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Article

Pre-treatment of the beta3-adrenergic receptor agonist BRL37344 reduces *in vivo* myocardial ischemia/reperfusion injury by improving AMPK and SIRT1 activity and by suppressing mTOR and p70S6K signaling pathways

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This study aimed to investigate the role and signaling pathways of β3-AR in myocardial ischemia/reperfusion (I/R) injury, which is one of the leading causes of death worldwide. 47 male rats were randomly divided into two main groups to evaluate infarct size and molecular parameters. Rats in both groups were randomly divided into 4 groups. Control (sham), I/R (30 min ischemia/120 min reperfusion), BRL37344 (BRL) (A) (5 µg/kg single-dose pre-treatment (preT) before I/R) and BRL (B) (5 µg/kg/day preT for 10 days before I/R). Infarct size was determined with triphenyltetrazolium chloride staining and analyzed with ImageJ program. The levels of AMPK, SIRT1, mTOR, and p70SK6 responsible for cellular energy and autophagy were evaluated by western blot. Infarct size increased in the I/R group (44.84 ± 1.47%) and reduced in the single-dose and 10-day BRL-treated groups (32.22 ± 1.57%, 29.65 ± 0.55%; respectively). AMPK and SIRT1 levels were decreased by I/R but improved in the treatment groups. While mTOR and p70S6K levels increased in the I/R group, they decreased with BRL preT. BRL preT protects the heart against I/R injury. These beneficial effects are mediated in part by activation of AMPK and SIRT1, inhibition of mTOR and p70S6K, and consequently protected autophagy.

Keywords: Adenosine monophosphate-activated protein kinase (AMPK). Beta3-adrenergic receptors. Myocardial Ischemia/Reperfusion. Mammalian target of rapamycin (mTOR). Sirtuin 1 (SIRT1).

INTRODUCTION

Acute myocardial infarction (AMI) is the leading cause of mortality and morbidity worldwide. AMI is a condition in which blood circulation is insufficient due to obstruction of the coronary arteries. During the occlusion period, necrosis starts in the tissues, and myocardial damages occur ultimately resulting in cell death. Therefore, early opening of the occluded coronary artery is vital in terms of decreasing complications and mortality rates. Paradoxically, reperfusion induces cardiomyocyte death independent of ischemia, known as myocardial reperfusion injury (Hausenloy, Yellon, 2013). Current mechanical and pharmacological treatments are insufficient to reduce myocardial ischemia/reperfusion (I/R) injury. New treatment approaches are needed to reduce I/R injury.

Third-generation beta-adrenergic receptors (β-ARs), defined as β3-ARs, were first cloned from human cDNA

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in 1989. The presence of β3-ARs in the heart was first reported in the early 1996s (Gauthier *et al*., 1996). β3-ARs become less insensitive to long-term catecholamine stimulation than β1 and β2. Stimulation of β3-ARs shows negative inotropic effect by antagonize the positive inotropic effect of β1- and β2-AR activation, thus acting as a compensatory "brake" to prevent excessive adrenergic activation (Gauthier *et al*., 1996; Moniotte *et al*., 2001). β3-ARs stimulation plays a critical role in heart diseases such as heart failure and MI (Balligand, 2016).

Recently, some studies have focused on the roles and effects of β3-ARs in heart diseases. β3-ARs stimulation is protective in ischemic heart disease (Salie *et al*., 2019; García-Prieto *et al*., 2014; Mutlu *et al*., 2018; Aragón *et al*., 2011). However, the protective mechanism of β3-ARs, which are promising for protective and metabolic diseases in the cardiovascular system, in I/R damage is not fully known.

This study aims to determine the protective role and molecular mechanism of the β3-ARs agonist BRL37344 (BRL) in *in vivo* myocardial I/R injury. For this purpose, the effects of BRL37344 on the necrosis area against cardiac I/R injury and the activities of AMP-activated protein kinase (AMPK), sirtuin1 (SIRT1), mechanistic target of rapamycin (mTOR), and p70SK6, which are may be possible signaling pathways in the cellular mechanism, were investigated.

MATERIAL AND METHODS

Ethics

The investigation conforms to the "*Guide for the Care and Use of Laboratory Animals"* published by the "US National Institutes of Health (NIH Publication No. 85-23, revised 1985)". This study was approved by the Local Ethics Committee of Firat University Experiments (Approval date: 06.12.2016, Number of meetings: 2017/22, Decision number: 249, Protocol number: 2017/33). 47 male rats of 8-10 weeks Sprague-Dawley (250-300 g) were used.

Animals

A total of 47 male rats were randomly divided into two main groups to evaluate infarct size and molecular parameters. For the evaluation of the infarct size (n = 23), the following four groups were investigated: control (sham) (n=5), I/R (30-min ischemia/120-min reperfusion) (n=6), BRL (A) single-dose (5 µg/kg 3 min before I/R) (n=6), and (B) repeated doses (5 µg/kg/day for 10 days before I/R) (n=6). For evaluation of molecular parameters (n = 24), levels in the following groups were studied: control, I/R, and BRL (A) and (B) as described above.

Maximum of 5 rats were kept in individual cages. The rats were housed in a 12h daylight/12h dark cycle, ventilated, and in rooms at constant temperature (21 ± 1ºC) and cages under the standards. Their feeding was provided by standard rat pellet feed and tap water. No special diet was applied.

In vivo Myocardial I/R Experiments

At the beginning of the experiment, urethane 300 mg/kg (Sigma Aldrich Inc. St. Louis, MO. USA) was administered intramuscularly. Experimental attempts were carried out under a heating lamp with the rats in the supine position. Cannulation was applied to the trachea to provide artificial respiration. Blood pressure was measured with the help of a cannula placed in the carotid artery, and ECG was measured with the help of electrodes attached to the extremities. A transducer (Biopac systems, MP36) and a recorder (Biopac systems MP36) were used. A left thoracotomy was performed by cutting the fourth rib to the left of the sternum. The rats were connected to a ventilation device (Harvard animal rodent ventilator) to maintain negative intra-thoracic pressure. The device provided positive pressure breathing with a volume of 1.5 ml/100 g and a pulse rate of 60 beats/min. The heart separated from the pericardium was gently exteriorized and a 6/0 silk suture was placed under the left main coronary artery (LAD) with a 10 mm round needle. The heart was quickly repositioned into the rib cage. It waited for 20 min for stabilization. The subject was excluded if the arrhythmia or mean blood pressure fell below 70 mm Hg before occlusion. Ischemia was approved by ST-segment elevation on ECG and a decrease in blood pressure. After 30 min of ischemia, the LAD was opened and 120 min of reperfusion was achieved. The hearts were taken for determining the

infarct size and evaluation of molecular parameters (Sahna *et al.*, 2005).

Drug Administration

The stock solution was prepared by dissolving 5 mg BRL (Sigma Chemical Company, St. Louis, MO, U.S.A.) in distilled water. The stock solution was kept in the fridge for the duration of the experiment. Fresh solutions were prepared by diluting with 0.9% NaCl daily. The BRL dose was determined as 5 µg/kg, taking into account the study of García-Prieto *et al.* (2014). 5 µg/kg BRL was injected ip at the end of the stabilization period to the BRL (A) group. For the BRL (B) group, 5 µg/kg/day BRL was administered ip for 10 days.

Evaluation of Infarct Size

The heart tissue was removed and kept in the freezer for 12-h. Frozen hearts were sliced in 2 mm thickness and incubated in a buffer (pH = 7.4) containing 1% triphenyl tetrazolium chloride (TTC) (VWR chemicals, UK) at 37ºC for 20 min. TTC reduced formazan pigments when NADPH, dehydrogenase, and diaphoresis are present in the tissue. Living tissues are dyed dark red because they contain these enzymes and cofactors, while infarct areas are not stained because they do not contain them. The infarct size was analyzed using the ImageJ program and expressed as a percentage of the heart tissue (Sahna *et al.*, 2005).

Western Blot Analysis

Homogenization of the heart tissue and western blot preparation procedures were performed according to the method applied by Pala *et al.* (2016) in their previous work. Primary antibodies anti-mTOR (sc517464), anti-p70S6K (ab9366), anti-SIRT1 (ab12193), p-AMPK (ab109402), (Abcam, Cambridge, UK) were diluted (1:1000) in the same buffer containing 0.05% Tween-20. Primary antibodies were incubated at 4°C overnight. The blots were washed and the secondary antibodies (horseradish peroxidase-linked goat antimouse immunoglobulin G) (ab7090) (Abcam, Cambridge, UK) were used to incubate the nitrocellulose membrane for 1 h at room temperature. Diaminobenzidine and hydrogen peroxide were used as the substrate. Protein loading was controlled using a β-actin antibody (A5316; Sigma-Aldrich, St Louis, MO, USA). All blots were performed at least three times. Proteins were analyzed densitometrically using ImageJ (National Institute of Health, Bethesda, USA) software (Pala *et al.*, 2016).

Statistical Analysis

Statistical Package for the Social Sciences (SPSS) 22.0 statistical program was used to evaluate the western blot and necrosis area data. The sample size was determined by power analysis that suggested at least 6 individuals with an alpha error of 0.05 and a β error of 0.20 (power = 0.80). Data were expressed as means ± SEM. The homogeneity of variances was checked with the Levene test. The normality assumption was analyzed using the Shapiro-Wilk test. One-way analysis of variance (ANOVA) was used for comparisons of three or more groups. The post hoc Tukey HSD test was used from multiple comparison tests. Statistical significance was accepted as $p<0.05$.

RESULTS

There was no significant difference in blood pressure values (mm Hg) between the groups. The necrosis area was calculated as the rate of the infarct area to the whole heart. The myocardial necrosis area due to I/R (44.85 ± 1.47%) was significantly reduced in the groups treated with the β3-ARs agonist BRL (A), (B) (32.22 ± 1.57%, 29.65 ± 0.55% respectively) (Figure 1). BRL pre-treatment (preT) provided significant protection in the heart by reducing the necrosis area by 28.17% (A) and 33.90% (B), respectively.

AMPK, SIRT1 levels were significantly ($p<0.05$) decreased in the I/R group when compared with control (54.65 ± 0.62%, 45.78 ± 1.59%, control as 100%; respectively). However, AMPK and SIRT1 levels were significantly ($p<0.05$) increased in the BRL single-dose (A) and 10-day treatment groups (B)

compared to the I/R group (76.24 ± 1.48% (A), 71.03 ± 0.86% (B) for AMPK; 73.11 ± 2.63% (A), 64.59 ± 2.14% (B) for SIRT1). (Figure 2A and 2B). There was no significant difference between the single-dose and 10-day treatment groups for AMPK.

mTOR, p70S6K levels were significantly (*p<0.05*) increased in the I/R group when compared with control (228.9 ± 3.60%, 189.0 ± 4.66%, control as 100%; respectively). However, mTOR and p70S6K levels were significantly (*p<0.05*) decreased in the BRL single-dose (A) and 10-day treatment groups (B) compared to the I/R group (139.4 ± 2.40% (A), 125.6 ± 2.01% (B) for mTOR; 144.8 ± 4.45% (A), 124.0 ± 2.90% (B) for p70S6K). (Figure 2C and 2D).

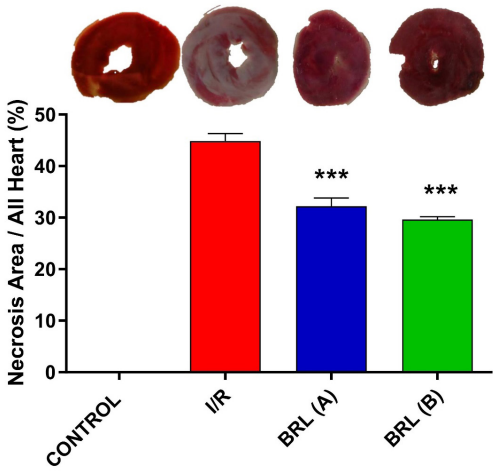

FIGURE 1 - Myocardial infarct size (%) at experimental groups. I/R were assessed 30-min ischemia followed by 120-min reperfusion. (I/R: Ischemia/Reperfusion, BRL (A): BRL37344 (5 µg/kg) single-dose before I/R, BRL(B): 5 µg/kg/day BRL37344 during 10 days before I/R.) (*** *p<0.001*).

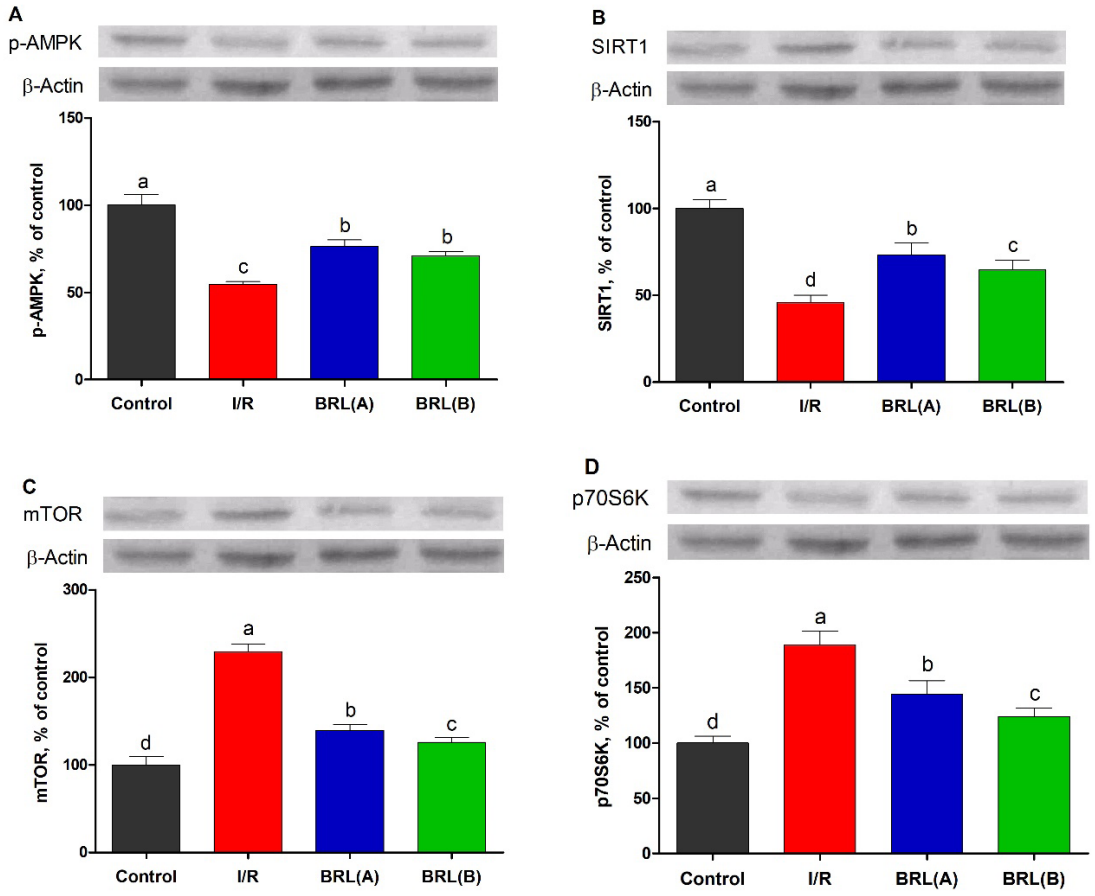

FIGURE 2 - Relative AMPK (A), SIRT1 (B), mTOR (C), p70S6K (D) protein expression (%) between groups. β-Actin was used as the reference protein. I/R were assessed 30-min ischemia followed by 120-min reperfusion. (I/R: Ischemia/Reperfusion, BRL (A): BRL37344 (5 µg/kg) single-dose before I/R, BRL(B): 5 µg/kg/day BRL37344 during 10 days before I/R). Values with different superscripts in the same column are statistically significantly different (*p<0.05*).

DISCUSSION

Determining new treatment strategies to reduce I/R injury is important to reduce myocardial damage therefore mortality. Niu *et al.* (2014) suggested that when they treated mice with the β3-AR agonist BRL and the β3-AR antagonist SR59230A 1 day after I/R operation, BRL significantly reduced the area of fibrosis, preserved cardiac function, and reduced cardiomyocyte apoptosis compared to SR59230A. In mice, BRL per-treatment, administered before reperfusion, reduced infarct size. Left ventricular function was significantly improved in the BRL-treated groups (García-Prieto *et al.*, 2014). PostT of BRL in I/R reduced apoptosis, reduced infarct size and improved cardiac function (Wang *et al.*, 2021). β3-ARs activation has an important effect on the improvement of heart function (Niu *et al.*, 2012). According to different studies, although cardio protective interventions applied before reperfusion have reduced I/R injury, this effect is insufficient. Because the damage that occurs during ischemia also contributes to I/R damage (Hausenloy, Tsang, Yellon, 2005). Implementation of cardio protective strategies previous to ischemia increases protection mainly for long ischemia duration (Murphy, Steenbergen, 2008). In this study, preT of the β3-AR selective agonist BRL significantly reduced the area of necrosis compared to I/R. There was no significance between a single dose and 10 days of treatment.

Generally, the role of β3-ARs in myocardial I/R injury has been investigated in *in vitro* models (Salie *et al.*, 2019; Mutlu *et al.*, 2018). The I/R pathogenesis in vivo includes the interaction of multiple mechanisms such as cardiac overload, oxidative stress damage, impaired autophagy, apoptosis, and these mechanisms cause heart damage (Morano *et al.*, 2017). *In vivo* models will be more useful to understand the roles of β3-ARs, which are part of the sympathetic nervous system, in I/R damage. To our knowledge, this is the first study to report the beneficial effects of a β3-ARs agonist in single-dose and repeated-dose preT in an *in vivo* model of myocardial I/R injury.

AMPK activation contributes to protective autophagy in the early phase of cardiac ischemia (Ma *et al.*, 2015). Additionally, AMPK activation significantly reduces *in vivo* myocardial I/R injury. AMPK activation of pharmacological drugs is considered a therapeutic target (Ma *et al.*, 2015). This study showed that AMPK levels were reduced by I/R but significantly improved by BRL preT. We suggest that β3-ARs protect the heart against I/R damage through AMPK activation. The enhanced AMPK levels with BRL may contribute to protecting the heart during I/R and reducing the necrosis area.

Sirtuin1 plays a protective role, particularly in heart diseases, by participating in processes related including regulation of energy production, autophagy, and cell survival (Tanno *et al.*, 2012). The results of this study are compatible with the general literature and show that SIRT1 levels decrease with I/R (Hsu *et al.*, 2010). BRL treatment significantly increased SIRT1 level in heart tissue. Single-dose of BRL increased SIRT1 levels by more than 10 days of treatment. Contrary to general findings, Alcendor *et al.* (2007) has noted that SIRT1 increases as a compensatory mechanism for heart damage induced by certain stresses, including cardiac aging, oxidative stress, and heart failure. Regulation of SIRT1 levels appears to be stimulus-specific and SIRT1's stress compensatory mechanism is not effective against I/R damage (Hsu *et al.*, 2010). More studies are needed to understand the role and effects of sirtuins on the heart cell (Tanno *et al.*, 2012). However, current studies indicate that sirtuin-activating compounds could be a promising future therapeutic target for heart failure. BRL can increase SIRT1 activity. One of the heart intracellular signaling pathways of β3-ARs may also be related to SIRT1.

During the ischemic phase, stimulation of cardiac autophagy is essential for energy recovery and cardiomyocyte survival (Ma *et al.*, 2015). ATP production decreases in the ischemia phase. Autophagy contributes to energy production, thus reducing the energy requirement during the myocardial ischemia phase. ATP gain is important for the survival of cardiomyocytes (Matsui *et al.*, 2007). In this case, in response to changes in the intracellular ATP/AMP ratio, AMPK is activated and phosphorylates TSC2, thereby inhibiting mTOR (Inoki, Zhu, Guan, 2003). Thus, although there is little direct evidence supporting that AMPK plays a critical role in regulating the autophagy of cardiac myocytes, the AMPK-mTOR pathway is

thought to be a key regulator of autophagy in reaction to energy deficiency (Meley *et al*., 2006). SIRT1 and AMPK can activate each other, but there is an inhibition relationship between SIRT1 and mTOR (Giovannini, Bianchi, 2017). While autophagy is induced through an AMPK-dependent mechanism during the ischemia period, it stimulates autophagy during the post-ischemia reperfusion period through a Beclin-1 dependent but AMPK independent mechanism. Autophagy shows different effects in ischemia and reperfusion periods. Inhibition of autophagy during ischemia increases cell death (Yan *et al*., 2005). While autophagy can be protective during ischemia, Beclin-1 induced autophagy during reperfusion may play a detrimental role (Matsui *et al*., 2007). A treatment that stimulates autophagy during ischemia may contribute to reducing the damage. BRL probably exerted its beneficial effects by preserving autophagy during ischemia. We propose a new mechanism for the cardioprotective effects of BRL.

In this study, preT of the β3-ARs agonist BRL was shown to be protective against myocardial I/R injury in vivo and reduce the area of necrosis. AMPK and SIRT1 levels responsible for processes such as cell survival, autophagy, energy metabolism, and reduction of oxidative stress decreased in I/R groups. The levels of mTOR and p70S6K responsible for stress reactions in heart tissue significantly increased in I/R. AMPK and SIRT1 levels were significantly increased, while mTOR and p70S6K levels were decreased in BRL single dose and 10-day preT groups. According to the results of this study, it is suggested that some of the beneficial effects of β3-ARs are mediated by the activation of AMPK and SIRT1 and the inhibition of mTOR and p70S6K directly and/or indirectly. Protected autophagy during the AMPK-SIRT1-mTOR/p70S6K signaling pathway mediated ischemia may be contributing to the beneficial effects of β3-ARs activation.

CONCLUSION

Single-dose and 10-day BRL preT activated β3-ARs signaling pathways, protecting the heart against *in vivo* myocardial I/R injury and significantly reducing the infarct size. BRL preT may contribute to the maintenance of autophagy by activating AMPK-SIRT1 and inhibiting the mTOR/p70S6K signal pathways in the early stages of ischemia. With this study, we add a new one to the action of the mechanisms of β3-ARs known to date. The effect of β3-ARs on mechanisms responsible for cellular energy and autophagy such as mTOR, AMPK, and SIRT1 may help us better understand the functional role of β3-ARs in the treatment of cardiac I/R injury.

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