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The paradigm of postconditioning to protect the heart

C. Penna, D. Mancardi, S. Raimondo, S. Geuna, P. Pagliaro *

Dipartimento di Scienze Cliniche e Biologiche dell'Università di Torino, Orbassano, Torino, Italy

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Abstract

Ischaemic preconditioning limits the damage induced by subsequent ischaemia/reperfusion (I/R). However, preconditioning is of little practical use as the onset of an infarction is usually unpredictable. Recently, it has been shown that the heart can be protected against the extension of I/R injury if brief (10–30 sec.) coronary occlusions are performed just at the beginning of the reperfusion. This procedure has been called *postconditioning* (*PostC*). It can also be elicited at a distant organ, termed *remote PostC*, by intermittent pacing *(dyssynchrony-induced PostC)* and by pharmacological interventions, that is *pharmacological PostC*. In particular, brief applications of intermittent bradykinin or diazoxide at the beginning of reperfusion reproduce PostC protection. PostC reduces the reperfusion-induced injury, blunts oxidant-mediated damages and attenuates the local inflammatory response to reperfusion. PostC induces a reduction of infarct size, apoptosis, endothelial dysfunction and activation, neutrophil adherence and arrhythmias. Whether it reduces stunning is not clear yet. Similar to preconditioning, PostC triggers signalling pathways and activates effectors implicated in other cardioprotective manoeuvres. Adenosine and bradykinin are involved in PostC triggering. PostC triggers survival kinases (RISK), including Aκt and extracellular signal-regulated kinase (ERK). Nitric oxide, *via* nitric oxide synthase and non-enzymatic production, cyclic guanosine monophosphate (cGMP) and protein kinases G (PKG) participate in PostC. PostC-induced protection also involves an early redox-sensitive mechanism, and mitochondrial adenosine-5′-triphosphate (ATP)-sensitive K^+ and PKC activation. Protective pathways activated by PostC appear to converge on mitochondrial permeability transition pores, which are inhibited by acidosis and glycogen synthase kinase-3β (GSK-3β). In conclusion, the first minutes of reperfusion represent a window of opportunity for triggering the aforementioned mediators which will in concert lead to protection against reperfusion injury. Pharmacological PostC and possibly remote PostC may have a promising future in clinical scenario.

Keywords: heart protection • ischaemia • reperfusion injury • postconditioning

*Correspondence to: Dr Pasquale PAGLIARO, MD, PhD,
Dipartimento di Scienze Cliniche e
Biologiche, Università di Torino,
Ospedale S. Luigi, Regione Gonzole,
10043 ORBASSANO, Torino, Italy.
Tel.: +39-11 6705430/5450
Fax: +39-11 9038639
E-mail: pasquale.pagliaro@unito.it

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Introduction

Coronary heart disease is expected to become the leading worldwide cause of death by 2020 [1]; acute myocardial infarction is a major cause of such mortality. In patients with acute myocardial infarction, rapid restoration of blood flow (reperfusion) is the most effective treatment for myocardial salvage, either by thrombolysis or primary percutaneous coronary intervention. However, it is now clear that reperfusion has the potential to induce additional lethal injuries that are not present at the end of the ischaemic period [2, 3].

During late 1980s researchers had already started to investigate whether or not pharmacologically modified reperfusates could reduce reperfusion injury [for review see 4]. Moreover, several studies have examined whether myocardial damage by ischaemia /reperfusion could be limited if reperfusion was initiated in a gentle (low pressure) manner as opposed to standard full flow (high pressure) [5–12]. For instance, Hori and colleagues [7] tested the hypothesis that post-ischaemic stunning could be favourably attenuated if perfusion pressure upon initial relief of ischaemia was increased slowly as opposed to abruptly. Indeed, fractional shortening at 3 hrs post-reperfusion was significantly improved in hearts that received gradual reperfusion *versus* controls [7]. Similarly, in a canine model of coronary bypass, Okamoto *et al*. [5] initiated reperfusion either abruptly with full systemic pressure, or at a low pressure (50 mmHg) for 20 min. before full systemic pressure was restored. Interestingly, initial low-pressure reperfusion was associated with smaller infarct size and improved recovery of regional contractile function [5]. In the 1997 it has been reported that the gradual reperfusion limited the necrosis which followed ischaemia/reperfusion, but was accompanied by an increased leukocyte adherence to the endothelium [10]. The authors of this study suggested that the means by which reperfusion is initiated may play an important role in determining cardiomyocytes survival and, ultimately, clinical outcome. Notably, the concept of gentle or graded reperfusion has not been limited to the heart, and has also been found to be beneficial in other organs [11]. Despite the aforementioned experimental data suggesting that low-pressure reperfusion can limit myocardial ischaemia/reperfusion injury, this strategy has received limited clinical and experimental attention. The reasons of which could be: (1) clinicians are resistant to in induce a marked lowering of blood pressure immediately after restoring coronary patency in the cardiac catheterization laboratory, which could prove to be particularly harmful in patients who are already haemodynamically unstable due to myocardial ischaemia, (2) an adequate training of emergency medical professionals to provide timely intervention is required, (3) graded reperfusion would be difficult to implement safely outside of the controlled conditions of bypass surgery, (4) despite infarct size reduction with graded reperfusion an increase in leukocyte adhesion has been reported [10], and last, but not least (5) most of the aforementioned experimental data were obtained in a historic period in which preconditioning mechanisms were little or not know at all.

Beginning with the knowledge on gradual reperfusion and preconditioning, a few years ago, Vinten-Johansen group [13] introduced the ischaemic Postconditioning (PostC) treatment against ischaemia/reperfusion injury. As ischaemic preconditioning, ischaemic PostC consists of brief periods of ischaemia reperfusion. PostC, in fact, consists of brief periods of reperfusions alternating with brief re-occlusion applied during the very early minutes of a reperfusion.

Emerging data are now showing that the mechanical interventions of ischaemic PostC may activate multiple and interacting components which can protect the heart against myocardial injury. It seems that part of the protective effects are due to fact that an abrupt reperfusion is avoided and part to the fact that intermittent reperfusion may elicit endogenous cardioprotective mechanisms, which can protect against the numerous series of mechanisms contributing to reperfusion injury (see below). Notably PostC reduces post-ischaemic leukocyte adhesion in the heart.

Besides these mechanical interventions (gradual reperfusion and PostC), several pharmacological interventions have been studied against reperfusion injury (see below). For instance, it has been reported that either hypoxic reperfusion or reperfusion at lower pH at the onset of reperfusion could reduce reperfusion injury by lowering the formation of reactive oxygen species (ROS) [for review see 4].

Therefore, although PostC with intermittent interruption of flow by no means represents the first attempt in altering the conditions of reperfusion it did attract and renew the interest of many scientists on this field. On the light of the knowledge acquired with

the studies in preconditioning, PostC represents a tool to win our battle against the ischaemia/reperfusion injury. As a matter of fact, most of the studies on PostC are made possible because of the knowledge acquired in more than 20 years of studies on preconditioning.

It is well known that ischaemic preconditioning (IP) can be obtained with brief periods of myocardial ischaemia before an infarcting/index prolonged ischaemia. Preconditioning limits the severity of the injuries brought about by a subsequent I/R episode. Thus, after IP, the extent of the area of a subsequent infarction is reduced by 30–80% *versus* matched controls with matched risk areas. Preconditioning also can be obtained with ischaemias of distant organs (remote preconditioning) or with pharmacological treatment before the index ischaemia (pharmacological preconditioning). Preconditioning protection is active for few hours (2–3 hrs) immediately after the preconditioning manoeuvres (early classical preconditioning) as well as for longer time (24–72 hrs) in the so-called second window of protection (late preconditioning) that starts being be active 24–48 after preconditioning manoeuvres [for reviews on preconditioning see 14–17].

Since IP triggers protective pathways before ischaemia, whereas PostC alters events after ischaemia, it has been hypothesized that the mechanisms by which preconditioning and PostC confer myocardial protection should be different [13, 18]. There is no doubt that triggering the preconditioned state prior to ischaemia may activate a "memory function" that results in a modification of heart phenotype which may include a prolonged activation of the adenosine signal and of kinases at reperfusion [19]. Yet, data suggest that pre- and PostC have much in common. In fact, several reports indicate that components of the so-called RISK (reperfusion injury salvage kinase) pathway play a role both in pre- and in PostC [14–17, 20–25]. Pre- and PostC also reduce the oxidant-induced injury; moreover, they attenuate the local inflammatory response to reperfusion [13, 26–30]. In particular, the first few minutes of reperfusion following the infarcting ischaemia appear crucial to both IP- and PostC-induced protection. In this review we will see that both IP and PostC recruit a similar signalling pathway at the beginning of reperfusion, including: (1) the ligand-receptor interaction, (2) the RISK pathway and other kinases activation, (3) a redox signalling intervention, (4) the mitochondrial KATP (mKATP) channel activation and (5) the prevention of the mitochondrial permeability transition pore (mPTP) opening.

It is particularly intriguing that this common protective pathway can be activated at the time of myocardial reperfusion. This should lead to the development of novel treatment strategies to improve the clinical outcomes of patients undergoing coronary angioplasty or thrombolytic treatments after an acute myocardial infarction.

However, some differences between the pathways activated by pre- and PostC may exist. For instance, Darling *et al*. [31] showed an increase of phospho-ERK, but not of PI3-kinase/Akt in PostC, while Yang *et al*. [32] showed that ERK is involved in post-, but not in preconditioning. We and others showed that a redox signalling may be involved in PostC, but the reactive species involved may not be the same of those involved in preconditioning [33]. These findings may explain a certain degree of additive protection between IP and PostC, as observed by Yang *et al*. [34]. More similarities and differences between pre- and PostC are summarized in Table 1 and Table 2 (later).

Importantly, PostC can also be elicited by a distant organ ischaemia/reperfusion, termed 'remote PostC' [35, 36]. The understanding of the mechanisms of PostC-protection, may pave the way to new pharmacological strategy against reperfusion damages. For instance, we recently reported the possibility to trigger protection with an intermittent pharmacological intervention in the early reperfusion, which resembles ischaemic PostC [33].

The aims of this review are: to describe the characteristics of PostC and the suggested principal mechanisms. Moreover, we aim to call attention to the possible clinical relevance of the pharmacological PostC and to the importance of the early phase of reperfusion. Finally, we emphasize the differences between triggering mechanism of endogenous cardioprotection in early reperfusion and protection obtained with treatment during reperfusion. Particular attention is dedicated to the role of nitric oxide and ROS.

Reperfusion injury

As above mentioned, reperfusion injury is not a mere worsening of the ischaemia-induced damage, but it is secondary to events that are specifically induced by

Table 1 Major advantages and disadvantages of preconditioning and postconditioning

	Advantages	Disadvantages
Preconditioning	• It can be induced by heart and remote ischaemia, and by pharmacological treatment. • It can be programmed in the case of surgical interventions or programmable angioplasty in which heart is jeopardized. • It has two periods of effectiveness with different features: a) The first window of protection (early preconditioning) is more effective in reducing infarct size extension. b) The second window of protection (late preconditioning) is more effective against stunning.	• Can not be used in the case of an unpredictable ischaemic event (e.g. AMI) and in the following procedures of coronary revascularization. • In some case ischaemic preconditioning has been seen to induce inflammatory responses. • In the presence of some pathophysiological conditions (*e.g.* diabetes, hypertension and aging) it is not uniquely protective.
Postconditioning	• Protocol of ischaemic and/or pharmacological PostC can be applied in patients with acute unpredictable myocardial infarction (AMI) during coronary angioplasty or thrombolytic treatment. • Opened the possibility to short lasting treatments in post-ischaemic phase: a) Pharmacological treatment (*i.e.* intermittent BK); b) Non-pharmacological/ non-ischaemic procedure (*i.e.* dyssynchrony-induced PostC) and c) Remote/distant organ ischaemic PostC.	• The protection against infarct size may be less marked than preconditioning. • The protection against stunning is not so clear. • It is not yet clear whether or not PostC is effective in the elderly and in some pathophysiological conditions.

For references see text.

reperfusion. In fact, reperfusion injury is due to complex mechanisms involving mechanical, extra-cellular and intracellular processes.

The pathogenesis of reperfusion injury has been reviewed elsewhere [3, 37–39] and is beyond the aims of the present review. However, a short description of the principal events of the processes involved in reperfusion injury may be useful for the reader.

In patients with acute myocardial infarction, it is now widely accepted that timely reopening of the occluded coronary artery is accompanied by a reduction of the extent of necrosis and by a major reduction of short- and long-term mortality. However, together with a definite protective effect on ischaemic myocardium, post-ischaemic reperfusion may bring with it unwanted consequences that may partly counteract its beneficial effects. This phenomenon has thus been named reperfusion injury.

Causes of reperfusion injury

It seems that in the myocardium ischaemia/reperfusion (I/R) can induce various forms of cell death, such as programmed cell death, apoptosis, oncosis and necrosis [40–42.]. Apoptosis can be caused by both prolonged ischaemia/hypoxia and by reperfusion [26, 42]. In contrast to programmed cell death, apoptosis and oncosis, which are pre-mortal process, necrosis is a post-mortal event. According to this viewpoint necrosis is not a form of cell death but the end stage of any cell death process [for review see 42.].

The mechanisms of reperfusion-induced cell death are not completely understood, but it seems that the occurrence of oxidative stress related to the generation of ROS may play an important role [43, 44]. ROS have downstream effects, which result in the initiation of a highly orchestrated acute inflammatory response through the release of cytokines, activation of vascular endothelial cells and leukocytes with expression of cell surface adhesion molecules, and up-regulation of a program of pro-inflammatory genes, which contribute to the onset and maintenance of post-ischaemic inflammation [26]. When the occlusion of the coronary branch that perfuse the ischaemic myocardium is removed, the superoxide anion (O_2-) production increases as a result of the activation of various enzymatic complexes. The superoxide anion and other ROS strongly oxidize the myocardial fibres already damaged by the ischaemia, thus favouring the apoptosis [45–48]. It reacts with the nitric oxide, forming peroxynitrite ($ONOO^-$). Therefore, $ONOO^-$, represents a sign of a reduced availability of nitric oxide [49, 50] and it participates with O_2- to the lesion of myocardium [51–53]. Superoxide anion dependent damages are reduced if O_2- is transformed in hydrogen peroxide (H_2O_2) by the superoxide-dismutase. However, since in presence of Fe^{2+} or Cu^{2+}, the H_2O_2 can be transformed in hydroxyl anion (HO-), which is more toxic than O_2- and H_2O_2, an increase in toxicity can occur.

Reperfusion injury is also due to cellular Ca^{2+} overload. The Ca^{2+} overload, which starts during ischaemia, is further increased during reperfusion. The overload of Ca^{2+} increases the cellular osmolarity favouring swelling (explosive swelling) of myocardiocytes; it can also favour the expression of proapoptotic elements from mitochondria [48]. It is noteworthy that altered cytosolic Ca^{2+} handling during ischaemia may induce structural fragility and excessive contractile activation upon reperfusion, as also indicated from a progressive increase of ventricular diastolic pressure and contraction band necrosis [3, 47, 54].

Ca^{2+} overload is also considered to be responsible for the opening of mPTP. Although, mPTP opening is strongly inhibited by acidosis during ischaemia, it is favoured by ATP depletion, oxidative stress and high intramitochondrial Ca^{2+} concentrations, conditions all occurring during myocardial reperfusion [55].

Intriguingly, the *nuclear factor kappa B* (NFkB) plays a double-edge sword role in cardioprotection. Activation of NFkB is essential for late preconditioning, in which NFkB is involved in the up-regulation of iNOS and COX-2 genes. [for reviews see 56, 57]. However, in the longer time the role NFkB is also important in reperfusion injury. It contributes to the exacerbation of the myocardium lesions sustaining inflammatory reactions. The activation of NFkB is induced from several agents included hydrogen peroxide [58–61]. NFkB determine an up-regulation of the genes responsible of the production of molecules of cellular adhesion. These molecules favour the adhesion of leukocytes to the endothelium and possibly the migration within the myocardium [60]. Moreover, the reduced nitric oxide availability determined by I/R participates to the activation of genes transcription codifying for molecules of cellular adhesion [50, 59].

Therefore, myocardial damages during reperfusion among others can be due to the cellular/mitochondrial overload of Ca^{2+}, to the liberation of ROS, to the activation of mPTP, to the reduced availability of nitric oxide and to the activation of the NFkB. The nitric oxide deficiency can also cause vasoconstriction and formation of micro-thrombi into the lumen of the small vessels [62, 63]. These mechanisms, combined with the adhesion of the leucocytes to the endothelium, can lead to the so-called '*no-reflow phenomenon'* [64].

In summary, reperfusion injury is due to several mechanisms that include Ca^{2+} overload, ROS generation, reduced availability of nitric oxide, mPTP opening and to the activation of the NFkB, which lead to the augmented expression of molecules of cellular adhesion, leukocyte infiltration and no-reflow phenomenon.

Effects of reperfusion injury

Among the outcomes of reperfusion injury are included: (1) endothelial and vascular dysfunction and the sequels of impaired coronary flow, which may concur with the 'no-reflow phenomenon'; (2) metabolic and contractile dysfunction; (3) arrhythmias; (4) cellular death by cellular swelling, apoptosis and contraction band necrosis.

One may anticipate that effective treatment during reperfusion may reduce myocardial injury. However, the complexity of mechanisms suggests that one single intervention aimed to contrast just one or two of these mechanisms may not be sufficient.

Definition of postconditioning

The concept of 'Ischaemic PostC' was first described by Vinten-Johansen's group [13]. This study was performed in a canine model of 1 hr coronary occlusion and 3 hrs reperfusion. In such study the PostC algorithm was 30 sec. of reperfusion followed by 30 sec. of coronary occlusion, which were repeated for three cycles at the onset of reperfusion. Although this seminal study used the term 'Ischaemic PostC', subsequent studies of these and other authors omit the term 'Ischaemic' because it is not clear whether the brief periods of ischaemia, the preceding and/or the subsequent periods of reperfusion, or their combination, provide the key stimulus for cardioprotection.

In general, PostC can be defined as intermittent interruption of coronary flow in the very early phase of a reperfusion, which leads to cardioprotection.

The duration and number of these stuttering periods of reperfusion and ischaemia has been one of the aims of early studies on this topic.

Postconditioning algorithm

PostC can be obtained by different protocols in terms of duration of the periods of reperfusion and ischaemia and/or in terms of number of cycles of I/R applied after an infarcting ischaemia. These different procedures/protocols have been called PostC algorithms by Vinten-Johansen [28]. Accordingly, in the present review we will use the term 'PostC algorithm' to refer to PostC procedure/protocol. In virtually in all of the species in which different PostC algorithms have been tested they have been protective, including humans, with the exception of a recent work conducted on a rodent model [65] (Table 2). However, many other groups observed a cardioprotective effect of PostC in rodent models [27], including mice lacking connexin 43 [66]. With respect to this, it is paradigmatic the porcine model. In fact, in swine there are two studies: in one study, Schwarz & Lagranha [67] reported that PostC obtained with three cycles 30 sec. I/R had no beneficial effect in open-chest pigs. In another study it seems that PostC with intermittent cycles of 1 min. I/R is effective also in swine [68]. Although, it has not been specifically studied, it seems that contrasting findings in this species are due to different PostC algorithms. Nevertheless, these studies and other studies which used different algorithms to induce PostC stress the importance of the duration of index I/Rs periods as well as PostC I/R algorithm. Another recent study suggested that the cardiac effects of PostC may even be detrimental and that this deleterious effect depends critically on the duration of the preceding period of index ischaemia rather than the employed algorithm [69].

Many studies have sought to identify PostC algorithms that effectively limit infarct size, and, though the *'ideal'* PostC regimen has not been established, the emerging consensus is that multiple (3–6) cycles of brief (10–30 sec.) intermittent reperfusion/re-occlusion are associated with cardioprotection (Fig. 1). The duration of intermittent cycles of I/R seems to be shorter in smaller species (*i.e.* 10–15 sec in rats and mice) and longer in larger species (30 sec in dogs and rabbits) and human (60–90 sec) [28, 70, 71].

In the rat hearts we compared a *'classical'* PostC algorithm which consisted of five cycles of 10 sec. reperfusion and occlusion with a *'modified'* algorithm of PostC (Fig. 1) which consisted of an initial 15 sec. reperfusion and then in a sequence of progressively shorter (20, 15, 10 and 5 sec.) periods of occlusions separated by progressively longer periods of reperfusions (20, 25 and 30 sec.). This modified *PostC* protocol was equally effective as the classical algorithm in reducing the infarct size [72, 73].

Importantly, in all species, the PostC stimulus must be applied immediately upon relief of sustained ischaemia to be protective: if initial complete reperfusion is allowed to continue for more than the first 60–90 sec., then protection against infarct size extension is lost [27, 32, 74].

Protective effects of postconditioning

Depending on species, models and other factors, PostC reduces infarct size by ~20–70% *versus* matched controls with matched risk areas. There is an emerging agreement across multiple models and species that PostC may reduce endothelial dysfunction and endothelial activation, thus leading to a reduced endothelia/leukocyte interaction and to a reduced ROS formation. It has been also observed a reduced incidence of apoptosis and arrhythmias.

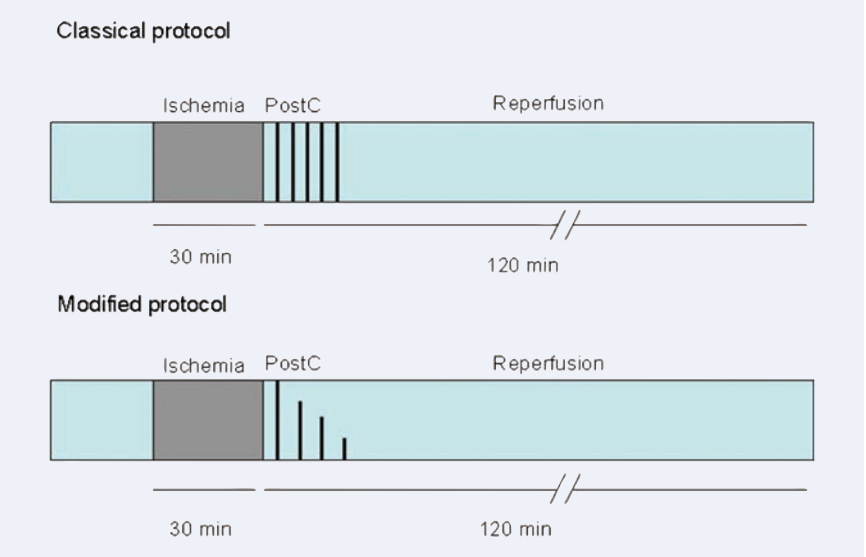

Fig. 1 Schematic representation of postconditioning protocols. In modified protocol the decreasing vertical bars represent shorter periods of ischaemia.

Whether PostC reduces post-ischaemic stunning it has not yet been clarified.

Infarct size reduction

In their seminal study Vinten-Johansen and coworkers [13] reported that PostC causes massive salvage of the myocardium. The infarct size was reduced by ~45% when the initial minutes of reperfusion were 'stuttered' compared to an abrupt and complete reperfusion. These findings have been confirmed by several laboratories including our laboratory [31, 75, 76] (Table 2). As said, in multiple species and models, PostC reduces infarct size by ~20–70% *versus* matched controls with matched risk areas [13, 34]. Studies from our laboratory and other laboratories confirmed the infarct size reduction in rat isolated heart model [20, 35, 72, 73, 76, 77]. We showed that in hearts perfused with constant flow the infarct size reduction by PostC is greater than that observed in the same model perfused at constant pressure [73] (see also below).

Reduction of apoptosis

Apoptosis is a genetically programmed cell death that occurs in reperfusion injury [41, 78–80]. The reduction in apoptosis may involve the inhibition of caspase-3 and caspase-9 and preservation of Bcl-2/Bax ratio. So far the only study that reported a reduction of apoptosis by PostC is of Zhao *et al*. [81] in which a reduced apoptosis was detected with TUNEL assay and the presence of DNA ladders in a model of isolated neonatal cardiomyocytes that underwent hypoxic PostC.

Reduction in endothelial dysfunction

The endothelial cell dysfunction is a common characteristic of various heart pathologies [82, 83]. In their seminal study Zhao *et al*. [13], reported that post-ischaemic endothelial dysfunction was attenuated by PostC. In this study, incremental doses of acetylcholine were used to evaluate the endothelial-dependent vasodilatation of coronary vessels isolated from the post-ischaemic region. The authors demonstrated that vasodilatation of post-conditioned vessels was improved with respect to that observed in post-ischaemic control vessels. The vasodilator response was similar to that observed in preconditioned vessels and to that observed in vessels from non-ischaemic region.

Reduction in endothelial activation, and neutrophil adherence

PostC decreases the expression of P-selectin, an adhesion molecule, on the surface of endothelial cells. Moreover it has been observed both a reduction in neutrophils adhesion on the post-conditioned

Table 2 Seminal studies in which postconditioning has been tested with positive or negative results

Authors	Model	Postconditioning algorithm	Primary endpoint	Cardio-protection	Mechanism
Zhao *et al.* [13]	*In vivo* dog	Three cycles of 30 sec.Rep/30 sec. ischaemia	Infarct size	Yes	Less oxidant injury
Halkos *et al.* [84]	*In vivo* dog	Three cycles of 30 sec. Rep/30 sec. ischaemia	Infarct size	Yes	Less oxidant injury
Kin *et al.* [27]	*In situ* rat	Six cycles of 10 sec. Rep/10 sec. ischaemia	Infarct size	Yes	Less oxidant injury
Kin *et al.* [77]	Isolated mouse heart Constant pressure (CP)	Three or six cycles of 10 sec. Rep/10 sec. ischaemia	Infarct size	Yes	Adenosine (Ade) retention; Ade A_{2a} receptor stimulation;
Penna *et al.* [73]	Isolated rat heart CP or constant flow (CF)	Six cycles of 10 sec.Rep/10 sec. ischaemia	Infarct size	Yes	NOS and GMPc production
Penna *et al.* [76]	Isolated rat heart CF	Six cycles of 10 sec. Rep/10 sec. ischaemia	Infarct size	Yes	ROS, mKATP and PKC activation
Philipp *et al.* [74]	*In vivo* rabbit	Four cycles of 30 sec. Rep/30 sec. ischaemia	Infarct size	Yes	Ade receptors, PI3-K activation
Tsang *et al.* [20]	Isolated rat heart CP	Six cycles of 10 sec.Rep/10 sec. Isch	Infarct size	Yes	PI3-K activation
Yang *et al.* [34]	Isolated rabbit heart CP	Six cycles of 10 sec. Rep/10 sec. Isch	Infarct size	Yes	Ade receptors; PI3-K, GC
Yang *et al.* [32]	*In vivo* rabbit	Four cycles of 30 sec. Rep/30 sec. Isch and 6 cycles of 30 sec. Rep/30 sec. Isch	Infarct size	Yes	ERK activation; NO production; mK_{ATP} channel
Darling *et al.* [31]	Isolated rabbit heart CP	Four cycles of 30 sec. Rep/ 30 sec. Isch	Infarct size	Yes	ERK activation; mitochondrial K_{ATP} channel
Argaud *et al.* [91]	*In vivo* rabbit and isolated mitochondria	Four cycles 1 min. Isch/1 min. Rep	Infarct size	Yes	Inhibition of mPTP
Sun *et al.* [85]	Isolated cardiomyocytes	Three cycles of 5 min. re-oxygenation/hypoxia	Cell death	Yes	ROS generation; oxidant injury and mitochondrial Ca^{+2} levels
Schwartz *et al.* [67]	*In vivo* pig	Three cycles of 30 sec. Rep/30 sec. Isch	Infarct size	No (see text)	N/A
Iliodromitis *et al.* [68]	*In vivo* pig	Two different PostC algorithms	Infarct size	Yes	Inhibition of mPTP
Dow and Kloner [65]	*In vivo* rat	Four different PostC algorithms	Infarct size	No (see text)	N/A
Couvreur *et al.* [86]	*In vivo* dog and rabbit	Three different PostC algorithms	Stunning	No	N/A
Sivaraman *et al.* [21]	Human atrial appendage	Four cycles of 30 or 60 sec. re-oxygenation/hypoxia	Stunning	Yes	RISK cascade
Penna *et al.* [33]	Isolated rat heart CF	Intermittent infusion of drugs	Infarct size	Yes/No	BK B2 receptors, NOS, ROS, mKATP, PKC activation

For acronyms and further explanations see the text.

coronary artery endothelium and accumulation of neutrophils in the area at risk [13]. A reduction in superoxide anion generation in the perivascular area has been also observed in the proximity of risk area of postconditioned hearts [67, 84]. Whether the reduced neutrophil accumulation, the subsequent ROS production and the pro-inflammatory response is a cause or an effect of necrosis, apoptosis and vascular injury is not clear. In fact, PostC exerts marked cardioprotection in leukocyte-free models (isolated buffer perfused hearts and isolated cardiomyocytes) [*e.g.* 21, 34, 73, 76, 85].

Reduction of stunning

The studies which tested whether PostC may improve post-ischaemic myocardial function after a post-infarcting ischaemia report either an improvement [13, 21, 34] or no effect [73] on cardiac performance. In the presence of an infarct, it is hard to distinguish whether the impairment of global function is due to necrosis and/or to myocardial contractile dysfunction or 'stunning' of viable tissue. To date the only study that tested *in vivo* whether PostC attenuates myocardial stunning in models of short ischaemias (*i.e.* 10–15 min. ischaemia), which usually induce stunning without cell death, reports that PostC does not prevent myocardial stunning [86]. In an *ex vivo* preparation of human atrial appendages hypoxic PostC seems to attenuate post-ischaemic dysfunction [21].

Anti-arrhythmic effects

As said, several investigations have demonstrated that it is mandatory to perform the PostC protocol as soon as possible after ischaemia to protect the heart against ischaemia reperfusion injury. However, Galagudza *et al.* [87] report that PostC performed 15 min. after the beginning of reperfusion still has a strong effect in limiting persistent reperfusion-induced tachyarrhythmia. The possibility to reduce reperfusion arrhythmias with the reintroduction of ischaemia is not completely novel. In fact, in 1994 Grech and Ramsdale [88] reported that coronary artery re-canalization by percutaneous transluminal coronary angioplasty (PTCA) induced an idioventricular rhythm, which was interrupted several times by the reinflation of the balloon and thus restoring sinus rhythm.

It has been reported that classical early PostC not only reduces infarct sizes in pigs, but also abolishes reperfusion arrhythmias [68]. Recently, Kloner and coworkers [89] have reported in a non-infarct regional ischaemia model in *in vivo* rats that PostC attenuates post-ischaemic ventricular arrhythmia. From these studies it seems that PostC with brief I/R can be used to limit reperfusion arrhythmias.

On the other hand, intermittent pacing (intermittent dyssynchrony-induced PostC) during early reperfusion reduces infarct size in two different species and models (isolated rabbit hearts and *in vivo* pig) [90]. The intermittent pacing was obtained in both models during the first minutes of reperfusion using few seconds of ventricular pacing alternated by few seconds of atrial pacing. It is noteworthy that dyssynchrony-protection is likely induced by modulation of local myocardial workload/metabolism, rather than graded reperfusion.

Potentiality of postconditioning

It has been reported that PostC-induced infarct size reduction persists up to 72 hrs [29, 91]. These are important studies because they demonstrate that the protection by PostC represents a long-term protective effect and not a mere attenuation of event involved in early reperfusion injury.

In some studies the protocol of classical preconditioning and PostC were combined in order to see whether or not the protection by these two protocols was additive, relative to the protection of each protocol alone. The results are inconsistent. In a canine model, Halkos *et al.* [84] showed that the combination of protocols is not additive for infarct size reduction, anion production nor for post-ischaemic endothelia dysfunction. Similar results were obtained by Tsang *et al.* [20] and by us [73] in isolated perfused rat hearts. However, Yang *et al.* [32] demonstrated in an *in vivo* rabbit model that the combination of the two protocols reduced infarct size significantly more than either manoeuvre alone. The different results may be due to species difference and/or different I/R and PostC protocols.

Recently, Bolli's group reported that cardioprotection induced by late preconditioning is enhanced by PostC *via* a COX-2-mediated mechanism in conscious rats [92]. It remains to de ascertained whether such additive effect between late preconditioning and PostC can be observed in other species and/or models.

Very few studies tested the differences between male and female hearts with regard to PostC effectiveness. In a specifically designed study it has been reported that while the PostC protective effect against stunning was observed in isolated male rat hearts after both 20 min. and 25 min. ischaemia, the protective effect was present in female rat hearts exposed to 20 min of ischaemia, but absent in those exposed to 25 min. ischaemia [93]. In a preliminary study, we observed that after 30 min. ischaemia the PostC protective effect against infarct is less effective in female than in male rat hearts [unpublished observations]. The importance of PostC warrants further studies to elucidate the mechanistic pathways and differences in males and female hearts.

It has been reported that cardioprotection by PostC is dependent on the PostC algorithm in aged and STAT3 (signal transducer and activator of transcription 3)-deficient hearts. Moreover it seems that the reduced levels of STAT3 with increasing age may contribute to the age-related loss of PostC protection [94].

Remote postconditioning

As stated, preconditioning can be obtained with ischaemias of distant organs (remote preconditioning) [95, 96]. Remote preconditioning of the heart was introduced by Przyklenk *et al*. [95], which observed that preconditioning ischaemia on one coronary artery territory can protect the non-treated myocardial territory (virgin myocardium) against a subsequent infarcting ischaemia. Later on, several studies indicated that a brief ischaemic insult on one organ releases endogenous factors that protect other organs against a prolonged ischaemic insult. This phenomenon, was termed remote preconditioning or preconditioning at a distance, and implicates an endocrine action, and/or neural-humoural signalling [96]. Recently, it has been reported that remote ischaemic PostC can protect the heart against acute myocardial infarction in pigs, rabbits and rats. Remote ischaemic PostC was induced by cycles of few minutes of I/R applied to a distal artery territory (femoral or renal artery) either immediately after the beginning of heart reperfusion (Pig) or just few minutes before heart reperfusion (Rabbits and Rats) [14, 35, 36, 97]. Very recently, it has been shown that remote PostC induced by transient limb ischaemia can prevent endothelial I/R injury in humans [98].

Pharmacological postconditioning

Several studies have demonstrated that many drugs including adenosine, adipocytokines, erythropoietin, bradykinin, genistein, insulin, natriuretic peptides, statins and volatile anaesthetics when administered at the time of reperfusion, reduce myocardial infarct size through the activation of the so called RISK pathway [14, 22, 23, 99]. Ultimately, this recruits various pathways that lead to the inhibition of mPTP opening [15, 23, 100, 101]. In fact Ciclosporin A, a mPTP blocker, can also be used at the time of myocardial reperfusion as pharmacological mimetic of PostC [101] (See also below 'Active mechanisms').

We recently introduced a sort of staccato treatment with either bradykinin (BK) or diazoxide to mimic ischaemic PostC [33]. In this study we were able to trigger the protective effect of PostC against infarct size using the drugs for few seconds at the beginning of reperfusion. We mimicked PostC with five cycles of 10 sec. drug infusion alternated with 10 sec. of simple reperfusion, exactly as ischaemic PostC is obtained alternating for a few seconds ischaemia and reperfusion. The downstream pathway activated by intermittent BK seems very similar to that activated by ischaemic PostC. This is a completely novel way to use the drugs during reperfusion. It is worthy for future experiments to test if other drugs can trigger PostC-like protection in this way. We have tried to reproduce these results using intermittent adenosine in lieu of BK. Unfortunately, intermittent adenosine was not as effective as intermittent BK [unpublished observations]. Overall these results teach us that not only the mechanism of action of the drug, but also timing and mode of infusion are very important in reperfusion.

Postconditioning the human heart

Ischaemic PostC has been shown to potentially reduce myocardial injury in patients undergoing primary coronary angioplasty for an acute myocardial infarction. In some studies PostC with stuttered 60 sec or 90 I/R appears to be protective [70, 71]. However, in all human studies that deal with I/R

injury, there are at least three facts/pitfalls to consider: the absence of matched control with matched risk area, the different duration of ischaemia and the surrogate end-points that need to be considered. Of course for ethical reasons, the end-point cannot be the infarct size, thus end-points such as enzyme release are studied. The absence of matched controls with matched risk area is a pitfall that can mislead the researcher. A smaller infarct size or a reduced enzyme release does not have any meaning if the risk area was also smaller. Notably, Staat *et al*. [71] used the area under the curve of CK release over the first 72 hrs of reperfusion as a surrogate evaluation of infarct size. Yet, they obtained left ventricle angiogram before re-opening the occluded coronary artery in order to estimate the size of the area at risk. Finally, duration of ischaemia, another major determinant of infarct size, was comparable between the control and post-conditioned groups.

Importantly, the results of above studies are confirmed in a retrospective study, in which it is reported that multiple balloon inflations during primary angioplasty may confer cardioprotection similarly to PostC [102]. Moreover, very recently, Sivaraman *et al*. [21] reported that in an *ex vivo* preparation, hypoxic PostC protects human atrial appendages against post-ischaemic dysfunction, and that this effect is dependent on the activation of the RISK pathway. This pathway, as mentioned above, plays a pivotal role in PostC protection as we will see in the following sections.

Postconditioning in diseased hearts

Many studies have suggested that certain diseases (*e.g*. hypertrophy, remodelling, heart failure, atherosclerosis, diabetic disorder) and also age might negatively impact the endogenous protective mechanisms of preconditioning [16]. To the best of our knowledge, so far, few studies analysed the role of diseases in PostC cardioprotection [103–106]. In particular, two studies from different laboratories suggested that in the presence of metabolic syndrome, the heart may be more resistant to PostC protection and that a stronger stimulus may be necessary to reduce infarct size [105, 106]. Yet, PostC has been shown to be able to attenuate I/R injury in both hypertrophied and remodelled heart [103, 104]. Interestingly, in the human study by Staat *et al*. [71], hypertension, hyperlipidaemia, and smoking habits each existed in more than 50% of patients, while 15% were diabetic patients. Although the samples were small, it seems to indicate that these conditions and risk factors did not prevent PostC protection. It seems that also the few patients older than 75 years which entered the study had benefit from PostC manoeuvres.

Since this issue is of paramount clinical importance, it will be mandatory to study the role of these conditions in animal models and humans.

Mechanisms involved in postconditioning

The mechanisms of protection by PostC were initially attributed mainly to improved endothelial function and to the events reducing the detrimental effects of lethal reperfusion injury, such as reduced oedema, reduced oxidative stress, reduced mitochondrial calcium accumulation, reduced endothelium damage and reduced inflammation. However, subsequent studies suggest that protection is mediated through the recruitment of signal transduction pathways as in the case of ischaemic preconditioning. Therefore, a distinction in passive and active mechanisms can be proposed (Fig. 2). Of course an intricate cross-talk among these events/mechanisms exists, thus this distinction can be useful for a better understanding of the phenomenon, but we must not forget that a single event/mechanism may not be effective if it occurs alone.

Passive mechanisms

Among passive mechanisms we can consider those strictly related with Starling force – hereafter named as ‘Mechanical mechanisms’ – and those related with reduced endothelial adhesion of leucocytes and subsequent reduction of inflammatory process that we call ‘Cellular mechanisms’.

Mechanical mechanisms

With regard to mechanical or haemodynamic mechanisms, it has been suggested that the stuttering of reperfusion and pressure during PostC manoeuvres may limit the Starling forces in a very important moment, thus limiting early oedema and consequent

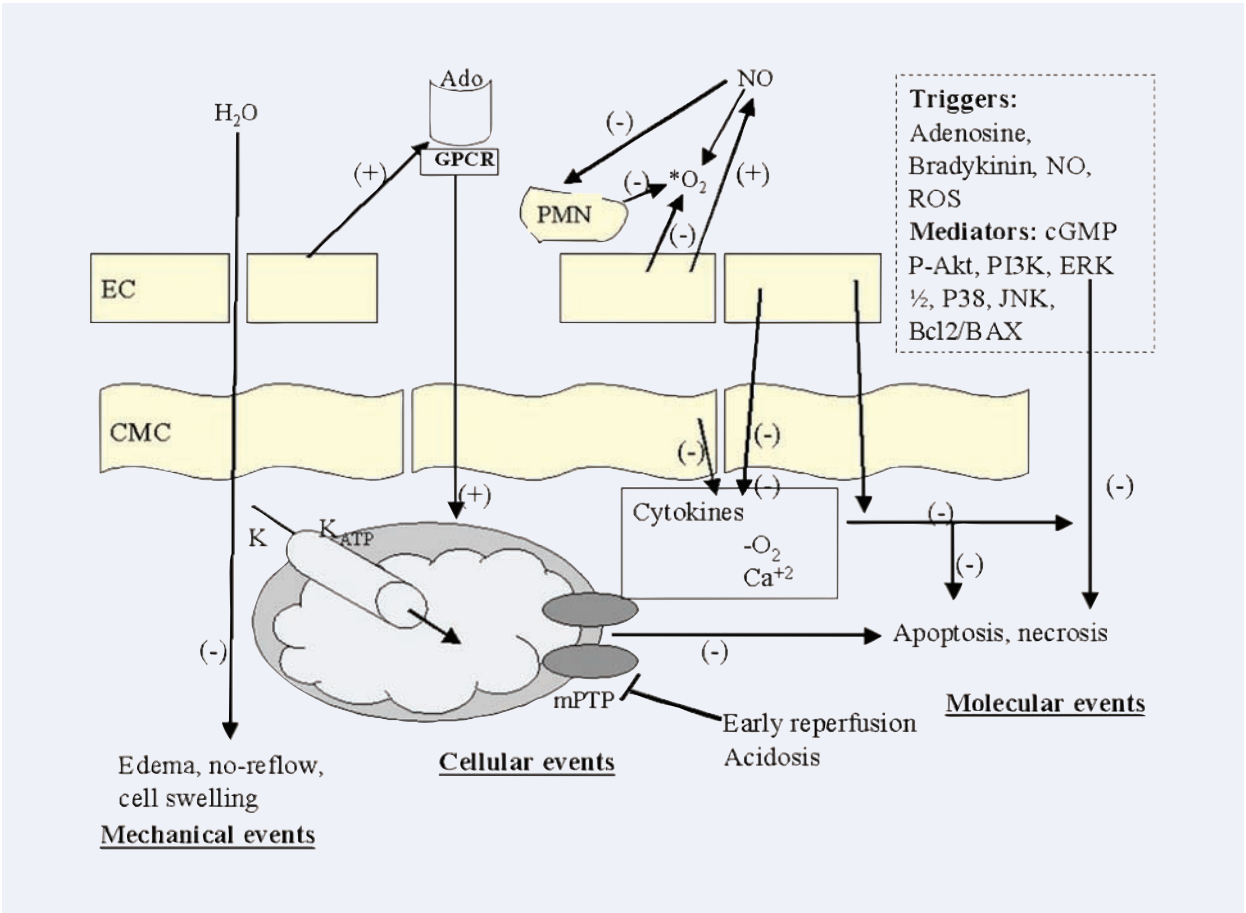

Fig. 2 Simplified schematic diagram of the proposed mechanisms of Postconditioning based on the studies currently available. EC, endothelial cells; CMC, cardiomyocytes; Ado, adenosine; CPCR, G protein-coupled receptors; other acronyms as in the text.

damages. In experiments performed in isolated heart models the effect of the PostC on the infarct area has been studied perfusing the hearts either with constant pressure or with constant flow. We compared the role of these two types of perfusion in affecting the infarct area during PostC. In the constant pressure model the infarct area was less reduced by PostC than it was with the model of the constant flow reperfusion after PostC [73]. Considering that during the short period of restoration of flow in the PostC manoeuvres the capillary pressure increases less in the constant flow model, than in the constant pressure model (*i.e.* at beginning of reperfusion in the constant flow model there is less 'hydrostatic' pressure and so less capillary pressure), it was argued that in the constant-flow model a reduced oedema and consequent reduced damages may explain the increased effectiveness of PostC. In other words, we believe the fact that in the constant flow model the effectiveness of PostC is greater than in the constant pressure model supports the idea that the reduction of Starling forces during PostC manoeuvres may play an important role in determining the protective effects.

Cellular mechanisms

Among the cellular mechanisms we consider acute inflammatory response. It occurs through the release of cytokines, activation of vascular endothelial cells

and leukocytes with expression of cell surface adhesion molecules, and up-regulation of a program of pro-inflammatory genes. PostC delays the onset and reduces the maintenance of post-ischaemic inflammation [13]. As stated before, whether this is a cause or an effect of PostC protection remains to be elucidated.

Active mechanisms (intramyocardiocyte mechanisms)

What are the intracellular mechanisms of the protection? Studies have identified a signalling pathway that is recruited at the time of reperfusion and which is similar in ischaemic preconditioning and PostC. This pathway includes the survival kinases phosphatidylinositol 3-kinase (PI3K)-Akt and Erk1/2, the major components of the reperfusion-injury salvage-kinase pathway, termed the RISK- pathway, which may influence the mPTP, a non-specific pore of the mitochondrial membrane whose opening in the first few minutes of myocardial reperfusion promotes cell death [14, 17, 22–24, 107]. Delayed washout of endogenously produced adenosine and activation of the adenosine receptor also seems required for PostC protection, [77] by activating the survival pathway. Thus delayed washout of adenosine in the setting of PostC might recruit RISK at the time of reperfusion through the activation of adenosine-responsive G-protein-coupled receptors. It seems that adenosine receptors are repopulated during PostC manoeuvres. While in murine hearts adenosine A_{2a} and A_3 subtypes [77] have been seen to be involved, in rabbit hearts PostC seems to depend on A_{2b} subtype [74]. An important role of the redox environment has been also observed [33, 76].

Therefore, similar to preconditioning, PostC has been proposed to be triggered by receptor stimulation, mediated by one or more complex and interrelated signal transduction pathways, and, ultimately, achieved *via* phosphorylation of one or more end-effectors of cardioprotection [17, 24].

Triggers

Ligands, such as adenosine [74, 77] and BK [33] which accumulate during PostC manoeuvres may initiate the cascade that lead to PostC protection. It has been recently reported that inhibition of opioid receptors with opioid receptor antagonists administered 5 min. before reperfusion in the absence or presence of PostC, reversed the infarct sparing effect of PostC in *in vivo* rat model [108]. The activation of protein kinases C and G (PKC and PKG) and opening of mitochondrial K_{ATP} channels after PostC (see below) would be consistent with the involvement of BK and endogenous opioids.

Nitric oxide and ROS may be included among the triggers. Nitric oxide is demonstrated to act both as a trigger and as a mediator of the preconditioning response in a variety of species. The role of endogenous NO in classic ischaemic preconditioning was controversial. Cohen and Downey's group suggested that exogenously administered NO could trigger the preconditioned state through a free radical-mediated process not shared by endogenous NO. Very recently these authors questioned whether their observation was due to a bias in the experimental model. These authors are now of the opinion that endogenous NO participates in triggering *in vivo* preconditioning [109].

Among the autacoids released by the ischaemic heart there is BK which may induce nitric oxide release (Fig. 3). It has been suggested that the mechanism whereby NO protects myocardium includes the activation of guanylate-cyclase [110]. As an inducer of the protection, nitric oxide may also directly open the mitochondrial KATP channels [111]. Therefore, nitric oxide acting on mitochondria may play a relevant role in protection both through activation of these channels and *via* modulation of respiratory chain; both mechanisms favor ROS signalling, which can trigger protection [112, 113]. A relevant role of nitric oxide may also be attributed to the endothelial protection brought about by this molecule [114–115] or to its role as antioxidant under certain conditions [116, 117].

The one-electron-reduction product of nitric oxide, HNO/NO^- (nitrosyl hydride/nitroxyl anion), has been scarcely studied in an I/R scenario. In our laboratory low doses of Angeli's salt, a donor of HNO/NO^-, have been seen to induce early/classical preconditioning against myocardial damages [118]. Intriguingly, the protective effects of HNO/NO^- generated by Angeli's salt were more potent than the protective effects induced by equimolar concentration of the pure nitric oxide donor diethylamine/nitric oxide (DEA/NO).

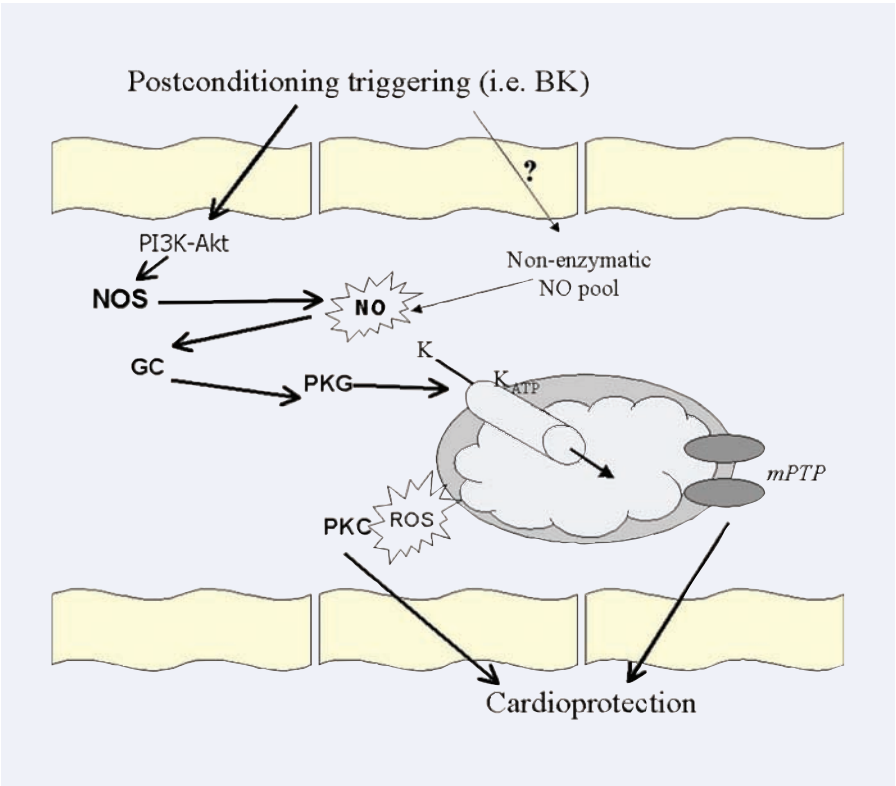

Fig. 3 Simplified schematic diagram of the proposed role of nitric oxide activated pathway in Postconditioning based on the studies currently available. Acronyms as in Figure 2 and in the text.

While the HNO/NO^- donor seems deleterious in reperfusion [119], there is evidence that NO may also be involved in the cardioprotection by ischaemic PostC. When the nitric oxide synthase (NOS) inhibitor N-omega-nitro-L-arginine methyl esther (L-NAME) was given 5 min. before start of reperfusion of *in situ* rabbit hearts, the infarct limiting effect was abolished [32]. We have shown that nitric oxide participates in PostC, but NOS inhibitors given for the entire reperfusion period only blunted the protective effect of PostC [73]. Paradoxically, the same inhibitor, given only during PostC manoeuvres completely blocked the protective effects [33]. At the moment, we do not have an explanation for this apparent paradox. In a previous study, we argued that nitric oxide may be produced in post-conditioned heart both by NOS and by non-enzymatic mechanisms. Nitric oxide can then activate the guanylyl cyclase to produce cyclic guanosine monophosphate (cGMP), which mediates protection [72, 73] (see also below). The infusion of a NOS inhibitor only during PostC manoeuvres may alter the equilibrium between ROS and nitric oxide thus leading to the production of the wrong kind of radical which does not trigger the protective pathway. It can be argued that in the absence of this protection the stronger limitation of nitric oxide production by NOS may be protective during reperfusion. In fact, data have demonstrated that NOS inhibitors can attenuate I/R damage [63, 120, 121]. Also, the different doses of nitric oxide inhibitors applied and the different basal levels of nitric oxide endogenously produced may explain these disparities.

The beneficial and deleterious effects of nitric oxide and nitrite in pathophysiological conditions and contradictory results about the effects of nitric oxide during reperfusion have been reviewed by Bolli in 2001 [122], Wink *et al*. in 2003 [123], Pagliaro in 2003 [57] and Schulz *et al*. in 2004 [124].

ROS could also be included among the triggers of PostC. In fact, ROS scavengers such as N-acetylcysteine and 2-mercaptopropionylglycine given during PostC manoeuvres prevent the protective effects [33, 76, 125, 126]. It is possible that the low pH during the PostC cycles prevents mPTP opening, while the intermittent oxygen bursts allow mitochondria to

make enough ROS in a moment in which other enzymes, able to produce massive quantity of ROS, are not yet re-activated. Then mitochondrial ROS may activate PKC and put the heart into a protected state. The importance of the role of acidosis in the triggering of PostC protection has been recently confirmed by two independent laboratories [100, 127]. Acidosis may also prevent mPTP opening in the early reperfusion (see below).

Recently, it has been reported that redox signalling and a low pH at the time of myocardial reperfusion are also required to mediate the cardioprotection triggered by ischaemic preconditioning [128].

Mediators

We considered ROS among triggers as they are necessary during PostC manoeuvres. Nevertheless, PostC activated the RISK pathway, with increased expression of the phosphorylated form of endothelial nitric oxide synthase (eNOS) as one of the results [20]. It is thus likely that after NOS activation cGMP is produced and PKG activated; then mitochondrial ATP-dependent potassium (mK_{ATP}) channels are opened and ROS produced. Therefore cGMP, PKG, mK_{ATP} and ROS may be considered as mediators of PostC protection, which are likely to be upstream to PKC activation. We demonstrated that cGMP production is increased during reperfusion of postconditioned hearts [72, 73]. Moreover we showed that in these hearts mK_{ATP} and PKC must be also active (*i.e.* they should not be blocked) during late reperfusion [76]. Regarding the role of mK_{ATP} channels a couple of papers indicate that the mK_{ATP} channel are important for PostC [34, 76]. In such studies two different mK_{ATP} channel blockers (glibenclamide and 5-hydroxydecanoate) abolished the protective effect of PostC [34, 76].

It is interesting that many of the RISK elements (*e.g.* PI3K/Akt and MEK1/2-ERK) involved in the signalling pathway in preconditioning protection against reperfusion injury have recently been documented also in PostC [17, 20, 24, 25, 28, 34]. Some differences, however, may exist between pre- and PostC (see also Table 1 and Table 2). Darling *et al.* [31] showed an increase of phospho-ERK, but not of PI3K/Akt in Post-C, while Yang *et al.* [34] showed that ERK is involved in PostC, but not in preconditioning. These findings may explain a certain degree of additive protection between ischaemic preconditioning and PostC, as observed by Yang *et al.* [34]. Yet in contrast with Yang *et al.* [34], Cao *et al.* [129] reported that ERK is present in preconditioning trigger pathway. The reasons for the differences are not clear. Different species and/or protocols may play a role [28]. A role may be also played by different methods of tissue sampling [31]. Besides protein kinase C, the possible roles for tyrosine kinase, and members of the MAPK family other than ERK1/2 in PostC has been suggested [14, 30].

Focal disorganization of gap junction distribution and down-regulation of connexin 43 (Cx43) are typical features of myocardial remodelling [130] and Cx43 – especially Cx43 localized in mitochondria – has been indicated as one key element of the signal transduction cascade of the protection by preconditioning. However, Cx43 does not seem to be important for infarct size reduction by PostC [66]. These results, together with the above reported differences on kinases activation by pre- and PostC, suggest a certain degree of differences between the protective pathways activated by these two procedures.

End effectors

mPTPs opening represents a fundamental step of reperfusion injury. Among the potential mechanisms responsible for mPTP opening during reperfusion, Ca^{2+} overload has received particular attention. In particular, mitochondrial Ca^{2+} overload occurring during ischaemia should bring mitochondria closer to the threshold at which mPTP opening takes place, favouring the occurrence of mPTP opening during reperfusion, a phenomenon described as mitochondrial priming [131]. Additionally, reduced mitochondrial Ca^{2+} overload during ischaemia has been pointed out as a potentially important mechanism of ischaemic and pharmacological preconditioning [132].

Neonatal rat cardiomyocytes subjected to 3 hrs hypoxia and 6 hrs of re-oxygenation, “hypoxic PostC” with alternating exposure to three cycles of 5 min. hypoxic and normoxic conditions preceding re-oxygenation reduced intracellular and mitochondrial Ca^{2+} loading compared to non-postconditioned cardiomyocytes. This was associated with a reduction in

cardiomyocyte death assessed by propidium iodide and lactate dehydrogenase release [85]. However, the signalling pathways and physiological consequences of this lower intracellular Ca^{2+} by PostC are not known at present, especially *in vivo*. For instance, it cannot be excluded that reduced mitochondrial Ca^{2+} overload could actually be a consequence of a more preserved Ca^{2+} handling by the sarcoplasmic reticulum in postconditioned cardiomyocytes rather than a cause of protection.

It has been reported that PostC reduces calcium-induced opening of the mPTP in mitochondria isolated from the myocardial area at risk [91]. PostC was also associated with a reduction in infarct size after both acute and long-term (72 hrs) reperfusion. Bopassa *et al*. [133] demonstrated in isolated perfused rat hearts that maintenance of mPTP closure was associated with PI3K activation, which is consistent with the activation of survival kinase pathways described above, but the functional involvement of these pathways and regulation of the mPTP *in vivo* is not yet clear. It seems that in the PostC scenario the inhibition of GSK3β contributes to the prevention of mPTP opening [55].

Taken together it would appear that the trigger pathway for PostC involves the following sequence of events: occupation of surface receptors (adenosine and possibly BK and opioid receptors), activation of NOS and non-enzymatic processes to make nitric oxide, activation of cGMP-dependent kinase (PKG), opening of mK_{ATP}, production of ROS and finally activation of PKC and MAPKs as well as inhibition of GSK3β which put the heart into a protected state. The protect state may include a central role of the prevention of mPTP opening by acidosis in the early phase and by the aforementioned mechanisms in the late reperfusion (Figs 2 and 3).

Cardioprotection by pre- and postconditioning is redox-sensitive

It has been already established that preconditioning triggering, that is the period that precedes the index ischaemia, is redox sensitive. This was demonstrated by both avoiding preconditioning with ROS scavengers and inducing preconditioning with ROS generators given before the index ischaemia [43, 118, 134–143]. Also, several metabolites, including acetylcholine, BK, opioids and phenylephrine, trigger preconditioning-like protection *via* a mKATP-ROS-dependent mechanism [48, 67, 68, 138, 144, 145]. As stated in the *Cause of reperfusion injury*, ROS are also implicated in the sequel of myocardial reperfusion injury [57, 64, 65, 146 and references therein]. These studies supported the paradigm that ROS may be protective in pre-ischaemic phase, but are deleterious in the post-ischaemic phase. Thus the main idea was that ROS play an essential, though double-edged, role in cardioprotection: they may participate in reperfusion injury or may play a role as signalling elements of protection in pre-ischaemic phase [10, 48, 57–59, 64–68, 76, 125, 136, 138, 139, 144–147]. The importance of ROS signalling (as opposed to excess ROS in the development of injury) has been examined closely in great detail in recent years [*i.e*. 139–143].

Intriguingly, and in contrast to the above-described theory of ROS as an obligatory part of reperfusion induced damage, some studies suggest the possibility that some ROS species at low concentrations could protect ischaemic hearts [148–154]. Yet, from the above reported mechanisms of PostC, it appears that also ischaemic PostC is a cardioprotective phenomenon that requires the intervention of redox signalling to be protective [33, 76, 125, 126]. Moreover, as mentioned, very recently it has been shown that redox signalling is also required at the time of myocardial reperfusion to mediate the cardioprotection elicited by ischaemic preconditioning [128]. Therefore, the role of ROS in reperfusion may be reconsidered as they are not only deleterious. This fact may help to understand the variability in the results of studies aimed at proving a role of ROS in reperfusion injury. For instance, negative results came from trials in which free radical scavengers such as recombinant human superoxide dismutase or vitamin E were administered to patients with coronary artery disease or risk factors for cardiovascular events [155, 156]. In addition to the dual role of ROS (beneficial *versus* deleterious), among the reasons why these scavengers did not show any consistent benefit in these human studies may be: (1) the type of ROS generated (*e.g*. superoxide dismutase only removes the superoxide and not the hydroxyl radical); (2) the site of ROS generation (*e.g*. most scavengers scarcely enter into the cells) and (3) the rate of reaction between two ROS and/or scavengers. The

importance of the rate of reaction can be understood if we consider that, despite a five times lower concentration of nitric oxide with respect to superoxide dismutase, 50% or more of the available superoxide will react with nitric oxide to form $ONOO^-$ instead of reacting with superoxide dismutase [157, 158].

Notwithstanding the evidence of a protective role of ROS signalling in reperfusion, we were unable to reproduce cardioprotection with ROS generation by purine/xanthine oxidase given at reperfusion [33]. Since ROS scavengers (N-acetyl-L-cysteine or 2-mercaptopropionylglycine), given at the beginning of reperfusion, abolished both IP- and PostC-induced protection [33, 76, 125, 126, 128] it is likely that the type, the concentration and/or the compartmentalization of ROS may play a pivotal role in triggering protection at reperfusion time. We are performing studies in the attempt to clarify this issue.

Conclusions

It seems that the different mechanisms of PostC counteract the multiple mechanisms involved in reperfusion injury. In fact, PostC is a mechanical manoeuvre imposed during the early moments of reperfusion that attenuates many manifestations of reperfusion, such as apoptosis and infarction as well as endothelial activation and dysfunction. The mechanical procedure of PostC may induce a multiplicity of events that together attenuate reperfusion injury at many cellular and intracellular sites, as schematically represented in Figure 2. The mechanical event of PostC increases endogenous 'triggers' of protection such as adenosine, BK and opioids that lead to cardioprotective effects *via* signal transduction pathways and through signalling molecules, in which NO-cGMP-PKG-mKATP-ROS-PKC pathway/signalling play a pivotal role. Meanwhile early acidosis prevents mPTP opening, thereafter their opening is prevented by kinases (*e.g.* GS3Kβ). PostC attenuates oxidants and oxidant-mediated injury, but may also preserve the signalling function of both oxidants and nitric oxide in the early moments of reperfusion. Finally, by preserving other anatomical and cellular structures (*e.g.* endothelium), PostC may prevent the insurgence of oedema and may enhance the endogenous cardioprotective mechanisms of myocardium.

PostC not only has been successfully tested in humans, but has also the huge merit to have stimulated researchers to further study the concept of reperfusion injury and to search novel interventions or treatment strategies that could be combined with existing clinical therapies to decrease myocardial damage in the setting of I/R.

All studies on PostC stress the importance of the reperfusion phase for the myocardial salvage by properly timed protective interventions. Future studies are needed to determine whether pharmacological agents may be applied to reproduce or enhance cardioprotection by PostC.

In our opinion there are differences between the manoeuvres that triggers endogenous protective signalling leading to protection and therapies that requires prolonged infusion of a drug. The possibility to trigger PostC-like protection with intermittent drug(s) infusion in the early reperfusion phase, opens new prospective to pharmacological intervention to limit reperfusion injury. Also, remote PostC and dyssynchrony-induced PostC opens new possibilities for cardioprotection in the clinical setting that deserve to be further tested in humans.

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