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Rapamycin protects heart from ischemia/reperfusion injury independent of autophagy by activating PI3 kinase-Akt pathway and mitochondria K_{ATP} channel

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Objectives: The purpose of this study was to investigate potential roles of rapamycin, a macrocytic lactone produced by *Streptomyces hygroscopicus*, in myocardial ischemia/reperfusion (I/R) injury. *Methods:* Male Wistar rats were pretreated with three different doses of rapamycin (0.25, 2, and 5 mg/kg). Then, isolated rat hearts were exposed to 40 min of global ischemia followed by 120 min of reperfusion using a Langendorff apparatus. Western blot analysis was used to examine changes in the expression levels of $ERK_{1/2}$ and Akt kinases and LC_3 –II/I (a marker of autophagy). The area of myocardial infarction and cardiac function were evaluated. *Results*: Our results demonstrated that rapamycin mediates cardioprotection in a dose-dependent manner in isolated rat hearts during myocardial I/R injury. Significant a autophagy was induced by rapamycin during I/R. Both, the mitochondrial K_{ATP}-channel blocker 5-hydroxydecanoate (5-HD) and the PI3K inhibitor LY294002 (LY) abolished the protection afforded by rapamycin completely, while the inhibitors alone did not influence the infarct size in control hearts. However, the ERK1/2 inhibitor PD98059(PD) and the blocker of autophagy 3-methyladenine (3-MA) had no effect on rapamycin-mediated cardioprtection. *Conclusions:* Cardioprotection afforded by rapamycin involves the PI3K pathway and the activation of mitochondrial K_{ATP}-channels, but is independent of rapamycin-induced autophagy. This study may have significant impact on clinical practice.

1. Introduction

According to the World Health Organization, acute coronary occlusion is the leading cause of morbidity and mortality in the Western world and will continue to be the major cause of death in the world by the year 2020 (Murray and Lopez 1997). Reperfusion therapy is beneficial to prevent cardiomyocyte death and contractile dysfunction after myocardial ischemia (MI). However, numerous studies have shown that reperfusion itself may enhance the injury, resulting in extension of infarct size after ischemia (i.e., ischemia/reperfusion (I/R) injury). Currently, It has been widely accepted that cardiac ischemic preconditioning (IP) can attenuate both contractile dysfunction and development of myocardial infarction following an I/R insult (Yellon and Downey 2003). However, IP has its limitations in that preconditioning mimetic must be applied before the index ischemic event, which is unpredictable and impractical in the clinical setting of acute myocardial infarction. Accordingly, pharmacological preconditioning was developed. Therefore, the identification and characterisation of agents that can protect the heart from the damaging effects of I/R are of considerable importance. In the past years, many pharmacological agents, such as opioids, bradykinin and adrenaline, have been shown to induce a preconditioning-like effect (Ryan et al. 1999; Goto et al. 1995; Liu et al. 1991). Recently, it was reported that rapamycin also possesses this effect. However, such a claim is not without its controversies (Khan et al. 2006). Further studies are required to elucidate the signaling pathways that lead to the cardioprotective effect of rapamycin and to demonstrate the clinical benefits of the drug in patients with coronary artery disease.

Rapamycin, a macrocytic lactone produced by *Streptomyces hygroscopicus*, has immunosuppressive, antimicrobial and antitumor properties. It binds intracellularly to FK506 binding protein and targets the protein kinase mammalian target of rapamycin (mTOR). An interesting question is whether rapamycin mediates all of its effects through mTOR. To date, there are no known alternative targets of rapamycin other than FK506 binding protein 12. It is also well known that inhibition of phosphorylation of mTOR by rapamycin can activate autophagy (Ravikumar et al. 2004). Autophagy is an evolutionary conserved process involved in degradation of long-lived proteins and excess or dysfunctional organelles. Autophagy occurs at low basal levels under normal conditions in the heart, but is rapidly upregulated in response to stress such as nutrient deprivation, hypoxia and pressure overload (Shintani and Klionsky 2004). It allows cells to recoup ATP and essentially building blocks for biosynthesis when they are starved of nutrients or when they are exposed to hypoxia. Many studies support the

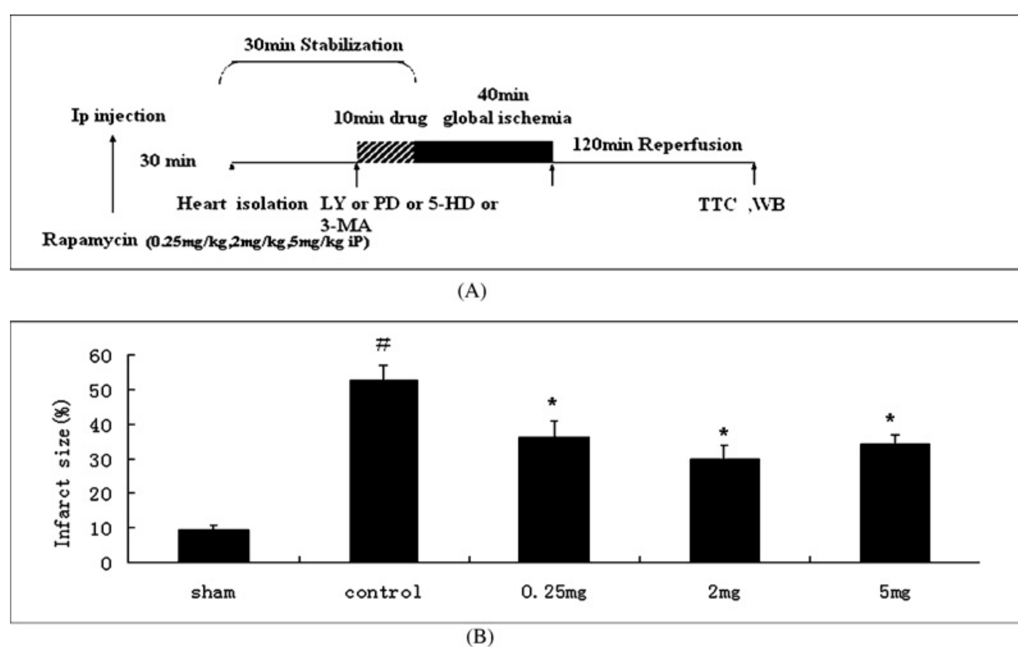

Fig. 1: Protocol and infarct size. A, Experimental protocol. B, rapamycin reduces infarct size in isolated heart. Results are given as mean ± SEM (each group n = 6 to 8). *$P < 0.05$ versus control group.# $P < 0.05$ versus sham group

hypothesis that upregulation of autophagy provides protection against I/R injury (Shintani and Klionsky 2004; Hamacher et al. 2007; Matsui et al. 2007). It is still unkown, whether rapamycin, as a potent stimulator of autophagy, provides protection against I/R injury by autophagy or by activating other signaling pathways. In the present study, we sought to delineate whether rapamycin mediates its protective effects via autophagy and to explore the possible mechanism of preconditioning-like protection of rapamycin against I/R injury.

2. Investigations and results

2.1. *Effect of rapamycin on myocardial infarct size*

To determine whether rapamycin is capable of attenuating myocardial infarction (MI) in this isolated mouse model of ischemia/reperfusion injury, we compared wild-type hearts, in the presence the solute vehicle, DMSO (final concentration <1%) injected 30 min before stabilization, with incremental doses of rapamycin. Over a dose range of 0.25 to 5 mg/kg, rapamycin significantly attenuated infarction, with maximal protection observed with the 2 mg/kg dose. Figure 1 B presents the average infarct sizes for the rapamycin-treated groups. Infarct size was significantly reduced in the rapamycin-treated groups (0.25, 2, 5 mg/kg Ra) compared with control group (36.5 ± 4.6%, 29.9 ± 4.1%, 34.3 ± 2.6% versus 52.54 ± 4.5%).

2.2. *Haemodynamic data*

The Table summarizes the CF, HR and LVDP data as recorded from the four experimental groups during the stabilization and reperfusion periods. Ischemia/reperfusion markedly reduced all functional variables and coronary flow. Hearts treated with rapamycin did not exhibit improvement in post-ischemic functional recovery and coronary flow as compared with hearts that remained untreated.

2.3. *The role of autophagy in rapamycin-treated groups*

To determine whether rapamycin induced autophagy in the heart or not, isolated rat hearts were exposed to different doses of rapamycin and the distribution of autophagosomes (LC3 dots) was assessed by immunostaining (Figure 2A). During the induction of autophagy, LC3 is proteolytically processed by Atg4 to expose a terminal glycine (LC3-I) and then is conjugated to phosphatidylethanolamine by Atg7, a specialized ubiquitin ligase. The lipidated LC3 is membrane-associated and has an altered mobility on SDS-PAGE (LC3-II). The conversion of LC3-I to LC3-II reflects autophagic flux. Rapamycin resulted in a significant increase in the ratio of LC3-II/I (Fig. 2B). 3-MA (an inhibitor of autophagy) pretreatment reduced rapamycin-induced autophagy. However, as shown in Figure 2C, 3-MA did not diminish rapamycin-induced cardioprotection (RM 38.7 ± 4.6% versus control group 52.54 ± 4.5%). These results demonstrated that although autophagy was remarkably induced by rapamycin during myocardial I/R, it did not play a key role in rapamycin-induced cardioprotection.

2.4. *Rapamycin activates phosphatidylinositol 3-kinase (PI3K)–Akt signaling pathways and induces opening of mitochondrial K_{ATP} channels in reperfused myocardium*

To explore the effect of rapamycin on reperfusion injury salvage kinases pathway, including phosphatidylinositol 3-kinase (PI3K)–Akt and extracellularsignal-regulated kinase (ERK), we investigated the phosphorylation states of $ERK_{1/2}$ and Akt in rapamycin-treated groups. Extracts were rapidly prepared in the presence of phosphatase and protease inhibitors. Proteins were separated by SDS-PAGE before western blotting with antibodies against the phosphorylated and total kinases, respectively. Representative blots and mean values for the ratio of phosphorylated to total protein are shown in Fig. 3. Significant increases in Akt phosphorylation relative to control hearts were detected in rapamycin-treated groups. However, no significant effects of rapamycin on $ERK_{1/2}$ phosphorylation were detected. The infarct reduction afforded by rapamycin was abolished completely in the presence of the PI3K inhibitor, LY294002 and the blocker of mitochondrial K_{ATP} channels, 5-HD (0.25 mg Ra 36.5 ± 4.6% versus 0.25 mg Ra + LY 48 ± 2%, $P < 0.05$; 0.25 mg Ra 36.5 ± 4.6% versus 0.25 mg Ra + 5-HD 52.8 ± 2.4%, $P < 0.05$; Fig. 4). The inhibitor of $ERK_{1/2}$, PD98059 did not influence infarct size in

Table: Effect of various pharmacological interventions on the cardiac performance and coronary flow

	Group	Baseline	Reperfusion		
			Immediate	60 min	120 min
CF (ml /min)	control	7.5 ± 0.7	$3.0 \pm 0.4^*$	$3.9 \pm 0.4^*$	$2.8 \pm 0.2^*$
	0.25 mg Ra	7.6 ± 0.6	$2.9 \pm 0.3^*$	$4.1 \pm 0.3^*$	$2.8 \pm 0.3^*$
	2 mg Ra	7.5 ± 0.1	$3.0 \pm 0.2^*$	$3.8 \pm 0.2^*$	$2.8 \pm 0.2^*$
	5 mg Ra	7.6 ± 0.3	$2.9 \pm 0.2^*$	$3.8 \pm 0.1^*$	$3.0 \pm 0.1^*$
HR (beats/min)	control	276 ± 8.0	$237 \pm 12^*$	$264 \pm 12^*$	$247 \pm 13^*$
	0.25 mg Ra	277 ± 10	$242 \pm 13^*$	$262 \pm 9.0^*$	$248 \pm 10^*$
	2 mg Ra	283 ± 13	$245 \pm 11^*$	$274 \pm 11^*$	$246 \pm 8.0^*$
	5 mg Ra	282 ± 10	$242 \pm 7^*$	$261 \pm 10^*$	$251 \pm 9.0^*$
LVDP (mmHg)	control	102 ± 5	$30 \pm 6^*$	$48 \pm 4.0^*$	$43 \pm 5.0^*$
	0.25 mg Ra	101 ± 5	$29 \pm 4^*$	$49 \pm 4.0^*$	$44 \pm 4.0^*$
	2 mg Ra	100 ± 4	$29 \pm 2^*$	$49 \pm 3.0^*$	$42 \pm 2.0^*$
	5 mg Ra	100 ± 5	$30 \pm 3^*$	$48 \pm 2.0^*$	$39 \pm 2.0^*$

Data are presented as mean ± SD (n ⩾ 6 for each group), * $p < 0.05$ VS respective baseline

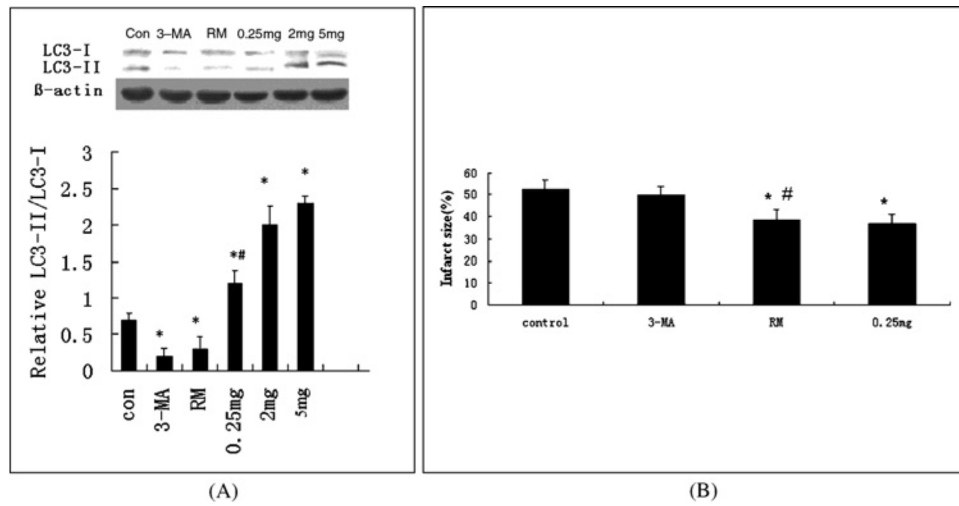

Fig. 2: Rapamycin induces autophagy and the role of autophagy in rapamycin-mediated cardioprotection. A.The gels represent the Western blot results of LC3-I and LC3-II protein expression in the groups of con, 3-MA, RM, 0.25 mg, 2 mg and 5 mg groups .Representative Results are from 4 independent experiments. values are given as means ± SEM, * $P < 0.05$ versus control group, # $p < 0.05$ versus RM. B. Infarct size. * $P < 0.05$ VS control group, # $p < 0.05$ versus 3-MA group,. Values are given as mean ± SEM (each group n = 6 to 8)

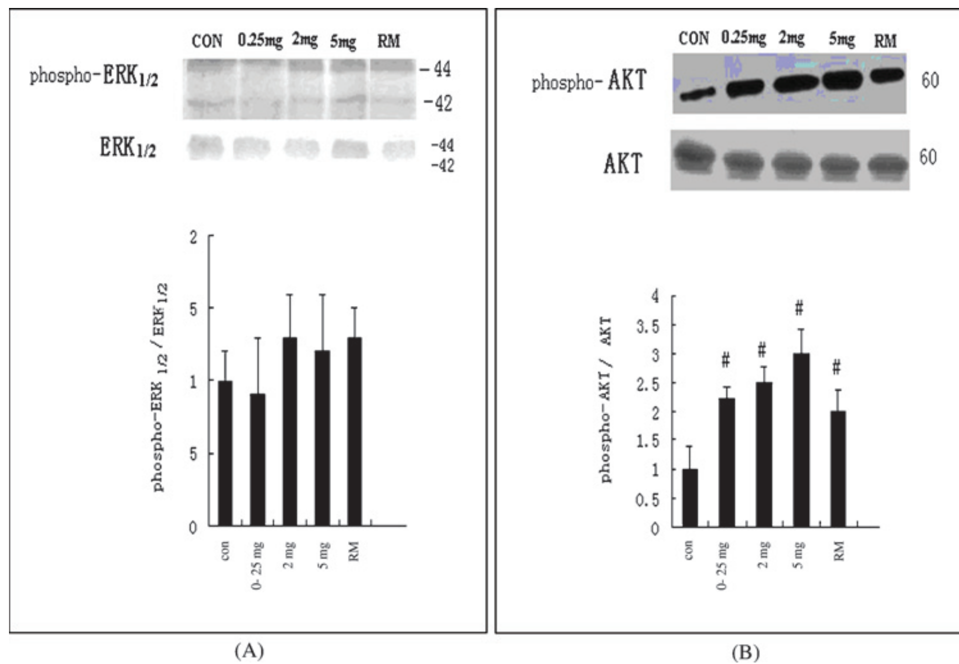

Fig. 3: Rapamycin–mediated signaling pathway in isolated I/R rat hearts. A. Phosphorylation of Extracellular signal-regulated kinase ($ERK_{1/2}$) in reperfused hearts. B. Phosphorylation of phosphatidylinositol 3-kinase (PI3K)–Akt in reperfused hearts. Values are given as means ± SEM (each group n = 4 to 5). # $P < 0.05$ versus control group. Representative results from 4-5 independent experiments were shown

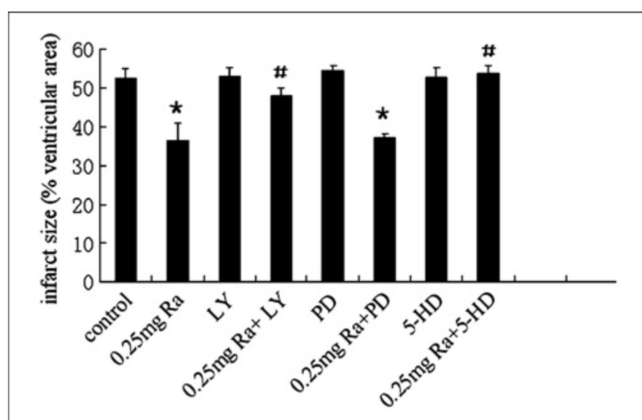

Fig. 4: Effects of 0.25 mg/kg Ra in the absence or presence of phosphatidylinositol 3-kinase inhibitor, LY 294002 (15 μmol/L), the blocker of 5-hydroxydecanoate (5-HD, 100 μmol/L) or the $ERK_{1/2}$ inhibitor, PD98059 (5 mmol/L) on infarct size. * $P < 0.05$ versus control group. # $P < 0.05$ versus 0.25 mg Ra group

control or rapamycin-treated group (DMSO 52.6 ± 2.3% versus PD + DMSO 54.6 ± 1.2%, $P > 0.05$; 0.25 mg Ra 36.5 ± 4.6% versus 0.25 mg Ra +PD 37.3 ± 0.9%, $p > 0.05$; Fig. 4). LY and 5-HD did not influence infarct size compared with control group (LY 53 ± 2.3%; 5-HD 52.8 ± 2.4% versus control 52.6 ± 2.3%, $p > 0.05$; Fig. 4).

3. Discussion

The main findings of this study are summarized in the following three points: (1) Rapamycin mediates cardioprotection in a dose-dependent manner in isolated rat hearts, (2) rapamycin exerted no deleterious effects on post-ischemic contractile dysfunction (stunning) in perfused rat hearts as measured after reperfusion and (3) cardioprotection afforded by rapamycin against I/R injury may be due to involve activations of the PI3 kinase (PI3K)-Akt signaling pathway and mitochondria K_{ATP} channels and appears to be independent of rapamycin-mediated autophagy.

Rapamycin (Sirolimus) is an immunosuppressant macrolide antibiotic which has been approved for clinical use in the prevention of renal transplant rejection and heart transplant rejection and vasculopathy (Kahan et al. 2000; Keogh et al. 2004). Rapamycin has also been used as a coating agent in stents used in coronary angioplasty to prevent restenosis and has been shown to be effective in preventing recurrent in-stent stenosis by intensive oral administration (OSIRIS Trial) (Hausleiter et al. 2004). However, many other properties of rapamycin are still not fully elucidated. Usually, rapamycin is used as an inhibitor of mTOR during I/R. Few studies have been directed toward examining the effect of rapamycin as a pharmacological preconditioning agent for use in myocardial I/R injury. As far as we are aware, the only other study is that of Khan et al. (2006), who studied the effect of rapamycin on I/R injury in Langendorff-perfused hearts. Their study demonstrated that pretreatment with rapamycin (0.25 mg/kg) during I/R reduced myocardial infarct size. Unfortunately, these authors did neither investigate the dose-response parameters of rapamycin upon cardioprotection function, nor was the potential for rapamycin to activate autophagy during I/R assessed in this report. In the present study, we estimated myocardial damage by means of direct measurements of infarct size, using triphenyltetrazolium chloride (TTC) staining and evaluated cardial function with CF, HR and LVDP. With our model we found that I/R markedly reduced all functional parameters or cardiac activity and coronary flow as summarized in the Table. Hearts which were treated with rapamycin exhibited no beneficial effects with regard to postischemic functional recovery, nor was there any difference in coronary flow as compared with control group. these results are in accord with previous findings (Khan et al. 2006). Besides, the present study shows that rapamycin mediates cardioprotection in a dose-dependent manner in isolated perfused rat hearts. Over a dose range of 0.25 to 5 mg/kg, rapamycin significantly attenuated infarction, with maximal protection observed with the 2 mg/kg dose. These findings at first seem divergent from observations made by Raphael et al. (2008). However, there are some clear explanations that reconcile these seemingly conflicting differences. First of all, species differences may be one of those factors. In their study, rabbits were used. However, we have employed rats in our study. Another compelling explanation may be due to the model of ischemia/reperfusion injury. In the present study, the Langendorff system has been employed because it permits the use of pharmacological interventions without any interference due to changes in systemic circulation. Others have also reported an absence of rapamycin effects upon reperfusion as rapamycin perfused during reperfusion had no effect on reducing infarct size (Kozma and Thomas 2002; Hausenloy et al. 2005). It seems likely that rapamycin is activating different mechanistic pathways to exert such differential effects upon cardiac function. During ischemia, rapamycin is capable of functioning as a cardioprotectant and while at reperfusion it may be involved in blocking survival through activation of kinase pathways. It is clear that much additional work on this topic is required to establish an effective concentration range of protection and the molecular mechanisms involved with the differential effects of rapamycin upon ischemia and reperfusion.

As a potent stimulator of autophagy by inhibiting mTOR, rapamycin provides protection against I/R injury in Langendorff perfused rat hearts (Khan et al. 2006). But the exact mechanistic relationship between autophagy and its activation by rapamycin is still unknown. Autophagy represents an important process in cardiac function which is responsible for the normal turnover of long lived proteins and organelles. Autophagy is a prominent feature of myocardial ischemia and reperfusion (Decker and Wildenthal 1980; Sybers et al. 1976) and although enhanced autophagy is often seen in dying cardiac myocytes, the functional significance of autophagy during I/R is not clear. Upregulation of autophagy has been reported to both protect and cause the death of cardiac cells (Hamacher et al. 2007; Matsui et al. 2007). To explore the role of autophagy activation by rapamycin, we used 3-MA, a specific inhibitor of autophagy, to block autophagy and measured autophagy responses by immunoprecipitation analysis of myocardial LC3, a specific marker of autophagy. Our results showed that 3-MA inhibited rapamycine-induced autophagy during I/R, but did not diminish the cardioprotective capacity of rapamycin. We can offer two potential reasons as to the complex role of autophagy in cardiac functions during I/R. First, autophagy may function differently during ischemia and reperfusion. These are supporting data from recent studies which suggest that autophagic activation during myocardial ischemia, when the heart is "starved", is beneficial. Conversely, autophagic induction following restoration of blood flow (reperfusion) may be harmful. Under such conditions, a bi-directional effect of autophagy as activated by rapamycin may result in these opposing effects upon cardiac function. A second possible explanation is that autophagic induction may activate different processes depending on the nature of the triggering stress and extent of autophagic activity. The dose of rapamycin used in our experiment may not have been sufficient to induce beneficial effects of autophagy. These possibilities await validation in future studies.

In view of the protective effect of rapamycin and its potential therapeutic importance, we have further investigated the mechanisms underlying the protection it mediates. Protection by many

pharmaceutical preconditioning agents during I/R involves activation of pro-survival kinases such as members of the MAPK family and the PI3K-Akt cascade (Tsang et al. 2004). In other research paradigms, like that in tumor models, it was reported that rapamycin can activate AKT (Shi et al. 2005). Based upon these findings, we propose that rapamycin may play its protective role by activating PI3K-Akt or $ERK_{1/2}$. In the present study, no increases in $ERK_{1/2}$ and $ERK_{1/2}$ phosphorylation by rapamycin was observed, however a clear increase in Akt phosphorylation was obtained with rapamycin-treated hearts during I/R. Therefore, our results demonstrate the existence of several intriguing similarities in the signaling pathways involved in the protective effects of rapamycin and ischemic preconditioning. Different mechanisms are to be suspected. Activation of Akt during I/R has been reported in numerous studies (Hausenloy and Yelon 2004; Yang et al. 2005). This activation is enhanced by protective events such as preconditioning and opioid agonists. In the present study, rapamycin enhanced the activation of p-Akt. Moreover, inhibition of either mitochondrial K_{ATP} channel or Akt activation abolished rapamycin-mediated cardioprotection. Therefore, it is possible that rapamycin is involved in the cross-talk between the survival kinases through inhibition of mTOR that may result in the upregulation of other pro-survival kinases such as the PI3K/Akt pathways to further affect the opening of mitochondrial K_{ATP} channels.

In conclusion, the present study shows that rapamycin has a strong protective effect against myocardial I/R injury in rats. This study is the first to demonstrate that rapamycin-mediated cardioprotection is independent of autophagy. The most probable mechanism involves the PI3K pathway and the activation of mitochondrial KATP-channels.

4. Experimental

4.1. Animals

Adult male wistar rats weighing 200–350 g were supplied by the Center for Experimental Animals of Haerbin Medical University. The project was approved by the committee of experimental animals of Haerbin Medical University. All procedures were carried out according to the National Institutes of Health guidelines for the care and use of the laboratory animals.

4.2. Drugs and chemicals

Rapamycin was purchased from Sigma-Aldrich and was dissolved in DMSO for intraperitoneal injection (final DMSO concentration < 1%). Unless specified otherwise, all other chemicals including 5-hydroxydecanoate (5-HD), 3-methyladenine (3-MA), LY294002, PD98059 and triphenyltetrazolium-chloride (TTC) were obtained from Sigma-Aldrich.

4.3. Treatment of animals and experimental protocols

The experimental protocols for these studies are presented in Fig. 1 A. The experimental protocol consisted of 30 min stabilization followed by 40 min global ischemia followed by 120 min reperfusion. During the last 10 min of stabilization, the hearts were randomized to intracoronary infusion of either K–H buffer or other drugs (3-MA, LY294002, PD98059, 5-HD) through a side arm of a three-way stopcock connected directly above the aortic cannula with a Harvard microdialysis syringe pump (model 22). All the drugs were dissolved in dimethyl sulfoxide (DMSO) and added to Krebs-Henseleit (K-H) bicarbonate buffer (in mmol: NaCl 118, KCl 4.7, $CaCl_2$ 1.7, $NaHCO_3$ 25, KH_2PO_4 0.36, $MgSO_4$ 1.2 and glucose 10) so that the final DMSO concentration was <0.005%. The heart tissues collected from animals were randomly assigned to one of the following treated groups: (1) Sham group (n = 6): isolated rat heart was perfused with K-H solution for 160 min after stabilization for 30 min; (2) control group (n = 8): injection of volume-matched DMSO (solvent for rapamycin, ip) 30 min prior to heart isolation and subjected to 30 min of stabilization on the Langendorff system then subjected to 40 min global ischemia followed by reperfusion for 120 min; (3) 0.25 mg Ra group (n = 7): injection of rapamycin (0.25 mg/kg, ip) 30 min prior to heart isolation and subjected to 30 min of stabilization on the Langendorff system then subjected to 40 min global ischemia followed by reperfusion for 120 min; (4) 2 mg/kg Ra group (n = 6): injection of rapamycin (2 mg/kg, ip) 30 min prior to heart isolation and subjected to 30 min of stabilization on the Langendorff system then subjected to 40 min global ischemia followed by reperfusion for 120 min; (5) 5 mg/kg Ra group (n = 7): injection of rapamycin (5 mg/kg, ip) 30 min prior to heart isolation and subjected to 30 min of stabilization on the Langendorff system then subjected to 40 min global ischemia followed by reperfusion for 120 min; (6) 3-MA group (n = 7): administration of 3-MA (an inhibitor of autophagy, 10 mmol/L) for 10 min before ischemia then subjected to 40 min global ischemia followed by reperfusion for 120 min; (7) RM group (n = 6): injection of rapamycin (0.25 mg/kg, ip) 30 min prior to heart isolation and hearts with the inhibitor of autophagy 3-MA (10 mmol/L), given for 10 min before ischemia then subjected to 40 min global ischemia followed by reperfusion for 120 min; (8) 0.25 mg rapamycin+ LY294002 (LY) group (n = 8): injection of rapamycin (0.25 mg/kg, ip) 30 min prior to heart isolation and hearts with the PI3K inhibitor LY (15 μmol/L), given for 10 min before ischemia then subjected to 40 min global ischemia followed by reperfusion for 120 min; (9) LY group (n = 7): hearts with LY given for 10 min before ischemia then subjected to 40 min global ischemia followed by reperfusion for 120 min; (10) 0.25 mg rapamycin +PD98059 (PD) group (n = 6): injection of rapamycin (0.25 mg/kg,ip) 30 min prior to heart isolation and hearts with the $ERK_{1/2}$ inhibitor PD98059 (5 μmol/L), given for 10 min before ischemia then subjected to 40 min global ischemia followed by reperfusion for 120 min; (11) PD group (n = 6): hearts with PD given for the 10 min before ischemia then subjected to 40 min global ischemia followed by reperfusion for 120 min; (12) 0.25 mg rapamycin +5-HD group (n = 6): injection of rapamycin (0.25 mg/kg,ip) 30 min prior to heart isolation and heart with the blocker of mitochondrial K_{ATP} channel 5-HD (100 μmol/L) given for 10 min before ischemia then subjected to 40 min global ischemia followed by reperfusion for 120 min; (13) 5-HD group (n = 6): heart with the blocker of mitochondrial K_{ATP} channel 5-HD given for 10 min before ischemia then subjected to 40 min global ischemia followed by reperfusion for 120 min. Additionally, heart tissues from 3 to 5 rats in related groups were used to perform Western blot analysis.

4.4. Isolation and preparation of heart tissue

Rats were anesthetized with an intraperitoneal injection of pentobarbital sodium (60 mg/kg body weight) and were given heparin intravenous (500 IU/kg). After thoracotomy, the heart tissue was excised, and placed in ice-cold perfusion buffer. Immediately after isolation of heart tissue, the aorta was cannulated and perfused according to procedures described by the Langendorff method. Specifically, the heart was perfused (at 37.8 °C) for a 5-min washout period at a constant perfusion pressure equivalent to 100 cm of water (10 kPa). The perfusion medium consisted of a modified Krebs-Henseleit (K-H) bicarbonate buffer was equilibrated with 95% O_2 and 5% CO_2 to achieve a pH of 7.4. The Langendorff preparation was switched to the working mode after the washout period, as previously described in detail by Yamamoto et al. (1984). Hearts were immersed in a chamber containing perfusate maintained at a constant 37 °C. During the perfusion period, hearts were not electrically paced.

4.5. Measurement of heart function

Before ischemia and during reperfusion, heart rate (HR) and coronary flow (CF) were monitored. The coronary flow rate was calculated by collecting the efflux perfusate within certain time period. Left ventricle developed pressure (LVDP) was measured by means of a non-elastic water-filled balloon inserted into the left ventricle via the left atrium (adjusted to obtain an end-diastolic pressure of 5-7 mm Hg) and connected to a pressure transducer (MLP844 Physiological Pressure Transducer, AD Instruments). Then LVDP and HR were registered and captured by the computer acquisition system.

4.6. Measurement of infarct size

Following 2 h reperfusion, hearts were lowered into the organ bath. Triphenyltetrazolium chloride (TTC) solution (1%) was injected down the side arm of the aortic cannula and infused into the coronary circulation. Once the hearts had been stained dark red they were removed, blotted dry and weighed. They were then frozen and stored at −80 °C. The following day, the heart tissues were defrosted and sliced into 1 mm sections in parallel to the atrioventricular groove and then fixed in 2% formaldehyde overnight. Slices were then compressed between plexiglass plates 0.57 mm apart and a magnified video image was generated by a specially modified camera. The image was digitized (Apple Macintosh 7500) and the area of infarction and ventricular area (the entire LV and RV were at risk) were planimetered using image analysis software (NIH Image v1.61). Graph paper (1 mm squares) was used to calibrate the image areas, which were then multiplied by the slice thickness (0.57 mm) to generate the planimetered volume.

4.7. *Western blot analysis*

Proteins prepared from rat hearts were quantified by Bio-Rad protein assay. For immunodetection, 50 µg of crude cytosol prepared as above were resolved on SDS-PAGE 10% denaturing gels and transferred to PVDF nylon membranes. The membranes were blocked with 5% nonfat dry milk in TNT buffer (in mM: NaCl 100, Tris·HCl 10 (pH 7.4) and 0.1% (Tween-20) for 1 h. The blots were then incubated with 200-fold diluted primary antibodies against LC3 (Novus Bio., Littleton, CO) at 4 °C overnight, or with 1,000-fold diluted primary antibodies against p-Akt (Serine 473), Akt, p-$ERK_{1/2}$ (Thr202/Thr204), $ERK_{1/2}$ (rabbit polyclonal) (Santa Cruz Biotechnology) at room temperature for 2 h. Membranes were washed with TNT buffer at room T and incubated with appropriate peroxidase-conjugated secondary antibody (1:2000 dilution). Immunoreactive bands were visualized by chemiluminescence (ECL kit, Amersham) on X-ray film. Each immunoblotting experiment was repeated three to five times and the results were averaged. To quantify the protein, intensity of 200 bands was assessed with Scion Image Software.

4.8. *Statistics*

The results were expressed as mean ± standard error of means (S.E.M.). For hemodynamics, time-dependent comparisons among groups were analyzed by repeated-measures ANOVA while the results of infarct size and SDS-PAGE electrophoresis were analyzed by one-way ANOVA followed by Tukey's multiple range tests as post-hoc analysis. A value of $p < 0.05$ was considered to be statistically significant.

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