH image, version 1.61.

Immunoblot analysis

At the end of the reperfusion period, tissue preparation was performed (Uecker *et al*. 2003). Briefly, LV specimens were pulverized and dissolved in lysis buffer (Cell signaling, USA). The solution was vigorously homogenized with Pyrex Potter-Elvehjem Tissue Grinders (BLD science, USA) 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, USA). Proteins were separated in a 12% SDS-polyacrylamide gel and transferred to PVDF membrane (Millipore Co, USA). 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- extracellular signal-regulated kinases (ERK 1/2), mouse anti-phospho ERK 1/2, mouse anti- B cell leukemia/lymphoma-2 (Bcl-2), mouse anti-cytochrome C (Santa Cruz Biotechnology, USA), rabbit anti- Bcl-2-associated X protein (Bax) (Assay Designs, USA) and mouse anti-β actin antibodies (Sigma, USA). 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, USA). β-actin gene was used as the standards for equal of the protein samples. The band intensities were quantified using a Photo-Image System (Molecular Dynamics, Sweden). Each experiment was performed at least three times.

RT-PCR analysis

At the end of the reperfusion period, tissue preparation was performed (Uecker *et al*. 2003). Briefly, LV samples were vigorously homogenized with Pyrex Potter-Elvehjem Tissue Grinders (BLD science, USA) in TRI Reagent (Sigma, USA). Total RNA was prepared by the UltraspectTM-II RNA system (Biotecx Laboratories, Inc., USA) 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, 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 genes related to Ca^{2+} homeostasis are shown in Table 1. The PCR condition was 94°C for 3 min, 94°C for 1 min, 41-49°C for 1 min and 72°C for 2 min, 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, UK)

Statistical analysis

Data are presented as mean (SD) or mean (SEM). Where results of blots and RT-PCR are shown, a representative experiment is depicted. Data analysis was performed with statistical software program Prism v3.0 (GraphPad Software, USA). Haemodynamics were analyzed using two-way analysis of variance (ANOVA) with Bonferroni post-hoc test for multiple comparisons if significant F ratio was obtained. Infarct sizes were analyzed between groups using ANOVA with Student-Newman-Keula post-hoc test for multiple comparisons. Statistical significance was defined as $P<0.05$ and $P<0.01$.

Results

Haemodynamic

The heart rate and mean arterial pressure are summarized in Table 2. The heart rates in the Post-R ($P < 0.05$) and Long-R groups ($P < 0.01$) were significantly lower compared to the I/R group after reperfusion. The heart rate in the Long-R groups after reperfusion was also significantly lower compared to the baseline value ($P < 0.05$). The mean arterial pressures significantly decreased at the end of ischemia ($P<0.01$) and after reperfusion ($P<0.05$) compared to the baseline value in the Long-R groups. The other values of heart rate and mean arterial pressure revealed no statistically significant differences in either inter- or intra-group comparisons.

Infarct Size Measurement

Mean infarct size of the LV in the I/R group was 43.6 (5.5)%. In the groups administrated with remifentanil, infarct sizes of the LV were significantly reduced to 20.1 (1.9)%, 18.8 (3.8)% and 19.3 (3.2)% in the Pre-R, Post-R and Long-R group, respectively (all $P<0.05$). No significant differences were observed between the remifentanil-administrated groups (Fig. 2).

Western blotting analysis on phosphorylation of ERK 1/2 and apoptosis related proteins

Phosphorylations of ERK 1/2 were significantly lower in the I/R group than in the Sham group. Phosphorylations of ERK 1/2 in the remifentanil-administrated groups were significantly improved

compared to the I/R group, but were lower than in the Sham group. Phosphorylations of ERK 1/2 were similar between the remifentanil-administrated groups (Fig. 3A).

Expression levels of pro-apoptotic proteins, Bax and cytochrome c, significantly increased and expression level of anti-apoptotic protein, Bcl-2 significantly decreased in the I/R group than in the Sham group. In the remifentanil-administrated groups, the expression levels of Bax and cytochrome c significantly decreased and the expression level of Bcl-2 significantly increased than in the I/R group. The expression levels of Bax, cytochrome c and Bcl-2 were similar among the remifentanil-administrated groups (Fig. 3B).

Gene expression of proteins influencing the calcium homeostasis

In the I/R group, gene expression for sarcoplasmic reticulum (SR) proteins including SR Ca^{2+}-ATPase (SERCA2a), phospholamban (PLB), ryanodine receptor 2 (RyR), and calsequestrin (CSQ) reduced than in the Sham group. In the remifentanil-administrated groups, mRNA transcription levels for SR proteins were significantly up-regulated than in the I/R group without differences between the groups. The Ca^{2+} entry across the membrane through L-type Ca^{2+} channels is balanced by the efflux of Ca^{2+} from the cell via the Na^{+}-Ca^{2+} exchanger (NCX) (Bers 2002). In the present results, levels of gene expression for L-type Ca^{2+} channels and NCX were unaltered by I/R with or without remifentanil (Fig. 4).

Discussion

In the current study, remifentanil significantly reduced myocardial infarct size without reference to the timing and duration of administration in I/R rat heart, which occurred in conjunction with the preserved phosphorylations of ERK 1/2 and anti-apoptotic protein Bcl2 and attenuated increased pro-apoptotic proteins, Bax and cytochrome c resulting from I/R. Remifentanil administration also resulted in preserved gene expressions of SR proteins.

Remifentanil is a fentanyl derivative with a unique pharmacokinetic profile of rapid and predictable recovery, gaining increasing popularity in cardiac anaesthesia. Similar to certain anaesthetic agent, remifentanil has also been found to possess cardioprotective effects against I/R, although the effect occurred at significantly higher dose than clinically used (Minto *et al.* 1997, Scott and Perry *et al*. 2005). Remifentanil was reported to exert cardioprotective effect via all three opioid receptors (Peart and Gross 2004, Yu *et al*. 2007). Activation of κ- and δ- opioid receptors was associated with activation of PKC and opening of mitochondrial K_{ATP} channel, which are important targets of cardioprotection in ischemic- and pharmacologic preconditioning (Zhang *et al*. 2005).

While other signal transduction pathways also play an important role in cardioprotection, however, little has been studied regarding mechanisms of remifentanil induced cardioprotection and we could observe that remifentanil acted through proteins involved in anti-apoptoic pathways of survival regardless of timing and duration of administration. Apoptosis has been linked with reperfusion-induced myocardial injury after reversible coronary occlusion and suggested as one of the key mechanisms in the

development of infraction in rat cardiac myocytes (Buja and Entman 1998, Haunstetter and Izumo 2000).

In the current study, remifentanil increased the phosphorylation of ERK 1/2 and anti-apoptotic proteins in I/R rat heart. ERK is activated in response to I/R, oxidative stress, and hypoxia, and is an established player in the anti-apoptotic defense network (Yue *et al*. 2000). As our results indicate, remifentanil preserved the phosphorylation of ERK 1/2 which were significantly reduced following I/R leading to improved viability of the rat heart exposed to I/R. In response to an apoptotic stimulus such as I/R, the pro-apoptotic protein, Bax, undergoes a conformational change that allows it to translocate to the mitochondria, where it induces cytochrome c release. Phosphorylation of ERK 1/2 inhibits the conformational change in Bax protein and cytochrome c- induced caspase activation, thereby preventing apoptosis (Tsuruta *et al.* 2004). The anti-apoptotic protein Bcl-2 attenuates cellular injury by inhibiting cytochrome c translocation (Kluck *et al.* 1997) and inhibits Bax translocation (Zhu *et al.* 2001). During ischemic preconditioning, an increase in Bcl-2 in association with a decrease in the pro-apoptotic protein Bax occurred in isolated rat heart (Lazou *et al*. 2006). In this study, we could observe that improved phosphorylations of ERK1/2, and associated recovery of expression of Bcl-2 and mitigated expression of Bax and mitochondrial cytochrome c release against I/R induced injury, which all indicate remifentanil induced a cardioprotective effect via anti-apoptosis pathways of survival in I/R rat heart.

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. The SR plays a central role in regulating the

intracellular Ca^{2+} concentration and contains SR Ca^{2+}-cycling proteins such as RyR, SERCA2a, PLB, and CSQ. The expression of SR genes significantly reduced in I/R rat heart in this study which was in accordance with the result of previous study (Temsah *et al.* 1999), and treatment with remifentanil preserved the levels of SR genes expression. These results suggest that remifentanil might modulate the expression of SR genes in I/R rat heart. Since we did not measure intracellular Ca^{2+} concentration, the real changes in intracellular Ca^{2+} concentration as consequences of remifentanil induced modulation of Ca^{2+} homeostasis could not be clarified in this study.

With regard to the effect of timing and duration of drug administration on the extent of myocardial protection, the combination of pre- and postconditioning with sevoflurane provided additive cardioprotection (Obal *et al.* 2005), whereas desflurane showed no additive cardioprotective effects (Haelewyn *et al.* 2004). As we had investigated the cardioprotective effects of remifentanil with different administration protocols, we observed similar protective effect between remifentanil preconditioning and postconditioning groups, and could not observe additive cardioprotective effects in continuously administrated group. These results suggest that cardioprotective signaling pathways may vary among anaesthetics but activated pathways during remifentanil pre- and postconditioining might be similar, which merit further studies addressing the precise mechanisms of this particular anaesthetic-induced cardioprotection.

Cardioprotective effects of remifentanil in this study were demonstrated at a dose of 6 μg kg^{-1} min^{-1} which is higher than the dose used in clinical practice (0.2-0.8 μg kg^{-1} min^{-1}) (Scott and Perry 2005).

Previous studies reported that remifentanil had cardioprotective effect against I/R injury with dose-dependency, and the maximum effect of remifentanil was 6 or 10 μg kg^{-1} min^{-1} (Yu *et al*. 2007, Zhang *et al*. 2005). Based on those studies, we choose the minimal concentration of remifentanil obtaining maximal cardioprotective effect. The pharmacologic characteristics of remifentanil may vary and difficult to compare between species and can be given in relatively high doses for a short period of time (Yu *et al*. 2007, Zhang *et al*. 2005).

The limitation of this study is that we did not determine the area at risk. In this study, we referred the infarct size measurement to the methods of our research (Hwang *et al.* 2004, Kang *et al.* 2006). Therefore we determined the infarct size by dividing the total necrotic area by the total LV area. Although this method was used in other study (Bazargan *et al.* 2008), determining the necrotic area by the area at risk can be more accurate.

In conclusion, remifentanil confers myocardial protection against injury in I/R rat heart without reference to the timing and duration of administration, which was conducted with preserved phosphorylations of ERK 1/2 and of signal transduction proteins against apoptotic cell death, and attenuated changes in expression of SR genes related to Ca^{2+} homeostasis.

Conflict of Interest

There is no conflict of interest.

Acknowledgments

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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’	
PMCA1	Sense: 5′-TGCCTTGTTGGGATTTCTCT-3′	351
	Antisense: 5′-CACTCTGGTTCTGGCTCTCC-3′	
NCX	Sense: 5′-TGTCTGCGATTGCTTGTCTC-3′	364
	Antisense: 5′-TCACTCATCTCCACCAGACG-3′	
SERCA2a	Sense: 5′-TCCATCTGCCTGTCCAT-3′	196
	Antisense: 5′-GCGGTTACTCCAGTATTG-3′	
Phospholamban	Sense: 5′-GCTGAGCTCCCAGACTTCAC-3′	339
	Antisense: 5′-GCGACAGCTTGTCACAGAAG-3′	
Ryanodine receptor 2	Sense: 5′-CCAACATGCCAGACCCTACT-3′	351
	Antisense: 5′-TTTCTCCATCCTCTCCCTCA-3′	

Calsequestrin	Sense: 5′-TCAAAGACCCACCCTACGTC-3′ Antisense: 5′-CCAGTCTTCCAGCTCCTCAG-3′	352

Table 2 Haemodynamic parameters.

	Baseline		**Ischemia**		**2 h after reperfusion**	
	HR	**MAP**	**HR**	**MAP**	**HR**	**MAP**
I/R	437 (52)	131 (14)	446 (67)	127 (17)	449 (35)	129 (15)
Pre-R	456 (47)	144 (7)	467 (57)	129 (8)	462 (48)	134 (10)
Post-R	397 (61)	129 (10)	402 (48)	132 (9)	347 (48)*	118 (15)
Long-R	424 (38)	135 (11)	384 (41)	101 (6)*‡	345 (40)**†	99 (25)†

HR, heart rate; MAP, mean arterial pressure; I/R, ischemia/reperfusion group (n = 7); Pre-R, remifentanil administrated during the preconditioning period (n = 6); Post-R, remifentanil administrated during the first 20 min of reperfusion (n = 7); Long-R, remifentanil administrated during the entire period (from the preconditioning to the first 20 min of reperfusion) (n = 9). Data are mean (SD). * P <0.05, ** P <0.01 vs. I/R group; † P <0.05, ‡ P <0.01 vs. baseline.

Figure

Fig. 1

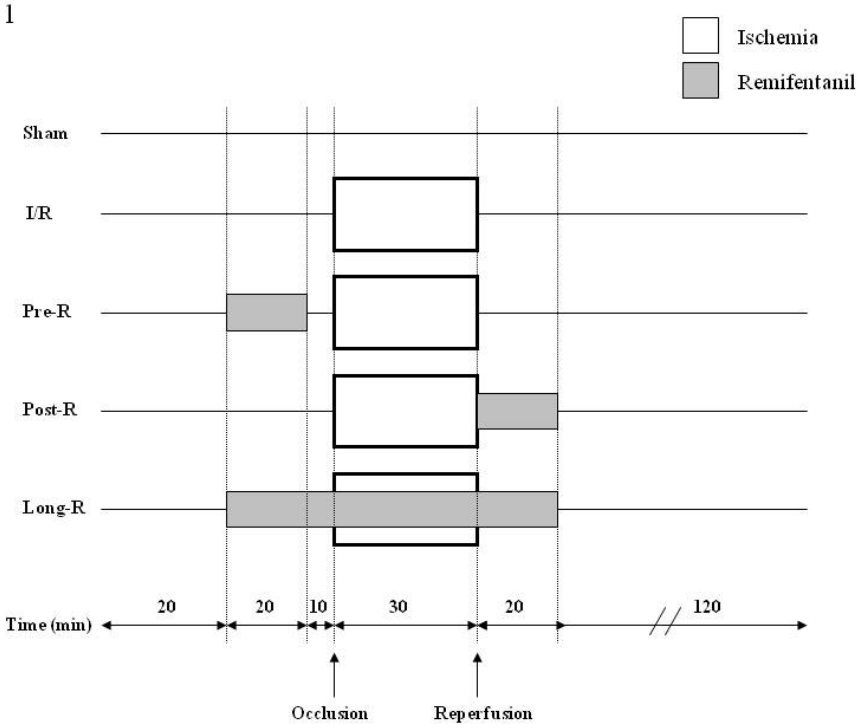


Fig 1 The experimental protocol. Occlusion of the left anterior (LAD) coronary artery was maintained for 30 min, followed by reperfusion. After 2 h, sampling was performed for infarct size, immunoblot, and RT-PCR respectively. Sham, the coronary suture was not tied, and remifentanil was not administrated; I/R, ischemia/reperfusion group (n = 7); Pre-R, remifentanil administrated during the preconditioning period (n = 6); Post-R, remifentanil administrated during the first 20 min of reperfusion (n = 7); Long-R, remifentanil administrated during the entire period (from the preconditioning to the first 20 min of reperfusion) (n = 9).

Fig. 2

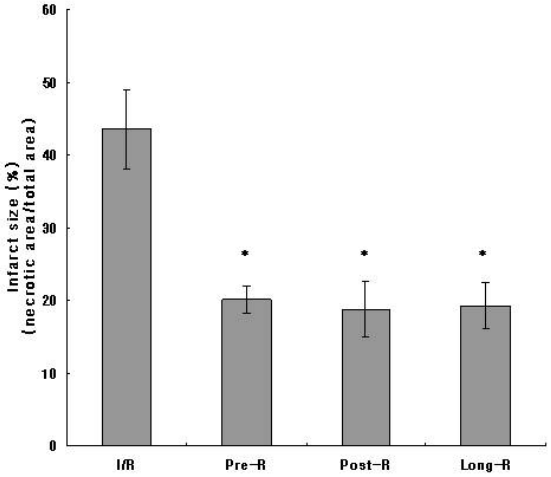
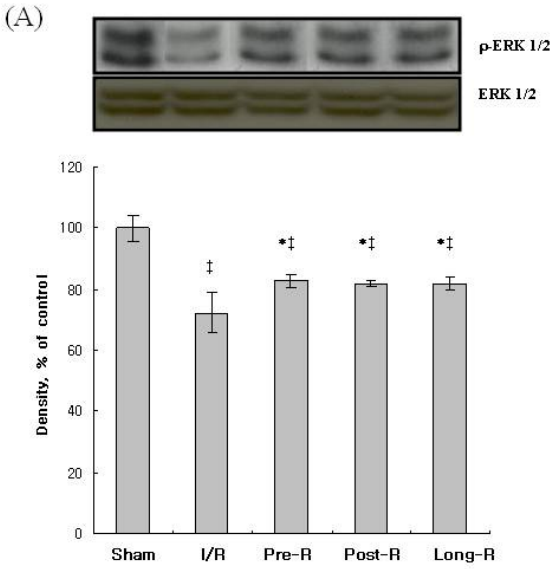


Fig 2 Infarct sizes of the left ventricle in each group. Infarct size was expressed as a ratio of the left ventricular area. Values are presented as mean (SEM). **P*<0.05 compared with the I/R group (*n* = 5-9 per group).

Fig. 3


(A)
ρ-ERK 1/2
ERK 1/2
Density, % of control
120
100
80
60
40
20
0
‡
*‡
*‡
*‡
Sham
I/R
Pre-R
Post-R
Long-R

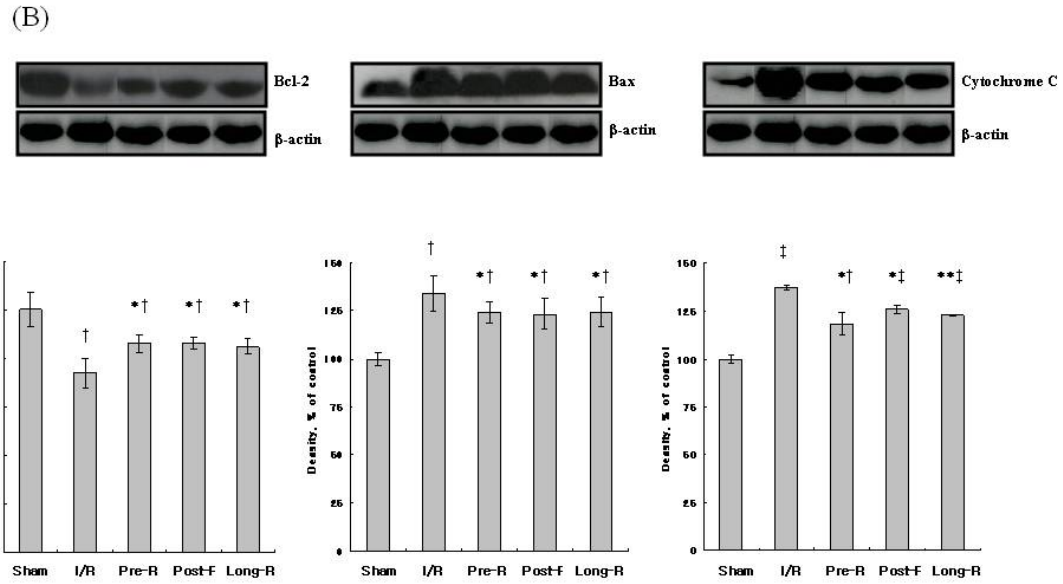
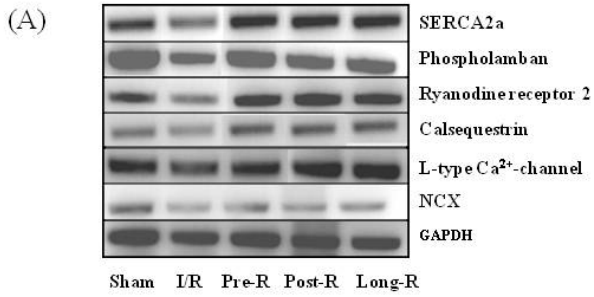

(B)
Bcl-2
β-actin
Bax
β-actin
Cytochrome C
β-actin
Density, % of control
†
*†
*†
*†
‡
*†
*‡
**‡
Sham
I/R
Pre-R
Post-R
Long-R

Fig 3 Western blot analysis of (A) Western blot analysis of ERK 1/2 and p-ERK 1/2. (B) Western blot analysis of Bcl-2, Bax, and cytochrome c. Each signal was quantified by scanning densitometry. Values are presented as mean (SEM). **P*<0.05 and ***P*<0.01 compared with the I/R group.; † *P*<0.05 and ‡ *P*<0.01 compared with the Sham group.

Fig. 4


(A)
SERCA2a
Phospholamban
Ryanodine receptor 2
Calsequestrin
L-type Ca2+-channel
NCX
GAPDH
Sham
I/R
Pre-R
Post-R
Long-R

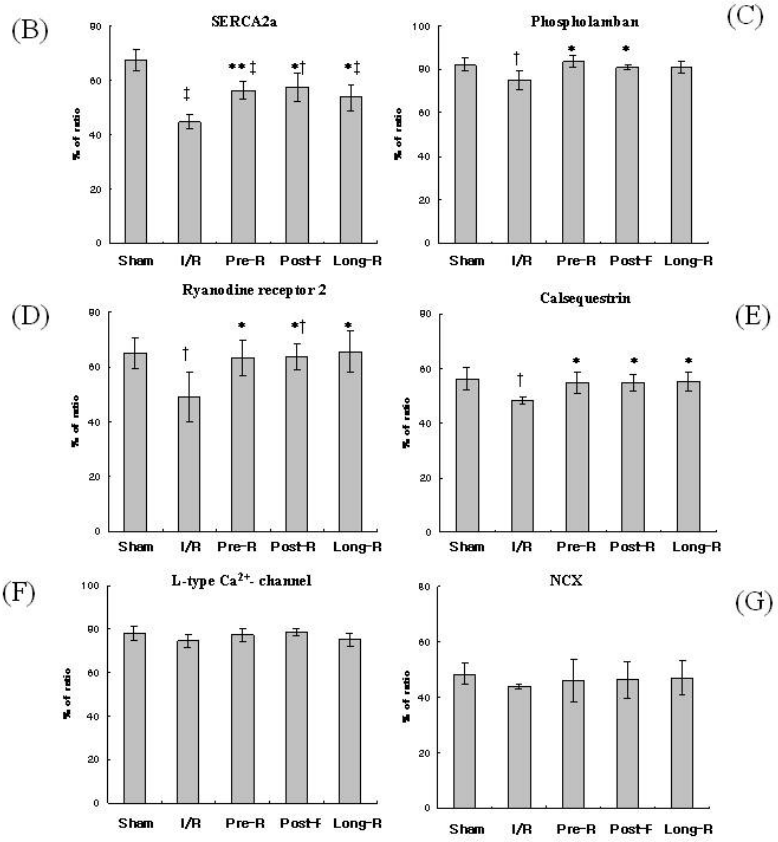

(B)
SERCA2a
(C)
Phospholamban
(D)
Ryanodine receptor 2
(E)
Calsequestrin
(F)
L-type Ca2+- channel
(G)
NCX
% of ratio
Sham
I/R
Pre-R
Post-R
Long-R

Fig 4 Analysis of the expressions of genes 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. Each value is the mean (SEM) of 5 hearts/group. **P*<0.05 and ***P*<0.01 compared with the I/R group; † *P*<0.05 and ‡ *P*<0.01 compared with the Sham group.