

Effects of Rotigaptide and RIC on Ischemia Reperfusion Injury in the *In Vitro* Rabbit Heart

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Abstract

Background: Remote Ischemic Preconditioning (rIPC) and the antiarrhythmic peptide analogue, Rotigaptide (ZP123), protects against myocardial ischemia-reperfusion injury through potentially similar mechanisms. We aimed to study whether the cardioprotective effects of Rotigaptide and rIPC interact.

Methods: We used male New Zealand White rabbit hearts mounted in a Langendorff system and exposed to 30 min of global no-flow ischemia and 120 min of reperfusion. A total of 48 rabbits were randomized into 6 groups: control (n=6), Rotigaptide (1 μM) before (n=9) or after (n=9) ischemia, rIPC (n=7), rIPC+Rotigaptide before (n=9) or after (n=8) ischemia. rIPC was induced by four cycles of 5-min ischemia and reperfusion on the left hind limb achieved by intermittent tourniquet occlusion. Primary endpoint was infarct size measured by tetrazolium staining.

Results: rIPC reduced infarct size compared to controls. Rotigaptide alone did not affect infarct size irrespective of administration before ischemia or during reperfusion. The combination of rIPC and Rotigaptide before ischemia reduced infarct size, whereas the effect of rIPC was abrogated by Rotigaptide when administered during reperfusion. No significant changes in hemodynamic recovery were observed when compared to control group.

Conclusion: In contrast to *in vivo* rIPC, *in vitro* Rotigaptide did not yield cardioprotection in our rabbit model, but Rotigaptide attenuated the effect of rIPC. These findings indicate that modification of myocardial gap junction is involved in cardioprotection by rIPC.

Keywords: Ischemia; Reperfusion; Myocardial infarction; Connexin 43; Rotigaptide; Remote ischemic

Introduction

Early revascularization is paramount to salvage threatened myocardium in ST-Elevation Myocardial Infarction (STEMI). Paradoxically, reperfusion itself may cause myocardial damage beyond the ischemic damage. Combined acute ischemia and reperfusion injury may lead to irreversible tissue injury and cell death and determines final infarct size. Protecting the heart beyond the myocardial salvage achieved by early revascularization is crucial as infarct size is directly linked to patient outcome [1].

One of the most promising concepts of cardioprotection in the clinical setting is Remote Ischemic Preconditioning (rIPC), which can be achieved by repeated periods of non-lethal ischemia and reperfusion in a distant organ or body part, e.g. a limb, before a sustained ischemic insult to the target organ. When applied during an evolving myocardial infarction, rIPC reduces troponin release [2-7] and increases myocardial salvage in patients admitted with STEMI admitted for Primary Percutaneous Coronary Intervention (pPCI) [8]. An alternative cardioprotective approach is pharmacological conditioning. Multiple pharmacological compounds have been shown to exert cardioprotection in animal models but the majority have failed to translate successfully into beneficial effect in clinical studies [9]. However, most of these drugs target only one of many signalling pathways involved in cardioprotection and do not fully replicate ischemic preconditioning, which may explain the absent success in clinical trials.

Rotigaptide reduces infarct size in experimental models of myocardial ischemia-reperfusion injury [10-12]. Rotigaptide has been demonstrated to prevent dephosphorylation of Cx43 during ischemic stress [13]. Sarcolemmal Cx43 contributes to

the dissemination of myocardial injury [14,15] and inhibition of sarcolemmal Cx43 is cardioprotective [16,17]. Cx43 is also present in mitochondria and, in contrast to sarcolemmal Cx43, opening of mitochondrial Cx43 channels before ischemia/reperfusion provides protection against ischemia/reperfusion injury by ischemic preconditioning [18]. Modulation of Cx43 protein expression and phosphorylation also seem to be an inherent component of rIPC [19], suggesting that Rotigaptide and rIPC may interact such that the effect of simultaneous administration may provide information about the mechanisms underlying rIPC.

The aim of the present study was to investigate the individual cardioprotective efficacy of Rotigaptide and rIPC and whether they have interacting cardioprotective effects against ischemia-reperfusion injury in rabbit model.

Materials and Methods

Adult male New Zealand White rabbits (2.5-3.6 kg) (n=48) were handled in accordance with institutional and national guidelines for animal research, The Animal Experiments Inspectorate in Denmark (Dyreforsøgstilsynet, Copenhagen, Denmark).

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Study groups

Animals were randomized into six different groups (Figure 1), control (n=6) and rIPC (n=7), Rotigaptide before ischemia or immediately at the onset of reperfusion (PreRoti, n=9 or PostRoti, n=9), or a combination of rIPC and Rotigaptide before ischemia or during reperfusion (PreRotirIPC, n=9 or PostRotirIPC, n=8).

After *in vivo* rIPC or a time-matched sham procedure according to protocol, the heart was excised. The isolated hearts were subjected to 20 min of stabilization, 10 min of Rotigaptide or vehicle administration, 30 min of global no-flow ischemia, and 120 min of reperfusion (2 h), a total of 180 min.

Experimental preparation

Peripheral intravenous access was obtained through a marginal ear vein. Anaesthesia was induced by a bolus of sodium pentobarbital (30 mg/kg). The rabbits were weighed and placed over a heat pad. Anaesthesia was maintained by a continuous infusion of 50 mg/h of sodium pentobarbital. When adequate depth of anaesthesia was obtained as evaluated by loss of the toe pinch reflex, a tracheostomy was performed and the rabbit was immediately connected to a volume-controlled ventilator (Ugo Basile 7025 rodent ventilator, Comerio, Italy). Arterial blood samples (Radiometer ABL700-serie) were taken from the central ear artery to assure appropriate ventilation and oxygenation.

A laparotomy and thoracotomy was performed. The animals were heparinized by a bolus of 100 IU/kg heparin i.v. (Leo Pharma, Copenhagen, Denmark) prior to cannulation. A thymectomy and excision of the pericardium and surrounding tissue was performed to reveal the aorta. A tourniquet was placed around the ascending aorta. The ascending aorta was cannulated with the heart in-situ, the tourniquet was tightened and retrograde perfusion with Krebs-Henseleit (KH) buffer was immediately commenced. The heart was then rapidly excised and mounted on the *ex-vivo*, Langendorff apparatus (Hugo Sachs Elektronik, Harvard Apparatus). The hearts were continuously retrogradely perfused at a constant pressure of 70 mmHg with KH buffer (118.5 mM NaCl; 25.0 mM NaHCO₃; 11.1 mM glucosemonohydrate; 1.2 mM MgSO₄; 2.0 mM CaCl₂; 1.2 mM KH₂PO₄; 4.7 mM KCl) at 37.0°C oxygenated with 95% O₂ and 5% CO₂ to maintain a pH level of 7.35-7.45. Once the heart was mounted on the Langendorff setting, excess connective tissue and fat was excised and aorta was secured with additional ligatures to maintain an adequate

coronary flow. An incision was made in the left atrial auricle where a balloon-tipped catheter (size 14, Hugo Sachs Electronics, March-Hugstetten, Germany) connected to a pressure transducer was inserted into the left ventricular cavity to allow for continuous measurements of the heart rate, diastolic and systolic pressure. The diastolic pressure was set to 7-12 mmHg by adjusting the balloon volume during stabilization. Hearts were submerged in a custom made preheated glass-cup to keep the myocardium at constant 37.0°C.

Coronary flow was measured continuously by an inline flow meter (Hugo Sachs Electronics, March-Hugstetten, Germany). Hemodynamic data and coronary flow measurements were acquired using a dedicated software platform (Notocord, Croissy sur Seine, France).

Cx43 modification

Rotigaptide was synthesized and supplied by Zealand Pharma. An effluent sample was acquired at the end of reperfusion for each Rotigaptide-protocol from sixteen random experiments after pre- and post-ischemic Rotigaptide administration. The samples were frozen at -80°C and subsequently analysed for verification of the target concentration in buffer solution during reperfusion.

Remote ischemic preconditioning

In vivo rIPC was performed using an occluding tourniquet on the left hind limb. Distal limb pallor was observed during occlusion of the arterial blood flow followed by reactive hyperaemia during reperfusion. Ultra-high frequency ultrasound (Vevo[®] 2100, VisualSonics Inc., Toronto, ON, Canada) with a 32-56 MHz linear array transducer was furthermore used to confirm occlusion in selected animals). rIPC was performed in anaesthetized animals as 4 cycles of 5 min of ischemia followed by 5 min of reperfusion followed by an additional 15 min of rest separating the rIPC procedure and the heart explant. During the rIPC protocol, adequate depth of anaesthesia, ventilation and stable body temperature was maintained. Control animals underwent the same procedure but the tourniquet was not tightened.

Infarct size: Immediately after the end of the perfusion protocol, the hearts were removed from the Langendorff apparatus, frozen at -80°C and manually sliced into approximately 1.5 mm slices. The slices were placed in individual tissue cassettes and incubated into a solution of 1% 2,3,5-Triphenyltetrazolium Chloride (TTC), (Sigma, St. Louis, Mo, USA) at 37°C for 5 min. The cassettes were then immersed in ice-cold water to discontinue the staining procedure and stored in

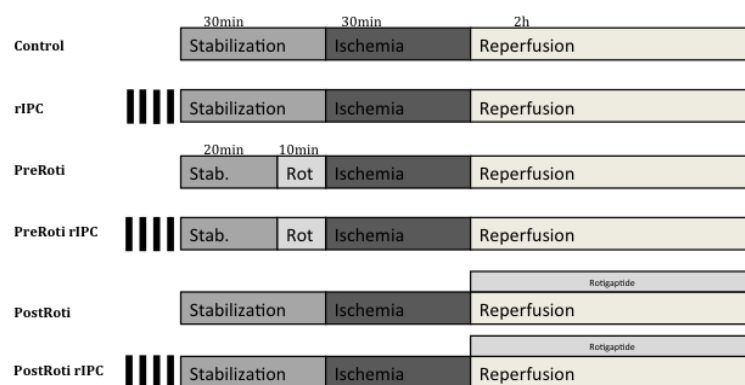


Figure 1: Study protocols: Displaying the protocols used in each group. rIPC: Remote ischemic preconditioning; PreRoti: Rotigaptide before ischemia; PreRotirIPC: Rotigaptide before ischemia+remote ischemic preconditioning; PostRoti: Rotigaptide after ischemia; PostRotirIPC: Rotigaptide after reperfusion+remote ischemic preconditioning.

4% formaldehyde solution overnight to enhance the vital from the infarcted area. The following day the slices were weighed and scanned in a flatbed scanner (Canon, 9000 F Mark II). Manual delineating the area of the left ventricle which is equal to the Area-at-Risk (AAR) in our global ischemia model and delineation of the area of the infarcted tissue was performed using image analysing software (Image J, NIH). Measurements were weighted with the mass of each individual slice and the infarct size/area-at-risk ratio (IS/AAR) was calculated. All tracings were executed by a single investigator and in a blinded manner.

Statistics: All statistical data are presented as mean \pm SEM. IS was analysed using one-way Analysis of Variance (ANOVA) followed by Bonferroni's post hoc test. Hemodynamic data were compared using two-way ANOVA with repeated measurements followed by Dunnett's post hoc test. Left Ventricular Developed Pressure (LVDP) was calculated by subtracting the LV diastolic pressure from the LV systolic pressure. One-way ANOVA was used to compare LVDP, Rate Pressure Product (RPP), Heart Rate, Coronary Flow (CF) and dP/dt max at specific timepoints. $P < 0.05$ was considered statistically significant. Statistical analyses were performed using GraphPad Prism6 (GraphPad Software Inc., San Diego, CA, USA).

Results

Infarct size

rIPC reduced Infarct Size (IS) compared to controls (32.7 ± 5.9 vs. $64.2 \pm 4.9\%$, $p = 0.0002$). Rotigaptide alone administered prior to (PreRoti) or after (PostRoti) ischemia had no effect on IS ($p > 0.99$). The combination of rIPC and Rotigaptide prior to ischemia (PreRotirIPC) reduced IS compared to controls (45.4 ± 3.3 vs. $64.2 \pm 4.9\%$, $p = 0.04$), although IS was insignificantly higher than rIPC alone (45.4 ± 3.3 vs. $32.7 \pm 5.9\%$, $p = 0.31$) suggesting an attenuation of the cardioprotective signal when administered before ischemia (Figure 2). We found no IS reduction by rIPC when Rotigaptide was administered during reperfusion (PostRotirIPC) as compared to controls (52.0 ± 5.9 vs. $64.2 \pm 4.9\%$, $p = 0.49$). Notably, this group had significantly larger

infarcts than the group receiving rIPC alone (52.0 ± 5.9 vs. $32.7 \pm 5.9\%$, $p = 0.027$) (Figure 2).

Hemodynamic recovery

The rIPC-group tended towards a better hemodynamic recovery of LVDP, RPP and dP/dt max although differences between groups were not significant (Table 1). Rotigaptide before ischemia or after reperfusion with rIPC (PreRotirIPC and PostRotirIPC) or without rIPC (PreRoti and PostRoti) did not improve hemodynamic recovery when compared to control (Table 1). Notably, when compared to rIPC alone, recovery of LVDP in the PostRotirIPC group was decreased with statistically borderline significance (17.8 ± 1.3 vs. 30.0 ± 2.0 mmHg, $p = 0.06$).

Rotigaptide concentrations

Mean concentrations of Rotigaptide in the perfusate after administration were: rIPC: below detection limit, preischemic administration: 0.13 ± 0.12 (range 0-0.53) nmol/l ($n = 9$) and postischemic administration: 200 ± 26 (range 100-283) nmol/l ($n = 7$).

Discussion

The main result of the present study is that Rotigaptide exerts no significant cardioprotective effect in our isolated rabbit heart model of ischemia and reperfusion injury. In addition, Rotigaptide seems to attenuate the cardioprotective effect of rIPC when administered before ischemia and abrogate the cardioprotective effect of rIPC when administered during reperfusion. These findings may indicate that modification of gap junctions with a predominant increment of gap junction intercellular communication at reperfusion may promote expansion of irreversible injury and hence attenuate the cardioprotective effect of rIPC.

Our results are in contrast to our previous experience in an *in vivo* pig model of catheter-induced occlusion of the left anterior descending artery [10]. We demonstrated a significant 57% IS reduction following

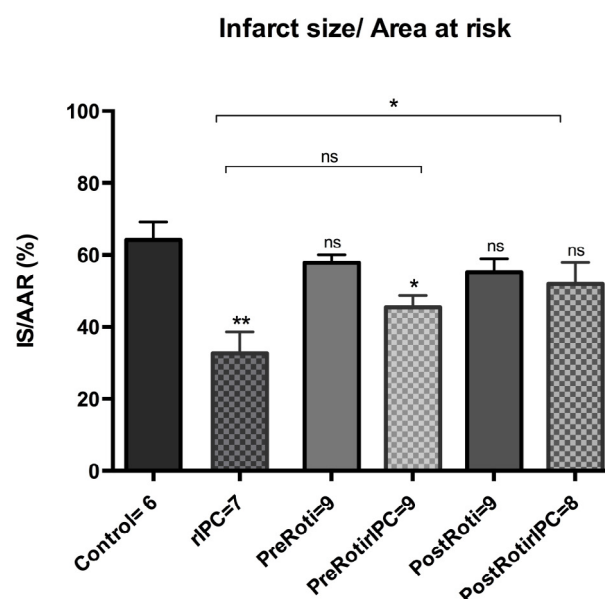


Figure 2: Infarct size: Data are presented as mean \pm SEM. Effect of rIPC and/or Rotigaptide administered before or after ischemia on infarct size (IS) as percentage of area-at-risk (AAR). NS non-significant. * $p \leq 0.05$; ** $p \leq 0.001$. rIPC: Remote ischemic preconditioning; PreRoti: Rotigaptide before ischemia; PreRotirIPC: Rotigaptide before ischemia+remote ischemic preconditioning; PostRoti: Rotigaptide after ischemia; PostRotirIPC: Rotigaptide after reperfusion+remote ischemic preconditioning.

	Baseline 11 min	Rotigaptide -1 min	Ischemia	Reperfusion		
				10 min	40 min	120 min
LVDP (mm Hg)						
CON	103 ± 7	108 ± 5	-	17 ± 3	28 ± 4	24 ± 4
rIPC	121 ± 5	121 ± 6		22 ± 5	34 ± 7	32 ± 7
PreRoti	102 ± 4	104 ± 4		14 ± 3	20 ± 2	16 ± 3
PreRotirIPC	113 ± 6	118 ± 6		13 ± 3	25 ± 2	21 ± 3
PostRoti	117 ± 4	119 ± 4		9 ± 2	21 ± 3	20 ± 3
PostRotirIPC	108 ± 6	105 ± 6		12 ± 2	20 ± 4	20 ± 4
RPP (BPM x mm Hg)						
CON	21406 ± 1664	22477 ± 796	-	2915 ± 453	5160 ± 644	4285 ± 728
rIPC	22047 ± 1506	21263 ± 1259		3684 ± 936	6371 ± 1302	5361 ± 1254
PreRoti	22250 ± 747	21903 ± 552		2264 ± 494	4109 ± 438	3246 ± 543
PreRotirIPC	23584 ± 763	23984 ± 698		2557 ± 508	4957 ± 408	3811 ± 549
PostRoti	23777 ± 1041	23502 ± 1085		1807 ± 355	3933 ± 667	3565 ± 588
PostRotirIPC	22441 ± 1556	21084 ± 1572		1869 ± 345	4175 ± 587	3746 ± 665
Heart rate (BPM)						
CON	209 ± 12	212 ± 16	-	176 ± 16	190 ± 19	186 ± 24
rIPC	186 ± 17	179 ± 14		181 ± 19	193 ± 16	175 ± 16
PreRoti	220 ± 7	211 ± 6		198 ± 29	207 ± 7	200 ± 9
PreRotirIPC	211 ± 9	207 ± 11		231 ± 24	202 ± 9	187 ± 10
PostRoti	203 ± 6	197 ± 6		218 ± 22	222 ± 27	199 ± 13
PostRotirIPC	207 ± 7	200 ± 8		173 ± 16	235 ± 33	192 ± 5
CF (mL/min)						
CON	48 ± 5	50 ± 3	-	40 ± 3	27 ± 3	20 ± 2
rIPC	55 ± 3	51 ± 2		44 ± 3	31 ± 3	24 ± 3
PreRoti	51 ± 2	48 ± 1		37 ± 2	28 ± 2	23 ± 1
PreRotirIPC	52 ± 4	52 ± 3		43 ± 3	31 ± 4	26 ± 4
PostRoti	53 ± 3	52 ± 3		40 ± 3	28 ± 2	23 ± 2
PostRotirIPC	53 ± 2	50 ± 2		43 ± 4	31 ± 2	24 ± 2
dp/dt max (mm Hg/ms)						
CON	103 ± 7	108 ± 5	-	17 ± 3	28 ± 4	24 ± 4
rIPC	121 ± 5	121 ± 6		22 ± 5	34 ± 7	32 ± 7
PreRoti	102 ± 4	104 ± 4		13 ± 3	20 ± 2	16 ± 3
PreRotirIPC	113 ± 6	118 ± 6		13 ± 3	25 ± 2	21 ± 3
PostRoti	117 ± 4	119 ± 4		9 ± 2	22 ± 3	18 ± 3
PostRotirIPC	108 ± 6	105 ± 6		12 ± 2	20 ± 4	20 ± 4

Table 1: Hemodynamic function. (rIPC: remote ischemic preconditioning; PreRoti: Rotigaptide before ischemia; PreRotirIPC: remote ischemic preconditioning + Rotigaptide before ischemia; PostRoti: Rotigaptide after reperfusion; PostRotirIPC: remote ischemic preconditioning + Rotigaptide after reperfusion; LVDP: left ventricular developed diastolic pressure; RPP: heart rate pressure product; BPM: beats per minute; CF: coronary flow; dp/dt: rate of rise of left ventricular pressure).

Rotigaptide administration intravenously as a 10 min bolus prior to reperfusion followed by a continuous intravenous infusion during 2 h of reperfusion [10]. Similarly, Haugan et al. showed that myocardial infarction induced by coronary artery ligation followed by administration of Rotigaptide during a three-week period could reduce IS in rats. Their study was conducted with three different concentrations (1.1 ± 0.3, 16 ± 4.5 and 230 ± 63 nM) of Rotigaptide and differed from our study by absence of reperfusion and thus did not investigate reperfusion injury. Compared with a vehicle group, only treatment with the intermediate dose of Rotigaptide reduced IS [11]. The results tended to respond in a bell-shaped dose-response relationship. Consequently, Rotigaptide was subsequently tested in dogs subjected to coronary occlusion in an *in vivo* model by Hennen and colleagues. Rotigaptide was administered in different groups receiving i.v. bolus of Rotigaptide 10 min before reperfusion and different concentrations throughout reperfusion (1 ng/kg bolus+10 ng/kg/h infusion, 10 ng/kg bolus+100 ng/kg/h and 100 ng/kg bolus+1000 ng/kg/h and 1000 ng/kg bolus+10 ng/kg/h). In this study, IS reduction was dose-dependent [12]. More recently, danegaptide a mimetic of the connexin 43 targeting peptide Rotigaptide also demonstrated cardioprotective capacity in an *in vivo* model [20].

The discrepancy between results in our *in vitro* studies and those observed in previous *in vivo* studies may relate to the different models. First, our study was an *in vitro* model in which we applied acute global ischemia rather than regional ischemia. Also, species differences (rat/dogs vs. rabbits) may have been of importance. While reduced gap junction intercellular communication by gap junction uncoupler heptanol may reduce cell-to-cell propagation of hypercontracture and cell death [16,21], heptanol failed to induce cardioprotection specifically in a rabbit model of ischemia/reperfusion injury [22]. Similarly, Rotigaptide increases atrial conduction velocity in a rabbit model of chronic volume overload induced atrial conduction velocity slowing. However, the reduction did not translate into a prevention of atrial tachyarrhythmia inducibility [23]. Together, these findings may indicate that the rabbit model is not optimal for studying the impact of Rotigaptide on ischemia reperfusion injury and arrhythmia mechanisms. Finally, we administered a single dose to reach our target concentration in the circulating buffer. Yet, we achieved circulating Rotigaptide concentrations in our *in vitro* model of 201 nmol/l, which was within the range of 157-322 nmol/l that was previously reported to yield cardioprotection in an *in vivo* dog model [12].

The mechanism underlying the cardioprotective effect of Rotigaptide in other species [11,12] is thought to involve modulation of Cx43 [24]. Its localization in the intercalated discs of the myocyte cell membrane [25,26], involves not only gap junctions but also non-junctional hemichannels. The additional presence in the cardiac mitochondria [18], favours these three positions as likely sites of action. Phosphorylation of serine residues regulates Cx43 activity associated with gap junction uncoupling during ischemia [13] while non-junctional Cx43 is involved in volume regulation [27], and contributes to development of edema during ischemia/reperfusion [28]. Inhibition of sarcolemmal Cx43 is cardioprotective [16,17]. The presence of Cx43 in the myocyte mitochondria [29,30], is mandatory for cardioprotection by ischemic preconditioning [31,32], by interaction with ATP-dependent potassium channels and formation of reactive oxygen species. Cardioprotection by ischemic preconditioning is associated with opening of mitochondrial Cx43 channels before ischemia/reperfusion [33].

The unpredictable response to Rotigaptide in various species may relate to dissimilar effect between the different sites of actions. Hence, the resultant agonist or antagonist action of connexin modulating peptides on endogenous connexins may depend on their predominant site of action [34]. During prolonged ischemia Cx43 is dephosphorylated followed by redistribution of Cx43 from the intercalated discs to the lateral cell borders [35,36], redistribution to the intercellular pool [37], and opening of hemichannels [38], which modify cardiomyocyte intercellular communication. AAP10, an unstable predecessor of Rotigaptide, seems to modulate Cx43 only in the intercalated discs while being inactive on Cx43 in the lateral cell borders [39]. Changes in the spatial distribution of Cx43 are observed during ischemia, including local ischemic preconditioning [36,40], and is associated with improved cellular survival. The attenuating effect of conditioning by Rotigaptide may be caused by its ability to redistribute and reorganize active Cx43 from intercellular gap junctions. Because lateralization of Cx43 from the gap junction following ischemia-reperfusion or modification by rIPC/local ischemic preconditioning [19,41], are not consistent findings and because the specific impact of Rotigaptide on mitochondrial Cx43 channels remain unknown, the exact mechanism are not completely clarified by our study. Even though Rotigaptide *per se* did not change ischemia-reperfusion injury, our data indicate that a modification of gap junctional conductance, presumably by a predominant increment, clearly attenuates the cardioprotective efficacy of rIPC supporting the concept of a “spread of injury” [16].

Rotigaptide concentrations during reperfusion was significantly higher by post- than by preischemic Rotigaptide administration because the circulating buffer was changed to a Rotigaptide free buffer at onset of ischemia. Our finding that preischemic administration of Rotigaptide attenuated, while postischemic administration almost abrogated the cardioprotective effect of rIPC are consistent with the difference in Rotigaptide concentration during reperfusion, when preischemically administered Rotigaptide was effectively removed by buffer substitution at onset of ischemia. Our findings also suggest that a considerable modification of ischemia-reperfusion injury takes place during reperfusion.

The absence in change of hemodynamic recovery in our study is in accordance with previous studies testing Rotigaptide and the new analogue danegaptide [1-13,20,42-44]. Although we did not observe statistically significant changes in post-ischemic hemodynamic outcome between the different groups the pattern followed the expected responses according to infarct size reduction. The lack of statistically

significant difference in end-LVDP between rIPC and controls are probably explained by relatively small group sizes.

Our study provided no mechanistic insight into the cardioprotective effect of Rotigaptide. Given the multiple phosphorylation sites [13,45], and potential intracellular actions of Cx43 and the lack of knowledge on the precise interaction of Rotigaptide with Cx43, it is not possible to define a potential target within the signal transduction cascade of cardioprotection.

In our power calculation, we anticipated an infarct size reduction by rotigaptide of 30% (from 60 to 40% of LV with a SD of 12%), a significance level of 5 % and a power of 0.80. Although we included the required minimum of 6 animals requested by our power calculation in each study group, our study yields 80% probability of detecting a 30% infarct size reduction. While the absence of infarct size reduction is most probable, we do not definitively exclude a cardioprotective effect of rotigaptide in an *in vitro* rabbit model.

Rotigaptide *per se* did not modify ischemia-reperfusion injury in our rabbit Langendorff model. Rotigaptide attenuated the effect of rIPC indicating that modification of myocardial gap junction function could be involved in cardioprotection by rIPC.

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