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REVIEW

Possible role of thromboxane A_2 in remote hind limb preconditioning-induced cardioprotection

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Abstract Remote hind limb preconditioning (RIPC) is a protective strategy in which short episodes of ischemia and reperfusion in a remote organ (hind limb) protects the target organ (heart) against sustained ischemic reperfusion injury. The present study was designed to investigate the possible role of thromboxane A_2 in RIPC-induced cardioprotection in rats. Remote hind limb preconditioning was performed by four episodes of 5 min of inflation and 5 min of deflation of pressure cuff. Occlusion of the hind limb with blood pressure cuff is most feasible, non-invasive, clinically relevant, and safe method for inducing RIPC. Isolated rat hearts were perfused on Langendorff apparatus and were subjected to global ischemia for 30 min followed by 120-min reperfusion. The levels of lactate dehydrogenase (LDH) and creatine kinase (CK) were measured in coronary effluent to assess the degree of myocardial injury. The extent of myocardial infarct size along with the functional parameters including left ventricular developed pressure (LVDP), dp/dt_{max}, and dp/dt_{min} were also measured. Ozagrel (thromboxane synthase inhibitor) and seratrodast (thromboxane A_2 receptor antagonist) were employed as pharmacological modulators of thromboxane A_2. Remote hind limb preconditioning significantly attenuated ischemia/reperfusion-induced myocardial injury and produced cardioprotective effects. However, administration of ozagrel and seratrodast completely abolished the cardioprotective effects of RIPC suggesting the key role of thromboxane A_2 in RIPC-induced cardioprotection. It may be concluded that brief episodes of preconditioning ischemia and reperfusion activates the thromboxane synthase enzyme that produces thromboxane A_2, which may elicit cardioprotection either involving humoral or neurogenic pathway.

Keywords Remote hind limb preconditioning · Cardioprotection · Thromboxane A_2 · Humoral · Neurogenic

Abbreviations

RIPC	Remote ischemic preconditioning
LDH	Lactate dehydrogenase
CK	Creatine kinase
IR	Ischemia reperfusion
TTC	Triphenyl tetrazolium chloride
KH	Krebs-Henseleit
2,4-DNPH	2,4-Dinitrophenylhydrazine
TXA_2	Thromboxane A_2
PCI	Percutaneous coronary intervention
CABG	Coronary artery bypass grafting

Introduction

Remote preconditioning is the phenomenon in which transient ischemic episodes to an organ at a distance from target organ afford protection to the heart against sustained ischemia/reperfusion injury (Przyklenk et al. 1993; Shimizu et al. 2009). Various studies including ours have reported that short episodes of occlusion/reperfusion of abdominal aorta (Erling et al. 2013), hepatic portal arteries (Noorbakhsh et al. 2015), and renal arteries (Diwan et al. 2008; Kant et al. 2008; Yoon et al. 2015) produce preconditioning of myocardium. It is documented to confer beneficial effects in patients undergoing various myocardial interventions, whereby alternate cycles of

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inflation and deflation of blood pressure cuff tied on the upper arm serve as the remote preconditioning stimulus. It reduces the postoperative myocardial injury in patients undergoing various myocardial interventions like heart valve surgery, coronary artery bypass graft surgery, abdominal aortic aneurysm repair, percutaneous coronary intervention, and drug-eluting stent implantation (Cheung et al. 2006; Hausenloy et al. 2007; Ali et al. 2007; Botker et al. 2010; Wu et al. 2014).

The application of ischemic episodes to different regions including hind limb, renal, abdominal, and hepatic produces cardioprotection to more or less at the same extent (Diwan et al. 2008; Donato et al. 2013; Paio et al. 2014). Accordingly, it may be hypothesized that some common tissue may respond to brief ischemic episodes during remote hind limb preconditioning (RIPC) to involve either humoral or neurogenic pathway and elicit cardioprotection. It may be hypothesized that blood and its components may be a common tissue that responds to ischemia by releasing some chemical mediators including thromboxane A_2. This contention of involvement of common tissue, not the remote organ itself, may also be justified due to the fact that hind limb is not a secretory tissue and is relatively resistant to short ischemia. Thromboxane A_2 is a biologically active metabolite of arachidonic acid produced from prostaglandin H_2 by thromboxane A_2 synthase (Narumiya 2001). It is produced by platelets, endothelial cells, macrophages, and cardiac tissues (Mehta and Mehta 1985). Thromboxane A_2 receptors are located on different regions of heart including right ventricle, left ventricle, right atria, and left atria (Katugampola 2001). These receptors are also localized on neurons, oligodendrocytes, spinal cord, and Schwann cells of sciatic nerves (Wacker et al. 2005; Muja et al. 2001). Thromboxane synthase is sensitive to ischemia and responds to latter by synthesizing and releasing thromboxane A_2 (Pettigrew et al. 1989; Schmitz et al. 1985). Previous studies have reported the involvement of thromboxane A_2 in ischemia-reperfusion-induced myocardial injury (Ito et al. 1990; Byrne et al. 1993). Intriguingly, the mediators or factors which produce deleterious effects such as ischemia, free radicals, and calcium are also well documented to confer tissue protection in the form of preconditioning (Weinbrenner et al. 2004; Penna 2009). Therefore, it may be hypothesized that thromboxane synthase responds to brief ischemic episodes of RIPC and release thromboxane A_2 into blood which may further activate its receptors on heart or nerves and may stimulate various preconditioning mediators to elicit cardioprotection.

Material and methods

Animals, drugs, and chemicals

Wistar albino rats (150–220 g) of both sexes were employed in the present study. They were fed on standard laboratory chow (Ashirwad Industries, Kharar, Mohali, India) and had free access to water and food. The experimental protocol was approved by Institutional Animal Ethics Committee, and care of the animals was carried out as per the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forest, Government of India (Reg. No. 2014/07/CPCSEA). Ozagrel hydrochloride (Sun Pharma Laboratories Ltd., India) and Seratrodast (Zuventus Healthcare Ltd., Mumbai, India.) were employed in the present study. Ozagrel (15 and 30 mg/kg) (Koumura et al. 2011; Satoh et al. 1996) and seratrodast (15 and 30 mg/kg) (Miyagawa et al. 2008; Horiguchi et al. 2002) were administered intraperitoneally. Creatine phosphokinase (CK) estimation kit was procured from Agappe Diagnostics Ltd., Kerala, India. All other chemicals were of analytical grade and were obtained from S.D. Fine Chemicals, Mumbai, India. All the drugs were dissolved in distilled water.

Remote hind limb preconditioning

Rat was anaesthetized with thiopental sodium (50 mg kg^{-1}, *i.p.*). A pressure cuff was tied on the hind limb of rat and was inflated with air up to 150 mm of Hg to produce ischemia in the limb, and the pressure was released for reperfusion. Four episodes of ischemia and reperfusion, each comprising of 5 min of inflation and 5 min of deflation of pressure cuff, were used to produce remote limb preconditioning (Zhu et al. 2013; Kharbanda et al. 2002).

Isolated rat heart preparation and measurement of hemodynamic parameters

Rat was heparinized (500 IU, i.p.) about 20 min before sacrificing the animal by cervical dislocation. Heart was rapidly excised and immediately mounted on Langendorff apparatus (Jaggi et al. 2007). Isolated heart was retrogradely perfused at constant pressure of 70 mm Hg with Krebs-Henseleit (KH) buffer (NaCl 118 mM, KCl 4.7 mM, $CaCl_2$ 2.5 mM, $MgSO_4{\cdot}7H_2O$ 1.2 mM, $NaHCO_3$ 25 mM, KH_2PO_4 1.2 mM, $C_6H_{12}O_6$ 11 mM), pH 7.4, maintained at 37 °C, bubbled with 95 % O_2 and 5 % CO_2. Flow rate was maintained at 6–8 ml/min. The heart was enclosed in a double wall jacket, the temperature of which was maintained by circulating water maintained at 37 °C. A fluid-filled latex balloon was inserted into left ventricle and was connected to a pressure transducer (AD Instruments, Australia) to record left ventricular developed pressure (LVDP) and its first derivatives dp/dt_{max} and dp/dt_{min}. Global ischemia was produced for 30 min by blocking the inflow of Krebs-Henseleit solution. It was followed by reperfusion for 120 min. Coronary effluent was collected at different time intervals, i.e., basal (immediately after

stabilization), 0, 5, and 30 min after reperfusion for biochemical estimations.

Assessment of infarct size

Heart was removed from Langendorff apparatus. The atria and the root of aorta were excised, and heart was kept overnight at 0 °C. The heart slices were incubated in 1 % triphenyltetrazolium chloride (TTC) at 37 °C in 0.2 M Tris buffer (pH 7.4) for 20 min. Infarct size was estimated in terms of % infarct size by weight and volume method (Diwan et al. 2008; Jaggi et al. 2007).

Estimation of lactate dehydrogenase levels

The levels of LDH were estimated in samples of coronary effluent collected after stabilization, immediately and 30 min after reperfusion using 2,4-DNPH method (King 1959).

Estimation of creatine kinase

The levels of CK were estimated in coronary effluent samples after stabilization and 5 min after reperfusion (Swanson and Wilkinson 1972).

Experimental protocol

Eight groups, each comprising six Wistar albino rats, were employed in the present study.

Group I (control): Rat heart was isolated and perfused on Langendorff apparatus. After 10-min stabilization, the heart was subjected to global ischemia for 30 min followed by reperfusion for 120 min.

Group II (remote limb preconditioning): A pressure cuff was tied on the hind limb of anaesthetized rat and was inflated to produce ischemia in the limb and deflated for reperfusion. Four episodes of ischemia and reperfusion, each comprising of 5 min of inflation and 5 min of deflation, were used to produce remote limb preconditioning. Immediately after the last episode of remote preconditioning, heart was isolated and subjected to 30-min global ischemia followed by 120-min reperfusion as described in group I.

Group III (ozagrel 15 mg kg^{-1} i.p. in remote limb preconditioning): Rat was administered ozagrel 30 min prior to performing RIPC. After RIPC, heart was subjected to ischemia and reperfusion as described in group I.

Group IV (ozagrel 30 mg kg^{-1} i.p. in remote limb preconditioning): Rat was administered ozagrel 30 min prior to performing RIPC. After RIPC, heart was subjected to ischemia and reperfusion as described in group I.

Group V (seratrodast 15 mg kg^{-1} i.p. in remote limb preconditioning): Rat was administered seratrodast 30 min prior to performing RIPC. After RIPC, heart was subjected to ischemia and reperfusion as described in group I.

Group VI (seratrodast 30 mg kg^{-1} i.p. in remote limb preconditioning): Rat was administered seratrodast 30 min prior to performing RIPC. After RIPC, heart was subjected to ischemia and reperfusion as described in group I.

Group VII (ozagrel 15 mg kg^{-1} i.p. per se): Ozagrel was administered 70 min before isolating the heart, and thereafter, the isolated rat heart was perfused on Langendorff apparatus. After 10-min stabilization, the heart was subjected to global ischemia for 30 min followed by reperfusion for 120 min.

Group VIII (seratrodast 15 mg kg^{-1} i.p. per se): Seratrodast was administered 70 min before isolating the heart, and thereafter, the isolated rat heart was perfused on Langendorff apparatus. After 10-min stabilization, heart was subjected to global ischemia for 30 min followed by reperfusion for 120 min.

Statistical analysis

The results were expressed as mean±standard error of mean (SEM). The statistical analysis for LDH, CK, % change in LVDP, dp/dt_{max}, and dp/dt_{min} was done using two-way ANOVA followed by Bonferonni's post hoc test, while the results of infarct size were analyzed by one-way ANOVA followed by Tukey's multiple range test for post hoc analysis. A value of $p<0.05$ was considered to be statistically significant.

Results

Administration of both ozagrel and seratrodast in control animals did not produce any significant preconditioning effect indicating the lack of pharmacological preconditioning effects of these drugs in the control animals.

Effect of pharmacological interventions on myocardial infarct size

Thirty minutes of global ischemia followed by 120 min of reperfusion produced significant myocardial injury in terms of increase in myocardial infarct size, both by volume and weight methods. Four alternate cycles of inflation (5 min) and deflation (5 min) of blood pressure cuff on hind limb in the form of RIPC significantly attenuated ischemia-reperfusion-induced increase in myocardial infarct size. However, administration of ozagrel (15 and 30 mg/kg i.p.) and seratrodast (15 and 30 mg/kg i.p.) abolished infarct sparing effect of RIPC in a significant manner. Per se administration of ozagrel and seratrodast

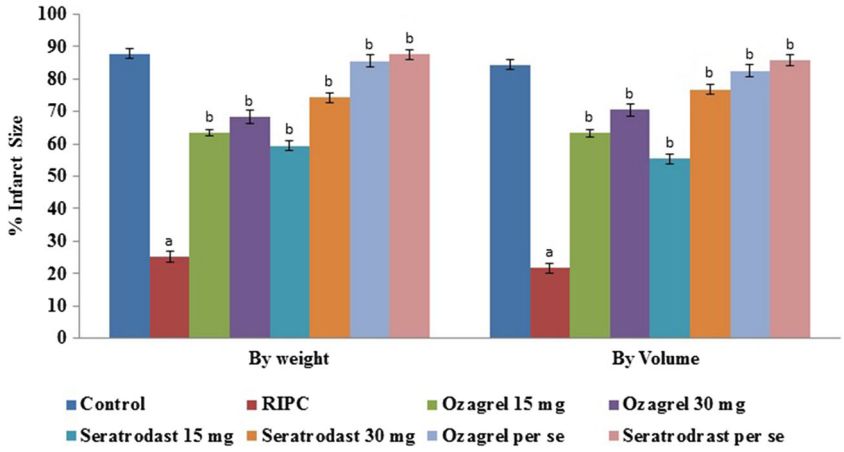

Fig. 1 Pharmacological effects of ozagrel and seratrodast on RIPC-induced reduction in infarct size by volume and weight method. Data were statistically analyzed by using one-way ANOVA followed by Tukey's multiple range test; values were expressed as mean± SEM with n=6 in each group [for weight, $F(7, 40)=399$; for volume, $F(7, 40)=592.9$; $p<0.05$], $^{a}p<0.05$ versus control; $^{b}p<0.05$ versus RIPC. *RIPC* remote ischemic preconditioning

(30 mg/kg) did not modulate infarct size in comparison to control (Fig. 1).

Effect of pharmacological interventions on release of LDH in coronary effluent

Thirty minutes of global ischemia followed by 120 min of reperfusion resulted in a significant increase in release of LDH, noted immediately and 30 min after reperfusion, in coronary effluent as compared to basal. RIPC significantly attenuated ischemia/reperfusion (I/R)-induced increase in release of LDH in coronary effluent in a significant manner. However, administration of ozagrel (15 and 30 mg/kg i.p.) and seratrodast (15 and 30 mg/kg i.p.) abolished RIPC-induced decrease in release of LDH in coronary effluent in a significant manner. Per se administration of ozagrel and seratrodast (30 mg/kg) did not modulate release of LDH in comparison to control (Fig. 2).

Effect of pharmacological interventions on release of CK in coronary effluent

Thirty minutes of global ischemia followed by 120-min reperfusion resulted in a significant increase in release of CK, noted at 5 min after reperfusion, in coronary effluent as compared to basal. RIPC significantly attenuated I/R-induced increase in release of CK in coronary effluent in a significant manner. Administration of ozagrel (15 and 30 mg/kg i.p.) and seratrodast (15 and 30 mg/kg i.p.) abolished RIPC-induced decrease in release of CK in coronary effluent in a significant manner. Per se administration of ozagrel and seratrodast (30 mg/kg i.p.) did not modulate release of CK in comparison to control (Fig. 3).

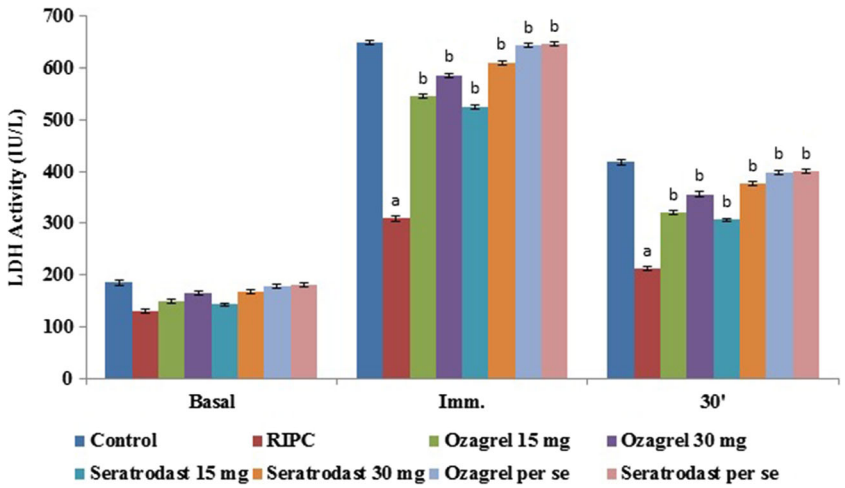

Fig. 2 Pharmacological effects of ozagrel and seratrodast on RIPC-induced reduction in LDH release in coronary effluent. *Basal* before global ischemia, *Imm* immediately after reperfusion, *30′* 30 min after reperfusion. Data were statistically analyzed by using two-way ANOVA followed by Bonfferoni test; values were expressed as mean±SEM with n=6 in each group [for treatment, $F(7, 40)=145.5$; for time, $F(2, 40)=250.24$; $p<0.05$], $^{a}p<0.05$ versus control; $^{b}p<0.05$ versus RIPC. *RIPC* remote ischemic preconditioning

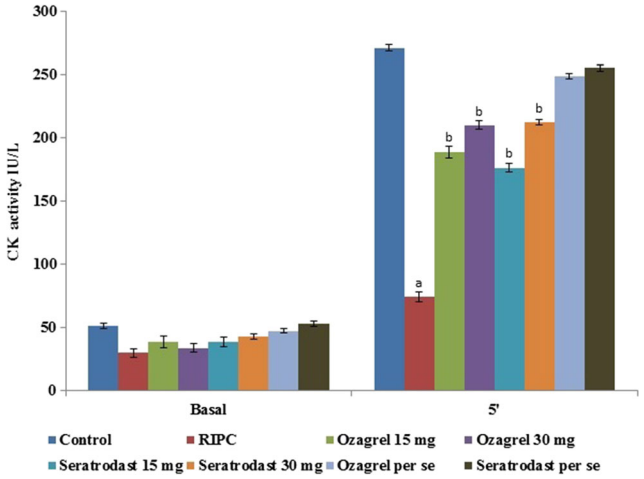

Fig. 3 Pharmacological effects of ozagrel and seratrodast on RIPC-induced reduction in CK release in coronary effluent. *Basal* before global ischemia, *5′* 5 min after reperfusion. Data were statistically analyzed by using two-way ANOVA followed by Bonfferoni test; values were expressed as mean±SEM with $n=6$ in each group [for treatment, $F(7, 40)=85.45$; for time, $F(1, 40)=550.65$; $p<0.05$], $^{a}p<0.05$ versus control; $^{b}p<0.05$ versus RIPC. *RIPC* remote ischemic preconditioning

Effect of pharmacological interventions on LVDP, dp/dt_{max}, and dp/dt_{min}

Index ischemia of 30 min resulted in marked decrease in LVDP (Fig. 4), dp/dt_{max} (Fig. 5), and dp/dt_{min} (Fig. 6) during reperfusion period. RIPC resulted in a marked attenuation of ischemia-reperfusion-induced decrease in LVDP, dp/dt_{max}, and dp/dt_{min} during reperfusion. Moreover, administration of ozagrel (15 and 30 mg/kg i.p.) and seratrodast (15 and 30 mg/kg i.p.) abolished the RIPC-induced improvement in LVDP, dp/dt_{max}, and dp/dt_{min} during reperfusion. Per se administration of ozagrel and seratrodast (30 mg/kg i.p. did not modulate % change in LVDP, dp/dt_{max}, and dp/dt_{min} in comparison to control (Figs. 4, 5, and 6).

Discussion

In the present study, 30 min of global ischemia followed by 120 min of reperfusion on isolated Langendorff system produced ischemia/reperfusion injury assessed in terms of alterations in the parameters that are important markers of myocardial injury such as increase in infarct size and release of myocardial enzymes (LDH and CK). LDH and CK are the cardiac enzymes released during ischemia-reperfusion-induced irreversible myocardial injury (Wei et al. 1985). Our previous studies have reported that peak release of LDH occurs immediately and 30 min after reperfusion, and peak release of CK occurs at 5 min of reperfusion (Diwan et al. 2008; Kant et al. 2008). Infarct size was measured macroscopically using TTC staining. During reperfusion injury, membrane damage causes loss of myocardial enzymes from the cardiac tissue due to which TTC stain shows no color and the infarcted tissue appears yellow in color. Moreover, ischemia-reperfusion injury significantly decreased functional contractility parameters including LVDP, dp/dt_{max}, and dp/dt_{min}. However, four short episodes of remote ischemia followed by reperfusion of hind limb significantly reduced ischemia-reperfusion-induced myocardial injury in terms of the reduction in release of myocardial enzymes (LDH and CK), infarct size, and improvement of hemodynamic parameters. These results have been supported by various previous studies of RIPC-induced cardioprotection (Patel 2002; Tokuna et al.

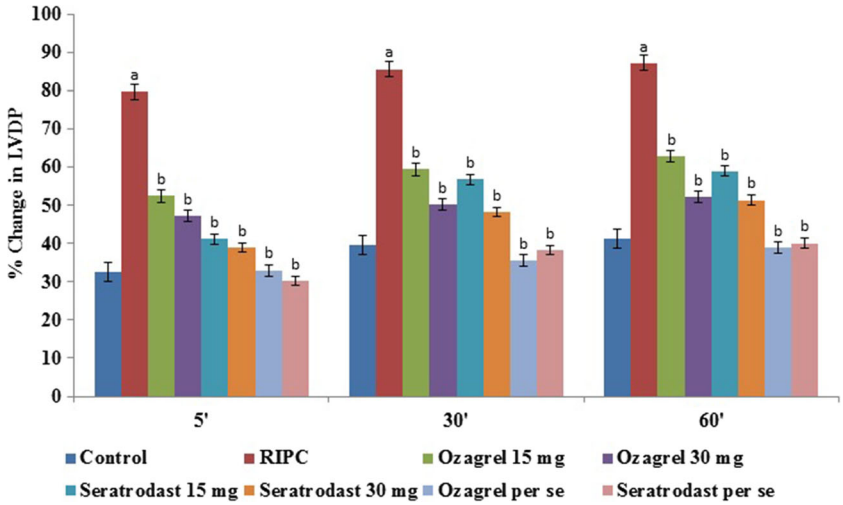

Fig. 4 Pharmacological effects of ozagrel and seratrodast on % change in LVDP. *5′* 5 min after reperfusion, *30′* 30 min after reperfusion, *60′* 60 min after reperfusion. Data were statistically analyzed by using two-way ANOVA followed by Bonfferoni test; values were expressed as mean± SEM with $n=6$ in each group [for treatment, $F(7, 40)=980.62$; for time, $F(2, 40)=1048.32$; $p<0.05$], $^{a}p<0.05$ versus control; $^{b}p<0.05$ versus RIPC. *RIPC* remote ischemic preconditioning, *LVDP* left ventricular developed pressure

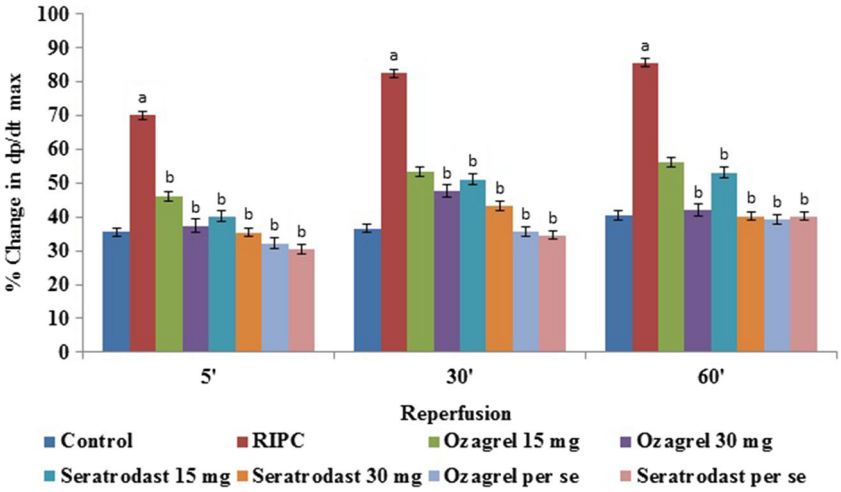

Fig. 5 Pharmacological effects of ozagrel and seratrodast on % change in dp/dt_{max}. *5′* 5 min after reperfusion, *30′* 30 min after reperfusion, *60′* 60 min after reperfusion. Data were statistically analyzed by using two-way ANOVA followed by Bonfferoni test; values were expressed as mean±SEM with $n=6$ in each group [for treatment, $F(7, 40)=225.32$; for time, $F(2, 40)=650.74$; $p<0.05$], $^{a}p<0.05$ versus control; $^{b}p<0.05$ versus RIPC. *RIPC* remote ischemic preconditioning

2002) and are also in consistent with the previous reports from our laboratory (Diwan et al. 2008; Kant et al. 2008).

Furthermore, in the present study, administration of ozagrel before instituting RIPC significantly attenuated cardioprotective effects of RIPC suggesting the role of thromboxane A_2 in RIPC-induced cardioprotection against ischemia-reperfusion injury. Ozagrel is a thromboxane A_2 synthase inhibitor and has been clinically employed as an antiplatelet agent (Loo et al. 1987). Thromboxane A_2 is an arachidonic acid metabolite generated in the activated platelets from prostaglandin H_2 by thromboxane synthase enzyme (Narumiya 2001; Reilly and Fitzgerald 1993). The thromboxane synthase enzyme is sensitive to ischemia and responds to latter by synthesizing and releasing thromboxane A_2 (Pettigrew et al. 1989). Furthermore, the presence of thromboxane A_2 receptors on different parts of the heart including right ventricle, left ventricle, right atria, left atria, and coronary arteries has been demonstrated (Katugampola 2001). Therefore, it is possible that during short episodes of remote preconditioning ischemia to hind limb, thromboxane A_2 may activate the heart-localized thromboxane A_2 receptors to produce the cardioprotective effects. This contention is further supported by the observation of our present study in which the administration of seratrodast, a thromboxane receptor antagonist, inhibited the cardioprotective effects of RIPC suggesting the involvement of thromboxane A_2 in RIPC-induced cardioprotection. The previous studies have shown the deleterious effects of thromboxane A_2 in ischemia-reperfusion injury (Ito et al. 1990; Byrne et al. 1993), while our present study demonstrates the preconditioning effects of thromboxane A_2. Indeed, the agents or factors which produce deleterious effects such as ischemia, free radicals, CGRP (Peng et al. 1996), bradykinin (Linz et al. 1996), angiotensin (Yang et al. 1998), and calcium are also well documented to confer tissue protection in the form of preconditioning (Weinbrenner et al. 2004; Penna 2009).

Although the selected pharmacological agents for the present study may block different prostaglandins including PGD_2, 9α, 11β-PGF_2, and $PGF_{2\alpha}$ receptors (Ashida et al. 1989;

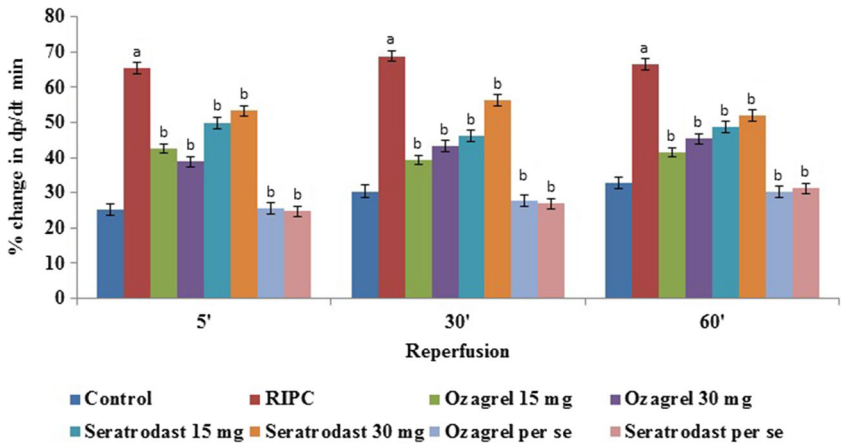

Fig. 6 Pharmacological effects of ozagrel and seratrodast on % change in dp/dt_{min}. *5′* 5 min after reperfusion, *30′* 30 min after reperfusion, *60′* 60 min after reperfusion. Data were statistically analyzed by using two-way ANOVA followed by Bonfferoni test; values were expressed as mean±SEM with $n=6$ in each group [for treatment, $F(7, 40)=120.43$ for time, $F(2, 40)=875.2$; $p<0.05$], $^{a}p<0.05$ versus control; $^{b}p<0.05$ versus RIPC. *RIPC* remote ischemic preconditioning

Kurokawa et al. 1994), they primarily modulate the functions of thromboxane A_2 and are employed clinically by virtue of this pharmacological property (Wei et al. 2004). Nevertheless, further studies in this direction may help to delineate the possible contribution of prostaglandin blockade in seratodast and ozagrel-mediated pharmacological actions in RIPC-subjected rats.

It is possible that during short episodes of remote preconditioning ischemia to hind limb, thromboxane A_2 is synthesized from the blood platelets and is transferred via blood (humoral pathway) to activate the heart-localized thromboxane A_2 receptors to produce the cardioprotective effects. However, there is also a possibility that remote preconditioning ischemia-induced thromboxane A_2 may activate the neurogenic pathway to trigger the cardioprotection. According to neurogenic theory, during hind limb occlusion (as a part of remote preconditioning), the peripheral sensory nerve fibers are stimulated and these afferent fibers convey the signals to the brain from which efferent transmit the cardioprotective signals to the heart (Gill et al. 2015). Studies have shown the important role of the sciatic, femoral, and spinal nerves in hind limb remote preconditioning as resection of these nerves has been associated with attenuated cardioprotective effects (Lim et al. 2010; Mastitskaya et al. 2012). Thromboxane A_2 receptors exist on the neurons, oligodendrocytes, spinal cord, and Schwann cells of sciatic nerves in rats (Muja et al. 2001). On this basis, it is possible to hypothesize that brief ischemic episodes of RIPC may activate platelets or endothelial cells to release thromboxane A_2 into the localized region of hind limb, which, in turn, may act on thromboxane A_2 receptors present on peripheral nerves (sciatic or spinal) to activate neurogenic pathway involved in RIPC-induced cardioprotection. Furthermore, studies have shown that thromboxane A_2 and its mimetic (U46619) stimulate vagal afferents (Carrithers et al. 1994; Karla et al. 1992), and stimulation of vagal nerve has been shown to confer myocardial protection from ischemia/reperfusion injury (Kawada et al. 2006). Nevertheless, further experimental investigations are required to elucidate the precise mechanism responsible for thromboxane A_2-mediated RIPC-induced cardioprotection.

Conclusion

Remote hind limb preconditioning-induced cardioprotective effects in ischemia/reperfusion-induced myocardial injury may be mediated through the release of thromboxane A_2. During brief ischemic episodes of remote preconditioning, thromboxane synthase may respond to ischemia by releasing thromboxane A_2 which may act on its heart-localized receptors (humoral pathway) or may activate its receptors present on sensory nerves (neurogenic pathway) to elicit cardioprotection.

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Compliance with ethical standards

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Conflict of interest The authors declare no conflict of interest.

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