

Heart Protection by Combination Therapy with Esmolol and Milrinone at Late-Ischemia and Early Reperfusion

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Abstract

Introduction The present study determined whether late-ischemia/early reperfusion therapy with the β_1 -adrenergic receptor (AR) blocker esmolol and phosphodiesterase III inhibitor milrinone reduced left ventricular (LV) myocardial infarct size (IS).

Methods and Results In an ischemia/reperfusion rat model (30-min ischemia/4-hr reperfusion), esmolol, milrinone or esmolol + milrinone were intravenous (IV) infused over 10 min (from the last 5min of ischemia to the first 5min of reperfusion). LV-IS were $48.9 \pm 8.9\%$, $41.5 \pm 5.4\%$, $25.8 \pm 7.7\%$ and $16.8 \pm 7.3\%$ for saline, esmolol, milrinone, and esmolol + milrinone, respectively ($n=12$ /group). Esmolol + milrinone further reduced LV-IS compared with esmolol or milrinone alone ($p < 0.05$). LV-IS-reduction induced by

esmolol + milrinone was eliminated in the presence of protein kinase A-(PKA)-inhibitor (Rp-cAMPS) or Akt-inhibitor (AKT 1/2 kinase inhibitor). In mixed rat ventricular cardiomyocyte cultures, intra-ischemic application of esmolol, milrinone or esmolol + milrinone reduced myocyte death rates by 5.5%, 13.3%, and 16.8%, respectively, compared with saline ($p < 0.01$). This cell protective effect by esmolol + milrinone was abrogated in the presence of PKA-inhibitor or Akt-inhibitor. Esmolol, milrinone or esmolol + milrinone increased myocardial PKA activity by 22%, 28% and 59%, respectively, compared with saline ($n=6$, $p < 0.01$). No non-specific adverse effect of Rp-cAMPS on myocytes was identified in a purified myocyte preparation during hypoxia/re-oxygenation. Antiapoptotic pathways were assessed by measuring myocardial phosphorylated Akt (pAkt) levels combined with terminal dUTP nick-end labelling staining analysis. Ten minutes following infusion of esmolol, milrinone or esmolol + milrinone, there were 1.7-, 2.7-, and 6-fold increase in tissue pAkt levels, respectively. This esmolol + milrinone induced pAkt activation was abolished in the presence of PKA inhibitor. Esmolol, milrinone and esmolol + milrinone reduced myocyte apoptosis rates by 22%, 37% and 60%, respectively, compared with saline ($p < 0.01$).

Conclusions Late-ischemia/early reperfusion therapy with esmolol+milrinone additively reduces LV-IS associated with robust activation of myocardial PKA and subsequent Akt-antiapoptotic pathway.

Key words Akt · Esmolol · Milrinone · PKA · Reperfusion injury

Introduction

While the translation of ischemic preconditioning to clinical application remains unrealistic for patients with acute

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myocardial infarction (AMI), recent research in ischemic postconditioning opens new opportunity for potentially accessible clinical treatment for AMI. Reperfusion injury salvage kinase (RISK) pathway has been implicated in the postconditioning for infarct-size (IS) reduction [1, 2]. Among several potential mechanisms, Akt activation has been implicated as one of the key signaling pathways underlying postconditioning mediated heart protection [1, 2], although species difference has been reported [3]. The mammalian heart possesses adrenoepitidergic neuroparacrine system consisting of intrinsic cardiac adrenergic (ICA) cells [4–6]. ICA cells synthesize and release epinephrine and calcitonin gene-related peptide (CGRP), stimulating myocardial β_2 -adrenoreceptor (β_2 -AR) and CGRP-receptors [5, 6], both of which being coupled to the adenylate cyclase-cAMP-dependent signaling pathway [7–9]. Concomitant stimulation of cardiac β_2 -AR/CGRP-receptors before ischemia or at the beginning of the reperfusion confers robust IS-reduction [10]. This adrenoepitidergic signaling is mediated through the activation of cAMP-dependent PKA [10] which, in turn, activates Akt, a powerful antiapoptotic signaling pathway [1, 2, 11, 12]. It is conceivable that medications sharing similar distal signaling mechanisms of β_2 -AR/CGRP-receptors may provide alternatives for the receptor-mediated cardioprotection. For instance, direct myocardial PKA activation with a phosphodiesterase (PDE) III inhibitor milrinone at reperfusion may confer equivalent cardioprotection to that conferred by β_2 -AR/CGRP-receptor stimulation, since milrinone is known to activate PKA [13, 14]. High dose of β_1 -AR blocker esmolol given at the onset of reperfusion reduces LV-IS [15]. This cardioprotective effect is likely mediated, in part, through the enhanced β_2 -AR signaling promoting myocyte survival [11, 12], since at early reperfusion, there is >100 fold increase in myocardial interstitial epinephrine release [16] exerting unopposed myocardial β_2 -AR stimulation in the presence of β_1 -AR blockade. Although, it has been reported that intra-ischemic PKA activation with milrinone reduces right ventricular IS in dogs [17], it has not been demonstrated whether intra-ischemic/reperfusion therapy with milrinone reduces LV-IS. Furthermore, the mechanism underlying post-ischemia milrinone-induced heart protection has not been elucidated. We have hypothesized that cardiac PKA activation with the selective β_1 -AR blocker esmolol in combination with milrinone at late-ischemia/early reperfusion decreases LV-IS. The present study had the following objectives: to determine if: 1) LV-IS can be additively reduced by cardiac PKA activation with combination therapy of esmolol + milrinone delivered at the beginning of reperfusion, in a manner dependent of PKA and Akt activation; 2) myocyte death rates can be reduced by intra-ischemic treatment with esmolol + milrinone in vitro, 3) combination therapy with esmolol +

milrinone additively enhance myocardial PKA and PKA-dependent Akt activation; and 4) esmolol + milrinone-induced Akt activation results in reduced myocyte apoptosis during ischemia/reperfusion.

Materials and methods

Effect of esmolol + milrinone on LV-IS-reduction The rat AMI model consisted of 30-min ischemia and 4-hr reperfusion [5, 6, 18]. Protocols for animal studies were approved by the Institutional Animal Care and the Use Committee of the University of Texas Medical Branch. Saline, esmolol (10 $\mu\text{g}/\text{kg}/\text{min}$), milrinone (5 $\mu\text{g}/\text{kg}/\text{min}$) or esmolol + milrinone were infused via the tail veins over 10-min (5-min before and after the onset of reperfusion, $n=12/\text{group}$). Arterial blood pressure (BP) and heart rate (HR) were monitored via an artery catheter (iWorx/CB Sciences, Inc, Dover, NH). It should be noted that to avoid significant hemodynamic changes, the milrinone dose applied in the present study is only 1/10 of that recommended loading dose for clinical heart failure treatment and esmolol dose is 1/5 of that maintenance dose for the treatment of clinical supraventricular tachycardia. To test whether esmolol + milrinone induced effect was dependent on PKA activation, LV-IS was assessed in the animals ($n=12$) who received PKA inhibitor Rp-cAMPS (2 mg/kg, infused at 10 min after coronary artery occlusion) followed by infusion of esmolol + milrinone. The effect of Rp-cAMPS alone on IS was tested in 8 animals. To determine whether esmolol's effect was mediated through the enhanced endogenous cardiac β_2 -AR stimulation, the effect of esmolol on IS was tested in the animals ($n=8$) pretreated with the selective β_2 -AR antagonist ICI-118,551 (200 $\mu\text{g}/\text{kg}/\text{min}$ for 5 min) infused at 10 min after the onset of ischemia. To further test whether the cardioprotective effect of esmolol + milrinone was dependent on Akt activation, IS was assessed in a separate group of animals ($n=12$) who received Akt inhibition with AKT 1/2 kinase inhibitor (Sigma, 2 mg/kg, infused at 10 min after the onset of ischemia) followed by administration of esmolol + milrinone. The effect of AKT 1/2 kinase inhibitor on IS was also tested separately ($n=8$).

Effect of esmolol + milrinone on myocyte protection in vitro To confirm the *in vivo* intra-ischemic myocyte-protection of esmolol + milrinone, we studied their effects in a well validated adult rat mixed cardiomyocyte culture preparation [5, 6]. After 24 hr culture, hypoxia was induced by layering mineral oil over media covering the cells for 2 hr followed by 60 min re-oxygenation. Saline, esmolol (1 nmol/L), milrinone (4.7 $\mu\text{mol}/\text{L}$) or esmolol + milrinone was individually applied to the myocyte culture at the 30 min after the onset of ischemia. The effects of esmolol +

milrinone on myocyte survival were also tested in the presence of Rp-cAMPS (10 $\mu\text{mol/L}$) or AKT 1/2 kinase inhibitor (10 $\mu\text{mol/L}$). Myocyte death rates were quantified by counting trypan blue-stained cells and expressed as a percentage of the total cells counted ($n=8/\text{group}$). The examiner who counted cells was blinded to the treatment assignments.

To determine whether Rp-cAMPS exerts any non-specific effects on ventricular myocyte, we tested the effect of Rp-cAMPS on isolated ventricular myocytes depleted with ICA cells. This is an important issue, since in the intact heart or cultured cardiocyte mixture, there are endogenous and constitutive adrenoceptor-mediated neuroparacrine-to-myocyte inputs (derived from ICA cells) via cAMP/PKA-dependent signaling [4–6]. Removal of ICA cells from myocytes allows direct assessment of the effect of Rp-cAMPS on myocytes without introducing confounding effect. In ICA cell-free myocyte preparation, we further tested whether Rp-cAMPS specifically blocked the effect of known PKA activators (ie, β_2 -AR agonist terbutaline plus CGRP) [6, 10]. The ICA cell-free myocytes were prepared by mechanical depletion of ICA cells from dissociated rat ventricular cardiocytes using magnetic purification method [4–6]. After 24-hr cell culture, saline, Rp-cAMPS (100 $\mu\text{mol/L}$), or the combination of CGRP (5 $\mu\text{mol/L}$) and β_2 -AR agonist terbutaline (10 nmol/L) were applied individually to ICA cell-free myocyte culture at 30 min after the onset of hypoxia. In addition, the effect of terbutaline + CGRP was tested in the presence of Rp-cAMPS applied 10-min before the administration of the drugs. Myocyte death rate was quantified by counting trypan blue-stained cells and expressed as a percentage of the total cells counted ($n=6/\text{group}$).

Effect of esmolol + milrinone on myocardial PKA and Akt In the same ischemia model, saline, esmolol, milrinone or esmolol + milrinone were IV infused over 10-min (5 min before and after the onset of reperfusion, $n=6/\text{group}$). Ten minutes into the reperfusion, LV tissues just below the ligation snare were removed and stored in liquid nitrogen for PKA and Akt assay. To standardize tissue preparation, the anterior half of LV myocardial tissue (3 mm below the ligation) extending to the apex was removed for the analysis. Tissue PKA activity was determined using a SignaTECT cAMP-dependent PKA assay system (Promega, Madison, WI). Western blot was performed to detect Akt activity (Ser 473). Dilution for anti-pAkt and anti-Akt (Santa Cruz Biotechnology, Santa Cruz, CA) was 1:1000 [15]. To determine whether the Akt activation is dependent on PKA signaling pathway, animals ($n=4$) were pretreated with Rp-cAMPS (2 mg/kg IV) 10 min after the onset of ischemia followed by the administration of esmolol +

milrinone. Then, myocardial pAkt activity was determined as described above.

TUNEL staining Immunofluorescent and terminal dUTP nick-end labeling (TUNEL) were performed in 4 groups of ischemic/reperfused rat hearts treated with saline, esmolol, milrinone or esmolol + milrinone. The methods for tissue preparation and TUNEL staining were described elsewhere [6]. Tissues were harvested at 4 hr after reperfusion for apoptosis determination. Adult rat cardiac myocyte is binucleated [19]. It is possible that only one of nuclei of myocyte exhibits TUNEL-positive staining when it undergoes apoptosis at early stage. Since one (not necessarily two) TUNEL-positive nuclei is required to define an apoptotic myocyte, the % apoptotic cells/section was estimated by counting the number of TUNEL-staining nuclei divided by $\frac{1}{2}$ total number of propidium iodide-positive nuclei in 10 randomly selected fields ($n=6$ slides/heart). Six hearts for each treatment group were studied. To confirm that apoptotic nuclei are truly belongs to true ventricular myocytes, additional TUNEL staining was performed in conjunction with immunohistochemical colocalization of myocyte-specific marker α -actinin using DeadEnd Fluorometric TUNEL System Kit (Promega, Madison, WI). Mouse anti- α -actinin (1:100 dilution, Abcam Cambridge MA,) was used as the primary antibody to counter label myocytes. Secondary antibody was Alexa Fluor 568 goat anti-mouse IgG (H + L) (1:1000 dilution, Invitrogen Corporation, Carlsbad, CA). TUNEL-positive nuclei within counter-stained myocytes were analyzed from control, esmolol, milrinone and esmolol + milrinone treated groups. Ten randomly selected fields were analyzed for each tissue section and six slides were counterstained for each heart ($n=6$ hearts/group). The number of apoptotic myocytes in control group was compared to esmolol, milrinone, and esmolol + milrinone treated group.

Data analysis Two-way ANOVA (saline vs. milrinone in the absence and presence of esmolol) was used for analyzing LV-IS, PKA and Akt activation and apoptosis in ischemia/reperfusion studies with the Bonferroni adjustment for intra-group comparisons. All data are presented as mean \pm SD. The significance level α is 0.05.

Results

Effect of esmolol + milrinone on LV-IS and myocyte protection Compared with saline control, 13%, 45% and 65% IS-reduction were observed in animals receiving reperfusion treatment of esmolol, milrinone or esmolol + milrinone, respectively ($n=12/\text{group}$, $p<0.01$, Fig. 1). IS-

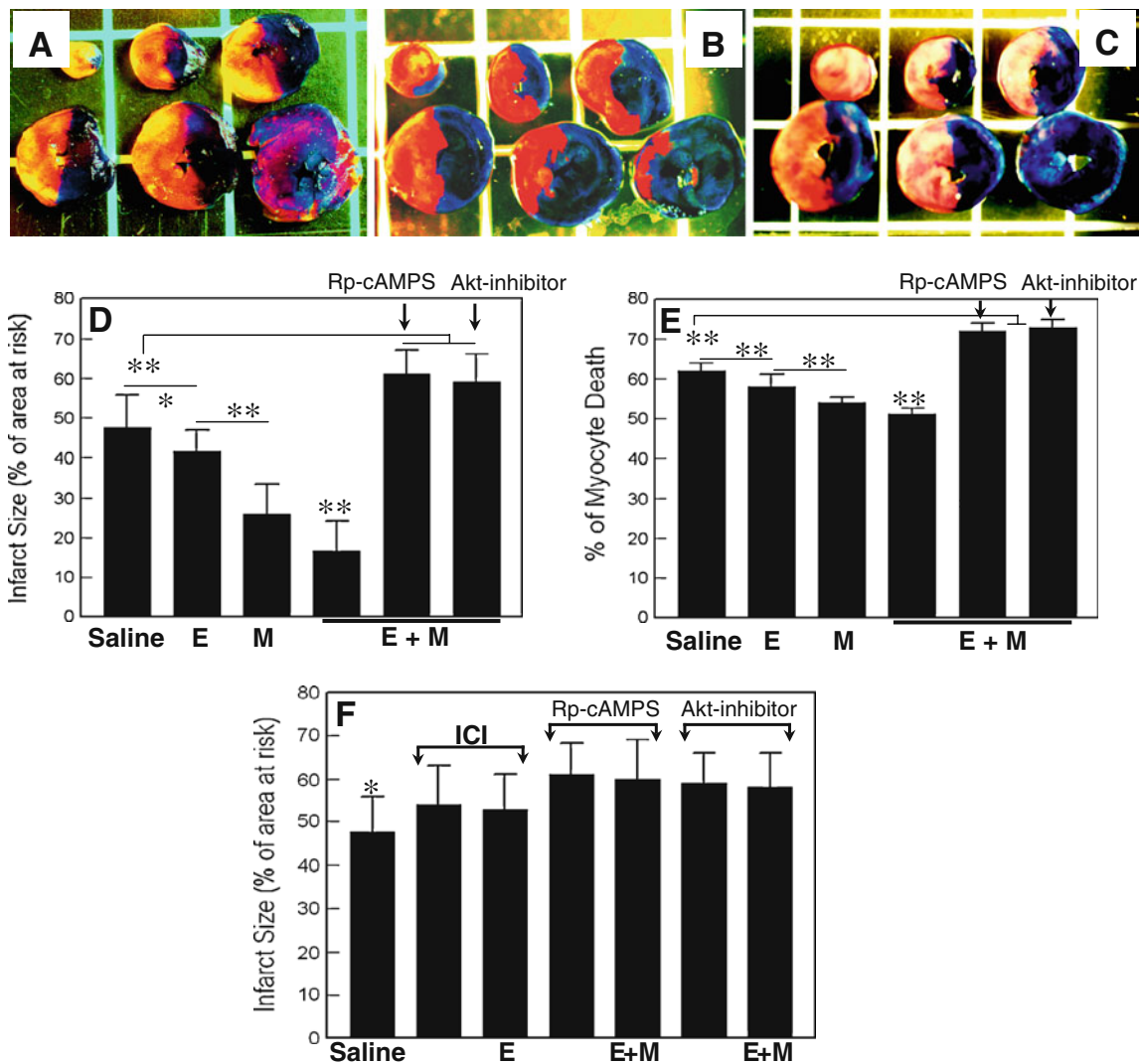


Fig. 1 Panels A, B and C show representative cases of IS in the animals treated with saline (a), esmolol + milrinone (E + M) (b) and E + M in the presence of Rp-cAMPS (c). Infarcted tissue is indicated with yellow color. Panel d shows the IS affected by the therapy with esmolol, milrinone or E + M in the absence and presence of Rp-cAMPS or AKT 1/2 kinase inhibitor ($n=12$ /group). IS in E + M-treated group is significantly smaller compared with all other groups (*: $p < 0.05$, **: $p < 0.01$). Panel E: the effects of intra-ischemic application of esmolol, milrinone or E + M in the absence and presence of Rp-

cAMPS or AKT 1/2 kinase inhibitor on myocyte death rates during hypoxia/re- O_2 in myocyte culture preparation ($n=8$ /group). Panel f: effects of β_2 -AR antagonist ICI-118,551 (ICI), Rp-cAMPS, or AKT-inhibitor alone on IS, comparing with saline ($n=8$ /group). Effects of esmolol, milrinone and esmolol + esmolol in the presence of their respective antagonists were plotted for comparison. Myocyte death rate in E + M treated group is significantly lower compared with all other groups (*: $p < 0.05$, **: $p < 0.01$)

reduction conferred by esmolol was abolished in the animals pretreated with the β_2 -AR antagonist ICI-118,551 which alone increased IS ($p < 0.05$, $n=8$), compared with saline (Fig. 1f). Esmolol + milrinone conferred 35% and 59% more IS reduction compared with milrinone or esmolol alone, respectively ($p < 0.05$). In the presence of Rp-cAMPS or AKT 1/2 kinase inhibitor, esmolol + milrinone conferred no reduction on IS. Rp-cAMPS or Akt inhibitor alone increased IS, compared with saline group ($p < 0.05$, $n=8$, Fig. 1f). There were no significant differences in animal weight and area-at-risk among all the groups (data not shown). There was no significant change

in arterial BP following esmolol + milrinone infusion. A 6% HR reduction was observed at 6 min following the completion of esmolol + milrinone infusion (Tables 1, 2, 3). There was no post-ischemic mortality in the animals receiving esmolol, milrinone or esmolol + milrinone. The mortality rates were 10%, 30% and 35% in saline, Rp-cAMPS, and Akt-inhibitor groups, respectively. In mixed myocyte cultures, treatment with esmolol, milrinone and esmolol + milrinone reduced cell death rates by 5.5%, 13.3% and 16.8%, respectively, compared with saline ($n=8$ /group, $p < 0.01$, Fig. 1). The myocyte death rates of esmolol + milrinone group in the presence of

Table 1 Hemodynamic changes following intravenous infusion of esmolol + milrinone

Esmolol + Milrinone	25-min into ischemia (5-min before reperfusion)	10-min after completion of esmolol + milrinone infusion	P
HR	241±29	227±16	<0.01
Systolic BP	140±16	135±13	NS
Diastolic BP	120±5	110±6	NS
HR × BP	33636±2669	30661±1675	<0.01

Rp-cAMPS or Akt inhibitor were higher than that of saline control (Fig. 1). In ICA cell-free myocyte preparation, the effect of Rp-cAMPS on myocytes was not different from that of saline during hypoxia/re-oxygenation ($p=ns$, $n=6$, Fig. 2). The myocyte protective effect conferred by terbutaline + CGRP was abolished in the presence of Rp-cAMPS (Fig. 2).

Effect of esmolol + milrinone on myocardial PKA and Akt activation Five min after completing infusion of esmolol, milrinone or esmolol + milrinone, there was 22%, 28% and 59% increase in ischemic LV tissue PKA activity, respectively, compared with control (Fig. 3). In parallel with PKA changes, esmolol, milrinone and esmolol + milrinone treatment increased pAkt levels in ischemic LV tissue by 1.7-, 2.7-, and 6-fold, respectively. There was no increase in cardiac pAkt activity in those animals pretreated with Rp-cAMPS. Compared with saline treatment, Rp-cAMPS or esmolol + milrinone in the presence of Rp-cAMPS induced 0.8- and 0.9- fold pAkt changes ($p=ns$, Fig. 4).

Effect of esmolol + milrinone on myocyte apoptosis Early reperfusion treatment of LV myocardium with esmolol, milrinone or esmolol + milrinone reduced overall cardiocyte apoptosis by 23%, 37% and 60%, respectively, compared with saline control (Fig. 5). Myocyte-specific TUNEL staining demonstrated 28%, 40% and 67% reduction in apoptosis in esmolol, milrinone and esmolol + milrinone

Table 2 Hemodynamic changes following intravenous infusion of esmolol

Esmolol	25-min into ischemia (5-min before reperfusion)	10-min after completion of esmolol infusion	P
HR	240±28	218±16	<0.01
Systolic BP	142±16	140±13	NS
Diastolic BP	120±8	116±6	NS
HR x BP	34080±2568	28340±1675	<0.01

Table 3 Hemodynamic changes following intravenous infusion of milrinone

Milrinone	25-min into ischemia (5-min before reperfusion)	10-min after completion of milrinone infusion	P
HR	241±29	246±28	<0.05
Systolic BP	141±16	137±13	NS
Diastolic BP	120±5	115±6	NS
HR × BP	33980±2615	33702±2642	NS

treatment group, respectively, compared with saline control (Fig. 5).

Discussion

We have demonstrated for the first time that brief IV infusion of esmolol + milrinone at late-ischemia and early-reperfusion (i.e., bridging from end of coronary occlusion to re-canalization) confers robust additive LV-IS-reduction. This cardioprotective effect is associated with the activation of PKA/Akt-dependent antiapoptotic signaling.

Effect of esmolol + milrinone on LV-IS Ischemic preconditioning with milrinone reduces LV-IS in a canine reperfused AMI model [14]. However, until proven otherwise, the pharmacological ischemic preconditioning cannot generalize the same mechanism and outcome when the same agent is administered in ischemic postconditioning setting. The cardioprotective effect of intra-ischemic milrinone administration has not been fully established. The cellular mechanisms underlying milrinone-mediated ischemic postconditioning are unknown. Inconsistent results have been published with respect to the effects of intra-ischemic administration of milrinone on the heart [17, 20]. Intra-ischemic milrinone infusion in a non-reperfused canine AMI model failed to reduce LV-IS [20]. In contrast, a recent study reported that intra-ischemic milrinone infusion reduced right ventricular IS in a canine ischemia-reperfusion model [17], although the latter study provided no data on the effect of milrinone on LV-IS [17]. The lack of milrinone-induced LV-IS-reduction in non-reperfused AMI [20] could be explained by the lack of reperfusion injury, a process that would have been effectively targeted by milrinone. In an isolated perfused rabbit heart model, milrinone had no effect on LV-IS [21]. This could be due to the fact that isolated hearts already underwent a brief episode of ischemia preconditioning during the heart isolation procedure thereby blunting subsequent milrinone's effect. The

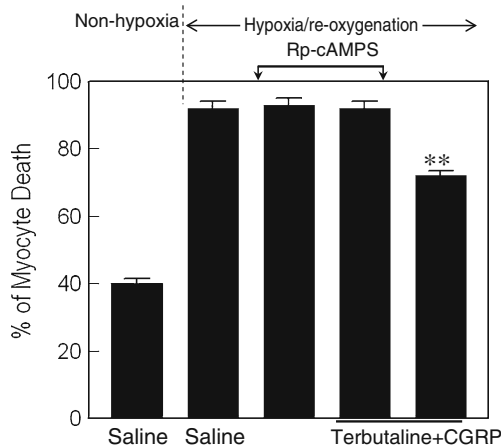


Fig. 2 Effect of Rp-cAMPS on myocyte survival during hypoxia/re-oxygenation in ICA cell-free ventricular myocyte culture. There is no difference in myocyte survival rates between saline and Rp-cAMPS groups ($p = ns$, $n=6/\text{group}$). Rp-cAMPS abolishes myocyte protective effect conferred by PKA agonists (Terbutaline + CGRP). Hypoxia/re-oxygenation markedly increases myocyte death rates compared with non-hypoxic condition in ICA cell-free myocyte culture. **: $p < 0.01$

present study is the first to show that late-ischemia/early reperfusion administration of milrinone reduced LV-IS. Furthermore, we demonstrated that early reperfusion administration of esmolol + milrinone conferred additive LV-IS-reduction. The cell protective effect conferred by esmolol + milrinone was confirmed in an isolated mixed myocyte preparation in the present study. Endogenous epinephrine derived from ICA cells confers significant myocardial protection against reperfusion injury through β_2 -AR stimulation [5, 6]. In consistent with this concept, the present study demonstrated that esmolol's IS-reduction is primarily mediated via the enhancement of cardiac β_2 -AR signaling. It has been demonstrated that myocardial interstitial epinephrine levels can increase by >100 fold in the early coronary

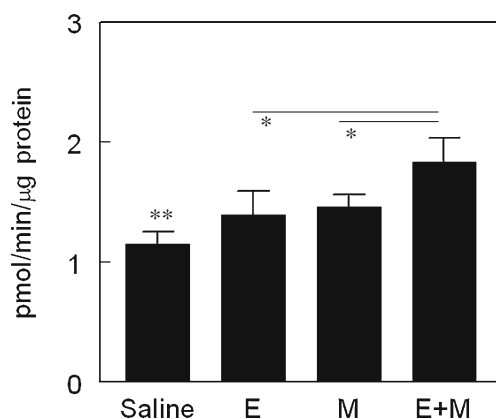


Fig. 3 Effects of reperfusion therapy with esmolol, milrinone or E + M on LV myocardial tissue PKA during ischemia/reperfusion ($n=6/\text{group}$). PKA activity in saline treated group is significantly lower than all other groups. *: $p < 0.05$, **: $p < 0.01$

reperfusion [16]. Thus, β_1 -AR blockade with esmolol during early reperfusion can exert unopposed β_2 -AR stimulation via endogenous epinephrine producing myocardial protection. Reduction in myocardial oxygen consumption by esmolol during ischemia can potentially contribute to LV-IS-reduction. However, cardioprotection conferred by esmolol + milrinone in the present study is unlikely due to its favorable hemodynamic profile as reflected by the low BP-HR double product, since esmolol alone which had comparable hemodynamic effect only produced modest LV-IS-reduction. The IS-reduction conferred by esmolol + milrinone can be reasonably attributed to their effects against reperfusion injury, since the drugs were given towards the very end of ischemia and the beginning of reperfusion.

Administration of Rp-cAMPS or Akt-inhibitor increases IS, compared with saline control. These data indicate that there is a basal tonic PKA-Akt signaling during ischemia/reperfusion in the heart. Disrupting this signaling pathway can result in catastrophic reperfusion injury. In the presence of Rp-cAMPS or Akt-inhibitor, esmolol/milrinone conferred no IS-reduction. This finding indicates that mechanism of esmolol/milrinone-induced heart protection is mediated through the PKA/Akt signaling cascade. The cardiac effects of Rp-cAMPS are unlikely to be non-specific in the present study. Our data (Fig. 2) indicates that Rp-cAMPS, in the absence of background extracellular ligand-mediated PKA stimulation, acts no more than saline on purified myocytes (depleted with ICA cells). In ICA cell-free myocyte preparation, Rp-cAMPS abolished myocyte protective effect conferred by terbutaline + CGRP, the agonists of β_2 -AR/CGRP-receptors known to stimulate PKA with IS reduction [6, 10]. These findings confirmed the specificity of Rp-cAMPS on PKA's inhibition and confirmed in vivo data that cardioprotection conferred by milrinone + esmolol is mediated specifically through the activation of cAMP-PKA dependent signaling pathway.

There are limited clinical therapies to reduce reperfusion injury in AMI [22]. Recent experiments demonstrated that cardiac PKA activation using concomitant β_2 -AR/CGRP-receptor stimulation with the receptor agonists (i.e., terbutaline and CGRP) at early reperfusion conferred additive cardioprotection with a 74% LV-IS-reduction compared with saline [10]. The present study investigates a more distal signaling step (i.e., skipping β_2 -AR/CGRP-receptor-G protein-coupling sequence) to directly activate downstream PKA. This strategy allows the most rapid PKA activation at the earliest reperfusion time. Milrinone is known to activate PKA in non-ischemic [13] and ischemic myocardium [14]. Esmolol may indirectly activate PKA at early reperfusion through enhanced endogenous β_2 -AR stimulation. Esmolol + milrinone infusion at 5-min before the onset of reperfusion offers a "priming" effect of PKA

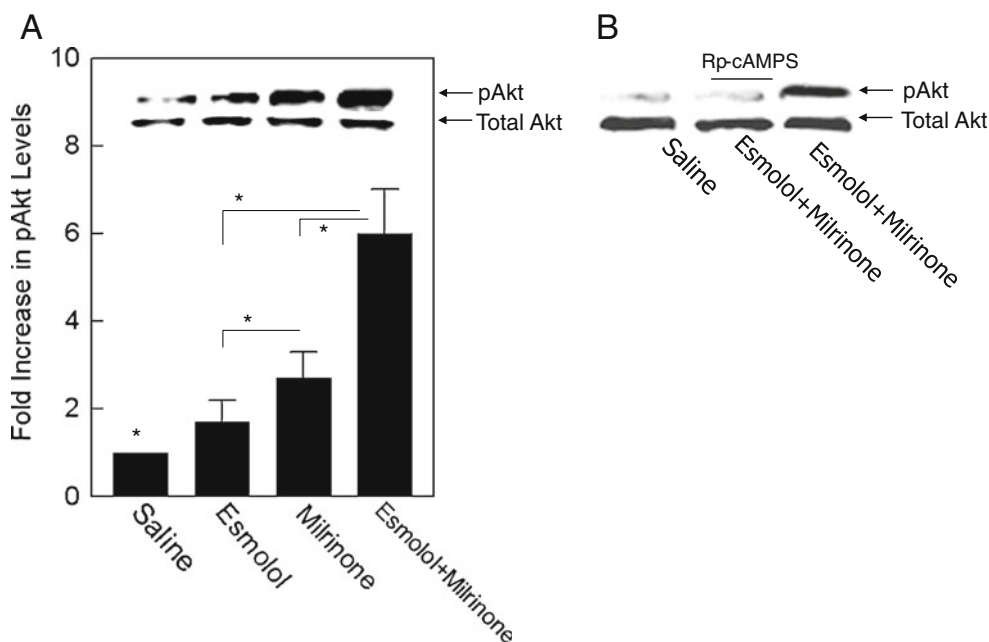


Fig. 4 Panel A: Effects of reperfusion therapy with esmolol, milrinone or E + M on myocardial tissue Akt activity during ischemia/reperfusion ($n=5/\text{group}$). The pAkt activity in saline treated group is significantly lower compared with all other group (**: $p < 0.01$). The phosphorylated Akt levels were normalized to total Akt. Insert: an individual case of myocardial tissue pAkt levels in response to different treatments. Panel B: A typical case demonstrating that in

the presence of PKA inhibitor Rp-cAMPS, E + M exerts no effect on myocardial pAkt activity. Overall, application of E + M increases myocardial pAkt activity by 7 ± 1 -fold, compared with saline ($p < 0.01$, $n=4/\text{group}$). In the presence of Rp-cAMPS, E + M has no effect on myocardial tissue pAkt activity during ischemia/reperfusion, compared with saline ($n=4/\text{group}$)

activation via collateral delivery of drugs to the ischemic zone. Even in the absence of collaterals to the ischemic zone, the fully circulated drugs in the blood stream can mount the quickest action upon reperfusion without missing the critical first seconds of “window-of-opportunity”.

Antiapoptotic pathways involved in esmolol + milrinone-mediated myocardial protection Milrinone increases cytosolic cAMP production which induces PKA activation [13, 14]. PKA activation can, in turn, activate Akt-dependent antiapoptotic pathway [6, 23]. It is unclear why the level of Akt activation is much more robust than those of PKA activation in the present study. We speculated that myocardial Akt activation may not be easily triggered at baseline despite relatively high basal PKA activity in living animals. Under extreme circumstances, further increase in PKA activity beyond a critically high threshold may trigger robust Akt activation. The present study has demonstrated proof-of-the-concept that esmolol + milrinone-induced Akt activation is dependent on PKA stimulation, since in the presence of PKA inhibition with Rp-cAMPS, milrinone + esmolol failed to activate Akt (Fig. 4). Furthermore, the down stream Akt activation plays a critical role in myocardial protection, since the inhibition of Akt abolished esmolol + milrinone-induced myocardial protection. The fact that the larger than control LV-IS following PKA or

Akt inhibition suggests that ischemia-reperfusion *per se* can trigger a small but significant amount of PKA/Akt-dependent antiapoptotic signaling which can be readily enhanced pharmacologically during the very early phase of reperfusion.

The β_1 -AR blocker is the standard of therapy for patients with AMI to improve long-term survival [24–26]. The mechanisms underlying cardioprotection of β_1 -AR blocker may not be simply attributed to its reducing myocardial oxygen consumption or blocking β_1 -AR signaling which is detrimental to the heart. It has been well recognized that β_1 -AR and β_2 -AR signaling are counter-regulatory [11, 12]. Myocardial β_1 -AR inhibition greatly enhances cardioprotective β_2 -AR signaling which activates antiapoptotic pathway [11, 12]. Selective β_1 -AR blockade with esmolol significantly enhanced milrinone-mediated myocardial protection. These results underscore the importance of intrinsic cardiac epinephrine- β_2 -AR signaling derived from ICA cells providing rich myocardial epinephrine [5, 6]. Furthermore, our findings indicate that activation of β_2 -AR in concert with direct PKA activation with milrinone confers powerful heart protection. Accumulating evidence supports a concept that apoptosis contributes substantially to the extent of IS in the reperfused AMI [2]. In agreement with this, the ratios of IS-reduction

Fig. 5 (a) Effects of reperfusion therapy with esmolol, milrinone or E + M on LV myocyte apoptosis during ischemia/reperfusion. Panels A-C show a case of total cardiocyte apoptosis in a saline-treated heart.

A: Propidium iodide-positive nuclei (red) of cardiocytes.

B: TUNEL-positive nuclei (green, arrows) in the same field. **C:** Superimposed panels A and B. Panels D-F show a case of cardiocyte apoptosis in an E + M-treated heart.

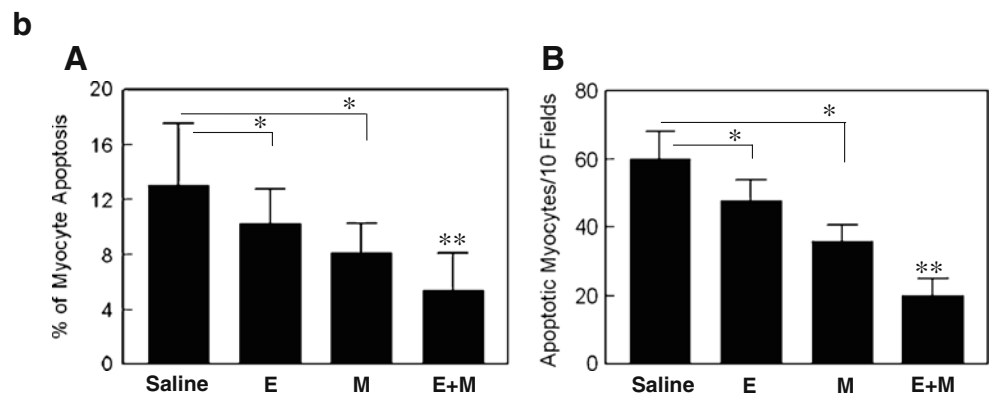
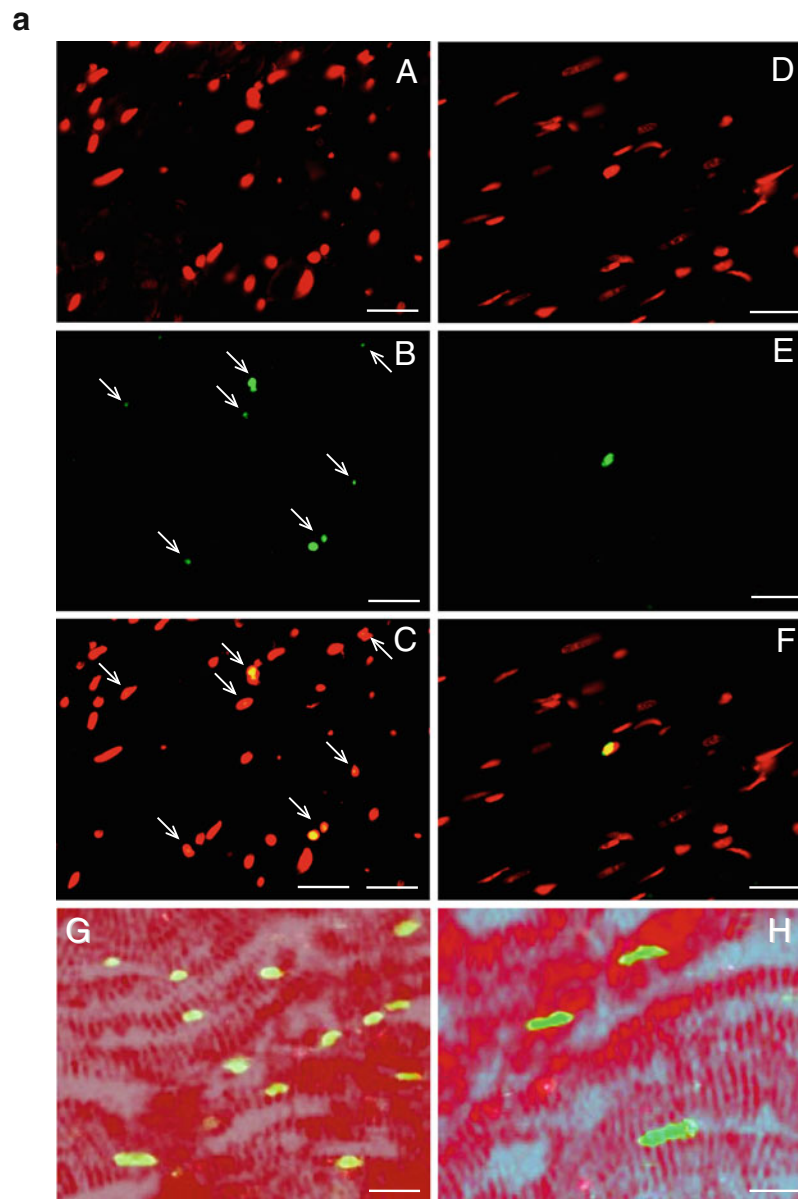
D: Propidium iodide-positive nuclei of cardiocytes.

E: TUNEL-positive nuclei in the same field of D.

F: Superimposed panels D and E. Panels

G-H: Immunohistochemical double staining confirms myocyte (red)-specific apoptosis in a field of saline-treated heart (G) and field esmolol + milrinone-treated heart (H). TUNEL positive nuclei are stained with green color. Scale bar=10 μm .

(b): A: Percentage changes in cardiocyte apoptosis in the hearts treated with saline, esmolol, milrinone and E + M, respectively ($n=6/\text{group}$). The cardiocyte apoptosis rate in hearts treated with E + M is smaller than all other groups ($p<0.01$). B: Comparison of myocyte-specific apoptosis among 4 different treatment groups. The extent of ventricular myocyte apoptosis in esmolol + milrinone group is significantly smaller than all other groups ($p<0.01$). *: $p<0.05$, **: $p<0.01$



between esmolol + milrinone and other treatment groups are in the similar orders of the ratios of myocyte apoptosis reduction in their counterparts. The absolute magnitudes of apoptosis do not match those of LV-IS. This discrepancy might be due to several reasons. First, the immunostaining might not be sensitive enough to detect all apoptotic nuclei, especially those of early phase. Second, myocyte necrosis certainly contributes to substantial amount of total LV-IS. Nevertheless, the present study provides proof-of-the-concept that anti-apoptotic effect elicited by esmolol + milrinone contributes significantly to the reduced reperfusion injury.

Perspectives There is no clinically approved drug that can effectively reduce reperfusion injury during AMI. For non-cardioprotective purpose, esmolol or milrinone was used individually with good tolerance in patients with AMI with or without reperfusion therapy [27, 28]. Although the cardioprotective efficacy of combination therapy with esmolol + milrinone has not been tested clinically, their remarkable preclinical LV-IS-limiting effect, if confirmed in human study, may make this novel combination a promising candidate for transition to the clinical arena.

Study limitation The present study cannot deduce that cardioprotective results of esmolol+milrinone are class effects of β_1 -AR blockers and PDE inhibitors. The reason for selecting esmolol vs. other β_1 -selective AR blockers is that esmolol possesses ultra short half-life providing favorable hemodynamic profile when combined with milrinone infusion. Further studies are needed to test whether other β_1 -AR blockers and PDE inhibitors confer similar cardioprotection. While the PKA phosphorylation was used to determine PKA activity in the present study, we have not assessed other molecular markers associated with PKA activation such as cytosolic cAMP levels or phosphorylation of the L-type calcium channels.

In conclusion, late-ischemia/early reperfusion therapy with combination of esmolol + milrinone confers additive LV-IS-reduction in experimental AMI. This cardioprotective effect is associated with a robust induction of antiapoptotic signaling pathway.

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Conflict of Interest No potential interest conflicts.

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