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Redox Balance and Cardioprotection

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Abstract

Coronary artery disease is a major cause of morbidity and mortality in the Western countries. Acute myocardial infarction is a serious and often lethal consequence of coronary artery disease, resulting in contractile dysfunction and cell death. It is well known that unbalanced and high steady state levels of reactive oxygen and nitrogen species (ROS/RNS) are responsible of cytotoxicity, which in heart leads to contractile dysfunction and cell death. Pre- and post-conditioning of the myocardium are two treatment strategies that reduce contractile dysfunction and the amount of cell death considerably. Paradoxically, ROS and RNS have been identified as a part of cardioprotective signaling molecules, which are essential in pre- and post-conditioning processes. S-nitrosylation of proteins is a specific posttranslational modification that plays an important role in cardioprotection, especially within mitochondria. In fact, mitochondria are of paramount importance in either promoting or limiting ROS/RNS generation and reperfusion injury, and in triggering kinase activation by ROS/RNS-signaling in cardioprotection. These organelles are also the targets of acidosis, which prevent mitochondrial transition pore opening, thus avoiding ROS induced ROS release. Therefore we will consider mitochondria as either targets of damage or protection from it. The origin of ROS/RNS and the cardioprotective signaling pathways involved in ROS/RNS-based pre- and post-conditioning will be explored in this article. A particular emphasis will be given to new aspects concerning the processes of S-nitrosylation in the cardioprotective scenario.

Key words: Ischemia/Reperfusion Injury; Mitochondria; Postconditioning; Preconditioning; Redox Signaling, S-Nitrosylation.

Introduction

Although high levels of reactive oxygen species (ROS) and reactive nitrogen species (RNS) induce structural modifications of the proteins, lipids and genes that impact on cell function and death, ROS/RNS can activate signaling pathways that contribute to *ischemic preconditioning* (IP) and *postconditioning* (PostC). IP and PostC are two cardioprotective strategies able to protect the heart from ischemia/reperfusion (I/R) injury [140, 151, 240] *via* activation of physiological mechanisms, which comprise ROS and RNS production by several processes, including mitochondrial enzyme activation/inactivation [45, 57, 81, 124, 160, 161, 167, 219]. It is well known that in these two cardioprotective strategies a pivotal role is also played by the gradual normalization of intracellular pH in the initial phase of reperfusion [45, 97, 165]. We will see that this favors redox signaling and the activation of a complex cascade of signal molecules. Moreover a role of paramount importance is played by the prevention of the opening of the so-called mitochondrial permeability transition pore (mPTP) in post-ischemic phase in which also acidosis and redox signaling play pivotal roles [43, 44, 97].

Myocardial cell death due to I/R is a major cause of morbidity and mortality in the Western world. In the past few decades, it has become clear from experimental studies that the myocardial damage due to I/R can be reduced. This has motivated intense study of the mechanisms of cardioprotection. An initial impulse to the concept of cardioprotection was given by the seminal studies of Braunwald and colleagues in the early seventies [130, 131]. The finding that the infarct size was related to ischemic time led to the corollary that the infarct size could be limited by initiating reperfusion as early as possible (from those studies we now say: *"Time is muscle"*). This point of view paved the way to revascularization by thrombolysis, angioplasty, stenting and coronary artery bypass grafting surgery. Therefore, there was a scientific rationale to develop therapies to reduce the infarct size. These therapies were aimed at attenuating the recognized contributors to acute ischemic injury. Studies became focused on pharmacological treatments during or after ischemia. Many of these approaches failed in clinical trials. In

particular, therapies with antioxidants failed in patients with cardiovascular diseases, despite the fact that many of these diseases including atherosclerosis, heart failure, and myocardial infarction appear to be related to altered redox regulation and increased *oxidative stress* [129]. The question then arises: why did these antioxidants studies fail? The answer to this question is not easy, as the reader can understand from the detailed discussion on this topic given elsewhere [206, 213, 215]. First of all, we must consider that a substantial lack of efficacy on cardiovascular morbidity and mortality has been demonstrated for different doses of "traditional" antioxidants, in diverse population groups and in different clinical trials [227]. This meta-analysis [227] clearly suggests that we need more hypothesis-driven clinical-trial designs. These should be guided by a rigorous and deeper understanding of the complex pathophysiology of ROS/RNS. Moreover, future research should develop newer antioxidant compounds, more specific, with a more favorable pharmacodynamic profile and/or impacting oxidative stress through different mechanisms. Yet, one fundamental issue is: what population is likely to benefit from antioxidant supplementation? The majority of the studies, so far, enrolled patients at high risk, characterized by established atherosclerotic damage, which is unlikely to recovery with antioxidant treatment. Besides populations under study, the role of other trial-design issues, such as duration of treatment, outcomes measured and concomitant therapy should not be underestimated. The failure of "traditional" antioxidants may also be partially explained by the difficulty to delivery these drugs to the sites of ROS generation at the right moment and in sufficient amount to quench oxidative stress [10, 75] and by the fact that chemical reactions between ROS, RNS and their targets may be faster than those with exogenous antioxidants [213]. Moreover, among the reasons of the failure of antioxidants, we can also suggest the fact that in the past their administration was based on the concept that ROS/RNS only have deleterious effects. Instead, as we know, some ROS/RNS play a role as intracellular mediators in physiological processes such as vasodilatation, cell growth and angiogenesis. Collectively, these considerations may provide an explanation to the failure of "traditional" antioxidants. Thus, rather than discarding the oxidative stress hypothesis, evidence suggests that we need more rigorous understanding

of the complex physiology of ROS/RNS and of the multifaceted pharmacology of antioxidants. As we will further discuss, an important role of ROS/RNS has also been shown in protective mechanisms against I/R injury, such as pre- and post-conditioning. Thus, in certain settings, ROS/RNS quenching might have deleterious implications that offset the impact on organ function. In other words, the concept of *redox balance* implies that the reactive species are also important determinants of the normal cellular function, signal transduction underlying epigenetic- and genetic-regulation, and determination of cellular phenotype, even in I/R scenario. Clearly interference with this physiological *redox signaling* leads to compromised cell function. Therefore, cellular function requires an optimal redox environment and alterations in redox potential have major cellular consequences. Importantly, the redox conditions of the cell may be considered a continuum that range from reductive to oxidative stresses, in which the biological extremes of the redox spectrum play pivotal roles in disease pathogenesis. The notion that reductive stress predisposes to cardiomyopathy as much as oxidative stress is gaining ground among biologists. Due to space constrain we cannot consider the reductive stress issue here but the reader is kindly redirect to a recent review on this topic [27].

There is no doubt that I/R perturbs the redox balance towards redox stress; however a simple use of a general antioxidant may not be the solution. In fact, as discussed above, several trials failed when antioxidants were used to limit injury due to ischemic coronary artery disease. Therefore, we think that it is mandatory to better understand the pathophysiology of redox activity in each pathological condition before we try to propose any specific antioxidant therapeutic strategy. This is especially true for cardioprotection against I/R injury in which a delicate beneficial-to-deleterious switch seems to be present. A better understanding of the complex regulatory systems, particularly in human hearts, is necessary to progress with this approach [86].

In the present review we bring readers up to date on biologically useful concepts in a constantly changing field. In particular, we will consider: *1)* the novel viewpoint of redox balance as a variable continuum, in

which chemistry and signaling role of redox species may vary depending on biological conditions (*e.g.* normal-perfusion, ischemia, reperfusion etc), *2)* the importance of redox signaling (oxidative and nitrosative signaling) in the physiological regulation of many processes, 3*)* the concept that a reactive species is not always deleterious and 4*)* the new concept that reactive species may be beneficial even in reperfusion.

Clearly, from 1986, when the *IP phenomenon* was described by Murry, Jennings and Reimer [140], the research in the field increased exponentially. In this seminal study the authors reported that 4 cycles of 5-min ischemia/5-min reperfusion prior to a 40-min coronary occlusion decreased infarct size by 75% compared to the control dogs with 40-min of coronary occlusion only. Then in 2003 another big impulse to research was given by the most improbable observation that the intermittent interruption of coronary flow in the very early phase of a reperfusion leads to cardioprotection [240]. Vinten-Johansen's group in such a study suggested that *PostC protocol* obtained with 30-s of reperfusion followed by 30-s of coronary occlusion repeated for three cycles at the very onset of reperfusion is as cardioprotective as preconditioning. It soon became clear that in these two cardioprotective strategies the redox signaling plays a role of vital importance. These strategies to protect the heart trigger protective pathways that modify cell membrane, cytosol and organelle composition and function. In particular, both pre- and post-conditioning include triggers, mediators and effectors converging on mitochondria, which are involved in many phases of the protective process. Here we explore particular aspects of these two strategies and especially those related with *redox stress* and *redox signaling*. In this context the role of *S-nitrosylation* of critical proteins, especially within mitochondria is rapidly gaining ground.

Ischemic preconditioning, a brief synopsis

Ischemic preconditioning has become the *'gold standard'* treatment by which other therapies are judged; it consists of a series of brief periods of I/R performed before the infarcting ischemia (mechanical preconditioning), and has shown that infarct size can be modified by triggering endogenous mechanisms

of protection. Preconditioning not only reduces infarct size, but also apoptosis, endothelial dysfunction and activation as well as neutrophil adhesion and inflammatory response. It also reduces stunning (the contractile depression which follows ischemia) and arrhythmias [21, 52, 149] (Figure 1).

It has been shown that preconditioning can also be induced by pharmacological treatments and a similar protection can be induced by exercise protocols [49]. Moreover preconditioning, improving endothelial function also ameliorates vascular responsiveness to endothelial-dependent vasodilatation. Finally, it has been shown that IP can slow mitochondrial metabolism [33, 49, 67, 150, 152, 157, 162].

When considering preconditioning it is useful to think in terms of triggers, memory, mediators and end-effectors. The current theory of IP protection considers a *trigger phase* in which agonists of surface receptor couple through multiple pathways to protein kinase C (PKC) activation *via* an important contribute of mitochondrial ROS/RNS signaling. There is an *ischemic phase* in which PKC and perhaps other kinases act as a *memory*, and there is a *reperfusion phase* in which the *mediators* and *end-effectors* are recruited [43]. Among mediators and end-effectors of protection the recruitment of protective pathways and prevention of the opening of mPTP play a role of paramount importance [98].

In the *trigger phase*, during the brief preconditioning periods of I/R, membrane receptor population occurs (*i.e.*, ligands are formed/released and in autocrine/paracrine fashion will target their receptors). This will lead, via a complex mechanism (see below), to the opening of mitochondrial ATP-sensible K^+ channels (mK_{ATP}) and consequently to mitochondrial ROS/RNS formation [155] ROS/RNS, together with a diacylglycerol (DAG) dependent mechanism, will lead to the activation and translocation to the membrane of PKCs, which will act as a memory leading to protection [102, 125]. In fact, it has been proposed that the activation of PKCs requires the physical translocation of the enzymes to their docking sites [99]. However, the hypothesis that IP is the result of translocation of cytosolic PKC into the membranes has been challenged by

other authors [198]. Although many studies have shown that various PKC isoforms are translocated in preconditioned myocardium, few have clearly and unequivocally correlated the translocation with the presence of a protected state. For a review on the translocation theory the reader may see [41].

The ROS/RNS formation is important; in fact ROS/RNS scavengers, given in this phase, will block preconditioning cardioprotection [2, 62, 146, 150, 199, 216, 217, 232, 235]. For example, scavenging of reactive species with ascorbic acid blunts the beneficial effect of ischemic preconditioning in pigs [199]. The ROS/RNS step explains why receptor population during ischemia without brief reperfusion does not protect. Although ROS can be formed also during ischemia, the brief reperfusion during preconditioning maneuvers is important for the re-introduction of oxygen and the formation of protective ROS/RNS. The origin of ROS/RNS and the role of ROS/RNS in I/R injury and cardioprotection will be considered more in deep in this brief review (see below).

The pathways that lead from receptor activation to ROS/RNS formation are quite complex (Figure 2). Several studies used activators, blockers and proteomic analyses to reveal the signal transduction events between the membrane receptors and the production of ROS/RNS with a signaling role. The reader is referred to other excellent reviews on this area [193, 233]. The sequence of intracellular events is summarized below.

The cardioprotection of *ischemic preconditioning* is induced by mechanical maneuvers (*i.e.* brief cycles of I/R) that trigger the release of various molecules (*e.g.* adenosine, acetylcholine, opiates and/or bradykinin). The cardioprotection can be also induced directly by the infusion of one of these agonists or other agents for few minutes; that is *pharmacological preconditioning* [80, 233]. The consequence of the coupling of some of these agonists to their receptors is the activation of phosphatidylinositol 3-kinase (PI3K) with a pivotal role of matrix-metalloproteinases in the transactivation of the epidermal growth factor receptor (EGFR). Briefly, the matrix metalloprotease may cleave the pro-heparin-binding EGF (pro-

HB-EGF) to liberate the soluble HB-EGF, which activates the EGFR resulting in EGFR dimerization. This leads to autophosphorylation of tyrosine residues on both EGFRs and binding of Src kinase to form a signaling module which activates PI3K [32, 63, 72, 114, 157, 207, 229].

Then PI3K activates protein kinase B (PKB or Akt) with the subsequent activation of nitric oxide synthase (NOS)/nitric oxide ($NO^{\bullet}$)/guanylyl cyclase (GC)/ guanosine monophosphate 3'5' (cGMP) pathway that in turn activates protein kinase G (PKG). PKG, in cooperation with *intra-mitochondrial* PKCε, will lead to mK_{ATP} channel opening, potassium entry and ROS/RNS formation. It seems that mK_{ATP} channel opening is first mediated by PKG phosphorylation of a mitochondrial outer membrane protein; thus leading to increased ROS, which then activate an intra-mitochondrial PKCε, which also phosphorylates mK_{ATP} channels and leave them in a prolonged open state [47, 64, 66]. This prolonged open state of mK_{ATP} may be co-responsible for "memory," which is seen with all preconditioning [47]. ROS will spread out of the mitochondria and will lead to kinase activation, including PKCs, which may represent the memory effect. In this ROS formation and spreading may play an important role the presence of connexin 43 (Cx43) in the mitochondrial inner membrane [17, 62, 84, 160, 182, 184] (see also below).

Therefore, in *ischemic (memory) phase* PKCs and possibly others kinases will act as a memory effect, which may include in the sensitization of the low-affinity A2b adenosine receptors (A2b-AR) [43]. Consequently, in reperfusion (*mediator phase*) A2bAR and other receptors can be reactivated by endogenous adenosine and other autacoids (*e.g.,* bradykinin and opioids) released by the previously ischemic cardiomyocytes. In this way cardioprotective pathways can be activated in reperfusion to avoid mPTP opening. In fact, it has been reported that mPTP is maintained in a closed state during ischemia and in non-protected (naïve) hearts typically opens at reperfusion, when calcium overload, ROS/RNS stress and pH recovery all occur. These are consequential to the restoration of blood flow to the ischemic heart and they are all recognized as potent triggers that increase the open probability of the mPTP. The opening of these pores will lead to cell death by different mechanisms including mitochondrial

destruction from mitochondrial swelling and membrane rupture and cytochrome-C release which induces programmed cell-death pathways [54, 148]. mPTP inhibition at the time of reperfusion appears pivotal to all strategies of cardioprotection thus far studied [18, 54] (Figure 3). However, despite intensive investigation, the precise molecular identity of the mPTP components remains unknown. Both the adenine nucleotide translocase (ANT) and the voltage-dependent anion channel (VDAC) are reported to be components of the mPTP, together with cyclophilin D (CyPD). Nevertheless, neither ANT nor VDAC seems to be an obligatory component of mPTP [8, 107]. Given the fact that mPTP structure remains elusive, mPTP inhibition is viewed with skepticism by some authors. With no doubts the identification of the actual molecular components of the mPTP may provide new strategies for mPTP inhibition and perhaps novel therapeutic targets for cardioprotection [77].

Therefore, given the importance of mPTP formation at reperfusion, one can speak of *reperfusion injury*: the damage that occurs typical in reperfusion phase. In other words, at reperfusion we can consider three populations of cells: *1)* cells that were killed by ischemia, *2)* cells that had sub-lethal injury and will survive, and *3)* cells that are alive but will die from mitochondrial pore opening. These latter cells are the target of preconditioning and, as we will see, they are also the target of postconditioning. Hence, both pre- and post-conditioning limit reperfusion injury.

The question arises: what inhibits transition pore opening? We will see the importance of the recruitment of protective pathways, such as the so-called Reperfusion Injury Salvage Kinases (RISK) pathway and Survival Activating Factor Enhancement (SAFE) pathway, the persistence of acidosis and possibly a new ROS/RNS signaling in reperfusion phase [78, 88, 90, 97, 151].

What turns on PI3K and RISK pathway at reperfusion? The answer is: adenosine, bradykinin and other autacoids that accumulated during ischemia. For brevity we will concentrate on adenosine and bradykinin.

The importance of adenosine in the reperfusion phase in preconditioned hearts has been demonstrated by the experiments of Solenkova *et al.*[202], who have shown that non-selective and selective adenosine receptor antagonists block preconditioning protection if given in reperfusion. Proteomic studies have also revealed the importance of PI3K/Akt and extracellular-signal-regulated kinase 1/2 (ERK1/2) which are considered the initiators of the RISK pathway [43, 78]. As said above, it is PKC that makes the preconditioned heart sensitive to its own adenosine. In fact, an activator of PKC given at reperfusion is able to induce protection, whereas both PKC antagonists and adenosine receptor blockers given at reperfusion abolish protection [171].

Therefore, in a nutshell we can say that in *preconditioning* the protective maneuvers are performed before the infarcting ischemia, but the protection does not occur against ischemia only. The protection is mainly against the reperfusion injury. The repopulation of receptors by autacoids and the activation of multiple kinases of the so called RISK pathway are critical events leading to prevention of mPTP opening and cell death. In the trigger phase mK_{ATP} channels and ROS/RNS signaling are important players; the actual protection occurs in the reperfusion rather than the ischemic phase, and reactivation of adenosine receptors, ROS/RNS signaling and multiple kinases are critical events, which lead to prevention of mPTP opening and limitation of reperfusion injury. Of note, an antioxidant given early in reperfusion may abort the cardioprotection by preconditioning (see below).

Some in the “cardioprotection community” might challenge various aspects of the above scenario. In this brief synopsis we cannot consider all the aspects and controversies on preconditioning. For instance, there are authors that challenged the role of autacoids, such as bradykinin [156] and researchers that even challenged the existence of diazoxide/glibenclamide-sensitive K_{ATP} channels in rat mitochondria [39]. There are also investigators that have failed to find any RISK-pathway relevance in preconditioning’s protection (this may be species-specific) [87, 200]. Moreover, it must be stressed that cardioprotection may also occur completely independent of RISK and SAFE pathways. For instance, neither modifications

of RISK signaling nor those of SAFE signaling contributed to the increased tolerance to myocardial I/R injury usually induced by Cx43 deficiency [188]. Also matrix-metalloproteinase inhibition has been seen to be cardioprotective, independent of Akt/ERK/CyPD/mPTP activity and to be additive to the protection observed following inhibition of mPTP opening [14]. These data are indicative of redundant parallel pathway(s) to protection in ischemic/reperfused heart. The reader is kindly directed to several reviews on these pathways and controversies [*e.g*. [81, 233]].

Points to be underlined:

How and when the IP stimulus generates the ROS signaling.

It has been proposed that ROS may be produced during brief preconditioning ischemia and that these ischemia-generated ROS may play an important signaling role. Therefore ischemia-generated ROS could be the actual ROS that is triggering preconditioning [10, 225].

Other authors are of the opinion that ROS that triggers protection are produced during the reperfusion phases of the preconditioning maneuvers [56].

It seems that mitochondrial ROS generation and limitation of mPTP formation are connected in a protective signaling pathway located inside the mitochondria [79]. In fact loss of mitochondrial Cx43 decreases ROS formation by Diazoxide, leading to a loss of pharmacological preconditioning-induced protection [84]. Moreover it has been suggested that PKG, PKCε1 and 2, mK_{ATP} opening, and Cx43 are all located within mitochondria and in concert regulate mitochondrial ROS production [47] Yet, it has been stressed that mPTP transient opening may be fundamental for the ROS signaling upon IP and pharmacological preconditioning with Diazoxide [76] or Isoproterenol [185]. However, the mitochondrial origin of protective ROS seems to be in contrast with the finding that nicotinamide adenine dinucleotide phosphate (NADPH) oxidase plays a pivotal role in ischemic preconditioning and that NADPH oxidase-deficient mice cannot be preconditioned [13]. 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 [161].

How ROS/RNS activate protein kinases

Several studies indicate that the protein kinase activation events that are initiated by I/R can be reproduced by oxidative agents [3, 9, 39, 40, 170] suggesting that ROS/RNS are among the principal responsible for the reversible protein kinase activation observed subsequently to I/R. In fact, ROS/RNS may induce either reversible or irreversible oxidation of proteins/enzymes (Figure 4). Reversible modifications include, for instance, modifications of cysteine residues such as disulfides, sulfenamides, protein SNO and S-glutathionylation. Sulfenamides can be further oxidized to sulfinic or sulfonic acid, which are generally considered irreversible modifications [37]. Besides cysteine residues other targets of ROS may be iron-sulfide centers and zinc-finger domains of proteins.

ROS, such as peroxide, may be responsible of kinase activation *via* tyrosine phosphorylation or *via* the induction of the release of zinc from zinc-finger domains of PKC [109, 110]. Besides kinase activation, the increased kinase activity and consequent phosphorylation may also be the result of the well-known inhibitor effect of ROS on phosphatases, whose enzymatic activity is abolished by oxidation of a cysteine residue in their active site [37, 186]. Alternatively, the PKC activation may be determined by a downstream product of hydroxyl radical, probably a product of phospholipid oxidation [66]. Protein kinase C activation, besides depending on redox action, is classically activated by lipid second messengers, such as DAG. Thus, one can wonder whether these two activation modes involve the same or alternate mechanisms. Recently, it has been suggested that both lipid activators and oxidation target the zinc-finger domains of PKC, supporting a unifying activation mechanism [238]. As reported above, PKCε activation contributes to the $NO^{\bullet}$ mediated cardioprotection. This activation has been associated

with the tyrosine nitration of PKCε [9]. Thus, both nitration [9, 221] and S-nitrosylation [53] may be involved in direct kinase regulation. Hence, $NO^{\bullet}$ and RNS may interfere with tyrosine phosphorylation in several ways: *1)* they can diminish the efficacy of a protein as a substrate for tyrosine kinases [71, 108], *2a)* can lead to their direct activation by increasing phosphorylation of kinases (*e.g.* focal adhesion kinase, Src kinase and mitogen activated protein kinases) [16, 136, 147] or *2b)* by inactivating phosphatases [212].

Finally, we must consider that irreversibly oxided/nitrated proteins may be preferentially degraded via ubiquitin mediated pathways [203], which could determine the stability/activity of enzymes in cells.

Therefore, while we highlight the activation of enzymes (*e.g.* kinases) by redox mechanisms, they could also affect the inhibition and/or degradation of other enzymes [164]. Clearly, much more work is needed to clarify the relative importance of each of the above proposed mechanisms.

How IP reduces the generation of detrimental ROS/RNS at reperfusion

One of the major mechanisms for the limitation of the formation of detrimental ROS/RNS at reperfusion is the avoidance of the long lasting opening of mPTP and the consequent phenomenon termed *ROS-induced ROS release* (RIRR). In fact mitochondria can respond to elevated ROS concentrations by increasing their own ROS production [25]. This oxidative stress is particularly exacerbated in the presence of pressure overload in ischemic-reperfused hearts [137].

Long-lasting mPTP opening and RIRR are favored by several factors, including calcium overload and pH recovery. Also the constitutive NOSs are calcium-dependent. Therefore preconditioning, by limiting this calcium overload and pH recovery may prevent detrimental ROS/RNS at reperfusion [44, 80]. It is of note that NOS function is also dependent on redox conditions and its S-glutathionylation. This can be important in cell signaling and I/R; for a review see [242].

The translocation of protective factors of both RISK and SAFE pathways to mitochondria may activate intra-mitochondrial protective mechanisms and may contribute to the limitation of ROS formation by these organelles [80]. Of course the ROS that trigger RIRR may have a different origin (see below), therefore other mechanisms may play a role, such as modification of the activity of endogenous antioxidants.

Remote preconditioning, a brief synopsis

It is worthwhile to spend few words on *remote ischemic preconditioning*, which has different but also similar aspects to classical preconditioning and may have an important clinical application [82].

Transient non-lethal ischemia and reperfusion of an organ (remote organ) may confer resistance to a subsequent episode of lethal I/R injury in another target organ or tissue. This intriguing phenomenon is called *remote ischemic preconditioning*. It was originally described as an intramyocardial protection, which can be "transferred" from the myocardium served by one coronary artery to another [174]. Then it was demonstrated that myocardial injury can be drastically reduced by applying brief ischemia and reperfusion to an organ or tissue distant from the heart before the onset of myocardial ischemia [68, 175]. Now we know that remote organ protection may be applied to several organs and may represent a general form of inter-organ protection against I/R injury, including also the limbs in humans. At least three hypotheses have been proposed to explain the occurrence of remote ischemic preconditioning [82]. A first hypothesis (*neural hypothesis*) considers the release of local autacoids at the site of remote preconditioning that in turn trigger neuronal reflexes protecting the target organ. The second hypothesis (*humoral hypothesis*) proposes that humoral factors (*e.g.* adenosine, Angiotensin I, bradykinin, CGRP, endocannabinoids, erythropoietin and opioids) or some as yet unidentified endogenous substance(s) generated in the remote organ or tissue. These are then carried through the circulation to the site of I/R injury where they exert their effects recruiting the various pathways of cardioprotection implicated in preconditioning. The third hypothesis (*systemic hypothesis*) suggests that transient ischemia and

reperfusion of an organ provokes a systemic protective response, which suppresses inflammation and apoptosis [82]. A role for ROS signaling in the remote organ and a reduction of redox stress in the target organ has been also proposed [93, 194]. In particular, it appears that remote ischemic preconditioning is triggered by a combination of increased $NO^{\bullet}$ synthesis, opening of mK_{ATP} channels and increased ROS production in remote tissue. Moreover, it seems that $NO^{\bullet}$ is working upstream and acts *via* activation of mK_{ATP} channels, which subsequently increases the production of ROS [93, 194]. Of note, it has been suggested that in man the activation of a single cell surface receptor may be sufficient to trigger preconditioning, while blockade of multiple pathways may be required to abolish the protective effect of remote preconditioning [156]. In fact, a recent study does not support a role for endogenous bradykinin as a mediator of I/R injury or remote ischemic preconditioning in human arms. In particular, these authors have demonstrated that systemic administration of a bradykinin B2 receptor antagonist has no effect on the endothelial vasomotor dysfunction induced by I/R injury or the protection conferred by remote ischemic preconditioning [156].

Postconditioning, a brief synopsis

Preconditioning can be useful in programmed cardio-surgery interventions. However, it is not useful in the patient with acute myocardial infarction because it must be instituted prior to ischemia [201]. What solved this quandary was the most improbable discovery that multiple cycles of reperfusion/ischemia after an infarcting ischemia could also induce a potent protection against I/R injury. This phenomenon has been called *postconditioning* and it opened a new avenue of research [240].

It is now clear that *ischemic postconditioning* reduces: infarct size, post-ischemic arrhythmias, apoptosis, endothelial-dysfunction and -activation. However it is not clear if it can reduce myocardial stunning [151, 164, 166] (Figure 1).

Initially, *ischemic postconditioning* was attributed to *passive* mechanisms, which considered postconditioning as a sort of gradual reperfusion leading to a reduced accumulation of neutrophils and a reduced ROS/RNS stress. This would reduce calcium overload and cell death [240].

However, it was soon clear that postconditioning is able to activate intrinsic protective pathways. This was demonstrated almost simultaneously by us and the group of Yellon using an isolated buffer perfuse heart model, in which, of course, there were no leucocytes: for this reason *active* intra-myocardial mechanisms have been highlighted [153, 218].

The picture about postconditioning cardioprotection includes now *passive and active mechanisms* that lead to prevention of mPTP opening and cardioprotection. Again the RISK pathway is involved [78] (Figure 5). We have shown that postconditioning is NOS and guanylyl cyclase dependent [158]. Most importantly, it has been shown that if we delay postconditioning maneuvers just for few minutes PostC is not longer protective [104]. The relevant time-window may be species-specific and may be conditioned by concomitant diseases and by the suited end-point (infarct size, stunning or arrhythmias) [201].

A few years after the discovery of postconditioning phenomenon we made “a really radical observation”: *ROS/RNS signaling is protective in early reperfusion* [167].

ROS/RNS are required in early reperfusion to trigger protection *via* PKC and mK_{ATP} activation. In fact cell permeant ROS-scavengers, an mK_{ATP} antagonist or a PKC blocker given in early reperfusion block postconditioning’s protection [167]. In other words postconditioning also uses the same steps seen in preconditioning trigger pathway. “A really radical observation” is the expression used by Cohen and Downey when they wrote a comment on our article and confirmed our proposal that ROS do trigger postconditioning’s protection using a similar model [57]. We confirmed the importance of ROS/RNS also in bradykinin induced postconditioning [160]. The importance of ROS/RNS in postconditioning

cardioprotection obtained with brief intermittent ischemic periods or pharmacological tools has been confirmed by other authors, *in vivo*[44].

A very important role in postconditioning cardioprotection may be played by the *delayed intracellular pH recovery* in postconditioned heart [97] (Figure 5). In fact, many research groups have proposed that postconditioning protects because it maintains acidosis during early reoxygenation which inhibits mPTP opening, directly or indirectly *via* calpain inhibition, and allows ROS/RNS signaling. This gives the heart enough time to trigger cardioprotective pathways and to *"precondition itself"* against reperfusion injury. In fact, in the absence of acidosis in early reperfusion (*i.e*. when pH recovers freely and swiftly) there is an over production of deleterious ROS. Instead the acidosis, limits, but does not eliminate, ROS production. Incidentally, this reduced ROS production is mandatory to trigger the cardioprotective processes. In fact, if the ROS production is impaired with the addition of antioxidants in this phase the protective maneuvers are ineffective (see below). Recently, the groups of Yellon and Downey have proposed that in *preconditioning* there is also a delayed recovery of pH and a ROS signaling in early reperfusion [81, 115]. We have shown the importance of bradykinin receptors [160], while the groups of Downey [42] and Vinten-Johansen [103] have shown the importance of different adenosine type 2 receptors subtypes in postconditioning.

Since the delayed recovery of pH has been given so much importance, the question arises: is acidic perfusion protective in early reperfusion? The answer is yes: brief acidosis in early reperfusion (BAER) is as protective as postconditioning. However, some concerns derive from the possibility that it might increase arrhythmias [183]. This observation might be species-specific and is in contrast with the strong anti-arrhythmic effect of postconditioning obtained with intermittent reperfusion/ischemia. In fact, acidic reperfusion reduces the incidence of ventricular fibrillation in rat heart models of regional ischemia [5, 95], and there are early reports which propose brief intermittent I/R as a maneuver against ventricular arrhythmias in reperfusion in different species [65, 105, 141], including pigs [96] and humans [73]. The

ROS-dependent protection of BAER has been also demonstrated by Downey Group [45]. We have also confirmed that BAER is protective and that this protection can be avoided by a ROS-scavenger [165]. Moreover, we have shown that in postconditioning and acidosis the reaction of $NO^{\bullet}$ with ROS to form RNS switches from *Nitration* to *S-nitrosylation* (SNO) of proteins [165]. In fact, 3-nitrotirosine levels are reduced and S-Nitrosylated proteins are increased at the 10^{th} min of reperfusion. As a matter of fact, it is now believed that SNO of critical proteins plays a very important role in nitric-oxide dependent cardioprotection. SNO of many proteins has been found in mitochondria [138] (see also below). Nevertheless, PostC increased cardiac 3-nitrotyrosine concentration in the normal but not in the cholesterol-fed heart when measured at the 5^{th} min of reperfusion. These data suggest that a balance between nitrosative stress and S-nitrosylation is involved in the triggering mechanism of PostC and that comorbidities may cause the loss of the cardioprotective effect of PostC, at least in part, *via* deterioration of the nitrosative trigger [116].

To sum up "*pre- and post-conditioning are united at reperfusion"* [78] with the important role of physiological mechanisms such as acidosis, ROS/RNS signaling and RISK activation. Importantly, pharmacological postconditioning can protect the heart, but this must be applied very soon in reperfusion, because the first minutes of reperfusion are critically important. The low pH during the postconditioning cycles prevents transition pore opening, mitochondria can make the right ROS/RNS to activate PKC and put the heart into a protected state; then PKC activates pathways that prevent transition pore opening after the pH is normalized.

As stated, there is another parallel pathway activated by pre and post-conditioning. This is *SAFE pathway* (Figures 2 and 5), which includes the activation of the cytokine tumor necrosis factor alpha (TNFα) and the transcription factor signal transducer and activator of transcription-3 (STAT-3). SAFE is now recognized as a pathway that can confer protection in ischemic pre and post-conditioning independently from RISK pathway. Nevertheless these two apparently parallel pathways (RISK and SAFE) may cross talk

and both converge on mitochondria [78]. Whether all protective strategies activate both RISK and SAFE pathways, or alternatively some strategies may activate one or the other, it is not clear at moment. For instance, we have evidences that ischemic PostC may activate both RISK and SAFE pathway. However, PostC with Diazoxide (an activator of mK_{ATP} channels) may trigger a redox-sensitive translocation to mitochondria of RISK elements only. Thus protection by Diazoxide does not need SAFE pathway activation [163].

We have seen the importance of acidosis, ROS/RNS signaling and protective pathways that prevent mPTP opening in early reperfusion. Protective pathways have mitochondria as point of convergence. The hope is that the discovery of these major components of protection may help to identify novel pharmacological targets for protecting the ischemic heart at the time of myocardial reperfusion.

Point to be underlined:

How the PostC stimulus generates the ROS signaling at reperfusion- given the overwhelming presence of detrimental ROS at this time

To address this issue, perhaps the best way is to report a paragraph of the Editorial of James M. Downey and Michael V. Cohen [57]:

"*One of our fellows asked if testing a free radical scavenger in postconditioning might be a worthwhile experiment. We quickly chastised him explaining that the Paul Schumacker's group had shown that the ROS burst during mK_{ATP} opening is less than a tenth of what is seen at reoxygenation after simulated lethal ischemia [232]. Any ROS produced by mitochondria following a prolonged period of ischemia would be like a sneeze in a hurricane – too small to be noticed. We assured him that we had to look elsewhere for what postconditioning does. We were wrong again! Penna and colleagues actually did our fellow's experiment and postconditioned their rat hearts in the presence of the cell permeant ROS scavenger N-acetyl-cysteine. Of course, it blocked protection and their results are published in this issue of*

Basic Research in Cardiology [167]. After reading this paper we finally tried the same intervention in our rabbit model with the same result. Penna and colleagues completed the story when they also found that PKC inhibitors aborted protection. The data in this paper imply that during postconditioning the heart literally preconditions itself! We guess that the low pH during the postconditioning cycles prevents mPTP opening, while the intermittent oxygen bursts allow mitochondria to make enough ROS to activate PKC and put the heart into a protected state. PKC then activates pathways to keep the transition pores from forming after the pH is normalized. Why don't the high levels of free radicals normally produced after release of a coronary occlusion do the job? Two possibilities come to mind. First their concentration may be too high to activate PKC without also directly causing transition pore opening at the same time. The second is that the wrong kind of radical may be produced by simple reperfusion".

It is now clear that in early reperfusion there is a redox signaling both in preconditioning [44] and postconditioning process [44, 167, 219] that it is best not to be disturbed for an appropriate protection. We are of the idea that both possibilities suggested by Downey and Cohen [57] are responsible of postconditioning protection.

Other possibilities of protection with brief ischemia are ***remote perconditioning*** *(also called remote peri-conditioning)* and ***remote postconditioning.*** The first can be defined as "the induction of brief, repetitive interruptions of blood flow at a remote site, *simultaneously* with sustained target organ ischemia" and the latter as "the induction of brief, repetitive interruptions of blood flow at a remote site, *immediately after* the target organ ischemia" [211]. Although, as above reported, several studies demonstrated a key role of ROS signaling in the mechanism of both *pre-* and *post-conditioning* procedures during the early phase of reperfusion [45, 57, 81, 124, 160, 161, 167, 219], it is not clear whether *remote per- and post-conditioning* include a protective role of ROS signaling during reperfusion and whether this signal occurs in remote or target organ [126, 191, 239]. However, very few studies considered these two procedures

(*remote per- and post-conditioning*) and further investigations are required to reach a conclusion on whether or not a ROS signaling in either remote or target organ is needed.

ROS may be good or bad depending on the environment

We have seen the central role of ROS in cardioprotection. Let's consider now some of the features that can make ROS good or bad. First of all let's make it clear that it is not possible to subdivide the various ROS as good or bad without taking into account their chemistry and the biological environment in which they operate. In fact, the reactive species that were classically considered as very bad (*i.e.* peroxynitrite and hydroxyl radical) are now considered responsible of some important steps in cardioprotection: see for example the works of Kupai *et al.* [116] and Garlid *et al.* [66]; see also below.

Normal metabolism is dependent upon a ROS, oxygen. The two unpaired electrons of oxygen spin in the same direction; thus oxygen is a bi-radical, but it is not a very dangerous radical. Other oxygen-derived free radical species, such as superoxide anion ($O_2^{-\bullet}$) and hydroxyl radical ($OH^{\bullet}$), or hydrogen peroxide (H_2O_2), are stronger oxidants and therefore potentially more dangerous. However, these ROS are normally produced during oxidative metabolism and energy production by the cells. They are involved in several processes such as, electron transport in mitochondria, enzyme-catalyzed reactions, signal transduction and gene expression. They are also involved in the activation of nuclear transcription factors, oxidative damage to molecules, cells and tissues, as well as in antimicrobial action and in long term processes of aging and inflammatory/degenerative diseases [10, 31, 58, 83, 91, 161].

Nitric oxide is another radical that can be produced by enzymatic and non-enzymatic reactions in the biological systems [196, 224]. Despite its radical nature, $NO^{\bullet}$ may have antioxidant properties [228, 229]. The antioxidant properties of $NO^{\bullet}$ can be also inferred by recent intriguing experimental models introduced by the group of Roberto Bolli. They used a mouse model of cardiomyocyte-restricted

overexpression of extracellular superoxide dismutase (ecSOD) [199] and a model with inducible NOS (iNOS) gene transfer [121]. In the model with cardiac-specific ecSOD overexpression, they observed an attenuated levels of ROS which was attributed to increased $NO^{\bullet}$ availability in response to I/R leading to protection against reperfusion injury [199].In the model with iNOS gene transfer, they observed long-term (1 year) cardioprotection against I/R injury, without negative functional consequences [121]. However caution must be used in the interpretation and translation to the clinic of this approach. In fact, iNOS expression in peripheral blood cells may mediate myocardial I/R injury [74].

It is well known that $NO^{\bullet}$ modulates cell function by activation of soluble GC (sGC) to form cGMP. However, in addition to activating cGMP-dependent signaling pathways, $NO^{\bullet}$ nitrosylates target proteins, which consists in the covalent attachment of an NO-moiety to a nucleophilic protein sulfhydryl resulting in S-nitrosothiol formation. Actually, $NO^{\bullet}$ is involved in several processes of pro-oxidation or anti-oxidation, as well as in a cornucopia of reaction leading to processes of nitrosation/nitrosylation or alternatively to processes of nitration to form RNS [151] (see below).

Point to be underlined:

How, why and when the role of ROS and RNS changes from physiological cell signaling into pathological contribution to disease

Both ROS and RNS are involved in normal cell regulation in which oxidants and redox status are important in signal transduction. Oxidative stress is increasingly seen as a major upstream component in virtually all pathological processes, such as inflammatory responses, cytostatic effects and cell death (apoptosis, necrosis and/or necroptosis), where ROS/RNS may be causal and/or secondary to the disease process [10, 151, 161, 164]. The biochemistry and biology of ROS/RNS is particularly complicated and it is not easy to differentiate a clear border between beneficial and deleterious effects. However, to simplify

the concepts, we can consider beneficial those reactions involving ROS/RNS that lead to *short-lasting and reversible modifications* of the targeted component, such as proteins, nucleic acids and lipids, so that we can speak of *redox signaling*. For example the effects of $NO^{\bullet}$ can be separated in two basic categories, *direct* and *indirect* effects [120, 230]. Usually direct effects are reversible and are mediated by $NO^{\bullet}$ when it binds directly to a site on the target molecule (*e.g.*, the reaction between $NO^{\bullet}$ and heme-containing proteins). These reactions are generally rapid and require submicromolar concentration of $NO^{\bullet}$. On the contrary we can consider those reactions that are *long lasting and/or irreversible* as deleterious (usually indirect reactions). They may damage targets that will compromise cell and organ function, so that we can speak of *redox stress*. For instance, $NO^{\bullet}$'s indirect effects may fall in this category when great concentrations of $NO^{\bullet}$ (greater than 1 microM) reacts first with ROS and then with DNA inducing irreversible modification and damage leading to mutations [230].

Of course the above is an oversimplification; in fact for example, we can have redox processes that damage a protein that is subsequently removed by the proteasome and/or autophagy and ultimately result in protection of the cell. For example, *autophagy* is very important in cardioprotection [70]. It is a physiological mechanism that comprises a group of processes, involving degradation of macromolecular complexes and organelles within the vacuole or the lysosome. During autophagy the cell can recycle obsolete cellular constituents and eliminate damaged organelles and protein aggregates [94, 226]. In the heart, autophagy is upregulated by ROS, hypoxia, and ischemic preconditioning. Thus, this ROS-dependent physiological mechanism plays a key role in enhancing the heart's tolerance to ischemia. In fact the upregulation of autophagy confers cardioprotection against I/R injury, whereas its inhibition is associated with a loss of protection conferred by pharmacological preconditioning [70].

Moreover, some ROS/RNS that is considered “bad or ugly”, may be involved in inducing important protective signaling. This is the case, for instance, of peroxynitrite, which is classically considered “bad”, but it is also responsible of the triggering of cardioprotection [116] (see also below). Nevertheless, both

ROS and RNS can directly react with glutathione to lower the levels of this substance, the cell's primary antioxidant. So that changes in redox status and depletion of antioxidants occurring during frank oxidative stress may be revealed by the thiol redox status. In fact oxidized glutathione (glutathione disulfide, GSSG) accumulates under conditions of oxidant exposure, and this alters the ratio of oxidized to reduced glutathione (GSH): an increased GSSG/GSH ratio may indicate oxidative stress.

Finally, S-nitrosylation may represent a shield against oxidative stress, so that nitrosylated protein may protect cells from an irreversible oxidation and a burst of ROS/RNS [138]. This shielding effect of S-nitrosylated proteins may occur, for instance, in the first seconds of reperfusion during PostC maneuvers [165]. S-nitrosylation of proteins is emerging as a multifaceted post-translational modification. Since SNO is the result of a transient reaction, it can either act as a signaling molecule itself or as an intermediate leading to other modifications. Moreover, specific SNO can play a role when occurring in a single cysteine site, such as calcium channels [123] and mitochondrial VDAC [163]; additionally it may have a role within the context of other SNO sites and other post-translational modifications [139, 151, 164]. In particular, SNO of some proteins has been suggested to reduce ROS generation, such as in the case of SNO and inhibition of mitochondrial complex I. In fact, compartmentalization and SNO occupancy may play an important role in the consequences of the protein SNO modifications [29, 127, 197, 214]. It seems that SNO carries promising implications for cardiac protection, which, however, needs future study to be further detailed and corroborated.

Sources of ROS and RNS: reactive oxygen and nitrogen species are products of multiple enzymes and reactions within and outside the cells. These are described below and illustrated in Figure 5.

In cardiomyocytes the principal source of ROS is the mitochondrion. Thirty percent of the cardiomyocyte's volume is occupied by mitochondria. They are localized under sarcolemma (subsarcolemmal mitochondria) and between myofilaments (intermyofibrillars mitochondria), closely related to the sarcoplasmic reticulum (SR) and also clustered around the nucleus. Such an organization facilitates the

functional interplay between mitochondrial ATP production, membrane function, SR-regulated Ca^{2+} homeostasis and myofilament dependent contraction [111, 181]. Intriguingly, subsarcolemmal and intermyofibrillars mitochondria have different composition and respond differently to Diazoxide, a drug supposed to promote ROS signaling through actions on mK_{ATP} channels, probably because of the absence of Cx43 in the intermyofibrillars mitochondria [18, 19, 84, 92, 182, 188].

During respiration, mitochondria produce ATP and ROS; within mitochondria the $O_2^{-\bullet}$ is the immediate product of multiple enzymes, including the enzymes and other components (*e.g.,* iron-sulfur clusters, quinones and cytochromes) of mitochondrial oxidative phosphorylation. It seems that $O_2^{-\bullet}$ is mainly generated at complexes I and III of the electron transport chain (ETC). Apart from the ETC, there are other sources of ROS in mitochondria, for example, enzyme systems such as 2-oxoglutarate, pyruvate dehydrogenase, and flavoprotein acyl-CoA dehydrogenase [113, 204]. While superoxide is continuously produced by respiring mitochondria, H_2O_2 is rapidly generated due to spontaneous and enzymatic $O_2^{-\bullet}$ dismutation by Mn-SOD. Moreover H_2O_2 can be directly formed by the action of p66Shc and monoamine oxidases. H_2O_2 can affect cell function by reacting with thiol residues in redox-sensitive proteins in either the mitochondria or cytoplasm. In fact, while H_2O_2 easily crosses the biological membranes, the negatively charged $O_2^{-\bullet}$ does not permeate the lipid bilayer of membranes. However, we should keep in mind that $O_2^{-\bullet}$ is able to pass through the pore of anion channels (AC), such as voltage-dependent mitochondrial anion channel (Figure 6).

Under redox balanced conditions, mitochondrial H_2O_2 production is limited by the scavenging capacity of the GSH and thioredoxin-2 (Trx2) antioxidant systems [48, 180]. The H_2O_2 scavenger, catalase, is present in very low concentrations in the mitochondria [177, 187] When the rate of ROS production exceeds the scavenging capacity of the antioxidant systems H_2O_2 can be transformed to the more dangerous $OH^{\bullet}$, which, however, when formed transiently and in small amounts may be protective [66].

The importance of the role of a balanced production of ROS within mitochondria may be inferred from studies in genetically-altered mice in which mitochondrial antioxidant levels have been modified.

Transgenic mice overexpressing mitochondrial peroxiredoxin III [134] or glutathione peroxidase [195] have shown a significant attenuation of post-ischemic adverse left ventricular remodeling. Similarly, mice with a mitochondrial-targeted overexpression of catalase have demonstrated a prolonged life span with improved cardiac function [192] and an attenuation of cardiac aging [50]. In contrast, mice with ablation of either the Trx2 or thioredoxin-reductase-2 gene confers a lethal embryonic phenotype [46, 144], and cardiac tissue-restricted ablation of TrxR2 results in fatal dilated cardiomyopathy [46]. Mice with complete deletion of mitochondrial Mn-SOD develop severe fatal dilated cardiomyopathy [122]. All together these data demonstrate that mitochondria have an enzymatic kit that will potentially produce toxic amounts of ROS, but at the same time they have the ability to mitigate their toxicity. In general, we can understand that there is a delicate balance between pro- and anti-oxidants and that when this balance is perturbed toxic effects prevail; otherwise ROS can have beneficial effects. There are a plethora of variables in play in the ischemic heart including anoxia and hypoxia, *vs* normoxia, ATP/ADP, Fe^{2+}/F^{3+}, $[Ca^{2+}]_i$ levels and pH, as well as intracellular compartments, to mention only few. Of course, we cannot consider in the detail the role of the single factor to determine the threshold separating beneficial from detrimental effects in this minireview, and the reader is aware of the complexity of the topic, which needs much more research.

NO-synthases may be coupled or uncoupled

Nitric oxide is produced by *coupled* NOSs, which convert L-arginine into L-cytrullin in the presence of O_2 and co-factors, such as flavins (FAD and FMN), NADPH and tetrahydrobiopterin. Nitric oxide production by NOS is believed to be regulated by the docking of the FMN domain in one subunit of the dimer onto the heme domain of the adjacent subunit. There are three different isoforms of NOS in the heart: cardiomyocytes constitutively express both neuronal and endothelial NOS isoforms (nNOS and eNOS,

respectively), whereas iNOS isoform may be expressed in various pathophysiologic situations. These NOS isoforms are localized to different microdomains in the cardiomyocytes, and are linked to selective signaling that is further impacted by heart disease. For instance, while eNOS is involved with cGMP-dependent modulation of β-adrenergic stimuli, nNOS is linked to sarcoplasmic reticular calcium cycling (the reader is kindly directed to specific reviews on this topic) [24, 34, 128, 190]. In this context, it is useful to recall that iNOS may play a protective role in the so called second window of protection of preconditioning [22, 121, 149]

A major post-translational cause of these enzymes pathophysiology is *NOS-uncoupling*. In fact, NOSs may be the source of ROS when they are uncoupled, resulting in the generation of $O_2^{-\bullet}$ and $OH^{\bullet}$ instead of $NO^{\bullet}$. *NOS-uncoupling* occurs under certain conditions such as scarcity or absence of the cofactor, tetrahydrobiopterin, and/or of the substrate, L-arginine, as well as in the presence of oxidation of the Zn^{2+}-thiolate center of NOS homodimer. NOS catalytic activity becomes ‘‘uncoupled’’ when the coupling between the reductase domain and L-arginine oxidation at the active site is lost and electron transfer from NADPH through the flavins to O_2 is not inhibited, resulting, in fact, in a formation of $O_2^{-\bullet}$ and $OH^{\bullet}$. It seems that supplementation with tetrahydrobiopterin may reverse *NOS-uncoupling* in some cardiovascular conditions [34].

Under stress conditions, eNOS translocates from the sarcolemma to the mitochondria [209] with an essential role of lipid rafts [51] or signalosomes [176]. There is still an ongoing debate on whether mitochondria also contain functional NOS (for review see: [172]) and contribute to overall $NO^{\bullet}$ generation in cardiomyocytes [24].

Also *NOS independent mechanisms* may generate $NO^{\bullet}$ [244] .For instance, nitrite can be a source of $NO^{\bullet}$ *via* several reduction mechanisms both under acidotic and highly reduced conditions [119, 245] as well as in normoxia if a sufficient amount of nitrite is available [243]. Moreover, heme-containing proteins, such as myoglobin, may be responsible for nitrite-dependent $NO^{\bullet}$ production [85]. Recently, it was reported

that another hemeprotein, *Cytoglobin* (an ubiquitous protein with an important role in oxidative/nitrosative signaling), mediates nitrite reduction and can play an important role in $NO^{\bullet}$ generation and sGC activation under hypoxic conditions [118]. $NO^{\bullet}$ may also be generated by the xanthine oxidoreductase [228]. Within mitochondria, the cytochrome-C oxidase forms nitrite from $NO^{\bullet}$ during normoxia [190], but will generate $NO^{\bullet}$ from nitrite during low oxygen tension and low pH [35]. In the presence of hypoxia, acidosis and reduced mitochondrial membrane potential, $NO^{\bullet}$ formation through NOS isoforms declines. In fact, *in vivo* the $NO^{\bullet}$ concentration during low-flow ischemia remains unaffected by the pharmacological blockade of NOS [132], supporting a NOS-independent $NO^{\bullet}$ formation in these conditions. For a more exhaustive list of enzymatic and non-enzymatic pathways for this endogenous reduction of nitrite see the reviews of Zweier JL and Gladwin MT groups [224, 243].

$NO^{\bullet}$ signaling depends upon sGC activation and cGMP formation, with the consequent action on cGMP-dependent enzymes (protein kinases and phosphodiesterases). However, the understanding of the importance of the so-called *cGMP-independent $NO^{\bullet}$ signaling* is increasing. These also include $NO^{\bullet}$ binding to cytochrome-C oxidase in the mitochondria and its functional consequences, and are mostly related to NO-mediated post-translational modifications and the reaction of $NO^{\bullet}$ with $O_2^{-\bullet}$. As a matter of fact, $O_2^{-\bullet}$ can react with $NO^{\bullet}$ to generate peroxynitrite ($ONOO^-$) and other RNS (*via* non-enzymatic reactions limited only by diffusion). These are a quite complex reactions and controversy exists on whether the reaction of $NO^{\bullet}$ with $O_2^{-\bullet}$ is bell-shaped or not [100, 241]. It seems that, in the absence of scavengers, $ONOO^-$ decomposes to yield nitrate (NO_3^-) and free radical intermediates: hydroxyl radical and nitrogen dioxide. In most biological systems, carbon dioxide is a likely scavenger of $ONOO^-$, yielding a short-lived nitrosoperoxycarbonate anion ($ONOOCO_2^-$). During the decomposition of $ONOOCO_2^-$, nitrate and carbon dioxide are formed, as well as nitrogen dioxide radical and carbonate radical anion ($CO_3^{\bullet-}$). However, the reaction of $NO^{\bullet}$ with $O_2^{-\bullet}$ yielding $ONOO^-$, is very fast and peroxynitrite is considered the major effector of *nitrosative stress*, which may modify the effect of $NO^{\bullet}$ from protective to deleterious

[60]. Although it is widely accepted that enhanced $ONOO^-$ formation is cytotoxic *via* nitrosative stress, physiologic levels of peroxynitrite may contribute to regulation of normal cellular functions *via* SNO of proteins, including mitochondrial (*e.g.* CyPD) and non-mitochondrial proteins (*e.g.* SERCA) [60, 69].

Nevertheless, it seems that many, or even the most, effects of $NO^\bullet$ are mediated by SNO of proteins, which is a covalent modification of a protein cysteine thiol by an NO-group that generates an S-nitrosothiol. It seems that environments that allow a transient nitration with a subsequent shift toward SNO of proteins may favor cardioprotection [165]. An increase of protein SNO has also been found to be necessary in nitrite-mediated cardioprotection. Yet, as seen above, $NO^\bullet$ can derive from nitrite under acidic conditions that occur during preconditioning ischemia, and this non-enzymatically generated $NO^\bullet$ would not be blunted by NOS inhibitors [168, 197, 208]. A number of S-nitrosylated proteins have been identified in cardiomyocytes [60, 69, 106, 138] and cardiomyocyte mitochondria [138, 165, 172]. Since SNO can be reversed by intracellular reductants such as glutathione or ascorbate [6], as well as by SOD [101, 210] dynamic S-nitrosylation/de-nitrosylation reactions seem essential in cardiovascular regulation [12].

NADPH oxidase

The Nox family NADPH oxidases are important sources of ROS also in the myocardium. Seven oxidase family members, which have distinct catalytic subunit (*i.e.,* Nox-1-5 and Duox1 and 2) and different additional protein subunits, have been described (for reviews see [11, 117, 189]). The two Nox isoforms which are importantly expressed and have functional effects in cardiac myocytes are Nox2 [15] and Nox4 [1, 30]. It seems that activated Nox2 is located predominantly on the plasma membrane, whereas Nox4 is found in cellular organelles [28, 36, 55, 220]. In particular, a mitochondrial and an endoplasmic reticulum (ER)-related perinuclear location has been described for Nox4 [1, 236]. Nox2 is normally quiescent and is *acutely* activated by various stimuli [11, 223]. Yet, Nox4 may be regarded as a slowly inducible isoform. In fact, Nox4 has constitutive low-level activity, and seems to be regulated largely by changes in its

expression level [11, 26, 117, 223]. While Nox2 generates predominantly $O_2^{\bullet-}$, Nox4 may generate predominantly H_2O_2 [55, 143].

The downstream effects of these two Nox isoforms seem divergent, with Nox2 mediating detrimental effects (redox stress), whereas Nox4 may facilitate beneficial processes (redox signaling) such as angiogenesis and adaptive hypertrophy. Using mouse models with selective gain and loss of function for these Nox isoforms, it has been suggested that a very different role is played by each one, roles that themselves vary depending upon the disease condition. For instance, deletion of Nox2 is protective against angiotensin II-induced cardiac fibrosis and hypertrophy, whereas deletion of Nox4 is detrimental. However, it should be noted that recent studies suggest that high levels of Nox4 may have detrimental effects via $O_2^{\bullet-}$ production. The reasons for this discrepancy remain to be elucidated (for reviews see [11, 117, 189, 237]).

Summary of Redox-Stress and Redox-Signaling in the Context of Cardioprotection

From the above description it is inferred that ROS/RNS may be detrimental (*redox stress*) or beneficial (*redox-signaling*). Irreversible oxidation of the target molecule is common during *redox-stress,* such as those occurring in lipid peroxidation. In contrast, *redox-signaling* entails one or more reactions, which are usually reversible and involve the oxidation of a signaling molecule by a reactive species. In other words, in redox-signaling, the reaction of the ROS/RNS with the target(s) is reminiscent of “on-off” signaling similar to phosphorylation. Among reversible redox signaling SNO of proteins is particularly relevant. It is a labile modification, and may be modified by the de-nitrosylation process. This consists of the removal of the nitroso-group and is an important aspect of S-nitrosylation signaling. In fact de-nitrosylation may limit the amount of nitrosylated proteins in order to avoid ‘excessive’’ S-nitrosylation, as can occur, for instance, when iNOS is produced. The de-nitrosylation was firstly considered as a spontaneous and non-

regulated process. Recently several non-enzymatic and enzymatic mechanisms of de-nitrosylation have been described *in vitro* and *in vivo* [for reviews see [133, 138, 205, 210] (see also below for the role of SNO in cardioprotection).

Several excellent reviews have published evidence supporting a role for ROS and RNS in cell signaling, described the general properties that define a second messenger and showed how ROS and RNS fit into this role [58, 61, 135, 169, 178, 230, 231]. As seen above ROS/RNS can be synthesized within or outside the mitochondria. Whatever the case, either *redox-stress* or *redox-signaling* can occur on both sides of mitochondrial membranes. *Redox signaling* can occur by mitochondria releasing H_2O_2 that modulates the activity of target proteins through the reversible oxidation of critical protein thiols [7, 142], thus altering the activity of enzymes, kinases, phosphatases and transcription factors in mitochondria, cytosol or nucleus [151]. Although it is beyond the aim of the present review, it is necessary to recall that the redox control of phosphatases and kinases is of paramount importance in the biological systems, see for example [23, 179] .

It must be borne in mind that the switch from redox-signaling to redox-stress (within and outside mitochondria) does not depend only by the type of ROS/RNS, but it also depend on amount of ROS/RNS and the vicinity of targets and antioxidants. Several isoforms of SOD degrade $O_2^{-\bullet}$ to H_2O_2, including manganese SOD (MnSOD) in the matrix, and copper/zinc SOD (CuZnSOD) in the intermembrane space and cytosol. In the matrix, H_2O_2 is further detoxified to water primarily by glutathione peroxidase and catalase. Nevertheless, transient $OH^{\bullet}$ formation may be one step in a protective pathway [66].

Although excessive ROS/RNS formation during reperfusion that follows infarcting ischemia may enhance cell death, ROS/RNS signaling during early reperfusion is essential for the protection by ischemic and some pharmacological pre- and post-conditioning. As outlined above, ROS/RNS signaling before index ischemia, *i.e.,* during brief pre-conditioning-ischemia [10, 223], and/or during the following reperfusion [56, 161] is clearly involved in the triggering of IP protection against I/R injury. ROS/RNS signaling is also

involved in the mediation phase of IP and in the triggering of PostC in the early reperfusion of conditioned hearts [44, 81, 124, 167]. For instance, beta-adrenergic preconditioning elicited by transient administration of Isoproterenol in rat hearts is dependent on generation of ROS during the triggering and mediation phases [185]. As mentioned above, opening of mK_{ATP} may be of pivotal importance for cardioprotection with pre- and post-conditioning protocols. The opening of mK_{ATP} channels may involve Cx43 and may be upstream of ROS/RNS signaling. In fact, it has been demonstrated that Diazoxide (a drug supposed to cause protection by increasing ROS production through actions on mK_{ATP} channels) may induce both pre- and postconditioning protection [62, 84, 160]. However, replacement of Cx43 by Cx32 in mice is characterized by loss of preconditioning protection, in particular when induced by Diazoxide [184]. Moreover, the failure of cardiomyocytes from $Cx43^{+/-}$ mice to be protected by Diazoxide, has been attributed to the attenuated ROS generation [84]. This last effect may be related to absence of Cx43 at the inner mitochondrial membrane of cardiomyocytes from these animals [17, 182]. Therefore, mK_{ATP} channel opening is upstream of ROS generation. It is thus likely that these ROS may cross the mitochondrial membrane and may react with $NO^{\bullet}$ to form RNS thus contributing to protection.

Cardioprotective procedures delay the post-ischemic recovery of intracellular pH that might prevent mPTP opening directly and indirectly (*i.e.,* by inhibiting calpain activation). In addition mPTP opening might be further prevented by a ROS/RNS signaling that appears to depend on acidosis, which may favor $NO^{\bullet}$ production and protein SNO. Redox signaling triggers a protective kinase cascade including PKC and converging on mPTP. So that, mPTP closure may be dependent on ROS/RNS signaling effects, both upstream, together acidotic effect, and downstream, depending on kinase effects [47, 54].

Therefore, mitochondria are involved at least in four different steps to limit ROS generation and reperfusion injury, and to trigger kinase activation by ROS-signaling:

1) in early reperfusion mitochondria are the targets of acidosis; thus preventing mPTP opening and avoiding RIRR;

2) the activation of mK_{ATP} channels allows the formation of small amounts of ROS: mitochondria are triggers or signal amplifiers;

3) mitochondria are targets of signaling pathways with translocation of several kinases on mitochondrial membranes as well as within the interspaces of membrane and the matrix; therefore they are end-effectors of protection (in terms of inhibition of mPTP opening and of reduced release of pro-apoptotic factors into the cytosol);

4) mitochondria are targets of damage and protection from it (in terms of their functional and morphological integrity).

Points to be underlined:

We should consider that mitochondria represent more than the 30% of the cardiomyocyte mass and ROS produced by RIRR in post-ischemic phase may be among the major cause of ROS generation.

The mitochondrial components involved in *damage and protection* are several, including those seen above. Specifically in cardiac protection we may have the mitochondrial translocation of some elements of RISK pathway and/or STAT3 of SAFE pathway [78, 89, 164]. These enzymes will induce phosphorylation of several components of mitochondria including the putative elements of mPTP and mK_{ATP} channels which will preserve mitochondrial integrity and will mediate protection. On the contrary, in non-protected hearts there is the intervention of death factors, such as PKCδ and Beclin-1, which may modify mitochondria function inducing all form of cell death [59, 112, 222]. In addition, in the reperfusion phase the abrupt recovery of pH, Ca^{2+} overload and the overwhelming presence of ROS may be responsible of mPTP formation and RIRR, which may induce *redox stress*. Interestingly, a lower threshold for triggering the mPTP opening in response to Ca^{2+} overload and an increased ROS generation has been associated to the higher susceptibility of late pregnant rodent hearts to I/R injury [120].

It is of note that, though PostC affects structural features of mitochondria, it does not influence mitochondrial respiration [154]. In particular, in mitochondria isolated from PostC hearts basal state 4 or ADP-stimulated state 3 respirations are not affected, thus excluding uncoupling or inhibition of the respiratory chain as a mechanism of mPTP inhibition [4]. Nevertheless, while basal respiration was not affected, ADP-stimulated respiration was increased after pharmacological PostC with morphine [145]. This last observation is in line with many reports showing that a mild degree of mitochondrial dysfunction confers protection against I/R injury [173]. It is also in line with the important role of mitochondrial Cx43 for oxygen consumption and ATP production. In fact inhibition or reduction of mitochondrial Cx43 specifically decreases complex I respiration [20]. Mitochondrial Cx43 is important for both ROS signaling and respiration in cardiac conditioning.

It may seem paradoxical that post-ischemic mitochondrial dysfunction does not add injury, but confers protection. For several authors, including us, this is the basis of the concept that cardioprotection is afforded by a partial degree of mitochondrial dysfunction which may lead to ROS signaling [38, 162, 173]. In fact, when oxygen supply is re-established after a prolonged ischemia, if something (*e.g.,* acidosis, Cyclosporin A) prevents prolonged mPTP opening and RIRR, the partial mitochondrial dysfunction may allow ROS signaling.

Overall, whereas ischemia and reperfusion damage mitochondria (*e.g.,* limited oxidative phosphorylation), mitochondria themselves may contribute to myocardial injury (*e.g.,* mPTP opening-induced cell death) and protection (*e.g.,* limiting mPTP opening and allowing redox signaling). Cardioprotection may occur because of a favorable reaction between ROS and RNS, leading to reversible modifications of targets. This may trigger beneficial signaling in the appropriate microdomains, such as, for example, S-nitrosylation of membrane and organelle enzymes and channels.

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Figure Legends

Figure 1. Ischemia/reperfusion injury and pre- and post-conditioning effects.

Ischemia/reperfusion injury is characterized by the development of contractile dysfunction, arrhythmias, and endothelial dysfunction. Pre- and post-conditioning (IP or PostC) cardioprotective effect results in a decrease of infarct size, of arrhythmias and an improvement of post-ischemic contractile function. Myocardial stunning (*i.e.* the transient mechanical dysfunction that persists after reperfusion despite restoration of normal coronary flow) may remain unaffected by ischemic conditioning [159, 234].

Figure 2. Proposed cardioprotection pathway triggered by preconditioning.

Brief periods (a few minutes) of ischemia (black box) intervalled by brief periods of re-perfusion (white boxes) are used to precondition the myocardium against a sustained infarcting ischemia followed by reperfusion. Preconditioning cardioprotective pathway is divided in: a *trigger phase,* in which Ade (adenosine), BK (bradykinin), and other surface receptors lead to mK_{ATP} opening, mitochondrial ROS/RNS formation and PKC activation; an *ischemic phase,* in which PKC acts as a memory; a *reperfusion phase,* in which signal transduction pathways (RISK and SAFE) act to maintain mPTP in a closed state. Redox signaling and a low pH at the time of myocardial reperfusion are required to mediate the cardioprotection elicited by ischemic preconditioning. The diagram here presented is a simplification of the complex pathways activated by conditioning protocol. Moreover many links between the arrows need more evidences to be confirmed.

Acronyms are reported in the text.

Figure 3. Mitochondrial permeability transition pore (mPTP) opening state during ischemia and reperfusion.

During ischemia the closed state of mPTP is maintained by intracellular acidosis (low pH) despite an increase of cellular levels of ROS, Ca^{2+} and inorganic phosphate (Pi), thus leading to limited cellular injury. During reperfusion mPTP opening is promoted by the recovery of pH towards normal values, elevated [Ca^{2+}] and bad ROS formation due to RIRR. Prolonged opened state induces irreversible injury and leads to cell death.

Figure 4. Oxidative modification of protein thiols.

Under oxidative stress, protein cysteines are subject to either reversible or irreversible oxidative modification. Protein cysteine residues are important targets in ROS/RNS signaling depending on intracellular pH. Protein cysteine residues with the pKA lower than intracellular pH display more thiolate anion which can rapidly react with ROS/RNS.

Reversible modifications: cysteine sulfenic acid, disulfide bond, *S*-nitrosylation, and *S*-glutathionylation formation play important roles in cellular redox regulation in the presence of other surrounding thiols (RSH), nitrogens (NHR) or GSH. When cellular reducing status returns to normal, cysteine modifications are reversed by antioxidant systems such as glutaredoxin (Grx) and Trx.

Irreversible modifications: protein thiols are oxidized to sulfinic and sulfonic acid, leading to loss of protein function and protein degradation, which will not necessarily lead to cell damage. Among irreversible modifications is included protein tyrosine nitration. See also text for further explanation.

Figure 5. Proposed cardioprotection pathway triggered by postconditioning.

Brief cycles (a few seconds) of ischemia/reperfusion (black and with boxes) immediately after a prolonged ischemia are used to condition the myocardium against ischemia/reperfusion injury. These maneuvers maintain acidosis in early reperfusion and allow the heart to activate cardioprotective signal pathway (RISK and SAFE), thus leading to mPTP closure and reduced ROS production. Redox signaling and a low pH at the time of intermittent reperfusion are required to mediate the cardioprotection elicited by ischemic postconditioning. The diagram here presented is a simplification of the complex pathways activated by conditioning protocol. Moreover many links between the arrows need more evidences to be confirmed.

INN, inner membrane, OUT, outer membrane of mitochondria; other acronyms are reported in the text.

Figure 6. ROS formation within and outside mitochondria

$O_2^{\cdot-}$ is mainly generated at complex I and III of the electron transport chain and dismutated to H_2O_2 by different isoforms of SOD: MnSOD in the matrix and CuZnSOD in the intermembrane space and cytosol. H_2O_2 is further detoxified to water by glutathione peroxidase (GPX), by catalase (CAT) and Trx2.

While H_2O_2 easily cross the bilayer lipid membranes, the $O_2^{\cdot-}$ needs a pore of AC. The thickness of broken lines represents the easiness to diffuse through the membrane. $O_2^{\cdot-}$ and H_2O_2 can react (Haber-Weiss reaction) and form the most dangerous $OH^{\cdot}$.

Superoxide anion can react with $NO^{\bullet}$ to generate $ONOO^{-}$ and other RNS. The switch from redox signaling to redox stress does not depend only by the type of ROS/RNS but also on the amount of ROS/RNS and

the vicinity of targets and antioxidants. INN, inner membrane, OUT, outer membrane of mitochondria; other acronyms are reported in the text.

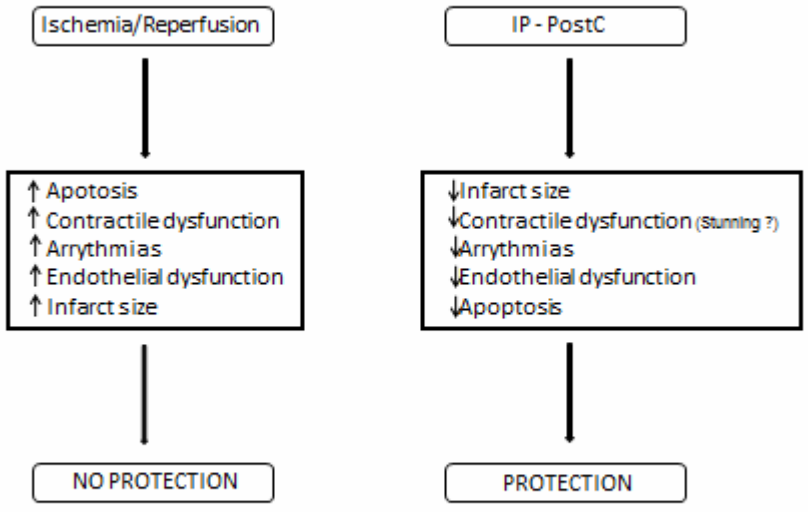
Ischemia/Reperfusion
IP - PostC
↑ Apotosis
↑ Contractile dysfunction
↑ Arrythmias
↑ Endothelial dysfunction
↑ Infarct size
↓Infarct size
↓Contractile dysfunction (Stunning ?)
↓Arrythmias
↓Endothelial dysfunction
↓Apoptosis
NO PROTECTION
PROTECTION

Fig.1

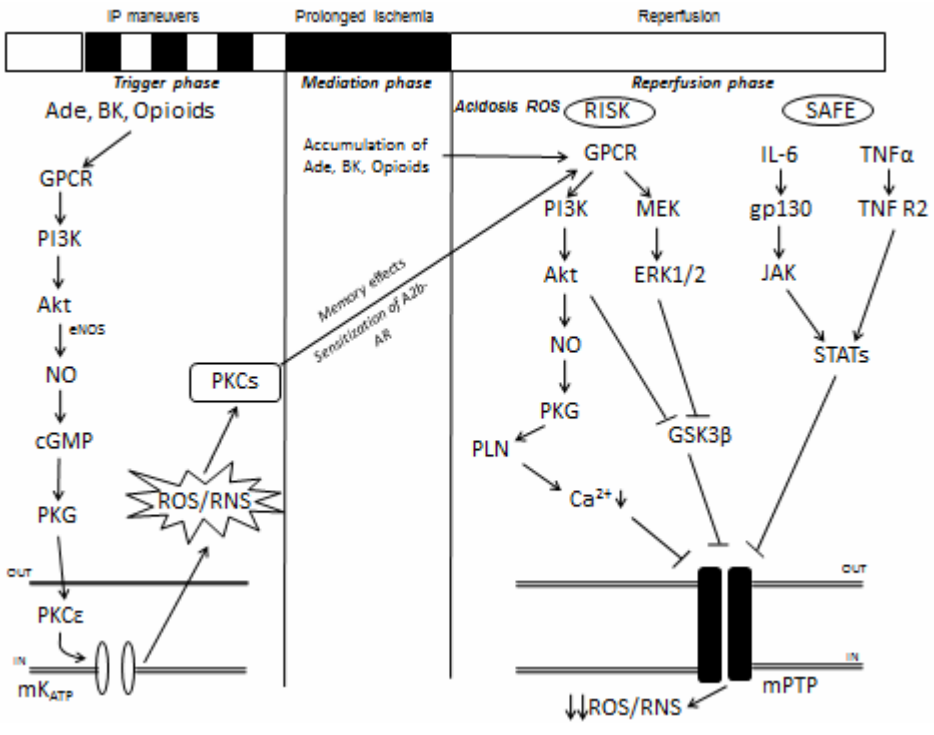
IP maneuvers
Prolonged Ischemia
Reperfusion
Trigger phase
Mediation phase
Reperfusion phase
Ade, BK, Opioids
GPCR
PI3K
Akt
eNOS
NO
cGMP
PKG
PKCε
IN
mK$_{ATP}$
OUT
PKCs
ROS/RNS
Memory effects
Sensitization of A2b-AR
Accumulation of Ade, BK, Opioids
Acidosis ROS
RISK
SAFE
GPCR
PI3K
MEK
Akt
ERK1/2
NO
PKG
PLN
Ca^{2+}
GSK3β
IL-6
TNFα
gp130
TNF R2
JAK
STATs
OUT
IN
mPTP
↓↓ROS/RNS

Fig.2

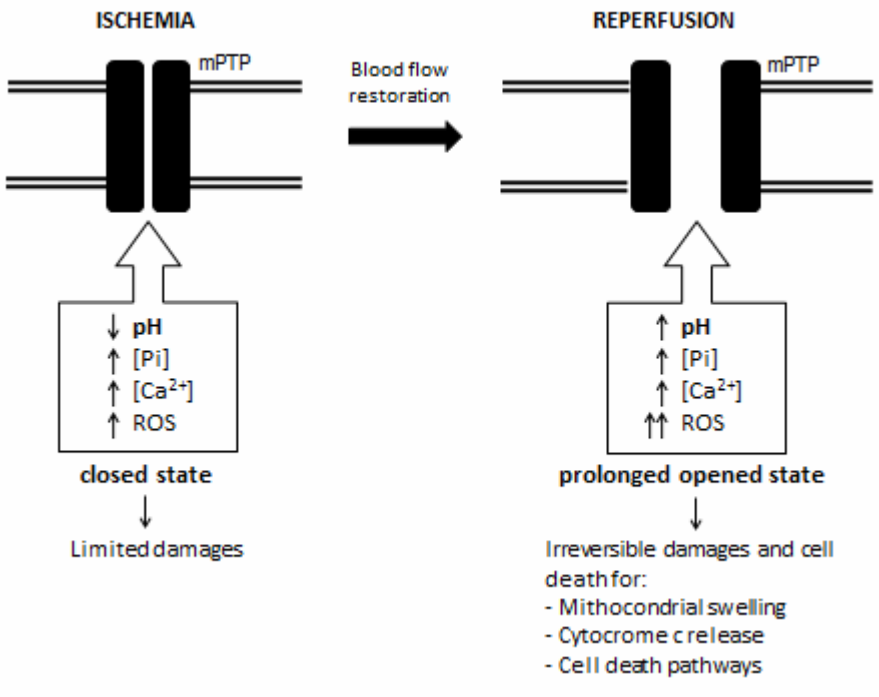
ISCHEMIA
mPTP
Blood flow restoration
REPERFUSION
mPTP
↓ pH
↑ [Pi]
↑ [Ca2+]
↑ ROS
closed state
Limited damages
↑ pH
↑ [Pi]
↑ [Ca2+]
↑↑ ROS
prolonged opened state
Irreversible damages and cell death for:
- Mithocondrial swelling
- Cytocrome c release
- Cell death pathways

Fig.3

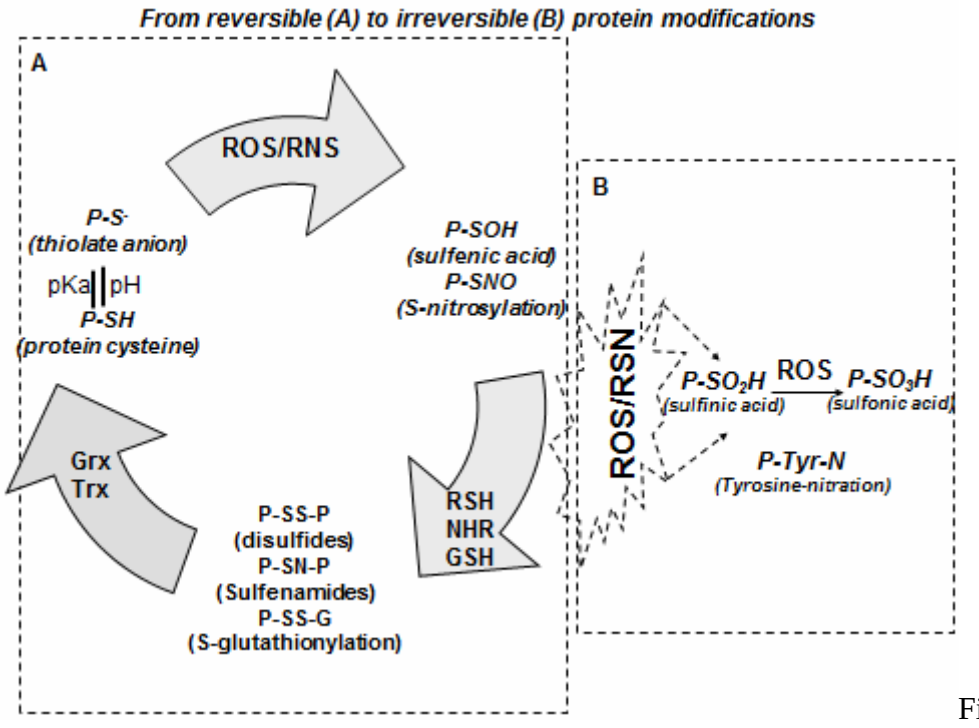
From reversible (A) to irreversible (B) protein modifications
A
ROS/RNS
P-S⁻
(thiolate anion)
pKa pH
P-SH
(protein cysteine)
P-SOH
(sulfenic acid)
P-SNO
(S-nitrosylation)
B
ROS/RSN
P-SO2H
(sulfinic acid)
ROS
P-SO3H
(sulfonic acid)
P-Tyr-N
(Tyrosine-nitration)
Grx
Trx
RSH
NHR
GSH
P-SS-P
(disulfides)
P-SN-P
(Sulfenamides)
P-SS-G
(S-glutathionylation)

Fig.4

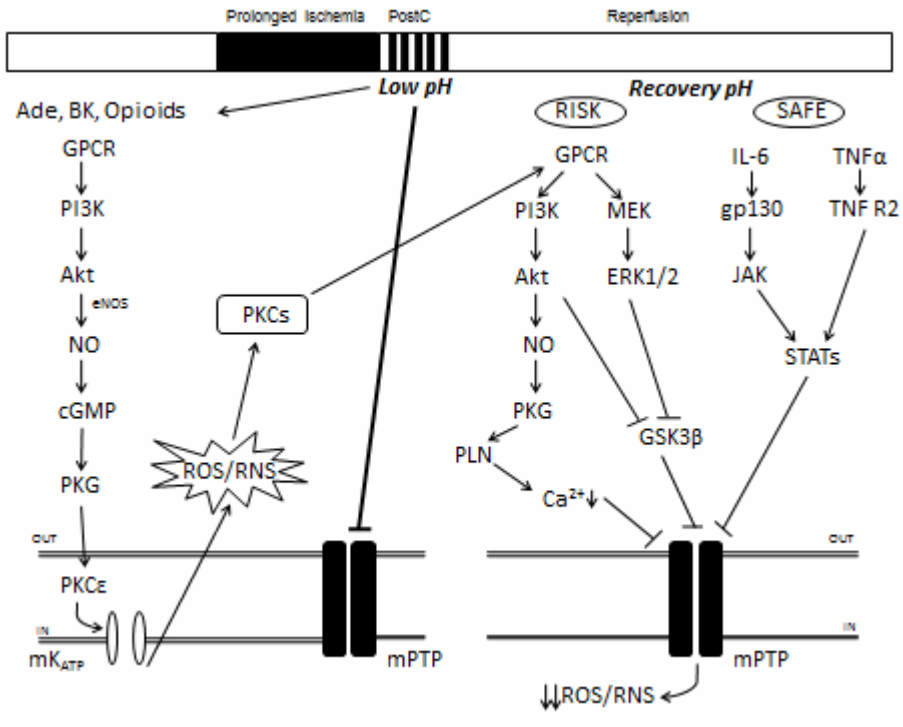
Prolonged Ischemia
PostC
Reperfusion
Low pH
Recovery pH
Ade, BK, Opioids
RISK
SAFE
GPCR
PI3K
Akt
eNOS
NO
cGMP
PKG
PKCε
PKCs
ROS/RNS
mK$_{ATP}$
mPTP
GPCR
PI3K
MEK
Akt
ERK1/2
NO
PKG
PLN
Ca^{2+}
GSK3β
IL-6
TNFα
gp130
TNF R2
JAK
STATs
OUT
IN
mPTP
ROS/RNS

Fig.5

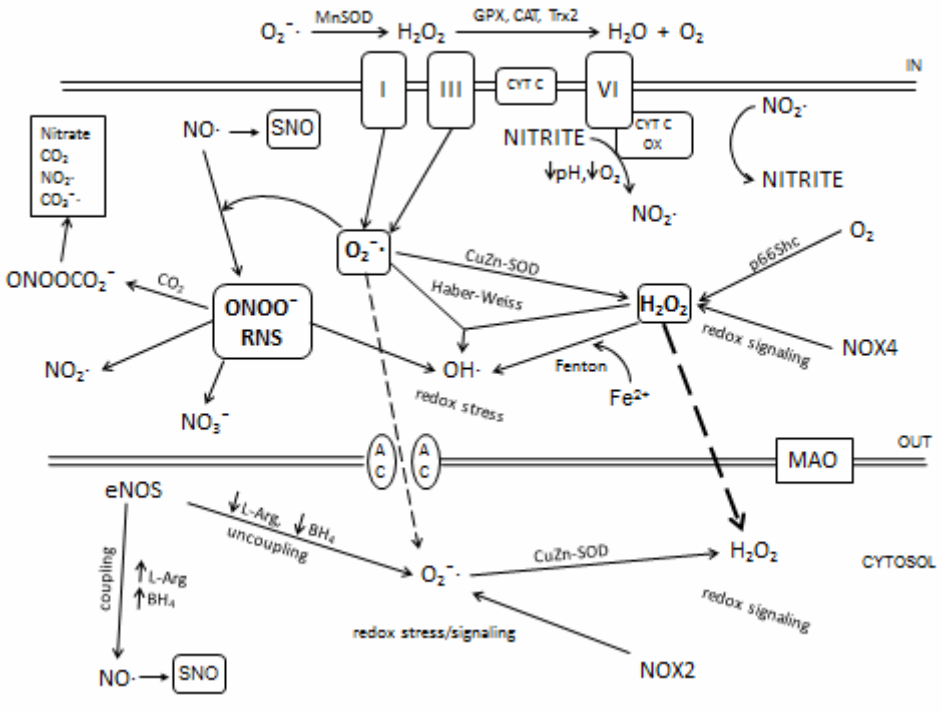
MnSOD
GPX, CAT, Trx2
IN
I
III
CYT C
VI
CYT C OX
NITRITE
NITRITE
Nitrate
SNO
Haber-Weiss
CuZn-SOD
p66Shc
NOX4
redox signaling
Fenton
redox stress
OUT
MAO
eNOS
uncoupling
coupling
L-Arg
CuZn-SOD
CYTOSOL
redox signaling
redox stress/signaling
NOX2
SNO

Fig.6