Is Postconditioning Effective in Prevention against Long-Lasting Myocardial Ischemia in the Rabbit?

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Summary

The goal of the study was to determine whether postconditioning protects against different ischemia durations in the rabbit. Rabbits were assigned to a 20-, 25-, 45- or 60-min coronary occlusion followed by 24-h of reperfusion. Rabbits received no further intervention (control) or were postconditioned with four cycles of 30-s occlusion and 30-s reperfusion after myocardial infarction. Plasma levels of troponin I were quantified throughout reperfusion. In control conditions, infarct sizes (% area at risk using triphenyltetrazolium chloride) after 20, 25, 45 and 60 min of coronary occlusions were 23±3, 51±4, 70±3 and 81±3 %, respectively. With 20 and 25 min occlusion, postconditioning reduced infarct size by 43±10 and 73±21 %, respectively. On the other hand, with 45 or 60 min occlusion, postconditioning had no significant effects on infarct size (61±3 and 80±2 % of area at risk). Preconditioning protocol was performed with 25- and 60-min coronary occlusion. As expected, preconditioning significantly reduced infarct size. In conclusion, in the rabbit, the cardioprotection afforded by postconditioning is limited to less than 45 min coronary occlusion.

Key words

Postconditioning • Preconditioning • Ischemia-reperfusion

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Introduction

Recently, a novel endogenous mechanism of cardioprotection called postconditioning has been described with very promising results (Zhao *et al.* 2003, Yang *et al.* 2004, Vinten-Johansen *et al.* 2005). Briefly, very short cycles (10-30 s) of ischemia and reperfusion, applied immediately after sustained ischemia, are able to limit the infarct size. To date, cardioprotection by postconditioning has been reported in several species, including the dog (Zhao *et al.* 2003, Halkos *et al.* 2004, Couvreur et al. 2006), rabbit (Yang *et al.* 2004, Philipp *et al.* 2006, Tissier *et al.* 2007), rat (Kin *et al.* 2004, Kerendi *et al.* 2005, Tang *et al.* 2006), and mouse (Kin *et al.* 2005, Heusch *et al.* 2006). On the other hand, Schwartz and Lagranha (2006) reported that postconditioning failed to prevent myocardial ischemia in pigs. On the contrary, Iliodromitis *et al.* (2006a) have shown that the postconditioning confers protection in pigs but requires more than four ischemia-reperfusion cycles.

In a recent landmark clinical study, Staat *et al.* (2005) postconditioned the human myocardium in patients undergoing primary percutaneous coronary intervention by repetitively inflating and deflating an angioplasty balloon within minutes of stenting. Using simple enzyme release as an index of infarction, they found that infarct size was significantly reduced by onethird, confirming the relevance of postconditioning in human.

Recently, Tang *et al.* (2006) have shown that the cardioprotection afforded by postconditioning is limited to mild to moderate myocardial injury in the rat. Indeed, the majority of postconditioning studies usually used animal models with mild to moderate degrees of ischemic injury. In other respects, a very recent study demonstrated that postconditioning may be deleterious when the ischemia duration was relatively short [15 or 30 min coronary artery occlusion (Manintveld *et al.* 2007)].

Thus, the specific aim of the present study was

to determine whether postconditioning protects against different ischemia durations in the rabbit. A second goal was to investigate whether postconditioning differs from preconditioning. Ischemic preconditioning is clearly one of the most reproducible and powerful cardioprotective maneuvers for reducing experimental myocardial infarct size (Murry *et al.* 1986, Ferdinandy *et al.* 2007) and it still affords a cardioprotection against long-lasting myocardial injury (Ferdinandy *et al.* 2007).

In our model, 24-h reperfusion was allowed before myocardial infarct size was measured. Indeed the duration of reperfusion is a key factor for a precise, robust determination of myocardial infarct size (Bohle *et al.* 1991, Tissier *et al.* 2002).

Methods

Animals were housed and tested in an Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC)-accredited facility in strict compliance with all applicable regulations. The protocol was carried out in compliance with French regulations and with local Ethical Committee guidelines for animal research.

Surgical preparation

New Zealand white male rabbits weighing between 2.0-2.4 kg were anesthetized with an intraperitoneal injection of ketamine (10-15 mg/kg) and xylazine (7 mg/kg), intubated with an endotracheal tube and mechanically ventilated with 100 % oxygen (at ~ 40 breaths per minute with tidal volume 10 ml/kg). A heating pad maintained rectal temperature between 38.5 and 39.5 °C. Arterial pH, pO_2, and pCO_2 were measured with blood gas analyzer (ABL 510, Radiometer, Denmark) throughout the experiment and values were maintained within a normal physiological range. Polyethylene catheter filled with saline was introduced in the right carotid artery to monitor arterial blood pressure and for blood sampling. An external electrocardiogram (ECG) was also recorded. The chest was opened *via* a left thoracotomy in the fourth intercostal space and the beating heart was exposed. After pericardium opening, a 4/0 silk thread was passed beneath a major branch of the left coronary artery. The ends of the ligature were passed through a short segment of propylene tubing to form a snare. Ischemia was induced by pulling the snare. The successful induction of ischemia was verified by ST segment elevation on the ECG and by visual inspection (cyanosis) of the heart. Reperfusion was

induced by releasing the snare and was verified by refilling of the artery. The chest was then closed in layers and a small tube was left in the thorax to evacuate air and fluids after surgery. Analgesia was achieved by using subcutaneous Temgesic administration (0.1 mg/kg).

Experimental protocol

Following stabilization of hemodynamics and blood gases, the rabbits were assigned to a 20-min (n=8), 25-min (n=7), 45-min (n=8) or 60-min (n=8) coronary artery occlusion. In each group, rabbits received no further intervention (control) or were postconditioned (n=7-8) with four cycles of 30-s reperfusion and 30-s occlusion immediately after myocardial infarction (Darling *et al.* 2005, Couvreur *et al.* 2006, Philipp *et al.* 2006, Tessier-Vetzel *et al.* 2006). A sham procedure, i.e. without ischemia was performed in an additional group of animals (n=4). Finally, to determine the effects of preconditioning on myocardial infarction two groups have been added. Two cycles of 5-min coronary artery occlusion and 10-min reperfusion were performed 10 min before 25-min (n=8) and 60-min (n=6) coronary occlusion. All animals underwent 24-h reperfusion. No anti-arrhythmic agents were given at any time.

Measurement of area at risk and infarct size

After 24-h reperfusion, animals were killed by sodium pentobarbital (160 mg/kg, i.p.) and the heart was rapidly excised. The ascending aorta was cannulated and infused retrogradely with saline followed by Evans blue (0.4 %) after ligation of the previously occluded artery (the original occlusive suture was left in place). The left ventricle was cut into 10-12 slices which were weighed and then incubated with 1 % 2, 3, 5-triphenyltetrazolium chloride at 37 °C for 10 min. Slices were fixed in 10 % formaldehyde and then photographed with a digital camera. Using a computerized planimetric program (Leica, Microsystems Imaging Solutions, Cambridge, UK) the area at risk and the infarcted zones were quantified. The area at risk was identified as the non-blue region and was expressed as percentage of the left ventricle weight. Infarcted area was identified as the triphenyltetrazolium-unstained tissue and was expressed as percentage of the area at risk.

Measurement of plasma levels of troponin I

Six blood samples were obtained: 5 min before coronary artery occlusion, and at 5 min, 30 min, 1 h, 2 h and 24 h reperfusion. The quantitative determination of

Table 1. Body weight, heart weight and area at risk.

Group	n	Body weight (kg)	Heart weight (g)	Area at risk / Left ventricle (%)
Sham	4	2.19 ± 0.05	5.9 ± 0.2	47 ± 3
Control 20	8	2.21 ± 0.04	5.8 ± 0.2	48 ± 3
PostC 20	7	2.27 ± 0.03	5.8 ± 0.1	53 ± 7
Control 25	7	2.35 ± 0.05	5.8 ± 0.3	52 ± 4
PostC 25	8	2.29 ± 0.07	5.5 ± 0.2	43 ± 3
PreC 25	8	2.29 ± 0.03	6.0 ± 0.1	54 ± 2
Control 45	8	2.19 ± 0.03	5.6 ± 0.1	55 ± 4
PostC 45	8	2.26 ± 0.03	5.8 ± 0.2	52 ± 5
Control 60	8	2.26 ± 0.03	6.0 ± 0.1	57 ± 2
PostC 60	8	2.26 ± 0.01	5.6 ± 0.2	54 ± 5
PreC60	6	2.33 ± 0.02	6.1 ± 0.2	54 ± 4

Values are means ± S.E.M. Control: coronary artery occlusion followed by reperfusion without postconditioning. 20, 25, 45, 60: duration of coronary artery occlusion (min). PostC: coronary artery occlusion followed by a postconditioning protocol of four 30-s occlusion / 30-s reperfusion cycles at the onset of reperfusion. PreC: two 5-min occlusion / 10-min reperfusion cycles before 25- and 60-min coronary artery occlusion.

troponin I in rabbit plasma was preformed by AxSYM System (Abbot Laboratories, Abbot Park, IL, USA) in a medical analysis laboratory. The AxSYM troponin I (3C29-20) assay was based on microparticle enzyme immunoassay technology. The analytical sensitivity of the AxSYM troponin I assay was given as 0.3 μg/l at the 95 % confidence level (n=29).

Measured parameters

Arterial pressure and heart rate were recorded before ischemia (baseline) during ischemia and up to 30 min reperfusion. Arterial pressure and ECG signals were digitized (500 Hz for blood pressure and 1000 Hz for ECG), simultaneously recorded and analyzed online by interactive software (Notocord-hem Evolution, Notocord Systems, Croissy sur Seine, France).

Statistical analysis

All values are reported as means ± S.E.M. Infarct size data were analyzed using two-way (ischemia time x treatment) ANOVA followed by *post-hoc* testing with Dunnett's test. Heart rate and arterial pressure were analyzed using two-way (time x treatment) ANOVA for repeated measures followed by Dunnett's test. Finally, troponin I data were analyzed using three-way ANOVA (ischemia time x treatment x time) followed by Dunnett's test. The relationships between infarct size and risk region size were compared among groups with an

analysis of covariance (ANCOVA) and were assessed by linear regression analysis using the least-squares method. ANOVA was performed using Sigma Stat version 2.03 and ANCOVA with SAS version 9.1. A probability of $P<0.05$ was considered statistically significant.

Results

A total of eighty rabbits were included in the present study. Body and heart weights and the sizes of area at risk are reported in Table 1. All values were similar among groups.

Hemodynamics

Hemodynamic data are summarized in Table 2. There were no significant differences between controls, preconditioning and postconditioning groups for any hemodynamic variable: heart rate and mean arterial pressure whatever the time of measurement (baseline, end or middle of ischemia and 30 min reperfusion). On the other hand, after 20 min ischemia, mean arterial pressure dropped in all groups, this hypotension was sustained after 30 min reperfusion. Heart rate increased in all groups during ischemia and was normalized after 30 min reperfusion.

Infarct size

As expected, no myocardial infarction (0.5±0.3 %

Table 2. Hemodynamic variables during the course of the experiment.

Variables	Groups	Baseline	20-min ischemia	30-min reperfusion
MAP	Sham	82 ± 3	74 ± 8	61 ± 5 *
MAP	Control 20	83 ± 2	60 ± 3 *	58 ± 2 *
MAP	Control 25	85 ± 2	56 ± 2 *	51 ± 2 *
MAP	Control 45	84 ± 3	58 ± 2 *	47 ± 2 *
MAP	Control 60	93 ± 3	65 ± 2 *	53 ± 2 *
MAP	PostC 20	80 ± 3	61 ± 3 *	57 ± 2 *
MAP	PostC 25	80 ± 4	63 ± 4 *	54 ± 2 *
MAP	PostC 45	84 ± 4	65 ± 4 *	54 ± 4 *
MAP	PostC 60	89 ± 3	66 ± 3 *	50 ± 2 *
MAP	PreC 25	88 ± 2	57 ± 2 *	51 ± 2 *
MAP	PreC 60	89 ± 3	63 ± 3 *	53 ± 4 *
HR	Sham	150 ± 10	127 ± 7	136 ± 7
HR	Control 20	135 ± 3	151 ± 8 *	122 ± 8
HR	Control 25	135 ± 4	166 ± 9 *	139 ± 10
HR	Control 45	144 ± 5	168 ± 6 *	142 ± 8
HR	Control 60	161 ± 4	202 ± 15 *	178 ± 9
HR	PostC 20	136 ± 9	161 ± 10 *	134 ± 7
HR	PostC 25	133 ± 6	145 ± 7 *	135 ± 11
HR	PostC 45	154 ± 7	162 ± 7 *	147 ± 9
HR	PostC 60	157 ± 7	192 ± 16 *	165 ± 9
HR	PreC 25	153 ± 6	172 ± 11 *	161 ± 6
HR	PreC 60	158 ± 2	154 ± 6	160 ± 9

Values are means ± S.E.M. Control: coronary artery occlusion followed by reperfusion without postconditioning. 20, 25, 45, 60: duration of coronary artery occlusion (min). PostC: coronary artery occlusion followed by a postconditioning protocol of four 30-s occlusion / 30-s reperfusion cycles at the onset of reperfusion. PreC: two 5-min occlusion / 10-min reperfusion cycles before 25- and 60-min coronary artery occlusion. HR: heart rate (bpm); MAP: mean arterial pressure (mmHg). * $P < 0.05$ vs. baseline.

of area at risk) was detected in sham-operated animals. The average infarct size, expressed as a percentage of the region at risk were 22.6±3.3, 50.7±4.4, 70.1±3.0 and 81.0±3.0 %, respectively with 20-, 25-, 45- or 60-min of coronary artery occlusion followed by 24-h reperfusion (Fig. 1). Preconditioning resulted in a 44±7 and 16±6 % reduction in myocardial infarct size with 25-min and 60-min coronary occlusion, respectively (Fig. 1). Postconditioning significantly reduced infarct size with only 12.8±2.5 and 34.2±4.0 % of the region at risk, respectively for coronary artery occlusion duration of 20 and 25 min. However, with a longer coronary artery occlusion duration (45 and 60 min), postconditioning had no significant cardioprotective effect compared to corresponding controls, since infarct size represented 61.2±3.0 and 79.8±2.4 % of region at risk, respectively. Thus, postconditioning reduced infarct size by 43±11, 33±8, 13±4 and 2±3 %, respectively for ischemia duration 20-, 25-, 45- and 60 min.

In all groups, the size of the infarction was positively and linearly related to the size of the region at risk (r=0.20; 0.36; 0.89; 0.69; 0.93; 0.85; 0.98; 0.98), respectively in groups control 20-min, postconditioning 20-min, control 25-min, postconditioning 25-min, control 45-min, postconditioning 45-min, control 60-min, postconditioning 60-min. As shown in Figure 2, the regression lines were significantly shifted downward and to the right by postconditioning for 20-min and 25-min ischemia, respectively (for both groups, P<0.05 by ANCOVA), indicating that for each given size of region at risk, the resulting infarction was smaller in the hearts of the postconditioned groups. In contrast, in postconditioning 45-min and 60-min group, the regression lines were not significantly shifted compared to control 45-min and 60-min.
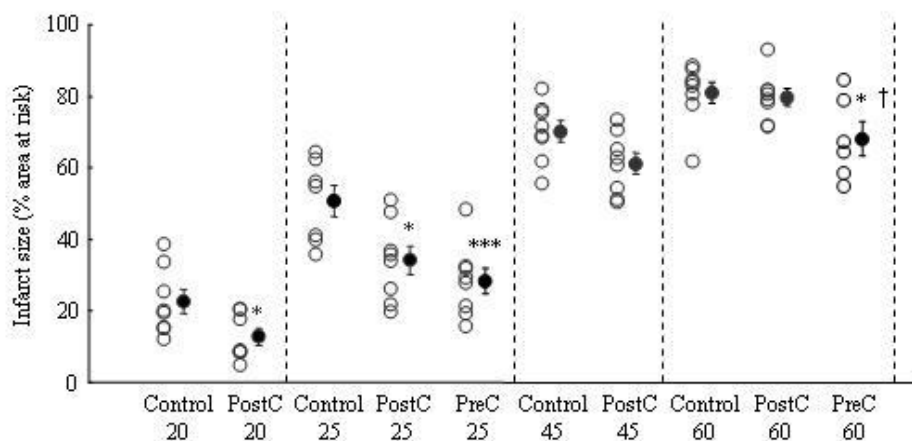
Fig. 1. Effects of postconditioning (with four cycles of 30-s occlusion and 30-s reperfusion) and preconditioning (with two 5-min occlusion and 10-min reperfusion) on infarct size (percentage of area at risk) produced by coronary artery occlusion of different durations and 24-h reperfusion. Open circles represent individual experiments and closed circles depict group means with S.E.M. * P<0.05 vs. corresponding control, † P<0.05 vs. postconditioning.
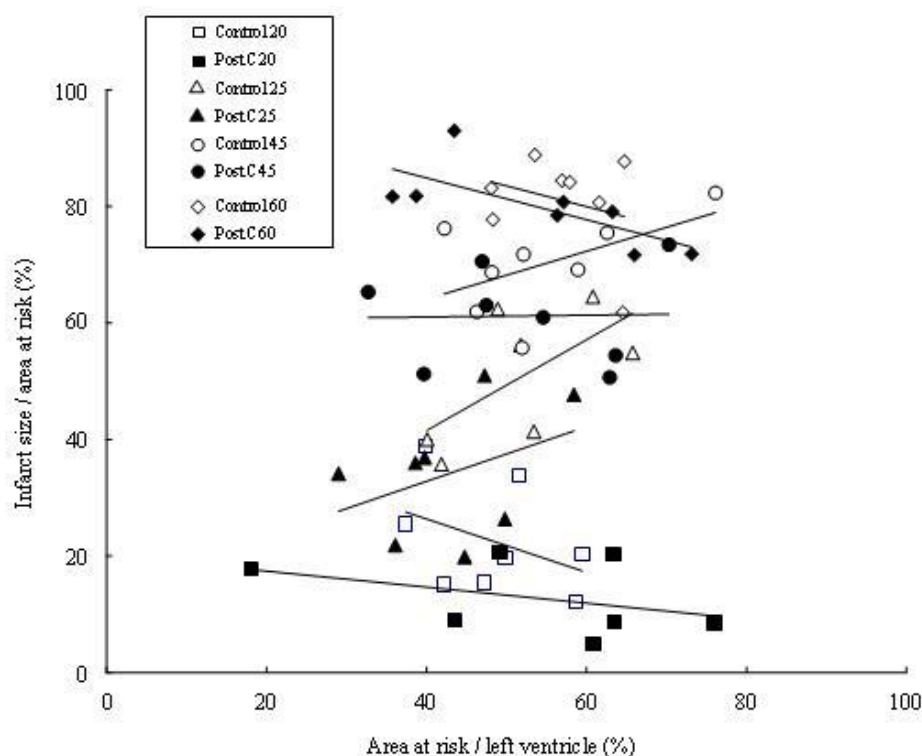

Fig. 2. Relationship between the infarct size / the area at risk (%) and the area at risk / left ventricle (%) in rabbits exposed to a 20-min (square), 25-min (triangle), 45-min (circle) or 60-min (diamond) coronary artery occlusion without further intervention (empty symbol) and with postconditioning protocol (full symbol). Both individual values and the regression lines obtained by linear regression analysis are represented. The linear regression equations were as follows: control 20, y=0.10 x +157 (r=0.20); postconditioning 20, y=0.07 x + 72 (r=0.36); control 25, y=0.90 x -501 (r=0.89); postconditioning 25, y=0.64 x − 325 (r=0.69); control 45, y=0.99 x −415 (r=0.93); post-conditioning 45, y=0.62 x − 9.4 (r=0.85); control 60, y=0.89 x +345 (r=0.98); postconditioning 60, y=1.53 x − 237 (r=0.98).

Plasma levels of troponin I

For all groups, from 5 min up to 24 h reperfusion, plasma troponin I levels significantly increased compared to initial values and were significantly higher than in sham-operated animals (data not shown). Maximal plasma troponin I levels appear at 2 h reperfusion for each group (Fig. 3). In groups with 20 min coronary artery occlusion, plasma troponin I levels only reached 43±10 μg/l after 2 h reperfusion in control conditions and surprisingly postconditioning failed to affect this biomarker, 49±9 μg/l (even if the area under the curves for troponin I tended to be lower in postconditioned group – 42988±10865 in vehicle group compared to 30730±7786 in postconditioned group). On the other hand with 25 min coronary artery occlusion, postconditioning significantly reduced the plasma troponin I level, 186±37 versus 73±21 μg/l (P<0.05). With a longer coronary artery occlusion duration, postconditioning was devoid of significant effect on plasma levels of troponin I – 263±42 vs. 209±38 μg/l with 45 min occlusion and 436±64 vs. 377±63 μg/l with 60 min (Fig. 3). As expected, preconditioning significantly reduced the plasma troponin I level, 90±17 μg/l with 25-min coronary occlusion (P<0.05, vs. 186±37 μg/l in control conditions) and tended to decrease this plasma level of this marker with 60-min coronary occlusion – 329±55 μg/l vs. 436±64 μg/l in control conditions (data not shown).
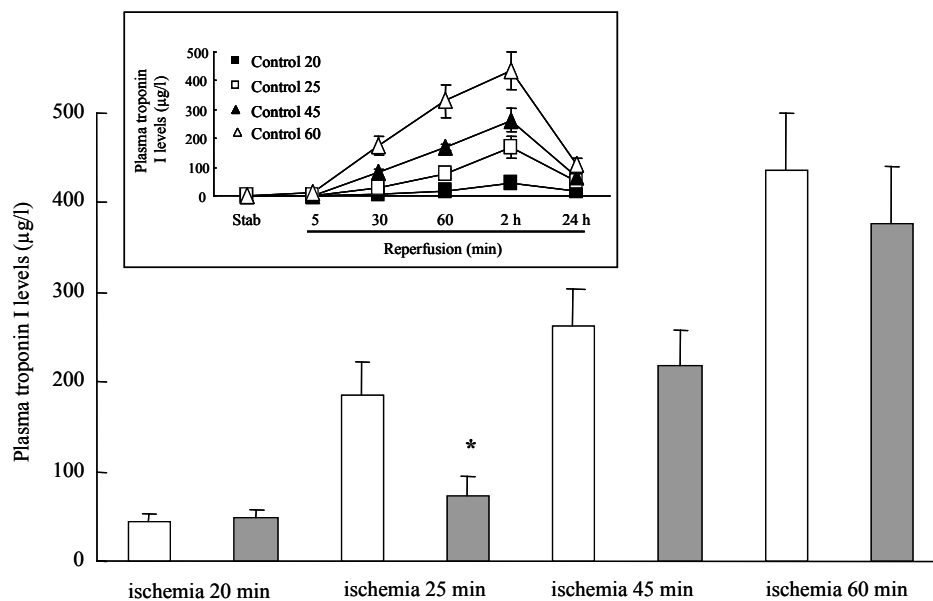
Fig. 3. Effects of postconditioning (grey columns) with four cycles of 30-s occlusion and 30-s reperfusion on plasma troponin I levels at 2-h reperfusion after coronary artery occlusion of different durations. Inset: time-course of the plasma troponin I levels during 24-h reperfusion in control groups. Data are means ± S.E.M. * P<0.05 vs. corresponding control (white columns).

Discussion

The results of the current investigation demonstrate that the cardioprotective effects of postconditioning in the rabbit are dependent on the coronary artery occlusion duration. A sequence of four cycles of 30-s coronary artery occlusion and 30-s reperfusion immediately after 20-min or 25-min ischemia markedly reduces infarct size, demonstrating cardioprotective effects on postconditioning. On the other hand, the absence of protection after 45 min coronary artery occlusion clearly suggests that postconditioning protects only against the myocardial injury caused by coronary occlusions shorter than 45 min.

In this study, myocardial infarction was quantified in rabbits by measuring firstly changes in plasma levels of the selective biochemical marker during reperfusion and secondly infarct size assessed by tetrazolium staining. It clearly appears from the literature that the duration of reperfusion is a key factor for a precise, robust determination of myocardial infarct size (Bohle *et al.* 1991, Tissier *et al.* 2002). Consequently, in our study, 24-h reperfusion was allowed before quantification of myocardial infarct size. Under these conditions, the present results demonstrate that myocardial infarct size increases progressively with the ischemic duration from 23 % of the region at risk after 20 min occlusion to 80 % after 60 min coronary occlusion.

Cardiac troponin I is often considered to be the "gold standard" biochemical test for the diagnosis of myocardial damage (Collinson *et al.* 2001, Hamm 2001).

Despite the presence of troponin in skeletal muscle, the myocardium contains cardiac troponin I and T isoforms that are not present in skeletal muscles, thus it is considered as a biomarker with a high specificity for cardiac tissue (Solaro and Rarick 1998). The importance of troponin I for the diagnosis of myocardial infarction comes from the fact that it may have higher absolute cardiac specificity than troponin T (Vallins *et al.* 1990). In the present study troponin I levels were significantly increased from 5 min to 24 h reperfusion. Postconditioning markedly reduced plasma levels of troponin I for a coronary artery occlusion duration of 25 min. To our knowledge, this is the first *in vivo* demonstration that postconditioning also protects against troponin I release. Indeed, Bopassa *et al.* (2006) demonstrated that postconditioning reduced troponin I release but in an isolated rat heart model. For 20 or 25 min coronary artery occlusion duration, postconditioning shifted downward the relationship between myocardial necrosis and plasma levels of troponin I, thus surprisingly with a postconditioning protocol the infarct size reduction appears more important than that of troponin I level.

In the present study, it is confirmed that a preconditioning (with 25-min and 60-min coronary occlusion) is capable to reduce the infarct size. For moderate durations of ischemia preconditioning and postconditioning are approximately equally protective.

From 45-min coronary artery occlusion, postconditioning failed to afford cardioprotection. This loss of protection has already been observed in rats by Tang *et al.* (2006). They showed that a postconditioning protocol which reduces infarct size after a 30-min

occlusion does not significantly reduce infarct size or creatine kinase after a 45-min or 60- min occlusion in conscious rats. Consequently, the protection afforded by postconditioning is relatively modest, being observed only when the ischemic insult is shorter than 45 min for at least two species: rats (Tang *et al.* 2006) and rabbits (the present study). Nevertheless, these findings are apparently at odds with those of Yang *et al.* (2004) who reported that in open-chest rabbits, postconditioning was still effective in reducing infarct size after a 45-min occlusion. The divergent results may be explained by the differences in reperfusion duration (3 h vs. 24 h). Interestingly, in hypercholesterolemic rabbits, 30-min coronary artery occlusion induced a larger infarct size (> 55 % of region at risk, higher than in normal rabbits) and in this case, postconditioning exerted no cardioprotective effects (Iliodromitis *et al.* 2006b). Furthermore, our data differ from a study of Manintveld *et al.* (2007) in which it was shown that postconditioning may even aggravate infarct size when applied after a relatively short duration of coronary artery occlusion (15-30 min) in rats. In our study, postconditioning is more effective with the shortest ischemia duration (20 min). These discrepancies could be explained both by the species differences (rat versus rabbit) and by reperfusion duration (2 h vs. 24 h).

The investigation of precise mechanisms of ischemic postconditioning is at an early stage (Ferdinandy *et al.* 2007). Five major mechanistic themes should be considered – the involvement of endogenous adenosine, the role of the NO/cGMP pathway, the involvement of mitochondrial K_{ATP} channels, the activation of reperfusion salvage kinase pathways (notably PI3K/Akt, MAPK/ERK and PKG) and inhibition of mitochondrial permeability transition pore (mPTP) opening at reperfusion. Depending on the species or the algorithm of postconditioning, it is possible for different systems to be more or less involved which could explain different results between studies.

A protection afforded by postconditioning has been reported in several species, but in the pig, more than four ischemia-reperfusion cycles were necessary to afford a cardioprotection (Iliodromitis *et al.* 2006a). Consequently, it would be interesting to investigate in our model a postconditioning protocol with more than four ischemic/reperfusion cycles with ischemia durations less than 45 min. Two aspects of postconditioning algorithm appear to be important, the number of cycles imposed and the duration of each cycle. Furthermore, it would seem that smaller body size requires shorter cycles, while models with larger body size require cycles of longer duration (Vinten-Johansen *et al.* 2005, Ferdinandy *et al.* 2007). In the present model, we have chosen a 30-s algorithm with four cycles, since in rabbits this postconditioning protocol has been reported to be very effective (Darling *et al.* 2005, Couvreur *et al.* 2006, Philipp *et al.* 2006, Tessier-Vetzel *et al.* 2006). The protective effects afforded by postconditioning were very comparable to those observed with ischemic preconditioning (Crisostomo *et al.* 2006). Unlike ischemic preconditioning, postconditioning offers the unique opportunity to be applied in clinical practice, since the episodes of brief ischemia-reperfusion can be performed at the time of reflow during the percutaneous transluminal coronary angioplasty procedure. Staat et al. (2005) demonstrated for the first time that postconditioning could become a much more clinically relevant form of therapy for treatment of acute coronary occlusion and reperfusion, even if our data suggest that the clinical interest of this intervention may be restricted by its limited cardioprotective efficacy.

In conclusion, we demonstrate for the first time that the ischemia duration is a key factor for the cardioprotection afforded by postconditioning in a rabbit model. Thus, a 45-min coronary artery occlusion is not protected by a standard postconditioning procedure. Therefore, the duration of ischemia should be considered for the putative application of postconditioning for clinical setting.

Conflict of Interest
There is no conflict of interest.

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