Rapid ventricular pacing-induced postconditioning attenuates reperfusion injury: effects on peroxynitrite, RISK and SAFE pathways

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Short running title: Rapid ventricular pacing-induced postconditioning

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Abstract

Background and purpose: Rapid ventricular pacing (RVP) applied before an index ischaemia has anti-ischaemic effects. Here we investigated whether RVP applied after index ischaemia attenuates reperfusion injury and whether peroxynitrite, RISK and SAFE pathways as well as HO-1 are involved in the mechanism of RVP-induced postconditioning.

Experimental approach: Langendorff perfused rat hearts were subjected to 30 min regional ischaemia and 120 min reperfusion with or without ischaemic postconditioning (6x10/10-s reperfusion/ischaemia; IPost) or RVP (6x10/10-s non-pacing/rapid pacing at 600 bpm) applied at the onset of reperfusion.

Key results: Meta-analysis of our previous studies revealed an association of longer reperfusion-induced ventricular tachycardia/fibrillation with decreased infarct size. In the present experiments testing if RVP is cardioprotective, we found that both IPost and RVP significantly decreased infarct size ($38 \pm 5\%$ and $27 \pm 5\%$ vs. $53 \pm 4\%$, $p < 0.05$), however, only RVP attenuated the incidence of reperfusion-induced ventricular tachycardia. Both postconditioning methods increased formation of cardiac 3-nitrotyrosine and superoxide, and non-significantly enhanced Akt phosphorylation at the beginning of reperfusion without affecting Erk1/2 and Stat3, while solely IPost induced HO-1. Application of brief ischaemia/reperfusion cycles or RVP without preceding index ischaemia also facilitated peroxynitrite formation, nevertheless, only brief RVP increased Stat3 phosphorylation.

Conclusions and implications: Application of short periods of RVP at the onset of reperfusion is cardioprotective and increases peroxynitrite formation similarly to IPost, and thus may serve as an alternative postconditioning method. However, downstream mechanisms of the protection elicited by IPost and RVP seem to be partially different.

Keywords: cardioprotection, conditioning, oxidative and nitrative stress, $ONOO^-$, protein kinase, MAPK, haem oxygenase

Abbreviations:

ANOVA (analysis of variance)
BSA (bovine serum albumin)
GAPDH (glyceraldehyde 3-phosphate dehydrogenase)
ECG (electrocardiogram)
HO-1 (haem oxygenase 1)
I/R (ischaemia/reperfusion)
IPost (ischaemic postconditioning)
LAD (left anterior descending coronary artery)
LDH (lactate dehydrogenase)
RIPA (radioimmunoprecipitation)
RISK (reperfusion injury salvage kinase)
SAFE (survival activating factor enhancement)
RVP (rapid ventricular pacing)
VF (ventricular fibrillation)
VT (ventricular tachycardia)
S.E.M. (standard error of mean)

Introduction

Ischaemic heart diseases including acute myocardial infarction are the leading cause of death in industrialized countries. Reperfusion therapy for infarction allows rapid return of blood flow to the ischaemic myocardium and decreases mortality rate. However, early reperfusion itself is accompanied by deleterious events: occurrence of life-threatening arrhythmias, no-reflow phenomenon, myocardial stunning and additional cell death (Yellon *et al.*, 2007). This paradoxical reperfusion injury caused by the restoration of blood flow and oxygen supply (Yamada *et al.*, 1990) leads to increased infarct size, impaired contractile function, and electric vulnerability, largely compromising clinical outcomes.

Ischaemic postconditioning (IPost) has emerged in the last decade as a potential therapeutic intervention for limiting reperfusion injury (Zhao *et al.*, 2003; Ovize *et al.*, 2010). The procedure is based on application of brief cycles of ischaemia/reperfusion (I/R) immediately after a prolonged ischaemia and it has been reported to reduce myocardial damage in both animal studies and in human clinical trials (Ovize *et al.*, 2010). Nevertheless, some studies have reported the ineffectiveness of IPost both in animals and in humans (Dow *et al.*, 2007; Hahn *et al.*, 2013). A possible explanation for the controversial results could be that the outcome of postconditioning may depend on several factors such as failure to achieve complete reperfusion during application of brief I/R cycles, the duration of index ischaemia, the algorithm of postconditioning manoeuvre, gender, age, and temperature (Skyschally *et al.*, 2009b). In addition, comorbidities like hyperlipidaemia (Kupai *et al.*, 2009) and diabetes (Miki *et al.*, 2012) may interfere with the infarct size-limiting effect of postconditioning. These confounding factors indicate the necessity to develop new alternative methods and models to induce postconditioning.

Heart rate is known to play a role in the development of I/R injury (Bernier *et al.*, 1989), and it was shown that induction of either slow- or rapid heart rate before ischaemia limits myocardial injury (Tosaki *et al.*, 1988; Bernier *et al.*, 1989; Hearse *et al.*, 1999). Moreover, we have shown previously that short periods of rapid ventricular pacing (RVP) applied before an index ischaemia has anti-ischaemic effects (pacing-induced preconditioning) (Ferdinandy *et al.*, 1997a; Ferdinandy *et al.*, 1997b; Ferdinandy *et al.*, 1998). However, the effect of short periods of RVP performed at the early phase of reperfusion has not been investigated so far.

The exact molecular mechanism of myocardial postconditioning is not entirely clear. Increasing evidence suggests that enhanced formation of cardiac peroxynitrite is involved in cardioprotection afforded by both pre- (Altug *et al.*, 2000; Altup *et al.*, 2001; Csonka *et al.*,

2001) and postconditioning (Kupai *et al.*, 2009; Li *et al.*, 2013). Kupai e*t al.* have reported first that IPost failed to decrease infarct size in the presence of a peroxynitrite decomposition catalyst, thereby suggesting essential triggering role of peroxynitrite in postconditioning-induced cardioprotection (Kupai *et al.*, 2009).

Therefore, here we aimed to investigate whether RVP applied after index ischaemia has any effect on markers of reperfusion injury and we studied the role of peroxynitrite in the mechanisms of postconditioning. Furthermore, we looked at activation of reperfusion injury salvage kinase (RISK) and survival activating factor enhancement (SAFE) pathways and haem oxygenase 1 (HO-1) as possible downstream targets of RVP-induced postconditioning.

Materials and methods

Male Wistar rats were used in our previous and present studies. The studies conform to the 'Guide for the care and use of laboratory animals' published by the US National Institutes of Health (NIH publication No. 85–23, revised 1996) and was approved by local ethics committees. The animals were kept at 12/12-hour light/dark cycle and had free access to standard laboratory chow and drinking water.

Isolated heart preparation

Isolated heart preparation was done as described in our previous studies with slight modifications (Ferdinandy *et al.*, 1997a; Kocsis *et al.*, 2012; Varga *et al.*, 2014). Rats were anaesthetised with diethyl ether, an anaesthetic not known to interfere with cardioprotection, and were given 500 $U \cdot kg^{-1}$ heparin intravenously. Hearts were then isolated and perfused according to Langendorff at 37 ºC with Krebs-Henseleit buffer containing NaCl 118 mM, $NaHCO_3$ 25 mM, KCl 4.3 mM, $CaCl_2$ 1.5 mM, KH_2PO_4 1.2 mM, $MgSO_4$ 1.2 mM, glucose 11 mM, gassed with 95% O_2 and 5% CO_2. Hydrostatic perfusion pressure was kept constant at 100 cmH_2O (9.8 kPa) throughout the experiments. Coronary flow was measured by collecting coronary effluent for a period of time and was expressed as $mL \cdot min^{-1}$.

A 3-0 silk suture was placed around the left anterior descending coronary artery (LAD) close to its origin and the snare was tightened by applying a 100 g hanging weight to induce regional index ischaemia. For IPost brief no-flow global ischaemia was performed by turning off the perfusion cannula. The presence of ischaemia was verified by monitoring coronary flow. Rapid ventricular pacing (600 bpm; 10 Hz) was performed by an electric stimulator (Experimetria, Budapest, Hungary) with double threshold square, 1 V, 1 mA and 5-ms impulses conducted by electrodes attached directly to the surface of the right ventricle

close to the apex and to the aortic cannula as described previously (Ferdinandy *et al.*, 1997a; Ferdinandy *et al.*, 1997b; Ferdinandy *et al.*, 1998). Heart rates were monitored (Isosys, Experimetria Inc., Budapest, Hungary) by recording epicardial electrocardiogram (ECG) throughout the whole duration of perfusion.

Relationship of the duration of reperfusion-induced ventricular tachyarrhythmia and infarct size: a meta-analysis

Meta-analysis was performed on ECGs and infarct size data from our six previous studies done in our laboratory on isolated rat hearts subjected to 30 min regional ischaemia and 120 min reperfusion [Figure 1A]. Reperfusion-induced arrhythmias were analysed in the first 10 min of reperfusion. Hearts presenting sustained (>10 min) tachyarrhythmia were excluded (n = 14). Three separate evaluations were done based on total duration of ventricular tachycardia (VT), ventricular fibrillation (VF), or VT+VF, respectively. Infarct size data were presented on the basis of duration (shorter or longer than 60 s) of VT, VF, or VT+VF. Infarct size data exceeding mean ± two standard deviations were excluded from the analysis (n = 6).

Experimental design 1: testing the cardioprotective effect of rapid ventricular pacing

To examine whether RVP applied at the onset of reperfusion induces cardioprotection, isolated hearts were perfused as shown on Figure 2A. Three experimental groups were designed: (1) ischaemia/reperfusion control, (2) ischaemic postconditioning, (3) and rapid ventricular pacing groups (n = 12 in each group). The I/R control group was subjected to 15 min equilibration period, followed by 30 min regional index ischaemia and 120 min reperfusion. IPost was induced by six consecutive cycles of 10 s reperfusion and 10 s no-flow global ischaemia at the onset of reperfusion. In the RVP group the spontaneous rhythm of hearts was replaced by 10-s pacing period (600 bpm; 10 Hz) in 6 alternating cycles during the first 2 min of reperfusion.

To assess the severity of cellular damage in the myocardium, the activity of lactate dehydrogenase (LDH) enzyme from coronary effluents (collected during the first 5 min of reperfusion) was measured using a LDH-P kit (Diagnosticum, Budapest, Hungary) (n = 5 in each group). The enzyme activity ($U \cdot mL^{-1}$) measured in an effluent was multiplied with the corresponding coronary flow ($mL \cdot min^{-1}$) to give LDH release expressed as $U \cdot min^{-1}$.

To determine infarct size, the LAD was reoccluded at the end of reperfusion and hearts were stained with 0.1% Evans-blue to determine area at risk (Csonka *et al.*, 2010). Hearts were then frozen at -20 °C and cut into approximately 2-mm thick slices. Each slice was

incubated at 37 °C for 10 min in 1% 2,3,4-triphenyl-tetrazolium-chloride solution dissolved in phosphate buffer (pH 7.4). Slices were then fixed in 10% formaldehyde and scanned. Infarct size was evaluated by planimetry (InfarctSize™ 2.4.b, Pharmahungary Group, Szeged, Hungary) and normalised to area at risk.

To assess reperfusion-induced tachyarrhythmias (VT and VF), ECG was recorded (Isosys, Experimetria Inc., Budapest, Hungary) during the entire perfusion protocol. Analysis of arrhythmias was carried out according to the original Lambeth conventions (Walker *et al.*, 1988).

Experimental design 2: investigating the role of peroxynitrite and possible downstream targets in rapid ventricular pacing-induced postconditioning

To assess the possible role of peroxynitrite in cardioprotection induced by ischaemic- or rapid ventricular pacing-induced postconditioning, in separate experiments, cardiac 3-nitrotyrosine, a well-known peroxynitrite marker was determined. To confirm increased peroxynitrite formation, cardiac superoxide anion was also measured. Furthermore, involvement of molecular mechanisms (i.e. RISK and SAFE pathways, HO-1) that have been implicated in cardioprotection (Hausenloy *et al.*, 2004; Lecour, 2009; Bak *et al.*, 2010) was also investigated as possible downstream targets of RVP-induced postconditioning.

Hearts were subjected to 15 min equilibration period, followed by 30 min regional ischaemia and 7 min reperfusion with or without IPost or RVP [Figure 4A]. At the end of reperfusion myocardial samples were taken from the ischaemic zone of the left ventricle for 3-nitrotyrosine measurement and western blot analysis (n = 5 in each group). Sampling was done by an oblique cut from the origin of the LAD toward the right side of the apical area that involves the majority of the anterior wall of the left ventricle as well as the apex of the heart. Samples were rapidly freeze-clamped, powdered with a pestle and mortar in liquid nitrogen, and stored in cryovials at -80 °C until further analysis. Sampling for *in situ* detection of superoxide anion was done in separate experiments (n = 3 in each group) using the same perfusion protocol [Figure 4A]. Approximately 3-mm thick transverse slices were cut from the middle of the ventricles, embedded in Tissue-Tek O.C.T. compound (Sakura Finetek, Zoeterwoude, Netherlands), carefully frozen in isopentane precooled in liquid nitrogen, and stored at -80 °C until sectioning with a microtome.

Cardiac free 3-nitrotyrosine content, a marker of peroxynitrite, was measured by enzyme-linked immunosorbent assay (Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's instructions (Kupai *et al.*, 2009; Kocsis *et al.*, 2012). Briefly, homogenates

were incubated overnight with nitrotyrosine acetylcholinesterase tracer and anti-nitrotyrosine rabbit IgG in microplates precoated with mouse anti-rabbit IgG. Ellman's reagent was used for development. Free nitrotyrosine content was normalised to protein content of cardiac homogenate and expressed as ng per mg protein.

Superoxide anion (O_2^-) is a reactive oxygen radical that reacts with nitric oxide to form peroxynitrite. The *in situ* fluorescent dihydroethidium staining was performed to evaluate intracellular production of superoxide anion (Varga *et al.*, 2013). Unfixed frozen heart sections (30 μm) were placed on glass slides and incubated in 10^{-6} mol·L^{-1} dihydroethidium (Sigma, St. Louis, MO, USA) in PBS buffer (pH 7.4) at 37 °C for 30 min in a dark humidified container. Fluorescence was then detected by a fluorescent microscope (Nikon, Japan) with a 590 nm long-pass filter. Images of the hearts were collected digitally (n = 20 in each heart), integrated density were evaluated by ImageJ 1.44p software and expressed in arbitrary unit.

The involvement of possible downstream targets in the mechanism of RVP-induced postconditioning was examined by standard Western blot techniques (Kocsis *et al.*, 2008; Fekete *et al.*, 2013). Tissue samples were homogenized with an ultrasonicator (UP100H Hielscher, Teltow, Germany) in RIPA buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.5% sodium deoxycholate, 5 mM EDTA, 0.1% SDS, 1% NP-40) supplemented with protease inhibitor cocktail (Sigma, St. Louis, MO, USA), PMSF, NaF and Na_3VO_4. The crude homogenates were centrifuged at 10,000 x g for 10 min at 4 °C. After quantification of protein concentrations of the supernatants using BCA Protein Assay Kit (Pierce, Rockford, IL, USA), 20 μg (50 μg for HO-1) reduced and denaturated protein was loaded and SDS-PAGE (10% gel, 90 V, 1.5 h) was performed followed by transfer of proteins onto nitrocellulose membrane (20% methanol, 35 V, 2 h). Membranes were blocked for 1 h in 5% w/v bovine serum albumin (BSA) at room temperature and then incubated with primary antibodies against phospho(Ser473)-Akt 1:500, Akt 1:2000, phospho(Thr202/Tyr204)-Erk1/Erk2 1:2000, Erk1/Erk2 1:1000, phospho(Tyr705)-Stat3 1:2000, Stat3 1:2000 (Cell Signaling, Beverly, MA, USA; overnight, 4 °C, 5% BSA) or HO-1 1:2000 (Enzo Life Sciences, Plymouth Meeting, PA, USA; 2 h, room temperature, 1% milk) or GAPDH 1:10,000 (Cell Signaling, Beverly, MA, USA; 1 h, room temperature, 1% milk). After incubation with HRP-conjugated secondary antibody 1:5000 (1:20,000 for GAPDH) (Dako Corporation, Santa Barbara, CA, USA; 1 h, room temperature, 1% milk), membranes were developed using enhanced chemiluminescence kit (Pierce, Rockford, IL, USA).

To further prove that both IPost and RVP protocols (i.e. application of brief ischaemia/reperfusion or rapid ventricular pacing) facilitate peroxynitrite formation, 3-

nitrotyrosine was measured in the absence of index ischaemia. Effect of the protocols on possible downstream targets of peroxynitrite (i.e. RISK and SAFE pathways) was also examined in the absence of preceding index ischaemia.

In this set of experiments, the time course of perfusion protocol was adjusted to the previous setup without index ischaemia [Figure 5A]. In the normoxic perfusion group (n = 8) hearts were perfused for 52 min. In the repeated brief I/R group (n = 7) hearts were subjected to 45 min perfusion followed by 6 x 10/10-s cycles of no-flow global I/R and 5 min reperfusion. In the repeated brief RVP group (n = 8), the spontaneous rhythm of the hearts was replaced by 10-s pacing period (600 bpm; 10 Hz) in 6 alternating cycles after 45 min perfusion. At the end of perfusion, cardiac free 3-nitrotyrosine level was determined and RISK as well as SAFE pathways were examined as described above.

Statistical analysis

Data were expressed as mean ± S.E.M and analysed with unpaired t-test, one-way analysis of variance (ANOVA), or Fisher's exact test as appropriate. If a difference was established in ANOVA, Fisher's Least Significant Difference (LSD) post hoc test was applied. Differences were considered significant at $p < 0.05$.

Results

Duration of reperfusion-induced ventricular tachycardia and/or fibrillation is associated with decreased infarct size

Meta-analysis of six separate studies previously performed in our laboratory using the same experimental protocol (i.e. isolated rat hearts subjected to I/R) showed that the presence of VT, VF, or VT+VF with a total duration of longer than 60 s in the first 10 min of reperfusion was associated with a markedly decreased infarct size [Figure 1B], respectively. In this analysis a larger area at risk was associated with longer than 60 s total duration of VT+VF [Figure 1C].

Rapid ventricular pacing exerts cardioprotective effect: limits the infarction and reperfusion-induced arrhythmias

In order to assess the possible cardioprotective effect of RVP, the extent of myocardial infarction (LDH release and infarct size) was measured and reperfusion-induced arrhythmias were analysed.

The post-ischaemic LDH release was significantly reduced by RVP [Figure 2B]. IPost also reduced LDH release, however, the difference did not reach the level of statistical significance [Figure 2B]. Infarct size was significantly decreased by both IPost and RVP [Figure 2C]. There was no difference in the area at risk of either experimental group [Figure 2D].

The incidence of VT and VF was not affected significantly by IPost in our present study [Figure 3]. In contrast, short periods of RVP decreased the incidence of reperfusion-induced VT without having a significant effect on VF [Figure 3].

There was no difference in animal weight, heart wet weight, baseline heart rate, coronary flow (baseline, beginning of ischaemia, end of reperfusion) between the experimental groups [Table 1]. In contrast to IPost, coronary flow at the onset of reperfusion was not changed by short periods of RVP compared to I/R control [Table 1].

Peroxynitrite is likely involved in rapid ventricular pacing induced-postconditioning

To obtain some mechanistic insight into the beneficial effect of RVP, cardiac 3-nitrotyrosine and superoxide were measured at the 7^{th} min of reperfusion following the 30 min index ischaemia.

Postconditioning induced either by IPost or by RVP significantly increased free cardiac 3-nitrotyrosine level (a marker of peroxynitrite formation) [Figure 4B]. Moreover, the peroxynitrite precursor superoxide anion was mildly, but significantly elevated in both postconditioning groups [Figure 4C].

To further prove that the postconditioning manoeuvres induce nitrative stress, cardiac 3-nitrotyrosine was measured after the postconditioning stimuli applied following normoxic perfusion without index ischaemia. The application of brief I/R cycles or periodic RVP increased the cardiac formation of 3-nitrotyrosine in the absence of index ischaemia [Figure 5B].

Downstream mechanisms of rapid ventricular pacing-induced cardioprotection differs from that of ischaemic postconditioning

To elucidate the possible downstream targets of RVP, RISK and SAFE pathways as well as HO-1 were investigated either in the presence or absence of index ischaemia.

Both postconditioning methods non-significantly enhanced Akt phosphorylation after index ischaemia at the beginning of reperfusion without affecting phosphorylation of Erk1/2 and Stat3 [Figure 4E, F]. Protein level of HO-1 was increased by IPost but not RVP

[Figure 4E, F]. In the absence of index ischaemia, applying short periods of RVP protocol increased Stat3 phosphorylation, in contrast to brief cycles of I/R [Figure 5C, D]. Phosphorylation of Akt and Erk 1/2 was not affected significantly by any of the interventions in the absence of index ischaemia [Figure 5C, D].

Discussion and conclusion

In our present study, using an isolated perfused rat heart model, we confirmed that IPost beneficially affects I/R injury. Moreover, we demonstrated for the first time in the literature that applying short periods of RVP at the onset of reperfusion also exerts cardioprotective effect as it attenuates reperfusion injury by decreasing infarct size and reperfusion-induced arrhythmias. We showed that RVP increased peroxynitrite formation either in the presence or absence of index ischaemia in a similar way to IPost. These findings suggest that the formation of peroxynitrite in early reperfusion is a key event in the development of cardioprotection elicited by IPost or RVP. However, we also demonstrated that the downstream mechanisms of RVP-induced cardioprotection and IPost seem to be partially different.

In a meta-analysis of our previous studies on isolated hearts subjected to I/R we analysed if there is an association between the duration of reperfusion-induced ventricular tachyarrhythmias (VT, VF, or VT+VF) and infarct size. It is well accepted in the literature that I/R induces cellular damage that makes the myocardium more susceptible to arrhythmogenesis, and thus reperfusion-induced arrhythmias are considered as indicators of I/R injury (Engelen *et al.*, 2003; Majidi *et al.*, 2009). For instance, Majidi *et al.* have reported that presence of reperfusion arrhythmia bursts in STEMI patients are associated with worse outcome (larger infarct size and decreased ejection fraction) (Majidi *et al.*, 2009). However, here we found surprisingly that longer than 60 s reperfusion-induced ventricular tachycardia/fibrillation was associated with decreased infarct size. In this analysis a larger area at risk was associated with longer total duration of VT+VF in accordance with literature data (Curtis *et al.*, 1989). Interpretation of these results is difficult since causality was not examined in these studies. A possible explanation for the results of our meta-analysis is that the size of infarction affects the occurrence of sustained VT and/or VF, while another possibility is that longer tachyarrhythmias at the beginning of reperfusion somehow attenuate infarct development. To the best of our knowledge, this latter approach has not been investigated in the literature, and therefore these findings served as a basis for our current

experimental study to investigate if exogenous application of controlled tachycardia induced by RVP at the onset of reperfusion is able to elicit cardioprotection.

Heart rate is known to play a role in the development of I/R injury (Bernier *et al.*, 1989) and its controlled modification may elicit cardioprotection. For instance, pharmacologically-induced bradycardia (Tosaki *et al.*, 1987), slow- (Tosaki *et al.*, 1988) or rapid (Ferdinandy *et al.*, 1998; Hearse *et al.*, 1999) pacing before ischaemia was reported to limit myocardial injury. Since the presence of longer reperfusion-induced tachyarrhythmias was associated with lower infarct size in our meta-analysis, we wanted to test whether exogenous rapid pacing exerts protection. To the best of our knowledge, we demonstrated for the first time in the literature that the application of short periods of rapid (600 bpm) ventricular pacing at the beginning of reperfusion reduces infarct size and reperfusion-induced arrhythmias.

In the present study, both RVP and classic IPost decreased infarct size. The beneficial effect of RVP on infarct size was further confirmed by a reduction of LDH release into coronary effluent. Infarct size is a key determinant of major clinical outcomes (mortality and morbidity of consequent heart failure) (Gibbons *et al.*, 2004), therefore, development of procedures which effectively decrease infarct size along with reperfusion therapy is in the focus of preclinical and clinical studies (Ovize *et al.*, 2010). IPost is a widely studied approach, and the infarct size reducing effect of this procedure was confirmed in various mice, rat, rabbit, dog, and swine animal models (Skyschally *et al.*, 2009b) as well as in clinical trials (Ovize *et al.*, 2010). However, some studies reported the ineffectiveness of IPost in animal models (Dow *et al.*, 2007; Skyschally *et al.*, 2009b) and in clinical trials (Hahn *et al.*, 2013). A possible explanation for the controversial results could be that the cardioprotective effect of IPost depends on several factors such as for instance (1) species, strain, gender, age of research animal; (2) experimental model and set up; (3) the duration of index ischaemia before reperfusion; (4) number and duration of brief I/R cycles; (5) technical difficulty to achieve complete reperfusion; (6) temperature; (7) presence of comorbidities. These confounding factors indicate the necessity to develop alternative methods of IPost and we suggest that RVP-induced postconditioning is a simple method that eliminates technical problems associated with induction of IPost.

Besides infarct size reduction, RVP-induced postconditioning decreased reperfusion-induced ventricular arrhythmias as well. Reperfusion therapy is accompanied by occurrence of arrhythmias (Krumholz *et al.*, 1991). Some of them are benign (e.g. accelerated idioventricular rhythm, the most common type) but other ones are potentially life-threatening malignant arrhythmias such as VT or VF that need to be managed in the clinical practice to

avoid fatal consequences. Based on literature data (Kloner *et al.*, 2006), IPost effectively decreases ventricular arrhythmias. However, in our present study, solely RVP-induced postconditioning reduced the incidence of reperfusion-induced VT with no significant effect on VF. The reason for the inability of RVP to improve post-ischaemic VF is not clear. However, one may speculate that some interacting triggers of reperfusion-induced VF (e.g. reactive oxygen intermediates and calcium) may interfere with the possible anti-VF effect of RVP (Hearse *et al.*, 1988).

Here we demonstrated that IPost and RVP-induced postconditioning enhanced peroxynitrite formation at the onset of reperfusion after an index ischaemia. In addition, postconditioning manoeuvres themselves (i.e. brief ischaemia/reperfusion and rapid ventricular pacing) increased peroxynitrite formation in the absence of the index ischaemia. Since peroxynitrite is reported as a possible trigger of IPost (Kupai *et al.*, 2009), based on our current results, we propose that the enhanced peroxynitrite formation also plays a role in triggering RVP-induced postconditioning. Back in 1997, Yasmin *et al.* reported that the level of peroxynitrite increases during reperfusion, which contributes to reperfusion injury in isolated rat hearts (Yasmin *et al.*, 1997). Further studies also confirmed that enhanced peroxynitrite formation plays a central role in numerous cardiovascular diseases by inducing oxidative, nitrative- and nitrosative stress (Pacher *et al.*, 2007). However, peroxynitrite was demonstrated to have physiological functions (Lefer *et al.*, 1997) and to play a role in triggering ischaemic preconditioning (Altug *et al.*, 2000; Altup *et al.*, 2001; Csonka *et al.*, 2001). We have previously reported for the first time that peroxynitrite is a trigger of IPost, since the peroxynitrite scavenger, FeTPPS interfered with the cardioprotective effect of IPost (Kupai *et al.*, 2009). Our results were confirmed by Li *et al.* showing that peroxynitrite is a key mediator of IPost *in vivo* (Li *et al.*, 2013). Nevertheless, the possible mechanisms lying downstream of peroxynitrite formation in postconditioning have not been elucidated.

Here we also looked at possible targets of endogenous peroxynitrite formation induced by IPost or by RVP. Several studies have reported that the activation of RISK (Akt, Erk1/Erk2) and SAFE (Stat3) pathways at the onset of reperfusion might play a role in the cardioprotective effect of IPost (Hausenloy, 2009; Lecour, 2009). In other studies overexpression of HO-1 was shown to reduce infarct size in the heart (Bak *et al.*, 2010) and was implicated in pulmonary and hepatic IPost (Xia *et al.*, 2009; Zeng *et al.*, 2011). In our present study, both IPost and RVP-induced postconditioning non-significantly enhanced Akt phosphorylation without affecting Erk1/2 and Stat3 at the beginning of reperfusion. Although several studies showed increased phosphorylation of Akt and/or Erk due to IPost (Tsang *et*

al., 2004; Yang *et al.*, 2004), some recent papers suggested that postconditioning did not activate RISK pathway in the early phase of reperfusion (Skyschally *et al.*, 2009a; Fekete *et al.*, 2013). We also found here that IPost but not RVP increased HO-1 protein in the heart. This effect of IPost on HO-1 is in agreement with findings of others in the lung and liver (Xia *et al.*, 2009; Zeng *et al.*, 2011). We also examined the effect of postconditioning manoeuvres (i.e. repeated brief cycles of ischaemia/reperfusion or rapid ventricular pacing) in the absence of a preceding index ischaemia and found no activation of the RISK pathway. In these experiments, Stat3 phosphorylation was increased only by short periods of RVP protocol. Taken together, our present results indicate that (1) the downstream mechanisms of RVP-induced cardioprotection and IPost are partially different, (2) HO-1 is likely not involved in the cardioprotective effect of RVP-induced postconditioning, and (3) the precise role of the RISK and SAFE pathways remains to be elucidated in future studies. Involvement of alternative pathways in the protective effect of RVP-induced postconditioning is likely, and may include for instance activation of NO-cGMP-PKG, sphingosine-, protein kinase C-, or CGRP-mediated pathways (Heusch *et al.*, 2008; Bice *et al.*, 2014). Since endogenous NO-cGMP play a role in protection against reperfusion injury by attenuating infarct size (Penna *et al.*, 2006) and reperfusion-induced VF (Pabla *et al.*, 1995; Pabla *et al.*, 1996), investigation of the exact role of NO in RVP would be interesting.

Although we clearly demonstrated that RVP induces cardioprotection when applied at the onset of reperfusion, some further limitations of our study may be considered. First, ventricular pacing was reported to have direct pro-arrhythmic effects caused by the stimulus itself independently from the heart rate (Nakata *et al.*, 1990). Although in our study ventricular pacing last only for short periods (6 x 10 s), and the incidence of reperfusion-induced VF was not increased in the RVP group when compared to I/R controls, consideration of pacing as an ectopic focus cannot be excluded. Second, in RVP-induced postconditioning ventricles were activated in a non-physiological way in the present *ex vivo* study. Although the atrio-ventricular conduction system of rats was reported to be suitable for reaching 600 bpm heart rate by atrial pacing in an *in vivo* model (Gonzalez *et al.*, 1998), further *in vivo* studies are needed to investigate the infarct size limiting effect of postconditioning induced by rapid atrial or ventricular pacing at different rates. Third, our study suggests that rapid heart rate at the early phase of reperfusion may contribute to initiation of adaptive molecular mechanisms to prevent I/R-induced cellular damage. However, further studies are needed to analyse (1) the precise molecular nature of these mechanisms and (2) if reperfusion-induced spontaneous arrhythmias also trigger adaptive

mechanisms in the myocardium. Our findings may also suggest that reperfusion-induced tachyarrhythmias require attention in future studies focusing on cardioprotection assessed by infarct size.

In conclusion, application of short periods of rapid ventricular pacing at the onset of reperfusion beneficially affects essential components of reperfusion injury: the infarct size and reperfusion-induced ventricular arrhythmias. In addition, RVP increases peroxynitrite formation, which likely plays a role in triggering cardioprotection similarly to IPost. Nevertheless, downstream mechanisms in RVP-induced protection seem to be partially different from that of IPost, and further research is needed to elucidate them. Since RVP exerted a similar cardioprotective effect to IPost, we feel that RVP-induced postconditioning may serve as an alternative experimental model of IPost. Moreover, RVP could be performed in more controlled manner than applying brief I/R cycles in IPost, which is an important technical advantage compared to IPost.

Acknowledgements

We are grateful to Nóra Bagi, Fatime Hawchar, Szilvia Török for their skilful technical assistance. We acknowledge the support of grants from the Hungarian Scientific Research Fund (OTKA K 79167), National Office for Research and Technology Grants (NKTH MED_FOOD, TÁMOP-4.2.1/B-09/1/KONV-2010-0005, TÁMOP-4.2.2.A-11/1/KONV-2012-0035). This work was also supported by János Bolyai Research Scholarship of the Hungarian Academy of Sciences (TC and CC).

Conflict of interest: not declared.

References

Altug S, Demiryurek AT, Kane KA, Kanzik I (2000). Evidence for the involvement of peroxynitrite in ischaemic preconditioning in rat isolated hearts. Br J Pharmacol 130**:** 125-131.

Altup S, Demiryurek AT, Ak D, Tungel M, Kanzik I (2001). Contribution of peroxynitrite to the beneficial effects of preconditioning on ischaemia-induced arrhythmias in rat isolated hearts. Eur J Pharmacol 415**:** 239-246.

Bak I, Czompa A, Juhasz B, Lekli I, Tosaki A (2010). Reduction of reperfusion-induced ventricular fibrillation and infarct size via heme oxygenase-1 overexpression in isolated mouse hearts*. J Cell Mol Med 14**:** 2268–2272.

Bernier M, Curtis MJ, Hearse DJ (1989). Ischemia-induced and reperfusion-induced arrhythmias: importance of heart rate. Am J Physiol 256**:** H21-31.

Bice JS, Baxter GF (2014). Postconditioning signalling in the heart: mechanisms and translatability. Br J Pharmacol. (In press)

Csonka C, Csont T, Onody A, Ferdinandy P (2001). Preconditioning decreases ischemia/reperfusion-induced peroxynitrite formation. Biochem Biophys Res Commun 285**:** 1217-1219.

Csonka C, Kupai K, Kocsis GF, Novak G, Fekete V, Bencsik P, et al. (2010). Measurement of myocardial infarct size in preclinical studies. J Pharmacol Toxicol Methods 61**:** 163-170.

Curtis MJ, Hearse DJ (1989). Reperfusion-induced arrhythmias are critically dependent upon occluded zone size: relevance to the mechanism of arrhythmogenesis. J Mol Cell Cardiol 21**:** 625-637.

Dow J, Kloner RA (2007). Postconditioning does not reduce myocardial infarct size in an in vivo regional ischemia rodent model. J Cardiovasc Pharmacol Ther 12**:** 153-163.

Engelen DJ, Gressin V, Krucoff MW, Theuns DA, Green C, Cheriex EC, et al. (2003). Usefulness of frequent arrhythmias after epicardial recanalization in anterior wall acute myocardial infarction as a marker of cellular injury leading to poor recovery of left ventricular function. Am J Cardiol 92**:** 1143-1149.

Fekete V, Murlasits Z, Aypar E, Bencsik P, Sarkozy M, Szenasi G, et al. (2013). Myocardial postconditioning is lost in vascular nitrate tolerance. J Cardiovasc Pharmacol 62**:** 298-303.

Ferdinandy P, Csonka C, Csont T, Szilvassy Z, Dux L (1998). Rapid pacing-induced preconditioning is recaptured by farnesol treatment in hearts of cholesterol-fed rats: role of polyprenyl derivatives and nitric oxide. Mol Cell Biochem 186**:** 27-34.

Ferdinandy P, Csont T, Csonka C, Torok M, Dux M, Nemeth J, et al. (1997a). Capsaicin-sensitive local sensory innervation is involved in pacing-induced preconditioning in rat hearts: role of nitric oxide and CGRP? Naunyn Schmiedebergs Arch Pharmacol 356**:** 356-363.

Ferdinandy P, Szilvassy Z, Horvath LI, Csont T, Csonka C, Nagy E, et al. (1997b). Loss of pacing-induced preconditioning in rat hearts: role of nitric oxide and cholesterol-enriched diet. J Mol Cell Cardiol 29**:** 3321-3333.

Gibbons RJ, Valeti US, Araoz PA, Jaffe AS (2004). The quantification of infarct size. J Am Coll Cardiol 44**:** 1533-1542.

Gonzalez NC, Clancy RL, Moue Y, Richalet JP (1998). Increasing maximal heart rate increases maximal O2 uptake in rats acclimatized to simulated altitude. J Appl Physiol (1985) 84**:** 164-168.

Hahn JY, Song YB, Kim EK, Yu CW, Bae JW, Chung WY, et al. (2013). Ischemic postconditioning during primary percutaneous coronary intervention: the effects of postconditioning on myocardial reperfusion in patients with ST-segment elevation myocardial infarction (POST) randomized trial. Circulation 128**:** 1889-1896.

Hausenloy DJ (2009). Signalling pathways in ischaemic postconditioning. Thromb Haemost 101**:** 626-634.

Hausenloy DJ, Yellon DM (2004). New directions for protecting the heart against ischaemia-reperfusion injury: targeting the Reperfusion Injury Salvage Kinase (RISK)-pathway. Cardiovasc Res 61**:** 448-460.

Hearse DJ, Ferrari R, Sutherland FJ (1999). Cardioprotection: intermittent ventricular fibrillation and rapid pacing can induce preconditioning in the blood-perfused rat heart. J Mol Cell Cardiol 31**:** 1961-1973.

Hearse DJ, Tosaki A (1988). Free radicals and calcium: simultaneous interacting triggers as determinants of vulnerability to reperfusion-induced arrhythmias in the rat heart. J Mol Cell Cardiol 20**:** 213-223.

Heusch G, Boengler K, Schulz R (2008). Cardioprotection: nitric oxide, protein kinases, and mitochondria. Circulation 118**:** 1915-1919.

Kloner RA, Dow J, Bhandari A (2006). Postconditioning markedly attenuates ventricular arrhythmias after ischemia-reperfusion. J Cardiovasc Pharmacol Ther 11**:** 55-63.

Kocsis GF, Pipis J, Fekete V, Kovacs-Simon A, Odendaal L, Molnar E, et al. (2008). Lovastatin interferes with the infarct size-limiting effect of ischemic preconditioning and postconditioning in rat hearts. Am J Physiol Heart Circ Physiol 294**:** H2406-2409.

Kocsis GF, Sarkozy M, Bencsik P, Pipicz M, Varga ZV, Paloczi J, et al. (2012). Preconditioning protects the heart in a prolonged uremic condition. Am J Physiol Heart Circ Physiol 303**:** H1229-1236.

Krumholz HM, Goldberger AL (1991). Reperfusion arrhythmias after thrombolysis. Electrophysiologic tempest, or much ado about nothing. Chest 99**:** 135S-140S.

Kupai K, Csonka C, Fekete V, Odendaal L, van Rooyen J, Marais de W, et al. (2009). Cholesterol diet-induced hyperlipidemia impairs the cardioprotective effect of postconditioning: role of peroxynitrite. Am J Physiol Heart Circ Physiol 297**:** H1729-1735.

Lecour S (2009). Activation of the protective Survivor Activating Factor Enhancement (SAFE) pathway against reperfusion injury: Does it go beyond the RISK pathway? J Mol Cell Cardiol 47**:** 32-40.

Lefer DJ, Scalia R, Campbell B, Nossuli T, Hayward R, Salamon M, et al. (1997). Peroxynitrite inhibits leukocyte-endothelial cell interactions and protects against ischemia-reperfusion injury in rats. J Clin Invest 99**:** 684-691.

Li J, Loukili N, Rosenblatt-Velin N, Pacher P, Feihl F, Waeber B, et al. (2013). Peroxynitrite is a key mediator of the cardioprotection afforded by ischemic postconditioning in vivo. PLoS One 8**:** e70331.

Majidi M, Kosinski AS, Al-Khatib SM, Lemmert ME, Smolders L, van Weert A, et al. (2009). Reperfusion ventricular arrhythmia 'bursts' predict larger infarct size despite TIMI 3 flow restoration with primary angioplasty for anterior ST-elevation myocardial infarction. Eur Heart J 30**:** 757-764.

Miki T, Itoh T, Sunaga D, Miura T (2012). Effects of diabetes on myocardial infarct size and cardioprotection by preconditioning and postconditioning. Cardiovasc Diabetol 11**:** 67.

Nakata T, Hearse DJ, Curtis MJ (1990). Are reperfusion-induced arrhythmias caused by disinhibition of an arrhythmogenic component of ischemia? J Mol Cell Cardiol 22**:** 843-858.

Ovize M, Baxter GF, Di Lisa F, Ferdinandy P, Garcia-Dorado D, Hausenloy DJ, et al. (2010). Postconditioning and protection from reperfusion injury: where do we stand? Position paper from the Working Group of Cellular Biology of the Heart of the European Society of Cardiology. Cardiovasc Res 87**:** 406-423.

Pabla R, Bland-Ward P, Moore PK, Curtis MJ (1995). An endogenous protectant effect of cardiac cyclic GMP against reperfusion-induced ventricular fibrillation in the rat heart. Br J Pharmacol 116**:** 2923-2930.

Pabla R, Curtis MJ (1996). Endogenous protection against reperfusion-induced ventricular fibrillation: role of neuronal versus non-neuronal sources of nitric oxide and species dependence in the rat versus rabbit isolated heart. J Mol Cell Cardiol 28**:** 2097-2110.

Pacher P, Beckman JS, Liaudet L (2007). Nitric oxide and peroxynitrite in health and disease. Physiol Rev 87**:** 315-424.

Penna C, Cappello S, Mancardi D, Raimondo S, Rastaldo R, Gattullo D, et al. (2006). Post-conditioning reduces infarct size in the isolated rat heart: role of coronary flow and pressure and the nitric oxide/cGMP pathway. Basic Res Cardiol 101**:** 168-179.

Skyschally A, van Caster P, Boengler K, Gres P, Musiolik J, Schilawa D, et al. (2009a). Ischemic postconditioning in pigs: no causal role for RISK activation. Circ Res 104**:** 15-18.

Skyschally A, van Caster P, Iliodromitis EK, Schulz R, Kremastinos DT, Heusch G (2009b). Ischemic postconditioning: experimental models and protocol algorithms. Basic Res Cardiol 104**:** 469-483.

Tosaki A, Balint S, Szekeres L (1988). Pacing and reperfusion induced arrhythmias: protection by slow heart rate in the rat heart. Cardiovasc Res 22**:** 818-825.

Tosaki A, Szekeres L, Hearse DJ (1987). Metoprolol reduces reperfusion-induced fibrillation in the isolated rat heart: protection is secondary to bradycardia. J Cardiovasc Pharmacol 10**:** 489-497.

Tsang A, Hausenloy DJ, Mocanu MM, Yellon DM (2004). Postconditioning: a form of "modified reperfusion" protects the myocardium by activating the phosphatidylinositol 3-kinase-Akt pathway. Circ Res 95**:** 230-232.

Varga ZV, Kupai K, Szucs G, Gaspar R, Paloczi J, Farago N, et al. (2013). MicroRNA-25-dependent up-regulation of NADPH oxidase 4 (NOX4) mediates hypercholesterolemia-induced oxidative/nitrative stress and subsequent dysfunction in the heart. J Mol Cell Cardiol 62**:** 111-121.

Varga ZV, Zvara A, Farago N, Kocsis GF, Pipicz M, Gaspar R, et al. (2014). MicroRNAs associated with ischemia-reperfusion injury and cardioprotection by ischemic pre- and postconditioning: protectomiRs. Am J Physiol Heart Circ Physiol 307**:** H216-227.

Walker MJ, Curtis MJ, Hearse DJ, Campbell RW, Janse MJ, Yellon DM, et al. (1988). The Lambeth Conventions: guidelines for the study of arrhythmias in ischaemia infarction, and reperfusion. Cardiovasc Res 22**:** 447-455.

Xia ZY, Gao J, Ancharaz AK (2009). Protective effect of ischemic postconditioning on lung ischemia-reperfusion injury in rats and the role of heme oxygenase-1. Chin J Traumatol 12**:** 162-166.

Yamada M, Hearse DJ, Curtis MJ (1990). Reperfusion and readmission of oxygen. Pathophysiological relevance of oxygen-derived free radicals to arrhythmogenesis. Circ Res 67**:** 1211-1224.

Yang XM, Proctor JB, Cui L, Krieg T, Downey JM, Cohen MV (2004). Multiple, brief coronary occlusions during early reperfusion protect rabbit hearts by targeting cell signaling pathways. J Am Coll Cardiol 44**:** 1103-1110.

Yasmin W, Strynadka KD, Schulz R (1997). Generation of peroxynitrite contributes to ischemia-reperfusion injury in isolated rat hearts. Cardiovasc Res 33**:** 422-432.

Yellon DM, Hausenloy DJ (2007). Myocardial reperfusion injury. N Engl J Med 357**:** 1121-1135.

Zeng Z, Huang HF, Chen MQ, Song F, Zhang YJ (2011). Contributions of heme oxygenase-1 in postconditioning-protected ischemia-reperfusion injury in rat liver transplantation. Transplant Proc 43**:** 2517-2523.

Zhao ZQ, Corvera JS, Halkos ME, Kerendi F, Wang NP, Guyton RA, et al. (2003). Inhibition of myocardial injury by ischemic postconditioning during reperfusion: comparison with ischemic preconditioning. Am J Physiol Heart Circ Physiol 285**:** H579-588.

Table 1. Morphological and *ex vivo* haemodynamic parameters.

	I/R	**IPost**	**RVP**
Animal weight (g)	367 ± 8	358 ± 10	345 ± 10
Heart wet weight (g)	1.28 ± 0.03	1.22 ± 0.04	1.30 ± 0.06
Basal heart rate (bpm)	301 ± 11	291 ± 12	304 ± 8
Coronary flow ($mL \cdot min^{-1}$)			
Before ischaemia	18.8 ± 1.5	16.7 ± 1.2	18.7 ± 1.1
Beginning of ischaemia[a]	10.7 ± 1.0	9.0 ± 0.8	11.5 ± 1.0
Beginning of reperfusion[b]	16.5 ± 1.0	8.7 ± 0.6*	17.9 ± 0.7
End of reperfusion	11.5 ± 1.5	9.9 ± 0.9	11.8 ± 1.5

[a] regional ischaemia

[b] 6 x 10 s global ischaemia was applied to induce IPost in the first 2 min of reperfusion. Coronary flow was measured by collecting coronary effluent for 2 min and then was expressed as $mL \cdot min^{-1}$.

Results are expressed as mean ± S.E.M. *$p < 0.05$ vs. I/R and RVP, one-way ANOVA.

I/R: ischaemia/reperfusion control, IPost: ischaemic postconditioning, RVP: rapid ventricular pacing

Figure legends

Figure 1. *Duration of reperfusion-induced ventricular tachycardia and/or fibrillation is associated with decreased infarct size: a meta-analysis.*

Flow chart of the meta-analysis *(A)* indicates that reperfusion-induced tachyarrhythmias and infarct size data from our previous studies on isolated rat hearts subjected to 30 min regional ischaemia and 120 min reperfusion were analysed in three separate ways considering the duration of either ventricular tachycardia (VT), ventricular fibrillation (VF) or both in the first 10 min of reperfusion. Results of the meta-analysis shows infarct size normalised to area at risk (*B*) and area at risk (*C*) in the presence of shorter (<60 s) or longer (>60 s) total durations of VT, VF, or VT+VF, respectively. Values are expressed as mean ± S.E.M. $*p < 0.05$ vs. corresponding <60 s groups, unpaired t-test.

Figure 2. *Rapid ventricular pacing reduces post-ischaemic LDH release and infarct size.*

Experimental protocol *(A)*, post-ischaemic LDH release *(B)*, infarct size normalised to area at risk (*C*), area at risk (*D*). Hearts were subjected to 15 min equilibration period, followed by 30 min regional ischaemia and 120 min reperfusion. Ischaemic postconditioning was induced by 6x10-s/10-s cycles of reperfusion/no-flow global ischaemia. In the rapid ventricular pacing group, the autonomic rhythm of the hearts was replaced by 10-s pacing period (600 bpm; 10 Hz) in 6 alternating cycles at the onset of reperfusion. Coronary effluent was collected during the first 5 min of reperfusion for LDH activity determination (n = 5 in each group), the measured activities were multiplied by the corresponding coronary flow to give LDH release. Infarct size was measured at the end of reperfusion (n = 12 in each group). Values are expressed as mean ± S.E.M. $*p < 0.05$ vs. I/R, one-way ANOVA.

Figure 3. *Rapid ventricular pacing attenuates reperfusion induced arrhythmias.*

Incidence of reperfusion-induced ventricular tachycardia *(A)* and fibrillation *(B)* are shown. $*p < 0.05$ vs. I/R, Fisher's exact test-. I/R: ischaemia/reperfusion control, IPost: ischaemic postconditioning, RVP: rapid ventricular pacing. VT = ventricular tachycardia, VF = ventricular fibrillation.

Figure 4. *Postconditioning by rapid ventricular pacing enhances formation of peroxynitrite and superoxide anion, effects on possible downstream targets.*

Experimental protocol *(A)*, level of free cardiac 3-nitrotyrosine *(B)*, representative images of *in situ* superoxide detection (*C*), quantification of *in situ* superoxide anion level *(D)*, representative images *(E)*, and quantification *(F)* of western blots of possible downstream targets. Hearts were subjected to 15 min equilibration period, followed by 30 min regional ischaemia and 7 min reperfusion with or without ischaemic postconditioning or rapid ventricular pacing. At the end of reperfusion myocardial samples were taken from the ischaemic zone of the left ventricle for biochemical analysis. The peroxynitrite marker, 3-nitrotyrosine was quantified by ELISA (n = 5 in each group). Transverse cardiac sections from three hearts per group were used for *in situ* detection of superoxide anion (n = 60 random images in each group). Activation of RISK (Akt, Erk1/2) and SAFE (Stat3) pathways as well as protein level of HO-1 was assessed by western blot. Values are expressed as mean ± S.E.M. $*p < 0.05$ vs. I/R, one-way ANOVA. p-Akt: phospho(Ser473)-Akt, p-Erk1: phospho(Thr202)-Erk1, p-Erk2: phospho(Tyr204)-Erk2, p-Stat3: phospho(Tyr705)-Stat3, HO-1: haem oxygenase 1, GAPDH: glyceraldehyde 3-phosphate dehydrogenase

Figure 5. *Postconditioning manoeuvres without a preceding index ischaemia enhances peroxynitrite formation, effects on possible downstream targets.*

Experimental protocol (A), level of free cardiac 3-nitrotyrosine *(B)*, and representative images *(C)* and quantification *(D)* of western blots of possible downstream targets. After 45 min normoxic perfusion, repeated (6 x 10/10-s) brief cycles of no-flow global ischaemia/reperfusion (n = 7) or rapid ventricular pacing at 600 bpm/spontaneous rhythm of the hearts (n = 8) were applied followed by 5 min perfusion. In the normoxic perfusion control group (n = 8), hearts were perfused for 52 min. At the end of perfusion, cardiac free 3-nitrotyrosine level was determined by ELISA and activation of RISK and SAFE pathways were examined by western blot. Values are expressed as mean ± S.E.M. $*p < 0.05$ vs. normoxic perfusion control, one-way ANOVA. I/R: ischaemia/reperfusion, RVP: rapid ventricular pacing. p-Akt: phospho(Ser473)-Akt, p-Erk1: phospho(Thr202)-Erk1, p-Erk2: phospho(Tyr204)-Erk2, p-Stat3: phospho(Tyr705)-Stat3, GAPDH: glyceraldehyde 3-phosphate dehydrogenase

Figure 1.

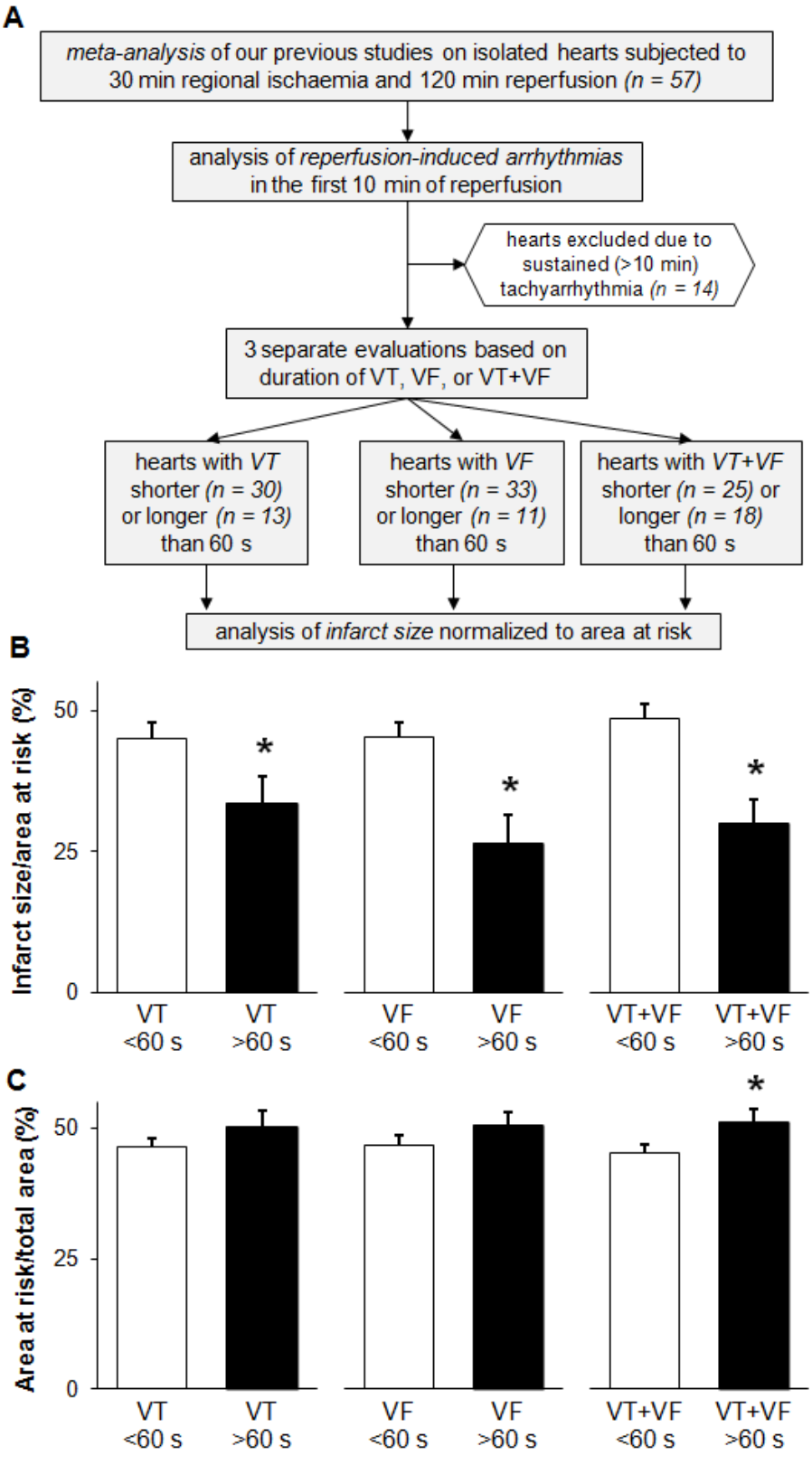

A
meta-analysis of our previous studies on isolated hearts subjected to 30 min regional ischaemia and 120 min reperfusion (n = 57)
analysis of reperfusion-induced arrhythmias in the first 10 min of reperfusion
hearts excluded due to sustained (>10 min) tachyarrhythmia (n = 14)
3 separate evaluations based on duration of VT, VF, or VT+VF
hearts with VT shorter (n = 30) or longer (n = 13) than 60 s
hearts with VF shorter (n = 33) or longer (n = 11) than 60 s
hearts with VT+VF shorter (n = 25) or longer (n = 18) than 60 s
analysis of infarct size normalized to area at risk
B
Infarct size/area at risk (%)
50
25
0
*
VT <60 s
VT >60 s
VF <60 s
VF >60 s
VT+VF <60 s
VT+VF >60 s
C
Area at risk/total area (%)
50
25
0
*
VT <60 s
VT >60 s
VF <60 s
VF >60 s
VT+VF <60 s
VT+VF >60 s

Figure 2.

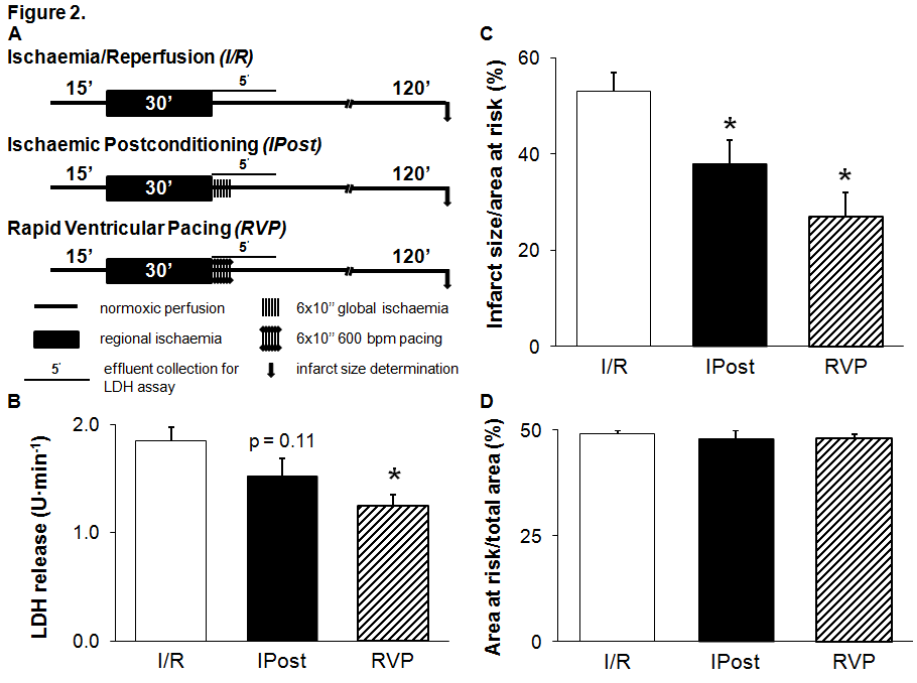


Figure 3.

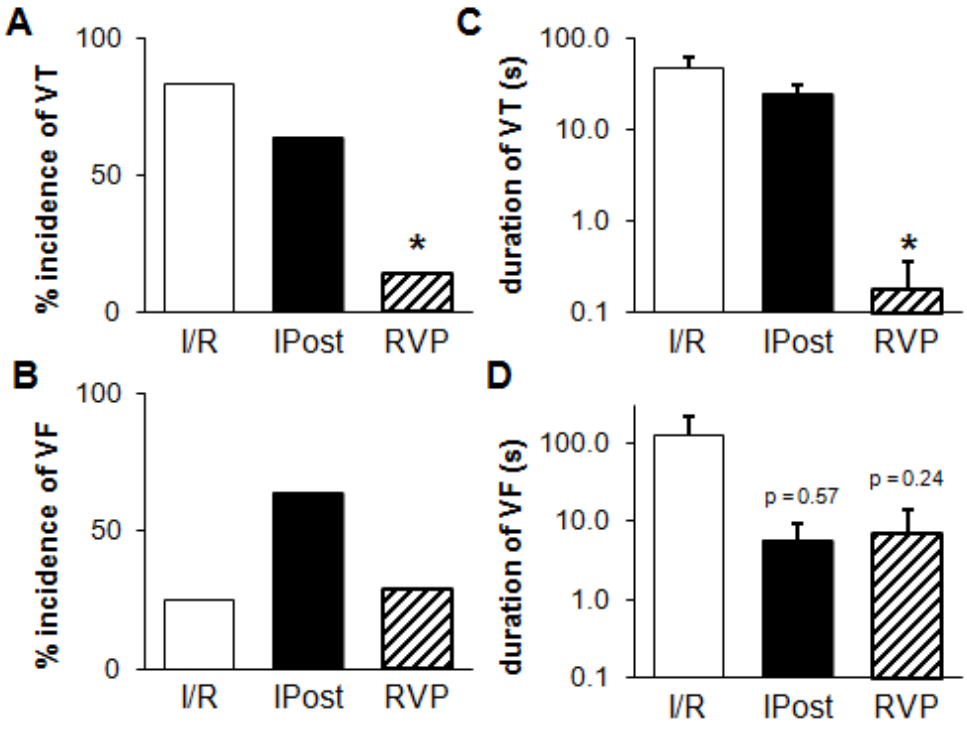


Figure 4.

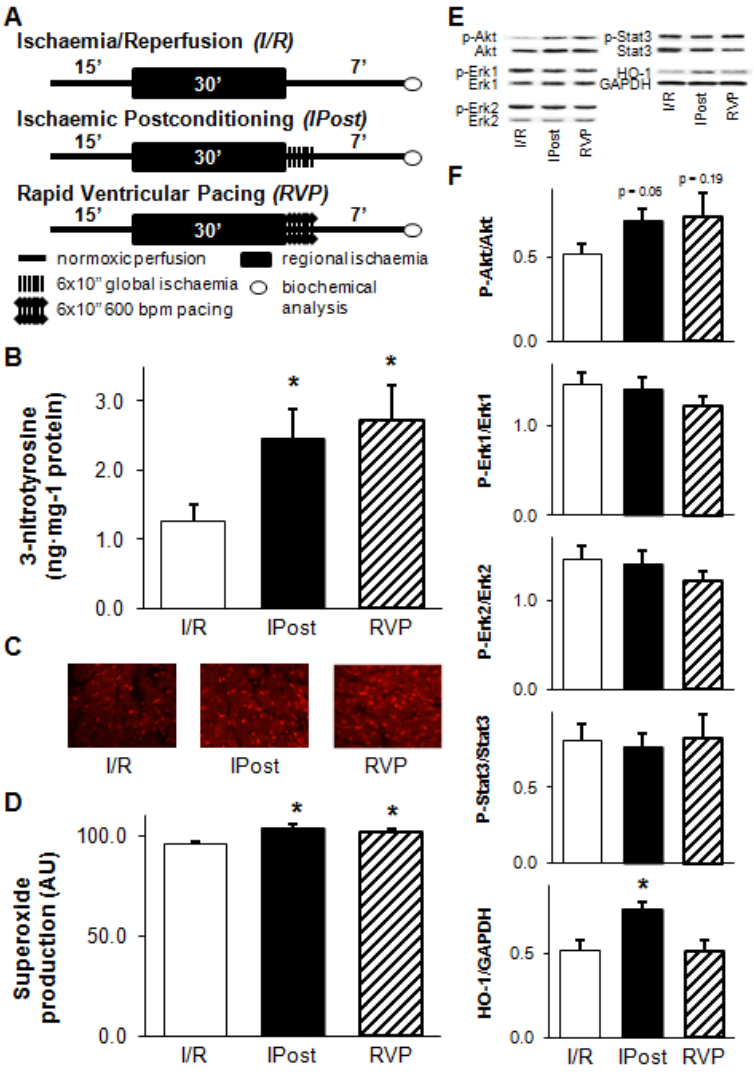
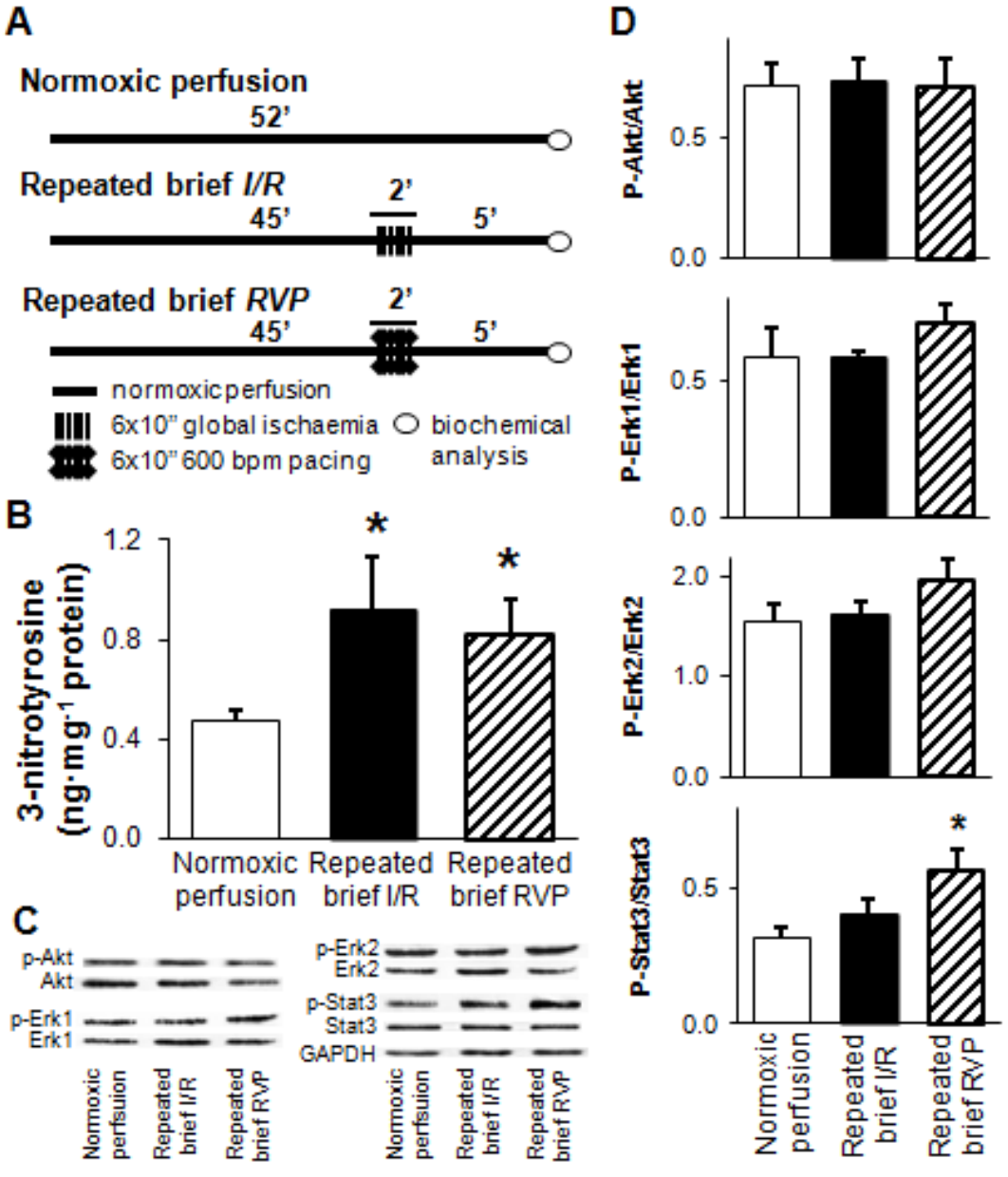

A
Ischaemia/Reperfusion (I/R)
15'
30'
7'
Ischaemic Postconditioning (IPost)
15'
30'
7'
Rapid Ventricular Pacing (RVP)
15'
30'
7'
normoxic perfusion
regional ischaemia
6x10" global ischaemia
biochemical analysis
6x10" 600 bpm pacing
B
3-nitrotyrosine (ng·mg-1 protein)
3.0
2.0
1.0
0.0
I/R
IPost
RVP
C
I/R
IPost
RVP
D
Superoxide production (AU)
100.0
50.0
0.0
I/R
IPost
RVP
E
p-Akt
Akt
p-Erk1
Erk1
p-Erk2
Erk2
p-Stat3
Stat3
HO-1
GAPDH
I/R
IPost
RVP
F
P-Akt/Akt
p = 0.06
p = 0.19
0.5
0.0
P-Erk1/Erk1
1.0
0.0
P-Erk2/Erk2
1.0
0.0
P-Stat3/Stat3
0.5
0.0
HO-1/GAPDH
0.5
0.0
I/R
IPost
RVP

Figure 5.


A
Normoxic perfusion
52'
Repeated brief I/R
2'
45'
5'
Repeated brief RVP
2'
45'
5'
normoxic perfusion
6x10" global ischaemia
6x10" 600 bpm pacing
biochemical analysis
B
3-nitrotyrosine (ng·mg-1 protein)
1.2
0.8
0.4
0.0
*
*
Normoxic perfusion
Repeated brief I/R
Repeated brief RVP
C
p-Akt
Akt
p-Erk1
Erk1
p-Erk2
Erk2
p-Stat3
Stat3
GAPDH
Normoxic perfsuion
Repeated brief I/R
Repeated brief RVP
D
P-Akt/Akt
P-Erk1/Erk1
P-Erk2/Erk2
P-Stat3/Stat3
0.5
0.0
2.0
1.0
*
Normoxic perfusion
Repeated brief I/R
Repeated brief RVP