



# Cardioprotective Effects of Beta3-Adrenergic Receptor ( $\beta$ 3-AR) Pre-, Per-, and Post-treatment in Ischemia–Reperfusion

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**Abstract** The  $\beta$ 3-AR (beta3-adrenergic receptor) is resistant to short-term agonist-promoted desensitization and delivers a constant intracellular signal, making this receptor a potential target in acute myocardial infarction (AMI).

**Aim** To investigate whether selective modulation of  $\beta$ 3-AR prior to or during ischemia and/or reperfusion may be cardioprotective.

**Methods** Isolated perfused rat hearts were exposed to 35-min regional ischemia (RI) and 60-min reperfusion. The  $\beta$ 3-AR agonist (BRL37344, 1  $\mu$ M) or antagonist (SR59230A, 0.1  $\mu$ M) was applied: (i) before RI (PreT) or (ii) last 10 min of RI (PerT) or (iii) onset of reperfusion (PostT) or (iv) during both PerT+PostT. Nitric oxide (NO) involvement was assessed, using the NOS inhibitor, L-NAME (50  $\mu$ M). Endpoints were functional recovery, infarct size (IS), cGMP levels, and Western blot analysis of eNOS, ERKp44/p42, PKB/Akt, and glycogen synthase kinase-3 $\beta$  (GSK-3 $\beta$ ).

**Results** Selective treatment with BRL significantly reduced IS. L-NAME abolished BRL-mediated cardioprotection. BRL (PreT) and BRL (PerT) significantly increased cGMP levels (which were reduced by L-NAME) and PKB/Akt phosphorylation. BRL (PostT) produced significantly increased cGMP levels, PKB/Akt, and ERKp44/p42 phosphorylation. BRL (PerT+PostT) caused significant eNOS, PKB/Akt, ERKp44/p42, and GSK-3 $\beta$  phosphorylation.

**Conclusion**  $\beta$ 3-AR activation by BRL37344 induced significant cardioprotection regardless of the experimental protocol. However, the pattern of intracellular signaling with each BRL treatment differed to some degree and suggests the involvement of cGMP, eNOS, ERK, GSK-3 $\beta$ , and particularly PKB/Akt activation. The data also suggest that clinical application of  $\beta$ 3-AR stimulation should preferably be incorporated during late ischemia or/and early reperfusion.

**Keywords** Myocardial ischemia · Reperfusion injury · Cardioprotection ·  $\beta$ 3-AR modulation

## Introduction

It has become clear that the myocardial response to ischemia–reperfusion can be manipulated to delay injury. However, it appears that the window of cardioprotection during reperfusion is very limited and although protection can be initiated at reperfusion, injury also occurs during ischemia, and the relative proportion of each event likely depends on the duration of ischemia [1]. Thus, if cardioprotective strategies can be initiated before or during ischemia, it is likely that they will enhance protection, especially with longer durations of ischemia. It is well-recognized that transient activation of the  $\beta$ -adrenergic signaling pathway before acute myocardial ischemia (AMI) with ligands such as isoproterenol, formoterol and dobutamine, elicits cardioprotection against subsequent long periods of ischemia [2, 3]. Primarily, the focus was on the  $\beta$ 1- and  $\beta$ 2-adrenergic receptors ( $\beta$ 1-AR,  $\beta$ 2-AR), but recently, the  $\beta$ 3-AR also emerged as a potential target in the treatment

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of heart disease and recent studies [2–5] support a significant role for  $\beta_3$ -AR modulation in reperfusion injury after AMI.

In heart failure,  $\beta_1$ - and  $\beta_2$ -AR levels are typically known to be downregulated while  $\beta_3$ -ARs, on the other hand, are upregulated or remain unchanged [6]. This is because  $\beta_3$ -AR lacks PKA phosphorylation sites and has fewer C-terminus serine and threonine residues than the  $\beta_1$ - and  $\beta_2$ -AR, making the  $\beta_3$ -AR resistant to short-term agonist-promoted desensitization [7]. Despite the fact that most of the available information about the  $\beta_3$ -ARs signaling has been obtained in studies of adipocytes, recent studies revealed that these signaling pathways could be consistently generated in cardiac tissues [8]. Therefore, increasing evidence puts the spotlight on the involvement of  $\beta_3$ -ARs, via eNOS, in the production of cyclic 3',5'-guanosine monophosphate (cGMP) [9]. A previous study by Gauthier et al. 2000 [8] has concluded that concurrent activation of cAMP-dependent positive ( $\beta_1$ - and  $\beta_2$ -ARs) and NO-dependent negative ( $\beta_3$ -ARs) inotropic pathways within the same cardiomyocyte would provide an integration-point between the adrenergic receptor-mediated stimulation of cardiac contraction. Accordingly, the  $\beta_3$ -ARs-mediated pathway would function as a countervailing “rescue” mechanism preventing cardiomyocyte damage from excessive  $\beta_1$ - and  $\beta_2$ -ARs stimulation [8].

The  $\beta_3$ -ARs are stimulated at high catecholamine concentrations and induce negative inotropic effects, serving as a “brake” to protect the heart from catecholamine overstimulation [10, 11] and once activated, the receptor would deliver a more sustained intracellular signal [12]. Subsequently, we postulate that selective  $\beta_3$ -AR stimulation prior to or during ischemia/reperfusion may be cardioprotective, whereas selective  $\beta_3$ -AR inhibition may prove useful in the end stages of sustained ischemia and early reperfusion. Therefore, in this study, we intended to investigate the possible cardioprotective effects of  $\beta_3$ -AR modulation prior to sustained ischemia, at the end of sustained ischemia, and/or early reperfusion.

## Materials and Methods

### Experimental Animals

Male Wistar rats (230 to 250 g) were used in this study. The handling of laboratory animals was in accordance with ethical guidelines as set out by the University of Stellenbosch, Faculty of Medicine and Health Sciences Ethics Committee and the South African National Standard for Care and Use of Animals for Scientific Purpose (SANS 10386: 2008). The rats had free access to food and water prior to experimentation. Rats were anesthetized with sodium pentobarbital (120 mg/kg) by intraperitoneal injection before removal of hearts.

### Perfusion Technique

Hearts were perfused as previously described [13]. Briefly, hearts were rapidly excised and arrested in ice-cold Krebs–Henseleit buffer, containing in millimolar per liter: NaCl 119; NaHCO<sub>3</sub> 24.9; KCl 4.74; KH<sub>2</sub>PO<sub>4</sub> 1.19; MgSO<sub>4</sub> 0.6; NaSO<sub>4</sub> 0.59; CaCl<sub>2</sub> 1.25; glucose 10; pH 7.4. The buffer was gassed with 95% O<sub>2</sub> and 5% CO<sub>2</sub> prior to and during the perfusion protocol and temperature was maintained at 36.5 °C. Hearts were mounted onto the aortic cannula of the Neely–Morgan perfusion system and retrogradely perfused (R) for 15 min to stabilize. The left atrium was cannulated to allow working heart perfusion (W) for 15 min with a preload of 15 cm and afterload of 100 cm H<sub>2</sub>O. The administration of drugs for 10 min was via a side-arm into the aortic cannula, while the heart was retrogradely perfused at a pressure of 100 cm H<sub>2</sub>O. In all experimental protocols, hearts were stabilized for a total of 50 min (15 min R, 15 min W, 10 min R, 10 min R for drug administration).

This was followed by 35-min regional ischemia (RI) and 60-min reperfusion (Rep) (10 min R, 20 min W, 30 min R). Percentage functional recovery of hearts was determined by expressing post-ischemic cardiac output (coronary flow + aortic flow rates, ml/min) as a percentage of pre-ischemic cardiac output. At completion of regional ischemia and reperfusion, the silk suture around the left anterior descending coronary artery (LAD) was permanently tied and 0.25% Evan's blue solution infused into the heart to stain viable tissues. Hearts were removed, frozen, cut into 2-mm-thick transverse tissue segments, and incubated in 1% triphenyl tetrazolium chloride (TTC) in phosphate buffer, pH 7.4 for 10 min to outline the area at risk, i.e., damaged but still functional tissues which take on a deep red coloration. Infarcted tissue areas were not stained and have a white color. The reaction with TTC was stopped by placing the tissue segments in 10% formalin. Tissue segments were subsequently placed between two glass plates and traced to outline the viable, the area at risk as well as infarcted area (included in the area at risk) in each ventricular section. The left ventricle area at risk (R) and the area of infarct (I) tissue were determined using computerized planimetry (UTHSCSA Image Tool, developed at the University of Texas Health Science Center at San Antonio, TX). The infarct size was expressed as a percentage of the area at risk (I/R %).

### Chemicals

The selective  $\beta_3$ -AR agonist, BRL37344 (BRL) (1  $\mu$ M); selective  $\beta_3$ -AR antagonist, SR59230A (SR) (0.1  $\mu$ M); and nitric oxide synthase inhibitor, nitro-L-arginine methyl ester hydrochloride, L-NAME (50  $\mu$ M), were obtained from Sigma-Aldrich. BRL37344 and SR59230A were dissolved in dimethylsulfoxide (DMSO) and the final concentration of

the DMSO in KREBS buffer was 0.0008% for both drugs. The concentrations of BRL37344 and SR59230A utilized in this study were based on previous studies where it was found to be effective at 1  $\mu$ M and 0.1  $\mu$ M for BRL37344 ( $\beta$ 3-AR agonist) and SR59230A ( $\beta$ 3-AR antagonist), respectively [14–16]. Antibodies (ERKp44/p42, phospho-ERKp44/p42 (Thr-202/Tyr-204), PKB/Akt, and phospho-PKB/Akt (Ser-473)) were purchased from Cell Signaling Technology, Beverly, MA, USA. Horseradish peroxidase labeled secondary antibody, ECL, and hyperfilm were from Amersham, Life Science.

## Experimental Groups and Protocols for Infarct Size Determination

**Key Abbreviations:** Non-pretreatment (NPT): no drugs applied

Pre-treatment (PreT): drug applied for 10 min before regional ischemia

Per-treatment (PerT): drug applied during the last 10 min of regional ischemia

Post-treatment (PostT): drug applied for 10 min at the onset of reperfusion

Per-treatment + post-treatment (PerT+PostT): drug applied during the last 10 min of regional ischemia and at the onset of reperfusion

**Non-pretreatment Protocol:** Hearts were stabilized for 50 min and subjected to 35-min regional ischemia (RI), followed by 60-min reperfusion ( $n = 6$ /group).

**Pre-treatment with  $\beta$ 3-AR Agonist BRL37344 or Antagonist SR59230A:** Hearts were stabilized for 50 min, thereafter pre-treated with either BRL or SR for 10 min (PreT), retrogradely applied, after which the hearts were subjected to 35-min regional ischemia and 60-min reperfusion ( $n = 6$  hearts for each drug) (Fig. 1a). Hemodynamic parameters were recorded at the end of the 15-min working heart mode, prior to regional ischemia, and compared with hemodynamic parameters at 30-min reperfusion after regional ischemia. After 1-h reperfusion, hearts were frozen for infarct size (IS) determinations.

**Per-treatment and Post-treatment with BRL37344 or SR59230A:** After the stabilization period, hearts were exposed to 35-min regional ischemia. BRL or SR was administered during the last 10 min of regional ischemia (PerT) (Fig. 1b). In another set of experiments, hearts were exposed to BRL or SR for a total period of 20 min, during the last 10 min of RI as well as the first 10 min of reperfusion (PerT + PostT), ( $n = 6$  hearts for each drug) (Fig. 1b).

**Post-treatment with BRL37344 or SR59230A:** Hearts in this group were stabilized for 50 min, subjected to 35-min regional

ischemia, and BRL or SR administered during the first 10 min of reperfusion (PostT) ( $n = 6$  hearts for each drug) (Fig. 1c).

**Per-treatment with BRL37344 and Post-treatment with SR59230A:** Hearts were stabilized for 50 min, subjected to 35-min RI, and BRL administered during the last 10 min of regional ischemia (PerT), followed by SR during the first 10 min of reperfusion (PostT) ( $n = 6$  hearts) (Fig. 1d).

**Assessment of Nitric Oxide Synthase, Using Nitric Oxide Synthase Inhibitor (L-NAME), in BRL37344 (PreT or PostT):**

Hearts were stabilized for 50 min and pre-treated with the nitric oxide synthase inhibitor (L-NAME) alone or in combination with BRL, for 10 min after which the hearts were subjected to 35-min regional ischemia and 60-min reperfusion. In the other group of experiments, L-NAME alone or in combination with BRL was applied for 10 min at the onset of reperfusion (Fig. 1e).

## Experimental Protocols for Western Blot Analysis

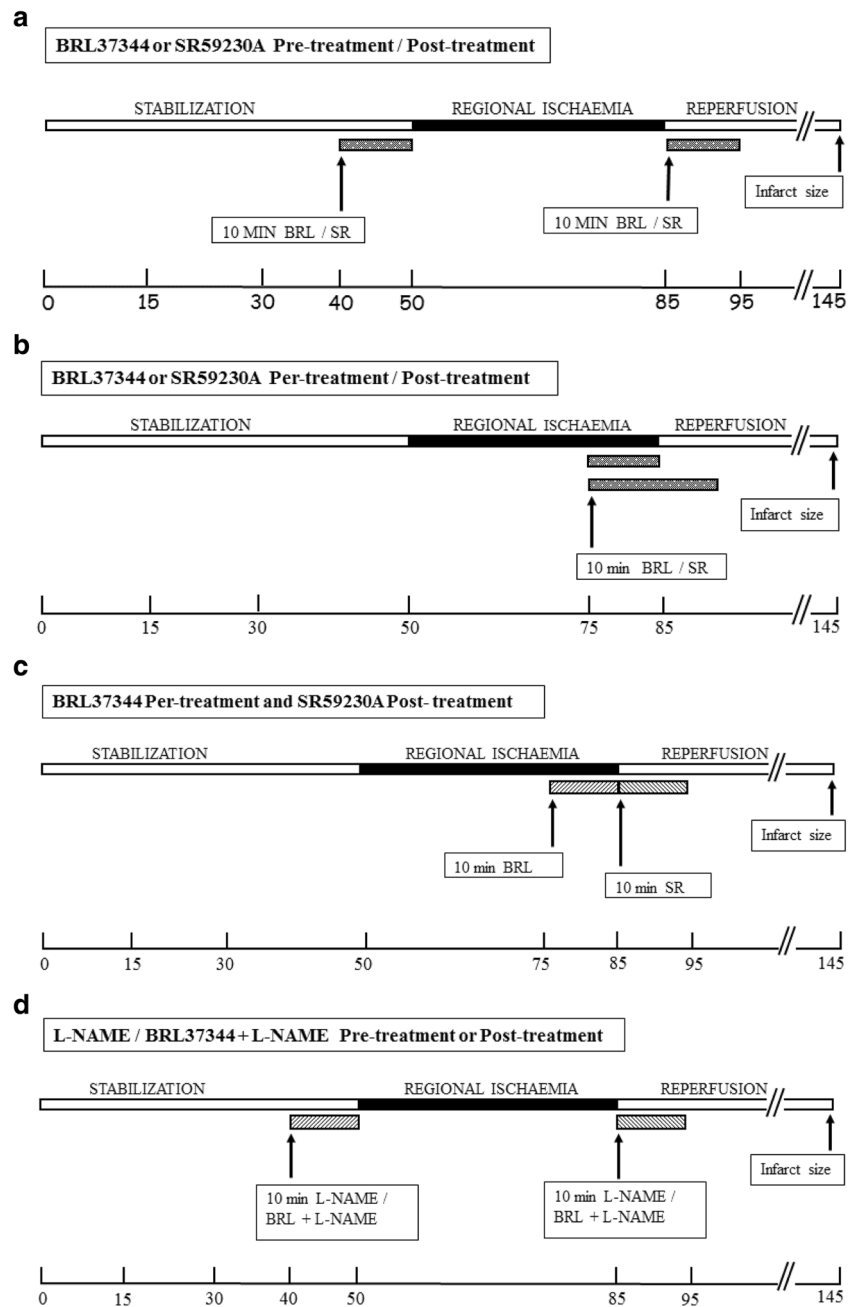
Non-pretreatment (NPT) hearts were stabilized for 50 min, followed by 35 min RI, after which the left ventricles were freeze-clamped at 10-min reperfusion ( $n = 6$ ). The following groups of hearts were exposed to a 50-min stabilization period and 35-min RI and subjected to subsequent treatments regimes: BRL (PreT) for 10 min prior to regional ischemia, BRL (PerT) for the last 10 min of regional ischemia, BRL (PostT) during the first 10 min of reperfusion, BRL (PerT+PostT) and BRL (PerT)+SR (PostT) ( $n = 6$  for each group). Negative control hearts (–ve C) were stabilized for 50 min and left ventricles freeze-clamped. Freeze-clamped left ventricular tissue was used for subsequent detection and measurement of total and phosphorylated ERKp44/p42, PKB/Akt, glycogen synthase kinase-3 $\beta$  (GSK-3 $\beta$ ), and eNOS by Western blot analysis using appropriate antibodies.

**Preparation of Lysates:** Ventricular tissue was pulverized and homogenized in 900  $\mu$ l lysis buffer using a Polytron homogenizer as described before [17]. Samples were centrifuged at 11,282g for 10 min to obtain the cytosolic fraction. The protein content was determined using the Bradford technique [18]. The lysates were diluted in Laemmli sample buffer.

## Western Immunoblot Analysis

Samples were subjected to electrophoresis on a 12% or 7.5% polyacrylamide gel (SDS–PAGE), depending on the size of the protein of interest, using the standard BIO-RAD Mini Protean III system. The separated proteins were transferred to an Immobilon membrane (Millipore) (Billerica, MA, USA: polyvinylidene fluoride (PVDF) membrane), using the Trans-Blot®Turbo™ Transfer

**Fig. 1** **a** Experimental protocols of BRL or SR (PreT) and time points of infarct size determination. **b** Experimental protocols of BRL or SR (PerT) and BRL or SR (PerT+PostT) and time points of infarct size determination. **c** Experimental protocols of BRL or SR (PostT) and time points of infarct size determination. **d** Experimental protocols of BRL (PerT) and SR (PostT) and time points of infarct size determination. **e** Experimental protocols of L-NAME or BRL+L-NAME (PreT) or (PostT) and time points of infarct size determination



system. Proper protein transfer and equal loading were routinely assessed using Ponceau-s staining and corrections were made if necessary. Non-specific binding sites on the membrane were blocked with 5% fat-free milk (5 g) in TBS-Tween (Tris-buffered saline + 0.1% Tween 20) for 1–2 h at room temperature and incubated overnight at 4 °C with the primary antibodies (Cell Signaling Technology, MA, USA) that recognize total or phosphorylated proteins: PKB/Akt, ERKp44/p42, GSK-3 $\beta$ , and eNOS. The membranes were washed with TBS-T (3  $\times$  5 min) and then incubated at room temperature with a diluted horseradish peroxidase-labeled secondary

antibody (Cell Signaling Technology). After thorough washing with TBS-T, membranes were covered with enhanced chemiluminescence ECL detective reagent for 1 min and briefly exposed with the Chemidoc MP Imager System with Image lab 5. Stain-Free membranes and the Chemidoc MP Imager System with Image lab 5 were used to validate protein transfer and equal loading of samples. Densitometry measurements were normalized to those of negative controls and quantified as fold increases. Cardiac microvascular endothelial cells treated with 500 nM Okadaic acid for 30 min were used as positive controls, for Western blotting of eNOS.

## cGMP Determinations

NPT, BRL (PreT), BRL (PerT), BRL (PostT), and BRL (PerT+PostT) hearts were freeze-clamped after each BRL treatment procedure ( $n=6$  for each group) with or without the nitric oxide synthase inhibitor (L-NAME), as described in the section for experimental groups and protocols. The left ventricles were excised, rapidly frozen in liquid nitrogen. A total of 30–35 mg of frozen tissue was pulverized and 0.5 ml of cold 5% trichloroacetic acid was added to each sample and homogenized. Samples were centrifuged at  $600\times g$  for 10 min at 4 °C. The supernatant was transferred and extracted with three volumes of water-saturated diethyl ether. Samples were centrifuged at  $4600\times g$  for 5 min at 4 °C. The top layer was removed and dried by evaporation in the fume hood overnight. The extracted samples were reconstituted with 250  $\mu$ l assay buffer and processed according to the instructions provided with the kit (Sigma-Aldrich, cGMP Enzyme Immunoassay kit, catalog number: cG201) for determination cGMP levels. Data are expressed as picomole/gram wet weight tissue and each sample was run in duplicate.

## Statistical Analysis

Results were expressed as mean  $\pm$  standard error of the mean (SEM). For multiple comparisons, one or two-way analysis of variance (ANOVA) was utilized (Graph Pad PrismPlus Version 5.0). Post hoc testing for differences between selected groups was done using Bonferroni's method. A  $p$  value of  $< 0.05$  was considered significant.

## Results

### Effect of $\beta$ 3-AR Modulation (Stimulation or Inhibition) on Infarct Size

The area at risk did not differ between the groups and averaged at  $51.8 \pm 0.8\%$  ( $n=60$ ) of left ventricle.

### Pre-treatment with $\beta$ 3-AR Agonist BRL37344 or Antagonist SR59230A

BRL37344 pre-treatment [BRL (PreT)] for a period of 10 min before 35-min regional ischemia (RI) significantly reduced infarct size (% infarct size per area at risk  $21.4 \pm 2.5$ , compared to NPT  $43.1 \pm 1.2$ ,  $p < 0.001$ ) (Fig. 2a). Pre-treatment with the vehicle, dimethylsulfoxide (DMSO) or the  $\beta$ 3-AR antagonist, SR59230A had no effect on infarct size (% infarct size per area at risk  $40.9 \pm 2.7$  and  $38.7 \pm 2.5\%$ , respectively compared to NPT group,  $43.1 \pm 1.0\%$ ) (Fig. 2a).

### Per-treatment with BRL or SR

BRL37344 per-treatment [BRL (PerT)] applied during the last 10 min of regional ischemia considerably reduced infarct size (% infarct size per area at risk  $14.9 \pm 2.3$ , compared to the NPT group  $41.5 \pm 1.4$ ,  $p < 0.001$ ) (Fig. 2b). The application of SR59230A in the same setting had no effect on infarct size (% of infarct size per area at risk  $40.9 \pm 2.7$  compared to NPT group  $41.5 \pm 1.4$ ).

### Post-treatment with BRL or SR

The application of BRL37344 during the first 10 min of reperfusion, BRL (PostT), caused a significant reduction in infarct size (% infarct size per area at risk  $19.0 \pm 1.8$ , compared to the NPT group  $42.5 \pm 0.5$ ,  $p < 0.001$ ). The application of SR59230A as a post-treatment (PostT) had no effect on infarct size (% infarct size  $38.1 \pm 2.4$  compared to NPT  $42.5 \pm 0.5$ ) (Fig. 3a).

### Per-treatment and Post-treatment with BRL or SR

Treatment of hearts with BRL37344 during the last 10 min of regional ischemia as well as during the first 10 min of reperfusion BRL (PerT+PostT) resulted in a significant reduction in IS when compared to NPT group (% infarct size per area at risk  $20.5 \pm 2.0$  vs.  $43.1 \pm 1.2$ ,  $p < 0.001$ ) (Fig. 3b). The groups treated with SR59230A had no effect on IS.

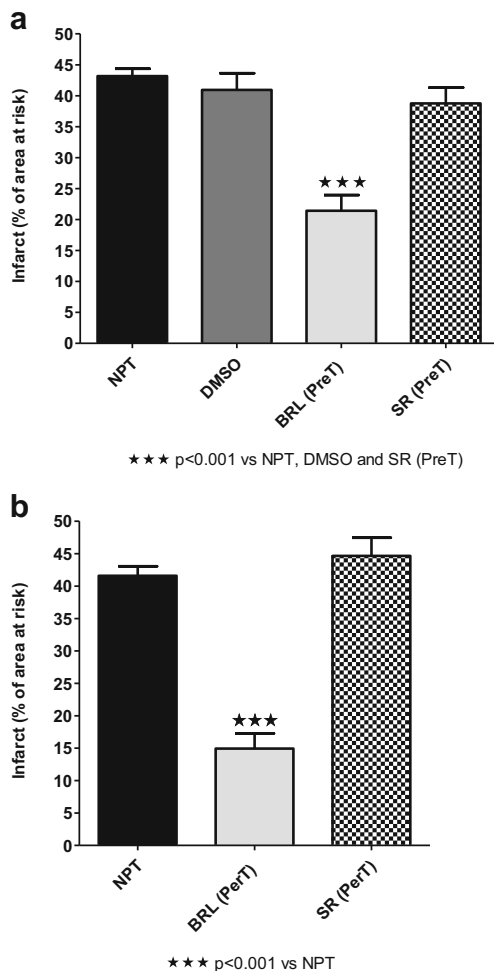
### Effect of NOS Inhibition with the Nitric Oxide Synthase Inhibitor, L-NAME

The reduction in infarct size elicited by the  $\beta$ 3-AR agonist, BRL37344 was abolished by L-NAME, when administered in combination with BRL37344 before regional ischemia, BRL+L-NAME (PreT) (% infarct size per area at risk:  $41.4 \pm 3.1$  vs. BRL (PreT)  $21.4 \pm 2.5$ ,  $p < 0.001$ ) or during first 10 min of reperfusion, BRL+L-NAME (PostT) (% infarct size per area at risk:  $35.75 \pm 3.54$  vs. BRL (PostT) ( $19.0 \pm 1.8$ ,  $p < 0.001$ ) (Fig. 4a, b). L-NAME, administered alone, before regional ischemia, L-NAME (PreT) or during reperfusion, L-NAME (PostT), had no effect on infarct size (% infarct size per area at risk  $39.8 \pm 3.6$  and  $34.7 \pm 2.2$ ), respectively.

## Hemodynamic Data

### Coronary Flow When BRL37344 Was Applied During Retrograde Perfusion

Coronary flow (CF) was calculated as a % change. BRL37344 retrogradely administered before RI or at the start of reperfusion significantly increased coronary flow (CF: % change/

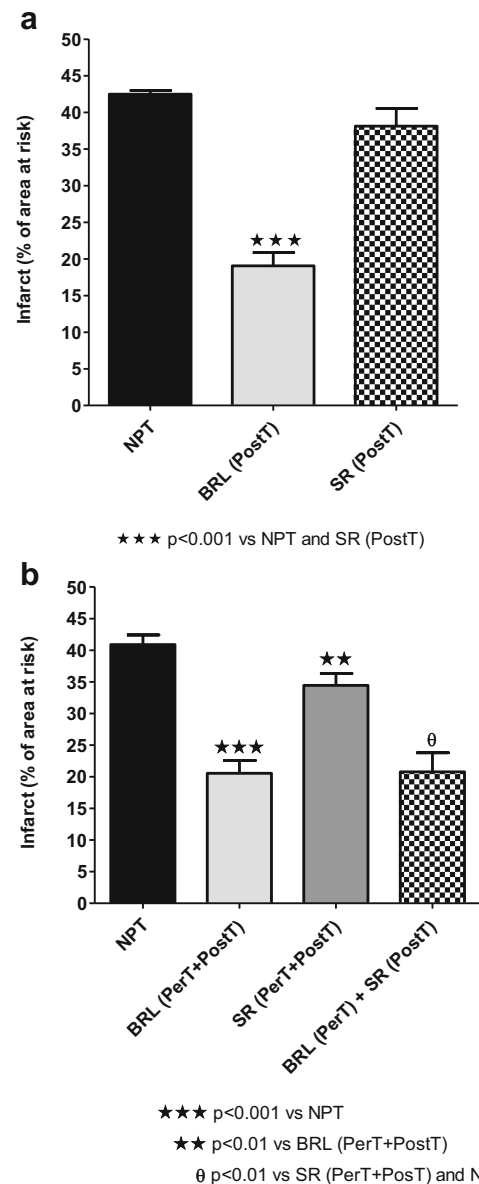


**Fig. 2** **a** Effect of pre-treatment with vehicle (DMSO), BRL (PreT), or SR (PreT) on IS after 35-min regional ischemia and 60-min reperfusion ( $n = 6$  per group). **b** Effect on IS when BRL (PerT) or SR (PerT) applied during the last 10 min of RI ( $n = 6$  per group)

increase  $104.50 \pm 4.53$ ,  $n = 12$ ) compared to non-treated hearts. However, administration of SR59230A was without effect on coronary flow.

### Hemodynamic Parameters and Percentages of Functional Recovery (Working Heart Model)

Functional performance before the onset of regional ischemia was similar in the eight groups studied (Table 1). All of these groups displayed significantly lower but similar functional recovery values after ischemia, except for the BRL (PerT)+SR (PostT) and SR (PerT+PostT) groups, which showed significantly reduced % recovery in terms of cardiac output when compared with the other groups after ischemia. For example, compared with the NPT group, the % recovery in cardiac output was significantly less (post-ischemic/pre-ischemic):  $16.3 \pm 10.3\%$  and  $21.3 \pm 9.7\%$ , respectively compared with NPT  $64.0 \pm 6.3\%$ ,  $p$  value  $< 0.01$ ) (Table 2). Similarly, the % total work (Wt) of these two groups followed the same trends



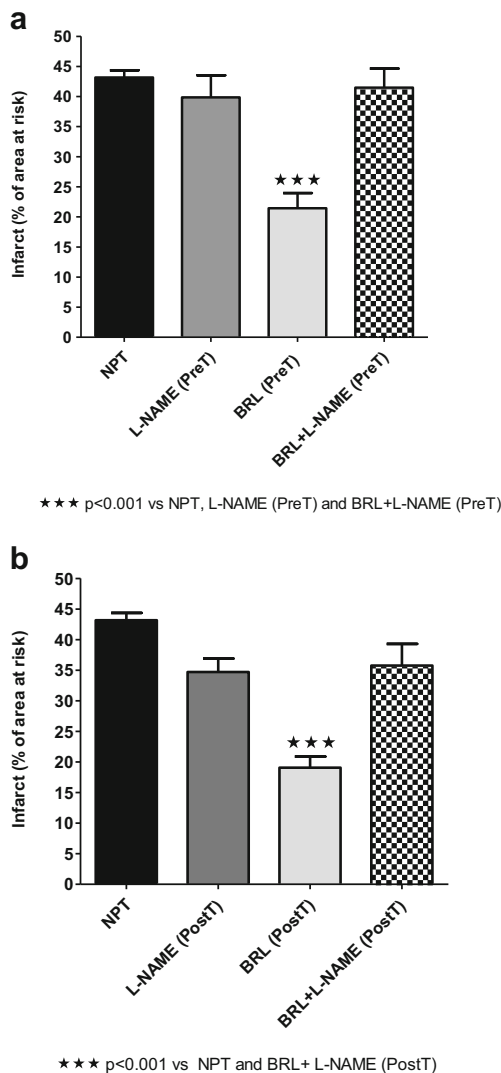
**Fig. 3** **a** Effect on IS when BRL (PostT) or SR (PostT) applied during the first 10 min of reperfusion ( $n = 6$  per group). **b** Effect on IS when BRL or SR applied during the last 10 min of RI as well as the first 10 min of reperfusion, BRL (PerT+PostT) and SR (PerT+PostT), respectively, and effect on IS when BRL was applied during the last 10 min of RI and SR at the onset of reperfusion, BRL (PerT)+SR (PostT) ( $n = 6$  per group)

(post-ischemic/pre-ischemic):  $14.7 \pm 9.3\%$  and  $22.0 \pm 9.8\%$ , respectively compared with NPT  $58.3 \pm 6.2\%$ ,  $p$  value  $< 0.01$ ) (Table 2).

### Effect of BRL37344 or SR59230A on PKB, ERKp44/p42, GSK-3 $\beta$ , and eNOS Activation

#### PKB/Akt Activation

Phosphorylation of PKB was significantly increased when BRL37344 was administered before RI [BRL (PreT)],



**Fig. 4** **a** Effects of L-NAME on IS when administered in combination with BRL before regional ischemia and reperfusion ( $n = 6$ ). **b** Effects of L-NAME on IS when administered in combination with BRL after regional ischemia at the onset of reperfusion ( $n = 6$ )

(fold increase (FI)  $2.9 \pm 0.2$ ,  $p < 0.01$  vs. NPT), or during the last 10 min of regional ischemia [BRL (PerT)] ( $FI 5.5 \pm 0.4$ ,  $p < 0.001$  vs. NPT and BRL (PreT) (Fig. 5a). BRL (PostT) significantly increased PKB compared to the NPT group ( $FI 5.3 \pm 0.4$ ,  $p < 0.05$  vs. NPT). A significant increase in PKB was also observed when BRL37344 was administered during the last 10 min of regional ischemia as well as at the onset of reperfusion for 10 min, [BRL (PerT+PostT)] ( $FI 14.2 \pm 3.7$ ,  $p < 0.01$  vs. NPT and  $p < 0.05$  vs. BRL (PostT) (Fig. 5b). Furthermore,  $\beta$ 3-AR stimulation with BRL37344 during late regional ischemia and  $\beta$ 3-AR inhibition with SR59230A at the start of reperfusion, [BRL (PerT)+SR (PostT)] significantly decreased PKB activation ( $FI 6.6 \pm 0.7$ ) when compared to the BRL (PerT+PostT) group ( $FI 13.9 \pm 2.9$ ,  $p < 0.05$ ) (Fig. 5b).

### ERK p44/42 Activation

BRL (PreT) or BRL (PerT) had no effect on ERK p44/p42 phosphorylation when compared to NPT (Fig. 6a). BRL37344 administered during reperfusion, BRL (PostT), significantly activated ERK p44/p42 ( $FI 3.9 \pm 0.34/3.6 \pm 0.4$ , respectively,  $p < 0.001$  and  $p < 0.01$  vs. NPT group) (Fig. 6b). ERK p44/p42 was also significantly increased when BRL37344 was applied during both regional ischemia and reperfusion BRL (PerT+PostT) ( $FI 4.9 \pm 0.3/4.6 \pm 0.1$ ,  $p < 0.001$  vs. NPT group) (Fig. 6b). Furthermore,  $\beta$ 3-AR stimulation during regional ischemia and inhibition at onset of reperfusion, BRL (PerT)+SR (PostT), did not affect ERK p44/p42 activation (Fig. 6b).

### Activation of Glycogen Synthase Kinase-3 $\beta$

No phosphorylation of GSK-3 $\beta$  was detected with BRL (PreT), (PerT) (Fig. 7a) or (PostT) (Fig. 7b). However, significant GSK-3 $\beta$  phosphorylation was observed when  $\beta$ 3-AR stimulation occurred during the end stage of regional ischemia and the onset of reperfusion, BRL (PerT+PostT) ( $FI 68.8 \pm 7.7$  vs. NPT  $1.3 \pm 0.4$ ,  $p < 0.001$ ).  $\beta$ 3-AR activation at the end of regional ischemia followed by  $\beta$ 3-AR inhibition at the beginning of reperfusion, [BRL (PerT)+SR (PostT)] resulted in phosphorylation of GSK-3 $\beta$ ; however, this phosphorylation was significantly reduced when compared to BRL (PerT+PostT) (Fig. 7b).

### Endothelial Nitric Oxide Synthase in $\beta$ 3-AR Modulation

The application of BRL37344 as a PreT or PerT did not result in any eNOS phosphorylation (Fig. 8a). BRL (PostT) caused marginal but not significant eNOS phosphorylation. BRL37344 administered during last 10 min of regional ischemia as well as the first 10 min of reperfusion [BRL (PerT+PostT)], resulted in a significant eNOS-Ser-1177 phosphorylation when compared to NPT and BRL (PostT) ( $FI 2.8 \pm 0.4$ ,  $p < 0.01$  and  $0.05$ , respectively). The inclusion of SR59230A with BRL as a post-treatment significantly increased eNOS-Ser-1177 phosphorylation, BRL (PerT)+SR (PostT) compared to NPT ( $FI 2.2 \pm 0.4$ ,  $p < 0.05$ ) (Fig. 8b). However, SR59230A post-treatment did not affect eNOS-Ser-1177 phosphorylation when compared to BRL (PerT+PostT) (Fig. 8b).

### cGMP Levels

To determine the role of the  $\beta$ 3-adrenergic receptor in cardioprotection, preference was given to determination of downstream tissue cGMP levels rather than determination of  $\beta$ 3-adrenergic receptor expression which is not necessarily an index of its activation. In addition, events

**Table 1** Hemodynamic parameters: functional performance after regional ischemia

Protocol name	Qe ml/min	Qa ml/min	CO ml/min	HR Beat/min	PSP mmHg	Wt mW
NPT	16.50 ± 1.31	46.17 ± 1.97	63.67 ± 3.28	302 ± 3.74	93.00 ± 1.03	13.21 ± 0.77
BRL (PreT)	13.00 ± 1.00	39.67 ± 2.65	58.67 ± 6.10	266 ± 17.21	89.83 ± 1.55	10.53 ± 0.77
BRL (PerT)	14.67 ± 0.98	44.67 ± 3.88	59.67 ± 5.04	295 ± 11.66	90.50 ± 1.70	12.15 ± 1.20
BRL (PostT)	16.00 ± 1.86	44.67 ± 2.95	61.17 ± 3.93	290 ± 10.82	92.83 ± 0.79	12.62 ± 0.87
BRL (PerT+PostT)	17.25 ± 0.45	51.75 ± 2.46	69.00 ± 2.95	306 ± 7.22	92.88 ± 0.89	14.27 ± 0.70
BRL (PerT)+SR (PostT)	16.00 ± 1.09	42.40 ± 1.93	58.40 ± 2.31	324 ± 24.43	87.80 ± 1.24	11.44 ± 0.56
SR (PreT)	14.67 ± 0.98	44.00 ± 2.58	58.67 ± 3.52	317 ± 13.16	90.33 ± 0.71	11.78 ± 0.72
SR (PerT)	16.00 ± 1.15	45.67 ± 3.02	61.67 ± 3.73	306 ± 18.02	91.33 ± 0.55	12.50 ± 0.75
SR (PostT)	14.67 ± 0.88	41.67 ± 3.32	56.33 ± 3.83	328 ± 28.90	88.17 ± 1.75	10.99 ± 0.92
SR (PerT+PostT)	14.44 ± 0.72	43.33 ± 1.52	57.78 ± 2.14	301 ± 19.44	91.44 ± 1.26	11.77 ± 0.51
L-NAME (PreT)	16.00 ± 0.51	45.67 ± 3.2	61.33 ± 3.63	299 ± 12.31	85.48 ± 3.19	12.07 ± 0.96
BRL (PreT)+L-NAME	18.33 ± 0.80	50.33 ± 1.5	68.67 ± 2.10	319 ± 14.04	83.83 ± 4.79	13.46 ± 0.61
L-NAME (PostT)	17.00 ± 1.23	42.33 ± 3.7	59.33 ± 3.67	294 ± 17.7	89.86 ± 3.40	12.10 ± 0.83
BRL (PostT)+L-NAME	17.67 ± 1.74	45.67 ± 0.6	63.33 ± 1.52	344 ± 33.15	88.67 ± 2.24	12.59 ± 0.42

downstream of cGMP have been proven to reduce calcium fluctuations, ventricular arrhythmias, hypercontracture damage, mitochondrial permeability transition pore (MPTP) opening, and ensuing cell death [19, 20]. The application of the  $\beta_3$ -AR agonist, BRL37344 as a BRL (PreT) (119.3 ± 2.7) as well as BRL (PerT) (110.9 ± 1.3) treatment, significantly increased cGMP levels when compared to NPT (73.4 ± 4.5,  $p < 0.001$  and 0.01, respectively) (Fig. 9). Furthermore, when L-NAME was applied together

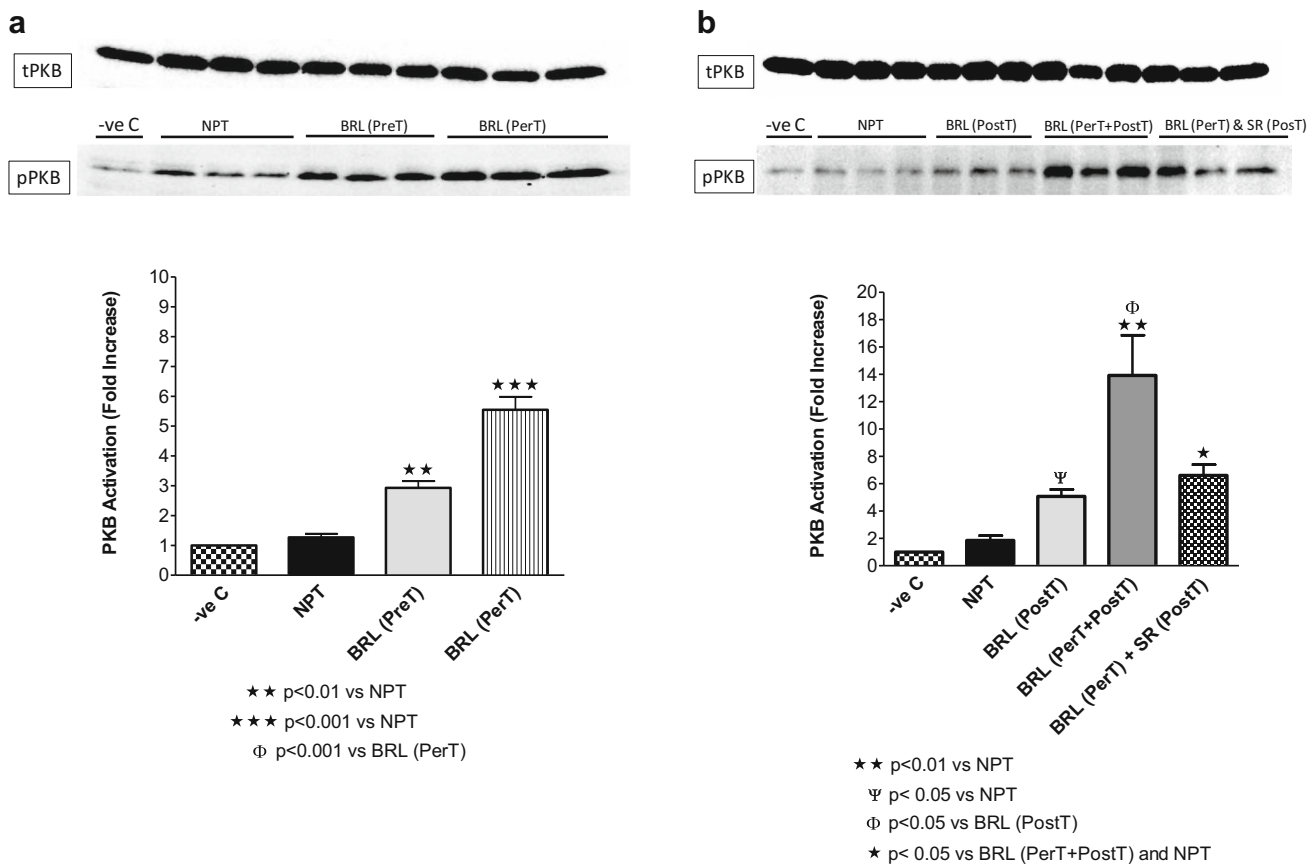
with BRL+L-NAME (PreT) (73.0 ± 3.5) and BRL+L-NAME (PerT) (85.0 ± 3.6), cGMP levels were significantly reduced compared to BRL (PreT) and BRL (PerT) ( $p < 0.01$  and 0.05, respectively) (Fig. 9). BRL (PostT) (96.9 ± 3.5) also significantly increased cGMP levels compared to NPT ( $p < 0.05$ ), which was not affected by L-NAME. Although BRL (PerT+PostT) caused an increase in cGMP levels, the change was not significant when compared to NPT nor did L-NAME affect cGMP levels.

**Table 2** Hemodynamic parameters (mean ± SEM) of the different treated groups after regional ischemia, \* $p < 0.01$  vs. NPT

Protocol name	Qe ml/min	Qa ml/min	CO ml/min	HR Beat/min	PSP mmHg	Wt mW
NPT	16.00 ± 1.03	24.67 ± 2.9	40.33 ± 3.73	311 ± 10.05	84.83 ± 0.98	7.578 ± 0.66
BRL (PreT)	13.83 ± 1.04	20.17 ± 3.6	33.33 ± 3.55	327 ± 33.30	85.00 ± 1.915	6.32 ± 0.73
BRL (PerT)	11.67 ± 3.40	14.67 ± 4.6	26.33 ± 7.47	247 ± 57.27	66.67 ± 13.40	4.74 ± 1.38
BRL (PostT)	12.33 ± 2.55	11.33 ± 3.0	23.67 ± 5.09	270 ± 56.54	68.00 ± 13.64	4.307 ± 0.94
BRL (PerT+PostT)	15.00 ± 0.75	15.50 ± 2.7	30.50 ± 2.99	318 ± 7.61	81.25 ± 0.97	5.543 ± 0.59
BRL (PerT)+SR (PostT)	3.20 ± 3.20*	2.80 ± 2.8*	6.00 ± 6.00*	69 ± 69.80*	16.20 ± 16.20*	1.07 ± 1.07*
SR (PreT)	12.33 ± 2.70	21.83 ± 5.3	34.17 ± 7.84	291 ± 59.30	70.67 ± 14.15	6.43 ± 1.48
SR (PerT)	11.33 ± 0.98	17.67 ± 2.8	29.00 ± 3.60	305 ± 8.08	81.50 ± 0.92	5.28 ± 0.70
SR (PostT)	13.92 ± 1.05	17.67 ± 3.3	31.58 ± 3.64	326 ± 25.70	82.00 ± 1.09	5.79 ± 0.70
SR (PerT+PostT)	8.11 ± 2.63*	10.00 ± 3.6*	18.11 ± 6.15*	167 ± 54.57*	46.56 ± 14.73*	3.36 ± 1.14*
L-NAME (PreT)	11.00 ± 2.3	19.08 ± 4.9	30.08 ± 7.03	247 ± 51.63	65.45 ± 13.11	5.29 ± 1.24
BRL (PreT)+L-NAME	9.66 ± 3.07	21.00 ± 6.6	30.67 ± 9.73	224 ± 71.55	50.67 ± 16.70	5.61 ± 1.77
L-NAME (PostT)	11.50 ± 2.9	22.00 ± 6.3	33.50 ± 8.99	271 ± 71.34	66.20 ± 16.73	6.20 ± 1.69
BRL (PostT)+L-NAME	10.40 ± 0.97	16.80 ± 2.9	27.20 ± 3.77	351 ± 29.15	81.60 ± 1.28	4.98 ± 0.82

Non-pretreatment (NPT) = no drugs applied. Pre-treatment (PreT) = drug applied for 10 min before regional ischemia. Per-treatment (PerT) = drug applied during the last 10 min of regional ischemia. Post-treatment (PostT) = drug applied for 10 min at the onset of reperfusion. Per-treatment + Post-treatment (PerT+PostT) = drug applied during the last 10 min of regional ischemia and at the onset of reperfusion. AO, CF, and Wt of all groups after RI were significantly decreased when compared with AO, CO, and Wt before RI ( $p < 0.001$ )





**Fig. 5** **a** PKB activation when BRL was administered before RI, BRL (PreT) or during the last 10 min of regional ischemia, BRL (PerT) ( $n = 6$  per group). **b** PKB activation when BRL was applied (i) at the beginning of reperfusion, BRL (PostT); (ii) during the last 10 min RI and at the onset

of reperfusion, BRL (PerT+PostT); and lastly (iii) BRL was administered during the last 10 min of RI followed by SR during the first 10 min of reperfusion, BRL (PerT)+SR (PostT) ( $n = 6$  per group)

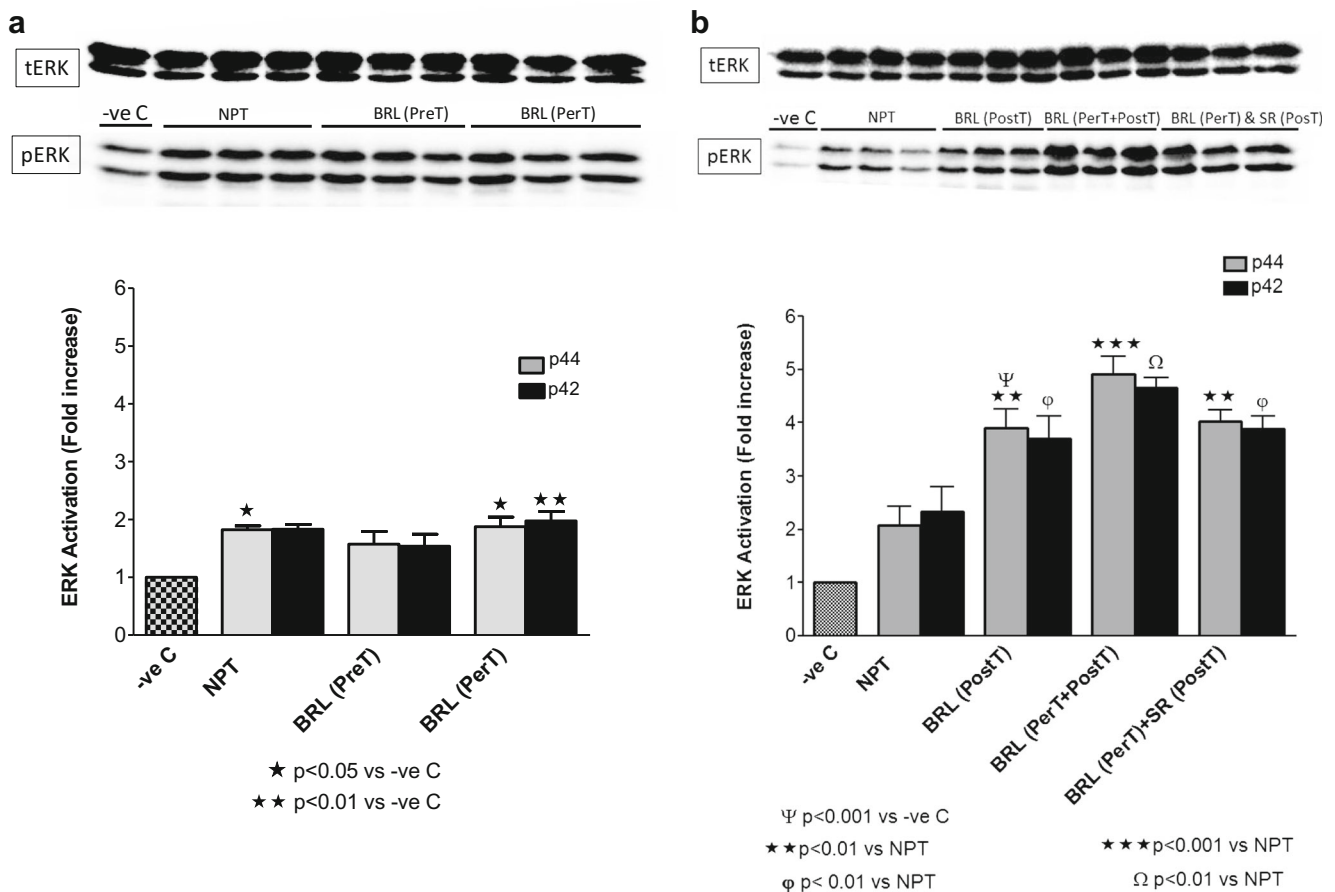
## Discussion

It is well-established that upregulation of the  $\beta_3$ -AR population and concomitant  $\beta_1$ - and  $\beta_2$ -ARs downregulation, in the failing human heart, are induced by increased catecholamine levels [21]. Ensuing  $\beta_3$ -AR activation delivers a more sustained intracellular signal [12] because of its resistance to short-term agonist-promoted desensitization [7], making this receptor an ideal target for therapeutic intervention. Previous studies have investigated the capability of cardioprotective interventions or drugs administered at the onset of reperfusion to reduce infarct size [22–24]. Although protection can be initiated at reperfusion, injury also occurs during ischemia and the relative contribution of each event also depends on the duration of ischemia [1]. Consequently, if cardioprotective strategies can be introduced before or during ischemia, it is possible that they will enhance protection, particularly with longer durations of ischemia [1]. Since  $\beta_3$ -ARs are stimulated at high catecholamine concentrations and induce negative inotropic effects, protecting the heart from catecholamine overstimulation [10, 11], their modulation prior to and/or at the end of sustained ischemia and/or early

reperfusion may prove to be a valuable cardioprotective strategy.

### Effect of $\beta_3$ -AR Stimulation/Inhibition on the Outcome of Ischemia/Reperfusion and the Consequent Impact on Infarct Size and Functional Recovery

The 35-min regional ischemia/60-min reperfusion periods employed in the present and numerous other studies done in our laboratory were found to be effective to induce the development ischemia/reperfusion-mediated damage [25–27]. The historical viewpoint of ischemia/reperfusion damage was highlighted in the review of Turer and Hill (2010) [28] emphasizing the crucial observation of Jennings and colleagues, 1960 [29] that reperfusion following ischemia was associated with myocardial injury. Histological changes seen following only 30–60 min of ischemia/reperfusion (I/R) were similar to the necrosis normally detected after 24 h of permanent coronary occlusion [29]. Also, reperfusion injury occurs within minutes of reperfusion and is additive to that component of cell death due to the ischemic event itself [30]. Whether



**Fig. 6** **a** ERKp44/p42 activation before regional ischemia, BRL (PreT) or during the last 10 min of regional ischemia, BRL (PerT) ( $n = 6$  per group). **b** ERKp44/p42 activation when BRL was applied (i) at the onset of reperfusion, BRL (PostT); (ii) during the last 10 min RI and the first

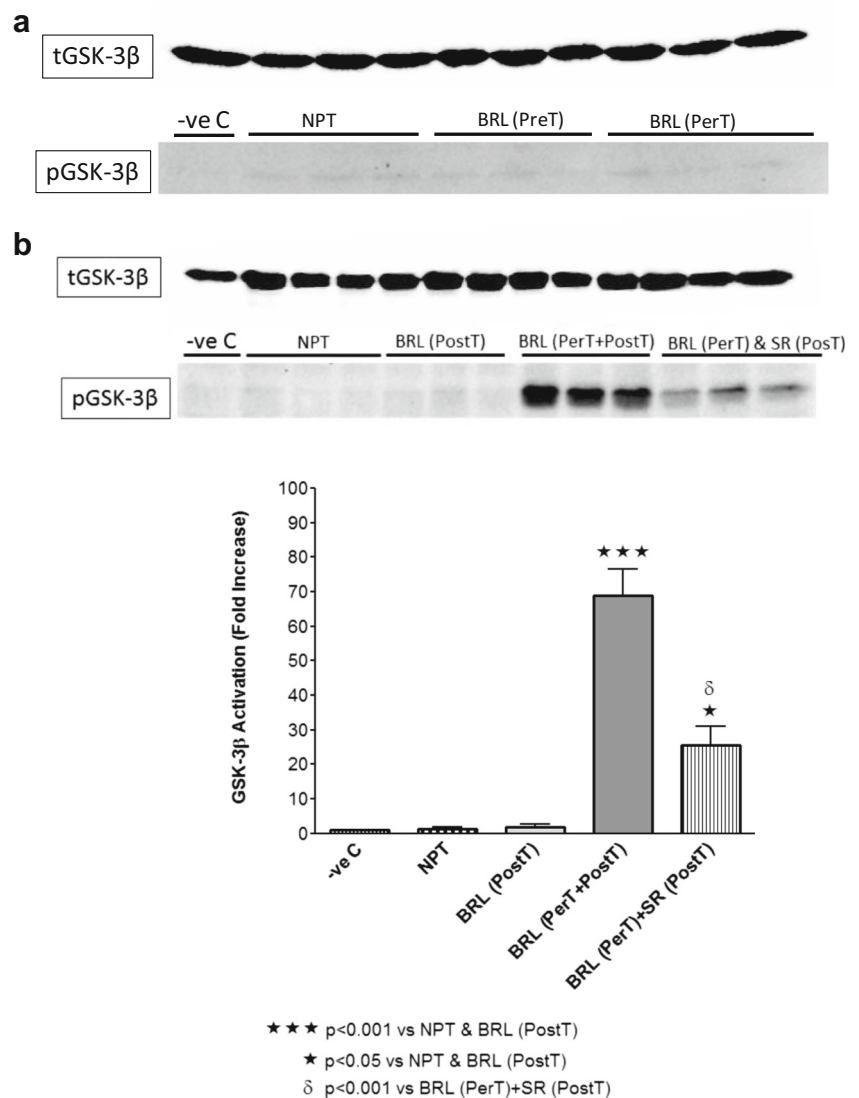
10 min reperfusion, BRL (PerT+PostT) and (iii) BRL was administered during regional ischemia and SR at the onset of reperfusion, BRL (PerT)+SR (PostT) ( $n = 6$  per group)

reperfusion is independently responsible for tissue injury, or merely accelerates the cell death otherwise destined for necrosis, will remain a matter of debate.

The current study provides conclusive evidence of the cardioprotective effects of  $\beta_3$ -AR stimulation with BRL37344, regardless of the time of administration during an ischemia/reperfusion protocol. This was clearly demonstrated by the significant reduction in infarct size, obtained after BRL37344 stimulation at different times during the experimental protocol [BRL (PreT) (Fig. 2a), BRL (PerT) (Fig. 2b) and BRL (PostT) (Fig. 3a). Other *in vivo* studies have recently also illustrated a reduction in infarct size with  $\beta_3$ -AR agonist administered during reperfusion, confirming our *ex vivo* data. For example, administration of BRL37344 during reperfusion after *in vivo* coronary artery ligation attenuated fibrosis and scar formation [31] and reduced infarct size in both small and large animal models of MI/R [5]. Also, it was recently demonstrated that nebivolol, a third-generation  $\beta$ -blocker, protects against myocardial infarction via stimulation of  $\beta_3$ -AR and the eNOS/nNOS pathway [32].

However, the reduction in infarct size was not associated with improvement in functional recovery during reperfusion (see “Results” section, Tables 1 and 2) and necessitates further investigation. Similarly, the study of Aragon and coworkers (2011) [4] also failed to demonstrate any significant improvement in cardiac function after reperfusion with nebivolol as well as with selective  $\beta_3$ -AR agonists, BRL37344 and CL316243. It can be, however, speculated that these findings may be due to concomitant myocardial stunning, namely post-ischemic mechanical contractile dysfunction that persists during reperfusion despite the restoration of flow, which counteracts the beneficial effects of infarct size reduction on mechanical performance. Myocardial stunning has been proposed to be due to either the generation of free radicals or calcium overload, leading to excitation-contraction uncoupling [33]. However, it was suggested that  $\beta_3$ -AR activates NOS, increases NO, and cGMP production [34]. Since increases in cGMP are known to be associated with a reduction in intracellular calcium concentration [35], this phenomenon should cause attenuation of myocardial stunning. However, since stunning persisted despite the reduction in infarct size,

**Fig. 7** **a** GSK-3 $\beta$  activation when BRL was administered before RI, BRL (PreT) or during the last 10 min of regional ischemia, BRL (PerT) ( $n = 6$  per group). **b** GSK-3 $\beta$  activation when BRL was applied (i) at the onset of reperfusion, BRL (PostT); (ii) during the last 10 min of RI as well as the first 10 min reperfusion, BRL (PerT+PostT); (iii) at the end of RI and inhibited with SR at the onset of reperfusion, BRL (PerT)+SR (PostT) ( $n = 6$  per group)



myocardial stunning could be attributed to ROS production during reperfusion and not calcium overload.

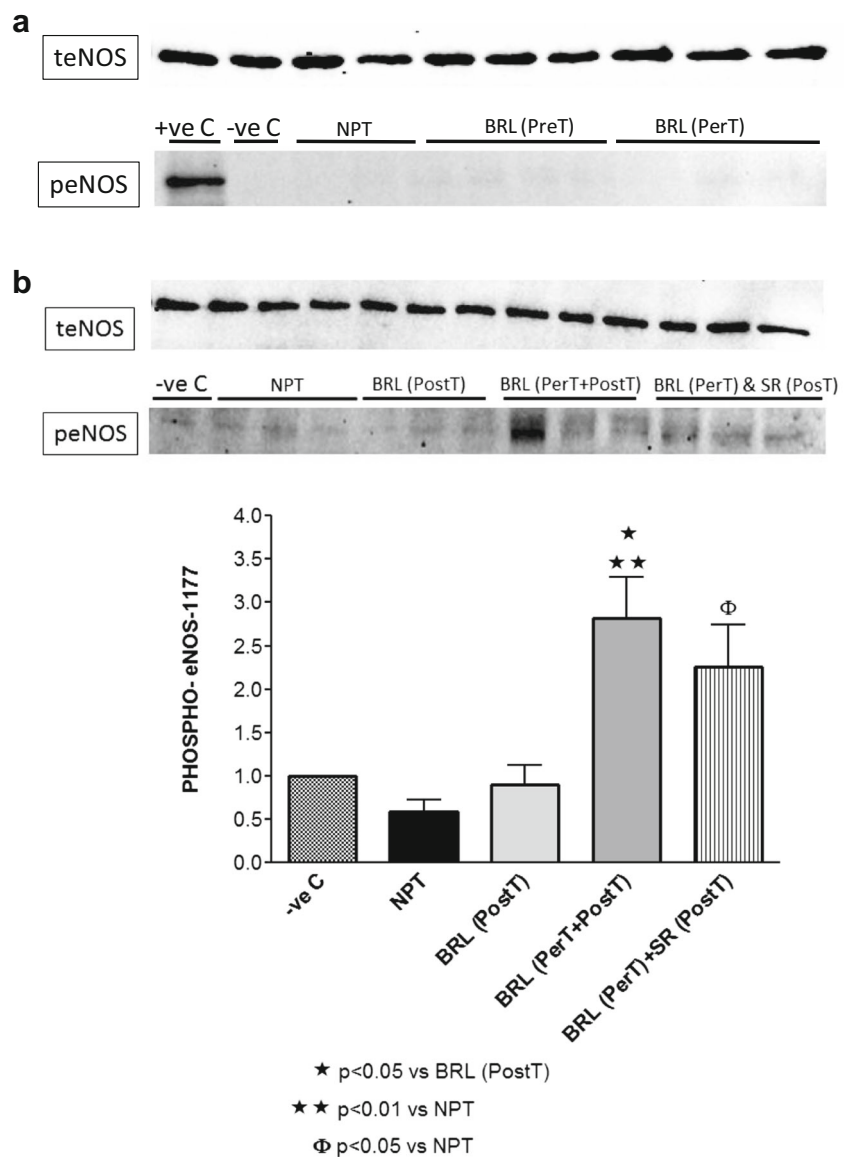
### $\beta$ 3-AR Stimulation, NOS, NO, and cGMP Activation

In the current study, the increased coronary flow (% increase  $104.50 \pm 4.53$ ) observed during administration of BRL37344 during retrograde perfusion of hearts suggest a vasodilatation effect, which may allude to the involvement of NOS and NO as important role players in  $\beta$ 3-AR-mediated cardioprotection. It was previously demonstrated that  $\beta$ 3-ARs expressed in human coronary endothelium mediate vasodilatation via NO production [36]. Subsequently, nebivolol, although it is a selective  $\beta$ 1-AR blocker, has been shown to produce vasodilation in both human and rodent coronary microarteries through stimulation of endothelial  $\beta$ 3-ARs and subsequent release of NO [36]. In the present study, the role of the NOS/NO/cGMP pathway was evaluated using the non-

specific NOS inhibitor, L-NAME at a concentration of 50  $\mu$ M, since it has been shown in our laboratory that this concentration significantly reduced cGMP levels [37]. L-NAME administered in combination with the  $\beta$ 3-AR agonist (BRL37344) prior to regional ischemia, [BRL+L-NAME (PreT)] or at the onset of reperfusion, [BRL+L-NAME (PostT)], abolished cardioprotection as illustrated by a significant increase in infarct size (Fig. 4a, b).

Western blot results consistently provided supportive evidence for the involvement of eNOS in the cardioprotection elicited by selective  $\beta$ 3-AR stimulation, particularly when BRL37344 was applied at the end of regional ischemia and continued into reperfusion, [BRL (PerT+PostT)] (Fig. 8a, b). eNOS activation persisted, when the BRL37344 was administered at the end of ischemia and SR59230A at the beginning of reperfusion with [BRL (PerT)+SR (PostT)] (Fig. 8a, b). Similar effects were reported by Niu and coworkers (2012) [11]. In contrast to the proposed dual activation of eNOS and

**Fig. 8** **a** eNOS-Ser-1177 activation after BRL was applied before regional ischemia, BRL (PreT) or at the end of regional ischemia, BRL (PerT) ( $n = 6$  per group). **b** eNOS-Ser-1177 activation with BRL administration at (i) onset of reperfusion, BRL (PostT); (ii) during the last 10 min of RI as well as the first 10 min reperfusion, BRL (PerT+PostT); (iii) at the end of RI and SR at the onset of reperfusion, BRL (PerT)+SR (PostT) ( $n = 6$  per group)

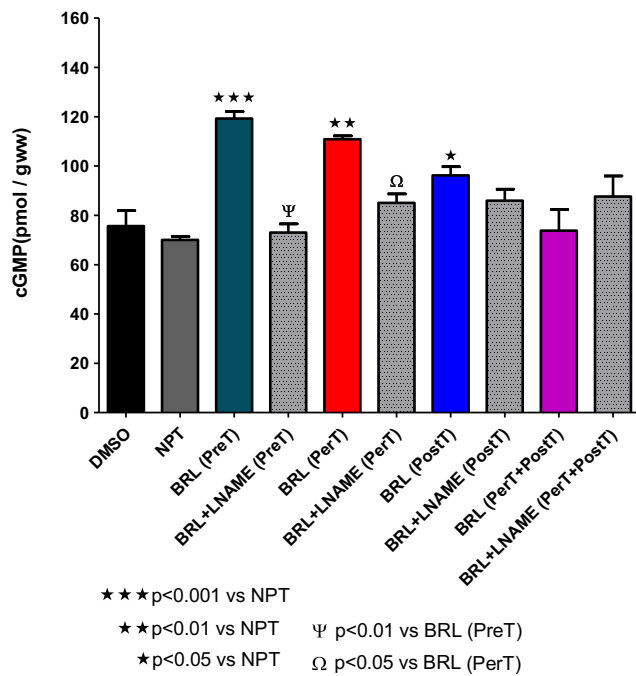


nNOS, other studies suggested that NO release, secondary to  $\beta_3$ -AR activation, was exclusively via eNOS [38, 39]. However, nNOS and/or iNOS have also been implicated in the  $\beta_3$ -AR-mediated cardioprotective signaling [40, 41].

Experimental evidence suggests that  $\beta_3$ -AR stimulation and cGMP/PKG activation ameliorate irreversible injury associated with ischemia–reperfusion and may be a valuable therapeutic target [42]. In this study, different strategies were used to target the  $\beta_3$ -AR, with BRL37344 (PreT) and BRL (PerT) (Fig. 2a, b) resulting in a reduction in infarct size, cardioprotection, and significant elevation in cGMP levels (Fig. 9). The NOS inhibitor L-NAME applied with BRL37344 (PreT) and BRL (PerT) significantly reduced cGMP levels confirming the involvement of NOS (Fig. 9). However, this was not associated with changes in eNOS phosphorylation (Fig. 8a). Although several studies suggested that

selective  $\beta_3$ -AR stimulation elicits its protection via NOS activating pathways and NO release [4, 36, 40], the exact NOS isoform involved is still being debated. However, this remains to be further elucidated since nNOS/iNOS activation was not evaluated in the present study, but indications are that iNOS is not activated by  $\beta_3$ -AR stimulation [4, 38]. Nevertheless, the involvement of an eNOS/NO-independent cGMP activation pathway to bring about cardioprotection cannot be ruled out [43, 44].

$\beta_3$ -adrenergic receptor-mediated cardioprotection was not always associated with eNOS activation, for example, BRL (PreT) and BRL (PerT)-mediated cardioprotection were not associated with eNOS activation but significantly increased cGMP levels and significantly increased phosphorylated PKB/Akt (Fig. 5a) but not ERKp44/p42 (Fig. 6a). On the other hand, BRL (PostT) produced marginal eNOS



**Fig. 9** cGMP levels of BRL (PreT), (PerT), (PostT) and BRL (PerT+PostT) with and without L-NAME

phosphorylation, significant cGMP levels, PKB/Akt (Fig. 5b), and ERKp44/p42 phosphorylation (Fig. 6b). In this regard, evidence implicates Akt/PKB and ERK p42/p44 MAPK in the phosphorylation of eNOS [45] and previous work demonstrated that the PI3-K/PKB pathway mediates increased eNOS phosphorylation at Ser1177 and NO production during hypoxia [46]. On the other hand, it was shown that phosphorylation of eNOS by ERK in vitro significantly reduced eNOS enzyme activity [47] and inhibition of MEK1/2 with PD98059 in pulmonary artery endothelial cells [48] attenuated eNOS activity [49]. Despite many studies, the mechanisms by which MEK/ERKp44/p42 and or Akt/PKB signaling affects eNOS activity remains to be established, especially when BRL37344 (PreT), BRL (PerT), and BRL (PostT)-mediated cardioprotection was achieved without the phosphorylation of GSK-3 $\beta$ .

Interestingly, the most significant changes in intracellular signaling were observed in the BRL (PerT+PostT) group namely, increased eNOS phosphorylation (Fig. 8b) associated with significant PKB/Akt (Fig. 5b) as well as ERKp44/p42 (Fig. 6b) activation and a 68-fold increase in phosphorylation of the downstream target, GSK-3 $\beta$  (Fig. 7b). We suggest that the ensuing phosphorylation and inactivation of GSK-3 $\beta$  lead toward the concomitant inhibition of MPTP opening, an event linked to cardioprotection [15]. It is of particular interest that activation of PKB/Akt was observed in all experimental protocols associated with cardioprotection, particularly in the BRL (Per+Post) where a 14-fold increased phosphorylation was observed (Fig. 5b). Both PKB and ERK stimulate ser1177 phosphorylation of eNOS [50], whereas ERKp44/

p42 has been implicated in eNOS activation by high density lipoprotein (HDL) which does not seem to involve ser1177 phosphorylation of eNOS [16]. BRL (PerT+PostT)-mediated cardioprotection was shown here to follow the more conformist manner of eNOS phosphorylation and activation of downstream cardioprotective targets, i.e., the NO-cGMP-PKG cascade [51].

As expected, stimulation of  $\beta$ 3-AR with BRL37344 during late regional ischemia and its inhibition with SR59230A at the onset of reperfusion [BRL (PerT)+SR (PostT)] resulted in a significant reduction in phospho-PKB/Akt compared to BRL (PerT+PostT) (Fig. 5b). However, the degree of PKB/Akt activation may have been adequate to provide suitable cardioprotection, since SR (PostT) could not abrogate the reduced IS and cardioprotection attained with BRL (PerT) (Fig. 3b). This could possibly be due to a too low concentration of the inhibitor or due to the presence of the agonist during per-treatment, which provided maximum cardioprotection. Also, stimulation of the  $\beta$ 3-AR during late regional ischemia and its inhibition with SR59230A at the onset of reperfusion, [BRL (PerT)+SR (PostT)] did not affect ERKp44/p42 activation (Fig. 6b). The significant ERKp44/p42 activation was also associated with a significant infarct size reduction (Fig. 3b) and accompanying eNOS activation (Fig. 8b), highlighting a crucial role for NO in ERKp44/p42 phosphorylation and BRL37344-mediated cardioprotection. These findings correlate well with the neonatal rat ventricular myocyte model of hypoxia/reoxygenation, where the nitric oxide (NO) donor S-nitrosoglutathione (10  $\mu$ M) rapidly (10 min) activated JNK and ERK, respectively [52].

## Summary

The cardioprotective effects of selective  $\beta$ 3-AR stimulation are well established and confirmed by the results obtained in the present study [4, 11, 33]. However, the novelty of this study was to establish the most appropriate time period to apply transient  $\beta$ 3-AR activation to bring about cardioprotection. The association between infarct size reduction and activation of cardioprotective signaling pathways were not always forthcoming, for example, the cardioprotective effect of  $\beta$ 3-AR stimulation before and during ischemia with BRL37344 (PreT) and BRL (PerT), respectively, was achieved without significant activation of eNOS, ERKp44/p42, or GSK-3 $\beta$  phosphorylation but significantly increased cGMP levels and PKB/Akt activation. On the other hand,  $\beta$ 3-AR stimulation at the beginning of reperfusion, BRL (PostT), marginally increased eNOS activation, significantly increased cGMP levels, PKB/Akt, and ERKp44/p42 phosphorylation. Interestingly, in all BRL protocols, cardioprotection was associated with increased phosphorylation and activation of PKB, while BRL (PerT+PostT)-mediated cardioprotection was associated with evidence of the

conventional  $\beta$ 3-AR/eNOS/NO/cGMP signaling cascade, associated with significant PKB/Akt, ERKp44/p42, and GSK-3 $\beta$  phosphorylation. Thus, it is concluded that  $\beta$ 3-adrenergic-mediated cardioprotection is largely dependent on the eNOS signaling pathway and ultimate activation of PKB. The data also suggest that clinical application of  $\beta$ 3-AR stimulation should preferably be incorporated during late ischemia or/and early reperfusion.

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### Compliance with Ethical Standards

**Experimental Animals** Male Wistar rats (230 to 250 g) were used in this study. The handling of laboratory animals was in accordance with the ethical guidelines as set out by the University of Stellenbosch, Faculty of Medicine and Health Sciences Ethics Committee and the South African National Standard for Care and Use of Animals for Scientific Purpose (SANS 10386: 2008). The rats had free access to food and water prior to experimentation. Rats were anesthetized with sodium pentobarbital (30 mg/rat) by intraperitoneal injection before removal of hearts.

**Conflict of Interest** The authors declare that they have no conflict of interest.

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