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Loss of Myocardial Ischemic Postconditioning in Adenosine A_1 and Bradykinin B_2 Receptors Gene Knockout Mice

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Abstract

Background—Ischemic postconditioning (PostC) is a recently described cardioprotective modality against reperfusion injury, through series of brief re-flow interruptions applied at the very onset of reperfusion. It is proposed that PostC can activate a complex cellular signaling cascade, in which cell membrane receptors could serve as the upstream triggers of PostC. However, the exact subtypes of such receptors remain controversial or uninvestigated. To this context, the purpose of present study was to determine the definitive role of adenosine A_1, bradykinin B_1 and B_2 receptors in PostC.

Methods and Results—The hearts isolated from adult male C57BL/6J wild-type mice or the mice lacking adenosine A_1, or bradykinin B_1 or B_2 receptors subjected to zero-flow global ischemia and reperfusion in a Langendorff model. PostC, consisting of 6 cycles of 10 sec of reperfusion and 10 sec of ischemia, demonstrated significantly reduced myocardial infarct size (22.8±3.1%, Mean±SEM) as compared with the non-PostC wild-type controls (35.1±2.8%, $P<0.05$). The infarct-limiting protection of PostC was absent in adenosine A_1 receptor knockout mice (34.9±2.7%) or bradykinin B_2 receptor knockout mice (33.3±1.7%) and was partially attenuated in bradykinin B_1 receptor deficient mice (25.6±2.9%; $P>0.05$). On the other hand, PostC did not significantly alter post-ischemic cardiac contractile function and coronary flow.

Conclusions—With the use of three distinctive strains of gene knockout mice, the current study has provided the first conclusive evidence showing PostC-induced infarct-limiting cardioprotection could be triggered by activation of multiple types of cell membrane receptors, which include adenosine A_1 and bradykinin B_2 receptors.

Keywords

Adenosine; Bradykinin; Infarction; Receptors; Reperfusion

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Disclosures None.

Introduction

Ischemic postconditioning (PostC) is a series of brief mechanical interruptions of reperfusion applied at the very onset of reperfusion[1]. Since the original description of PostC in an *in vivo* dog model in 2003[2], this cardioprotective phenomenon has been confirmed by a number of research groups in several mammalian species including rat[3–5], mouse[3;6;7], rabbit[8–12], dog[9], and human[13]. This novel cardioprotective strategy has received considerable interests, mainly due to its potential clinical applicability as a post-ischemic intervention to reduce the cellular damages caused by the pathological factors during the initial minutes of reperfusion. In terms of the timing of intervention, PostC has an advantage over ischemic preconditioning, which has to be applied prior to a sustained ischemic event whose occurrence is usually not easy to predict precisely. In contrast, PostC apparently does not have the same pretreatment timing restrain for preconditioning and therefore it can be used during the routine interventional reperfusion procedures in the patients suffering acute myocardial infarction.

It is now widely accepted that PostC has interrelated passive and active components in its underlying cellular protective mechanisms1;5;14. During the PostC maneuvers, the washout of intra-coronary release of adenosine (possibly bradykinin as well) was significantly delayed[3]. Furthermore, the PostC-induced intermittent accumulation/release of adenosine and bradykinin could also facilitate the activation of their corresponding receptors on cardiac cell membranes1;14, which in turn triggers the signaling cascade of PostC. Nevertheless, the exact subtypes of adenosine and bradykinin receptors involved in triggering PostC remain controversial or unknown1;3;11;14. To resolve this issue, the present study was undertaken to determine the efficacy of PostC in reducing myocardial infarct size in strains of mice lacking adenosine A_1, bradykinin B_1 and B_2 receptors.

Materials and Methods

Animals

Adult male C57BL/6J wild-type (C57-WT) mice and homozygous (−/−) bradykinin B_2 receptor knockout (B2-KO) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Adult male adenosine A_1 receptor knockout (A1-KO) mice were bred in the Virginia Commonwealth University (VCU) animal facility using the breeding pairs of homozygous (−/−) A1-KO mice, a generous gift from Dr. Jurgen Schnermann (NIDDK, Bethesda, MD, USA)[15]. Adult male homozygous (−/−) bradykinin B_1 receptor knockout (B1-KO) mice were generated at the Max-Delbrück-Center for Molecular Medicine, Berlin-Buch, Germany according to the published methods[16]. The B1-KO mice at age of 6 weeks were transferred to VCU animal facility. Because all of the gene-knockout mice used in this study were generated from C57BL/6J background, C57-WT mice were used as the common control group for A1-KO, B1-KO, and B2-KO groups. The animal care and experiments were conducted in conformity with the guidelines on humane use and care of laboratory animals for biomedical research published by NIH (No. 85-23, revised 1996) and the rodent experiment protocol was approved by the Institutional Animal Care and Use Committee of VCU.

Model of global ischemia-reperfusion in Langendorff isolated mouse heart

The methodology of Langendorff isolated buffer-perfused mouse heart preparation was previously described in details[17;18]. In brief, the mouse was anesthetized with pentobarbital sodium (100 mg/kg, 33 IU heparin; *i.p.*) and the heart was quickly removed from the thorax and placed in icecold buffer. The aortic opening was rapidly cannulated (time delay <3 min) and tied on a 20-gauge blunt needle connected to Langendorff perfusion system. The heart

was retrogradely perfused at constant pressure of 55 mm Hg with modified Krebs-Henseleit buffer containing (in mmol/L): 118 NaCl, 24 $NaHCO_3$, 2.5 $CaCl_2$, 4.7 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 11 glucose, and 0.5 EDTA. The buffer was continuously gassed with 95% O_2 + 5% CO_2 (pH ~7.4) and warmed by a heating bath/circulator. The heart temperature was continuously monitored and maintained at 37±0.5°C. Ventricular function was measured by a force-displacement transducer (model FT03, Grass) attached to the apex with surgical thread and metal hook. The resting tension was adjusted to ~0.30 g. The contractile force was continuously recorded with a PowerLab 8SP computerized data acquisition system connected to the force transducer. Coronary flow rate was calculated by timed collection of the outflow perfusate. No electrical pacing was applied to the heart.

Experiment groups and protocol

As illustrated inFig. 1, C57-WT control mice and the three strains of receptor deficient mice (*i.e.* A1-KO, B1-KO, B2-KO) were randomized into the following 8 experiment groups. Group 1: *C57-WT* (n=8), C57-WT subjected to 20 min of no-flow global ischemia and 30 min of reperfusion; Group 2: *C57-WT+PostC* (n=8), C57-WT subjected to ischemia and PostC intervention (6 cycles of 10 sec of ischemia and 10 sec of reperfusion) during the initial 2 min of reperfusion; Group 3: *A1-KO* (n=8), A1-KO subjected to ischemia and reperfusion; Group 4: *A1-KO+PostC* (n=7), A1-KO subjected to ischemia and PostC; Group 5: *B1-KO* (n=9), B1-KO subjected to ischemia and reperfusion; Group 6: *B1-KO+PostC* (n=9), B1-KO subjected to ischemia and PostC; Group 7: *B2-KO* (n=7), B2-KO subjected to ischemia and reperfusion; and Group 8: *B2-KO+PostC* (n=7), B2-KO subjected to ischemia and PostC.

Measurement of infarct size

At the end of experiment, the heart was immediately removed from Langendorff apparatus, weighed, and frozen at −20°C. The frozen heart was manually cut into seven to eight transverse slices (~1 mm thickness), which were incubated in 10% TTC for 30 min at room temperature. TTC was then replaced with 10% formaldehyde for 3–4 hours of fixation before infarct size was measured using a computerized morphometry system (Bioquant 98). The risk area was calculated as total ventricular area minus cavities. Infarct size was calculated as % of risk area.

Data analysis and statistics

The experimental data were presented as the group means and standard errors (SEM). The difference among experimental groups was analyzed with two-way ANOVA followed by Bonferroni post-hoc test for pair-wise comparison. This analysis examines the effects of two variables (*i.e.* Factor 1: PostC intervention and Factor 2: animal genetic background), both individually and together, on the experimental response. $P<0.05$ was considered as statistically significant.

Results

Animal exclusion record and morphometric characteristics

Out of 66 mice originally used in the present study, three mice (*i.e.* 4.5% of the total) were excluded due to persistent arrhythmia or poor pre-ischemic function. The body weight and heart weight were significantly higher in A1-KO and B1-KO mice as compared with the non-PostC C57-WT mice, indicating a genetic background related variation in body/heart mass (Table 1).

Myocardial infarct size

As shown inFigures 2 and 3, PostC resulted in reduction of infarct size following ischemia-reperfusion in C57-WT mice (22.8±3.1%), as compared with the wild-type control mice without PostC (35.1±2.8%; $P<0.05$). Infarct size in the non-PostC A1-KO and B2-KO was not different from the C57-WT controls ($P>0.05$). However, the infarct-limiting protection of PostC was completely abolished in mice lacking either adenosine A_1 receptors or bradykinin B_2 receptors (Figure 3; $P>0.05$ *vs.* the corresponding non-PostC A1-KO or B2-KO). Genetic deletion of bradykinin B_1 receptors also did not alter myocardial infarct size (*i.e.* 30.9±3.6% in B1-KO *vs.* 35.1±2.8% in C57-WT; $P<0.05$). PostC was able to marginally reduce infarct size to 25.6±2.9% in B1-KO (Figure 3; $P>0.05$ *vs.* non-PostC B1-KO group), indicating PostC was partially attenuated in the mice deficient of bradykinin B_1 receptors.

Baseline and post-ischemic cardiac contractile function

There was no significant difference in the pre-ischemia baseline levels of ventricular developed force and rate-force product among the eight experimental groups (Table 1; $P>0.05$), despite the baseline contractile function tended to be lower in B2-KO groups. Following the global ischemia-reperfusion, the developed force and rate-force product were remarkably depressed in all of the eight experimental groups, whereas heart rate remained at a constant level similar to the pre-ischemic values at both early (5 min) and late (30 min) time points of reperfusion (Tables 1 and 2;Figure 4). Interestingly, PostC did not improve the post-ischemic cardiac contractile function at either 5 min or 30 min of reperfusion in any strain of the wild-type and gene knockout mice (Table 2 and Figure 4; $P>0.05$). It is notable that the early post-ischemic functional recovery was remarkably improved in B1-KO group (*i.e.* 89.8±10.6% of pre-ischemic baseline for rate-force product *vs.* 58.9±12.6% in C57-WT group; seeFigure 4B), although it failed to reach the statistical significance mainly due to the high intra-group variability for the contractile function parameters ($P>0.05$ with two-way ANOVA). Furthermore, this trend of functional improvement in B1-KO mice disappeared at the end of 30 min reperfusion (Figure 4D).

Baseline and post-ischemic coronary flow

Genetic deletion of adenosine A_1, or bradykinin B_1 or B_2 receptors did not alter the pre-ischemic and post-ischemic coronary flow rate ($P>0.05$;Table 1 andFigure 5). There was only a slight trend towards higher post-ischemic coronary flow in A1-KO mice (Figure 5). PostC also had no effect on the post-ischemic coronary flow as compared with the corresponding non-PostC groups ($P>0.05$;Figure 5).

Discussion

The present study has demonstrated the existence of PostC against myocardial ischemia-reperfusion injury in C57BL/6J wild-type mice. The six cycles of 10 sec of reperfusion and 10 sec of ischemia PostC maneuvers applied at the very onset of reperfusion significantly reduced infarct size in the globally ischemic-reperfused mouse hearts (Figures 2 and 3). The degree of infarct size reduction of PostC was approximately 35% from the non-PostC controls, which appears to be less potent than the ~42% reduction observed in our previous ischemic preconditioning study in ICR outbred mice[17] performed in Langendorff perfused hearts with similar duration of ischemia-reperfusion. These results are comparable to the previous studies demonstrating efficacy of PostC in mice with 31% reduction in infarct size[6] and 37% reduction in cardiac troponin I release – a specific marker for cardiac cell necrosis[7]. These results also support the concept that protective efficacy of PostC is somewhat less robust as compared to ischemic preconditioning based on the findings in conscious rats[4] and anesthetized rabbits[8]. Furthermore, the infarct-limiting effect of PostC

was not associated with any improvement in ventricular contractile function at 5 min or 30 min of reperfusion (Table 2 and Figure 4). This dissociation of infarct size and ventricular function was previously observed by our laboratory and others in various settings of preconditioning[17–19] and PostC[9;19]. Some previous studies did report significant improvement in cardiac function following PostC[3;7].

More importantly, the present study is the first investigation using adenosine A_1, bradykinin B_1 or B_2 receptors knockout mice to determine the possible participation of these membrane receptors in triggering PostC. This is because the genetic deletion of a particular receptor gene could circumvent many confounding factors involved with the pharmacological antagonists of membrane receptors, such as suboptimal dose, target tissue availability, receptor affinity and selectivity, and other non-specific effects of the drugs. To our knowledge, there were only two previous PostC studies in which the mice with targeted deletion of connexin-43[6] or adenosine A_{2A} receptors[7] were used.

Adenosine A_1 receptors and PostC

Despite a general agreement for the involvement of adenosine receptors in cardioprotection at reperfusion such as PostC[12], the exact receptor subtype(s) that are responsible for triggering PostC remain controversial. One study addressed this issue by using the blockers of adenosine A_1 (DPCPX); A_{2A} (ZM241385) or A_3 (MRS1523) receptors at the onset of reperfusion in an *in vivo* rat model and the authors concluded that A_{2A} and A_3, but not A_1 receptors are important for PostC-induced cardioprotection[3]. The role of adenosine A_{2A} receptors was recently confirmed by abrogation of PostC in mice lacking A_{2A}[7]. However, another *in vivo* rabbit study reported blockade of PostC by an A_{2B} receptor antagonist, MRS1754, but not by DPCPX and A_{2A} receptor antagonist, 8-(13-chlorostyryl)caffeine[11]. In contrast, the present study clearly indicated complete loss of PostC-induced cardioprotection in A1-KO mice (Figure 3), which strongly suggest that adenosine A_1 receptors are also essential for eliciting PostC. Interestingly, the findings are supported by a recent publication[19], which reported that administration of A_1 blocker DPCPX (200 nmol/L) abolished PostC-induced infarct size reduction in isolated rabbit hearts subjected to global ischemia-reperfusion. In addition, activation of A_1 receptors by endogenous adenosine at reperfusion has been shown to protect the mouse heart[20]. To explain this apparent discrepancy, we would suspect that the intravenous administration of DPCPX shortly before or at the onset of reperfusion may not effectively block A_1 receptors in cardiomyocytes when PostC was employed immediately after reperfusion in the two *in vivo* studies[3;11].

Bradykinin B_1 receptors and PostC

The pathophysiological function of bradykinin B_1 receptors in ischemia-reperfusion injury remains ambiguous. From a few previous studies on B1-KO mice, both of beneficial[21] and detrimental[22;23] effects of B_1 receptors on myocardial infarction and/or remodeling have been proposed. Our current study also yielded a blurry picture on the role of B_1 receptors in PostC. We found that PostC produced a marginally smaller infarct size in B1-KO mice (25.6±2.9%) than the non-PostC B1-KO mice (30.9±3.6%; $P>0.05$;Figure 3). These results suggest that PostC was only partially attenuated in the absence of B_1 receptors. Therefore, we believe that bradykinin B_1 receptors do not play role in triggering PostC.

Bradykinin B_2 receptors and PostC

Contrary to the above-mentioned controversies concerning the role of adenosine A_1 and bradykinin B_1 receptors in myocardial reperfusion injury and cardioprotection, there is a unanimous agreement on the crucial importance of bradykinin B_2 receptors in myocardial protection, such as ischemic and pharmacologic preconditioning[24;25]. The present study further supports the notion that bradykinin B_2 receptors are also indispensable in PostC,

because the infarct-limiting effect of PostC observed in C57-WT mice was completely lost in B2-KO mice (Figure 3). Our results also showed that genetic deficiency of bradykinin B_2 receptors did not significantly modify myocardial tolerance to ischemia-reperfusion injury, indicated by the similar infarct size (*i.e.* 35.1±2.8% in C57-WT *vs.* 31.3±3.0% in B2-KO; $P>0.05$;Figure 3). Similar results were observed after *in vivo* ischemia-reperfusion in B2-KO mice[23;25]. Furthermore, a recent study showed that pharmacological inhibition of B_2 receptors by administration of HOE140 or WIN64338 blocked the infarct size reduction afforded by PostC or intermittent bradykinin infusion at the onset of reperfusion[26]. These results further suggest that the intact presence of bradykinin B_2 receptors at the early onset of reperfusion is critical for transmitting the cell survival signals of PostC.

Role of other G protein-coupled receptors in PostC

It is notable that other types of G protein-coupled receptors could also involve with the signaling cascades of PostC. As we previously discussed, in addition to the involvement of adenosine A_1 demonstrated by our present study and others[19], other subtypes of adenosine receptors were also shown to be indispensable for PostC by several research groups. These G protein-coupled receptors include adenosine A_{2A}[3;7], A_{2B}[11], and A_3[3]. Furthermore, a few recent studies suggested that opioid receptors are the likely participants in PostC signaling[27–29], which essentially confirmed the concept originally proposed by Gross and colleagues that pharmacological activation of opioid receptors at the early phase of reperfusion is as protective as preconditioning with the opioid receptor agonists in the rat heart[30]. However, it remains unclear how these G protein-coupled receptors cross-talk with each other, which leads to loss of Post-induced cardioprotection in the absence of one of these receptors.

Conclusion

The present study using three distinctive strains of gene knockout mice has provided conclusive evidence for essential role of both adenosine A_1 and bradykinin B_2 receptors on infarct-limiting protection of PostC in globally ischemic-reperfused mouse hearts. Future studies are necessary to elucidate the exact signaling cascades following the PostC-induced activation of each of these membrane receptor subtypes that ultimately lead to the cytoprotective phenotype against cardiac reperfusion injury.

Acknowledgments

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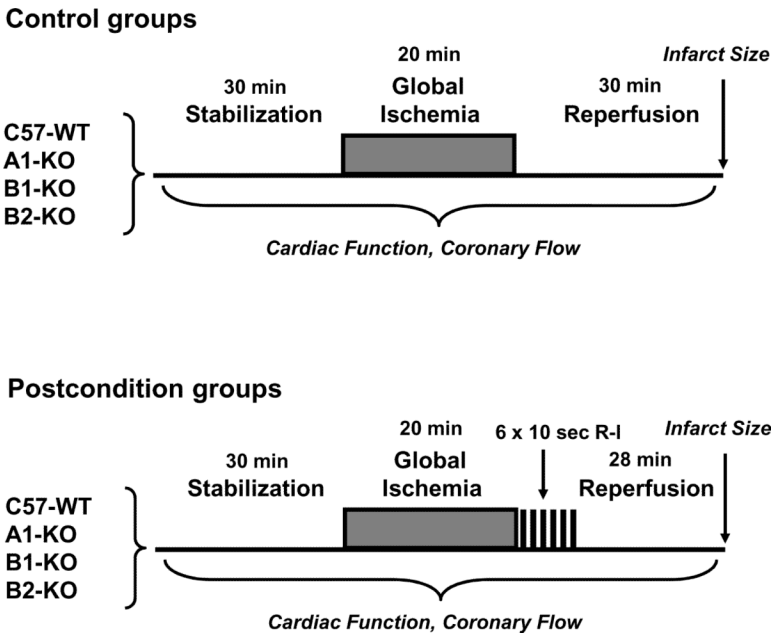

Figure 1.

Diagrammatical description of the experiment groups and protocol of global ischemiareperfusion with or without postconditioning in Langendorff isolated perfused heart model. See *Methods* for abbreviations.

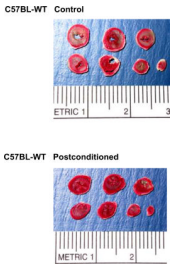

Figure 2.
Representative pictures of tetrazolium chloride-stained heart sections from C57BL/6J wild-type mice subjected to global ischemia-reperfusion alone (top) or with postconditioning (bottom). The infarct areas are in pale color and the viable tissues are in red color.

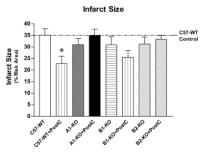

Figure 3.

Myocardial infarct size (Mean±SEM). *indicates $P<0.05$ versus C57-WT group (two-way ANOVA with Bonferroni post-hoc test). See *Methods* for detailed description of the experiment groups and sample numbers.

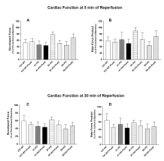

Figure 4.

Post-ischemic cardiac function, indicated by Developed Force (A and C) and double product of heart rate and developed force (Rate-Force Product; B and D; Mean±SEM) at 5 min (top) or 30 min (bottom) of reperfusion. No statistically significant difference was found among the 8 experiment groups.

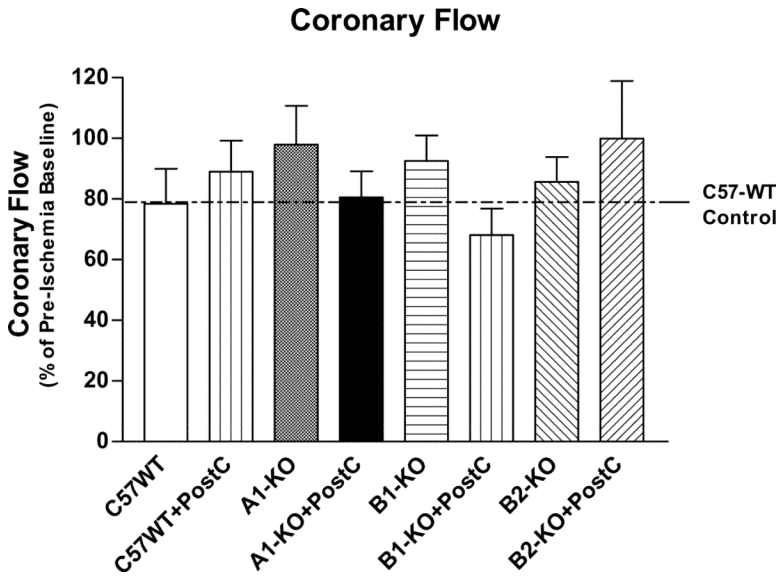

Figure 5.
Post-ischemic coronary flow (Mean±SEM). No statistically significant difference in this index of coronary vasodilation was observed among the 8 experiment groups.

Table 1

Morphometric Characteristics and Baseline Cardiac Function of the Mice

	C57-WT (n = 8)	C57-WT + PostC (n = 8)	A1-KO (n = 8)	A1-KO + PostC (n = 7)	B1-KO (n = 9)	B1-KO + PostC (n = 9)	B2-KO (n = 7)	B2-KO + PostC (n = 7)
Body weight, g	28.1±2.3	31.1±0.6	35.9±2.5*	35.4±1.4	35.2±1.2*	36.3±2.2	30.1±2.8	30.8±2.5
Heart wet weight, mg	205±9	215±6	259±13*	266±7#	261±18*	249±13	218±20	213±18
Coronary flow, ml/min	1.6±0.2	1.8±0.2	1.8±0.2	2.3±0.2	1.6±0.2	2.0±0.2	2.0±0.3	1.8±0.1
Heart rate, bpm	345±22	351±16	316±29	343±20	317±23	301±22	349±30	351±23
Developed force, g	0.81±0.11	0.96±0.18	1.03±0.21	0.84±0.19	0.76±0.15	1.00±0.20	0.59±0.14	0.59±0.12
Rate-force product, g×bpm	284±46	339±67	306±55	285±63	234±46	290±63	188±30	217±56

Values are Mean±SEM. Abbreviation: bpm – beats per minute.

*indicates $P<0.05$ versus C57-WT group

#indicates $P<0.05$ versus C57-WT+PostC group (two-way ANOVA with Bonferroni post-hoc test).

Table 2

Post-Ischemic Cardiac Function

	C57-WT (n = 8)	C57-WT + PostC (n = 8)	A1-KO (n = 8)	A1-KO + PostC (n = 7)	B1-KO (n = 9)	B1-KO + PostC (n = 9)	B2-KO (n = 7)	B2-KO + PostC (n = 7)
At 5 min of Reperfusion								
Heart rate, bpm	368±25	339±18	336±18	362±29	360±23	308±22	341±22	347±27
Developed force, g	0.48±0.13	0.47±0.14	0.53±0.19	0.43±0.16	0.61±0.15	0.58±0.18	0.27±0.10	0.41±0.14
Rate-force product, g×bpm	182±46	168±53	178±62	168±66	216±53	195±69	85±25	158±61
At 30 min of Reperfusion								
Heart rate, bpm	345±26	309±17	324±19	321±26	288±24	301±32	354±24	334±24
Developed force, g	0.53±0.15	0.48±0.13	0.55±0.20	0.42±0.16	0.55±0.14	0.59±0.19	0.27±0.14	0.28±0.09
Rate-force product, g×bpm	190±53	151±45	172±59	143±55	148±36	164±62	85±36	103±37

Values are Mean±SEM. Abbreviation: bpm – beats per minute. No significant difference was found between the groups for any of the functional parameters (two-way ANOVA with Bonferroni post-hoc test).