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Review

Cardioprotection: A radical view
Free radicals in pre and postconditioning

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ABSTRACT

A series of brief (a few minutes) ischemia/reperfusion cycles (ischemic preconditioning, IP) limits myocardial injury produced by a subsequent prolonged period of coronary artery occlusion and reperfusion. Postconditioning (PostC), which is a series of brief (a few seconds) reperfusion/ischemia cycles at reperfusion onset, attenuates also ischemia/reperfusion injury. In recent years the main idea has been that reactive oxygen species (ROS) play an essential, though double-edged, role in cardioprotection: they may participate in reperfusion injury or may play a role as signaling elements of protection in the pre-ischemic phase. It has been demonstrated that preconditioning triggering is redox-sensitive, using either ROS scavengers or ROS generators. We have shown that nitroxyl triggers preconditioning *via* pro-oxidative, and/or nitrosative stress-related mechanism(s). Several metabolites, including acetylcholine, bradykinin, opioids and phenylephrine, trigger preconditioning-like protection *via* a mitochondrial K_{ATP}-ROS-dependent mechanism. Intriguingly, and contradictory to the above mentioned theory of ROS as an obligatory part of reperfusion-induced damage, some studies suggest the possibility that some ROS at low concentrations could protect ischemic hearts against reperfusion injury. Yet, we demonstrated that ischemic PostC is also a cardioprotective phenomenon that requires the intervention of redox signaling to be protective. Emerging evidence suggests that in a preconditioning scenario a redox signal is required during the first few minutes of myocardial reperfusion following the index ischemic period. Intriguingly, the ROS signaling in the early reperfusion appear crucial to both preconditioning- and postconditioning-induced protection. Therefore, our and others' results suggest that the role of ROS in reperfusion may be reconsidered as they are not only deleterious.

1. Aim

This review is focused on acute injury and cardioprotection. Acute cardiac ischemia/reperfusion injury is due to cardiac dysfunction and cell death (apoptosis, autophagy and necrosis), but does not include the inflammatory response, which follows necrosis. Acute cardioprotection is mainly mediated by activation of signaling pathways and post-translational modification of proteins. In particular, this review focuses on two well-defined mechanisms of cardioprotection, namely preconditioning and postconditioning. We first examine how free radicals promote cardiac injury and how, paradoxically, they can also protect myocardium against ischemia/reperfusion injury in preconditioning and postconditioning scenarios. With respect to redox-mechanisms in preconditioning we consider the so-called early phase or first window of protection only.

The mechanisms involved in the so-called second window or delayed protection will be only mentioned. Since they are mainly mediated by gene induction and de-novo protein synthesis, the reader is referred to other extensive reviews [1–12].

2. Definition of free radicals

Free radicals are molecules and atoms with unpaired electrons in their outer shell. They are highly reactive and are formed in processes that involve oxygen. Free radicals that originate from oxygen are called reactive oxygen species (ROS), whereas free radicals that originate from the reaction of oxygen with nitrogen are considered a subclass of free radicals and are called reactive nitrogen species (RNS). Oxygen free radicals include superoxide anion (O_2^{-}) and hydroxyl radical ($OH^{\bullet}$), while RNS include nitric oxide ($NO^{\bullet}$) and peroxynitrite ($ONOO^{-}$), the latter being originated by a reaction of O_2^{-} with $NO^{\cdot}$. RNS also include nitrogen dioxide ($NO_2^{\bullet}$) and nitrosyl hydride (HNO) [13]. Such RNS may have biologic activities distinct from those of the parent molecules [e.g. [14–38]. In particular, in a biological system where O_2 or superoxide are present, $NO^{\bullet}$ may be considered a reactive radical, when involved in the so-

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called "indirect effects". Yet, $NO^{\bullet}$ "direct effects" are defined as those reactions occurring between $NO^{\cdot}$ and specific biological molecules. [39]. Accordingly, $NO^{\cdot}$ may act as an important oxidative biological signaling molecule in a large variety of diverse physiological processes including cardioprotection, smooth muscle relaxation, blood pressure regulation, neurotransmission, defense mechanisms, immune regulation, and platelet function [36–38].

Hydrogen peroxide (H_2O_2), which is not a free radical, is often discussed with ROS because it is very reactive. It can be formed from superoxide anion and can generate hydroxyl radicals. In fact, strong oxidants are produced through Fenton-type reactions of H_2O_2 with transition metal complexes [for reviews see [14–24].

3. Sources of ROS and RNS

Several enzymes and biochemical processes can produce ROS and RNS. The major sources of oxidative stress in cardiovascular system are the following: (*a*) the enzymes xanthine oxidoreductase, (*b*) NAD(P)H oxidase (multisubunit membrane complexes), (*c*) nitric oxide synthases (NOSs) and (*d*) the mitochondrial cytochromes [14–27]. Actually, in the myocardium the main source of ROS is located in mitochondria. It seems that these organelles can produce a low amount of ROS from respiratory complex and can produce a large quantity of ROS from Monoamine Oxidase (MAO) and p66Shc [28]. Even NOSs can produce O_2^{-} and $OH^{\cdot}$, instead of $NO^{\cdot}$ under certain conditions, as in the absence of tetrahydrobiopterin (BH_4). Yet, it has been proposed that when $NO^{\cdot}$ is produced, HNO is the primary product, which subsequently is transformed to $NO^{\cdot}$ in the presence of superoxide dismutase (SOD) [9,19,20,24,29–33]. Usually, $NO^{\cdot}$ is generated in biological tissues by specific NOSs (constitutive and inducible forms), which metabolize L-arginine to citrulline with the formation of $NO^{\cdot}$ *via* a five electron oxidative reaction [1,2,9,10,14,16,18–24,31–36]. Mitochondria also contain a constitutive isoform of NOS [17,26,36].

Hemoglobin can also be considered a source of free radicals. In particular NOSs and hemoglobin are the principal sources of RNS, including $NO^{\bullet}$ and $NO^{\bullet}$ modified cysteine thiols (SNOs) in amino acids, peptides, and proteins. SNOs together with other biological reactions they convey $NO^{\bullet}$ bioactivity in processes that are collectively known as non-enzymatic sources of $NO^{\cdot}$ [12,39,40].

It has been suggested that triggering ROS alone is not sufficient for massive ROS release from mitochondria, but that the involvement of the mitochondrial permeability transition pore (mPTP) may be required. Zorov et al. [41], working with isolated adult rat cardiomyocytes, "triggered" ROS release *via* intracellular photoactivation of tetramethylrhodamine compounds. These triggered ROS were associated with mitochondrial depolarization along with mPTP induction and a consequent large burst of ROS from mitochondria. Thus a positive feedback loop of "ROS-induced ROS release" has been suggested [41].

3.1. Defense mechanisms

Biological systems have several defense mechanisms against ROS, which in some circumstances can be overcome and in others may be strengthened by therapies and protective maneuvers. These mechanisms may be enzymatic or non-enzymatic in nature. Enzymes include catalase, glutathione peroxidase, thioredoxin reductase and superoxide dismutase, while non-enzymatic antioxidants include coenzyme Q, thioredoxins, some vitamins and minerals, as well as omega 3 fatty acids [14,19,20,23,42]. Nitric oxide can also be considered an antioxidant under certain conditions [14,19,20,23]. For instance, $NO^{\cdot}$ protects against exogenously applied $ONOO^{-}$ *via* secondary reactions [14,16,23,31,43]. Actually most ischemia/reperfusion studies found a depletion of myocardial non-enzymatic antioxidants (including $NO^{\cdot}$) with either a decrease, an increase or no change in activities of myocardial enzymatic antioxidants [44–55]. Antioxidants may also play a role as triggers of signaling cascade [54]. The emerging picture is that cells are protected against ischemia/reperfusion injury when they maintain a delicate balance between the protective oxidant/antioxidant signaling versus detrimental effects for reviews see [44,54,55].

Traditionally free radicals have been considered useless by-products of metabolism, deleterious to biological systems. It is now clear that low concentrations of some ROS and RNS play a pivotal role in signaling cascades that are essential for many biological responses including cardioprotection (see below). First let's consider the generation of free radicals in ischemia/reperfusion, then their deleterious role, and finally their beneficial/signaling effects.

4. Role of free radicals in ischemia and reperfusion

4.1. Generation of free radicals during ischemia

It is likely that the generation of ROS in hearts during ischemia is occurring. Even in a global model of ischemia tissue oxygen concentration does not immediately fall to zero, therefore an initial generation of ROS is possible. In fact, production of ROS during ischemia has been observed in isolated cardiomyocytes and isolated hearts [44–46]. However, caution must be used in drawing conclusions since radicals can be generated in these models by the oxygen present in the ambient/solution and not present in intact ischemic myocardium. Nevertheless, the levels of ROS generated during ischemia are generally low, and their pathological significance is uncertain. Yet, the amount and type of ROS may be heterogeneous depending on the level of antioxidants and residual tissue oxygenation, which will depend on factors such as collateral flow and pre-ischemic treatments. It has been proposed that these ischemia-generated ROS may play an important signaling role [47] and that they could be the actual ROS that is triggering preconditioning [48] (see below).

4.2. Generation of free radicals during reperfusion

Reactive oxygen species are known to be produced in large quantities in the first few minutes of post-ischemic reperfusion as well as under inflammatory conditions. Since ROS and RNS can interact, several alternative outcomes are possible [e.g. 14–24].

It is generally accepted that steadily high concentrations of free radicals contribute to the pathogenesis of several diseases, especially when normal defense mechanisms are impaired. These are usually age-related, multi-causal diseases such as malignancy, infections, diabetes, arteriosclerosis, etc. Thus free radicals may contribute to initiation, manifestation and complications of specific diseases.

In 1973, Hearse et al. published a paper [49] in which they demonstrated that after anoxia, reoxygenation of isolated rat hearts resulted in a massive release of cardiac enzymes and cell death. Following Hearse's paper the term "oxygen paradox" was introduced and caused an explosion of studies investigating the effects of ROS on the heart.

4.3. Mechanisms of reperfusion-induced cell death

These mechanisms are not completely understood, but it is likely that the occurrence of oxidative stress related to the generation of free radicals might play an important role [5,8–10,12,28,44,50–52].

When the occlusion of the coronary branch that perfuses the ischemic 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 fibers already damaged by the ischemia, thus favoring the apoptosis [49–61]. In reperfusion, O_2^{-} reacts with the nitric oxide,

forming peroxynitrite ($ONOO^-$). Therefore, $ONOO^-$, may represent a sign of a reduced availability of $NO^\bullet$ [62,63] and it may participate with O_2^- to the lesion of myocardium [62–68]. In particular, high $ONOO^-$ concentrations are considered to be highly cytotoxic [66–68]. The contribution of peroxynitrite generation to myocardial and vascular dysfunction during ischemia and reperfusion, myocarditis, chronic heart failure, and various other cardiovascular pathologies has recently been reviewed [64,65,68]. Superoxide anion dependent damages are reduced if O_2^- is transformed into hydrogen peroxide (H_2O_2) by the superoxide dismutase. However, since in the presence of Fe^{2+} or Cu^{2+}, the H_2O_2 can be transformed into hydroxyl radical ($OH^\bullet$) and hydroxyl anion (HO^-), which are more toxic than O_2^- and H_2O_2, an increase in toxicity can occur. In addition $ONOO^-$ cytotoxicity can occur, which can be reduced by the addition of $NO^\cdot$ *via* a secondary reaction [16,19,20,43].

The oxidative injury due to increased free radical formation leads to changes in membrane permeability, membrane lipid bilayer disruption and functional modification of various cellular proteins. In fact, oxidative stress is associated with modifications of membrane phospholipids and proteins leading to peroxidation and oxidation of thiol groups [9,14,15,19,20,30,32,69,70]. In addition to cellular protein and lipid damage, alterations in myocyte function due to increased oxidative stress are associated with the effects of free radicals on subcellular organelles. For instance, it has been suggested that oxygen free radicals may influence Ca^{2+} movements in the cell by depressing the activity of both the sarcolemmal ATP-independent Ca^{2+}-binding and the activity of the ecto-ATPase. Yet, sarcolemma incubated for 1 min with superoxide results in 15% reduction in the sarcolemmal ATP-dependent Ca^{2+} accumulation and Ca^{2+}-stimulated ATPase activities. These effects may be oxygen-radical species specific [71,72]. Therefore, myocardial damages during ischemia/reperfusion can be due to a "vicious circle" with a massive quantity of ROS inducing the cellular and mitochondrial overload of Ca^{2+}, with consequent release of a massive quantity of ROS. Ultimately, these may contribute to the activation of mPTP, to the reduced availability of $NO^\cdot$ and to the activation of the transcription factor NFκB (For the role of NFκB see reviews [e.g. 1–12]).

The mPTP opening may represent the moment of "no return" for cell death. Although, mPTP opening might be caused by ischemia in the absence of reperfusion [73], the conditions associated with reperfusion, such as massive ROS accumulation, pH normalization and [Ca^{2+}] rise, create an ideal scenario for mPTP opening [3–7,74–76]. $NO^\cdot$ deficiency can also cause vasoconstriction and formation of micro-thrombi into the lumen of the small vessels [38,43,50,77,78]. These mechanisms, combined with the adhesion of the leucocytes to the endothelium, induced by ROS, can lead to the so-called "no-reflow phenomenon" [43,50,78]. As a consequence, free radicals have also downstream effects, resulting in the initiation and progression 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-ischemic inflammation [78–81].

Since cardiac oxidative injury, initiated by ischemia, occurs mainly during reperfusion, the relative proportion of injury occurring during ischemia versus reperfusion is likely to depend on the duration of ischemia. As we will see, this fact make cardiac protection in reperfusion possible.

In 1996, a study on the angiotensin II mediated vasoconstriction [82], demonstrated that ROS are not just general toxins, but they also act as second messengers in discrete signal transduction pathways. Then, the signaling role of ROS (as opposed to excess ROS in the development of injury) started to emerge. Nevertheless, free radicals are still considered by many physicians as dangerous/useless by-products of metabolism that should be eliminated whenever possible. However, scavengers of free radicals were not effective in making the heart resistant to cell death in ischemia/reperfusion [80,83–86]. In fact, not all redox conditions are detrimental to the heart. In recent years the importance of ROS signaling has been examined closely in great detail [i.e. [9,10,12,38,87–89].

5. Preconditioning

In 1986, ischemic preconditioning (IP) was first described by Murry et al. [90]. It consists of brief periods (a few minutes) of ischemia, separated from one another by brief periods (a few minutes) of reperfusion just prior to a sustained period of ischemia followed by reperfusion (see Fig. 1). Preconditioning limits the severity of the ischemia/reperfusion injury. 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 reduces ischemia/reperfusion arrhythmias and may reduce contractile dysfunction. At first, preconditioning protection lasts a few hours (2–3 h) immediately after the preconditioning maneuvers. This is the so-called early classical preconditioning or first window of protection, which is followed by a period without protection lasting about 12–24 h. Then, the protection reappears and lasts 24–72 h in the so-called second window of protection (late preconditioning). Preconditioning can also be obtained by inducing an ischemia on distant organs (remote preconditioning) or by pharmacological treatment before the index ischemia (pharmacological preconditioning) [for reviews see 1–12,91].

Cardioprotection by ischemic preconditioning is triggered by autacoids such as adenosine, bradykinin, opioids and platelet activating factor, produced as a response to the cycles of brief ischemia/reperfusion [for reviews see 1–12,91]. Their receptors couple to signal transduction pathways that ultimately inhibit the formation of mPTP during the reperfusion phase following the infarcting ischemia (Fig. 2) [92–96]. In fact, the opening of mPTP completely disrupts mitochondrial function and invariably leads to cell death by either necrosis or apoptosis. It is likely that a large number of cells are killed by this mechanism during reperfusion [73–76,92,94–97]. In fact, infarcts in preconditioned hearts are smaller than the size of those in non-preconditioned hearts which went an identical ischemic insult. A similar, but not identical, mechanism operates in postconditioned hearts (see below).

Cardioprotection by preconditioning requires a complex signaling cascade, which includes the opening of mitochondrial ATP-sensitive potassium channels (mitoK_{ATP}) [98–105]. In fact, diazoxide, a mitoK_{ATP} opener, pharmacologically preconditions the heart [98,100]. Intriguingly the diazoxide-induced preconditioning can be completely blocked when the drug is administered with a free radical scavenger, such as N-acetyl-cysteine (NAC) or mercaptopropionyl glycine (MPG). It is remarkable that these antioxidant compounds given during preconditioning maneuvers also prevent the protective effects of ischemic preconditioning. These results [98,100] confirmed that redox signaling is involved in cardioprotection by preconditioning.

6. Role of free radicals in preconditioning

Redox signaling in preconditioning is still not completely understood, but it is widely accepted that transient, low concentrations of

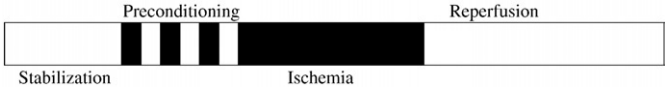

Fig. 1. Schematic representation of ischemic preconditioning protocol. Ischemic preconditioning (IP) limits the severity of the ischemia/reperfusion injury. Infarct area is reduced by 30–80% versus matched controls with matched risk areas. IP also reduces ischemia/reperfusion arrhythmias and may reduce post-ischemic contractile dysfunction.

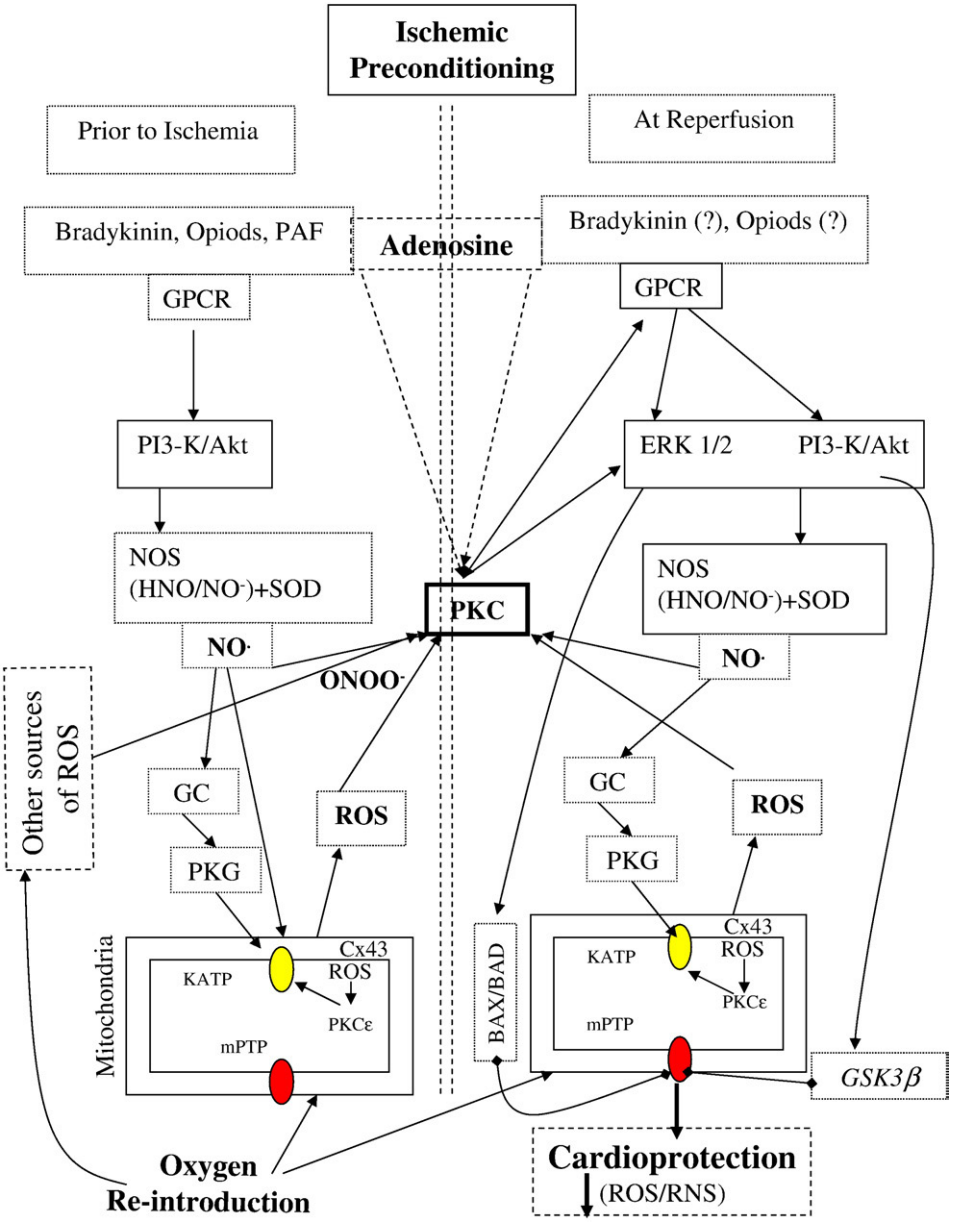

Fig. 2. Schematic representation of ischemic preconditioning pathway. This scheme shows the different components of the cardioprotective pathway activated prior ischemia and at the time of myocardial reperfusion by ischemic preconditioning. The two phases are schematically separated by vertical dashed lines. Prior to ischemia, many of the signaling pathway converge on PKC, which may represent the memory of the preconditioning. During the reperfusion, pathways converge on mPTP which are considered the end effector of preconditioning cardioprotection. A ROS signaling is considered in the two phases. Adenosine pathway acts *via* GPCR, but does not require ROS to activate PKC. More details are provided in the text. Acronyms are as in the text.

ROS and/or RNS may trigger protective mechanisms. Some RNS (i.e. $NO^{\bullet}$, HNO, $ONOO^{-}$) and ROS (i.e. O_2^{-} and H_2O_2) may be included among the triggers of preconditioning, and it is likely that they collaborate in inducing cardioprotection.

Even in 1988 Murry et al. [106] had linked ROS signaling to IP. They reported in a preliminary study that the *i.v.* administration of the free radical scavengers SOD and catalase had prevented preconditioning with ischemia in many but not all dog hearts. These data suggested that free radicals exerted a protective rather then deleterious effect in ischemia/reperfusion scenario. Among the first studies to show that exogenous and endogenous free radicals are involved in the myocardial protection by preconditioning were the studies by Baines et al. [107] and Tritto et al. [60]. Subsequently, Vanden Hoek et al. [48] demonstrated a ROS mechanism for "paradoxical" cardiomyocyte-protection with oxidants, and increased cell death due to antioxidants. They reported a loss of preconditioning protection and increased cell death when the antioxidant MPG was delivered only during the preconditioning period (then washed out prior to ischemia/reperfusion). Moreover, they reported that hydrogen peroxide (15 μM) alone given in lieu of hypoxic preconditioning for 10-min resulted in protection against cell death.

In situ, the source of ROS during ischemic preconditioning appears to be the mitochondria. In rabbit myocytes it has been demonstrated that maneuvers which cause potassium ions to enter mitochondria can cause ROS production. This type of mitoK_{ATP}-dependent ROS production can be abrogated if the electron transport chain is blocked by myxothiazol [88]. Yet, blocking mitochondrial MAO (monoamine oxidase) abrogates cardioprotection by preconditioning (Di Lisa F, unpublished observations). Recently, it has been reported that isolated mitochondria can be made resistant to mPTP opening by pretreatment with mitoK_{ATP} openers or potassium ionophores. This protection is both protein kinase C (PKC)- and ROS-dependent,

suggesting that the entire process, including ROS generation and PKCε activation, resides within the mitochondria [97,108]. In fact, Costa et al. [108] suggested that protein kinase G (PKG) may phosphorylate an unknown target at the mitochondrial outer membrane that would lead to activation of a PKCε pool within the intermembrane space. These authors also reported that PKG inhibits mPTP opening through a mechanism involving activation of two mitochondrial pools of PKCε, PKCε1 and PKCε2 [97]. The former will open mitoK$_{ATP}$, leading to a modest increase in matrix H_2O_2. H_2O_2 would promote further PKCε1 activation, and activating PKCε2 would inhibit mPTP formation. It seems that ROS released in response to mitoK$_{ATP}$ opening can limit injury *via* activation of a number of other kinases (e.g. p38-MAPK, JNK, TK and ERK 1/2) and can act on connexin-43 [3,4,7,8,75,85].

In cardiomyocytes connexin-43 is also expressed in the inner membrane of mitochondria [109–111]. Loss of mitochondrial connexin-43 decreases ROS formation by diazoxide, leading to a loss of pharmacological preconditioning-induced protection [110]. Therefore, PKG, mitochondrial PKCε1 and 2, mitoK$_{ATP}$ opening, connexin-43, ROS generation, and inhibition of mPTP formation are connected in a protective signaling pathway located inside the mitochondria. However, this seems to be in contrast with the finding that NADPH oxidase plays a pivotal role in ischemic preconditioning and that NADPH oxidase-deficient mice cannot be preconditioned [112]. It cannot be excluded that cooperation exists between different sources of ROS in preconditioning. Nevertheless, the cooperation between different sources of ROS in triggering preconditioning is not universally accepted, and little agreement exists among scientists about the phase (ischemia or reperfusion) and the nature of ROS involved in preconditioning triggering. The role of mitochondria seems universally accepted, but the source within the mitochondria is not clear. It has been suggested that ROS signaling occurs during the occlusion phases of preconditioning stimulus. Studies have demonstrated that superoxide and hydrogen peroxide [44–46] production begins very early during preconditioning ischemia/anoxia and ceases with the reintroduction of oxygen. Carmody and Cotter [47] and Vanden Hoek et al. [48] proposed that ischemia-generated oxidants from mitochondria are the source of ROS that trigger preconditioning.

It has been suggested that MPG is a cell-permeant ROS scavenger that removes hydroxyl radical and peroxynitrite very effectively and blocks the protective effects of preconditioning e.g. [97–101]. Based on these considerations, the group of Downey [113] used MPG to test whether the ROS that triggers protection are produced during the ischemic or the reperfusion phases of the preconditioning maneuvers. These authors concluded that protective redox signaling occurs when oxygen is reintroduced following the brief preconditioning coronary occlusion. In fact, when MPG was infused immediately before preconditioning and it was present in the myocardium during the brief coronary occlusion, but not during the subsequent reperfusion, preconditioning was still effective. However, when MPG was infused during the reperfusion phase of the preconditioning maneuver, protection was completely blocked. These data are in agreement with the fact that an occlusion followed by reperfusion is required to precondition the heart and with the observation that reperfusing the heart with hypoxic perfusate during preconditioning maneuvers increases the size of a subsequent infarction [113].

The putative target of ROS in preconditioning-induced redox signaling is the PKC (possibly PKCε2); in fact hearts can be preconditioned by simply infusing free radicals into the coronary arteries and that protection can be blocked by a PKC antagonist [60]. Recently, it has been reported that ROS can activate PKC *in vitro* by reacting with thiol groups associated with the zinc finger region of the molecule [114]. PKC is known to act (either directly or indirectly) on several proteins that are associated with the membranes of the mitochondria, including the mPTP, mitoK$_{ATP}$ channel, BAX/BAD and Bcl-2 [3–8,76,97,108].

In summary, the main idea of the preconditioning paradigm (Fig. 2) is that during the ischemic phase autacoids are released, activate their receptors, and lead to the opening of mitoK$_{ATP}$ *via* a NO$^{\cdot}$-mediated signaling; while during reperfusion phase the reintroduction of oxygen leads to ROS formation by mitochondria. Kinases such as PI3K, PKB (Akt), PKG and PKC are also involved either in the trigger (prior to index ischemia) or mediator (at reperfusion) phase. Therefore, in early preconditioning, signaling pathways converge again on the mitochondria to produce cardioprotection [6,28,76,94–105,107–112,115–118].

After we had shown that ROS played a pivotal role in postconditioning signaling [86, comments on 119] (see below), studies by different groups reported that ROS produced during the initial phase of reperfusion which followed a long-lasting infarcting ischemia (index ischemia) were necessary for a preconditioned heart to be effectively protected. A ROS scavenger added in the perfusate at the end of an infarcting ischemia blocks cardioprotection in hearts that have been previously preconditioned [120,121]. Nevertheless, cardioprotection by a direct PKC activator given at reperfusion is unaffected by the ROS scavenger MPG. These observations are consistent with the idea that during the reperfusion phase a new protective signaling is required. This includes the activation of several kinases, which are called reperfusion injury salvage kinases (RISK) [3,4,8,75,93,95,120,121–123]. Therefore, the paradigm is that the production of autacoids during infarcting ischemia and the re-population of autacoid receptors during the reperfusion phase may lead to the activation of RISK, the opening of mitoK$_{ATP}$ *via* a NO$^{\cdot}$-mediated signaling, and again to ROS formation by mitochondria, which play an important signaling role in the reperfusion phase (Fig. 2). Thus, protective redox signaling take places during the early reperfusion phase. It is likely that this signaling occurs before the massive ROS burst, which characterizes reperfusion injury.

In brief, ROS signaling during preconditioning maneuvers and during the early reperfusion phase, which follows an index ischemia, makes the heart resistant to reperfusion injury. The reperfusion injury in a control/naive heart includes massive ROS production and mPTP opening. In a preconditioned heart whether this massive production does not occur or is less injurious is not very clear. Since "ROS-induced ROS release" requires mPTP opening [41], it is more likely that in protected hearts (where mPTP opening does not occur) a massive ROS production does not take place. So that in protect hearts RISK, mitoK$_{ATP}$, and ROS signaling, in concert, lead to protection including mPTP closure. In other words, when mPTP formation is avoided ROS production is reduced because ROS-induced ROS release does not occur. However, an improvement of antioxidant properties, including increased NO$^{\cdot}$ synthesis, plays also a pivotal role [14,19,23,31,44,54,70].

Regarding nitric oxide and RNS, it has been demonstrated that they can act both as a trigger and as a mediator of the preconditioning response in a variety of species. NO$^{\cdot}$ can be produced during preconditioning maneuvers and during the reperfusion phase as well. The role of endogenous NO$^{\cdot}$ in classic ischemic preconditioning was controversial. In the early 1990 s NO$^{\cdot}$ donors were believed to decrease myocardial necrosis and reperfusion-induced endothelial dysfunction [125]. Similar observations were made concomitantly in the gut mesentery [126]. Protective effects of NO$^{\cdot}$ donors were also later demonstrated in brain and liver ischemia [18,127]. Cohen and Downey's group suggested that exogenously administered NO$^{\cdot}$ could trigger the preconditioned state through a free radical-mediated process not shared by endogenous NO$^{\cdot}$. 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$^{\cdot}$ participates in triggering *in vivo* preconditioning [128]. The ambiguity (exogenous versus endogenous) was in part a result of the extrapolation of *in vivo* pathogenic conditions from *in vitro* toxicological experiments. A retrospective analysis of 92 studies evaluating the modulatory effects of NO$^{\cdot}$ on the severity of ischemia/

reperfusion injury in non-preconditioned myocardium showed beneficial effects of exogenous or endogenous $NO^{\bullet}$ in the majority (67%) of the contributions [1]. In the ischemic heart, $NO^{\bullet}$ can provide protection through several mechanisms including inhibition of platelet aggregation [129] and neutrophil activity and adhesion [130] in a cGMP-dependent manner. The effect of $NO^{\bullet}$, either through exposure to $NO^{\bullet}$ donors or L-arginine, is proposed to be dependent on the stage of ischemia/reperfusion, with maximal protection against myocardial injury occurring with drug administered either immediately before or during the onset of reperfusion [1,2,77]. Furthermore, infarct size and post-ischemic myocardial functional recovery in endothelial NOS knockout mice are worse than in wild-type [131–134]. In addition, endothelial NOS-deficient hearts show a transient (about 1 h) increase in contractility in the early periods of reperfusion. In these hearts, bolus administration of $NO^{\bullet}$ donors before the index ischemia prevents the hypercontractile response during early reperfusion while significantly reducing myocardial damage [133]. While prevention of hypercontractility may depend on the effect of high concentration of NO and cGMP in myocardium, protection is likely to be due to the antioxidant properties of $NO^{\bullet}$, which not only safeguards against chemical insult from massive ROS (or RNS) [14,19,23,70] but also exerts other beneficial effects. For example, $NO^{\bullet}$ is a powerful vasodilator and may improve blood flow during reperfusion [9–11,18,134–136]. $NO^{\bullet}$ also inhibits inositol-1,4,5-trisphosphate signaling, thereby reducing calcium overload [137], and mediates PKC translocation at reperfusion, thus protecting contractile function in isolated rat heart [138]. Although the reaction of $NO^{\bullet}$ with O_2 produces $ONOO^{-}$ [67,69], which is considered to be cytotoxic [54,139], $NO^{\bullet}$ donors confer vascular protection against exogenously applied $ONOO^{-}$ [43] *via* secondary reactions [14–16]. Nitric oxide also modulates myocardial oxygen consumption in the normal and failing heart [36,140,141].

The role of $NO^{\bullet}$ is now fully recognized in preconditioning, and its effects are well-defined, particularly in the second window of protection [for review see 1–12]. Intriguingly, in a comparative study of the activity of Angeli's salt, an HNO donor, with that of the pure $NO^{\bullet}$ donor diethylamine/$NO^{\cdot}$ (DEA/$NO^{\cdot}$), we demonstrated that equimolar (1 μM) amount of HNO appeared to be more effective than $NO^{\bullet}$ as a preconditioning agent [142]. In fact, in isolated rat hearts post-ischemic contractility was similarly improved with ischemic preconditioning or pre-exposure to Angeli's salt, compared with control or DEA/NO pre-treated hearts. Similar results were obtained regarding the limitation of infarct size and lactate dehydrogenase release. Moreover, the preconditioning features of Angeli's salt appeared to be specific to HNO signaling because the HNO scavenger NAC (4 mM) completely reversed the beneficial effects of Angeli's salt. These data suggest that reactive nitrogen oxide species are not only necessary but also sufficient in triggering myocardial protection against reperfusion through species-dependent, pro-oxidative, and/or nitrosative stress-related mechanisms. In fact, the rapid reaction of thiols with HNO produces RSNHOH species [21,31]. This intermediate can then react with a reduced thiol to form an oxidized disulfide. Therefore, biomolecules that can be oxidized by $ONOO^{-}$ may also be converted to the same end-products following exposure to Angeli's salt through either direct binding of HNO or indirectly *via* its oxidized intermediate. The enhanced, thiol-dependent protection induced by HNO compared to equimolar $NO^{\bullet}$ suggests a mechanism involving either an oxidative alteration or the involvement of critical thiols or metals [32,142]. Therefore, it is not surprising that using NAC a complete inhibition of the protective effect exerted by Angeli's salt can be obtained without significant alterations to any other of the studied parameters. The effect of Angeli's salt on thiols is in line with the fact that free radicals can activate PKC *in vitro* by reacting with its thiol groups [114]; thiols are also oxidized by $ONOO^{-}$ which has been indicated as preconditioning inducer [113]. Whether HNO *per se* or its oxidative product is responsible for preconditioning remains to be elucidated as does whether the isolated heart model can be extrapolated to *in vivo* conditions and to a more sustained response (i.e., late preconditioning).

7. Definition of postconditioning

Although it has been shown that preconditioning can make ischemic myocardium resistant to ischemia/reperfusion injury, the need for a pretreatment could limit its clinical application. During late 1980s and 1990s researchers intensively investigated whether or not pharmacologically modified reperfusates could reduce reperfusion injury. Moreover, several studies examined whether myocardial damage by ischemia/reperfusion could be limited if reperfusion was initiated in a gentle (low pressure) manner as opposed to standard full flow (high pressure) [143–150]. It soon became clear that the window of opportunity during reperfusion is very limited. The efficacy of these mechanical or pharmacological interventions initiated later than 5–10 min after the beginning of reperfusion resulted in markedly limited addition of cardioprotection to reperfusion *per se*.

These observations together with the data on preconditioning prompted the Vinten-Johansen group [151] to study cardioprotection with stuttering reperfusion immediately after an infarcting ischemia (Fig. 3). This procedure was called ischemic postconditioning (PostC). PostC can be defined as intermittent interruption of coronary flow in the very early phase of a reperfusion, which leads to cardioprotection. Vinten-Johansen group [151] was the first to describe a role for PostC against infarct size. In such a study the PostC protocol was 30 s of reperfusion followed by 30 s of coronary occlusion, which were repeated for three cycles at the very onset of reperfusion. Then, also for PostC, it became clear that the window of opportunity is very limited. PostC initiated later than 1–3 min after the beginning of reperfusion resulted ineffective in inducing cardioprotection [152–156].

Depending on the species, models and other factors, PostC reduces infarct size by ~20–70% versus matched controls with matched risk areas [3,4,12,92,155,156]. Unlike preconditioning, PostC does not protect against stunning [157,158]. There is an emerging agreement across multiple models and species that PostC may reduce apoptosis, necrosis, endothelial dysfunction and endothelial activation, thus leading to a reduced endothelia/leukocyte interaction and to a reduced ROS inflammatory formation. A reduced incidence of reperfusion arrhythmias has been also observed [159–161]. It is noteworthy that already by 1994 Grech and Ramsdale [162] had reported that coronary artery re-canalization by percutaneous transluminal coronary angioplasty (PTCA) induced an idioventricular rhythm, which was interrupted several times by the re-inflation of the balloon and thus restoring sinus rhythm.

It is now widely accepted that PostC has interrelated passive and active components in its underlying cellular protective mechanisms [3,4,12,91,155,156]. During the PostC maneuvers, the washout of intracoronary release of adenosine is significantly delayed so that it can take part in protection [152]. An involvement of kinins and opiods in the early reperfusion phase has also been suggested [91,163–165]. These autacoids acting on their cardiac sarcolemmal receptors can triggers the signaling cascade of PostC [4,12,91,155,156]. In fact, postconditioning involves signal transduction pathways, which are

Fig. 3. Schematic representation of ischemic postconditioning protocol. Ischemic postconditioning (PostC) limits the severity of the ischemia/reperfusion injury. Infarct area is reduced by 20–70% versus matched controls with matched risk areas. PostC also reduces reperfusion arrhythmias and may reduce post-ischemic endothelial dysfunction.

similar to those seen in preconditioning [4,12,91,155,156]. In particular, autacoids can trigger a complex protective signaling pathway (Fig. 4) including RISK signaling [4,12,91,155,156,163–170]. The exact subtypes of adenosine, opioids, and bradykinin receptors involved in triggering PostC are still a matter of controversy. With regard to the role of adenosine in a ischemia/reperfusion scenario a plethora of complex and conflicting results is reported. These conflicting results are also due to the presence of different adenosine receptors (A_1, A_{2A}, A_{2B}, A_3) with different degrees of sensitivity during normoxia, ischemia, and reperfusion [for reviews see 91,171,172]. Despite a general agreement on the involvement of adenosine receptors in cardioprotection at reperfusion, the exact timing and receptor subtype(s) that are responsible for triggering PostC remain controversial [91,171,172]. The role of adenosine A_{2A} receptors was recently confirmed by abrogation of PostC in mice lacking A_{2A} [173]. However, another *in vivo* rabbit study reported a blockade of PostC by an A_{2B} receptor antagonist, MRS1754, but not by A_1 antagonist, DPCPX, nor A_{2A} receptor antagonist, 8-(13-chlorostyryl)-caffeine [153]. Yet, in an *in vivo* rat model using the blockers of adenosine A_1 (DPCPX), A_{2A} (ZM241385) or A_3 (MRS1523) receptors at the onset of reperfusion, Kin et al. [152] concluded that A_{2A} and A_3, but not A_1 receptors are important for PostC-induced cardioprotection. In contrast, in a recent study Donato et al. [174] reported that the administration of the A_1 blocker, DPCPX, abolished PostC-induced reduction of infarct size in hearts subjected to global ischemia/reperfusion, regardless they were isolated from normal or hypercholesterolemic rabbits. These data were confirmed by a recent study in which a complete loss of PostC-induced cardioprotection was observed in A_1-knockout mice

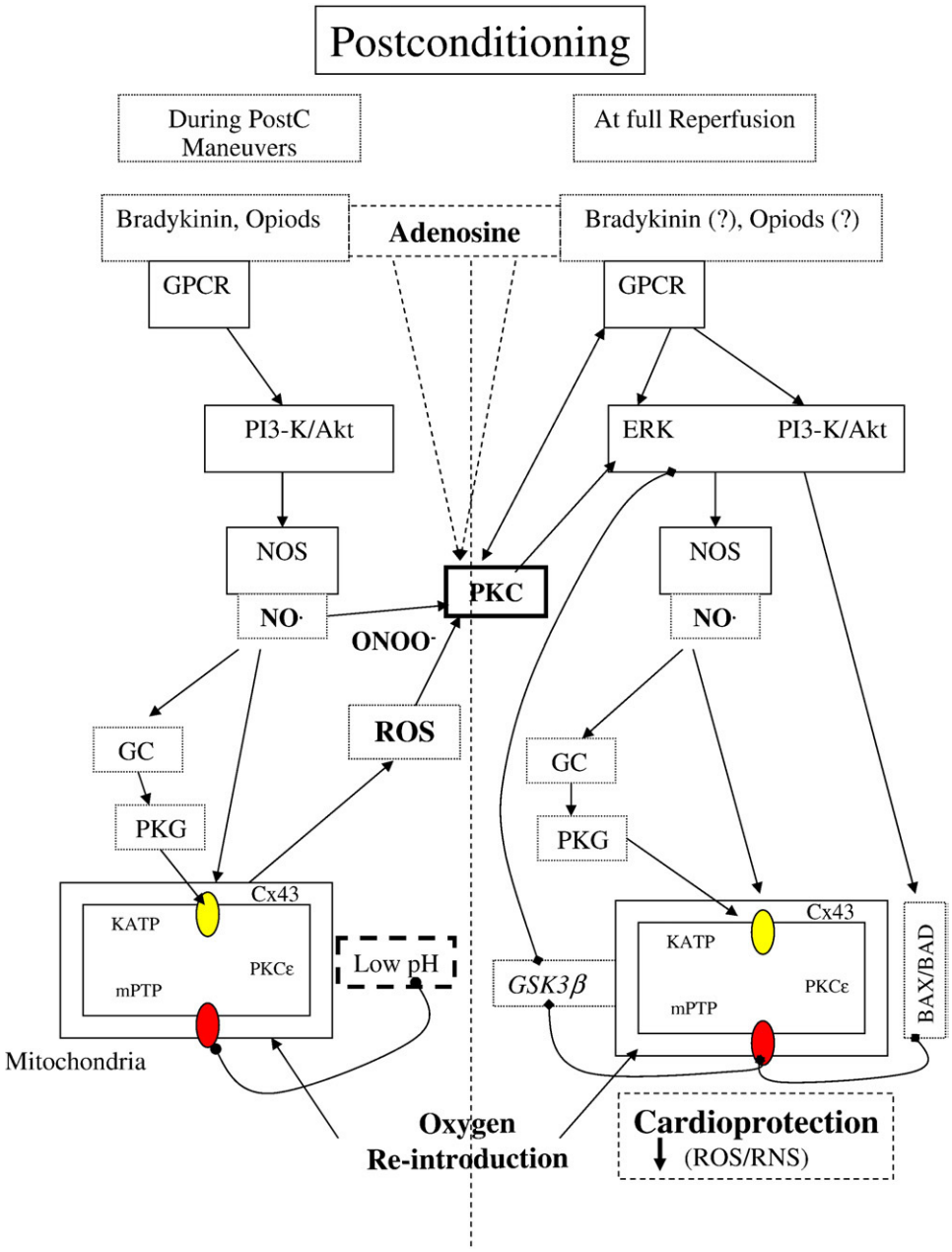

Fig. 4. Schematic representation of ischemic postconditioning pathway. This scheme shows the different components of the cardioprotective pathway activated during and after (at the time of full reperfusion) postconditioning maneuvers. The two phases are schematically separated by vertical dashed line. During postconditioning maneuvers many of the signaling pathway converge on PKC, which may represent the memory of the postconditioning, at the same time the low pH prevent mPTP opening. During the reperfusion, protective pathways converge on mPTP which are considered the end effector of postconditioning cardioprotection. A ROS signaling is considered during PostC maneuvers. It is likely that adenosine pathway acts *via* GPCR, but does not require ROS signaling. More details are provided in the text. Acronyms are as in the text.

[175]. These last reports strongly suggest that adenosine A_1 receptors are essential for cardioprotection at reperfusion. In our recent publication [167], we confirmed the involvement of adenosine receptors in PostC in isolated rat hearts. In fact, a three min blockade of all adenosine receptors with 8-SPT prevented PostC protection. However, neither adenosine infusion for three min (i.e. without intermittence) nor intermittent-adenosine application in the early reperfusion phase triggered PostC protection. We suggested that adenosine coupling with receptors is necessary, but not enough, for postconditioning protection. It is likely that other concomitant events (i.e. intermittent bradykinin B_2 and/or opioid receptor activation with ROS production) are necessary to induce postconditioning protection. This hypothesis of concomitant ROS release is also supported by the observations that ROS signaling is mandatory in PostC and that adenosine (possible *via* A_{2B} receptors) may induce preconditioning without ROS production [99]. It is, thus, possible, that adenosine *per se* does not trigger protection because it does not trigger ROS signaling; yet adenosine receptor occupation may be necessary to support PKC activation by PostC (see below).

If the role of adenosine is controversial and unclear, there is, instead, a unanimous agreement on the crucial importance of bradykinin B_2 receptors in myocardial protection, such as ischemic and pharmacological preconditioning [12,91,99,104,163,164,175–177]. We were the first to show that pharmacological inhibition of B_2 receptors by administration of HOE140 or WIN64338 blocks the infarct size reduction afforded by PostC or intermittent bradykinin infusion at the onset of reperfusion [163]. The notion that bradykinin B_2 receptors are indispensable in PostC, is further supported by a recent study in which the infarct-limiting effect of PostC observed in wild-type mice was completely lost in B_2-knockout mice [175]. This study also showed that genetic deficiency of bradykinin B_2 receptors did not significantly modify myocardial tolerance to ischemia/reperfusion injury, as indicated by the similar infarct size observed in wild-type and knockout mice in the absence of PostC. Similar results were observed after *in vivo* ischemia/reperfusion in B_2-knockout mice [176,177]. These results further suggest that the intact presence of bradykinin B_2 receptors at the early onset of reperfusion is critical for transmitting the cell survival signals of PostC. Moreover, bradykinin-induced preconditioning requires ROS production [99,104], which is also crucial for PostC-induced cardioprotection [86,119,163].

8. Role of free radicals in postconditioning

The importance of a reduced endothelial activation, neutrophil adherence and consequently redox-sensible mechanisms in PostC was shown by Zhao et al. [151]. PostC decreases the expression of P-selectin, an adhesion molecule, on the surface of endothelial cells, with both a reduction in neutrophil adhesion on the postconditioned coronary artery endothelium and a reduced accumulation of neutrophils in the area at risk [151]. A reduction in superoxide anion generation in the perivascular area has also been observed in the proximity of risk area of postconditioned hearts [178–180]. 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 was not clear. It now seems clear that the reduced neutrophil accumulation and its reduced ROS production is an effect of PostC protection. As a matter of fact, PostC exerts a marked cardioprotection in leukocyte-free models (isolated buffer perfused hearts and isolated cardiomyocytes) [e.g. [86,152,158,163,167,169,181,182].

8.1. Cardioprotection by postconditioning is redox-sensitive

The above findings of a reduced ROS production in PostC [151,178–180] are in line with the idea that massive ROS production is implicated in the sequel of myocardial reperfusion injury. As mentioned above, it has already been established that preconditioning triggering, i.e. the period that precedes the index/infarcting ischemia, is redox-sensitive (i.e. requires ROS signaling). This was demonstrated by both avoiding preconditioning with ROS scavengers and inducing preconditioning with ROS generators given before the index ischemia [e.g. 52,60,98–100,103–107,110]. Also, many G-protein-coupled receptor (GPCR) activators, including acetylcholine, bradykinin, opioids and phenylephrine, trigger preconditioning-like protection *via* a mitoK$_{ATP}$-ROS-dependent mechanism [e.g. 52,60,98–100,103–107,110]. All these studies supported the paradigm that ROS may be protective in pre-ischemic phase, but are deleterious in the post-ischemic 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 signaling elements of protection in pre-ischemic phase [e.g. 4–11].

Intriguingly, and in contrast with the above-described theory of ROS as an obligatory part of reperfusion-induced damage, some studies in the 1960–70s [183–187] and in more recent years [188,189] suggested the possibility that some ROS species at low concentrations could protect ischemic hearts. Based on this suggestion we were the first to consider that ROS could also be included among the triggers of PostC [86,119]. Moreover, as reported above, very recently it has been shown that redox signaling is also required at the time of myocardial reperfusion (i.e. after infarcting ischemia) to mediate the cardioprotection elicited by ischemic preconditioning [120,121].

Therefore, the role of free radicals in reperfusion may be reconsidered as they are not only deleterious. This fact may also help in understanding the variability in the results of studies aimed at proving a role of ROS in reperfusion injury [83–85]. In fact, we and other authors have shown that ROS scavengers such as NAC and MPG given during the PostC maneuvers prevented the protective effects [86,119,163,165]. It is possible that the low pH during the PostC cycles prevents mPTP opening, while the intermittent oxygen bursts produced during PostC maneuvers 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 (Fig. 4). The importance of the role of acidosis in triggering PostC protection has been confirmed by two independent laboratories [74,95,190]. Recently, it has been reported that redox signaling and a low pH at the time of myocardial reperfusion are also required to mediate the cardioprotection triggered by ischemic preconditioning [120].

From the above reported mechanisms of PostC, it appears that ischemic PostC is a cardioprotective phenomenon that requires the intervention of redox signaling to be protective. However, we have been unable to reproduce cardioprotection with ROS generation by purine/xanthine oxidase given at reperfusion [163]. Since ROS scavengers (NAC and MPG), given at the beginning of reperfusion, abolished both IP- and PostC-induced protection [86,119–121,163,165], 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. Our present studies aim to clarify this issue. Recent preliminary reports by the Ferdinandy Group [191] as well as by our group [192] point towards an important role for $ONOO^-$. These observations stress the importance of $NO^\cdot$ biochemistry in redox conditions.

As mentioned above, the mechanism by which $NO^\bullet$ protects myocardium includes the activation of guanylate-cyclase [1–12,31,36]. As an inducer of the protection, $NO^\bullet$ may also directly open the mitoK$_{ATP}$ channels [102,193]. Therefore, $NO^\bullet$ acting on mitochondria may play a relevant role in protection both through activation of these channels and *via* modulation of the respiratory chain; both mechanisms favor ROS signaling, which can lead to protection [31,88,103–111]. A relevant role of $NO^\bullet$ may also be attributed to the endothelial protection brought about by this molecule [10,11,38,125,132,136,194] as well as to its role as an antioxidant under certain conditions [9,14,19,20,195]. Moreover, as discussed above, one of the pivotal steps in

cardioprotection is redox dependent-PKC activation by reaction with thiol groups associated with the zinc finger region of the molecule [114]. The involvement of $ONOO^-$ in this reaction/activation has been widely considered in late preconditioning [for reviews see 2,7,10] but not adequately considered in postconditioning yet.

Also the one-electron-reduction product of $NO^\bullet$, HNO (nitrosyl hydride), has been scarcely studied in an ischemia/reperfusion scenario. As mentioned above, we have shown that low doses of Angeli's salt, a donor of HNO, induce early/classical preconditioning against myocardial damages, which were more potent than the protective effects induced by equimolar concentration DEA/NO [142], yet the HNO donor seems deleterious in reperfusion [196]. However, there is evidence that $NO^\bullet$ may also be involved in the cardioprotection by ischemic PostC. When the NOS inhibitor N^ω-nitro-L-arginine methyl esther (L-NAME) was given 5 min before the beginning of reperfusion to *in situ* rabbit hearts, the infarct-limiting effect was abolished [154]. We have shown that $NO^\bullet$ participates in rat heart PostC, but NOS inhibitors given for the entire period of reperfusion only blunted, but did not suppress, the protective effect of PostC [166]. Paradoxically, the same inhibitor, given only during PostC maneuvers suppressed the protective effects [163]. At present we have not yet found an explanation for this apparent paradox. In our previous study, we argued that $NO^\bullet$ might be produced in postconditioned heart by both NOS and non-enzymatic mechanisms. Nitric oxide can then activate the guanylyl cyclase to produce cGMP and/or to react with O_2^- to form $ONOO^-$, which mediate the protection [166] (also see below). The infusion of a NOS inhibitor only during PostC maneuvers [163] may alter the equilibrium between ROS and $NO^\bullet$ thus leading to the production of the wrong kind of radicals, which do not trigger the protective pathway. It can be argued that in the absence of this protection the stronger limitation of $NO^\bullet$ production by NOS inhibitors may be protective during reperfusion. In fact, it has been demonstrated that NOS inhibitors can attenuate ischemia/reperfusion damage [1,65,77]. The different doses of NOS inhibitors applied and the different basal levels of $NO^\bullet$ endogenously produced may explain these disparities. Recently, a significant role for nitrite in ischemia/reperfusion injury has been suggested. Nitrite is an oxidation product of $NO^\bullet$ which was considered inert. However, mice treated with sodium nitrite at the end of myocardial ischemia showed marked reduction in infarct size compared with nitrate-treated mouse hearts, an effect abolished by the $NO^\bullet$ scavenger, 2-phenyl-4,4,5,5,-tetramethylimidazoline-1-oxyl 3-oxide (PTIO) [197]. These authors proposed that the post-ischemic myocardial environment might allow for the acidic reduction of nitrite to $NO^\bullet$. Alternatively, xanthine oxidoreductase may catalyze such a transformation of nitrite during hypoxic/acidotic conditions [198]. The beneficial and deleterious effects of $NO^\bullet$ and nitrite in pathophysiological conditions and the contradictory results about the effects of $NO^\bullet$ during reperfusion have been reviewed by several authors [1,9,10,65,77,199].

In summary, it appears that the trigger pathway for PostC involves the following sequence of events: occupation of surface receptors (i.e. adenosine, bradykinin and opioid receptors), activation of NOS and non-enzymatic processes to produce $NO^\bullet$, activation of cGMP-dependent kinase (PKG), opening of $mitoK_{ATP}$, production of ROS and possibly synthesis of $ONOO^-$, and finally activation of PKC and MAPKs as well as inhibition of GSK3β, which put the heart into a protected state [3–7,76,93–95,123]. The protected state includes a central role of the prevention of mPTP opening by acidosis in the early phase of reperfusion and by the aforementioned mechanisms (RISK) (Fig. 4). We consider ROS among the triggers, as they are necessary during PostC maneuvers. Nevertheless, PostC activated the RISK pathway, with increased expression of the phosphorylated form of eNOS (p-eNOS) as one of the results [3–7]. It is, thus, likely that after NOS activation cGMP is produced and PKG activated, then $mitoK_{ATP}$ channels are opened and ROS may be produced by mitochondria. Therefore cGMP, PKG, $mitoK_{ATP}$ and possibly ROS may be considered as mediators of PostC protection, which are likely to converge on PKC activation. As said, this activation may depend on thiol reaction. Hence, it is likely that acidosis transiently prevents mPTP opening until PKC is activated and GSK3β inhibited to put the heart into a protected state *via* long-term mPTP closure. The role of RISK and GSK3β in PostC protection has recently been questioned [200,201] and a role for JAK/STAT pathway has been proposed [202,203]. We suggested an important role for cyclooxygenase (COX) and prostacyclin in PostC [164]. Thus the role of RISK, GSK3β, COX and JAK/STAT pathways in PostC need to be studied further. This also suggests a redundancy of mechanisms that are not mutually excluding each other, but include an involvement of ROS signaling.

8.2. In conclusion

Although the mechanisms of cardioprotection by pre and postconditioning are similar, important differences go beyond the simple intervention of mechanisms before and/or after ischemia. It is likely that the ROS signaling involved in pre- and post-ischemic phases are not the same. Since exogenous ROS are able to induce pre but not postconditioning, it is also likely that compartmentalization plays a pivotal role in the latter case. In particular, the concomitance of acidosis, $NO^\bullet$ formation, mPTP inhibition and ROS generation seems mandatory in PostC.

From a clinical point of view, situations in which the ischemic event can be predicted are limited, so that the practical application of preconditioning maneuvers is very low. Yet, postconditioning can be applied in different clinical situations (e.g. percutaneous coronary intervention, coronary artery bypass grafting, and cardiac surgery) when reperfusion is initiated. Initial clinical studies are in agreement with the success and extent to which PostC reduces infarct size and myocardial injury, even in the presence of other pathological conditions [124,204–207].

The possibility to induce PostC pharmacologically would be of paramount importance. Indeed, in the literature, several studies are focused on the effects of cardioprotective drugs administered either before or during the index ischemia or at the onset of reperfusion. Some of the drugs leading to myocardial salvage if given at reperfusion include PKC activators, adenosine, bradykinin, erythropoietin, nitric oxide donors and opiods. However, the strategy of adding reperfusion therapeutics to save myocardium has not yet been adopted into clinical practice. We opened a new strategy mimicking PostC with very brief intermittent periods of drug infusion in the early reperfusion [163,164,167]. Understanding the PostC mechanisms may improve the protective results of drug administration that is otherwise limited by systemic hemodynamic side effects or other harmful or unwanted side effects of the agents. The beneficial role of free radicals even in early reperfusion, as demonstrated by pre and postconditioning studies, open the future to mimetic agents that can induce cardioprotection without interfering with early redox conditions (ROS signaling) triggered by protective maneuvers.

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